

10 YEARS OF NEUROPHARMACOLOGY

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10 YEARS OF NEUROPHARMACOLOGY

Topic Editor:

Nicholas Barnes, University of Birmingham, United Kingdom



Image: Professor Nicholas M. Barnes
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I am delighted to write a preface for this Celebratory eBook published in the Specialty Section Neuropharmacology, within the journal *Frontiers in Pharmacology*. The eBook delivers a compendium of high impact papers published previously in the journal over the last ten years; at the time of writing this collection of 20 papers has been viewed 700k times with over 75k downloads. Clearly this Section is delivering a useful resource to the scientific and medical research community!

The scope of the Specialty Section Neuropharmacology is deliberately broad. We encourage submission of high quality and impactful papers in the field of neuropharmacology along the full length of the translational escalator; from cellular and molecular investigations to clinical trials. I believe this is a real strength of the Section and helps attract a diverse readership that builds the stakeholder network to promote further engagement of the Section with the research community. I have no doubt that this has contributed to the growth of the Section with submissions and accepted papers displaying 10-year (2009-2019) compound annual growth rates (CAGRs) of 58% and 78%, respectively. Vitaly, high quality, scientific rigour and impact are fundamental to a paper being accepted and this inevitably leads to a relatively high level of rejection, but the transparency of the review process means a fair decision is reached.

Essential for the handling and considered review of submitted papers, the Specialty Section Neuropharmacology has a dynamic cohort of Associate Editors that have been selected carefully (81 at the time of writing) that represent the full spectrum of neuropharmacology as well as being widespread geographically around the globe. The Associate Editors are supported by nearly 500 Review Editors that ensures

informed review – indeed the relatively open and interactive review process often adds considerably to the accepted version of the paper.

As we celebrate the success of *Frontiers in Pharmacology* and the Specialty Section Neuropharmacology with this eBook, we must also recognise and be thankful for the hard work and dedication of those in the Editorial Office; their enthusiasm and willingness to help is outstanding and they form an integral component of the overall team that delivers the success.

I look forward to further success for the Specialty Section Neuropharmacology.

Happy reading!

Professor Nicholas M. Barnes, FBPhS
Specialty Chief Editor, Neuropharmacology

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Pharmacotherapy for fibromyalgia

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Fibromyalgia (FM) is a chronic disorder characterized by multifocal pain and other associated somatic symptoms including fatigue, insomnia, cognitive/memory problems, and even psychological distress. It appears that 2–4% of the general population suffers from FM. FM negatively impacts the physical functioning of its patients, as evidenced by difficulties with multiple daily activities, as well as affecting emotional health, social functioning, and health related quality of life. This review will discuss the potential theories that possibly contribute to the pathogenesis of FM, although the precise mechanism is unknown. The evolution of the assessment of FM will also be examined, with the waning use of tender point examinations and the appearance of new simple, practical diagnostic criteria. Although non-pharmacologic therapeutic options (exercise, education, cognitive-behavioral therapy) have been shown to be extremely effective in FM, the focus of this article will be on pharmacologic strategies. Non-Food and Drug Administration (FDA) approved as well as FDA approved agents will be presented. Each agent's therapeutic "niche" in FM management will be discussed based on its pharmacologic profile, patient responsiveness, and tolerability. Finally a clinical algorithm will be presented for the step-wise management of pain and other associated symptoms of FM.

Keywords: pharmacotherapy, fibromyalgia, pregabalin, duloxetine, milnacipran, efficacy, pain

INTRODUCTION

Fibromyalgia (FM) is a central pain disorder that seems to involve altered afferent processing, resulting in augmentation of peripheral stimuli, especially the nociceptive types. The "core" symptoms seen in FM and many other central sensitization disorders include multifocal pain, fatigue, insomnia, cognitive/memory problems, and psychological distress. However, FM patients may experience a multitude of other symptoms, including dysesthesias, stiffness, poor balance, oral/ocular symptoms (e.g., keratoconjunctivitis sicca), headaches, sexual dysfunction, and impaired physical function (**Figure 1**).

Chronic widespread pain (CWP) may occur with no other associated symptoms, generally referring to persistent pain ≥3 months with multiple locations in multiple extremities (usually upper and lower/right and left side of body), spine/axial skeleton, head, and/or thoraco abdominopelvic regions. FM includes CWP, but also includes other symptoms, notably fatigue, sleep disturbance, stiffness, hyperalgesia, impaired functioning, and cognitive or memory problems.

There is growing support that FM is part of a much larger continuum that has been called many things, including functional somatic syndromes, medically unexplained symptoms, chronic multisymptom illnesses, somatoform disorders, and perhaps most appropriately, central sensitivity syndromes (CSS; Smith et al., 2011). Yunus (1984) showed FM to be associated with tension type headache, migraine, and irritable bowel syndrome (IBS). There may be a fair amount of clinical overlap between these syndromes. The more recent term CSS as proposed by Yunus (2008) is the preferred term to globally group these entities together in, because it is felt that this may represent the best nosological term at present

for these syndromes [e.g., chronic fatigue syndrome, vulvodynia/chronic pelvic pain, IBS, interstitial cystitis, temporomandibular disorder (TMD), FM].

Groups of individuals with these CSS conditions (e.g., FM, IBS, interstitial cystitis, headaches, TMD, etc.) display diffuse hyperalgesia (increased pain in response to normally painful stimuli) and/or allodynia (pain in response to normally non-painful stimuli; Langemark et al., 1989; Maixner et al., 1995; Clauw et al., 1997; Giesecke et al., 2004, 2005; Ness et al., 2005; Rodrigues et al., 2005). Many of these conditions have also been shown to demonstrate more sensitivity to many stimuli other than pain (i.e., auditory, Gerster and Hadj-Djilani, 1984; Geisser et al., 2007, visual), and the aggregate data suggest that these individuals have a fundamental problem with pain or sensory amplification rather than an structural or inflammatory condition in the specific body region where the pain is being experienced (Smith et al., 2011).

Non-pharmacologic therapeutic options are extremely important in the management of this disorder, however we will briefly touch upon this aspect of treatment as pharmacologic strategies are the focus of this article. In this narrative review of the current available literature, the authors each separately performed a review using MEDLINE/PubMed, and EMBASE as sources in a non-systematic fashion and search terms (FM, pathophysiology, treatment, criteria). Abstracts were screened for relevance with additional sources identified via manual search of bibliographies and reference lists. The searches were restricted to the English language. Observational studies (e.g., cohort and case control studies) and open-label studies were excluded from the review.

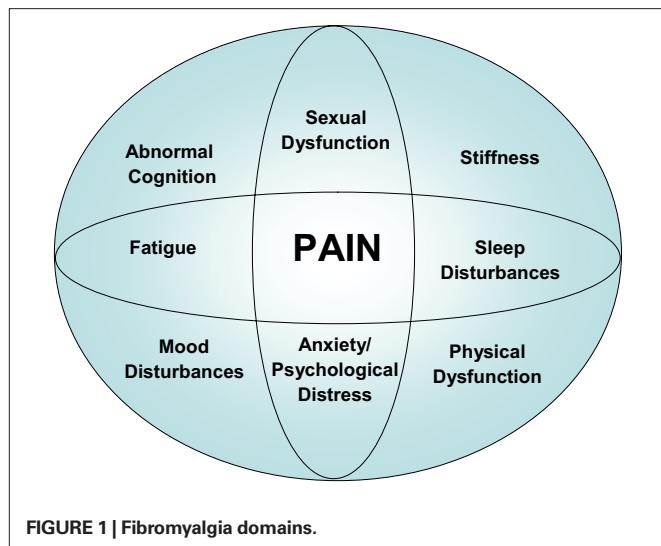


FIGURE 1 | Fibromyalgia domains.

FIBROMYALGIA SYNDROME

It appears that 2–4% (Wolfe et al., 1995; Miedema et al., 1998) of the population suffers from FM, with the disorder being two times more prevalent among women than men. This latter statement may be attributed to the fact that women tend to be more tender than men. The disorder is predominantly diagnosed in patients aged 20–60 years (mean age, 49 years; Wolfe et al., 1995; Miedema et al., 1998). FM negatively impacts the physical functioning of its patients, as evidenced by difficulties with multiple daily activities (Bennett et al., 2007; Jones et al., 2008). Sixty-two percentage of patients have difficulty climbing stairs, 55% have difficulty walking two blocks, and 35% have difficulty with activities of daily life (ADLs; Bennett et al., 2007). FM can also negatively affect personal relationships, career, and mental health (Bernard et al., 2000).

PATHOPHYSIOLOGY OF FIBROMYALGIA

A quantitative sensory testing study in 85 FM patients and 40 matched controls found that the patients had altered heat and cold thresholds and a reduced tolerance for pain, as well as a reduced nociceptive reflex threshold, a measure of central excitability (Desmeules et al., 2003). There appears to be significant support for central sensitization in the generation of FM symptoms (Burgmer et al., 2009; Woolf, 2011). Staud et al. (2001) showed temporal summation and after sensations of the pain elicited by repetitive cutaneous thermal stimuli and repetitive mechanical stimuli to muscles in patients with FM. Two years later, Staud et al. (2003a) found that temporal summation occurred at substantially lower forces and at a lower frequency of stimulation in FM patients than in control subjects, and that painful after sensations were greater in amplitude and more prolonged. The enhanced experimental pain in FM patients was shown to contribute to the variance of the clinical pain (Staud et al., 2003b). The year after Staud et al. (2004) showed that the maintenance of experimentally induced pain in FM patients requires significantly less frequent stimulation than in normal controls, and concluded that this heightened sensitivity to very low frequency inputs contributes to the persistent pain in these patients. Staud et al. (2007) demonstrated 3 years later that the temporal summation of pain and

its maintenance was widespread, and could be equally elicited from hands or feet, leading to the conclusion that central sensitization in these patients was generalized across the neuraxis. Staud et al. also concluded that enhanced neural mechanisms in FM are not the result of selective enhancement at cortical levels (Staud et al., 2008a) and peripheral sensitization does not significantly contribute to the enhanced temporal summation of thermal pain in FM patients, based on thermal thresholds (Staud et al., 2008b).

Although there is no direct evidence, it is hypothetically conceivable that microglial activation may contribute to FM pathophysiology (Smith, 2009; Younger and Mackey, 2009). Microglial activation could lead to thalamic changes (Pattany et al., 2002; Zhao et al., 2007) with resultant abnormal processing of ascending input in FM (Smith, 2009). Thalamic changes in FM appear to be supported by neuroimaging studies (Burgmer et al., 2009; Diers et al., 2011) and altered thalamic blood flow present in chronic pain states may normalize upon pain relief (Di Piero et al., 1991; Hsieh et al., 1996). Microglial activation coupled with thalamic changes may trigger neuronal hyperexcitability which in conjunction with diminished or inefficient descending inhibitory pathways in FM [as evidenced by reduced diffuse noxious inhibitory controls (DNIC) in FM patients; Staud et al., 2003c; de Souza et al., 2009; Normand et al., 2011; or the now “preferred” term in place of DNIC, conditioned pain modulation (CPM); Yarnitsky, 2010; Yarnitsky et al., 2010], may lead to central sensitization with the subsequent development of chronic pain (Smith, 2009).

The precise mechanisms responsible for FM are unknown, but most likely involve alterations in pain and sensory processing systems. In particular, it is thought that patients with FM have inefficient descending inhibitory pathways, which normally function as endogenous analgesic systems to ameliorate pain in healthy subjects. These descending inhibitory pathways are mediated in part by the neurotransmitters serotonin and norepinephrine (Figure 2).

Studies demonstrate that patients with FM have lower cerebrospinal fluid (CSF) levels of metabolites of biogenic amines (e.g., serotonin and norepinephrine; Russell et al., 1992). Further evidence comes from treatment studies which reveal that any agent that simultaneously raises both serotonin and norepinephrine [e.g., tricyclic antidepressants (TCAs), duloxetine, milnacipran, tramadol] has been shown to be efficacious in treating FM.

In addition both the subunit genes of the serotonergic receptors, HTR3A and HTR3B, have been assessed for variations in sequence in FM patients (Coaccioli et al., 2008). However, statistical analysis determined that the various polymorphisms are probably not correlated to the disorder (Coaccioli et al., 2008). Polymorphisms in catechol-O-methyltransferase, the enzyme that inactivates catecholamines, have also been recently examined for importance in FM (Gürsoy et al., 2003). Low activity COMT genotype (LL) and intermediate activity COMT genotype (LH) were both more frequently found in FM (Gürsoy et al., 2003).

Dysregulation of dopaminergic transmission has been proposed to potentially play a part in FM pathogenesis (Coaccioli et al., 2008). The pain-suppression system, activated by an acute stress, is mediated by activation of the mesolimbic dopamine neurons arising from the cell bodies of the ventral tegmental area and projecting to the nucleus accumbens (Coaccioli et al., 2008). It is proposed that exposure to prolonged stress produces both a reduction in dopamine output from the nucleus accumbens

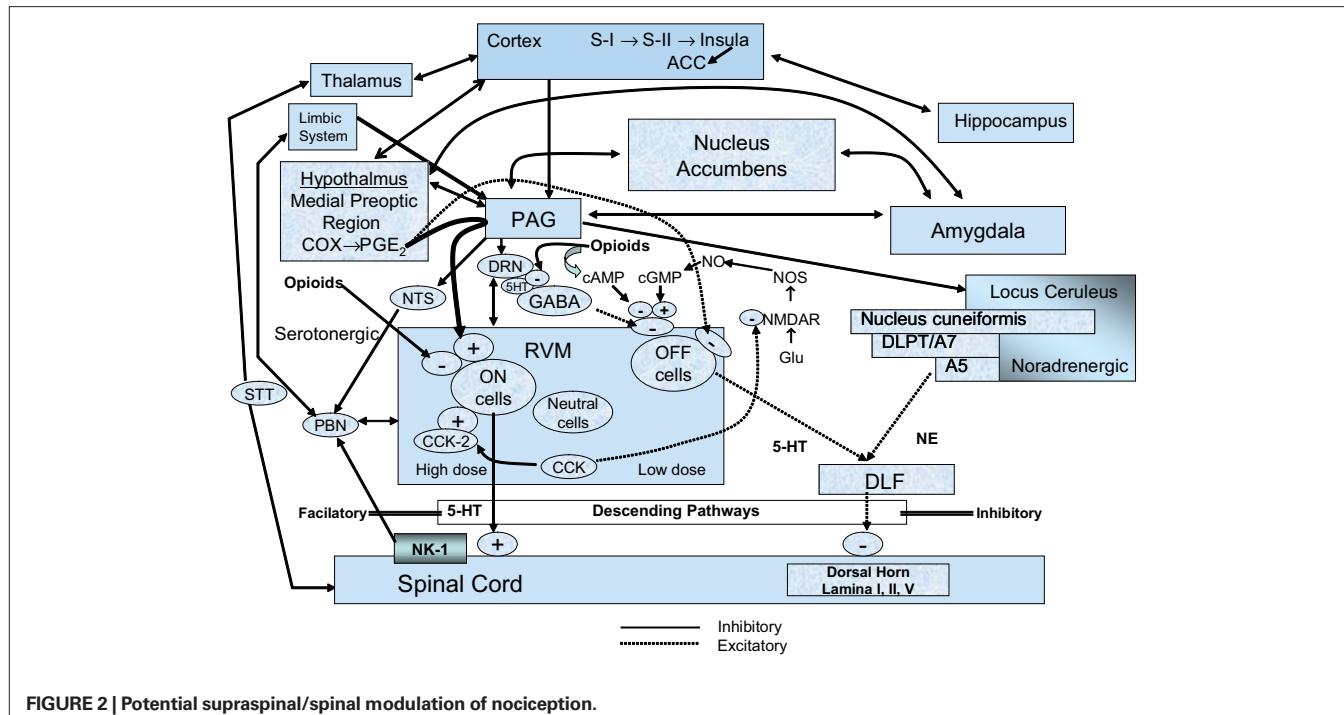


FIGURE 2 | Potential supraspinal/spinal modulation of nociception.

and the development of persistent hyperalgesia (Coaccioli et al., 2008). A pilot study examined this theory, demonstrating reduced presynaptic dopamine activity in FM patients using positron emission tomography with 6-18-fluoro-L-DOPA as a tracer (Wood et al., 2007).

Another mechanism thought to play a role in the pathophysiology of FM is the presence of augmented pain pathways in these patients. These pathways are mediated in part by substance P and the excitatory amino acid glutamate (Xu et al., 1992). Studies demonstrate that patients with FM have significantly higher concentrations of substance P in CSF compared with healthy subjects (Vaerø et al., 1988; Russell et al., 1994; Bradley et al., 1996; Bradley and Alarcón, 1999; Lui et al., 2000). CSF levels of glutamate are also twice as high in patients with FM compared with healthy controls (Sarchielli et al., 2007a). Furthermore, levels of the neurotrophic factors brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were increased in CSF of FM patients, but was not found to be specific to FM (also found in patients with chronic migraine; Sarchielli et al., 2007b).

Other biological abnormalities exist in FM, with possible relevance to its pathophysiology. In particular, anti-inflammatory cytokines were found to be decreased in FM patients, indicating that low levels of these protective cytokines could potentially be a risk factor for FM (Uçeyler et al., 2006). Interestingly, multi-modal pain therapy appears to modify cytokine profiles in FM (Wang et al., 2008).

Brain imaging studies also support the existence of central pain augmentation in patients with FM (Gracely et al., 2002). Gracely et al. (2002) performed a study utilizing functional MRI (fMRI) in patients with FM in 2002. When stimuli of equal magnitude were administered to both FM and healthy subjects, there was increased regional blood flow in FM patients compared with controls. The

regions exhibiting increased activity included the primary and secondary somatosensory cortex, the insula, and the anterior cingulate cortex, all areas which exhibit increased blood flow when normal subjects experience pain (Gracely et al., 2002).

Although it appears that the predominant mechanisms contributing to FM are largely central in nature; there may be peripheral mechanisms at play as well. Multiple studies have demonstrated differences in skin biopsies of FM patients vs. healthy controls (Kim et al., 2008) including: ballooning of Schwann cells (Kim et al., 2008), mitochondrial abnormalities (Cordero et al., 2010), and abnormal over expression of mastocytes (Blanco et al., 2010). Affaitati et al. (2011) revealed that identification and local targeted treatment of “peripheral pain generators” in the myofascial connective tissues or joints which coexisted in some patients with FM, resulted in significant benefit of the CWP of FM. Staud published that FM pain is likely to be at least partially maintained by peripheral impulse input from deep tissues, since injection of local anesthetics into painful muscles normalizes somatic hyperalgesia in FM patients (Staud et al., 2009; Staud, 2010).

DIAGNOSIS AND ASSESSMENT OF FIBROMYALGIA

The American College of Rheumatology (ACR) criteria require that an individual possess both a history of CWP and ≥ 11 of 18 possible tender points on physical examination. However, these criteria are used predominantly for research/epidemiologic purposes. The use of tender points as diagnostic criteria is beginning to fade as it fails to recognize the presence of other symptoms that need to be addressed to optimally manage FM patient (Carville et al., 2008). Wolfe (2003) conducted a study in which they mailed surveys to 12,799 patients with either RA, osteoarthritis (OA), or FM. They found that pain present in 19 primarily non-articular sites differentiated FM from the other two disorders (Wolfe, 2003; Wilke, 2009).

This study led to the proposal of new simple, practical criteria for the clinical diagnosis of FM. Through a multicenter study of 829 previously diagnosed FM patients and controls, the authors were able to develop a case definition of FM, develop criteria, and construct a symptom severity (SS) scale (Wolfe et al., 2010). Interestingly approximately 25% of FM patients did not satisfy the ACR criteria at the time of the study. The most important diagnostic variables were found to be the widespread pain index (WPI; a measure of the number of painful body regions) and categorical scales for cognitive symptoms, unrefreshed sleep, fatigue, and other somatic symptoms. The categorical scales were summed to create an SS scale (Wolfe et al., 2010). A new case definition of FM was developed by combining the WPI and SS scale: (WPI \geq 7 and SS \geq 5) or (WPI 3–6 and SS \geq 9; Wolfe et al., 2010). This new case definition of FM correctly classifies 88.1% of cases classified by the ACR criteria, without the use of a physical or tender point examination. The SS scale enables assessment of SS in currently or previously diagnosed FM patients, and may potentially be useful in the longitudinal evaluation of patients with marked symptom variability (Wolfe et al., 2010). It is important to note that these new criteria are certainly not meant to supplant the concept of a tender point or to not establish the presence of multifocal tenderness/mechanical hyperalgesia by a thorough physical examination.

The new American College of Rheumatology (ACR) criteria accomplish the following: remove tender points from the criteria and as the central element in the FM definition; change the case definition of FM; recognize the importance of a quantitative measure of widespread pain, the WPI; incorporate key FM symptoms into the criteria; and provide severity scales to measure the extent of widespread pain and SS (Wolfe, 2010). The new ACR criteria replace the 11 tender point dichotomy as well as the widespread pain dichotomy with the continuous WPI scale that provides much more information about pain threshold and pain extent (Wolfe, 2010). The new ACR criteria introduced the SS scale, which is a summary score from scales measuring the extent of fatigue, unrefreshed sleep, cognitive problems, and multiplicity of symptoms. The SS score correlates with the WPI at 0.733 and the tender point count at 0.680, and is used as part of new FM criteria (Wolfe, 2010).

The Fibromyalgia Impact Questionnaire (FIQ) is a validated, disease-specific composite measure that was developed to determine the range of symptoms experienced by FM patients and responses to therapy (Bennett, 2005). It was updated in 1997 and 2002 to reflect experience with using the instrument and to clarify the scoring system (Bennett, 2005). It includes 20 questions that assess functionality with ADLs, work difficulty, general feelings of well-being, sleep quality, and the severity of symptoms including pain, fatigue, depression, anxiety, and stiffness (Bennett, 2005). Bennett et al. (2009a) performed an analysis which demonstrated that a 14% change in the FIQ total score represented a statistically and clinically meaningful difference for the patient. The results of this analysis should enhance the utility of the FIQ for clinical and research purposes (Bennett et al., 2009a).

The Revised Fibromyalgia Impact Questionnaire (FIQR) is an updated version of the FIQ that has good psychometric properties, is easy to score, and can be completed in less than 2 min (Bennett et al., 2009b). It has the same three domains as the FIQ: function, overall impact, and symptoms. It differs from the FIQ

in that it has modified function questions and includes questions pertaining to memory, tenderness, balance, and environmental sensitivity. All questions are graded on a 0–10 numerical scale (Table 1).

Each of the three domains of the FIQR correlated well with the related domains of the FIQ ($r = 0.69$ – 0.88 , $p < 0.01$). The total scores of the FIQR and the FIQ were also closely correlated ($r = 0.88$, $p < 0.001$). There was good correlation between the FIQR and comparable domains in the Medical Outcomes Study Short Form 36 (SF-36), with a multiple regression analysis showing that the three FIQR domain scores predicted the eight SF-36 subscale scores (Bennett et al., 2009b).

NON-PHARMACOLOGIC TREATMENT OF FIBROMYALGIA

Non-pharmacologic approaches such as exercise, education, and cognitive-behavioral therapy (CBT) have a positive impact in FM, but it is felt that these treatments appear to be underutilized in usual clinical practice (Williams, 2005; Chou et al., 2007).

Several studies have shown that exercise is beneficial in FM patients, especially with respect to reducing physical symptoms and improving functional capacity (Jones and Liptan, 2009). Exercise modalities studied included land and water aerobics, strength training, flexibility training, and various combinations of these (Jones and Liptan, 2009). The strongest evidence demonstrating benefit in FM is for aerobic and mixed-type exercises, with growing evidence for positive effects from strength training (Jones et al., 2002; Figueroa et al., 2008; Valkeinen et al., 2008; Thomas and Blotman, 2010). Busch et al. (2008) systematically reviewed 34 studies assessing the efficacy of exercise in FM. Meta-analysis of six of those studies provided moderate-quality evidence that aerobic-only exercise at intensity levels recommended by the American College of Sports Medicine has positive effects on global-well-being, physical functioning, and potentially on pain (Busch et al., 2008).

Patient education has also been analyzed as a therapeutic option for FM patients. Rooks et al. (2007) completed a randomized controlled trials (RCT) with 207 participants with FM who were randomized to four groups: (1) aerobic and flexibility training group; (2) strength, aerobic, and flexibility training group; (3) the Fibromyalgia Self-Help Course; or (4) a combination of the previous three. The combination group was found to provide the most benefit (Rooks et al., 2007). Thus, education may be useful for FM patients when utilized with other multi-modal interventions.

Cognitive-behavioral therapy combines aspects of both cognitive and behavioral interventions. Catastrophic thoughts, which are beliefs that the worst possible outcome is going to occur, are associated with increased pain severity, reduced functional capacity, and affective distress in FM patients (Giesecke et al., 2005). Cognitive therapy focuses on taking catastrophic thoughts and reframing them into more positive beliefs (Hassett and Gevirtz, 2009). Behavioral therapy, in contrast, stresses the importance of operant behavioral change over inner thoughts and feelings (Hassett and Gevirtz, 2009). Its goals are to increase adaptive behavior through positive and negative reinforcement, and to extinguish maladaptive behavior through punishment (Hassett and Gevirtz, 2009). Studies have demonstrated that both OBT and CBT are effective modalities in treating FM (Thieme et al., 2006; Thieme and Gracely, 2009).

Table 1 | Revised Fibromyalgia Impact Questionnaire (FIQR).

Domain 1 directions: for each of the following nine questions, check the one box that best indicates how much your fibromyalgia made it difficult to do each of the following activities over the past 7 days

Brush or comb your hair	No difficulty	<input type="checkbox"/>	Very difficult										
Walk continuously for 20 min	No difficulty	<input type="checkbox"/>	Very difficult										
Prepare a homemade meal	No difficulty	<input type="checkbox"/>	Very difficult										
Vacuum, scrub, or sweep floors	No difficulty	<input type="checkbox"/>	Very difficult										
Lift and carry a bag full of groceries	No difficulty	<input type="checkbox"/>	Very difficult										
Climb one flight of stairs	No difficulty	<input type="checkbox"/>	Very difficult										
Change bed sheets	No difficulty	<input type="checkbox"/>	Very difficult										
Sit in a chair for 45 min	No difficulty	<input type="checkbox"/>	Very difficult										
Go shopping for groceries	No difficulty	<input type="checkbox"/>	Very difficult										

Domain 2 directions: for each of the following two questions, check the one box that best describes the overall impact of your fibromyalgia over the past 7 days

Fibromyalgia prevented me from accomplishing goals for the week	Never	<input type="checkbox"/>	Always										
I was completely overwhelmed by my fibromyalgia symptoms	Never	<input type="checkbox"/>	Always										

Domain 3 directions: for each of the following 10 questions, check the one box that the best indicates the intensity of your fibromyalgia symptoms over the past 7 days

Please rate your level of pain	No pain	<input type="checkbox"/>	Unbearable pain										
Please rate your level of energy	Lots of energy	<input type="checkbox"/>	No energy										
Please rate your level of stiffness	No stiffness	<input type="checkbox"/>	Severe stiffness										
Please rate the quality of your sleep	Awoke rested	<input type="checkbox"/>	Awoke very tired										
Please rate your level of depression	No depression	<input type="checkbox"/>	Very depressed										
Please rate your level of memory problems	Good memory	<input type="checkbox"/>	Very poor memory										
Please rate your level of anxiety	No anxious	<input type="checkbox"/>	Very anxious										
Please rate your level of tenderness to touch	No tenderness	<input type="checkbox"/>	Very tender										
Please rate your level of balance problems	No imbalance	<input type="checkbox"/>	Severe imbalance										
Please rate your level of sensitivity to loud noises, bright lights, odors, and cold	No sensitivity	<input type="checkbox"/>	Extreme sensitivity										

Scoring:

Step 1. Sum the scores for each of the three domains (function, overall, and symptoms).

Step 2. Divide domain 1 score by three, divide domain 2 score by one (that is, it is unchanged), and divide domain score 3 by two.

Step 3. Add the three resulting domain scores to obtain the total Revised Fibromyalgia Impact Questionnaire score.

Adapted from Bennett et al. (2009a).

Bernardy et al. (2010) recently performed the first meta-analysis of the efficacy of CBT in FM. The systematic review included 14 out of 27 studies with 910 subjects and a median treatment time of 27 h over a median time range of 9 weeks. The primary endpoints were pain, sleep, fatigue, and health related quality of life (HRQOL). Secondary endpoints included depressed mood, self-efficacy pain, and healthcare-seeking behavior (Bernardy et al., 2010). They demonstrated that CBT reduced depressed mood and self-efficacy pain post-treatment, but had no significant effects on pain, fatigue, sleep, or HRQOL after treatment or at follow-up. Furthermore, OBT was shown to significantly reduce the number of physician visits at follow-up. Thus CBT may be most beneficial in helping FM patients cope with pain and depression on their own and somewhat reduce dependence on health care providers (Bernardy et al., 2010).

PHARMACOLOGIC TREATMENT OF FIBROMYALGIA

The majority of clinical trials evaluating FM therapy have included antidepressants of one class or another, especially the older, TCAs. Amitriptyline is a TCA tertiary amine with prominent therapeutic effects from monoamine reuptake (serotonin > norepinephrine) and anticholinergic/sedative side effects mediated by receptor affinity at acetylcholine, muscarinic, and histamine 1 receptors (Smith and Barkin, 2010; **Figure 3**).

Its usual half-life is 31–46 h, and its metabolism is through CYP450 2C19, 1A2, and 2D6 (Smith and Barkin, 2010).

Uçeyler et al. (2008) performed a meta-analysis on the efficacy of antidepressants for treating FM. The authors found amitriptyline, studied in 13 RCTs, to provide a moderate magnitude of relief to FM patients (pain reduction by mean of 26%, improvement in QOL by 30%; Uçeyler et al., 2008).

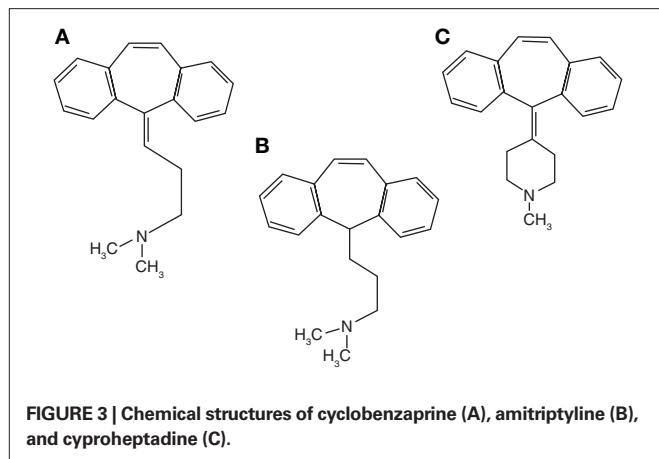


FIGURE 3 | Chemical structures of cyclobenzaprine (A), amitriptyline (B), and cyproheptadine (C).

Nishishinya et al. (2008) recently performed a systematic review specifically on the efficacy of amitriptyline in the treatment of FM. Ten RCTs were identified, and the overall study quality was moderate to high (Nishishinya et al., 2008). Amitriptyline 25 mg/day (six RCTs) demonstrated a therapeutic response compared with placebo in the domains of pain, sleep, fatigue, and overall patient and investigator impression (Nishishinya et al., 2008). This benefit was generally seen at 6–8 weeks of treatment but no significant effect was observed at 12 weeks (Nishishinya et al., 2008). Amitriptyline 50 mg/day (four RCTs) did not appear to demonstrate a significant therapeutic effect compared with placebo (Nishishinya et al., 2008). Neither dose of amitriptyline seemed to have an effect on tender point count (Nishishinya et al., 2008). The authors concluded that there is some evidence to support the short-term efficacy of amitriptyline 25 mg/day in FM (Nishishinya et al., 2008). However, there appeared to be no significant evidence to support the efficacy of amitriptyline at higher doses or for periods >8 weeks (Nishishinya et al., 2008).

Cyclobenzaprine is a centrally acting muscle relaxant which has a TCAs nucleus analog similar to amitriptyline and imipramine (Smith and Barkin, 2010; **Figure 3**). It exhibits norepinephrine and serotonin reuptake blockade as well as central and peripheral anticholinergic effects. The usual half-life is 32 h in capsules and 18 h in tablets. Metabolism of cyclobenzaprine is by CYP 3A4, 1A2, and 2D6 (Smith and Barkin, 2010). Cyclobenzaprine has been used to treat the musculoskeletal component and improves sleep in FM patients (Goldenberg, 1989).

Tofferi et al. (2004) systematically reviewed the effectiveness of cyclobenzaprine in the treatment of FM. Five randomized, placebo-controlled trials were identified. Endpoints included global improvement, treatment effects on pain, fatigue, sleep, and tender points over time. The odds ratio for global improvement with therapy was 3.0 [95% confidence interval (95% CI) 1.6–5.6] with a pooled risk difference of 0.21 (95% CI 0.09–0.34), which calculates to 4.8 (95% CI 3.0–11) individuals needing treatment for one patient to experience symptom improvement. Pain improved early on, but there did not appear to be any significant improvement in fatigue or tender points at any time. The authors concluded that cyclobenzaprine-treated patients were three times as likely to report overall improvement and to report moderate reductions in individual symptoms, particularly sleep (Tofferi et al., 2004).

Most TCAs increase CNS levels of serotonin and norepinephrine by directly blocking their reuptake. Many TCAs bind to multiple receptors, and thus may have many adverse effects, especially at higher doses. In general, secondary amines (e.g., nortriptyline, desipramine) are tolerated somewhat better than tertiary amines (e.g., amitriptyline, imipramine, doxepin). Tolerability can be improved by starting at lower doses (e.g., 10 mg of amitriptyline or 5 mg of cyclobenzaprine), giving the dose shortly before bedtime, and gradually increasing the titration over time.

It appears that selective serotonin reuptake inhibitors (SSRIs) in general are poor agents in producing analgesia in most pain states. Highly SSRIs (e.g., citalopram) have not been shown to produce significant analgesia. Otto et al. (2008) conducted a randomized, double-blinded, placebo-controlled, cross-over trial to evaluate if escitalopram 20 mg would relieve pain in painful neuropathy. Total pain and various pain symptoms were reduced during the treatment ($p = 0.001$ – 0.024). The number needed to treat (NNT) to obtain good/complete pain relief was 6.8 (Otto et al., 2008). Otto demonstrated that escitalopram did possess pain-alleviating properties in painful neuropathy, but a clinically relevant effect was found in only a few patients. Therefore, he concluded that escitalopram should not be recommended as a standard treatment in neuropathic pain (Otto et al., 2008). SSRIs that are less selective for serotonin reuptake (e.g., fluoxetine, paroxetine, sertraline) may somewhat affect norepinephrine reuptake and provide some relief in FM, but at higher than average doses as they are less potent than TCAs or serotonin–norepinephrine reuptake inhibitors (SNRIs; Fishbain et al., 2000).

Most of the SNRIs clinically available for the treatment of FM have more of a significant impact on serotonin compared with norepinephrine activity. SNRIs tend to be better tolerated than older TCAs. Venlafaxine, the first SNRI available in the US, tends to have clinically significant effects on norepinephrine reuptake only when used at higher doses (Sayar et al., 2003).

Sayar et al. (2003) conducted a study to evaluate the efficacy of venlafaxine in the treatment of patients with FM. Fifteen patients with FM were assessed prior to and after treatment with fixed-dose venlafaxine 75 mg/day for 12 weeks. Primary endpoints were the FIQ total score and pain score. The authors found a significant improvement in the mean intensity of pain ($F = 14.3$; $p = 0.0001$) and in the disability caused by FM ($F = 42.7$; $p = 0.0001$). Thus, venlafaxine could potentially be beneficial in FM patients when used at these higher doses (Sayar et al., 2003).

Duloxetine and milnacipran are two SNRIs that are approved by the Food and Drug Administration (FDA) for the treatment of FM in the US (in 2008 and 2009, respectively).

Duloxetine inhibits serotonin reuptake significantly more than norepinephrine reuptake (in an approximate 10:1 ratio; Stahl et al., 2005). The standard dosing to aim for is 60 mg/day, which in selected patients can be increased to 120 mg/day based on responsiveness and tolerability. Duloxetine is the (+)-(S) isomer of the racemic mixture with structural similarities to both fluoxetine and atomoxetine (Smith and Barkin, 2010). It possesses a secondary amine structure unlike venlafaxine, the first approved SNRI, which possesses a tertiary amine structure. It has a usual half-life of 8–17 h. Its metabolic pathways include cytochrome P450 1A2 and 2D6. Approximately 70% of duloxetine is renally excreted as

metabolites, with <1% as the parent compound. Metabolites found in plasma and urine include 4-hydroxy duloxetine glucuronide and 5-hydroxy, 6-methoxy duloxetine sulfate, neither of which appear to be significantly pharmacologically active (Curran, 2009). Approximately 20% of duloxetine is excreted in the feces, possibly representing hepatobiliary secretion. Duloxetine exhibits a high degree of protein binding (90%) and binds primarily to albumin and alpha-1-acid glycoprotein (Smith and Barkin, 2010).

Arnold et al. (2004) conducted a multicenter (18 centers), randomized, double-blinded, placebo-controlled trial assessing the efficacy of duloxetine in FM patients with or without concurrent major depressive disorder (MDD). After single-blinded placebo treatment for 1 week, patients were randomized to either duloxetine 60 mg twice daily ($n = 104$) or placebo ($n = 103$) for 12 weeks. Co-primary endpoints included the FIQ total score and FIQ pain score. Compared with placebo-treated subjects, duloxetine-treated subjects improved significantly more ($p = 0.027$) on the FIQ total score, but not significantly more on the FIQ pain score ($p = 0.130$). The FIQ pain score, however, might be limited in its capacity as an endpoint in that subjects must recall and rate their pain over the prior week, which may be more difficult to recall than pain over the past 24 h (Arnold et al., 2004).

Another multicenter (21 centers), randomized, double-blinded, placebo-controlled trial conducted by Arnold et al. (2005) assessed the efficacy of duloxetine exclusively in the treatment of females with or without MDD. The women were randomized to one of three treatment groups for a 12-week duration: duloxetine 60 mg/day ($n = 118$), duloxetine 60 mg twice daily ($n = 116$), or placebo ($n = 120$). The primary endpoint was pain severity as measured by the BPI average pain severity score. Compared with placebo, both duloxetine-treated groups improved significantly more ($p < 0.001$) on the BPI average pain severity score. A significantly higher percentage of duloxetine-treated patients had a decrease of $\geq 30\%$ in this score [duloxetine 60 mg/day (55%; $p < 0.001$); duloxetine 60 mg twice daily (54%; $p = 0.002$); placebo (33%); Arnold et al., 2005].

A third study conducted by Russell et al. (2008) also examined the efficacy of duloxetine for reducing pain severity in patients with or without current MDD over a 6-month period. It was a multicenter, randomized, double-blinded, placebo-controlled trial in which 520 patients were randomized to one of four groups: duloxetine 20 mg/day, 60 mg/day, 120 mg/day, or placebo. The co-primary endpoints were the BPI average pain severity score and PGI-I score. Compared with placebo, patients treated with duloxetine 120 mg/day improved significantly more on the co-primary endpoints at 3 months [change in BPI score (-2.31 vs. 1.39 , $p < 0.001$) and PGI-I score (2.89 vs. 3.39 , $p = 0.004$)] and at 6 months [change in BPI score (-2.26 vs. 1.43 , $p = 0.003$) and PGI-I score (2.93 vs. 3.37 , $p = 0.012$)]. Compared with placebo, patients treated with duloxetine 60 mg/day also demonstrated significantly improved co-primary endpoints at 3 months and BPI score at 6 months (Russell et al., 2008).

A study conducted by Chappell et al. (2009a) evaluated the efficacy of duloxetine in FM over a 1-year period. It was a Phase III study which consisted of an 8-week open-label period followed by a 52-week double-blinded period. Patients received duloxetine 30 mg/day for 1 week, then 60 mg/day for 7 weeks, and were subsequently randomized to either 60 or 120 mg/day. The endpoints included the BPI average pain severity and interference item scores,

the FIQ total score, the PGI-I score, the CGI-I score, the mean of the tender points pain thresholds, the number of tender points with a low threshold ($\leq 4 \text{ kg/cm}^2$), and the SDS. Significant pain reduction was observed as assessed by numerous endpoints during the open-label phase of the study. This reduction in pain severity persisted throughout the double-blinded phase, as evidenced by additional mean decreases in the BPI average pain score within both duloxetine groups (Chappell et al., 2009a).

Another study conducted by Chappell et al. (2009b) analyzed the effectiveness of duloxetine ($n = 162$) compared with placebo ($n = 168$) in the treatment of FM patients for 6 months. It was a Phase III, parallel, double-blinded, placebo-controlled trial in which patients were initially randomized to duloxetine 60 mg/day or placebo. The co-primary endpoints were BPI average pain score and the PGI-I score. The BPI average score and PGI-I score both demonstrated greater numerical improvement in duloxetine-treated compared with placebo-treated groups, but the differences were not statistically significant (BPI average score $p = 0.053$, PGI-I $p = 0.073$). However, a significant treatment-by-investigator interaction was observed for these variables which could not be fully explained. Duloxetine-treated patients did improve significantly on secondary endpoints which can be considered important factors in assessing treatment efficacy in patients with FM (Chappell et al., 2009b).

Arnold et al. (2009) pooled data from four of the prior RCTs so as to enable the assessment of precise treatment effects. Changes in the BPI average pain severity scores demonstrated significantly greater improvement in duloxetine-treated vs. placebo-treated patients at week 1 and continuing through week 12 ($p < 0.001$). Duloxetine also showed significantly greater improvement compared with placebo on the BPI severity scores for least pain, worst pain, and pain right now and on the mean of the pain interference scores. Finally, duloxetine was statistically superior to placebo with respect to improvement in CGI-S scores ($p < 0.001$), FIQ total scores ($p < 0.001$), HAMD₁₇ total scores ($p = 0.003$), PGI-I scores ($p < 0.001$), and QOL endpoints. The authors concluded that duloxetine 60–120 mg/day effectively improved FM symptoms and may offer benefits beyond pain relief, as evidenced by improvement in secondary endpoints (Arnold et al., 2009).

Choy et al. (2009) pooled data from the prior five RCTs to reliably assess the safety and tolerability of duloxetine in the treatment of patients with FM. The most commonly reported pooled treatment emergent adverse events (TEAEs) with duloxetine were nausea (33.4%), headache (25.2%), dry mouth (19.2%), insomnia (16.9%), fatigue (12.3%), constipation (16.7%), diarrhea (12.9%), and dizziness (15.1%). Most TEAEs were mild to moderate in severity and emerged early in treatment. About 20% of patients discontinued due to TEAEs in both the short-term and 1-year studies. Serious adverse events (SAEs) were uncommon, and there were no significant differences in SAEs between groups. Mean changes in vital signs and weight were small. Although duloxetine's noradrenergic effect suggests that it may slightly increase heart rate, only 0.5% of patients in the 3- and 6-month studies, 0.1% of patients enrolled for 6 months or more, and 0.6% of patients in the 1-year study had a clinically relevant increase in pulse rate. Rates of clinically significant laboratory and EKG changes were low, with the exception of ALT values being greater than five times the upper

limit of normal in duloxetine-treated patients (0.6%) compared with placebo-treated patients (0%). However, the lack of cases that met criteria for Hy's rule during either short- or long-term use suggests that the risk of hepatotoxicity for duloxetine in FM is very low. In the 1-year study, four patients (1.1%) had suicide-related behavior. However, without the presence of a placebo, this rate is difficult to interpret, especially because high rates of suicide have been demonstrated for patients with widespread pain syndromes like FM (Choy et al., 2009).

Milnacipran is the only currently FDA approved SNRI that inhibits norepinephrine reuptake more than serotonin reuptake. The standard dosing to aim for is 100 mg/day (50 mg BID), which in selected patients can be increased to 200 mg/day based on responsiveness and tolerability. Milnacipran is a chiral compound with an active portion being the D-isomer (Smith and Barkin, 2010). The usual half-life of milnacipran is 6–8 h for the parent compound and 8–10 h for D-milnacipran, the active isomer; thus twice-daily dosing is recommended. Milnacipran's metabolism has a limited hepatic contribution and is eliminated primarily by glucuronidation to an inactive metabolite. Elimination is predominantly renal (50–60% as unchanged parent compound in urine) with a small amount excreted in the feces (5% or less; Smith and Barkin, 2010). Early studies demonstrated milnacipran's efficacy for treating pain and other associated symptoms of FM (Vitton et al., 2004; Gendreau et al., 2005).

Arnold et al. (2010) conducted a study assessing the efficacy of milnacipran for the treatment of FM. It was a 12-week, double-blinded, placebo-controlled trial in which 1,025 patients were randomized to milnacipran 100 mg/day ($n = 516$) or placebo ($n = 509$). Patients underwent 4–6 weeks of flexible-dose escalation followed by 12 weeks of stable-dose treatment. Two composite responder definitions were utilized as primary endpoints. The 2-measure composite responders achieved $\geq 30\%$ improvement in pain and a rating of "very much improved" or "much improved" on the PGI-C (Change) scale. The 3-measure composite responders satisfied the above criteria while also demonstrating improvement on the SF-36 Physical Component Summary score. A significantly greater proportion of milnacipran-treated patients compared with placebo-treated patients showed statistically significant improvements, as evidenced by 2-measure composite responder criteria ($p < 0.001$) and 3-measure composite responder criteria ($p < 0.001$; Arnold et al., 2010).

Branco et al. (2010) performed a randomized, double-blinded, placebo-controlled, multicenter study examining the effectiveness of milnacipran in the treatment of FM. Eight hundred eighty-four patients were randomized to placebo ($n = 449$) or milnacipran 200 mg/day ($n = 435$) for 17 weeks. The primary endpoint was a 2-measure composite responder analysis. If the responder analysis was positive, FIQ was included as an additional primary endpoint. At the end of week 16, milnacipran 200 mg/day showed significant improvements compared with placebo in the 2-measure composite responder criteria ($p = 0.0003$) and FIQ total score ($p = 0.015$; Branco et al., 2010).

In an article by Goldenberg et al. (2010) the authors wanted to assess the durability of the therapeutic response to milnacipran for FM via a randomized, double-blinded, 6-month extension study. A total of 449 patients who successfully completed a 6-month lead-in study enrolled in this 6-month extension study. Patients initially

receiving milnacipran 200 mg/day during the lead-in study were maintained at this dose ($n = 209$). Patients initially assigned to placebo or milnacipran 100 mg/day were re-randomized to two groups: 100 mg/day ($n = 48$) or 200 mg/day ($n = 192$) of milnacipran for an additional 6 months. Endpoints included visual analog pain ratings, FIQ total score, and PGI-C score. Patients continuing on milnacipran demonstrated a sustained reduction in pain over the entire 12-month period. Additional benefits were maintained as evidenced by FIQ and PGI-C scores. Patients initially assigned to placebo or milnacipran 100 mg/day and re-randomized to milnacipran 200 mg/day experienced further improvements in mean pain scores, FIQ total scores, and PGI-C scores at 1-year (Goldenberg et al., 2010).

Mease et al. (2009) performed a study which evaluated the efficacy of milnacipran in the treatment of FM. It was a 27-week, double-blinded, multicenter trial in which 888 patients were randomized to one of three groups: placebo, 100 mg/day, or 200 mg/day of milnacipran. "FM responders" were considered 3-measure composite responders, while "FM pain responders" were considered 2-measure composite responders. After 3-month stable-dose treatment, a significantly higher percentage of milnacipran-treated patients met criteria as FM responders vs. placebo-treated patients (milnacipran 200 mg/day, $p = 0.017$; milnacipran 100 mg/day, $p = 0.028$). A significantly higher percentage of patients treated with milnacipran 200 mg/day also met criteria as FM pain responders vs. placebo-treated patients ($p = 0.032$). Significant pain reductions were observed after week 1 with both milnacipran doses (Mease et al., 2009).

Clauw et al. (2008) conducted a study which analyzed the effectiveness of milnacipran for the treatment of FM over a 15-week period. It was a multicenter, double-blinded, placebo-controlled trial in which 1,196 patients were randomized to either placebo ($n = 401$), milnacipran 100 mg/day ($n = 399$), or 200 mg/day ($n = 396$). The two primary endpoints were rates of FM composite responders and FM pain composite responders. Compared with placebo, significantly greater proportions of milnacipran-treated subjects were FM composite responders (100 mg/day: $p = 0.01$; 200 mg/day: $p = 0.02$) and FM pain composite responders (100 mg/day: $p = 0.03$; 200 mg/day: $p = 0.004$). Furthermore, milnacipran was shown to significantly improve pain after 1 week of treatment (100 mg/day: $p = 0.004$; 200 mg/day: $p = 0.04$; Clauw et al., 2008).

Finally, Geisser et al. (2011) pooled results from two of the prior RCTs to determine more precise treatment effects for milnacipran in FM. Once again the primary endpoints were a 2- and 3-measure composite response analysis. Additionally, a pooled analysis of mean changes from baseline pain scores was conducted in order to evaluate the effectiveness of milnacipran over time. At 3 months, composite responder rates were significantly higher in milnacipran-treated subjects compared with placebo-treated subjects (2- and 3-measure composite responder analyses: $p \leq 0.001$, both doses vs. placebo). These improvements were not dependent upon baseline pain severity. Similar composite responder results were observed in patients who continued treatment for up to 6 months. Significant improvements in mean pain scores were seen with both doses of milnacipran compared with placebo as early as 1 week after treatment and were sustained for up to 6 months (Geisser et al., 2011).

In the pooled analysis of data the most commonly reported adverse events with milnacipran treatment were nausea (100 mg/day 34.5%, 200 mg/day 40.1%, placebo 20.4%), headache (100 mg/day 18.6%, 200 mg/day 18.3%, placebo 14.1%), and constipation (100 mg/day 16.2%, 200 mg/day 16.1%, placebo 4.0%; Geisser et al., 2011). Greater than 90% of the adverse events reported in each treatment group were classified as either mild or moderate in severity. Furthermore, milnacipran treatment did result in slight increases in heart rate (100 mg/day 5.5%, 200 mg/day 6.5%, placebo 1.1%) and blood pressure (100 mg/day 6.6%, 200 mg/day 4.5%, placebo 1.9%). Patients at 6-month visits who received milnacipran 100 and 200 mg/day tended to lose more weight (-1.16 and -0.97 kg, respectively) compared with patients receiving placebo (-0.06 kg; $p < 0.05$, both doses vs. placebo; Geisser et al., 2011).

Pregabalin, approved for the treatment of FM in the US in 2007, is a gamma-aminobutyric acid (GABA) analog which binds to the alpha-2-delta subunit of calcium ion channels. The half-life of pregabalin is 5.5–6.7 h in the presence of a normal CrCl (Smith and Barkin, 2010). The dosing for this agent, however, is dependent upon the patient's CrCl because elimination is a function of renal clearance. Decremental dosing changes are recommended in patients with impaired renal function. Dosing secondary to side effects is based on 1-week intervals focusing on patient responsiveness and tolerability. Pregabalin's metabolism is negligible (not by CYP450 or Phase II metabolism; Barkin, 2008). Its metabolite is an N-methylated derivative. It is renally excreted, with 98% or greater as the unchanged parent compound. No plasma protein binding has been reported (Smith and Barkin, 2010).

Crofford et al. (2005) conducted a study in which the efficacy of pregabalin for the treatment of FM was evaluated. It was a multicenter, double-blinded, placebo-controlled trial in which 529 patients were randomized to one of four groups for 8 weeks: placebo, pregabalin 150, 300, and 450 mg/day. The primary endpoint was the comparison of end point mean pain scores, derived from daily diary ratings of pain intensity, among each of the pregabalin groups and the placebo group. Pregabalin 450 mg/day significantly reduced the average severity of pain in the primary analysis compared with placebo (-0.93 on a 0–10 scale; $p \leq 0.001$), and significantly more patients in this group had $\geq 50\%$ improvement in pain at the end point (29 vs. 13% in the placebo group; $p = 0.003$; Crofford et al., 2005).

In the FREEDOM study performed by Crofford et al. (2008) pregabalin's efficacy of durability was assessed in a multicenter, double-blinded, placebo-controlled, 32-week trial. The trial included a 6-week open-label phase followed by a 26-week double-blinded phase. During open-label weeks 1–3, patients received escalating doses of pregabalin to determine their optimal doses. During open-label weeks 4–6, patients received their optimal fixed doses (i.e., 300, 450, 600 mg/day). Two hundred eighty-seven patients were randomized to placebo, and 279 patients were randomized to pregabalin. The primary endpoint was time to loss of therapeutic response (LTR), defined as $< 30\%$ reduction in pain or worsening of FM. Time to LTR was longer for pregabalin compared with placebo ($p < 0.0001$). Kaplan–Meier estimates of time-to-event showed half the placebo group had LTR by day 19; half the pregabalin group still had not lost response by trial end. One hundred

seventy-four (61%) placebo patients met LTR criteria compared with 90 (32%) pregabalin patients at the end of the double-blinded phase (Crofford et al., 2008).

Mease et al. (2008) conducted a study which examined the efficacy of pregabalin for symptomatic pain relief and for the management of FM. It was a multicenter, double-blinded, placebo-controlled trial in which 748 FM patients were randomized to placebo, pregabalin 300, 450, or 600 mg/day for 13 weeks. The primary endpoint for symptomatic pain relief was comparison of mean pain scores among each pregabalin group and placebo. The primary endpoint for management of FM included mean pain scores, PGI-C score, and FIQ total score. Patients in all pregabalin groups demonstrated statistically significant improvement in mean pain score and in PGI-C score compared with placebo. Improvements in FIQ total score were numerically but not significantly greater than those for placebo (Mease et al., 2008).

Arnold et al. (2008) conducted an RCT which analyzed the effectiveness of pregabalin monotherapy in patients with FM. After 1 week of single-blinded placebo therapy, 750 patients were randomized to placebo, pregabalin 300, 450, or 600 mg/day for 14 weeks. The primary endpoint was comparison of mean pain scores, derived from daily diary ratings of pain intensity on a 0–10 scale, among each of the pregabalin groups and placebo. If positive, additional primary endpoints included the PGI-C score and the FIQ total score. Compared with placebo-treated subjects, mean changes in pain scores in pregabalin-treated subjects were significantly greater ($p < 0.001$: 300 mg/day, -0.71 ; 450 mg/day, -0.98 ; 600 mg/day, -1.00). Compared with placebo, significantly more pregabalin-treated subjects reported improvement in PGI-C score ($p < 0.01$ for all three doses) and significant improvements in FIQ total score for the 450-mg/day ($p = 0.004$) and 600-mg/day ($p = 0.003$) doses (Arnold et al., 2008).

Finally, Straube et al. (2010) performed a meta-analysis of five pregabalin trials ($n = 3,808$) in FM utilizing company trial reports. Significant benefit of pregabalin over placebo was seen for a variety of endpoints including mean pain and sleep scores, the proportion of patients achieving at least 50% pain reduction, and most of the individual domains of short form 36.

The meta-analysis demonstrated that pregabalin-treated patients (≥ 300 mg/day) experienced more somnolence, dizziness, $> 7\%$ weight gain, and discontinuations due to adverse events compared with placebo-treated patients. For dizziness and discontinuation due to adverse events there was a significant dose–response relationship. For somnolence, nausea, and weight gain there was no significant dose dependence. There was no significant difference between pregabalin and placebo in the rate of SAEs (approximately 2% in each case; Straube et al., 2010).

As of yet, there have not been any direct head-to-head comparisons of the three FDA approved drugs for FM. Häuser et al. (2010), however, recently compiled data from 11 RCTs enrolling 6,388 patients, which indirectly compared the benefits and harms of duloxetine, milnacipran, and pregabalin specifically in FM. The endpoints analyzed were reductions in pain, fatigue, sleep disturbance, depressed mood, HRQOL, and adverse events. They found that all three drugs were superior to placebo except for the following symptom-types: duloxetine for fatigue, milnacipran for sleep disturbances, and pregabalin for depressed mood. Häuser et al. (2010)

found the pooled NNTs for a 30% pain reduction to be as follows: duloxetine 7.2, milnacipran 19, and pregabalin 8.6. The authors showed that there was no significant difference among the three drugs in achieving a minimum 30% reduction in pain and discontinuation rates due to adverse events were similar (Häuser et al., 2010). There were substantial differences in symptom-type alleviated and adverse effects produced for each particular drug. Duloxetine and pregabalin were superior to milnacipran for pain and sleep disturbance. Duloxetine was superior to milnacipran and pregabalin for depressed mood. Milnacipran and pregabalin were superior to duloxetine for fatigue. The risk of headache and nausea was higher with duloxetine and milnacipran compared with pregabalin. The risk of diarrhea was higher with duloxetine compared with milnacipran and pregabalin. The most frequent adverse effects noted in pregabalin-treated patients were weight gain and peripheral edema. Rare but SAEs reported were liver failure and suicidality for duloxetine and milnacipran, and heart failure for pregabalin. Häuser et al. (2010) found the numbers needed to harm (NNHs) for discontinuation due to adverse effects to be as follows: duloxetine 14.9, milnacipran 7.6, and pregabalin 7.6.

Gabapentin is another alpha-2-delta ligand and antiepileptic drug structurally similar to pregabalin, but not approved for the treatment of FM. Its usual half-life is 5–7 h in normal renal function. Gabapentin is almost entirely eliminated renally as the parent compound as a result of negligible metabolism and requires renal dosing (Smith and Barkin, 2010). Despite not being approved, this agent has shown potential benefit in clinical trials. Arnold et al. (2007) performed a randomized, double-blinded, placebo-controlled trial examining the effectiveness/safety of gabapentin in treating FM. Patients were randomized to either gabapentin 1,200–2,400 mg/day ($n = 75$) or placebo ($n = 75$) for 12 weeks. The primary endpoint was the BPI average pain severity score. The authors concluded that gabapentin 1,200–2,400 mg/day is safe and efficacious for the treatment of pain and other symptoms associated with FM (Arnold et al., 2007).

Gamma-hydroxybutyrate (also known as sodium oxybate), a precursor of GABA known to possess strong sedative qualities, has been shown to improve fatigue, pain, and sleep architecture in FM (Scharf et al., 2003). Russell et al. (2009) randomized 118 patients with FM (92 of which completed the study) after discontinuing their pre-study FM medications to receive 4.5 or 6.0 g of sodium oxybate or placebo once per night for 8 weeks. The primary endpoint was a composite score in three co-primary self-reported measures: patient's pain rating (in daily electronic diaries) on a visual analog scale, the FIQ score, and the PGI-C score. Significant benefit was observed with both doses of sodium oxybate with regards to changes in the primary endpoint and subjective sleep quality. Improvements in patient pain ratings correlated well with sleep outcomes. Sodium oxybate was well-tolerated overall, with dose-related nausea ($\leq 28\%$ of patients) and dizziness ($\leq 18\%$ of patients) resolving with continued therapy (Russell et al., 2009).

Though speculative, it is conceivable that agents such as pramipexole and tizanidine may possess beneficial effects for patients with FM and co-existing restless leg syndrome or spasticity, respectively. Pramipexole is a dopamine agonist utilized for Parkinson's disease that is also useful for the treatment of restless leg syndrome (Bennett, 2001). Pramipexole may improve both pain and sleep in

FM patients (Holman and Myers, 2005). Tizanidine is a centrally acting alpha-2-adrenergic agonist that may possess muscle-relaxing effects in patients with spasticity. Tizanidine potentially may provide benefit for FM patients by reductions in pain, improvement in sleep, and improvement in QOL measures (Russell et al., 2002). Furthermore, tizanidine treatment reduced substance P levels in the CSF of FM patients.

Tramadol is a compound that possesses weak analgesic effects by binding to mu-opioid receptors, but its major pain-relieving effects are through serotonin-norepinephrine reuptake inhibition. The activity of the drug is focused on the M1 metabolite (O-desmethyltramadol), which yields six times more potent analgesia than the parent compound. The usual half-life of tramadol is 8 and 9 h for the M1 metabolite. It is metabolized by CYP450 enzymes 2B6, 2D6, and 3A4 (Smith and Barkin, 2010). Tramadol seems to be beneficial for the treatment of FM both alone and as a fixed-dose combination with acetaminophen (Russell et al., 2000; Bennett, 2001; Bennett et al., 2003).

Tapentadol is an agent that has not yet been studied for the treatment of FM. It does, however, possess some opioid effects as well as inhibits the reuptake of norepinephrine. Tapentadol exhibits extensive hepatic first pass effects, and its metabolism is largely hepatic primarily by the Phase II pathway (85%; glucuronidation, conjugation) and minor (15%) Phase I CYP450 oxidation (2C9), 2C19 (13%), 2D6 (2%). Thus there is minimal risk of any CYP450 drug interactions (Smith and Barkin, 2010). Non-analgesic N-desmethyl and OH-tapentadol metabolites follow with metabolism by conjugation (Smith and Barkin, 2010). Phase II metabolism is a high-capacity/low-affinity system providing water soluble, inactive metabolites for renal elimination. Tapentadol is 99% renally excreted: 70% as inactive metabolites and only 3% as the parent compound. It has a plasma protein binding of 20% and thus no significant plasma protein-binding interactions (Smith and Barkin, 2010).

Ultimately the management of FM can be approached in a step-wise manner, utilizing the most tried and true strategies initially and resorting to less well-studied agents with potential side effects based on individual patient responsiveness. This may be illustrated by a potential speculative schematic which represents a "step-ladder" type approach to the clinical management of FM (Smith and Barkin, 2010; **Figure 4**).

CONCLUSION

Fibromyalgia is a disorder characterized by CWP as well as other somatic symptoms. It appears to impart marked morbidity, negatively impacting physical functioning, HRQOL, sleep, emotional health, and social well-being, thus requiring prompt diagnosis and treatment. Non-FDA approved agents, such as amitriptyline and cyclobenzaprine, have been utilized in the "off-label" management of FM. There is evidence to support the short-term use of amitriptyline 25 mg/day, but higher doses for longer periods do not appear to be efficacious (Nishishinya et al., 2008). Cyclobenzaprine, which is structurally similar to amitriptyline, seems to be effective for the musculoskeletal component and improves sleep (Goldenberg, 1989). Agents such as SSRIs (Otto et al., 2008) and opioids appear to demonstrate little efficacy in FM. The three FDA approved agents, pregabalin, duloxetine, and

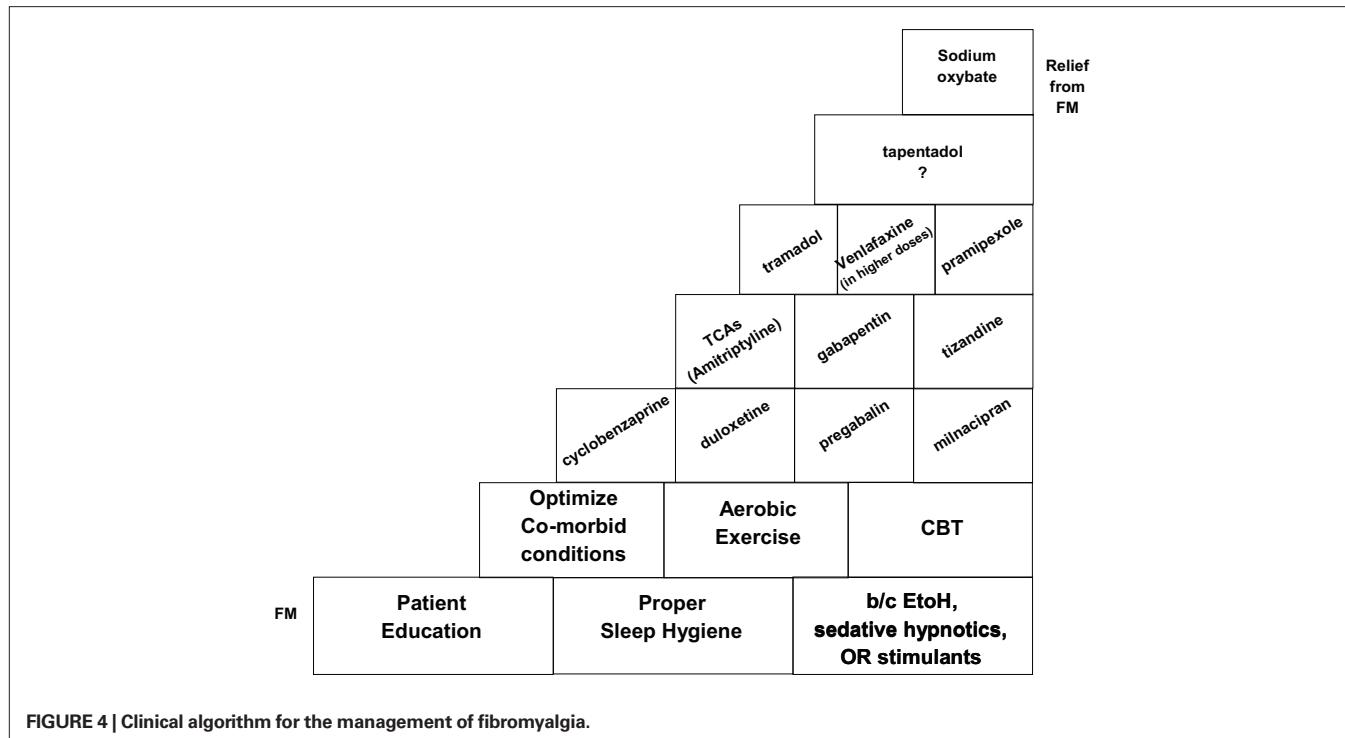


FIGURE 4 | Clinical algorithm for the management of fibromyalgia.

milnacipran, were shown to be superior to placebo except for the following symptom-types: duloxetine for fatigue, milnacipran for sleep disturbances, and pregabalin for depressed mood (Häuser et al., 2010). Other centrally acting agents may also show benefit in FM patients with a predominant symptom-type. For example, gamma-hydroxybutyrate, with its strong sedative qualities, may be clinically useful for FM patients with insomnia/sleep disturbance (Scharf et al., 2003; Russell et al., 2009). Pramipexole, a dopamine agonist used for Parkinson's disease, could be potentially useful for FM patients with concomitant restless leg syndrome (Bennett, 2001; Holman and Myers, 2005). Tramadol, which possesses some analgesic activity, may be utilized for FM patients with a significant pain component to their disease (Russell et al., 2000; Bennett,

2001; Bennett et al., 2003). Tizanidine, an alpha-2-adrenergic agonist muscle relaxant, could be potentially used for FM patients with spasticity. Based on these observations, choice of treatment medication should be tailored to fit individual patient needs and preferences. Finally, there is growing evidence supporting the effectiveness of aerobic exercise, education, and CBT in the treatment of FM. They should be considered, along with other multi-modal interventions, for more frequent utilization in clinical practice (Williams, 2005; Chou et al., 2007).

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REFERENCES

- Affaitati, G., Costantini, R., Fabrizio, A., Lapenna, D., Tafuri, E., and Giamberardino, M. A. (2011). Effects of treatment of peripheral pain generators in fibromyalgia patients. *Eur. J. Pain* 15, 61–69.
- Arnold, L. M., Clauw, D. J., Wohlreich, M. M., Wang, F., Ahl, J., Gaynor, P. J., and Chappell, A. S. (2009). Efficacy of duloxetine in patients with fibromyalgia: pooled analysis of 4 placebo-controlled clinical trials. *Prim. Care Companion J. Clin. Psychiatry* 11, 237–244.
- Arnold, L. M., Gendreau, R. M., Palmer, R. H., Gendreau, J. F., and Wang, Y. (2010). Efficacy and safety of milnacipran 100 mg/day in patients with fibromyalgia: results of a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* 62, 2745–2756.
- Arnold, L. M., Goldenberg, D. L., Stanford, S. B., Lalonde, J. K., Sandhu, H. S., Keck, P. E. Jr., Welge, J. A., Bishop, F., Stanford, K. E., Hess, E. V., and Hudson, J. I. (2007). Gabapentin in the treatment of fibromyalgia: a randomized, double-blind, placebo-controlled, multicenter trial. *Arthritis Rheum.* 56, 1336–1344.
- Arnold, L. M., Lu, Y., Crofford, L. J., Wohlreich, M., Detke, M. J., Ivengat, S., and Goldstein, D. J. (2004). A double-blind, multicenter trial comparing duloxetine with placebo in the treatment of fibromyalgia patients with or without major depressive disorder. *Arthritis Rheum.* 50, 2974–2984.
- Arnold, L. M., Rosen, A., Prichett, Y. L., D'Souza, D. N., Goldstein, D. J., Iyengar, S., and Wernicke, J. F. (2005). A randomized, double-blind, placebo-controlled trial of duloxetine in women with fibromyalgia. *J. Funct. Syndr.* 1, 79–92.
- Bennett, R. M., Friend, R., Jones, K. D., Ward, R., Han, B. K., and Ross, R. L. (2009a). The Revised Fibromyalgia Impact Questionnaire (FIQR): validation and psychometric properties. *Arthritis Res. Ther.* 11, R120.
- Bennett, R. M., Bushmakov, A. G., Cappelleri, J. C., Zlateva, G., and Sadosky, A. B. (2009b). Minimal clinically important difference in the fibromyalgia impact questionnaire. *J. Rheumatol.* 36, 1304–1311.
- Bennett, R. M., Jones, J., Turk, D. C., Russell, I. J., and Matallana, L. (2007). An internet survey of 2,596 people with fibromyalgia. *BMJ Musculoskeletal Disord.* 8, 27. doi: 10.1186/1471-2474-8-27

- Bennett, R. M., Kamin, M., Mrim, R., and Rosenthal, N. (2003). Tramadol and acetaminophen combination tablets in the treatment of fibromyalgia pain: a double-blind, randomized, placebo-controlled study. *Am. J. Med.* 114, 537–545.
- Bernard, A. L., Prince, A., and Edsall, P. (2000). Quality of life issues for fibromyalgia patients. *Arthritis Care Res.* 13, 42–50.
- Bernardy, K., Füber, N., Köllner, V., and Häuse, W. (2010). Efficacy of cognitive-behavioral therapies in fibromyalgia syndrome – a systematic review and metaanalysis of randomized controlled trials. *J. Rheumatol.* 37, 1991–2005.
- Blanco, I., Béritze, N., Argüelles, M., Cárcaba, V., Fernández, F., Janciakiene, S., Oikonomopoulou, K., de Serres, F. J., Fernández-Bustillo, E., and Hollenberg, M. D. (2010). Abnormal overexpression of mastocytosis in skin biopsies of fibromyalgia patients. *Clin. Rheumatol.* 29, 1403–1412.
- Bradley, L. A., and Alarcón, G. S. (1999). Is Chiari malformation associated with increased levels of substance P and clinical symptoms in persons with fibromyalgia? *Arthritis Rheum.* 42, 2731–2732.
- Bradley, L. A., Alberts, K. R., Alarcón, G. S., Alexander, M. T., Mount, J. M., Weigert, D. A., Liu, H. G., Blalock, J. E., Aaron, L. A., Alexander, R. W., San Pedro, E. C., Martin, M. Y., and Morell, A. C. (1996). Abnormal brain regional cerebral blood flow and cerebrospinal fluid levels of substance P in patients and non-patients with fibromyalgia. *Arthritis Rheum.* 39, 1109.
- Branco, J. C., Zahransson, O., Perrot, S., Mainguy, Y.; Multinational Coordinator Study Group. (2010). A European multicenter randomized double-blind placebo-controlled monotherapy clinical trial of milnacipran in treatment of fibromyalgia. *J. Rheumatol.* 37, 851–859.
- Burgmer, M., Pogatzki-Zahn, E., Gaubitz, M., Wessoleck, E., Heuft, G., and Pfleiderer, B. (2009). Altered brain activity during pain processing in fibromyalgia. *Neuroimage* 44, 502–508.
- Busch, A. J., Schachter, C. L., Overend, T. J., Peloso, P. M., and Barber, K. A. (2008). Exercise for fibromyalgia: a systematic review. *J. Rheumatol.* 35, 1130–1144.
- Carville, S. F., Arendt-Nielsen, S., Bliddal, H., Blotman, F., Branco, J. C., Buskila, D., Da Silva, J. A., Danneskiold-Samsøe, B., Dincer, F., Henriksson, C., Henriksson, K. G., Kosek, E., Longley, K., McCarthy, G. M., Perrot, S., Puszczewicz, M., Sarzi-Puttini, P., Silman, A., Späth, M., Choy, E. H.; EULAR. (2008). EULAR evidence-based recommendations for the management of fibromyalgia syndrome. *Ann. Rheum. Dis.* 67, 536–541.
- Chappell, A. S., Littlejohn, G., Kajdasz, D. K., Scheinberg, M., D'Souza, D. N., and Moldofsky, H. (2009a). A 1-year safety and efficacy study of duloxetine in patients with fibromyalgia. *Clin. J. Pain* 25, 365–375.
- Chappell, A. S., Bradley, L. A., Wiltse, C., Detke, M. J., D'Souza, D. N., and Spaeth, M. (2009b). A six-month double-blind, placebo-controlled, randomized clinical trial of duloxetine for the treatment of fibromyalgia. *Int. J. Gen. Med.* 1, 91–102.
- Chou, R., Huffman, L. H.; American Pain Society; American College of Physicians. (2007). Nonpharmacologic therapies for acute and chronic low back pain: a review of the evidence for an American Pain Society/American College of Physicians clinical practice guideline. *Ann. Intern. Med.* 147, 492–504.
- Choy, E. H., Mease, P. J., Kajdasz, D. K., Wohlreich, M. M., Crits-Christoph, P., Walker, D. J., and Chappell, A. S. (2009). Safety and tolerability of duloxetine in the treatment of patients with fibromyalgia: pooled analysis of data from five clinical trials. *Clin. Rheumatol.* 28, 1035–1044.
- Clauw, D. J., Mease, P., Palmer, R. H., Gendreau, R. M., and Wang, Y. (2008). Milnacipran for the treatment of fibromyalgia in adults: a 15-week, multicenter, randomized, double-blind, placebo-controlled, multiple-dose clinical trial. *Clin. Ther.* 30, 1988–2004.
- Clauw, D. J., Schmidt, M., Radulovic, D., Singer, A., Katz, P., and Bresette, J. (1997). The relationship between fibromyalgia and interstitial cystitis. *J. Psychiatr. Res.* 31, 125–131.
- Coaccioli, S., Varrassi, G., Sabatini, C., Marinangeli, F., Giuliani, M., and Puxeddu, A. (2008). Fibromyalgia: nosography and therapeutic perspectives. *Pain Pract.* 8, 190–201.
- Cordero, M. D., Moreno-Fernández, A. M., Carmona-López, M. I., Sánchez-Alcázar, J. A., Rodríguez, A. F., Navas, P., and de Miguel, M. (2010). Mitochondrial dysfunction in skin biopsies and blood mononuclear cells from two cases of fibromyalgia patients. *Clin. Biochem.* 43, 1174–1176.
- Crofford, L. J., Mease, P. J., Simpson, S. L., Young, J. P. Jr., Martin, S. A., Haig, G. M., and Sharma, U. (2008). Fibromyalgia relapse evaluation and efficacy for durability of meaningful relief (FREEDOM): a 6-month, double-blind, placebo-controlled trial with pregabalin. *Pain* 136, 419–431.
- Crofford, L. J., Rowbotham, M. C., Mease, P. J., Russell, I. J., Dworkin, R. H., Corbin, A. E., Young, J. P. Jr., LaMoreaux, L. K., Martin, S. A., Sharma, U.; Pregabalin 1008–105 Study Group. (2005). Pregabalin for the treatment of fibromyalgia syndrome: results of a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* 52, 1264–1273.
- Curran, M. P. (2009). Duloxetine: in patients with fibromyalgia. *Drugs* 69, 1217–1227.
- de Souza, J. B., Potvin, S., Goffaux, P., Charest, J., and Marchand, S. (2009). The deficit of pain inhibition in fibromyalgia is more pronounced in patients with comorbid depressive symptoms. *Clin. J. Pain* 25, 123–127.
- Desmeules, J. A., Cedraschi, C., Rapiti, E., Baumgartner, E., Finckh, A., Cohen, P., Dayer, P., and Vischer, T. L. (2003). Neurophysiologic evidence for a central sensitization in patients with fibromyalgia. *Arthritis Rheum.* 48, 1420–1429.
- Di Piero, V., Jones, A. K., Iannotti, F., Powell, M., Perani, D., Lenzi, G. L., and Frackowiak, R. S. (1991). Chronic pain: a PET study of the central effects of percutaneous high cervical cordotomy. *Pain* 46, 9–12.
- Diers, M., Schley, M. T., Rance, M., Yilmaz, P., Laufer, L., Rukwied, R., Schmelz, M., and Flor, H. (2011). Differential central pain processing following repetitive intramuscular proton/prostaglandin E(2) injections in female fibromyalgia patients and healthy controls. *Eur. J. Pain* (in press).
- Figueroa, A., Kingsley, J. D., McMillan, V., and Panton, L. B. (2008). Resistance exercise training improves heart rate variability in women with fibromyalgia. *Clin. Physiol. Funct. Imaging* 28, 49–54.
- Fishbain, D. A., Cutler, R., Rosomoff, H. L., and Rosomoff, R. S. (2000). Evidence-based data from animal and human experimental studies on pain relief with antidepressants: a structured review. *Pain Med.* 1, 310–316.
- Geisser, M. E., Gracely, R. H., Giesecke, T., Petzke, F. W., Williams, D. A., and Clauw, D. J. (2007). The association between experimental and clinical pain measures among persons with fibromyalgia and chronic fatigue syndrome. *Eur. J. Pain* 11, 202–207.
- Geisser, M. E., Palmer, R. H., Gendreau, R. M., Wang, Y., and Clauw, D. J. (2011). A pooled analysis of two randomized, double-blind, placebo-controlled trials of milnacipran monotherapy in the treatment of fibromyalgia. *Pain Pract.* 11, 120–131.
- Gendreau, R. M., Thorn, M. D., Gendreau, J. F., Kranzler, J. D., Ribeiro, S., Gracely, R. H., Williams, D. A., Mease, P. J., McLean, S. A., and Clauw, D. J. (2005). Efficacy of milnacipran in patients with fibromyalgia. *J. Rheumatol.* 32, 1975–1985.
- Gerster, J. C., and Hadj-Djilani, A. (1984). Hearing and vestibular abnormalities in primary fibrositis syndrome. *J. Rheumatol.* 11, 678–680.
- Giesecke, J., Reed, B. D., Haefner, H. K., Giesecke, T., Clauw, D. J., and Gracely, R. H. (2004). Quantitative sensory testing in vulvodynia patients and increased peripheral pressure pain sensitivity. *Obstet. Gynecol.* 104, 126–133.
- Giesecke, T., Gracely, R. H., Williams, D. A., Geisser, M. E., Petzke, F. W., and Clauw, D. J. (2005). The relationship between depression, clinical pain, and experimental pain in a chronic pain cohort. *Arthritis Rheum.* 52, 1577–1584.
- Goldenberg, D. L. (1989). Treatment of fibromyalgia syndrome. *Rheum. Dis. Clin. North Am.* 15, 61–71.
- Goldenberg, D. L., Clauw, D. J., Palmer, R. H., Mease, P., Chen, W., and Gendreau, R. M. (2010). Durability of therapeutic response to milnacipran treatment for fibromyalgia. Results of a randomized, double-blind, monotherapy 6-month extension study. *Pain Med.* 11, 180–194.
- Gracely, R. H., Petzke, F., Wolf, J. M., and Clauw, D. J. (2002). Functional magnetic resonance imaging evidence of augmented pain processing in fibromyalgia. *Arthritis Rheum.* 46, 1333–1343.
- Gürsoy, S., Erdal, E., Herken, H., Madenci, E., Alaşehirli, B., and Erdal, N. (2003). Significance of catechol-O-methyltransferase gene polymorphism in fibromyalgia syndrome. *Rheumatol. Int.* 23, 104–107.
- Hassett, A. L., and Govirtz, R. N. (2009). Nonpharmacologic treatment for fibromyalgia: patient education, cognitive-behavioral therapy, relaxation techniques, and complementary and alternative medicine. *Rheum. Dis. Clin. North Am.* 35, 393–407.
- Häuser, W., Petzke, F., and Sommer, C. C. (2010). Comparative efficacy and harms of duloxetine, milnacipran, and pregabalin in fibromyalgia syndrome. *J. Pain* 11, 505–521.
- Holman, A. J., and Myers, R. R. (2005). A randomized, double-blind, placebo-controlled trial of pramipexole, a dopamine agonist, in patients with fibromyalgia receiving concomitant medications. *Arthritis Rheum.* 52, 2495–2505.
- Hsieh, J. C., Stahle-Backdahl, M., Hagermark, O., Stone-Elander, S., Rosenquist, G., and Ingvar, M. (1996). Traumatic nociceptive pain activates the hypothalamus and the periaqueductal gray: a positron emission tomography study. *Pain* 64, 303–314.

- Jones, J., Rutledge, D. N., Jones, K. D., Matallana, L., and Rooks, D. S. (2008). Self-assessed physical function levels of women with fibromyalgia: a national survey. *Womens Health Issues* 18, 406–412.
- Jones, K. D., Burckhardt, C. S., Clark, S. R., Bennett, R. M., and Potempa, K. M. (2002). A randomized controlled trial of muscle strengthening versus flexibility training in fibromyalgia. *J. Rheumatol.* 29, 1041–1048.
- Jones, K. D., and Liptan, G. L. (2009). Exercise interventions in fibromyalgia: clinical applications from the evidence. *Rheum. Dis. Clin. North Am.* 35, 373–391.
- Kim, S. H., Kim, D. H., Oh, D. H., and Claud, D. J. (2008). Characteristic electron microscopic findings in the skin of patients with fibromyalgia – preliminary study. *Clin. Rheumatol.* 27, 407–411.
- Langemark, M., Jensen, K., Jensen, T. S., and Olesen, J. (1989). Pressure pain thresholds and thermal nociceptive thresholds in chronic tension-type headache. *Pain* 38, 203–210.
- Lui, Z., Welin, M., Bragee, B., and Nyberg, F. (2000). A high-recovery extraction procedure for quantitative analysis of substance P and opioid peptides in human cerebrospinal fluid. *Peptides* 21, 853–860.
- Maixner, W., Fillingim, R., Booker, D., and Sigurdsson, A. (1995). Sensitivity of patients with painful temporomandibular disorders to experimentally evoked pain. *Pain* 63, 341–351.
- Mease, P. J., Clauw, D. J., Gendreau, R. M., Rao, S. G., Kranzler, J., Chen, W., and Palmer, R. H. (2009). The efficacy and safety of milnacipran for treatment of fibromyalgia: a randomized, double-blind, placebo-controlled trial. *J. Rheumatol.* 36, 398–409.
- Mease, P. J., Russell, I. J., Arnold, L. M., Florian, H., Young, J. P. Jr., Martin, S. A., and Sharma, U. (2008). A randomized, double-blind, placebo-controlled, phase III trial of pregabalin in the treatment of patients with fibromyalgia. *J. Rheumatol.* 35, 502–514.
- Miedema, H. S., van der Linden, S. M., Rasker, J. J., and Valkenburg, H. A. (1998). National database of patients visiting rheumatologists in The Netherlands: the standard diagnosis register of rheumatic diseases. A report and preliminary analysis. *Br. J. Rheumatol.* 37, 555–561.
- Ness, T. J., Powell-Boone, T., Cannon, R., Lloyd, L. K., and Fillingim, R. B. (2005). Psychophysical evidence of hypersensitivity in subjects with interstitial cystitis. *J. Urol.* 173, 1983–1987.
- Nishishinya, B., Urrutia, G., Walitt, B., Rodriguez, A., Bonfill, X., Alegre, C., and Darko, G. (2008). Amitriptyline in the treatment of fibromyalgia: a systematic review of its efficacy. *Rheumatology (Oxford)* 47, 1741–1746.
- Normand, E., Potvin, S., Gaumond, I., Cloutier, G., Corbin, J. F., and Marchand, S. (2011). Pain inhibition is deficient in chronic widespread pain but normal in major depressive disorder. *J. Clin. Psychiatry* 72, 219–224.
- Otto, M., Bach, F. W., Jensen, T. S., Brosen, K., and Sindrup, S. H. (2008). Escitalopram in painful polyneuropathy: a randomized, placebo-controlled, cross-over trial. *Pain* 139, 275–283.
- Pattany, P. M., Yezierski, R. P., Widerstrom-Noga, E. G., Bowen, B. C., Martinez-Arizala, A., Garcia, B. R., and Quencer, R. M. (2002). Proton magnetic resonance spectroscopy of the thalamus in patients with chronic neuropathic pain after spinal cord injury. *AJNR Am. J. Neuroradiol.* 23, 901–905.
- Rodrigues, A. C., Nicholas, V. G., Schmidt, S., and Mauderli, A. P. (2005). Hypersensitivity to cutaneous thermal nociceptive stimuli in irritable bowel syndrome. *Pain* 115, 5–11.
- Rooks, D. S., Gautam, S., Romeling, M., Cross, M. L., Stratigakis, D., Evans, B., Goldenberg, D. L., Iverson, M. D., and Katz, J. N. (2007). Group exercise, education, and combination self-management in women with fibromyalgia: a randomized trial. *Arch. Intern. Med.* 167, 2192–2200.
- Russell, I. J., Kamin, M., Bennett, R. M., Schnitzer, T. J., Gren, J. A., and Katz, W. A. (2000). Efficacy of tramadol in treatment of pain in fibromyalgia. *J. Clin. Rheumatol.* 6, 250–257.
- Russell, I. J., Mease, P. J., Smith, T. R., Kajdasz, D. K., Wohlreich, M. M., Detke, M. J., Walker, D. J., Chappell, A. S., and Arnold, L. M. (2008). Efficacy and safety of duloxetine for treatment of fibromyalgia in patients with or without major depressive disorder: results from a 6-month, randomized, double-blind, placebo-controlled, fixed-dose trial. *Pain* 136, 432–444.
- Russell, I. J., Michalek, J. E., Xiao, Y., Haynes, W., Vertiz, R., and Lawrence, R. A. (2002). Therapy with a central alpha 2-adrenergic agonist (tizanidine) decreases cerebrospinal fluid substance P, and may reduce serum hyaluronic acid as it improves the clinical symptoms of the fibromyalgia syndrome. *Arthritis Rheum.* 46, S614.
- Russell, I. J., Orr, M. D., Littman, B., Vipraio, G. A., Albourek, D., Michalek, J. E., Lopez, Y., and MacKillip, F. (1994). Elevated cerebrospinal fluid levels of substance P in patients with the fibromyalgia syndrome. *Arthritis Rheum.* 37, 1593–1601.
- Russell, I. J., Perkins, A. T., Michalek, J. E., and Oxybate SXB-26 Fibromyalgia Syndrome Study Group. (2009). Sodium oxybate relieves pain and improves function in fibromyalgia syndrome: a randomized, double-blind, placebo-controlled, multicenter clinical trial. *Arthritis Rheum.* 60, 299–309.
- Russell, I. J., Vaeroy, H., Javors, M., and Nyberg, F. (1992). Cerebrospinal fluid biogenic amine metabolites in fibromyalgia/fibrositis syndrome and rheumatoid arthritis. *Arthritis Rheum.* 35, 550–556.
- Sarchielli, P., Di Filippo, M., Nardi, K., and Calabresi, P. (2007a). Sensitization, glutamate, and the link between migraine and fibromyalgia. *Curr. Pain Headache Rep.* 11, 343–351.
- Sarchielli, P., Mancini, M. L., Floridi, A., Coppola, F., Rossi, C., Nardi, K., Acciaresi, M., Pini, L. A., and Calabresi, P. (2007b). Increased levels of neurotrophins are not specific for chronic migraine: evidence from primary fibromyalgia syndrome. *J. Pain* 8, 737–745.
- Sayar, K., Aksu, G., Ak, I., and Tosun, M. (2003). Venlafaxine treatment of fibromyalgia. *Ann. Pharmacother.* 37, 1561–1565.
- Scharf, M. B., Baumann, M., and Berkowitz, D. V. (2003). The effects of sodium oxybate on clinical symptoms and sleep patterns in patients with fibromyalgia. *J. Rheumatol.* 30, 1070–1074.
- Smith, H. S. (2009). *The Pathophysiology of Fibromyalgia as part of National Lecture Series*. Baltimore: John Hopkins Medical Institute.
- Smith, H. S., and Barkin, R. L. (2010). Fibromyalgia syndrome: a discussion of the syndrome and pharmacotherapy. *Am. J. Ther.* 17, 418–439.
- Smith, H. S., Harris, R. E., and Clauw, D. J. (2011). Fibromyalgia: an afferent processing disorder leading to a complex pain generalized syndrome. *Pain Physician* (in press).
- Stahl, S. M., Grady, M. M., Moret, C., and Briley, M. (2005). SNRIs: their pharmacology, clinical efficacy, and tolerability in comparison with other classes of antidepressants. *CNS Spectr.* 10, 732–747.
- Staud, R. (2010). Is it all central sensitization? Role of peripheral tissue nociception in chronic musculoskeletal pain. *Curr. Rheumatol. Rep.* 12, 448–454.
- Staud, R., Bovee, C. E., Robinson, M. E., and Price, D. D. (2008a). Cutaneous C-fiber pain abnormalities of fibromyalgia patients are specifically related to temporal summation. *Pain* 139, 315–323.
- Staud, R., Craggs, J. G., Perlstein, W. M., Robinson, M. E., and Price, D. D. (2008b). Brain activity associated with slow temporal summation of C-fiber evoked pain in fibromyalgia patients and healthy controls. *Eur. J. Pain* 12, 1078–1089.
- Staud, R., Cannon, R. C., Mauderli, A. P., Robinson, M. E., Price, D. D., and Vierck, C. J. Jr. (2003a). Temporal summation of pain from mechanical stimulation of muscle tissue in normal controls and subjects with fibromyalgia syndrome. *Pain* 102, 87–95.
- Staud, R., Robinson, M. E., Vierck, C. J. Jr., Cannon, R. C., Mauderli, A. P., and Price, D. D. (2003b). Ratings of experimental pain and pain-related negative affect predict clinical pain in patients with fibromyalgia syndrome. *Pain* 105, 215–222.
- Staud, R., Robinson, M. E., Vierck, C. J. Jr., and Price, D. D. (2003c). Diffuse noxious inhibitory controls (DNIC) attenuate temporal summation of second pain in normal males but not in normal females or fibromyalgia patients. *Pain* 101, 167–174.
- Staud, R., Nagel, S., Robinson, M. E., and Price, D. D. (2009). Enhanced central pain processing of fibromyalgia patients is maintained by muscle afferent input: a randomized, double-blind, placebo-controlled study. *Pain* 145, 96–104.
- Staud, R., Price, D. D., Robinson, M. E., Mauderli, A. P., and Vierck, C. J. Jr. (2004). Maintenance of windup of second pain requires less frequent stimulation in fibromyalgia patients compared to normal controls. *Pain* 110, 689–696.
- Staud, R., Robinson, M. E., and Price, D. D. (2007). Temporal summation of second pain and its maintenance are useful for characterizing widespread central sensitization of fibromyalgia patients. *J. Pain* 8, 893–901.
- Staud, R., Vierck, C. J., Cannon, R. L., Mauderli, A. P., and Price, D. D. (2001). Abnormal sensitization and temporal summation of second pain (wind-up) in patients with fibromyalgia syndrome. *Pain* 91, 165–175.
- Straube, S., Derry, S., Moore, R. A., and McQuay, H. J. (2010). Pregabalin in fibromyalgia: meta-analysis of efficacy and safety from company clinical trial reports. *Rheumatology (Oxford)* 49, 706–715.
- Thieme, K., Flor, H., and Turk, D. (2006). Psychological pain treatment in fibromyalgia syndrome: efficacy of operant behavioural and cognitive behavioural treatments. *Arthritis Res. Ther.* 8, 121–132.
- Thieme, K., and Gracely, R. H. (2009). Are psychological treatments effective for fibromyalgia pain? *Curr. Rheumatol. Rep.* 11, 443–450.
- Thomas, E. N., and Blotman, F. (2010). Aerobic exercise in fibromyalgia: a practical review. *Rheumatol. Int.* 30, 1143–1150.

- Tofferi, J. K., Jackson, J. L., and O'Malley, P. G. (2004). Treatment of fibromyalgia with cyclobenzaprine: a meta-analysis. *Arthritis Rheum.* 51, 9–13.
- Uçeyler, N., Häuser, W., and Sommer, C. (2008). A systematic review on the effectiveness of treatment with antidepressants in fibromyalgia syndrome. *Arthritis Rheum.* 59, 1279–1298.
- Uçeyler, N., Valenza, R., Stock, M., Schedel, R., Sprotte, G., and Sommer, C. (2006). Reduced levels of antiinflammatory cytokines in patients with chronic widespread pain. *Arthritis Rheum.* 54, 2656–2664.
- Værøy, H., Helle, R., Førre, O., Kåss, E., and Terenius, L. (1988). Elevated CSF levels of substance P and high incidence of Raynaud phenomenon in patients with fibromyalgia: new features for diagnosis. *Pain* 32, 21–26.
- Valkeinen, H., Alen, M., Hakkinen, A., Hannonen, P., Kukkonen-Harjula, K., and Häkkinen, K. (2008). Effects of concurrent strength and endurance training on physical fitness and symptoms in postmenopausal women with fibromyalgia: a randomized controlled trial. *Arch. Phys. Med. Rehabil.* 89, 1660–1666.
- Witton, O., Gendreau, M., Gendreau, J., Kranzler, J., and Rao, S. G. (2004). A double-blind placebo-controlled trial of milnacipran in the treatment of fibromyalgia. *Hum. Psychopharmacol.* 19, S27–S35.
- Wang, H., Moser, M., Schiltenswolf, M., and Buchner, M. (2008). Circulating cytokine levels compared to pain in patients with fibromyalgia – a prospective longitudinal study over 6 months. *J. Rheumatol.* 35, 1366–1370.
- Wilke, W. S. (2009). New developments in the diagnosis of fibromyalgia syndrome: say goodbye to tender points? *Cleve. Clin. J. Med.* 76, 345–352.
- Williams, D. A. (2005). “Cognitive and behavioral approaches to chronic pain,” in *Fibromyalgia and Other Control Pain Syndromes*, eds D. J. Wallace and D. J. Clauw (Philadelphia, PA: Lippincott Williams & Wilkins), 343–352.
- Wolfe, F. (2003). Pain extent and diagnosis: development and validation of the regional pain scale in 12,799 patients with rheumatic disease. *J. Rheumatol.* 30, 369–378.
- Wolfe, F. (2010). New American College of Rheumatology criteria for fibromyalgia: a twenty-year journey. *Arthritis Care Res. (Hoboken)* 62, 583–584.
- Wolfe, F., Clauw, D. J., Fitzcharles, M. A., Goldenberg, D. L., Katz, R. S., Mease, P., Russell, A. S., Russell, I. J., Winfield, J. B., and Yunus, M. B. (2010). The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. *Arthritis Care Res. (Hoboken)* 62, 600–610.
- Wolfe, F., Ross, K., Anderson, J., Russell, I. J., and Hebert, L. (1995). The prevalence and characteristics of fibromyalgia in the general population. *Arthritis Rheum.* 38, 19–28.
- Wood, P. B., Patterson, J. C. II, Sunderland, J. J., Tainter, K. H., Glabus, M. F., and Lilien, D. L. (2007). Reduced presynaptic dopamine activity in fibromyalgia syndrome demonstrated with positron emission tomography: a pilot study. *J. Pain* 8, 51–58.
- Woolf, C. J. (2011). Central sensitization: implications for the diagnosis and treatment of pain. *Pain* 152, s2–s15.
- Xu, X. J., Dalsgaard, C. J., and Wiesenfeld-Hallin, Z. (1992). Spinal substance P and N-methyl-D-aspartate receptors are coactivated in the induction of central sensitization of the nociceptive flexor reflex. *Neuroscience* 51, 641–648.
- Yarnitsky, D. (2010). Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): its relevance for acute and chronic pain states. *Curr. Opin. Anaesthesiol.* 23, 611–615.
- Yarnitsky, D., Arendt-Nielsen, L., Bouhassira, D., Edwards, R. R., Fillingim, R. B., Granot, M., Hansson, P., Lautenbacher, S., Marchand, S., and Wilder-Smith, O. (2010). Recommendations on terminology and practice of psychophysical DNIC testing. *Eur. J. Pain* 14, 339.
- Younger, J., and Mackey, S. (2009). Fibromyalgia symptoms are reduced by low-dose naltrexone: a pilot study. *Pain Med.* 10, 663–672.
- Yunus, M. B. (1984). Primary fibromyalgia syndrome: current concepts. *Compr. Ther.* 10, 21–28.
- Yunus, M. B. (2008). Central sensitivity syndromes: a new paradigm and group nosology for fibromyalgia and overlapping conditions, and the related issue of disease versus illness. *Semin. Arthritis Rheum.* 37, 339–352.
- Zhao, P., Waxman, S. G., and Hains, B. C. (2007). Modulation of thalamic nociceptive processing after spinal cord injury through remote activation of thalamic microglia by cysteine cysteine chemokine ligand 21. *J. Neurosci.* 27, 8893–8902.

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History of innate immunity in neurodegenerative disorders

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The foundations of innate immunity in neurodegenerative disorders were first laid by Del Rio Hortega (1919). He identified and named microglia, recognizing them as cells of mesodermal origin. Van Furth in 1969 elaborated the monocyte phagocytic system with microglia as the brain representatives. Validation of these concepts did not occur until 1987 when HLA-DR was identified on activated microglia in a spectrum of neurological disorders. HLA-DR had already been established as a definitive marker of immunocompetent cells of mesodermal origin. It was soon determined that the observed inflammatory reaction was an innate immune response. A rapid expansion of the field took place as other markers of an innate immune response were found that were made by neurons, astrocytes, oligodendroglia, and endothelial cells. The molecules included complement proteins and their regulators, inflammatory cytokines, chemokines, acute phase reactants, prostaglandins, proteases, protease inhibitors, coagulation factors, fibrinolytic factors, anaphylatoxins, integrins, free radical generators, and other unidentified neurotoxins. The Nimmerjahn movies demonstrated that resting microglia were constantly active, sampling the surround, and responding rapidly to brain damage. Ways of reducing the neurotoxic innate immune response and stimulating a healing response continue to be sought as a means for ameliorating the pathology in a spectrum of chronic degenerative disorders.

Keywords: HLA-DR, Alzheimer disease, Parkinson disease, complement, neuroinflammation

BACKGROUND

Pio Del Rio Hortega, one of the greatest of all neuroscientists, established the basic foundation of neuroinflammation with his classic 1919 paper "El tercer elemento de los centros nerviosos" (Del Rio Hortega, 1919). He had developed an ammoniacal silver carbonate modification of the Golgi technique. With this new method he was able to identify small spidery cells which he named microglia. He recognized that they were of mesodermal origin, and that they migrated to the brain in late embryonic life. He also recognized their phagocytic capacity by examining their reaction to stab wounds. He categorized their morphology as resting, amoeboid, and reactive. By further modifying his technique, he later recognized another type of glial cell which was of epithelial origin and had a sparse cytoplasm. He named these cells oligodendroglia, perhaps because of the difficulty he encountered in staining them.

These two glial cell types made up "the third element" which had baffled Ramon y Cajal. Cajal, Hortega's mentor and employer, had clearly identified astrocytes with his methodology, and knew there were still unclassified glial cells. He thought the cells identified by Hortega might be another astrocytic type that was resistant to staining. He objected to Hortega's desire to publish his results on oligodendroglia, and when Hortega went ahead, believing others would make the same discovery, Cajal dismissed him. Hortega's own account of these travails, which he never published, were obtained after his death, later appearing in part in Haymaker and Adams' treatise on the histopathology of the nervous system (Haymaker and Adams, 1980a, pp. 484–485).

Hortega's travails were not limited to his falling out with Cajal. The Madrid laboratory he had set up after his departure from Cajal was bombed out in the Spanish civil war. He moved for a time to

Paris, and then to Oxford, but was uncomfortable in these locations. As a Spaniard opposed to Franco, he emigrated to Buenos Aires in 1938 where, in 1945, he died of cancer.

His findings and their interpretation were still being actively challenged long after his death. Some scientists supported his conclusions, others did not. Wilder Penfield was the first prominent investigator to uphold Hortega. He described, in the first issue of the American Journal of Pathology, the various stages of microglia, going from resting to reactive (Penfield, 1925). Numerous others did differing experiments, interpreting microglia cells as being of epithelial origin with unknown function. A detailed account of the continuing controversy, which extended for over six decades, appears in Haymaker and Adams (1980b, Chapter VI). This unjustified controversy may help to explain why Hortega never received a Nobel prize for his epic work.

The next giant step was taken by Ralph van Furth. He elaborated the concept of a monocyte phagocytic system to explain the origin and function of resident phagocytes throughout the body. The Memoranda endorsing the concept, which was introduced at the Conference on Mononuclear Phagocytes held in Lieden in 1969, is available on the web Chttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC2480884/pdf/bullwho00193-0157.pdf. He and his colleagues had studied labeled monocytes and their marrow precursors and followed their appearance as typical macrophages in various organs, including brain (Van Furth and Cohn, 1968). This concept replaced the vague theory of a reticuloendothelial system introduced by Aschoff (1924) which, despite its shortcomings, had become entrenched in medical teaching.

While van Furth's concept of a monocyte phagocytic system was widely accepted, its relationship to brain microglia was not.

Fujita in particular opposed the idea that microglia were the brain representatives of this system. He injected tritiated thymidine into chick embryos and identified labeled brain cells which he interpreted as glioblasts of subependymal origin (Fujita, 1965). He did later studies of human embryonic brain tissue and reached a similar conclusion, that brain microglia were of epithelial and not mesenchymal origin (Fujita, 1973; Fujita and Kitamura, 1973).

Oemichan et al. (1979) and Wood et al. (1979) also concluded that brain microglia could not be phagocytes of monocytic origin. The reason was that they could not detect the same surface antigens on microglial cells that they were able to detect on peripheral monocytes. The idea became established that inflammation of the brain did not involve microglia, and did not occur unless there was invasion of the brain by peripheral monocytes, which then became transformed into macrophages.

IDENTIFICATION OF HLA-DR POSITIVE MICROGLIA IN HUMAN BRAIN

The entry of our laboratory into the neuroinflammatory field was entirely serendipitous. Several researchers had suggested that AD might result from a herpes infection entering through the nasal passages because of involvement of the rhinencephalon (Ball, 1982). Our effort over some years to detect herpes virus in the CNS had failed, although we were able to detect it in some cases in the trigeminal nucleus, the source of herpes labialis (Walker et al., 1989). We sought the advice of local immunologists regarding what might be a broader indicator of a viral infection, and were counseled to look for HLA-DR since this class II glycoprotein would be responsible for presenting viral epitopes to T-cells. We were even presented with a gift of the HB-104 cell line, known to produce high levels of antibodies against HLA-DR.

The results astonished us. A plethora of cells was visible in AD tissue with a morphology that was completely unfamiliar to us. However we were able to confirm that they were microglia by showing them to the founder of our laboratory, Dr. William Gibson, who had been a student and biographer of Hortega (Prados and Gibson, 1946). The morphology was identical to that published by Del Rio Hortega (1919), permitting us to sense in a very small way the excitement he must have experienced.

As many frustrated investigators well know, peer reviewers, whether evaluating applications for research grants, or papers submitted to journals, are typically well versed in the dogmas of the day. Findings that radically conflict with established views are too often dismissed. Our data conflicted with two dogmas. The first was that microglia were of epithelial origin and were not immunocompetent cells. The second was that brain inflammation was not a characteristic of AD. A pathological journal rejected our initial manuscript, reasoning that the absence of a neuropathologist as an author explained the faulty conclusions. A peer reviewer for a renewal of our grant dismissed it with the comment “the hypothesis is ridiculous.” Nevertheless, our first paper appeared as a brief report in *Neuroscience Letters* (McGeer et al., 1987).

This final validation of Hortega depended on the availability of HB-104, a powerful and highly specific monoclonal antibody. The field of immunohistochemistry had been opened by Cesar Milstein’s work on the technique for producing monoclonal antibodies. He was a co-winner of the Nobel prize in 1984 for this accomplishment. Then, as now, the ability to visualize brain

biochemistry through the eyes of immunohistochemistry depends on the strength and specificity of the antibodies employed. HB-104 was a particularly good antibody, permitting us to make a clear distinction, which had eluded other investigators, between HLA-DR positive microglia and GFAP positive astrocytes.

We were not the first to report class II staining of brain cells. Lampson and Hickey (1986) had reported class II activity in occasional cell bodies of human brain with the “morphologic appearance of microglia or astrocytes.” Similarly deTribolet et al. (1984) reported HLA-DR positive cells in the white matter of normal brain which they interpreted as being astrocytes (deTribolet et al., 1984). Rogers et al. reported HLA-DR positive microglia and astrocytes in AD in an abstract at the Society of Neuroscience meeting in 1986 but, as in our case, formal publication was held up by disbelief in the findings. Their work finally appeared in 1988 (Luber-Narod and Rogers, 1988; Rogers et al., 1988).

These data led to a number of unanswered questions. What was the source of the inflammation in AD? Was it a special phenomenon or a general one that applied to many chronic neurological disorders? If it was a general phenomenon, was it localized to the brain or did it involve the peripheral immune system? Was it helpful or harmful to ongoing degenerative processes? What were the implications for treatment? Exploration of these questions led to the opening up of an important new field of neuroscience, namely neuroinflammation.

ESTABLISHMENT OF NEUROINFLAMMATION AS A DISTINCT FIELD OF NEUROSCIENCE RESEARCH

The question as to whether or not the presence of activated microglia in brain was a special one applying to AD, or a general phenomenon was quickly answered. HLA-DR activated microglia were observed in a variety of degenerative neurological conditions, including Parkinson disease (PD), Pick disease, ALS, Huntington disease, multiple sclerosis, AIDS encephalopathy, parkinsonism dementia of Guam, and the Shy–Drager syndrome (McGeer et al., 1988a). Their phagocytic function was easily demonstrated by melanin being observed within the HLA-DR positive microglia of the SN (McGeer et al., 1988b). Each of these diseases has a differing etiology, so the activated microglial response had to be the consequence of initiating factors in each condition and not the fundamental cause.

At this same time, the laboratory of Kreutzberg in Germany was carrying out work of a more fundamental nature. His team was utilizing the facial nerve axotomy model to examine the CNS reaction to a sterile lesion outside of the CNS. They found activated microglia enveloping damaged neurons of facial nerve cell bodies, with phagocytosis of dead cells taking place. They concluded that microglia might function as antigen presenting cells and thus be the effector cells responsible for recruitment of lymphocytes to the brain resulting in an inflammatory reaction. A review of their results appeared in the first volume of *Glia* (Streit et al., 1988) and a later review (Kreutzberg, 1996).

Further groundwork was laid by four key papers which appeared in a special issue of *Glia* in 1993 devoted to microglia. They were by Ling and Wong (1993) describing the origin and nature of microglia; Dickson et al. (1993) detailing cytokines and microglia in Alzheimer disease and AIDS; Banati et al. (1993)

on the cytotoxicity of microglia; and McGeer et al. (1993) on microglia in neurodegenerative diseases generally.

Since our laboratory and that of Joe Rogers, who had founded the Sun Health Research Institute, had identified HLA-DR activated microglia in AD, we joined forces for further investigation of the phenomenon, particularly the role of complement. The name complement was introduced by Paul Ehrlich in the 1890's to explain the heat labile factor in serum which helped antibodies to kill microorganisms. As a result, complement was considered to be a peripherally generated system for assisting the activity of antibodies. It was therefore believed that complement factors would only be found as an accompaniment to immunoglobulin antibodies.

Eikelenboom and Stam (1982) were the first to report the presence of complement factors in AD senile plaques. Previously it had been reported that immunoglobulins were associated with amyloid deposits, which was consistent with prevailing theory (Ishii and Haga, 1975). Our laboratory was able to detect the opsonizing components of the classical complement pathway in association with plaques, and the membrane attack complex in association with dystrophic neurites, consistent with bystander lysis occurring in AD (McGeer et al., 1989).

Working with Joseph Rogers, we were unable to detect immunoglobulins in AD tissue using a host of antibodies. Then Rogers explored the idea that beta amyloid protein itself might be an activator of complement. In a classic paper he and his colleagues demonstrated that immunoglobulin immunostaining did not colocalize with complement, and that beta amyloid protein and its N-terminal fragments bound to C1q directly, thus initiating the complement cascade independently of antibodies (Rogers et al., 1992). But what was the source of the complement proteins? It was soon determined from RNA studies that brain itself was the source (Johnson et al., 1992; Walker and McGeer, 1992). Meanwhile evolutionary studies were underway, establishing that the complement system could be traced back at least as far as horseshoe crabs and that it was the mainstay of innate immunity in most primitive organisms (Zhu et al., 2005). It far predated the antibody producing adaptive immune system which is an invention of higher vertebrates.

Two principles which had broad implications for the developing field of neuroinflammation emerged from this joint endeavor. The first was establishing that complement was part of the innate immune system and further that it had the potential of exacerbating the pathology through formation of the membrane attack complex. The second was showing that the inflammatory reaction in AD did exacerbate the pathology. We decided to explore whether patients on long term anti-inflammatory therapy were relatively spared from AD. We selected rheumatoid arthritic patients because onset of the disease typically appears at an earlier age than AD and involves aggressive anti-inflammatory therapy. The results showed an estimated sixfold sparing of AD in rheumatoid arthritic patients compared with age matched general populations (McGeer et al., 1990). Many subsequent epidemiological studies, in which consumption of NSAIDs in particular were targeted, confirmed these general findings (McGeer and McGeer, 1995).

Rogers was then motivated to establish a Neuroinflammation Working Group of 37 investigators to assemble the rapidly

accumulating data on inflammation in AD. The conclusion of the group was that the data represented "a virtual textbook of inflammatory mediators." Included were complement proteins and their regulators, inflammatory cytokines, chemokines, acute phase reactants, prostaglandins, proteases, protease inhibitors, coagulation factors, fibrinolytic factors, integrins, anaphylatoxins, free radical generators, and other unidentified neurotoxins (Akiyama et al., 2000). All are products of the innate immune system of brain, with major contributions coming from neurons, astrocytes, microglia, and endothelial cells.

Parkinson disease was another chronic degenerative disorder where activated microglia were detected in association with the SN lesions (McGeer et al., 1988b). An accidental model of PD provided new insights into the consequences of chronic neuroinflammation. Langston et al. (1984) had identified a group of young drug users who suddenly developed a parkinsonian syndrome. The causative agent (MPP+) was a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant in the street drug they were using. The condition was progressive, and autopsy studies on those who had died from this exposure years previously showed neuroinflammation of the SN similar to that observed in PD (Langston et al., 1999). We found a parallel situation in monkeys. They showed nigral degeneration and activated microglia in the SN 5.5–15 years after systemic exposure to MPTP (McGeer et al., 2003). These findings represent the clearest example of how neuroinflammation, once initiated, can persist, and cause continuing neurodegeneration.

Activated microglia are the pivotal cells. Their functioning *in vivo* has been remarkably demonstrated by the movies of Nimmerjahn et al. (2005). They developed mice transgenic for a green fluorescent protein in microglial cells and used two-photon microscopy through a window in the skull to observe their behavior. Microglial cells in the normal state were found not to be dormant, as implied by their traditional designation as resting, but were extremely active, continuously extending, and retracting their processes to sense their environment. When activated by a laser lesion of a capillary, they surrounded the lesion and phagocytosed the leaking blood.

The innate immune system is the body's first line of defense. As the Nimmerjahn movies demonstrate, it can act immediately. As shown in chronic degenerative diseases, as well as the MPTP model, it can maintain its activity indefinitely without significant engagement of the adaptive immune system. The adaptive immune system is slower to react but more powerful and specific in attacking targets. It depends upon appropriate presentation of epitopes to lymphatic organs so that lymphocytes can be cloned to attack targets where that epitope is exposed. There is a long list of diseases where the adaptive immune system directs self attack on healthy tissues. These conditions are known as autoimmune disorders. They differ from AD and other chronic neurodegenerative disorders where the adaptive immune system does not become significantly engaged. To distinguish between the two, we have suggested that such diseases be described as autotoxic disorders (McGeer and McGeer, 2000).

In theory, the self destruction in autotoxic disorders should be milder and more amenable to therapeutic intervention than the self destruction in autoimmune disorders. The question is how to

ameliorate the autotoxic response? Anti-inflammatory and anti-oxidant approaches have been the most widely utilized to date. But another, and potentially more effective method may be possible. That is to transform microglia from the attack mode, which has been so well characterized, to a healing mode. Such a transformation might result in enhanced phagocytotic activity, coupled with a switch from expressing inflammatory cytokines such as IL-1 and TNF to expressing anti-inflammatory cytokines such as IL-4 and IL-10. In the process the beneficial effects of phagocytosis might be enhanced. For example, suppressing the CD-40/CD 40L interaction in transgenic mice enhances the phagocytic activity of microglia and increase A β clearance (Tan et al., 2002). Clearly there is much still to be learned. It can be said that, despite the huge expansion of activity that has taken place in recent years, it is still a field in its infancy.

SUMMARY

Our understanding of the innate immune system in brain commenced with recognition of a single marker, HLA-DR, on a single cell type, microglia, in a single disorder, Alzheimer disease. Twenty

five years later, more than a thousand innate immune system markers have been identified which are associated with neurons, astrocytes, oligodendrocytes, and endothelial cells, as well as microglia.

They include, but are not limited to, complement proteins and their regulators, cytokines, chemokines, acute phase reactants, prostaglandins, proteases, protease inhibitors, coagulation factors, fibrinolytic factors, anaphylatoxins, integrins, and free radical generators. They are found in a spectrum of neurological diseases. Some stimulate inflammation, others inhibit it. Shifting the balance from a mode of attack to one of healing holds promise of having significant therapeutic benefit in a spectrum of degenerative diseases. Clearly there is much still to be learned. It can be said that, despite the huge expansion of activity that has taken place in the last 25 years, it is still a field in its infancy.

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REFERENCES

- Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., Cooper, N. R., Eikelenboom, P., Emmerling, M., Fiebich, B. L., Finch, C. E., Frautschi, S., Griffin, W. S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I. R., McGeer, P. L., O'Banion, M. K., Pachter, J., Pasinetti, G., Plata-Salamon, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F. L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G., Wyss-Coray, T., and Neuroinflammation Working Group. (2000). Inflammation and Alzheimer's disease. *Neurobiol. Aging* 21, 383–421.
- Aschoff, L. (1924). Das reticuloendothelial system. *Ergeb. Inn. Med. Kinderheilkd.* 26, 1.
- Ball, M. J. (1982). Limbic predilection in Alzheimer dementia: is reactivated herpes virus involved. *Can. J. Neurol. Sci.* 9, 303–306.
- Banati, R. B., Gehrmann, J., Schubert, P., and Kreutzberg, G. W. (1993). Cytotoxicity of microglia. *Glia* 7, 111–118.
- Del Rio Hortega, P. (1919). El tercer elemento de los centros nerviosos. *Bol. Soc. Esp. Biol.* 9, 69–129.
- deTribolet, N., Hamou, M. F., Mach, J.-P., Carrel, S., and Schreyer, M. (1984). Demonstration of HLA-DR antigens in normal human brain. *J. Neurol. Neurosurg. Psychiatr.* 47, 417–418.
- Dickson, D. W., Lee, S. C., Mattiace, L. A., Yen, S. H. C., and Brosnan, C. (1993). Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. *Glia* 7, 75–83.
- Eikelenboom, P., and Stam, F. C. (1982). Immunoglobulins and complement factors in senile plaques. *Acta Neuropathol.* 57, 239–242.
- Fujita, S. (1965). An autoradiographic study on the origin and fate of the sub-pial glioblast in the embryonic chick spinal cord. *J. Comp. Neurol.* 124, 51–59.
- Fujita, S. (1973). Genesis of glioblasts in the human spinal cord as revealed by Feulgen cytophotometry. *J. Comp. Neurol.* 151, 25.
- Fujita, S., and Kitamura, T. (1973). Origin of brain macrophages and the nature of so-called microglia. *Acta Neuropathol. Suppl.* 6, 291–296.
- Haymaker, W., and Adams, R. D. (1980a). *Histology and Histopathology of the Nervous System*. Springfield, IL: C. C. Thomas, 484–485.
- Haymaker, W., and Adams, R. D. (1980b). *Histology and Histopathology of the Nervous System*, Chapter VI. Springfield, IL: C. C. Thomas, 481–559.
- Ishii, T., and Haga, S. (1975). Identification of components of immunoglobulins in senile plaques by means of fluorescent antibody technique. *Acta Neuropathol.* 32, 157–162.
- Johnson, S. A., Lampert-Etchells, M., Pasinetti, G. M., Rozovsky, I., and Finch, C. E. (1992). Complement mRNA in the mammalian brain: responses to Alzheimer's disease and experimental brain lesioning. *Neurobiol. Aging* 13, 641–648.
- Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19, 312–318.
- Lampson, L. A., and Hickey, W. F. (1986). Monoclonal antibody analysis of MHC expression in human brain biopsies: tissue ranging from "histologically normal" to that showing different levels of glial tumor involvement. *J. Immunol.* 136, 4054–4062.
- Langston, J. W., Forno, L. S., and Tetrud, J., Reeves, A. G., Kaplan, J. A., and Karluk, D. (1999). Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. *Ann. Neurol.* 46, 598–605.
- Langston, J. W., Irwin, I., Langston, E. B., and Forno, L. S. (1984). 1-Methyl-4-phenylpyridium ion (MPP $^{+}$): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. *Neurosci. Lett.* 48, 87–92.
- Ling, E. A., and Wong, W. C. (1993). The origin and nature of ramified and ameboid microglia – a historical review and current concepts. *Glia* 7, 9–18.
- Luber-Narod, J., and Rogers, J. (1988). Immune system associated antigens expressed by cells of the human central nervous system. *Neurosci. Lett.* 94, 17–22.
- McGeer, P. L., Akiyama, H., Itagaki, S., and McGeer, E. (1989). Activation of the classical complement pathway in brain tissue of Alzheimer patients. *Neurosci. Lett.* 107, 341–346.
- McGeer, P. L., Akiyama, H., Itagaki, S., and McGeer, E. (1988a). Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. *Acta Neuropathol.* 76, 550–557.
- McGeer, P. L., Itagaki, S., and McGeer, E. G. (1988b). Rate of cell death in parkinsonism indicates active neuropathological process. *Ann. Neurol.* 24, 574–576.
- McGeer, P. L., Itagaki, S., Tago, H., and McGeer, E. G. (1987). Reactive microglia in patients with senile dementia of the Alzheimer types are positive for the histocompatibility glycoprotein HLA-DR. *Neurosci. Lett.* 79, 195–200.
- McGeer, P. L., Kawamata, T., Walker, D. G., Akiyama, H., Tooyoma, I., and McGeer, E. G. (1993). Microglia in degenerative neurological disease. *Glia* 7, 84–92.
- McGeer, P. L., and McGeer, E. G. (1995). The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative disorders. *Brain Res. Rev.* 21, 195–218.
- McGeer, P. L., and McGeer, E. G. (2000). Autotoxicity and Alzheimer disease. *Arch. Neurol.* 57, 789–790.
- McGeer, P. L., Rogers, J., McGeer, E. G., and Sibley, J. (1990). Does anti-inflammatory treatment protect against Alzheimer disease? *Lancet* 335, 1037.
- McGeer, P. L., Schwab, C., Parent, A., and Doudet, D. (2003). Presence of reactive microglia in monkey substantia nigra years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration. *Ann. Neurol.* 54, 599–604.
- Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308, 1314–1318.

- Oemichan, M., Wietholter, H., and Greaves, M. F. (1979). Immunological analysis of human microglia: lack of monocytic and lymphoid membrane differentiation antigens. *J. Neuropathol. Exp. Neurol.* 38, 99–103.
- Penfield, W. (1925). Microglia and the process of phagocytosis in gliomas. *Am. J. Pathol.* 1, 77–89.
- Prados, M., and Gibson, W. C. (1946). *Pio del Rio Hortega*, 1882–1945. *J. Neurosurg.* 3, 275–284.
- Rogers, J., Cooper, N. R., Webster, S., Schultz, J., McGeer, P. L., Styren, S. D., Civin, W. H., Brachova, L., Bradt, B., Ward, P., and Lieberburg, I. (1992). Complement activation by β -amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10016–10020.
- Rogers, J., Luber-Narod, J., Sturen, C. D., and Civin, W. H. (1988). Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol. Aging* 9, 339–349.
- Streit, W. J., Graeber, M. B., and Kreutzberg, G. W. (1988). Functional plasticity of microglia: a review. *Glia* 1, 301–307.
- Tan, J., Town, T., Crawford, F., Mori, T., DelleDonne, A., Crescentini, R., Obregon, D., Flavelli, R. A., and Mullan, M. J. (2002). Role of CD40 ligand in amyloidosis in transgenic Alzheimer's mice. *Nat. Neurosci.* 5, 1288–1293.
- Van Furth, R., and Cohn, Z. A. (1968). The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* 128, 415–435.
- Walker, D. G., and McGeer, P. L. (1992). Complement gene expression in human brains: comparison between normal and Alzheimer disease cases. *Brain Res. Mol. Brain Res.* 14, 109–116.
- Walker, D. G., O'Kusky, J. R., and McGeer, P. L. (1989). In situ hybridization analysis for herpes simplex virus nucleic acids in Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* 3, 123–131.
- Wood, G. W., Gollahan, K. A., Tilzer, S. A., Vats, T., and Morantz, R. A. (1979). The failure of microglia in normal brain to exhibit mononuclear phagocyte markers. *J. Neuropathol. Exp. Neurol.* 38, 369–376.
- Zhu, Y., Thangamani, S., and Ho, B. And Ding, J. L. (2005). The ancient origin of the complement system. *EMBO J.* 24, 382–394.

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The ketogenic diet as a treatment paradigm for diverse neurological disorders

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Dietary and metabolic therapies have been attempted in a wide variety of neurological diseases, including epilepsy, headache, neurotrauma, Alzheimer disease, Parkinson disease, sleep disorders, brain cancer, autism, pain, and multiple sclerosis. The impetus for using various diets to treat – or at least ameliorate symptoms of – these disorders stems from both a lack of effectiveness of pharmacological therapies, and also the intrinsic appeal of implementing a more “natural” treatment. The enormous spectrum of pathophysiological mechanisms underlying the aforementioned diseases would suggest a degree of complexity that cannot be impacted universally by any single dietary treatment. Yet, it is conceivable that alterations in certain dietary constituents could affect the course and impact the outcome of these brain disorders. Further, it is possible that a final common neurometabolic pathway might be influenced by a variety of dietary interventions. The most notable example of a dietary treatment with proven efficacy against a neurological condition is the high-fat, low-carbohydrate ketogenic diet (KD) used in patients with medically intractable epilepsy. While the mechanisms through which the KD works remain unclear, there is now compelling evidence that its efficacy is likely related to the normalization of aberrant energy metabolism. The concept that many neurological conditions are linked pathophysiological to energy dysregulation could well provide a common research and experimental therapeutics platform, from which the course of several neurological diseases could be favorably influenced by dietary means. Here we provide an overview of studies using the KD in a wide panoply of neurologic disorders in which neuroprotection is an essential component.

Keywords: **ketogenic diet, neuroplasticity, epilepsy, neurological disorders**

INTRODUCTION

The ketogenic diet (KD) is now a proven therapy for drug-resistant epilepsy (Vining et al., 1998; Neal et al., 2008), and while the mechanisms underlying its anticonvulsant effects remain incompletely understood (Hartman et al., 2007; Bough and Stafstrom, 2010; Rho and Stafstrom, 2011), there is mounting experimental evidence for its broad neuroprotective properties and in turn, emerging data supporting its use in multiple neurological disease states (Baranano and Hartman, 2008). Even in patients with medically refractory epilepsy who have remained seizure-free on the KD for 2 years or more, it is not uncommon for clinicians to observe that both anticonvulsant medications and the diet can be successfully discontinued without recrudescence of seizures (Freeman et al., 2007). This intriguing clinical observation forms the basis of the hypothesis that the KD may possess anti-epileptogenic properties.

This review article explores the rationale for using the KD and related dietary treatments in neurological disorders outside of epilepsy, and summarizes the clinical experience to date. An underlying theme of such diet-based therapies is that nutrients and metabolic substrates can exert profound effects on neuronal plasticity, modifying neural circuits and cellular properties to enhance

and normalize function. At a fundamental level, any disease in which the pathogenesis is influenced by abnormalities in cellular energy utilization – and this implies almost every known condition – would theoretically be amenable to the KD. It is important to acknowledge that much of the data discussed here are preliminary and anecdotal, and hence need to be validated by well-controlled prospective studies. Nevertheless, that diet and nutrition should influence brain function should not be altogether surprising, and there are already abundant clinical and laboratory data linking defects in energy metabolism to a wide variety of disease states (Waldbauern and Patel, 2010; Roth et al., 2011; Schiff et al., 2011). Thus, the potential for interesting and novel applications of the KD and related dietary therapies is almost limitless (Stafstrom, 2004).

NEUROPROTECTIVE ROLE OF THE KD

Over the past decade, investigators have identified numerous mechanisms through which the KD may provide neuroprotective activity. While a comprehensive discussion of such mechanisms is beyond the scope of this chapter, a brief discussion is warranted as such actions are intimately related to disorders that share the common feature of progressive neurodegeneration and/or cellular

bioenergetic dysfunction. The reader is referred to recent reviews for more details on this subject (Gasior et al., 2006; Acharya et al., 2008; Masino and Geiger, 2008).

Two hallmark features of KD treatment are the rise in ketone body production by the liver and a reduction in blood glucose levels. The elevation of ketones is largely a consequence of fatty acid oxidation. Specific polyunsaturated fatty acids (PUFAs) such as arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid, might themselves regulate neuronal membrane excitability by blocking voltage-gated sodium and calcium channels (Voskuyl and Vreugdenhil, 2001), reducing inflammation through activation of peroxisome proliferator-activated receptors (PPARs; Cullingford, 2008; Jeong et al., 2011), or inducing expression of mitochondrial uncoupling proteins which reduce reactive oxygen species (ROS) production (Bough et al., 2006; Kim do and Rho, 2008). Ketone bodies themselves have been shown to possess neuroprotective properties, by raising ATP levels and reducing ROS production through enhanced NADH oxidation and inhibition of mitochondrial permeability transition (mPT; Kim do et al., 2007). Along similar lines of improved bioenergetics, the KD has been shown to stimulate mitochondrial biogenesis, resulting in stabilized synaptic function (Bough et al., 2006).

The second major biochemical feature of the KD is the decrease in glycolytic flux. Reduction of glycolysis is an essential feature of calorie restriction, which has been shown to suppress seizures (Greene et al., 2001) as well as prolong the lifespan of numerous species, including primates (Kemnitz, 2011; Redman and Ravussin, 2011). While the link between calorie restriction and KD mechanisms remain controversial (Yamada, 2008; Maalouf et al., 2009), it is clear that both treatments result in reduction of blood glucose, likely involving reduced glycolytic flux. In that regard, 2-deoxy-D-glucose (2DG), an analog of glucose that blocks phosphoglucose isomerase and hence inhibits glycolysis, has been shown to block epileptogenesis in the rat kindling model by decreasing the expression of brain-derived neurotrophic factor (BDNF) and its principal receptor, tyrosine kinase B (TrkB; Garriga-Canut et al., 2006). Several other important mechanisms contribute to the neuroprotective consequences of calorie restriction, including improved mitochondrial function and decreased oxidative stress (similar to that seen with ketones and PUFAs), decreased activity of pro-apoptotic factors, and inhibition of inflammatory mediators such as interleukins and tumor necrosis factor alpha (TNF α ; Maalouf et al., 2009).

In the end, there are likely many other mechanisms that could contribute to the neuroprotective properties of the KD. Many of these mechanisms are thought to relate principally to the KD's anticonvulsant effects, but some if not all of them could contribute to cellular homeostasis and preventing neuronal injury or dysfunction. An important caveat, however, is that yet unidentified mechanisms may operate in diseases outside of epilepsy, and this possibility presents further opportunities for examining the pleiotropic effects of this metabolism-based therapy at a mechanistic level.

THE KD IN EPILEPSY

There is no longer any doubt that the KD is effective in ameliorating seizures in patients, especially children, with medically

refractory epilepsy (Vining, 1999; Neal et al., 2008; Freeman et al., 2009). After its introduction in 1920, the KD was used as a first or second-line treatment for severe childhood epilepsy. With the introduction of anticonvulsant medications in convenient pill form, the use of the KD waned, only to resurge later in the early 1990s, due largely to the efforts of concerned parents who brought the diet back to greater public awareness (Wheless, 2008). Recent years have witnessed a remarkable surge in research on the KD, including basic science efforts as well as clinical protocols and trials (Kim do and Rho, 2008; Neal et al., 2008; Kessler et al., 2011). The KD has now become an integral part of the armamentarium of most major epilepsy centers throughout the world (Kossoff and McGrogan, 2005).

THE KD IN AGING

Aging involves the gradual decrease in function, and at times outright degeneration, of neurons and neural circuits. It is possible that by altering energy metabolism with the KD, rates of degeneration of certain neural structures and functions might be slowed (Balietti et al., 2010a). However, KDs may induce differential morphological effects in structures such as the hippocampus, perhaps as a consequence of region-specific neuronal vulnerability during the late aging process (Balietti et al., 2008). Specifically, it has been shown that the medium-chain triglyceride (MCT) form of the KD may induce detrimental synaptic changes in CA1 stratum moleculare, but beneficial effects in the outer molecular layer of the dentate gyrus (Balietti et al., 2008). In MCT-fed aged rats compared to aged rats receiving a normal diet, mitochondrial density and function in cerebellar Purkinje cells were significantly increased, suggesting that the KD can rescue age-related mitochondrial dysfunction (Balietti et al., 2010b). These observations imply certain risks, but also potential benefits of the KD for the aging brain. However, the fact that the KD reduces oxidative stress and its downstream consequences provides a reasonable rationale for considering this type of treatment to retard the adverse consequences during aging (Freemantle et al., 2009). As an example, T-maze and object recognition performance were improved in aged rats by KD administration, suggesting a potential functional benefit in cognition (Xu et al., 2010). Finally, it should be noted that because of its similarities to calorie restriction (as noted above), the KD is likely to involve other neuroprotective mechanisms that could ameliorate pathological aging – especially when occurring in the context of neurodegeneration (Contestabile, 2009).

THE KD IN ALZHEIMER DISEASE

There is growing realization that neuronal excitability is enhanced in patients with Alzheimer disease (AD; Noebels, 2011; Roberston et al., 2011). While the essential pathological processes of AD involves neuronal degeneration with accumulation of abnormal cellular products such as fibrillary plaques and tangles, recent evidence points to alterations in the function of extant neural circuits and mitochondrial homeostasis (Kapogiannis and Mattson, 2011). This view is bolstered by the higher incidence of seizures in patients with AD as compared to the unaffected population (Palop and Mucke, 2009). Therefore, there is a rationale for hypothesizing that the KD might have a beneficial role in patients with

AD (Baietti et al., 2010a), in addition to the potential benefits to the aging process as noted above. One should note, importantly, that if ketone bodies are indeed the primary mediators that counter aging and neurodegeneration in AD, implementation of the KD should be tempered by known age-related differences in the production and extraction of ketones (i.e., this is more efficient in young animals), as well as age-specific regional differences in ketone utilization within the brain (Nehlig, 1999).

Clinical studies to date have been equivocal but promising. A randomized double-blind, placebo-controlled trial of a MCT KD resulted in significantly improved cognitive functioning in APOe4-negative patients with AD but not in patients with a APOe4 mutation (Henderson et al., 2009). In this study, the primary cognitive end-points measured were the mean change from baseline in the AD Assessment Scale-Cognitive subscale, and global scores in the AD Cooperative Study—Clinical Global Impression of Change (Henderson et al., 2009). This significant clinical improvement was considered to be secondary to improved mitochondrial function, since ketone bodies (specifically, beta-hydroxybutyrate or BHB) have been shown to protect against the toxic effects of β -amyloid on neurons in culture (Kashiwaya et al., 2000). Alternatively, the KD may actually decrease amounts of β -amyloid deposition (VanderAuwera et al., 2005). Interestingly, other diets such as the Mediterranean diet are showing some promise in AD (Gu et al., 2010), possibly through a reduction in systemic inflammation and improved metabolic profiles.

Recent studies have shown a closer linkage of AD to epilepsy. For example, animal models of AD exhibit neuronal hyperexcitability and enhanced propensity to seizures (Palop et al., 2007; Roberson et al., 2011); these models may ultimately allow for detailed analyses of both cognitive and anticonvulsant effects of the KD or other dietary manipulations such as calorie restriction. Transgenic AD mice fed 2DG demonstrated better mitochondrial function, less oxidative stress, and reduced expression of amyloid precursor protein and β -amyloid compared to control animals (Yao et al., 2011).

Another pathophysiological mechanism hypothesized to operate in AD ties together altered mitochondrial function and glucose metabolism, i.e., accumulation of advanced glycation endproducts (AGE; Srikanth et al., 2011). AGE accumulation is a process of normal aging that is accelerated in AD; proteins are non-enzymatically glycosylated and this cross-linking of proteins accentuates their dysfunction. One proposed mechanism is increased ROS and free radical formation, which, as discussed above, hampers mitochondrial function. The intriguing possibility that AGE inhibitors (e.g., aminoguanidine, tenilsetam, carnosine) could act in concert with the KD or antioxidants in retarding AD progression remains speculative at this time.

Thus, there is growing evidence that the KD may be an effective treatment for AD through a variety of metabolism-induced mechanisms that reduce oxidative stress and neuroinflammation, and enhance bioenergetic profiles – largely through enhanced mitochondrial functioning. However, caution should be exercised in extrapolating findings in animals to humans, as discrepancies in terms of both clinical efficacy and untoward side-effects have been noted. For example, adverse reactions to calorie restriction have been reported in some rodent models (Maalouf et al., 2009),

and in hippocampus, abnormal morphological synaptic changes have been observed in CA1 stratum moleculare (Baietti et al., 2008).

THE KD IN PARKINSON DISEASE

The primary pathophysiology in Parkinson disease (PD) is excitotoxic degeneration of dopaminergic neurons in the substantia nigra, leading to abnormalities of movement, and to an increasing extent, in cognition and other cortical functions. How could the KD benefit patients with PD? Based on the recognition that ketone bodies may bypass defects in mitochondrial complex I activity that have been implicated in PD, a small clinical study demonstrated that 5 of 7 affected patients showed improved scores on a standard PD rating scale (Vanitallie et al., 2005); however, given the small sample size, a placebo effect cannot be ruled out. In animal models of PD produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), BHB administration ameliorated the mitochondrial respiratory chain damage that ordinarily results from that toxin (Kashiwaya et al., 2000). Additional evidence supporting the potential benefits of ketone bodies in PD is provided by *in vitro* experiments demonstrating the protective effects of these substrates against mitochondrial respiratory chain dysfunction induced exogenously by complex I and II inhibitors rotenone and 3-nitropropionic acid, respectively (Kim do et al., 2010), and even anti-inflammatory actions of the KD on MPTP-induced neurotoxicity (Yang and Cheng, 2010). It would be of interest to determine whether commercially available treatments that augment ketonemia – e.g., the MCT-based formulation used in a recent Alzheimer's clinical trial (Henderson et al., 2009) – might benefit patients with PD.

THE KD IN AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive disease due to degeneration of motor neurons of the cortex and anterior horn of the spinal cord. As a consequence, voluntary motor activity gradually deteriorates, leaving the affected individual profoundly weak despite largely retained cognitive functioning. The essential pathophysiological mechanisms that underlie this relentless disorder are yet to be fully elucidated, but similar to other neurodegenerative disorders, the involvement of energy-producing systems likely play a role and mitochondrial dysfunction probably contributes to disease pathogenesis. In this regard, the KD may be a promising adjunctive treatment for this devastating disease (Siva, 2006), as evidenced in a mouse model of ALS, produced by knocking out the gene encoding the copper/zinc superoxide dismutase SOD1-G93A, causing progressive muscle weakness and death by respiratory failure. Administration of a KD to these mutant mice led to both histological (higher motor neuron counts) and functional improvements (preserved motor function on the rotarod test) compared to non-KD fed animals (Zhao et al., 2006). However, the KD did not extend survival time compared to non-KD fed control mice. Mitochondria from these mutant mice demonstrated increased ATP synthesis, countering the inhibition of complex I of the electron transport chain. It is important to note that approximately 20% of the familial cases of ALS have SOD1 mutations, and hence the possibility arises that the KD may be of benefit to patients with ALS.

One potentially important consideration in this regard – applicable to all neurodegenerative diseases – is determining whether timing of intervention is crucial for a protective effect by KD treatment. Neurological disorders in late stages of progression may have such extreme neuronal dysfunction and death to allow a “refueling” with metabolic substrates to help recover integrity and function. Certainly, this appears to be the case in a small pilot study of KD treatment in patients with Lafora body disease (Cardinali et al., 2006).

THE KD IN CANCER

Cells that exhibit the most active metabolic rates (i.e., cancer cells) are most sensitive to the lack of metabolic energy to fuel their activity, a well-recognized biochemical phenomenon known as the Warburg effect. Theoretically, depriving rapidly dividing, highly metabolic cancer cells of their usual fuel supply, e.g., glucose (by use of the KD or 2DG), could be clinically therapeutic (Aft et al., 2002; Pelicano et al., 2006; Otto et al., 2008). Despite this well documented cellular observation, the KD has only recently been considered as a clinical treatment in the oncology field.

Pioneering work by Seyfried et al. (2011) over the past decade has shown that animals with experimentally produced brain tumors placed on a KD exhibit markedly decreased tumor growth rates, and these remarkable effects appear to be a consequence of calorie restriction (i.e., reduced blood glucose levels) rather than KD-induced ketosis (i.e., fatty acid oxidation) as the principal mechanism. Other investigators have found similar effects of the KD in animal models. One group found that the KD reduces ROS production in malignant glioma cells, and gene microarray expression profiling demonstrated that the KD induces an overall reversion to patterns seen in non-tumor specimens and a reduction in the expression of genes encoding signal transduction pathways and growth factors known to be involved in glioma growth (Stafford et al., 2010). It is also interesting to note that PPAR α -activated by nutrients such as fatty acids – is now a target for developing anti-cancer drugs that target mitochondrial metabolism (Grabacka et al., 2010).

While clinical validation of this phenomenon is not yet forthcoming, there are several case reports suggesting that the KD may be efficacious in humans with brain tumors. Nebeling et al. (1995) reported beneficial effects of an MCT-based diet in two pediatric patients with advanced stage malignant astrocytomas. More recently, Zuccoli et al. (2010) described a case study of an elderly woman with glioblastoma multiforme who was treated with standard radiotherapy plus concomitant temozolomide therapy together with a calorie-restricted KD, and a complete absence of brain tumor tissue was noted on FDT-PET and MRI imaging after 2 months of treatment – results the authors attributed in part to the adjunctive dietary treatment. Further, in a pilot trial of the KD in 16 patients with advanced metastatic tumors, six individuals reported improved emotional functioning and less insomnia, indicating that in some instances, the KD may lead to improved quality of life (Schmidt et al., 2011). In contrast, a retrospective examination of five patients with tuberous sclerosis complex treated with the KD indicated either a lack of tumor suppression or further tumor growth (Chu-Shore et al., 2010). Thus, it may be that distinct tumor types within different organ systems may respond

differently to the KD or other dietary treatments and that such differences may reflect variations in the metabolic vulnerability of specific tumor types, perhaps through intrinsic differences in the expression of metabolism-related genes (Stafford et al., 2010).

THE KD IN STROKE

To date, no clinical trials of the KD have been performed in patients with stroke, but several animal studies of hypoxia-ischemia support the potential beneficial effect of the diet. Most of these models entail pre-treatment with the KD (or with BHB), resulting in decreased structural and functional damage from the stroke. For example, Tai et al. (2008) utilized a cardiac arrest model in rats and found significantly reduced Fluoro-Jade staining in animals that underwent 25 days of pre-treatment with the KD. These investigators later determined that these effects were not due to involvement of plasmalemmal ATP-sensitive potassium channels (Tai et al., 2009), which have been implicated in ketone body action (Ma et al., 2007). Other researchers have hypothesized that the neuroprotective properties of ketone bodies might be related to up-regulation of hypoxia inducible factor (HIF1- α) which is important in angiogenesis and anti-apoptotic activity (Puchowicz et al., 2008). In that study, pre-treatment with BHB (via intraventricular infusion, followed by middle cerebral artery occlusion) led to significant increases in brain succinate content, as well as elevations in HIF1- α and Bcl-2, an anti-apoptotic protein. To be clinically meaningful, of course, a positive effect must be demonstrable after, and not before, an ischemic event. Nevertheless, such studies imply that biochemical alterations that favor energy metabolism would be protective against acute forms of severe brain injury.

THE KD IN MITOCHONDRIAL DISORDERS

As mentioned above, given the growing evidence that the KD enhances mitochondrial functioning and biogenesis (Bough et al., 2006; Maalouf et al., 2009; Kim do et al., 2010), it is logical to ask whether patients with known mitochondrial cytopathies might derive a benefit from the KD and/or ketone bodies such as BHB. At the same time, it must be considered that inherent mitochondrial dysfunction might predispose individuals to adverse toxicities from high fatty acid loads that could overwhelm β -oxidation within the mitochondrial matrix. Experimental data described above attest to significant improvements in mitochondrial function, and many lines of evidence point to the rationale of therapeutically targeting mitochondrial bioenergetics for other disease states (Wallace et al., 2010), but is there any clinical evidence in patients with intrinsic mitochondrial disorders? Kang et al. (2007) reported that the KD was both safe and effective in 14 pediatric patients with established mitochondrial defects in complexes I, II, and IV, all of whom had medically intractable epilepsy. These authors observed that half of these patients became seizure-free on the KD, and only four patients failed to respond. Hence, these preliminary data suggest that the KD is not necessarily contraindicated in patients with mitochondrial respiratory chain abnormalities. However, KD treatment is not recommended in individuals with primary carnitine deficiencies [including mutations in carnitine palmitoyl transferase (CPT) I or II and mitochondrial translocase] and fatty acid β -oxidation abnormalities (e.g., medium-chain acyl dehydrogenase deficiency; Kossoff et al.,

2009). Thus, it is critical to determine the specific mitochondrial defect when considering treatment with the KD, to avert clinical deterioration.

THE KD IN BRAIN TRAUMA

Unfortunately, the incidence of brain injury is increasing in both civilian and military contexts. Brain injury, either due to a penetrating injury or to blunt/blast trauma, can lead to severe cognitive and motor consequences. Further, the occurrence of epilepsy months to years following brain trauma adds to the morbidity of affected individuals, and speaks to the emergence of hyperexcitable neuronal circuits over time. Hence, in light of the clinical problem of post-traumatic epileptogenesis and the fact that the KD can reduce seizure activity, the notion has emerged that dietary therapy might ameliorate brain injury and possibly, long-term consequences such as epilepsy.

Several recent animal studies support this idea, and investigators have principally focused on ketone bodies (Prins, 2008a). Using a controlled cortical impact (CCI) injury model, Prins et al. (2005) showed that pre-treatment with a KD significantly reduced cortical contusion volume in an age-related manner that correlated with maturation-dependent differences in cerebral metabolism and ketone utilization. Later, they showed that cognitive and motor functioning was also improved with KD treatment (Appelberg et al., 2009). Further, using a weight drop model, Hu et al. (2009) showed that the KD pre-treatment reduced Bcl-2 (also known as Bax) mRNA and protein levels 72 h after trauma, indicating that apoptotic neurodegeneration could be prevented with this diet. Consistent with these observations, it was found that fasting – which shares the key feature of ketosis with the KD – led to significant tissue sparing in brain following CCI injury, and that again ketosis (with improved mitochondrial functioning) rather than the relative hypoglycemia seen with fasting was the important determinant of neuroprotection (Davis et al., 2008).

With respect to anti-epileptogenesis following head injury, the data regarding KD effects are mixed. KD treatment – either before or after fluid percussion injury in rats – did not alter later seizure sensitivity to fluorothyl, even though the degree of hippocampal cell loss was reduced by pre- but not post-treatment (Schwartzkroin et al., 2010). Similarly, in the lithium–pilocarpine model of temporal lobe epilepsy, KD treatment prior to induction led to morphological neuroprotection in the hippocampus but did not affect latency to onset of spontaneous recurrent seizures (Linard et al., 2010). In contrast, Jiang et al. (2012) recently reported that the KD increased after-discharge thresholds and reduced generalized seizure occurrence in a rat amygdala kindling model. Thus, at this juncture, there is no consensus regarding whether the KD is anti-epileptogenic following a variety of traumatic insults and manipulations. However, given the recent finding that the KD inhibits the mammalian target of rapamycin (mTOR) pathway (McDaniel et al., 2011), which has been linked to modulation of epileptogenesis (McDaniel and Wong, 2011), further studies in different animal models are clearly warranted. What is unambiguous, nevertheless, is the age-dependence of the effects of the KD in ameliorating the consequences of head injury (Prins, 2008b; Deng-Bryant et al., 2011).

THE KD IN PSYCHIATRIC DISORDERS (DEPRESSION)

Mood stabilizing properties of the KD have been hypothesized (El-Mallakh and Paskitti, 2001), but no clinical studies have been conducted as of this writing. The potential role of the KD in depression has been studied in the forced choice model of depression in rats, which led to a beneficial effect similar to that afforded by conventional antidepressants (Murphy et al., 2004; Murphy and Burnham, 2006).

THE KD IN AUTISM

Autism is a neurodevelopmental disorder that affects language development and social function. The heterogeneous etiologies leading to autism spectrum disorders, plus the uncertainty about what causes autism in the majority of “idiopathic” cases, has hampered the development of a universally beneficial treatment, aside from symptomatic treatment of autism-related behaviors such as aggression or anxiety. Now, limited clinical evidence raises the intriguing possibility that the KD might be helpful to alleviate some of the abnormal behaviors seen in children with autism spectrum disorders. Using a KD variant consisting of MCT, 10 of 18 autistic children demonstrated moderate or significant behavioral improvement (by a blinded rater) after a 6-month trial of providing the diet for 4 weeks of KD diet treatment alternating with 2 weeks of normal diet, in 6-week cycles (Evangelou et al., 2003). This study was carried out on the island of Crete, where the frequency of autism is high but the possibility of genetic inbreeding is also significant. Therefore, these findings need to be interpreted cautiously and larger longitudinal studies are needed. The potential involvement of adenosine, an endogenous neuromodulator and anticonvulsant, in ameliorating autistic behaviors raises the possibility of overlap with KD mechanisms (Masino et al., 2011). As a caveat, many children with autism poorly tolerate changes in dietary and other routines, which could impact implementation of dietary therapies, which require strict adherence.

THE KD IN MIGRAINE

Migraine is a paroxysmal neurological disorder having considerable clinical phenotypic overlap with epilepsy (Rogawski, 2008). Although the intrinsic mechanisms underlying seizures and migraine attacks differ in many fundamental respects, there are theoretical reasons to consider the KD for chronic migraine. Both disorders involve paroxysmal excitability changes in the brain, and there is considerable overlap in the array of pharmacological agents used to treat these conditions. Although it might seem unlikely that an individual with migraine would undertake such a complicated dietary regimen as the KD, in light of suboptimal alternatives, this choice is worthy of consideration, particularly in the medically refractory population (Maggioni et al., 2011).

Interestingly, the first report of using the KD for migraine came in 1928, only a few years after the diet's first use for epilepsy (Schnabel, 1928). Nine of 28 patients reported “some improvement,” although the validity of this clinical study is uncertain and some patients admitted poor compliance. Compliance might be better with the less restrictive modified Atkins diet, which has also shown promise for migraine treatment (Kossoff et al., 2010). Other case reports exist but there are no large clinical series or trials. Notwithstanding this limitation, laboratory investigations

have found that both short-term and long-term treatment with either MCT or long-chain triglyceride forms of the KD resulted in a significant reduction in the velocity of cortical spreading depression (CSD) velocity in immature rats (de Almeida Rabello Oliveira et al., 2008). Another intriguing aspect of this study was the observation that triheptanoin – an anaplerotic substrate that enhances tricarboxylic acid cycle function – had a notable effect in retarding CSD, consistent with a later report that triheptanoin supplementation raised pentylenetetrazol tonic seizure threshold and delayed the development of corneal kindled seizures (Willis et al., 2010).

SUMMARY

Despite the relative lack of clinical data, there is an emerging literature supporting the broad use of the KD (and its variants)

against a variety of neurological conditions. These preliminary studies are largely based on the fundamental idea that metabolic shifts may lead to neuroprotective actions (Gasior et al., 2006; Maalouf et al., 2009). How can a simple dietary change lead to improvement in disorders with such a huge span of pathophysiological mechanisms? Alterations in energy metabolism appear to be a common theme. So while the mechanisms through which the KD exerts such effects are likely diverse (Maalouf et al., 2009; Rho and Stafstrom, 2011), there may indeed be one or more common final pathways that are mechanistically shared. Ultimately, the details of how that altered metabolism reduces neuronal excitability, abrogates ongoing neurodegeneration, or mitigates functional disability remain unknown. Herein lay rich opportunities for further investigation, in both the laboratory and the clinic, in the broad realm of translational neurosciences.

REFERENCES

- Acharya, M. M., Hattiangady, B., and Shetty, A. K. (2008). Progress in neuroprotective startegies for preventing epilepsy. *Prog. Neurobiol.* 84, 363–404.
- Aft, R. L., Zhang, F. W., and Gius, D. (2002). Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death. *Br. J. Cancer* 87, 805–812.
- Appelberg, K. S., Hovda, D. A., and Prins, M. L. (2009). The effects of a ketogenic diet on behavioral outcome after controlled cortical impact injury in the juvenile and adult rat. *J. Neurotrauma* 26, 497–506.
- Baliotti, M., Casoli, T., DiStefano, G., Giorgetti, B., Aicardi, G., and Fattoretti, P. (2010a). Ketogenic diets: an historical antiepileptic therapy with promising potentialities for the aging brain. *Ageing Res. Rev.* 9, 273–279.
- Baliotti, M., Giorgetti, B., DiStefano, G., Casoli, T., Platano, D., Solazzi, M., Bertoni-Freddari, C., Aicardi, G., Lattanzio, F., and Fattoretti, P. (2010b). A ketogenic diet increases succinic dehydrogenase (SDH) activity and recovers age-related decrease in numeric density of SDH-positive mitochondria in cerebellar Purkinje cells of late-adult rats. *Micron* 41, 143–148.
- Baliotti, M., Giorgetti, B., Fattoretti, P., Grossi, Y., DiStefano, G., Casoli, T., Platano, D., Solazzi, M., Orlando, F., Aicardi, G., and Bertoni-Freddari, C. (2008). Ketogenic diets cause opposing changes in synaptic morphology in CA1 hippocampus and dentate gyrus of late-adult rats. *Rejuvenation Res.* 11, 631–640.
- Baranano, K. M., and Hartman, A. L. (2008). The ketogenic diet: uses in epilepsy and other neurologic illnesses. *Curr. Treat. Options Neurol.* 10, 410–419.
- Bough, K. J., and Stafstrom, C. E. (2010). “The ketogenic diet: scientific principles underlying its use,” in *Epilepsy: Mechanisms, Models, and Translational Perspectives*, eds J. M. Rho, R. Sankar, and C. E. Stafstrom (Boca Raton, FL: CRC Press), 417–439.
- Bough, K. J., Wetherington, J., Hassel, B., Pare, J. F., Gawryluk, J. W., Greene, J. G., Shaw, R., Smith, Y., Geiger, J. D., and Dingledine, R. J. (2006). Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann. Neurol.* 60, 223–235.
- Cardinali, S., Canafoglia, L., Bertoli, S., Franceschetti, S., Lanzi, G., Tagliabue, A., and Veggiotti, P. (2006). A pilot study of a ketogenic diet in patients with Lafora body disease. *Epilepsy Res.* 69, 129–134.
- Chu-Shore, C. J., Major, P., Camposano, S., Muzykewicz, D., and Thiele, E. A. (2010). The natural history of epilepsy in tuberous sclerosis complex. *Epilepsia* 51, 1236–1241.
- Contestabile, A. (2009). Benefits of caloric restriction on brain aging and related pathological states: understanding mechanisms to devise novel therapies. *Curr. Med. Chem.* 16, 350–361.
- Cullingford, T. (2008). Peroxisome proliferator-activated receptor alpha and the ketogenic diet. *Epilepsia* 49(Suppl. 8), 70–72.
- Davis, L. M., Pauly, J. R., Readnower, R. D., Rho, J. M., and Sullivan, P. G. (2008). Fasting is neuroprotective following traumatic brain injury. *J. Neurosci. Res.* 86, 1812–1822.
- de Almeida Rabello Oliveira, M., da Rocha Ataide, T., de Oliveira, S. L., de Melo Lucena, A. L., de Lira, C. E., Soares, A. A., de Almeida, C. B., Ximenes da Silva, A. (2008). Effects of short-term and long-term treatment with medium- and long-chain triglycerides ketogenic diet on cortical spreading depression in young rats. *Neurosci. Lett.* 434, 66–70.
- Deng-Bryant, Y., Prins, M. L., Hovda, D. A., and Harris, N. G. (2011). Ketogenic diet prevents alterations in brain metabolism in young but not adult rats after traumatic brain injury. *J. Neurotrauma* 28, 1813–1825.
- El-Mallah, R. S., and Paskitti, M. E. (2001). The ketogenic diet may have mood-stabilizing properties. *Med. Hypotheses* 57, 724–726.
- Evangelou, A., Vlachonikolis, I., Mihailidou, H., Spilioti, M., Skarpalezou, A., Makaronas, N., Prokopiou, A., Christodoulou, P., Liapi-Adamidou, G., Helidonis, E., Sbyrakis, S., and Smeitink, J. (2003). Application of a ketogenic diet in children with autistic behavior: pilot study. *J. Child Neurol.* 18, 113–118.
- Freeman, J. M., Kossoff, E. H., Freeman, J. B., and Kelly, M. T. (2007). *The Ketogenic Diet – A Treatment for Children and Others with Epilepsy*. New York: Demos Publications.
- Freeman, J. M., Vining, E. P., Kossoff, E. H., Pyzik, P. L., Ye, X., and Goodman, S. N. (2009). A blinded, crossover study of the efficacy of the ketogenic diet. *Epilepsia* 50, 322–323.
- Freemantle, E., Vandal, M., TremblayMercier, J., Plourde, M., Poirier, J., and Cunnane, S. C. (2009). Metabolic response to a ketogenic breakfast in the healthy elderly. *J. Nutr. Health Aging* 13, 293–298.
- Garriga-Canut, M., Schoenike, B., Qazi, R., Bergendahl, K., Daley, T. J., Pfender, R. M., Morrison, J. F., Ockuly, J., Stafstrom, C., Sutula, T., and Roopra, A. (2006). 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat. Neurosci.* 9, 1382–1387.
- Gasior, M., Rogawski, M. A., and Hartman, A. L. (2006). Neuroprotective and disease-modifying effects of the ketogenic diet. *Behav. Pharmacol.* 17, 431–439.
- Grabacka, M., Pierzchalska, M., and Reiss, K. (2010). Peroxisome proliferator activated receptor α ligands as anti-cancer drugs targeting mitochondrial metabolism. *Curr. Pharm. Biotechnol.* PMID: 21133850.
- Greene, A. E., Todorova, M. T., McGowan, R., and Seyfried, T. N. (2001). Caloric restriction inhibits seizure susceptibility in epileptic EL mice by reducing blood glucose. *Epilepsia* 42, 1371–1378.
- Gu, Y., Luchsinger, J. A., Stern, Y., and Scarmeas, N. (2010). Mediterranean diet, inflammatory and metabolic biomarkers, and risk of Alzheimer’s disease. *J. Alzheimers Dis.* 22, 483–492.
- Hartman, A. L., Gasior, M., Vining, E. P. G., and Rogawski, M. A. (2007). The neuropharmacology of the ketogenic diet. *Pediatr. Neurol.* 36, 281–292.
- Henderson, S. T., Vogel, J. L., Barr, L. J., Garvin, F., Jones, J. J., and Costantini, L. C. (2009). Study of the ketogenic agent AC-1202 in mild to moderate Alzheimer’s disease: a randomized, double-blind, placebo-controlled, multicenter trial. *Nutr. Metab. (Lond.)* 6, 31.
- Hu, Z. G., Wang, H. D., Qiao, L., Yan, W., Tan, Q. F., and Yin, H. X. (2009). The protective effect of the ketogenic diet on traumatic brain injury-induced cell death in juvenile rats. *Brain Inj.* 23, 459–465.

- Jeong, E. A., Jeon, B. T., Shin, H. J., Kim, N., Lee, D. H., Kim, H. J., Kang, S. S., Cho, G. J., Choi, W. S., and Roh, G. S. (2011). Ketogenic diet-induced peroxisome proliferator-activated receptor- γ activation decreases neuroinflammation in the mouse hippocampus after kainic acid-induced seizures. *Exp. Neurol.* 232, 195–202.
- Jiang, Y., Yang, Y., Wang, S., Ding, Y., Guo, Y., Zhang, M. M., Wen, S. Q., and Ding, M. P. (2012). Ketogenic diet protects against epileptogenesis as well as neuronal loss in amygdaloid-kindling seizures. *Neurosci. Lett.* 508, 22–26.
- Kang, H. C., Lee, Y. M., Kim, H. D., Lee, J. S., and Slama, A. (2007). Safe and effective use of the ketogenic diet in children with epilepsy and mitochondrial respiratory chain complex defects. *Epilepsia* 48, 82–88.
- Kapogiannis, D., and Mattson, M. P. (2011). Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease. *Lancet Neurol.* 10, 187–198.
- Kashiwaya, Y., Takeshima, T., Mori, N., Nakashima, K., Clarke, K., and Veech, R. L. (2000). D-beta-hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5440–5444.
- Kemnitz, J. W. (2011). Calorie restriction and aging in nonhuman primates. *ILAR J.* 52, 66–77.
- Kessler, S. K., Neal, E. G., Camfield, C. S., and Kossoff, E. H. (2011). Dietary therapies for epilepsy: future research. *Epilepsy Behav.* 22, 17–22.
- Kim do, Y., Davis, L. M., Sullivan, P. G., Maalouf, M., Simeone, T. A., van-Brederode, J., and Rho, J. M. (2007). Ketone bodies are protective against oxidative stress in neocortical neurons. *J. Neurochem.* 101, 1316–1326.
- Kim do, Y., and Rho, J. M. (2008). The ketogenic diet and epilepsy. *Curr. Opin. Clin. Nutr. Metab. Care* 11, 113–120.
- Kim do, Y., Vallejo, J., and Rho, J. M. (2010). Ketones prevent synaptic dysfunction induced by mitochondrial respiratory complex inhibitors. *J. Neurochem.* 114, 130–141.
- Kossoff, E. H., Huffman, J., Turner, Z., and Gladstein, J. (2010). Use of the modified Atkins diet for adolescents with chronic daily headache. *Cephalalgia* 30, 1014–1016.
- Kossoff, E. H., and McGrogan, J. R. (2005). Worldwide use of the ketogenic diet. *Epilepsia* 46, 280–289.
- Kossoff, E. H., Zupec-Kania, B. A., Amark, P. E., Ballaban-Gil, K. R., Bergqvist, C. A. G., Blackford, R., Buchhalter, J. R., Caraballo, R. H., Cross, H. J., Dahlin, M. G., Donner, E. J., Klepper, J., Jehle, R. S., Kim, H. D., Liu, C. Y. M., Nation, J., Nordli, D. R. Jr., Pfeifer, H. H., Rho, J. M., Stafstrom, C. E., Thiele, E. A., Turner, Z., Wirrell, E. C., Wheless, J. W., Viggiani, P., and Vining, E. P. (2009). Optimal clinical management of children receiving the ketogenic diet: recommendations of the International Ketogenic Diet Study Group. *Epilepsia* 50, 304–317.
- Linard, B., Ferrandon, A., Koning, E., Nehlig, A., and Raffo, E. (2010). Ketogenic diet exhibits neuroprotective effects in hippocampus but fails to prevent epileptogenesis in the lithium-pilocarpine model of mesial temporal lobe epilepsy in adult rats. *Epilepsia* 51, 1829–1836.
- Ma, W., Berg, J., and Yellen, G. (2007). Ketogenic diet metabolites reduce firing in central neurons by opening K(ATP) channels. *J. Neurosci.* 27, 3618–3625.
- Maalouf, M., Rho, J. M., and Mattson, M. P. (2009). The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. *Brain Res. Rev.* 59, 293–315.
- Maggioni, F., Margoni, M., and Zanchin, G. (2011). Ketogenic diet in migraine treatment: a brief but ancient history. *Cephalgia* 31, 1150–1151.
- Masino, S. A., and Geiger, J. D. (2008). Are purines mediators of the anticonvulsant/neuroprotective effects of ketogenic diets? *Trends Neurosci.* 31, 273–278.
- Masino, S. A., Kawamura, M. Jr., Plotkin, L. M., Svedova, J., DiMario, F. J. Jr., and Eigsti, I. M. (2011). The relationship between the neuromodulator adenosine and behavioral symptoms of autism. *Neurosci. Lett.* 500, 1–5.
- McDaniel, S. S., Rensing, N. R., Thio, L. L., Yamada, K. A., and Wong, M. (2011). The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. *Epilepsia* 52, e7–e11.
- McDaniel, S. S., and Wong, M. (2011). Therapeutic role of mammalian target of rapamycin (mTOR) inhibition in preventing epileptogenesis. *Neurosci. Lett.* 497, 231–239.
- Murphy, P., and Burnham, W. M. (2006). The ketogenic diet causes a reversible decrease in activity level in Long-Evans rats. *Exp. Neurol.* 201, 84–89.
- Murphy, P., Likhodii, S., Nylen, K., and Burnham, W. M. (2004). The antidepressant properties of the ketogenic diet. *Biol. Psychiatry* 56, 981–983.
- Neal, E. G., Chaffe, H., Schwartz, R. H., Lawson, M. S., Edwards, N., Fitzsimmons, G., Whitney, A., and Cross, J. H. (2008). The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. *Lancet Neurol.* 7, 500–506.
- Nebeling, L. C., Miraldi, F., Shurin, S. B., and Lerner, E. (1995). Effects of a ketogenic diet on tumor metabolism and nutritional status in pediatric oncology patients: two case reports. *J. Am. Coll. Nutr.* 14, 202–208.
- Nehlig, A. (1999). Age-dependent pathways of brain energy metabolism: the suckling rat, a natural model of the ketogenic diet. *Epilepsy Res.* 37, 211–221.
- Noebels, J. L. (2011). A perfect storm: converging paths of epilepsy and Alzheimer's dementia intersect in the hippocampal formation. *Epilepsia* 52(Suppl. 1), 39–46.
- Otto, C., Kaemmerer, U., Illert, B., Muehling, B., Pfetzer, N., Wittig, R., Voelker, H. U., Thiede, A., and Coy, J. F. (2008). Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides. *BMC Cancer* 8, 122. doi:10.1186/1471-2407-8-122
- Palop, J. J., Chin, J., Roberson, E. D., Wang, J., Thwin, M. T., Bien-Ly, N., Yoo, J., Ho, K. O., Yu, G. Q., Kreitzer, A., Finkbeiner, S., Noebels, J. L., and Mucke, L. (2007). Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. *Neuron* 55, 697–711.
- Palop, J. J., and Mucke, L. (2009). Epilepsy and cognitive impairments in Alzheimer disease. *Arch. Neurol.* 66, 435–440.
- Pellicano, H., Martin, D. S., Xu, R.-H., and Huang, P. (2006). Glycolysis inhibition for anticancer treatment. *Oncogene* 25, 4633–4646.
- Prins, M. L. (2008a). Cerebral metabolic adaptation and ketone metabolism after brain injury. *J. Cereb. Blood Flow Metab.* 28, 1–16.
- Prins, M. L. (2008b). Diet, ketones, and neurotrauma. *Epilepsia* 49(Suppl. 8), 111–113.
- Prins, M. L., Fujima, L. S., and Hovda, D. A. (2005). Age-dependent reduction of cortical contusion volume by ketones after traumatic brain injury. *J. Neurosci. Res.* 82, 413–420.
- Puchowicz, M. A., Zechel, J. L., Valeviro, J., Emancipator, D. S., Xu, K., Pandik, S., LaManna, J. C., and Lust, W. D. (2008). Neuroprotection in diet-induced ketotic rat brain after focal ischemia. *J. Cereb. Blood Flow Metab.* 28, 1907–1916.
- Redman, L. M., and Ravussin, E. (2011). Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. *Antioxid. Redox Signal.* 14, 275–278.
- Rho, J. M., and Stafstrom, C. E. (2011). The ketogenic diet: what has science taught us? *Epilepsy Res.* doi:10.1016/j.epilepsies.2011.05.021
- Roberson, E. D., Halabiksy, B., Yoo, J. W., Yao, J., Chin, J., Yanm, F., Wu, T., Hamto, P., Devidze, N., Yu, G. Q., Palop, J. J., Noebels, J. L., and Mucke, L. (2011). Amyloid- β /Fyn-induced synaptic, network, and cognitive impairments depend on tau levels in multiple mouse models of Alzheimer's disease. *J. Neurosci.* 31, 700–711.
- Rogawski, M. A. (2008). Common pathophysiological mechanisms in migraine and epilepsy. *Arch. Neurol.* 65, 709–714.
- Roth, J., Szulc, A. L., and Danoff, A. (2011). Energy, evolution, and human diseases: an overview. *Am. J. Clin. Nutr.* 93, 875S–883S.
- Schiff, M., Bénit, P., Coulibaly, A., Loublier, S., El-Khoury, R., and Rustin, P. (2011). Mitochondrial response to controlled nutrition in health and disease. *Nutr. Rev.* 69, 65–75.
- Schmidt, M., Pfetzer, N., Schwab, M., Strauss, I., and Kämmerer, U. (2011). Effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer: a pilot trial. *Nutr. Metab. (Lond.)* 8, 54.
- Schnabel, T. G. (1928). An experience with a ketogenic diet in migraine. *Ann. Int. Med.* 2, 341–347.
- Schwartzkroin, P. A., Wenzel, H. J., Lyeth, B. G., Poon, C. C., Delance, A., Van, K. C., Campos, L., and Nguyen, D. V. (2010). Does ketogenic diet alter seizure sensitivity and cell loss following fluid percussion injury? *Epilepsy Res.* 92, 74–84.
- Seyfried, T. N., Marsh, J., Shelton, L. M., Huysestruyt, L. C., and Mukherjee, P. (2011). Is the restricted ketogenic diet a viable alternative to the standard of care for managing malignant brain cancer? *Epilepsy Res.* [Epub ahead of print].
- Siva, N. (2006). Can ketogenic diet slow progression of ALS? *Lancet Neurol.* 5, 476.
- Srikantan, V., Maczurek, A., Phan, T., Steele, M., Westcott, B., Juskiw, D., and Münch, G. (2011). Advanced glycation endproducts and their

- receptor RAGE in Alzheimer's disease. *Neurobiol. Aging* 32, 763–777.
- Stafford, P., Abdelwahab, M. G., Kimdo, Y., Preul, M. C., Rho, J. M., and Scheck, A. C. (2010). The ketogenic diet reverses gene expression patterns and reduces reactive oxygen species levels when used as an adjuvant therapy for glioma. *Nutr. Metab. (Lond.)* 7, 74.
- Stafstrom, C. E. (2004). Dietary approaches to epilepsy treatment: old and new options on the menu. *Epilepsy Curr.* 4, 215–222.
- Tai, K. K., Nguyen, N., Pham, L., and Truong, D. D. (2008). Ketogenic diet prevents cardiac arrest-induced cerebral ischemic neurodegeneration. *J. Neural Transm.* 115, 1011–1017.
- Tai, K. K., Pham, L., and Truong, D. D. (2009). Intracisternal administration of glibenclamide or 5-hydroxydecanoate does not reverse the neuroprotective effect of ketogenic diet against ischemic brain injury-induced neurodegeneration. *Brain Inj.* 23, 1081–1088.
- VanderAuwera, I., Wera, S., VanLeuven, F., and Henderson, S. T. (2005). A ketogenic diet reduces amyloid beta 40 and 42 in a mouse model of Alzheimer's disease. *Nutr. Metab. (Lond.)* 2, 28.
- Vanitallie, T. B., Nonas, C., DiRocco, A., Boyar, K., Hyams, K., and Heymsfield, S. B. (2005). Treatment of Parkinson disease with diet-induced hyperketonemia: a feasibility study. *Neurology* 64, 728–730.
- Vining, E., Freeman, J., Ballaban-Gil, K., Camfield, C., Camfield, P., Holmes, G., Shinnar, S., Shuman, R., Trevalan, E., and Wheless, J. (1998). A multicenter study of the efficacy of the ketogenic diet. *Arch. Neurol.* 55, 1433–1437.
- Vining, E. P. G. (1999). Clinical efficacy of the ketogenic diet. *Epilepsy Res.* 37, 181–190.
- Voskuyl, R. A., and Vreugdenhil, M. (2001). "Effects of essential fatty acids on voltage-regulated ionic channels and seizure thresholds in animals," in *Fatty Acids: Physiological and Behavioral Functions*, eds D. Mostofsky, S. Yehuda, and N. Jr. Salem (Totowa, NJ: Humana Press), 63–78.
- Waldbaum, S., and Patel, M. (2010). Mitochondrial dysfunction and oxidative stress: a contributing link to acquired epilepsy? *J. Bioenerg. Biomembr.* 42, 449–455.
- Wallace, D. C., Fan, W., and Procaccio, V. (2010). Mitochondrial energetics and therapeutics. *Annu. Rev. Pathol.* 5, 297–348.
- Wheless, J. W. (2008). History of the ketogenic diet. *Epilepsia* 49(Suppl. 8), 3–5.
- Willis, S., Stoll, J., Sweetman, L., and Borges, K. (2010). Anticonvulsant effects of a triheptanoin diet in two mouse chronic seizure models. *Neurobiol. Dis.* 40, 565–572.
- Xu, K., Sun, X., Eroku, B. O., Tsipis, C. P., Puchowicz, M. A., and LaManna, J. C. (2010). Diet-induced ketosis improves cognitive performance in aged rats. *Adv. Exp. Med. Biol.* 662, 71–75.
- Yamada, K. A. (2008). Calorie restriction and glucose regulation. *Epilepsia* 49(Suppl. 8), 94–96.
- Yang, X., and Cheng, B. (2010). Neuroprotective and anti-inflammatory activities of ketogenic diet on MPTP-induced neurotoxicity. *J. Mol. Neurosci.* 42, 145–153.
- Yao, J., Chen, S., Mao, Z., Cadena, E., and Brinton, R. D. (2011). 2-Deoxy-D-glucose treatment induces ketogenesis, sustains mitochondrial function, and reduces pathology in female mouse model of Alzheimer's disease. *PLoS ONE* 6, e21788. doi:10.1371/journal.pone.0021788
- Zhao, Z., Lange, D. J., Voustianiouk, A., MacGrogan, D., Ho, L., Suh, J., Humala, N., Thiagarajan, M., Wang, J., and Pasinetti, G. M. (2006). A ketogenic diet as a potential novel therapeutic intervention in amyotrophic lateral sclerosis. *BMC Neurosci.* 7, 29. doi:10.1186/1471-2202-7-29
- Zuccoli, G., Marcello, N., Pisanello, A., Servadei, F., Vaccaro, S., Mukherjee, P., and Seyfried, T. N. (2010). Metabolic management of glioblastoma multiforme using standard therapy together with a restricted ketogenic diet: case report. *Nutr. Metab. (Lond.)* 7, 33.

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Barrier mechanisms in the developing brain

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The adult brain functions within a well-controlled stable environment, the properties of which are determined by cellular exchange mechanisms superimposed on the diffusion restraint provided by tight junctions at interfaces between blood, brain and cerebrospinal fluid (CSF). These interfaces are referred to as "the" blood-brain barrier. It is widely believed that in embryos and newborns, this barrier is immature or "leaky," rendering the developing brain more vulnerable to drugs or toxins entering the fetal circulation from the mother. New evidence shows that many adult mechanisms, including functionally effective tight junctions are present in embryonic brain and some transporters are *more* active during development than in the adult. Additionally, some mechanisms present in embryos are not present in adults, e.g., specific transport of plasma proteins across the blood-CSF barrier and embryo-specific intercellular junctions between neuroependymal cells lining the ventricles. However developing cerebral vessels appear to be more fragile than in the adult. Together these properties may render developing brains more vulnerable to drugs, toxins, and pathological conditions, contributing to cerebral damage and later neurological disorders. In addition, after birth loss of protection by efflux transporters in placenta may also render the neonatal brain more vulnerable than in the fetus.

Keywords: blood-brain barrier, blood-CSF barrier, epithelial cell transport, endothelial cell transport, cerebrospinal fluid, fetus, newborn

INTRODUCTION

Understanding the role of blood-brain barrier mechanisms in normal brain development and possible deleterious effects should these mechanisms be dysfunctional is important from the clinical perspective of whether or not drugs or toxins, once they cross the placenta, may have access to the vulnerable developing brain. A reason given by regulatory bodies in US and European Union for caution in giving drugs to pregnant women or infants is "immaturity" of the blood-brain barrier^{1,2}.

One historical reason for belief in barrier immaturity comes from teleological thinking that fetal brains would not need a barrier, because the fetus is protected by a placenta (Barcroft, 1938). The developing brain is necessarily immature compared to that of the adult, but the real question should be about the functional status of the blood-brain barrier mechanisms in embryos, fetuses, and infants, compared to adults.

There is a widespread belief amongst pediatricians, neurologists, neuroscientists, and neurotoxicologists that "the" blood-brain barrier in the embryo, fetus, and newborn is "immature" implying that it is poorly formed, leaky, or even absent. Statements about the immaturity of the blood-brain barrier frequently seem to be made without evidence, or by reference to an earlier review that also lacks any evidence (e.g., Järup, 2003; Costa et al., 2004; Watson et al., 2006). This seems to be particularly common in the neurotoxicology literature and in toxicology reports (see review by Ek et al., 2012).

Abbreviations: BCRP, breast cancer resistance protein; CSF, cerebrospinal fluid.

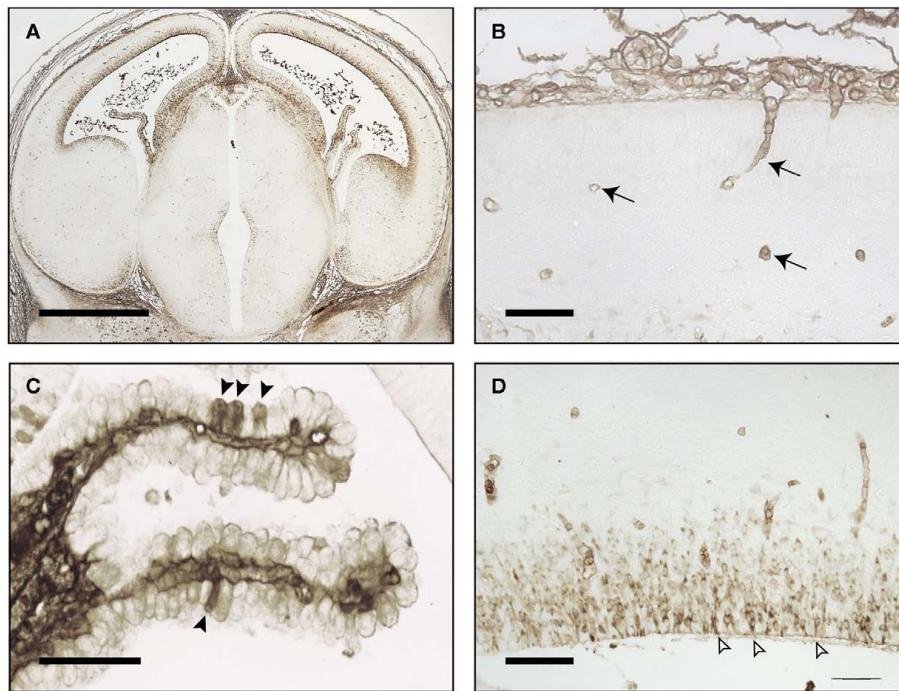
¹<http://www.atsdr.cdc.gov/csem/pediatric/docs/pediatric.pdf>

²www.emea.europa.eu

FUNCTIONAL SIGNIFICANCE OF EARLY DEVELOPMENT OF BRAIN BARRIERS

Without the diffusion restraint provided by intercellular junctions in brain barrier interfaces it would not be possible to establish efflux or influx mechanisms such as those controlling ionic gradients between blood and cerebrospinal fluid (CSF; Bito and Myers, 1970; Bradbury et al., 1972; Amtorp and Sørensen, 1974; and see Saunders, 1992 for review). In effect, these junctions convert the transport properties of individual cerebral endothelial and choroid plexus epithelial cells into those of the whole interfaces separating blood from brain. Thus transfer from blood to brain and CSF occurs across these interfaces and is known to be present from early stages of brain development for a wide range of metabolically important molecules: glucose (Dermietzel et al., 1992; Vannucci, 1994; Vannucci et al., 1994; Bauer et al., 1995), amino acids (Braun et al., 1980; Cornford et al., 1982; Pardridge and Mietus, 1982; Lefauconnier and Trouvé, 1983), and hormones (Hagenbuch, 2007). The molecular structure of tight junctions of the blood-brain barrier proper in the adult brain has been extensively studied and reviewed (e.g., Abbott et al., 2010).

Figure 1 illustrates the brain of an E16 rat brain immunostained with antibodies to total plasma protein. There is no evidence for any proteins escaping the vasculature, demonstrating functional effectiveness of the blood-brain barrier even so early in development. At this stage of rat development in the brain there are very few blood vessels and the cortex only becomes well vascularized after birth. In contrast, choroid plexuses are already a significant size (**Figure 1C**) and grow rapidly during the remainder of gestation. It appears that in early stages of brain development the

**FIGURE 1 | Embryonic day E16 rat brain stained for endogenous plasma proteins.**

(A) Low power coronal section. Immunostaining in CSF, choroid plexus, and ventricular zone, also few blood vessels in cortex. **(B)** Higher power of cortex: immunostaining in mesenchymal tissue outside brain surface and blood vessels (arrows), not in brain parenchyma. **(C)** Choroid

plexus: immunostaining in some epithelial cells (arrowheads) and stroma. **(D)** Immunostaining in neuroependymal cells and blood vessels, not in brain parenchyma outside neuroependyma. Open arrows: sites of strap junctions. Images reproduced from Saunders and Habgood (2011). Scale bars: **(A)**, 100 μ m; **(B,D)**, 25 μ m; **(C)**, 50 μ m.

choroid plexuses are the main portals of molecular transfer from blood into brain (Johansson et al., 2008; Liddelow et al., 2009).

Thus far from being incomplete or “leaky,” barriers in developing brain are adapted to fetal environments. However, it is important to distinguish functional effectiveness of early tight junctions at the blood–brain barrier from the evidence for their continued molecular and structural organization. This may relate to increased hydrostatic pressure that occurs during fetal development as systemic blood pressure rises in parallel with plasma protein concentration that provides the “colloid” osmotic pressure important for fluid exchange across capillaries.

EARLY STUDIES OF THE BLOOD–BRAIN BARRIER IN THE DEVELOPING BRAIN

DYE EXPERIMENTS

Ehrlich (1885) and Goldmann (1909) showed that parenteral injections of trypan blue and other acidic dyes resulted in staining of almost all tissues except the brain. Biedl and Kraus (1898) and Lewandowsky (1900) found that even the much smaller molecules: bile and sodium ferrocyanide (demonstrated with Prussian blue reaction) only had toxic effects when injected directly into the brain. It was these early experiments that led to the concept of the brain being protected by a mechanism called the blood–brain barrier, a term that appears to have been first used by Lewandowsky (1900). These experiments were soon followed by similar ones using embryos or newborns of various species. Most of the early

reports of experiments using trypan blue gave the same result as in adults, i.e., most of the brain was not stained apart from the circumventricular organs (Wislocki, 1920, guinea pig embryo; Stern and Peyrot, 1927; Stern and Rapoport, 1928; Stern et al., 1929, newborn rats, rabbits, and guinea pigs). Stern et al. (1929) stressed the importance of not injecting too much dye. This may explain one of the few early reports of brain staining following dye injection by Penta (1932) because he used multiple and such large injections that most of his experimental animals (newborn rabbits) died from the toxic effects of the dye (see Saunders, 1992). Behnson (1927) is perhaps the most frequently cited paper supposedly showing staining of the brain following dye injections in postnatal mice. However, his paper did not actually show this but instead his results illustrated that the sites of dye entry in the adult animals corresponded precisely to sites of maximal accumulation in the young mice. Rather curiously Behnson (1927) chose to illustrate his findings with drawings of sagittal sections using adult mouse brain for both adult and postnatal (2–3 week old) animals. Since areas of the brain such as the cerebral cortex are not yet fully developed at 2–3 weeks of age, the distribution of dye at this age would have appeared more widespread than in the adult, without actually being so. In the brain region with the least mature blood vessels (cerebral cortex) there was no dye staining of the brains of the younger mice. A key often overlooked reference in the field is the one in which both rabbit and human fetal material was studied using trypan blue injections (Gröntoft, 1954). The study showed

clearly that in human fetuses (5–26 cm long) obtained from legal abortions, providing the dye was injected within 10 min following placental separation, the brains remained dye-free indicating that the blood–brain barrier was impermeable to the dye; at later times or in aborted embryos that could not be examined until some time after delivery, the brains stained blue following dye injection. Gröntoft (1954) considered this to be due to an effect of asphyxia on the blood–brain barrier following the death of the fetuses. He confirmed this under controlled experimental conditions in rabbit fetuses in which he was able to study the brains at predetermined times after injection and death of the fetuses.

SODIUM FERROCYANIDE

Sodium ferrocyanide is a small molecular weight compound (mol wt 204) about the same size as sucrose (342 Da; molecular radius 5.1 Å). When treated with acid it gives a blue color (Prussian blue reaction). In what was probably the first brain barrier experiment in an embryo, Weed (1917) injected sodium ferrocyanide into the neural tube of pig embryos (19 mm, E20; term is 114 days) and showed that the blue staining following treatment with slightly acidic solutions of iron salts (Prussian blue reaction) was confined to the neural tube and did not penetrate out into rest of the embryo. This paper seems to have been overlooked by the blood–brain barrier field, perhaps because it is the converse of a classical barrier experiment in which the marker was injected parenterally (cf. Stern and Peyrot, 1927; Stern and Rapoport, 1928; Stern et al., 1929).

In spite of the above studies clearly showing lack of penetration of small molecular compounds even into an embryonic brain, there are nevertheless numerous reports claiming an absence or immaturity of the blood–brain barrier in the fetus and neonate continue to be published (see Ek et al., 2012 for review).

There are several well-known fetal-specific mechanisms that are different from the adult that reflect adaptation to a fetal-specific environment (e.g., fetal hemoglobin, Palis et al., 2010). Similarly, during development, brain barriers demonstrate some important differences, particularly in transport mechanisms, many of which have only recently begun to be described. These differences probably reflect mechanisms important for brain development rather than deficiencies compared to adult brain. This review will summarize the main brain barrier mechanisms in the adult and during development. In the last part of the review we shall consider evidence that blood–brain barrier mechanisms, while functionally effective in the developing brain, may nonetheless be more susceptible than in the adult to adverse circumstances and that damage to brain barrier mechanisms during development may lead to neurological and neuropsychological dysfunction in later life.

BRAIN BARRIERS

The main interfaces across which exchange occurs between the blood and the internal environment of the brain (brain interstitial fluid and CSF) are illustrated in **Figure 2**. There are five main barrier interfaces involved: (i) the blood–brain barrier proper at the level of the endothelium of the cerebral blood vessels; (ii) the arachnoid barrier between the CSF in the subarachnoid space and the dura; (iii) the pia/glia limitans between the CSF and extracellular fluid of the brain, which is much more complex in the embryo;

(iv) the CSF–brain barrier, which is only a significant barrier in the embryo, created by separation of the ventricular system from the extracellular fluid of the brain by strap junctions in the neuroependyma; and (v) the blood–CSF barrier at the level of the choroid plexus epithelial cells (**Figure 2**).

CELLULAR CONSTITUENTS OF BARRIER INTERFACES IN THE DEVELOPING BRAIN

The principal morphological basis of these barriers lies in intercellular junctions that provide a diffusional restraint between compartments.

The neurovascular unit

The term blood–brain barrier has a long history, but it has become increasingly recognized that it does not adequately encompass the wide range of morphological features and functional characteristics that it is now known to involve. For this reason the term “neurovascular unit” (Neuwelt, 2004) is being increasingly used. It comprises the endothelial cells, pericytes, microglia, astrocytes, and basement membrane that are characteristic of the cerebral vasculature. The term refers more to the close anatomical and functional association of these different cell types without implying any specific mechanisms.

Endothelial cells

Two features characterize the cerebral endothelial cells that constitute the blood–brain barrier. These are circumferential tight junctions, which occlude the intercellular space between adjacent endothelial cells and a lack of pinocytotic vesicles in the cytoplasm (Brightman and Reese, 1969). There are several reports that there are many more vesicles in the endothelial cells of developing brain vasculature (Donahue and Pappas, 1961; Dziegielewska et al., 1979) with a decline as development proceeds. In contrast Stewart and Hayakawa (1987, 1994) reported that vesicles were virtually absent in early cerebral blood vessels. This discrepancy could perhaps be due to differences in fixation or to different classes of blood vessels being examined. Dziegielewska et al. (1979) also reported that following intravenous Alcian blue injection in fetal sheep, the dye (which is electron-dense and binds to plasma albumin) could be seen within numerous vesicles and tubules in the cytoplasm of the endothelial cells; dye was also present in the basement membrane, but not inside tight junctions. However, this aspect of brain barrier development has otherwise been little studied. In contrast the question of whether cerebral interendothelial tight junctions in early brain development are as impermeable to markers such as dyes or horseradish peroxidase has attracted a lot of attention, with conflicting results being reported over many years. Thus some early studies using transmission electron microscopy and/or freeze fracture, showed well-formed tight junctions between cerebral endothelial cells from early in vascularization of the brain (Møllgård and Saunders, 1975; Møllgård et al., 1979; Bass et al., 1992; Bauer et al., 1993). Some studies claimed that cerebral endothelial tight junctions in fetal mouse and rat brain showed ultrastructural features such as the proportion of the junction composed of zonulae occludens which increased, while junctional clefts decreased, and expanded junctional clefts virtually disappeared (Stewart and Hayakawa, 1987, 1994). In a freeze fracture study,

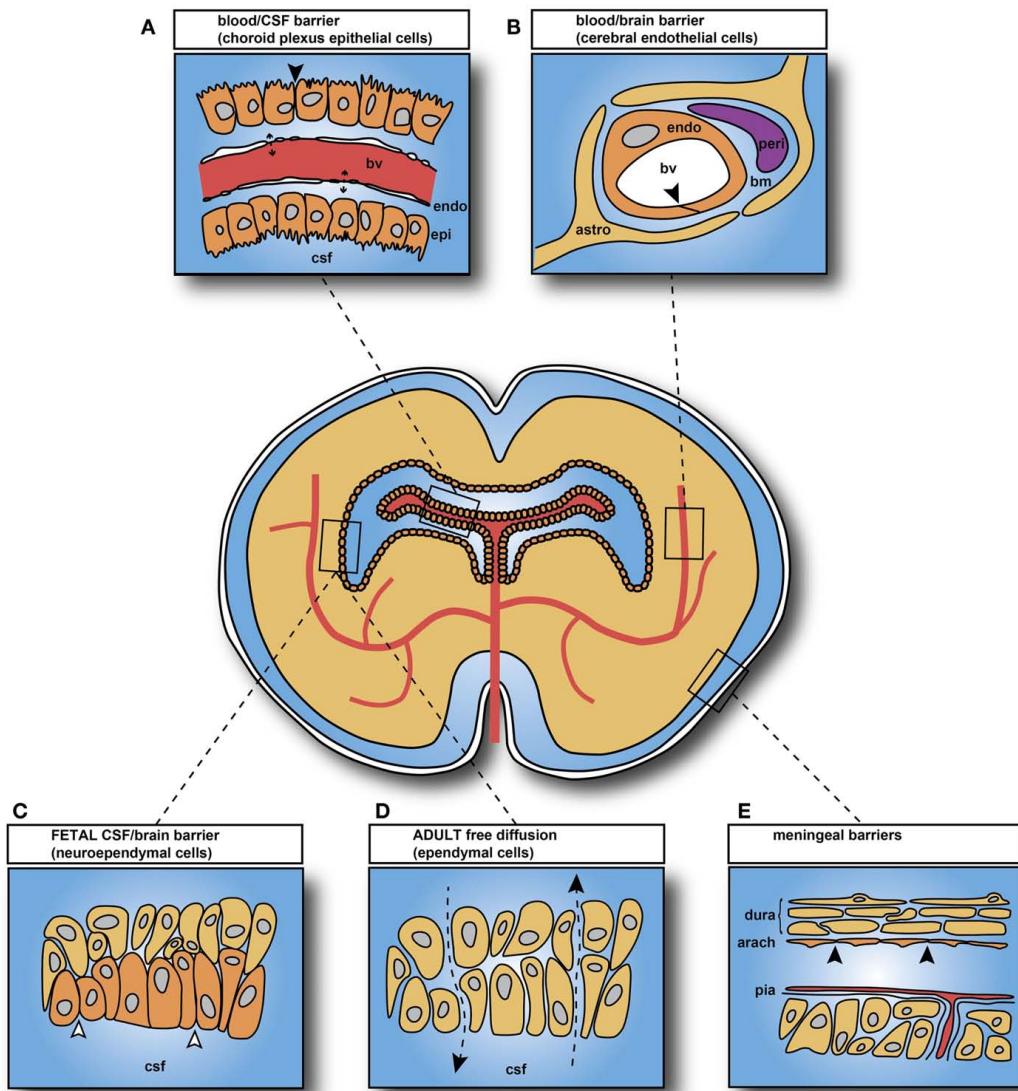


FIGURE 2 | Brain barrier interfaces. **(A)** Blood–cerebrospinal fluid (CSF) barrier: tight junctions between choroid plexus epithelial cells. **(B)** Blood–brain barrier: tight junctions between endothelial cells. **(C)** CSF–brain barrier only present in embryos and fetuses: strap junctions between neuroependymal cells. **(D)** CSF–brain interface in adult: gap junctions between ependymal cells, with free diffusion pathway (Arrowheads and broken lines). **(E)** Arachnoid barrier. In adult: tight junctions between cells of the inner layer of the arachnoid membrane and between endothelial cells of

pial blood vessels. In embryos: additional membrane specializations at the CSF–pial interface (Möllgård et al., 1987). Abbreviations: bv, blood vessel; endo, endothelial cell; epi, epithelial cell; bm, basement membrane; peri, pericyte; astro, astrocyte (astrocytes not yet differentiated in brain when blood vessels first appear; thus they cannot contribute to tight junction formation in early brain development). Black arrowheads: sites of tight junctions; open arrowheads: sites of strap junctions. Redrawn from Saunders et al. (2008).

Kniesel et al. (1996) identified changes in strand pattern during cerebral vascular development, which they suggested correlated with greater permeability and subsequent developmental decline in blood–brain barrier permeability. Stewart and Hayakawa (1987) assessed blood–brain barrier permeability by measuring peroxidase activity in homogenized brain at different ages. However, the choroid plexuses are disproportionately larger in the developing brain (Johansson et al., 2008) and transfer proteins across their epithelial cells (see below). It is unclear whether choroid plexus was removed from the brain samples and the possibility that HRP may have entered the brain via the plexuses was not considered. Kniesel

et al. (1996) did not include any permeability studies in parallel with their ultrastructural observations, but relied on comparisons with *in vitro* cultures of cerebral endothelial cells to support their conclusion that changes in freeze fracture replicas with age correlated with supposed greater blood–brain barrier permeability in the developing brain.

Later studies (Nitta et al., 2003; Ek et al., 2006) appear to confirm the earlier findings of well-formed tight junctions in the early stages of vascularization of the brain. The advent of small molecular sized water-soluble probes that can be visualized at the electron microscopical level has shown that these junctions are

indeed functionally tight to small molecules (Ek et al., 2006). Thus in these more recent light microscopical and ultrastructural studies using amounts of tracer that only increased circulating blood volume and protein concentration to a limited extent (<10%) the tight junctions of endothelial cells in cerebral blood vessels and in embryos and neonates were found to restrict the passage of low molecular weight molecules (Ek et al., 2006; Johansson et al., 2006; Daneman et al., 2010a). In contrast, some earlier studies in the developing brain claimed leakiness of cerebral blood vessels, but used large injection volumes or concentrations of the tracer horseradish peroxidase (e.g., Wakai and Hirokawa, 1978a,b; Risau et al., 1986; Risau and Wolburg, 1990; see Saunders, 1992 for review). The more recent findings of Ek et al. (2006), Johansson et al. (2006), and Daneman et al. (2010a) show that the physical basis for the brain barrier mechanism is already present and functionally effective from very early in development.

Immunolocalization of tight junctional proteins, such as claudin 5 and occludin, shows that these are present in brain barrier interfaces soon after blood vessels invade the brain in mammals (Ek et al., 2006; Daneman et al., 2010a) including humans (Virgintino et al., 2004). Many of the molecular studies of tight junction proteins have been carried out using *in vitro* preparations, often of cultures of artificial cell lines such as Madin–Darby bovine kidney (MDBK) cells and human intestinal epithelial cells (T84; Furuse et al., 1994, 2001; Itoh et al., 2001) mouse embryonic feeder cells (Saitou et al., 1998) MDCK cells (Sonoda et al., 1999; Colegio et al., 2003; Blasig et al., 2006). These studies have provided valuable insights into the molecular structure of tight junctions, which could then be followed up in material from normal brain endothelia and epithelia *in vitro* (Hirase et al., 1997; Haseloff et al., 2005; Cohen-Kashi Malina et al., 2009) and *in vivo* (Nitta et al., 2003; Furuse and Tsukita, 2006; Sadowska et al., 2009; Xie et al., 2010). However, when used to assess barrier interface permeability *in vitro* this has usually been done indirectly by measuring transepithelial or transendothelial resistance (TER), which is assumed to equate to a low resistance pathway via tight junctions (Frömlter and Diamond, 1972). It is not clear how well these *in vitro* systems reflect the situation *in vivo*, but it is becoming increasingly clear that they may not always do so, resulting in a number of misleading conclusions. As pointed out by Armulik et al. (2011), it was considered for some years on the basis of *in vitro* studies that pericytes played an important role in development and maintenance of cerebral interendothelial cell tight junctions; however two studies of transgenic mice with deficient pericytes showed that in these animals an increase in vascular permeability correlated with increased endothelial transcytosis rather than tight junction permeability (Armulik et al., 2010; Daneman et al., 2010b) as is discussed in the next section.

Pericytes

Daneman et al. (2010b) compared mice with null and hypomorphic alleles of Pdgfrb (platelet-derived growth factor receptor- β) which have defects in pericyte generation. They showed that pericytes are necessary for formation of a functionally effective blood–brain barrier and that pericyte coverage of blood vessels determines relative vascular permeability. Pericytes were found to regulate functional formation of tight junctions and vesicle

trafficking in CNS endothelial cells, however, they did not induce blood–brain barrier specific gene expression in cerebral endothelial cells; rather they inhibited the expression of molecules that increase vascular permeability and infiltration of immune cells. These effects on blood–brain barrier formation occurred a week before the differentiation of astrocytes, the cells that many have suggested are responsible for induction of tight junctions during blood–brain barrier formation (see next section).

Astrocytes

In the adult brain astrocytic end feet encircle almost the entire circumference of endothelial cells of cerebral capillaries (Caley and Maxwell, 1970; Xu and Ling, 1994). They are an important component of the neurovascular unit and are thought to make significant contributions to blood–brain barrier functions (Abbott et al., 2006). Their role in the developing brain has been more controversial. When *in vitro* blood–brain barrier models were first being developed it was found that the presence of either cultured astrocytes or conditioned medium from cultured astrocytes produced cells with more complex tight junctions (Tao-Cheng et al., 1987) and this was essential for the preparation of cerebral endothelial monolayers with high transendothelial resistance (Dehouck et al., 1990; Rubin et al., 1991). A study that has been particularly influential in discussion of the possible role of astrocytes in development of blood–brain barrier properties in the immature brain was that of Janzer and Raff (1987). These authors cultured cerebral endothelial cells in the anterior chamber of the eye and reported that only in the presence of astrocytes were blood vessels formed that retained dye within their lumena. They compared astrocyte implants with fibroblast implants and found that the vessels associated with the fibroblast grafts were “leaky” to Evans blue. Janzer and Raff (1987) interpreted their results as evidence that blood vessels with impermeable tight junctions were formed in the presence of astrocytes. However, this was not confirmed by electron microscopy; without ultrastructural evidence a claim of tight junction formation was unwarranted. The study was repeated by Holash et al. (1993) who did include electronmicroscopical observations. They found that astrocytes implanted into the anterior chamber formed grafts that were poorly vascularized and when examined by electron microscopy the iridial blood vessels associated with the astrocyte grafts did not change their ultrastructural characteristics to those of brain capillaries. In addition, grafted fibroblasts formed invasive masses that were well vascularized with fenestrated (non-barrier) blood vessels. Holash et al. (1993) suggested that it was the contrast in dye penetration into these well vascularized fibroblast grafts compared to the poorly vascularized astrocyte grafts that led to the incorrect conclusion of barrier formation in vessels penetrating the astrocyte grafts. However, this study is rarely cited in contrast to the paper of Janzer and Raff (1987), a depressing reflection that even scientists will cite the evidence that supports their preconceived ideas, not to mention the undue influence of some journals in which a particular study is published. A limitation of studies of blood–brain barrier interfaces *in vitro* is that the endothelial cells used were generally from adult brains and would therefore already have their adult properties; however it may be that their role is in maintenance of tight junctions rather than in their initial formation.

What seems to have been overlooked by the proponents of the idea that astrocytes are essential for tight junction formation in the blood vessels of the developing brain is that there are no astrocytes present in the developing brain when it is first vascularized (Caley and Maxwell, 1970; Daneman et al., 2010a). As first shown by Stewart and Wiley (1981) using chick-quail chimeras, tight junction formation in cerebral vessels is induced by some factor in the neural tissue of the developing brain. The vessels are tight to proteins and small molecules from as early as vessels first grow into the neural tissue (Bauer et al., 1993; Ek et al., 2006). It is not yet clear what the induction factor(s) is/are. However, as indicated above it seems that the pericytes make an important contribution to tight junction formation (Daneman et al., 2010b). The main period of differentiation of astrocytes and the encirclement of capillaries occur in rodents in the first 3 weeks of postnatal life; this is also the main period of vascularization of the developing brain in rodents (Caley and Maxwell, 1970). Thus it is possible that astrocytes contribute to tight junction–induction during this period of intense vascularization.

Basement membrane

The basement membrane surrounds all cerebral capillaries and is an important component of the neurovascular unit. Bär and Wolff (1972) have given a detailed ultrastructural description of formation of the basement membrane around capillaries from E14 in rat cerebral cortex. The basement membrane is thought to be formed by secretion of constituents by cerebral endothelial cells and by pericytes (Stratman and Davis, 2011) embedded in this structure (**Figure 1B**). There is also evidence that astrocytes contribute at later stages in vascularization of the brain (del Zoppo and Milner, 2006). The main constituents of the basement membrane are type IV collagen from endothelial cells and type I collagen from pericytes as well as fibronectin, thrombospondin (Canfield et al., 1989), and agrin (Barber and Lieth, 1997). In the developing brain basement membrane is apparent at least as early as E20 in rat fetuses and becomes denser and wider in the postnatal brain (Donahue and Pappas, 1961). In human fetuses it has been identified in cerebral capillaries at least as early as 8 weeks gestation in a detailed immunohistochemical study of the distribution of laminin $\beta 1$ and laminin $\beta 2$ chains (Roediger et al., 2010).

Wnt/ β CATENIN PATHWAY IN BLOOD–BRAIN BARRIER DEVELOPMENT

A major contribution to understanding the early stages of blood–brain barrier mechanism development was the publication of three papers implicating the Wnt/ β catenin pathway in some important features of blood–brain barrier function (Liebner et al., 2008; Stenman et al., 2008; Daneman et al., 2009). Wnt is an acronym for wingless (wg) first identified in *Drosophila* and INT-1, first identified in virally induced mammary tumors in mice. They are the two founding member genes of the Wnt signaling pathway. Thus far three major pathways downstream from Wnt have been identified. Of these the Wnt/ β catenin pathway seems to be important for aspects of angiogenesis and blood–brain barrier development. The Wnt/ β catenin pathway is also referred to as the canonical Wnt signaling pathway (canonical: “Of the nature of a general rule or standard formula” Oxford English Dictionary). Signaling via Wnt involves a complex molecular mechanism the end result of

which is blocking of intracellular mechanisms that would normally result in ubiquitination and proteasome-dependent degradation (Rudloff et al., 2011). Several studies have shown that interference with the Wnt/ β -catenin pathway results in reduced vessel numbers, loss of capillary beds, and formation of hemorrhagic vascular malformations; in addition Wnt/ β -catenin signaling has also been shown to regulate the expression of the blood–brain barrier-specific glucose transporter glut-1 (Stenman et al., 2008; Daneman et al., 2009). In a study using different transgenic mice at postnatal ages and primary cultures of mouse brain endothelial cells (the age of the animals from which these were derived was not specified) Liebner et al. (2008) reported that Wnt/ β -catenin signaling was important for the regulation of two key tight junction proteins, claudin 3 and claudin 5. In contrast, Daneman et al. (2009) reported no effect of down regulation of the Wnt/ β -catenin pathway on regulation of the tight junction proteins, occludin and claudin 5 in E11.5 endothelial-specific β -catenin mutants. It is not clear whether this difference was due to the mutants used or the age at which they were examined. The Wnt/ β -catenin pathway was found to be active in many endothelial cells of developing brain from as early as E9.5; expression declined after E15.5 to a lower level that was maintained in the neonatal period, but by adulthood expression was rare (Liebner et al., 2008).

There is general agreement that Wnt ligands are present in early neural progenitors of the ventricular zone with some regional specificity (Stenman et al., 2008; Daneman et al., 2009). Thus vascular Wnt activation temporally correlates with the expression of Wnt7a and Wnt7b in the developing forebrain and in the ventral and intermediate spinal cord; Wnt4 in the dorsal and intermediate spinal cord; and Wnt1, Wnt3, and Wnt3a throughout the dorsal neural tube (Daneman et al., 2009).

MENINGEAL BARRIER

At the blood–brain interface over the outer surface of the brain within the pia-arachnoid, the blood vessels also have tight junctions between the endothelial cells, but their cellular transport properties have been little studied. Other important interfaces are between the CSF and brain interstitium at the inner (ventricular) and outer (subarachnoid) spaces. In the adult, cells lining these interfaces are linked by gap junctions, which do not significantly hinder intercellular passage of molecules. However, in the early stages of brain development the cells lining these interfaces are more heterogeneous and are linked by strap junctions, which occlude the intercellular space except to the smallest molecules; this additional barrier provides a specific internal milieu for the developing brain, in contrast to free exchange between CSF and brain present in the adult (Fossan et al., 1985; Saunders, 1992; Balslev et al., 1997a).

CHOROID PLEXUSES

The molecular make-up of tight junctions of the blood–CSF barrier is less well-known (Wolburg et al., 2001). A recent study of tight junction protein expression in mouse embryos (E15) and adult choroid plexus (Liddelow et al., 2012) has shown that several key junctional genes are expressed at a higher level in embryos than in the adults, whereas for several other genes the reverse is the

case (**Table 1**). This is consistent with previous findings that the fundamental functional basis of this barrier, namely occlusion of the paracellular diffusion pathway, is well established from the earliest stages of differentiation of the choroid plexuses (Bauer et al., 1993; Ek et al., 2003, 2006).

APPARENT INCREASED PERMEABILITY OF BARRIERS IN DEVELOPING BRAIN

SMALL LIPID INSOLUBLE MOLECULES

Studies have been carried out in fetuses and newborn of many species using classical radiolabeled physiological permeability markers, sucrose and inulin. These all showed that the earlier in development experiments were conducted, the higher was the apparent permeability (expressed as brain–plasma and CSF–plasma ratios e.g. Habgood et al., 1993). This was interpreted by some as evidence for brain barrier “immaturity” (see Saunders, 1992, for review of earlier studies). However, extensive ultrastructural studies show that the tight junctions at brain barrier sites are formed very early in development (Møllgård and Saunders, 1975; Møllgård et al., 1976; Tauc et al., 1984; Ek et al., 2001, 2003, 2006). This discrepancy between well-formed tight junctions and higher apparent permeability in the developing brain has only recently been resolved by the use of small lipid insoluble molecules (mainly dextrans of different molecular size) which can be visualized at the electron microscopical level (Ek et al., 2001, 2003, 2006). At least in short term experiments, tight junctions in cerebral blood vessels and choroid plexus epithelial cells (plexus blood vessels are fenestrated and allow the movement of molecules between the blood and basement membrane) are impermeable to molecules as small as sucrose even when blood vessels first penetrate the brain (**Figure 3**). The observed drop in the concentration ratios can be explained by the initial rapid increase in ventricular volume (occurring as part of normal brain development), which dilutes the entering permeability markers and a low rate of CSF secretion.

Table 1 | Tight junction protein genes enriched in mouse lateral ventricular choroid plexus.

Gene symbol	GenBank ID	Fold change
TRANSMEMBRANE		
(A)		
<i>Pcdh18</i>	BC052198	6.9
<i>Cdh5</i>	BC054790	4.3
<i>Cmtm3</i>	AY241870	4.0
<i>Cdh2</i>	AB008811	3.4
<i>Jam3</i>	BC024357	3.2
<i>Cldn11</i>	BC021659	2.0
(B)		
<i>Igfsf5</i>	BC004806	9.6
<i>Cldn2</i>	BC085494	4.3
<i>Marveld3</i>	BC025851	4.2
<i>Cldn12</i>	BC024057	2.1

List of proteins known to be associated with tight junctions whose genes were up-regulated in either the embryo (A) or the adult (B) expressed as fold change compared to levels in other age. From Table 4 in Liddelow et al. (2012).

Subsequent opening of the inner ventricular system to the subarachnoid space and onset of CSF drainage via arachnoid villi (Jones, 1980; Jones and Sellars, 1982; Jones and Bucknall, 1988) with increasing CSF secretion add to further dilution of markers entering CSF. A similar process affects brain distribution of markers because of the presence of a transient CSF–brain barrier at the level of the neuroependymal cells lining the cerebral ventricles (Fossan et al., 1985; Møllgård et al., 1987).

PROTEIN PERMEABILITY

The protein concentration in fetal CSF is high compared to the adult (Dziegielewska and Saunders, 1988; Saunders et al., 1999). Some authors have interpreted this as evidence that brain barriers (both blood–CSF and blood–brain barrier) are immature in the embryo (Adinolfi et al., 1976; Adinolfi and Haddad, 1977; Ramey and Birge, 1979; Adinolfi, 1985). However, there is good experimental evidence that this high protein concentration is a result of transcellular transfer of plasma proteins across choroid plexus epithelial cells (Dziegielewska et al., 1980, 1991; Habgood et al., 1992; Knott et al., 1997; Liddelow et al., 2009, 2011a) reinforced by the slow turnover of CSF in the developing brain (Bass and Lundborg, 1973; Johanson and Woodbury, 1974) which would be expected to allow proteins entering the CSF via the choroid plexuses to accumulate to a greater extent than in the adult (Johansson et al., 2008). These authors also provide a novel

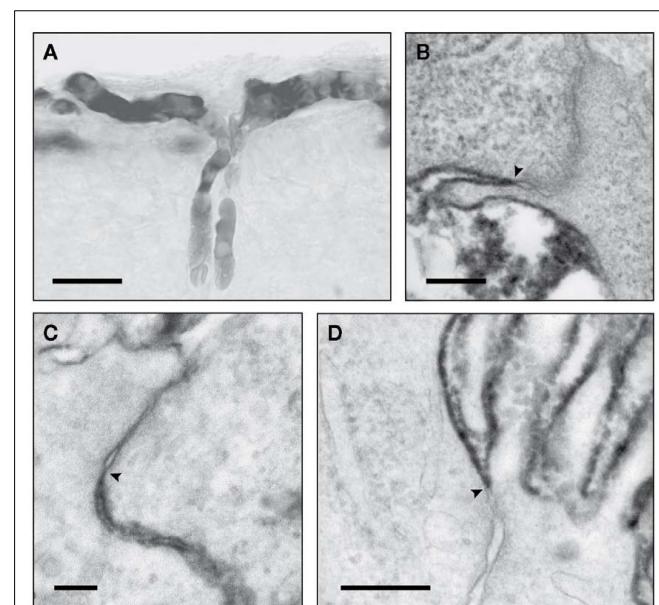


FIGURE 3 | Light (LM) and electronmicrographs (EM) of early developing brain blood vessels and choroid plexus, illustrating functional effectiveness of tight junctions. **(A)** (LM) and **(B)** (EM) of newborn opossum injected intraperitoneally (i.p.) with 3 kDa biotin dextran. Reaction product confined to vessel lumen **(A)** not passing through tight junctions [**(B**, arrowhead]. **(C)** Newborn opossum choroid plexus injected i.p. with 3 kDa biotin dextran which does not pass through tight junction (arrowhead). **(D)** E15 rat, tracer injected into lateral ventricle. Choroid plexus tight junctions (arrowhead): no passage of tracer between cells. Images: **(A,B)** from Ek et al. (2006); **(C,D)** from Ek et al. (2003). Scale bars: **(A)**, 25 μ m; **(B)**, 200 nm; **(C)**, 100 nm; **(D)**, 300 nm.

interpretation of the results by pointing out that the content of protein in the CSF should be taken into account rather than concentration. Concentration is a function of amounts and the volume in which the protein is distributed. Changes in the volume of distribution would change the concentration without altering the actual amount (see previous section). A general and approximate calculation comparing the amounts of protein in fetal and adult CSF of several species demonstrated that in adult CSF there is actually more protein than in the fetus in spite of CSF protein concentration being much higher in the younger brain (Johansson et al., 2008).

PROTEIN TRANSPORT FROM BLOOD TO CSF ACROSS THE CHOROID PLEXUSES

Transcellular transfer of plasma proteins was first described over 30 years ago (Dziegielewska et al., 1980). It was apparent from the outset that the transfer exhibited a striking degree of developmentally regulated specificity. In particular it was shown that the level of transport of albumin depended upon the species of albumin and the animal species in which it was studied. It is important to make the distinction between proteins made by the choroid plexus itself, e.g., transthyretin. (Schrieber et al., 1990), and those proteins transferred between the blood and the CSF. However, transthyretin is also transferred from blood plasma (Dziegielewska et al., 1980). Thus in the original experiments, fetal sheep (E60, term is 150 days) transferred their own albumin and bovine albumin to the same steady state level, whereas human and chicken albumins appeared to be discriminated to the extent that their steady state CSF/plasma concentration ratios were about half that of native albumin. A similar phenomenon has been described for albumins in embryonic and neonatal rats (Habgood et al., 1992; Johansson et al., 2006) postnatal opossum (Knott et al., 1997) and in postnatal mice (unpublished). A common characteristic of these experiments is that later in development the species specificity for different albumins disappears (Dziegielewska et al., 1980; Habgood et al., 1992; Knott et al., 1997) but it could also be experimentally abolished by chemical modification of the protein (Habgood et al., 1992; Knott et al., 1997).

Recent application of physiological and molecular techniques to the study of specific protein transport in the choroid plexus revealed that the plexus epithelial cells contain a number of receptor-/protein-binding-like molecules that have an affinity for albumin and may be the mechanism by which protein is transferred from blood to CSF (Liddelow et al., 2011b). Three genes: *Sparc*, Glycophorin A (*Gypa*), and C (*Gyc*), were identified as those whose gene products are candidates to target plasma proteins to choroid plexus cells. *Sparc* and *Gypa* were identified by immunocytochemistry in choroid plexus epithelial cells in the embryo, subcellular distribution consistent with transport of albumin from blood to CSF, as is illustrated in Figure 4. In adult plexus this pattern of immunostaining was absent. This mechanism has been shown to be more specific for individual plasma proteins early in brain development and responsive to changes in concentrations of proteins in plasma presumably as part of a normal homeostatic mechanism (Liddelow et al., 2009). In contrast to the one-way (blood to CSF) transport of proteins across the choroid plexus epithelial cells, inert dextrans are transported in both

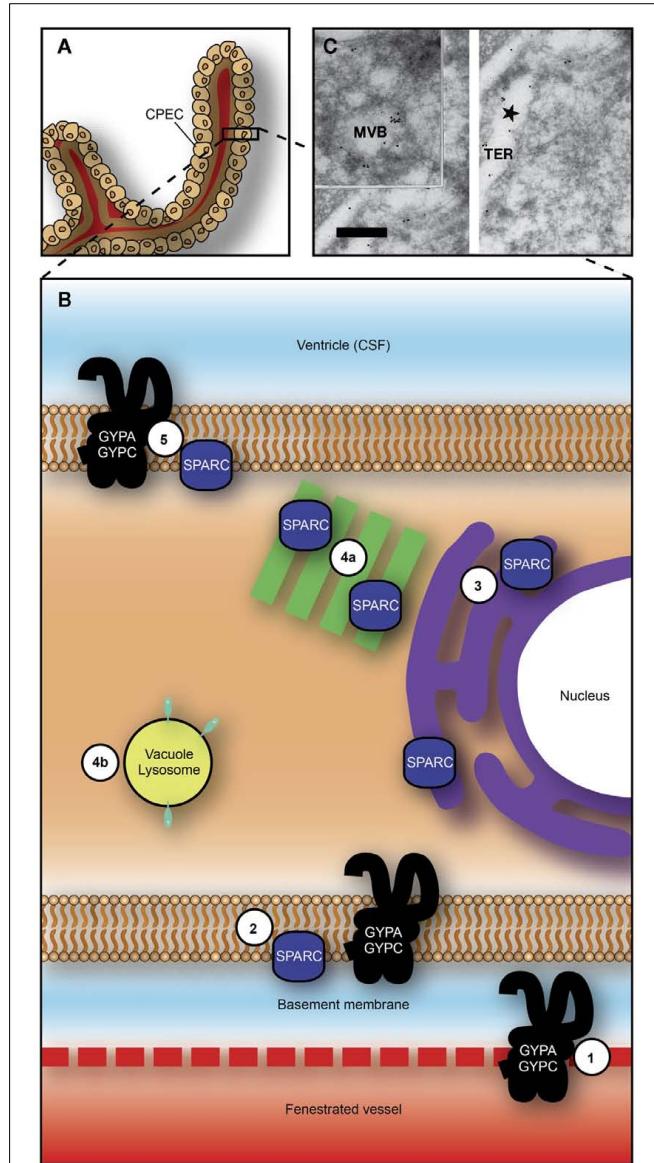


FIGURE 4 | Proposed transepithelial pathway for albumin through choroid plexus epithelial cells. **(A)** Whole choroid plexus showing single layer of epithelial cells sitting on thick basement membrane (see also Figure 1C). **(B)** Suggested routes of albumin from plasma into CSF across the choroid plexus epithelium. GYPA/C in endothelial cells may deliver albumin to basement membrane (1) from where it can be taken up into plexus epithelium by GYPA/C or SPARC (2). Albumin may then travel along a SPARC-specific pathway through tubulocisternal endoplasmic reticulum [3, and see (C)] and Golgi (4a), or via a VAMP-mediated pathway in vacuoles, lysosomes, or multivesicular bodies [4b, and see (C)]. On apical surface of plexus epithelium, GYPA/C may be involved in efflux of protein from the cell into CSF (5). In adult, lack of immunoreactivity in endoplasmic reticulum and Golgi and increased expression of gene products for VAMP molecules, suggest that majority of transport occurs via VAMP-mediated vesicular transport (4b). **(C)** Transmission electron micrograph of ultracryosection from E60 fetal sheep choroid plexus (Balslev et al., 1997b). Immunolabeled human albumin 6 nm particles and sheep albumin 12 nm gold particles are shown to co-localize within the tubulocisternal endoplasmic reticulum. Abbreviations: CPEC, choroid plexus epithelial cell; CSF, cerebrospinal fluid; GYPA, glycophorin A; GYPC, glycophorin C; MVB, multivesicular body; TER, tubulocisternal endoplasmic reticulum. Scale bar: 0.2 μm in (C). Image from Liddelow et al. (2012).

directions (blood to CSF and CSF to blood) with twice as many cells staining for dextrans when administered intraventricularly than intraperitoneally (**Figure 5**).

The proteins transported into CSF across the choroid plexuses have been suggested to have three functions in brain development: (i) some of the proteins are taken up by neural cells present in the ventricular zone that are in contact with the CSF and may have some specific involvement in features of brain development such as mitosis, migration, and differentiation (Stolp et al., 2011), (ii) they may act as carriers for growth factors, hormones and vitamins, (iii) the high concentration of proteins in CSF early in brain development may exert a colloid osmotic pressure within the ventricles, thus promoting fluid transfer across the choroid plexuses and contributing to developmental expansion of the ventricles (essential

for normal brain growth, see Saunders et al., 1999). However, it also needs to be considered that proteins such as albumin are known carriers of heavy metals and drugs, thus in the presence of such toxic molecules this normal mechanism may render the developing brain more vulnerable to such agents (Saunders et al., 2010; Ek et al., 2012).

UPTAKE FROM CSF INTO BRAIN

Once proteins have transferred across the choroid plexus into CSF, some are taken up into cells in the brain. For example some neuroependymal cells lining the cerebral ventricles take up proteins such as albumin and the fetal protein fetuin (Dziegielewska et al., 2000). The initial cells that form the first layers of the neocortex in the embryo take up fetuin via apical dendrites that make contact

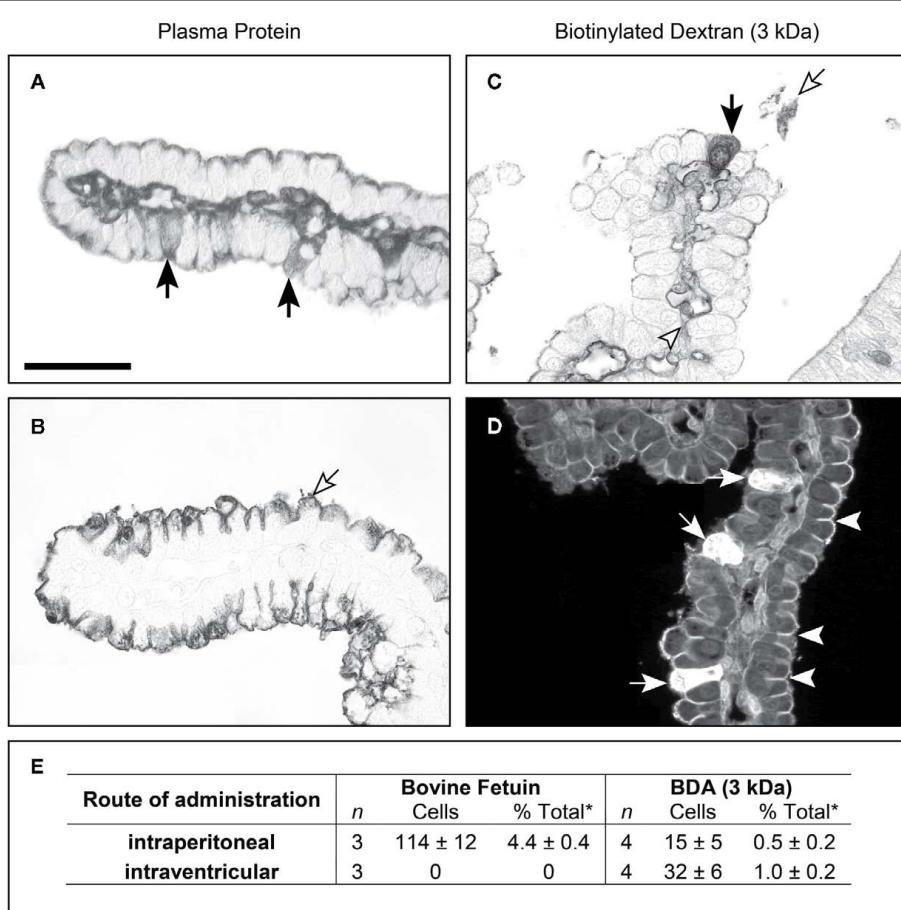


FIGURE 5 | Cellular localization of fetuin and inert biotin dextran (3 kDa) in postnatal (P9) *Monodelphis domestica*. Light micrograph showing the localization of bovine fetuin (**A,B**) detected with its antibodies and biotin dextran (**C,D**) detected with ABC (ABC kit, Vector Laboratories), in coronal sections of lateral ventricular choroid plexus. (**A**) Twenty-four hours after intraperitoneal injection of bovine fetuin, specific epithelial cells of the plexus (filled arrows) were found containing the protein. (**B**) Bovine fetuin was injected into the lateral ventricle and left for 10 min. The protein was not detected in any cells of the plexus, or in the lumen of blood vessels in the plexus stroma. Protein can be seen on the CSF side of the epithelial cells, precipitated on the brush border (unfilled arrow). (**C**) Forty-five minutes after intraperitoneal injection with BDA (3 kDa), the probe can be seen in specific epithelial cells of the choroid plexus (filled arrow), as well as in the blood

vessel lumen (arrowhead) and precipitated in the CSF (unfilled arrow). (**D**) Ten minutes after intraventricular injection with Fluorescein-conjugated BDA (3 kDa), more epithelial cells take up the probe (filled arrows) following CSF injection compared with intraperitoneal injection (**C**). Penetration of the fluorescent probe between epithelial cells is stopped by the presence of tight junctions (examples highlighted by arrowheads). (**E**) Uptake of bovine fetuin and BDA (3 kDa) into choroid plexus epithelial cells in P9 *Monodelphis* following intraperitoneal or intracerebroventricular injection; mean \pm SEM, numbers of immunostained cells. P9 *Monodelphis* injected with fetuin and BDA (3 kDa), biotinylated dextran amine MW 3 kDa. Scale: 50 μ m (**A–D**). From Liddelow et al. (2009) Figure 3 and Table 7.

with the dorsal surface of the cortex (Dziegielewska et al., 2000). However, this uptake has been little studied and it is not clear whether the proteins themselves are functionally important or bound ligands such as hormones and growth factors. A recent publication indicated that the number of plasma protein positive cells in the ventricular zone of a fetal mouse can be increased following an inflammatory response of the dam indicating that protein uptake into the brain can be physiologically responsive to its changing environment of Stolp et al. (2011).

INFLUX MECHANISMS ACROSS BRAIN BARRIERS IN THE DEVELOPING BRAIN

AMINO ACID TRANSPORT

Davson (1967) summarized the available evidence on exchange of metabolically important materials between blood and brain in developing animals. On the one hand he accepted Bakay's (1953) results using ^{32}P , which showed decreasing uptake with age, as a qualitative indication of the state of the blood–brain barrier. On the other hand he pointed out that greater metabolic incorporation in the developing brain would also contribute to the experimental findings. Bakay (1953) himself discussed both mechanisms and reconciled them by suggesting that his results indicated the presence of a blood–brain barrier in the fetus (rabbit) but that it was more permeable than in the adult. There were similar discussions of the results of experiments studying the entry of amino acids into the developing brain (Himwich et al., 1957; Roberts et al., 1959; Purpura and Carmichael, 1960; Lajtha and Toth, 1961; Seta et al., 1972; Baños et al., 1978) in which the entry of several

amino acids was found to be greater in younger animals than in the adult. Kuttner et al. (1961) attempted to get round the difficulty of distinguishing between cerebral endothelial cell transport and metabolic incorporation into brain tissue by studying the uptake of α -aminoisobutyric acid, which is transported but is metabolically inert. They reported a much greater uptake of this amino acid in neonatal rabbits compared to adults but interpreted this as indicating “lesser effectiveness” of the blood–brain barrier for amino acids in young animals; this interpretation was reiterated by others (e.g., Lee, 1971). Later studies using the Oldendorf (1971) short pass technique or modifications of the method, allowed separation of entry into the brain from metabolic incorporation. This was done by exposing the cerebral circulation of developing animals to test amino acids and other metabolically active compounds for only a brief period. These experiments showed that many amino acids and other metabolically active compounds were transported into the developing brain at much higher rates than in the adult and was interpreted as reflecting the greater metabolic demand of the developing brain rather than immaturity of the blood–brain barrier (Braun et al., 1980; Cornford et al., 1982; Pardridge and Mietus, 1982; Lefauconnier and Trouvé, 1983). However, some authors have continued to suggest that this greater uptake may reflect barrier immaturity (see Watson et al., 2006).

It is now clear that the transport mechanisms in the brain barrier interfaces determine the composition of the internal environment of developing brain and supply essential nutrients and other molecules important for growth and differentiation of the brain. **Figure 6** summarizes which inward (blood–brain) transport

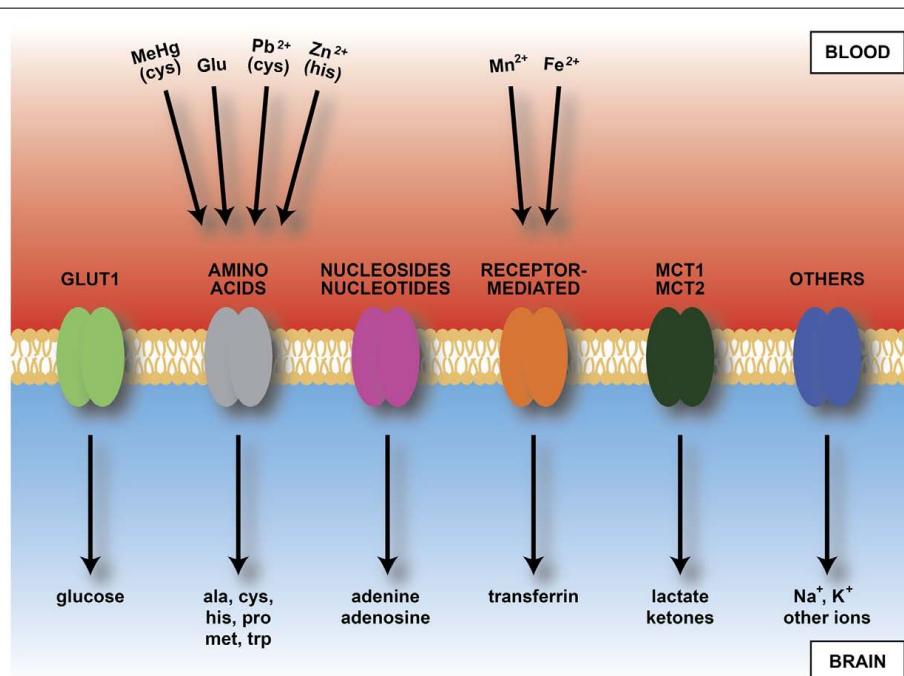


FIGURE 6 | Diagram of main inward transporters in cerebral endothelial cells. Heavy metals bind to some amino acids and transferrin receptors. Because of vulnerability of developing brain to heavy metals this transport may contribute to fetal or newborn neurotoxicity. Compare **Table 2**, which shows transporters expressed in endothelial cells from

developing brain, including those up-regulated compared to adult.
Abbreviations: ala, alanine; cys, cysteine; Fe²⁺, iron; Glu, glutamate; his, histidine; MCT, monocarboxylate transporter; MeHg, methyl mercury; met, methionine; Mn²⁺, manganese; Pb²⁺, lead; pro, proline; trp, tryptophan; Zn²⁺, zinc.

mechanisms have been shown to be functional in the fetal and newborn brain. Much of the evidence for function of many of these mechanisms was published some time ago and is outlined above (see also reviews by Saunders, 1992; Saunders et al., 2010; Saunders and Habgood, 2011; Liddelow et al., 2012).

The information that has been lacking until recently is that on the presence and expression level of transporters in the brain barrier interfaces in the developing brain. This is now available from expression studies of isolated cerebral endothelial cells from neonatal mice (Daneman et al., 2010a) and from choroid plexus of fetal mice compared to the adult (Liddelow et al., 2012). **Table 2** (from Daneman et al., 2010a) shows a summary of influx transporters that are up-regulated in the neonatal mouse compared to

the adult. **Table 3** shows similar data from fetal mouse choroid plexus. It is technically difficult to demonstrate how functionally active these genes are because embryos of common experimental animals are very small. In newborn rats and rabbits several amino acid transporters were shown to be functional at a higher rate than in the adult (Saunders and Habgood, 2011). Another example of greater transport in developing brain is inward transport of phosphorylated lysosomal enzymes by the transporter M6P/IGF2R in newborn mice. This transport is progressively lost with age and by adulthood is undetectable (Urayama et al., 2008). The findings also help to explain a number of important previous observations on developmentally different effects of amino acids on brain function. For example it was reported many years ago that glutamate is toxic to the brain if administered in the neonatal period (Olney and Ho,

Table 2 | Expression of transporters in endothelial cells from neonatal mouse brain.

Gene ID	Description
BBB ENRICHED INFLUX TRANSPORTERS	
(A)	
Slc1a1	Glutamate
Slc1a4	Alanine, serine, cysteine, threonine
Slc2a1	Glucose
Slc6a6	Taurine, β -alanine
Slc6a17	AAs, betaine, taurine, creatine
Slc7a1	Cationic AAs
Slc7a3	Cationic AAs
Slc7a5	Neutral AAs
Slc12a6	K^+ / Cl^-
Slc16a1	Monocarboxylates
Slc16a2	Thyroid hormones
Slc16a4	Monocarboxylates
Slc19a3	Thiamine
Slc25a20	Acylcarnitines
Slc25a33	Putative
Slc30a1	Zinc
Slc31a1	Copper
Slc35f2	Putative
Slc38a3	Glutamate, Na^+
Slc38a5	Neutral AAs
Slc39a10	Zinc
Slc40a1	Iron
Slc46a3	Putative
Slco1a4	Organic anions (e.g., bile acids)
Slco1c1	Thyroid hormones
Slco2b1	Prostaglandins, organic anions
(B)	
Abca5	
Abcb1a	Multidrug resistance protein 1
Abcc4	Multidrug resistance associated protein 4
Abcg2	Breast cancer resistance protein

(A) Influx transporters. Up-regulated glutamate transport probably explains neuro-toxic effects of glutamate in immature brain, rather than barrier deficiency (compare **Figure 6**). (B) Efflux transporters (compare **Figure 7**). Data from Daneman et al. (2010a). AA, amino acid.

Table 3 | Expression of influx transporters in embryonic mouse choroid plexus.

Gene symbol	GenBank ID	Fold change
SOLUTE CARRIERS		
(A)		
Slc16a10	BC052877	66.8
Slc6a15	AY149280	11.4
Slc40a1	AF231120	9.6
Slc7a11	AY766236	7.1
Slc4a1	BC053429	5.5
Slc6a13	BC029637	4.6
Slc1a4	BC043483	4.4
Slc38a4	AY027919	4.2
Slc6a6	L03292	4.1
Slc4a4	AF141934	4.1
Slc7a1	M26687	4.1
Slc39a8	BC006731	3.3
(B)		
Slc5a5	AF235001	13.6
Slc39a4	BC023498	9.6
Slc41a2	NM_177388	8.5
Slc24a4	AY156046	7.8
Slc28a3	BC013783	6.9
Slc24a5	AB085629	6.1
Slc9a7	BC058750	5.8
Slc6a17	AY155578	3.5
Slco1c1	AY007379	5.2
Slc4a10	AK220501	5.0
Slc39a14	AB177995	4.0
Slc35f3	BC115965	3.9
Slc13a4	BC089161	3.9
Slc37a2	AF121081	3.5
Slco1a5	AF240694	3.4
Slc39a12	BC089362	3.3
Slc46a1	BC057976	3.2
Slc25a35	BC019996	3.1
Slc22a5	AF110417	3.0

Solute carriers up-regulated in either the embryo (A) or the adult (B). From Table 5 in Liddelow et al. (2012).

Table 4 | Comparison of expression of influx transporters in mouse E15 choroid plexus and published reports on transport function in the developing brain.

Transporter	Transport function
<i>Slc16a10</i>	Iodothyronines T3, T4 ¹
<i>Slc6a15</i>	Neutral amino acids ²
<i>Slc40a1*</i>	Iron ³
<i>Slc7a11</i>	Cysteine, glutamate ²
<i>Slc4a1</i>	Anion transporter ⁴ , ($\text{Cl}^- - \text{HCO}_3^-$ exchange) ⁵
<i>Slc6a13</i>	GABA transporter ⁶
<i>Slc1a4</i>	Glutamate, neutral amino acids ⁷
<i>Slc38a4</i>	Acidic and neutral amino acids ^{2,7}
<i>Slc6a6</i>	Taurine ²
<i>Slc4a4</i>	$\text{Na}^+ - \text{HCO}_3^-$ cotransporter ⁴
<i>Slc7a1</i>	Acidic amino acids ²
<i>Slc39a8</i>	Zinc transporter ⁸

Only *Slc4a4*, *Slc7a11*, and *Slc40a1* have previously been identified in choroid plexus. Superscript numbers indicate published studies showing transport into developing brain or CSF. *Gene product ferroportin-1 identified in choroid plexus.

¹Porterfield and Hendrich (1992), ²Lefauconnier and Trouvé (1983), ³Morgan and Moos (2002), ⁴Damkier et al. (2010), ⁵Amtorp and Sørensen (1974), ⁶Al-Sarraf (2002), ⁷Al-Sarraf et al. (1997), ⁸Chowanadisai et al. (2005). From Table 6 in Liddelow et al. (2012).

1970) which some attributed to “immaturity” of the blood–brain barrier (Viña et al., 1997). However, it can now be seen that the barrier contribution to toxicity is much more likely to be due to greater transport by, e.g., *Slc1a4*, see **Table 4**, which summarizes data on expression of influx transporters and published reports on transport function in the developing brain.

AQUAPORINS

There is also good evidence for appearance of the key water channel aquaporin-1 in very early choroid plexus epithelial cells (Johansson et al., 2005). The development of ion gradients between CSF and plasma in the fetal brain suggests that at least some ion pumps are active across the blood–CSF barrier (see Saunders, 1992). Molecular expression studies in embryonic choroid plexus confirm that some ion exchange mechanisms are present early in development (Johansson et al., 2007; Liddelow et al., 2012) and probably also across the blood–brain barrier early in development (Daneman et al., 2010a). Little is known of what happens if these mechanisms become dysfunctional or develop abnormally.

EFFLUX MECHANISMS ACROSS BRAIN BARRIERS IN THE DEVELOPING BRAIN

An important mechanism in the adult brain at both the blood–brain and blood–CSF barriers are ATP-binding cassette (ABC) efflux transporters (Hartz and Bauer, 2011). These transporters are summarized in **Figure 7**. They exclude a large number of toxic but also potentially therapeutic compounds from the brain; thus knowledge of their presence and effectiveness in the developing brain is essential for assessing what risk drugs and toxins may pose. Immunohistochemical studies of P-glycoprotein in human fetuses report its presence in cerebral endothelial cells as early as

Table 5 | Efflux (ABC) transporter expression in embryonic (A) and adult (B) mouse choroid plexus.

Gene	Other IDs	Array	qPCR
(A)			
<i>Abcb3</i>	TAP2	–	16.5
<i>Abcb6</i>	UMAT, MTABC3	2.6	8.1
<i>Abcg2</i>	BCRP	–	15.8
<i>Abcg5</i>	Sterolin1, White3	14.1	44.8
<i>Abcg8</i>	Sterolin2, White4	3.4	20.3
(B)			
<i>Abca2</i>		3.5	22.4
<i>Abca4</i>	ABCR, RP19, RIM	9.7	12.9
<i>Abca5</i>		2.5	1.4
<i>Abca7</i>	ABCX	4.5	–
<i>Abcb9</i>	TAPL	2.8	1.8
<i>Abcc1</i>	MRP, MRP1	2.3	–

Most enriched genes during development of the mouse lateral ventricular choroid plexus in the embryo or adult. Expression of a further 35 genes was detected at both ages with no difference in expression levels (not shown). Array targets were considered enriched with fold changes equal or greater than 2. From Table 7 in Liddelow et al. (2012).

8 weeks gestation (Schumacher and Møllgård, 1997). Expression and immunohistochemical studies of brain and choroid plexuses in embryonic and adult rats (Ek et al., 2010) have shown that known key efflux transporters, multidrug resistance-associated proteins 1 and 4, P-glycoprotein and breast cancer resistance protein (BCRP) are expressed early in both brain and in choroid plexus epithelial cells and that their expression is differentially regulated both with respect to individual efflux transporters and age. It is particularly striking that BCRP is expressed at the highest level in embryonic rat choroid plexus (20-fold compared to adult). In the brain practically no change in expression level for BCRP between the embryo and adult was found (Ek et al., 2010). Many of these efflux mechanisms are present and functionally effective in the placenta (see Saunders et al., 2010). Data on efflux transporters in embryonic and adult mouse cerebral endothelial cells and choroid plexus are summarized in **Tables 3B** and **5**. Little is known about the function of efflux transporters in the human newborn brain, but it may be that because of the loss of the protection provided by the placenta, after birth the neonatal brain may be more vulnerable to entry of drugs and toxins.

NEUROPATHOLOGY OF BARRIER MECHANISMS IN THE DEVELOPING BRAIN

INFLAMMATION

The blood vessels in the developing brain are undoubtedly more fragile than in the adult, which probably explains many of the claims of barrier immaturity or leakiness, stemming from experiments in which excessive volumes of fluid have been injected into fetuses (see Saunders, 1992 for review). A specific example of susceptibility of cerebral blood vessels in the developing brain is the effect of lipopolysaccharides on permeability of blood vessels in white matter at a critical stage of brain development

(Stolp et al., 2005a,b). These blood vessels show a leakage of plasma proteins, demonstrated in postnatal rats and opossums at a stage of brain development equivalent to 22–28 weeks gestation in humans (Stolp and Dziegielewska, 2009). In a clinical context if a mother develops an infection, the fetus may be born prematurely and in some cases white matter damage has been observed with the development of cerebral palsy (Dammann and Leviton, 1997; Yoon et al., 2000). Leakage of proteins from plasma into white matter in the presence of uterine infection has been suggested to be part of the etiology (Stolp and Dziegielewska, 2009). The possible role of barrier dysfunction in the developing brain and subsequent development of neurological/neuropsychiatric disorders such as schizophrenia, Alzheimer's disease and multiple sclerosis has been reviewed (Stolp and Dziegielewska, 2009). Most studies seem to have concentrated on changes in permeability to large or small molecules and in properties of tight junctions. The possibility of functionally important changes in influx (Figure 6) or efflux (Figure 7) mechanisms following a pathological insult to the developing brain scarcely seems to have been considered.

KERNICTERUS

Ek et al. (2012) have discussed some possible reasons for the persisting belief amongst for, example neuropathologists and some physiologists and pediatricians that the blood–brain barrier in the developing brain is immature or “leaky.” A general reason was given by Barcroft (1938) who expressed the view “There is no reason why the brain of the embryo should require an environment of very great chemical constancy. It will of course require a certain minimum of the various materials necessary for growth, but

otherwise on first principles we might suppose that the good things of life may exist in and may vary in the fetal blood to an extent much greater than the maternal.” Bakay (1956) expressed a similar view. However, teleological arguments are hardly a rigorous way of determining the mechanism of physiological functions. It seems likely that the proposal that the blood–brain barrier in the fetus and newborn is immature stems in part from a view that prevailed in the middle of the last century that kernicterus (brain damage from excess unconjugated bilirubin in the circulating blood) occurred in prematurely born infants but less commonly in term babies and never in adult, because of immaturity of the blood–brain barrier (e.g., Bakay, 1953; Lee, 1971). This appears to have been first proposed by Spatz (1934, cited by Davson, 1967). Once it became realized that unconjugated bilirubin binds to plasma albumin it was increasingly appreciated that a key determinant of whether or not kernicterus occurred was whether or not this binding capacity was exceeded (e.g., Bakay, 1968). However, some have continued to cite claims of immaturity of the blood–brain barrier to small molecules as contributing to kernicterus in newborn infants (Barrett et al., 2010). This overlooks the fact that unconjugated bilirubin is lipid soluble and will enter the brain unless bound to plasma albumin. Also greater permeability to small molecules in the immature brain is apparent rather than real as it is due to slow turnover of CSF (see Apparent Increased Permeability of Barriers in Developing Brain above). The commonest cause of kernicterus is erythroblastosis fetalis when excessive amounts of bilirubin are generated from breakdown of incompatible red blood cells in the case of a Rhesus positive baby in a Rhesus negative mother. This tends to be more frequent and more severe in prematurely born infants. However,

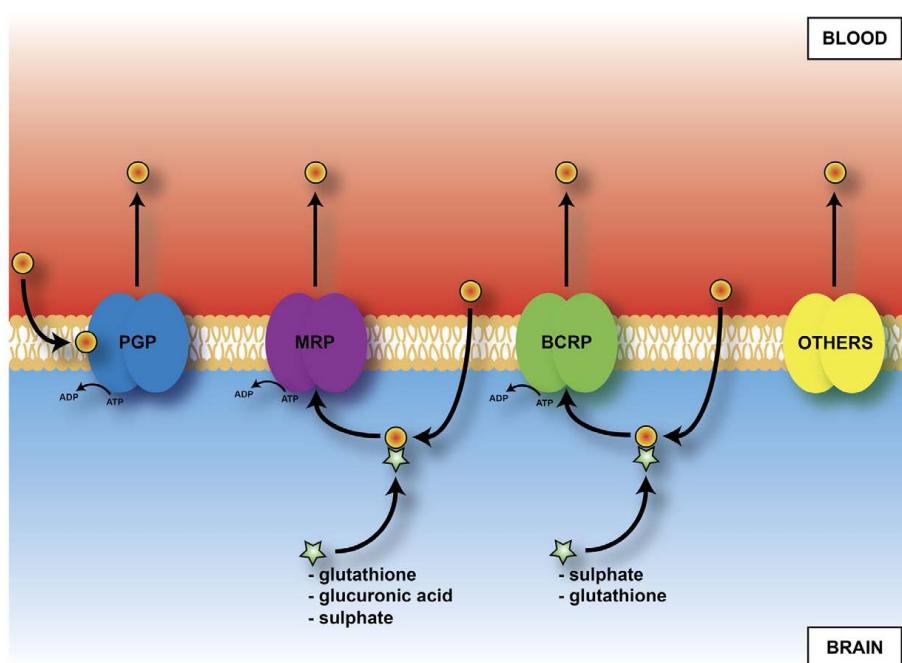


FIGURE 7 | Diagram of main outward transporters in cerebral endothelial cells. Some, e.g., PGP (P-glycoprotein) prevent entry. For others, e.g., MRP

(multidrug resistance-associated protein), ligand (drug or toxin) combines with glutathione, glucuronic acid or sulfate in cells before efflux.

there seems to be no clear-cut level of unconjugated bilirubin at which all babies will, or will not suffer from kernicterus and it has become increasingly clear that factors in addition to bilirubin binding to albumin are involved (Wennberg, 2000; Wennberg et al., 2006). Although immaturity of the blood–brain barrier in the sense of “leakiness” is not one of these, there are other brain barrier mechanisms that may contribute. Thus bilirubin is a substrate for the efflux transporter P-glycoprotein and possibly also other efflux transporters (Yokooji et al., 2010; Gazzin et al., 2011). Although P-glycoprotein is expressed in fetal brain endothelial cells early in gestation its levels are higher in the adult (Schumacher and Møllgård, 1997; Virgintino et al., 2008; Ek et al., 2010) so it is possible that the efflux capacity of P-glycoprotein is exceeded in the presence of high levels of unconjugated bilirubin. Other efflux transporters are present in fetal cerebral endothelial cells and choroid plexus that are expressed at higher levels in the fetus than in the adult (Ek et al., 2010, see previous section) but it is not clear if bilirubin is also a substrate for these transporters. Another factor is that as described above there is a developmentally regulated transport of albumin from blood to CSF across the epithelial cells of the choroid plexus in the fetal brain. This albumin would presumably carry any bound unconjugated bilirubin into the CSF and thence the

brain unless removed by the efflux transporters in the choroid plexuses.

CONCLUSION

Recent evidence confirms that the brain develops within a well-controlled internal environment. Tight junctions and many of the transport mechanisms (both inward and outward) are already present in the cellular interfaces between the blood, brain, and CSF, very early in development. Some properties of these barrier mechanisms and their susceptibility to disruption may lead to brain damage and later neurological disorders. We hope that this review will contribute to laying to rest the myth of the “leaky” or “immature” blood–brain barrier and focus attention on the need to understand better the level of function of barrier mechanisms that protect the brain from exposure to drugs and toxins, so that clinical advice will be based on the reality of evidence rather than teleological belief.

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REFERENCES

- Abbott, N. J., Patabendige, A. A., Dolman, D. E., Yusof, S. R., and Begley, D. J. (2010). Structure and function of the blood–brain barrier. *Neurobiol. Dis.* 37, 13–25.
- Abbott, N. J., Rönnbäck, L., and Hanssonet, E. (2006). Astrocyte–endothelial interactions at the blood–brain barrier. *Nat. Rev. Neurosci.* 7, 41–53.
- Adinolfi, M. (1985). The development of the human blood–CSF–brain barrier. *Dev. Med. Child Neurol.* 27, 532–537.
- Adinolfi, M., Beck, S. E., Haddad, S. A., and Seller, M. J. (1976). Permeability of the blood–cerebrospinal fluid barrier to plasma proteins during foetal and perinatal life. *Nature* 259, 140–141.
- Adinolfi, M., and Haddad, S. A. (1977). Levels of plasma proteins in human and rat fetal CSF and the development of the blood–CSF barrier. *Neuropadiatrie* 8, 345–353.
- Al-Sarraf, H. (2002). Transport of ¹⁴C-gamma-aminobutyric acid into brain, cerebrospinal fluid and choroid plexus in neonatal and adult rats. *Brain Res. Dev. Brain Res.* 139, 121–129.
- Al-Sarraf, H., Preston, J. E., and Segal, M. B. (1997). Changes in the kinetics of the acidic amino acid brain and CSF uptake during development in the rat. *Brain Res. Dev. Brain Res.* 102, 127–134.
- Amtorp, O., and Sørensen, S. C. (1974). The ontogenetic development of concentration differences of protein and ions between plasma and cerebrospinal fluid in rabbits and rats. *J. Physiol.* 243, 387–400.
- Armulik, A., Genové, G., Mæe, M., Nisancioglu, M. H., Wallgard, E., Niaudet, C., He, L., Norlin, J., Lindblom, P., Strittmatter, K., Johansson, B. R., and Betsholtz, C. (2010). Pericytes regulate the blood–brain barrier. *Nature* 468, 557–561.
- Armulik, A., Genové, G., and Betsholtz, C. (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* 21, 193–215.
- Bakay, L. (1953). Studies on blood–brain barrier with radioactive phosphorus. III. Embryonic development of the barrier. *AMA Arch. Neurol. Psychiatry* 70, 30–39.
- Bakay, L. (1956). “Development of the blood–brain barrier,” in *The Blood–Brain Barrier, with Special Regard to the use of Radioactive Isotopes*, Chap. 7, ed. L. Bakay (Springfield, IL: Thomas), 77–149.
- Bakay, L. (1968). Changes in barrier effect in pathological states. *Prog. Brain Res.* 29, 315–339.
- Balslev, Y., Saunders, N. R., and Møllgård, K. (1997a). Ontogenetic development of diffusional restriction to protein at the pial surface of the rat brain; an electron microscopical study. *J. Neurocytol.* 26, 133–148.
- Balslev, Y., Dziegielewska, K. M., Møllgård, K., and Saunders, N. R. (1997b). Intercellular barriers to and transcellular transfer of albumin in the fetal sheep brain. *Anat. Embryol.* 195, 229–236.
- Baños, G., Daniel, P. M., and Pratt, O. E. (1978). The effect of age upon the entry of some amino acids into the brain, and their incorporation into cerebral protein. *Dev. Med. Child Neurol.* 20, 335–346.
- Bär, T. H., and Wolff, J. R. (1972). The formation of capillary basement membranes during internal vascularization of the rat's cerebral cortex. *Z. Zellforsch. Mikrosk. Anat.* 133, 231–248.
- Barber, A. J., and Lieth, E. (1997). Agrin accumulates in the brain microvascular basal lamina during development of the blood–brain barrier. *Dev. Dyn.* 208, 62–74.
- Barcroft, J. (1938). *The Brain and its Environment. 1. The Activity of the Brain in Mid-Foetal Life*. New Haven: Yale University Press.
- Barrett, K. E., Barman, S. M., Boitano, S., and Brooks, H. (2010). *Ganong's Review of Medical Physiology*, 23rd Edn. New York: McGraw Hill, 530.
- Bass, N. H., and Lundborg, P. (1973). Postnatal development of bulk flow in the cerebrospinal fluid system of the albino rat: clearance of carboxyl-(14C)inulin after intrathecal infusion. *Brain Res.* 52, 323–332.
- Bass, T., Singer, G., Slusser, J., and Liuzzi, F. J. (1992). Radial glial interaction with cerebral germinal matrix capillaries in the fetal baboon. *Exp. Neurol.* 118, 126–132.
- Bauer, H., Sonnleitner, U., Lametschwandtner, A., Steiner, M., Adam, H., and Bauer, H. C. (1995). Ontogenetic expression of the erythroid-type glucose transporter (Glut 1) in the telencephalon of the mouse: correlation to the tightening of the blood–brain barrier. *Brain Res. Dev. Brain Res.* 86, 317–325.
- Bauer, H. C., Bauer, H., Lametschwandtner, A., Amberger, A., Ruiz, P., and Steiner, M. (1993). Neovascularization and the appearance of morphological characteristics of the blood–brain barrier in the embryonic mouse central nervous system. *Brain Res. Dev. Brain Res.* 75, 269–278.
- Behnsen, G. (1927). Über die farbstoffspeicherung im zentralnervensystem der weissen maus in verschiedenen alterzuständen. *Z. Zellforsch. Mikrosk. Anat.* 4, 515–572.
- Biedl, A., and Kraus, R. (1898). Übereiner bisher unbekannte toxische Wirkung der Gallensauren auf das Zentralnervensystem. *Zentralblatt. Inn. Med.* 19, 1185–1200.
- Bito, L. Z., and Myers, R. E. (1970). The ontogenesis of haematoencephalic cation transport processes in the rhesus monkey. *J. Physiol.* 208, 153–170.

- Blasig, I. E., Winkler, L., Lassowski, B., Mueller, S. L., Zuleger, N., Krause, E., Krause, G., Gast, K., Kolbe, M., and Piontek, J. (2006). On the self-association potential of transmembrane tight junction proteins. *Cell. Mol. Life Sci.* 63, 505–514.
- Bradbury, M. W., Crowder, J., Desai, S., Reynolds, J. M., Reynolds, M., and Saunders, N. R. (1972). Electrolytes and water in the brain and cerebrospinal fluid of the foetal sheep and guinea-pig. *J. Physiol. (Lond.)* 227, 591–610.
- Braun, L. D., Cornford, E. M., and Oldendorf, W. H. (1980). Newborn rabbit blood-brain barrier is selectively permeable and differs substantially from the adult. *J. Neurochem.* 34, 147–152.
- Brightman, M. W., and Reese, T. S. (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* 40, 48–77.
- Caley, D. W., and Maxwell, D. S. (1970). Development of the blood vessels and extracellular spaces during postnatal maturation of rat cerebral cortex. *J. Comp. Neurol.* 138, 31–47.
- Canfield, A. E., Schor, A. M., Loskutoff, D. J., Schor, S. L., and Grant, M. E. (1989). Plasminogen activator inhibitor-type I is a major biosynthetic product of retinal microvascular endothelial cells and pericytes in culture. *Biochem. J.* 259, 529–535.
- Chowanadisai, W., Kelleher, S. L., and Lönnedal, B. (2005). Zinc deficiency is associated with increased brain zinc import and LIV-1 expression and decreased ZnT-1 expression in neonatal rats. *J. Nutr.* 135, 1002–1007.
- Cohen-Kashi Malina, K., Cooper, I., and Teichberg, V. I. (2009). Closing the gap between the in-vivo and in-vitro blood-brain barrier tightness. *Brain Res.* 1284, 12–21.
- Colegio, O. R., Van Itallie, C., Rahner, C., and Anderson, J. M. (2003). Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture. *Am. J. Physiol. Cell Physiol.* 284, C1346–C1354.
- Cornford, E. M., Braun, L. D., and Oldendorf, W. H. (1982). Developmental modulations of blood-brain barrier permeability as an indicator of changing nutritional requirements in the brain. *Pediatr. Res.* 16, 324–328.
- Costa, L. G., Aschner, M., Vitalone, A., Syversen, T., and Soldin, O. P. (2004). Developmental neuropathology of environmental agents. *Annu. Rev. Pharmacol. Toxicol.* 44, 87–110.
- Damkier, H. H., Brown, P. D., and Praetorius, J. (2010). Epithelial pathways in choroid plexus electrolyte transport. *Physiology* 25, 239–249.
- Dammann, O., and Leviton, A. (1997). Maternal intrauterine infection, cytokines and brain damage in the preterm newborn. *Pediatr. Res.* 42, 1–8.
- Daneman, R., Agalliu, D., Zhou, L., Kuhner, F., Kuo, C. J., and Barres, B. A. (2009). Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 106, 641–646.
- Daneman, R., Zhou, L., Agalliu, D., Cahoy, J. D., Kaushal, A., and Barres, B. A. (2010a). The mouse blood-brain barrier transcriptome: a new resource for understanding the development and function of brain endothelial cells. *PLoS ONE* 5, e13741. doi:10.1371/journal.pone.0013741
- Daneman, R., Zhou, L., Kebede, A. A., and Barres, B. A. (2010b). Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468, 562–566.
- Davson, H. (1967). *Physiology of the Cerebrospinal Fluid*. London: Churchill, 231–239.
- Dehouck, M.-P., Méresse, S., Delorme, P., Fruchart, J.-C., and Cecchelli, R. (1990). An easier, reproducible, and mass-production method to study the blood-brain barrier in vitro. *J. Neurochem.* 54, 1798–1801.
- del Zoppo, G. L., and Milner, R. (2006). Integrin–matrix interactions in the cerebral microvasculature. *Arterioscler. Thromb. Vasc. Biol.* 26, 1966–1975.
- Dermietzel, R., Krause, D., Kremer, M., Wang, C., and Stevenson, B. (1992). Pattern of glucose transporter (Glut 1) expression in embryonic brains is related to maturation of blood-brain barrier tightness. *Dev. Dyn.* 193, 152–163.
- Donahue, S., and Pappas, G. D. (1961). The fine structure of capillaries in the cerebralcortex of the rat at various stages of development. *Am. J. Anat.* 108, 331–347.
- Dziegielewska, K. M., Daikuhara, Y., Ohnishi, T., Waite, P. M. E., Ek, J., Habgood, M. D., Lane, M. A., Potter, A., and Saunders, N. R. (2000). Fetuin in the developing neocortex of the rat: distribution and origin. *J. Comp. Neurol.* 423, 373–388.
- Dziegielewska, K. M., Evans, C. A. N., Malinowska, D. H., Møllgård, K., Reynolds, J. M., Reynolds, M. L., and Saunders, N. R. (1979). Studies of the development of brain barrier systems to lipid insoluble molecules in fetal sheep. *J. Physiol.* 292, 207–231.
- Dziegielewska, K. M., Evans, C. A. N., Malinowska, D. H., Møllgård, K., Reynolds, M. L., and Saunders, N. R. (1980). Blood-cerebrospinal fluid transfer of plasma proteins during fetal development in the sheep. *J. Physiol.* 300, 457–465.
- Dziegielewska, K. M., Habgood, M. D., Møllgård, K., Stagaard, M., and Saunders, N. R. (1991). Species-specific transfer of plasma albumin from blood into different cerebrospinal fluid compartments in the fetal sheep. *J. Physiol.* 439, 215–237.
- Dziegielewska, K. M., and Saunders, N. R. (1988). “The development of the blood-brain barrier: proteins in fetal and neonatal CSF, their nature and origins,” in *Handbook of Human Growth and Developmental Biology*. Vol. 1. *Neural, Sensory, Motor and Integrative Development*, eds E. Meisami and P. S. Timiras (Boca Raton: CRC Press), 169–191.
- Ehrlich, P. (1885). *Das sauerstoffbedürfnis des organismus. Eine farbenanalytische studie*. Berlin: Hirschwald.
- Ek, C. J., Dziegielewska, K. M., Habgood, M. D., and Saunders, N. R. (2012). Barriers in the developing brain and Neurotoxicology. doi:10.1016/j.neuro.2011.12.009. [Epub ahead of print].
- Ek, C. J., Habgood, M. D., Dziegielewska, K. M., and Saunders, N. R. (2003). Structural characteristics and barrier properties of the choroid plexuses in developing brain of the opossum (*Monodelphis domestica*). *J. Comp. Neurol.* 460, 451–464.
- Ek, C. J., Habgood, M. D., Dziegielewska, K. M., and Saunders, N. R. (2006). Functional effectiveness of the blood-brain barrier to small water-soluble molecules in developing and adult opossum (*Monodelphis domestica*). *J. Comp. Neurol.* 496, 13–26.
- Ek, C. J., Wong, A., Liddelow, S. A., Johansson, P. A., Dziegielewska, K. M., and Saunders, N. R. (2010). Efflux mechanisms at the developing brain barriers: ABC-transporters in the fetal and postnatal rat. *Toxicol. Lett.* 197, 51–59.
- Ek, J., Habgood, M. D., Dziegielewska, K. M., and Saunders, N. R. (2001). Permeability of the blood-brain and blood-CSF barriers to small molecular weight lipid insoluble markers during postnatal development in the opossum, *Monodelphis domestica*. *J. Physiol.* 536, 841–853.
- Fossan, G., Cavanagh, M. E., Evans, C. A. N., Malinowska, D. H., Møllgård, K., Reynolds, M. L., and Saunders, N. R. (1985). CSF-brain permeability in the immature sheep fetus: a CSF-brain barrier. *Brain Res. Dev. Brain Res.* 18, 113–124.
- Frömler, E., and Diamond, J. (1972). Route of passive ion permeation in epithelia. *Nat. New Biol.* 235, 9–13.
- Furuse, M., Furuse, K., Sasaki, H., and Tsukita, S. (2001). Conversion of Zonulae Occludentes from Tight to Leaky Strand Type by Introducing Claudin-2 into Madin-Darby Canine Kidney I Cells. *J. Cell Biol.* 153, 263–272.
- Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, Y., Tsukita, S., and Tsukita, S. (1994). Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J. Cell Biol.* 127, 1617–1626.
- Furuse, M., and Tsukita, S. (2006). Claudins in occluding junctions of humans and flies. *Trends Cell Biol.* 16, 181–188.
- Gazzin, S., Berengeno, A. L., Strazielle, N., Fazzari, F., Raseni, A., Ostrow, J. D., Wennberg, R., Ghersi-Egea, J.-F., and Tiribelli, C. (2011). Modulation of Mrp1 (ABCC1) and Pgp (ABCb1) by bilirubin at the blood-CSF and blood-brain barriers in the Gunn rat. *PLoS ONE* 6, e16165. doi:10.1371/journal.pone.0016165
- Goldmann, E. E. (1909). Die aussere und innere Sekretion des gesunden und kranken Organismus im Lichte der “vitalen Farbung.” *Beitr. Klin. Chir.* 64, 192–265.
- Gröntoft, O. (1954). Intracranial haemorrhage and blood-brain barrier problems in the newborn: a pathologico-anatomical and experimental investigation. *Acta Pathol. Microbiol. Scand. Suppl.* 100, 8–109.
- Habgood, M. D., Knott, G. W., Dziegielewska, K. M., and Saunders, N. R. (1993). The nature of the blood-CSF barrier permeability decrease during postnatal brain development in the rat. *J. Physiol.* 468, 73–83.
- Habgood, M. D., Sedgwick, J. E. C., Dziegielewska, K. M., and Saunders, N. R. (1992). A developmentally regulated blood-cerebrospinal fluid transfer mechanism for albumin in immature rats. *J. Physiol.* 456, 181–192.

- Hagenbuch, B. (2007). Cellular entry of thyroid hormones by organic anion transporting polypeptides. *Best Pract. Res. Clin. Endocrinol. Metab.* 21, 209–221.
- Hartz, A. M. S., and Bauer, B. (2011). ABC transporters in the CNS – an inventory. *Curr. Pharm. Biotechnol.* 12, 656–673.
- Haseloff, R. F., Blasig, I. E., Bauer, H. C., and Bauer, H. (2005). In search of the astrocytic factor(s) modulating blood-brain barrier functions in brain capillary endothelial cells in vitro. *Cell. Mol. Neurobiol.* 25, 25–39.
- Himwich, W. A., Petersen, J. C., and Allen, M. L. (1957). Hematocephalic exchange as a function of age. *Neurology* 7, 705–710.
- Hirase, T., Staddon, J. M., Saitou, M., Ando-Akatsuka, Y., Itoh, M., Furuse, M., Fujimoto, K., Tsukita, S., and Rubin, L. L. (1997). Occludin as a possible determinant of tight junction permeability in endothelial cells. *J. Cell. Sci.* 110, 1603–1613.
- Holash, J. A., Noden, D. M., and Stewart, P. A. (1993). Re-evaluating the role of astrocytes in blood-brain barrier induction. *Dev. Dyn.* 197, 14–25.
- Itoh, M., Sasaki, H., Furuse, M., Ozaki, H., Kita, T., and Tsukita, S. (2001). Junctional adhesion molecule (JAM) binds to PAR-3: a possible mechanism for the recruitment of PAR-3 to tight junctions. *J. Cell Biol.* 154, 491–497.
- Janzer, R. C., and Raff, M. C. (1987). Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* 325, 253–257.
- Järup, L. (2003). Hazards of heavy metal contamination. *Br. Med. Bull.* 68, 167–182.
- Johanson, C. E., and Woodbury, D. M. (1974). “Changers in CSF flow and extracellular space in the developing rat,” in *Drugs and the Developing Brain*, eds A. Vernadakis and N. Weiner (New York: Plenum), 281–287.
- Johansson, P. A., Burnstock, G., Dziegielewska, K. M., Guida, E., McIntyre, P., and Saunders, N. R. (2007). Expression and localization of P2 nucleotide receptor subtypes during development of the lateral ventricular choroid plexus of the rat. *Eur. J. Neurosci.* 26, 3319–3331.
- Johansson, P. A., Dziegielewska, K. M., Ek, C. J., Habgood, M. D., Liddelow, S. A., Potter, A. M., Stolp, H. B., and Saunders, N. R. (2006). Blood-CSF barrier function in the rat embryo. *Eur. J. Neurosci.* 24, 65–76.
- Johansson, P. A., Dziegielewska, K. M., Ek, C. J., Habgood, M. D., Møllgård, K., Potter, A., Schuliga, M., and Saunders, N. R. (2005). Aquaporin-1 in the choroid plexuses of developing mammalian brain. *Cell Tissue Res.* 322, 353–364.
- Johansson, P. A., Dziegielewska, K. M., Liddelow, S. A., and Saunders, N. R. (2008). The blood-CSF barrier explained: when development is not immaturity. *Bioessays* 30, 237–248.
- Jones, H. C. (1980). Intercellular pores between the ependymal cells lining the roof of the 4th cerebral ventricle in mammalian fetuses. *Z. Kinderchir.* 31, 309–316.
- Jones, H. C., and Bucknall, R. M. (1988). Inherited prenatal hydrocephalus in the H-Tx rat: a morphological study. *Neuropathol. Appl. Neurobiol.* 14, 263–274.
- Jones, H. C., and Sellars, R. A. (1982). The movement of fluid out of the cerebral ventricles in fetal and neonatal rats. *Z. Kinderchir.* 37, 130–133.
- Kriesel, U., Risau, W., and Wolburg, H. (1996). Development of blood-brain barrier tight junctions in the rat cortex. *Brain Res. Dev. Brain Res.* 96, 229–240.
- Knott, G. W., Dziegielewska, K. M., Habgood, M. D., Li, Z. S., and Saunders, N. R. (1997). Albumin transfer across the choroid plexus of South American opossum (*Monodelphis domestica*). *J. Physiol.* 499, 179–194.
- Kuttner, R., Sims, J. A., and Gordon, M. W. (1961). The uptake of a metabolically inert amino acid by brain and other organs. *J. Neurochem.* 6, 311–317.
- Lajtha, A., and Toth, J. (1961). The brain barrier system-II. Uptake and transport of amino acids by the brain. *J. Neurochem.* 8, 216–225.
- Lee, J. C. (1971). Evolution of the concept of the blood-brain barrier phenomenon. *Prog. Neuropathol.* 1, 84–145.
- Lefauconnier, J.-M., and Trouvé, R. (1983). Developmental changes in the pattern of amino acid transport at the blood-brain barrier in rats. *Brain Res.* 283, 175–182.
- Lewandowsky, M. (1900). Zur lehre der cerebrospinal flüssigkeit. *Z. Klin. Med.* 40, 480–494.
- Liddelow, S., Dziegielewska, K. M., Ek, C. J., Johansson, P. A., Potter, A., and Saunders, N. R. (2009). Cellular transfer of macromolecules across the developing choroid plexus of *Monodelphis domestica*. *Eur. J. Neurosci.* 29, 253–266.
- Liddelow, S., Dziegielewska, K. M., Noor, N., Potter, A. M., and Saunders, N. R. (2011a). Modification of choroid plexus protein transfer from blood to cerebrospinal fluid in response to altered plasma protein composition during development. *Eur. J. Neurosci.* 33, 391–400.
- Liddelow, S. A., Dziegielewska, K. M., Møllgård, K., Phoenix, T. N., Temple, S., VandeBerg, J. L., and Saunders, N. R. (2011b). Sparc/osteonectin, an endogenous mechanism for targeting albumin to the blood-CSF interface during brain development. *Eur. J. Neurosci.* 34, 1062–1073.
- Liddelow, S. A., Temple, S., Møllgård, K., Gehwold, R., Wagner, A., Bauer, H., Bauer, H.-C., Phoenix, T. N., Dziegielewska, K. M., and Saunders, N. R. (2012). Molecular characterisation of transport mechanisms at the developing mouse blood-CSF interface: a transcriptome approach. *PLoS ONE*. (in press).
- Liebner, S., Corada, M., Bangsow, T., Babbage, J., Taddei, A., Czupolla, C. J., Reis, M., Felici, A., Wolburg, H., Fruttiger, M., Taketo, M. M., von Melchner, H., Plate, K. H., Gerhardt, H., and Dejana, E. (2008). Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J. Cell Biol.* 183, 409–417.
- Møllgård, K., Balslev, Y., Lauritzen, B., and Saunders, N. R. (1987). Cell junctions and membrane specializations in the ventricular zone (germinal matrix) of the developing sheep brain: a CSF-brain barrier. *J. Neurocytol.* 16, 433–444.
- Møllgård, K., Lauritzen, B., and Saunders, N. R. (1979). Double replica technique applied to choroid plexus from early foetal sheep: completeness and complexity of tight junctions. *J. Neurocytol.* 8, 139–149.
- Møllgård, K., Malinowska, D. H., and Saunders, N. R. (1976). Lack of correlation between tight junction morphology and permeability properties in developing choroid plexus. *Nature* 264, 293–294.
- Møllgård, K., and Saunders, N. R. (1975). Complex tight junctions of epithelial and of endothelial cells in early foetal brain. *J. Neurocytol.* 4, 453–468.
- Morgan, E. H., and Moos, T. (2002). Mechanism and developmental changes in iron transport across the blood-brain barrier. *Dev. Neurosci.* 24, 106–113.
- Neuwelt, E. A. (2004). Mechanisms of disease: the blood-brain barrier. *Neurosurgery* 54, 131–140.
- Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., and Tsukita, S. (2003). Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J. Cell Biol.* 161, 653–660.
- Oldendorf, W. M. (1971). Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am. J. Physiol.* 221, 1629–1639.
- Olney, J. W., and Ho, O. L. (1970). Brain damage in infant mice following oral intake of glutamate, aspartate or cysteine. *Nature* 227, 609–611.
- Palis, J., Malik, J., McGrath, K. E., and Kingsley, P. D. (2010). Primitive erythropoiesis in the mammalian embryo. *Int. J. Dev. Biol.* 54, 1011–1018.
- Pardridge, W. M., and Mietus, L. J. (1982). Kinetics of neutral amino acid transport through the blood-brain barrier of the newborn rabbit. *J. Neurochem.* 38, 955–962.
- Penta, P. (1932). Sulla colorazione vitale del sistema nervoso negli centrale animali neonati. *Riv. Neurol.* 5, 62–80.
- Porterfield, S. P., and Hendrich, C. E. (1992). Tissue iodothyronine levels in fetuses of control and hypothyroid rats at 13 and 16 days gestation. *Endocrinology* 131, 195–200.
- Purpura, D. P., and Carmichael, M. W. (1960). Characteristics of blood-brain barrier to gamma-aminobutyric acid in neonatal cat. *Science* 131, 410–412.
- Ramey, B. A., and Birge, W. J. (1979). Development of cerebrospinal fluid and the blood-cerebrospinal fluid barrier in rabbits. *Dev. Biol.* 68, 292–298.
- Risau, W., Hallmann, R., and Albrecht, U. (1986). Differentiation-dependent expression of proteins in brain endothelium during development of the blood-brain barrier. *Dev. Biol.* 117, 537–545.
- Risau, W., and Wolburg, H. (1990). Development of the blood-brain barrier. *Trends Neurosci.* 13, 174–178.
- Roberts, R. B., Flexner, J. B., and Flexner, L. B. (1959). Biochemical and physiological differentiation during morphogenesis. XXIII. Further observations relating to the synthesis of amino acids and proteins by the cerebral cortex and liver of the mouse. *J. Neurochem.* 4, 78–90.
- Roediger, M., Miosge, N., and Gersdorff, N. (2010). Tissue distribution of the laminin $\beta 1$ and $\beta 2$ chain during embryonic and fetal human development. *J. Mol. Histol.* 41, 177–184.

- Rubin, L. L., Hall, D. E., Porter, S., Barbu, K., Cannon, C., Horner, H. C., Janatpour, M., Liaw, C. W., Manning, K., Morales, J., Tanner, L. I., Tomaselli, K. J., and Bard, F. (1991). A cell culture model of the blood-brain barrier. *J. Cell Biol.* 115, 1725–1735.
- Rudloff, S., Messerschmidt, D., and Kemler, R. (2011). “Wnt signaling in development,” in *Intercellular Signaling in Development and Disease: Cell Signaling Collection*, Chap. 21, eds E. A. Dennis and R. A. Bradshaw (San Diego: Academic Press), 215–220.
- Sadowska, G. B., Malaeb, S. N., and Stonestreet, B. S. (2009). Maternal glucocorticoid exposure alters tight junction protein expression in the brain of fetal sheep. *Am. J. Physiol. Heart Circ. Physiol.* 298, H179–H188.
- Saitou, M., Fujimoto, K., Do, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., Noda, T., and Tsukita, S. (1998). Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. *J. Cell Biol.* 141, 397–408.
- Saunders, N. R. (1992). “Ontogenetic development of brain barrier mechanisms,” in *Physiology and Pharmacology of the Blood Brain-Barrier. Vol. 103. Handbook of Experimental Pharmacology*, ed. M. W. B. Bradbury (Berlin: Springer-Verlag), 327–369.
- Saunders, N. R., Ek, C. J., Habgood, M. D., and Dziegielewska, K. M. (2008). Barriers in the brain: a renaissance? *Trends Neurosci.* 31, 279–236.
- Saunders, N. R., and Habgood, M. D. (2011). Understanding barrier mechanisms in the developing brain to aid therapy for the dysfunctional brain. *Future Neurol.* 6, 187–199.
- Saunders, N. R., Habgood, M. D., and Dziegielewska, K. M. (1999). Barrier mechanisms in the brain II immature brain. *Clin. Exp. Pharmacol. Physiol.* 26, 85–91.
- Saunders, N. R., Habgood, M. D., and Dziegielewska, K. M. (2010). “Neurotoxicology of barriers in the developing brain,” in *Developmental Neurotoxicology*, Chap. 3, eds J. Harry, H. Tilson (New York: Informa Health Care), 50–78.
- Schrieber, G., Aldred, A. R., Jaworowski, A., Nilsson, C., Achen, M. G., and Segal, M. B. (1990). Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. *Am. J. Physiol.* 258, R338–R345.
- Schumacher, U., and Møllgård, K. (1997). The multidrug-resistance P-glycoprotein (Pgp, MDR1) is an early marker of blood–brain barrier development in the microvessels of the developing human brain. *Histochem. Cell Biol.* 108, 179–182.
- Seta, K., Sershen, H., and Lajtha, A. (1972). Cerebral amino acid uptake in vivo in newborn mice. *Brain Res.* 47, 415–425.
- Sonoda, N., Furuse, M., Sasaki, H., Yonemura, S., Katahira, J., Horiguchim, Y., and Tsukita, S. (1999). *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J. Cell Biol.* 147, 195–204.
- Stenman, J. M., Rajagopal, J., Carroll, T. J., Ishibashi, M., McMahon, J., and McMahon, A. P. (2008). Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* 322, 1247–1250.
- Stern, L., and Peyrot, R. (1927). Le fonctionnement de la barrière hémato-encéphalique aux divers stades de développement chez les diverses espèces animales. *C. R. Soc. Biol. (Paris)* 96, 1124–1126.
- Stern, L., and Rapoport, J. L. (1928). Les rapports entre l’augmentation de la perméabilité de la barrière hémato-encéphalique et les altérations de son substratum morphologique. *C. R. Soc. Biol. (Paris)* 98, 1515–1517.
- Stern, L., Rapoport, J. L., and Lokschina, E.-S. (1929). Le fonctionnement de la barrière hémato-encéphalique chez les nouveau nés. *C. R. Soc. Biol. (Paris)* 100, 231–223.
- Stewart, P. A., and Hayakawa, E. M. (1987). Interendothelial junctional changes underlie the developmental “tightening” of the blood-brain barrier. *Brain Res. Dev. Brain Res.* 32, 271–281.
- Stewart, P. A., and Hayakawa, E. M. (1994). Early ultrastructural changes in blood-brain barrier vessels of the rat embryo. *Brain Res. Dev. Brain Res.* 78, 25–34.
- Stewart, P. A., and Wiley, M. J. (1981). Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail-chick transplantation chimeras. *Dev. Biol.* 84, 183–192.
- Stolp, H. B., and Dziegielewska, K. M. (2009). Review: role of developmental inflammation and blood-brain barrier dysfunction in neurodevelopmental and neurodegenerative diseases. *Neuropathol. Appl. Neurobiol.* 35, 132–146.
- Stolp, H. B., Dziegielewska, K. M., Ek, C. J., Habgood, M. D., Lane, M. A., Potter, A. M., and Saunders, N. R. (2005a). Breakdown of the blood-brain barrier to proteins in white matter of the developing brain following systemic inflammation. *Cell Tissue Res.* 320, 369–378.
- Stolp, H. B., Dziegielewska, K. M., Ek, C. J., Potter, A. M., and Saunders, N. R. (2005b). Long-term changes in blood-brain barrier permeability and white matter following prolonged systemic inflammation in early development in the rat. *Eur. J. Neurosci.* 22, 2805–2816.
- Stolp, H. B., Dziegielewska, K. M., Saunders, N. R., Anthony, D. C., and Molnár, Z. (2011). Reduced ventricular proliferation in the foetal cortex following maternal inflammation in mouse. *Brain* 134, 3236–3248.
- Stratman, A. N., and Davis, G. E. (2011). Endothelial cell-pericyte interactions stimulate basement membrane matrix assembly: influence on vascular tube remodeling, maturation, and stabilization. *Microsc. Microanal.* 14, 1–13.
- Tao-Cheng, J. H., Nagy, Z., and Brightman, M. W. (1987). Tight junctions of brain endothelium in vitro are enhanced by astroglia. *J. Neurosci.* 7, 3293–3299.
- Tauc, M., Vignon, X., and Bouchaud, C. (1984). Evidence for the effectiveness of the blood–CSF barrier in the fetal rat choroid plexus. A freeze-fracture and peroxidase diffusion study. *Tissue Cell* 16, 65–74.
- Urayama, A., Grubb, J. H., Sly, W. S., and Banks, W. A. (2008). Mannose 6-phosphate receptor-mediated transport of sulfamidase across the blood-brain barrier in the newborn mouse. *Mol. Ther.* 16, 1261–1266.
- Vannucci, S. J. (1994). Developmental expression of GLUT1 and GLUT3 glucose transporters in rat brain. *J. Neurochem.* 62, 240–246.
- Vannucci, S. J., Seaman, L. B., Brucklacher, R. M., and Vannucci, R. C. (1994). Glucose transport in developing rat brain: glucose transporter proteins, rate constants and cerebral glucose utilization. *Mol. Cell. Biochem.* 140, 177–184.
- Viña, J. R., DeJoseph, M. R., Hawkins, P. A., and Hawkins, R. A. (1997). Penetration of glutamate into brain of 7-day-old rats. *Metab. Brain Dis.* 12, 219–227.
- Virgintino, D., Errede, M., Girolamo, F., Capobianco, C., Robertson, D., Vimercati, A., Serio, G., Di Benedetto, A., Yonekawa, Y., Frei, K., and Roncali, L. (2008). Fetal blood-brain barrier P-glycoprotein contributes to brain protection during human development. *J. Neuropathol. Exp. Neurol.* 67, 50–61.
- Virgintino, D., Errede, M., Robertson, D., Capobianco, C., Girolamo, F., Vimercati, A., Bertossi, M., and Roncali, L. (2004). Immunolocalization of tight junction proteins in the adult and developing human brain. *Histochem. Cell Biol.* 122, 51–59.
- Wakai, S., and Hirokawa, N. (1978a). Development of the blood-brain barrier to horseradish peroxidase in the chick embryo. *Cell Tissue Res.* 195, 195–203.
- Wakai, S., and Hirokawa, N. (1978b). Development of blood-cerebrospinal fluid barrier to horseradish peroxidase in the avian choroidal epithelium. *Cell Tissue Res.* 214, 271–278.
- Watson, R. E., Desesso, J. M., Hurtt, M. E., and Cappon, G. D. (2006). Postnatal growth and morphological development of the brain: a species comparison. *Birth Defects Res. B Dev. Reprod. Toxicol.* 77, 471–484.
- Weed, L. H. (1917). The development of the cerebrospinal fluid spaces in pig and in man. *Contrib. Embryol.* 5, 41–52.
- Wennberg, R. P. (2000). The blood-brain barrier and bilirubin encephalopathy. *Cell. Mol. Neurobiol.* 20, 97–109.
- Wennberg, R. P., Ahlfors, C. E., Bhutani, V. K., Johnson, L. H., and Shapiro, S. M. (2006). Toward understanding kernicterus: a challenge to improve the management of jaundiced newborns. *Pediatrics* 117, 474–485.
- Wislocki, G. B. (1920). Experimental studies on fetal absorption. I. The vitality stained fetus. *Contrib. Embryol.* 11, 45–60.
- Wolburg, H., Wolburg-Buchholz, K., Liebner, S., and Engelhardt, B. (2001). Claudin-1, claudin-2 and claudin-11 are present in tight junctions of choroid plexus epithelium of the mouse. *Neurosci. Lett.* 307, 77–80.
- Xie, J., Farage, E., Sugimoto, M., and Anand-Apte, B. (2010). A novel transgenic zebrafish model for blood-brain and blood-retinal barrier development. *BMC Dev. Biol.* 10, 76. doi: 10.1186/1471-213X-10-76
- Xu, J., and Ling, E.-A. (1994). Studies of the ultrastructure and permeability of the blood-brain barrier in the

- developing corpus callosum in postnatal rat brain using electron dense tracers. *J. Anat.* 184, 227–237.
- Yokooji, T., Mori, N., and Murakami, T. (2010). Modulated function of tissue efflux transporters under hyperbilirubinemia in rats. *Eur. J. Pharmacol.* 636, 166–172.
- Yoon, B. H., Romero, R., Park, J. S., Kim, C. J., Kim, S. H., Choi, J. H., and

Han, T. R. (2000). Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of 3 years. *Am. J. Obstet. Gynecol.* 182, 675–81.

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Antidepressant effects of ketamine: mechanisms underlying fast-acting novel antidepressants

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Newer antidepressants are needed for the many individuals with major depressive disorder (MDD) that do not respond adequately to treatment and because of a delay of weeks before the emergence of therapeutic effects. Recent evidence from clinical trials shows that the NMDA antagonist ketamine is a revolutionary novel antidepressant because it acts rapidly and is effective for treatment-resistant patients. A single infusion of ketamine alleviates depressive symptoms in treatment-resistant depressed patients within hours and these effects may be sustained for up to 2 weeks. Although the discovery of ketamine's effects has reshaped drug discovery for antidepressants, the psychotomimetic properties of this compound limit the use of this therapy to the most severely ill patients. In order to develop additional antidepressants like ketamine, adequate preclinical behavioral screening paradigms for fast-acting antidepressants need to be established and used to identify the underlying neural mechanisms. This review examines the preclinical literature attempting to model the antidepressant-like effects of ketamine. Acute administration of ketamine has produced effects in behavioral screens for antidepressants like the forced swim test, novelty suppression of feeding and in rodent models for depression. Protracted behavioral effects of ketamine have been reported to appear after a single treatment that last for days. This temporal pattern is similar to its clinical effects and may serve as a new animal paradigm for rapid antidepressant effects in humans. In addition, protracted changes in molecules mediating synaptic plasticity have been implicated in mediating the antidepressant-like behavioral effects of ketamine. Current preclinical studies are examining compounds with more specific pharmacological effects at glutamate receptors and synapses in order to develop additional rapidly acting antidepressants without the hallucinogenic side effects or abuse potential of ketamine.

Keywords: ketamine, antidepressants, depression, animal models, BDNF

INTRODUCTION

Major depressive disorder (MDD) is a serious public health problem and one of the most common psychiatric disorders, with a lifetime prevalence of 17% in the United States (Kessler et al., 2005). Although the currently available antidepressants provide a measurable degree of therapy, approximately 50% of individuals diagnosed with MDD do not respond adequately to first-line treatment with conventional antidepressants (Trivedi et al., 2006; Fava et al., 2008). Moreover, the 3–4 week delay in the onset of therapeutic efficacy is particularly difficult for patients with persistent suicidal ideation. Patients that emerge as treatment resistant, defined as failing two or more trials of medication, are more severely ill with comorbid anxiety disorders and are at increased risk of suicide for an extended period of time (Joffe et al., 1993; Souery et al., 2007; Schosser et al., 2012). Therefore, there is a pressing medical need to develop rapidly acting therapeutics that are capable of immediately relieving the depressive symptomatology, and persisting in their action as an antidepressant, for patients unable to respond to conventional therapies.

Recently it has been demonstrated that the NMDA receptor antagonist ketamine has rapid-acting and transient antidepressant effects in patients that are treatment resistant (Mathew et al., 2012). However, the discovery of ketamine is no panacea. The psychotomimetic properties and abuse potential of ketamine necessitate caution in promoting this particular compound as a general treatment for MDD. Understanding the underlying mechanism of action of ketamine linked to behavioral improvement is of significant importance for the development of novel, more improved antidepressants beyond the use of ketamine. This review will focus on the molecular alterations and animal behavior studies that have been used to measure potential correlates of the antidepressant effects of ketamine. As ketamine produces clinical antidepressant effects with a different time course and apparently different neurochemical mechanism than conventional antidepressants, the results of these studies have revealed new paradigms that can be used to identify novel compounds which may have a similar therapeutic potential and time course as ketamine in targeting treatment resistant depression (TRD).

KETAMINE—CLINICAL TRIALS

The initial clinical trials were double blind crossover studies that utilized a single infusion of ketamine (0.5 mg/kg) administered intravenously over a 40 min period (Berman et al., 2000; Zarate et al., 2006). Berman et al reported decreases in depressive symptomatology, which emerged progressively over the first 3 days in all of the eight patients that were treated; one patient continued to show antidepressant-like effects 2 weeks post-infusion. Similarly, Zarate and colleagues reported a significant and rapid alleviation of depressive symptoms in 12 individuals on the first day, with six subjects exhibiting symptom alleviation for at least 1 week; two of these subjects continued to show antidepressant effects 2 weeks post-single ketamine infusion. Subsequent studies reported significant efficacy of ketamine in reducing suicidal ideation in individuals exhibiting TRD (Diazgranados et al., 2010). Moreover, a proof of concept trial conducted in treatment-resistant bipolar patients revealed a more rapid onset of antidepressant effects following ketamine infusion concomitant to their valproate and lithium treatment compared to previous studies conducted in MDD patients. However, the alleviation of depressive symptoms in the bipolar study persisted for only 3 days compared to the 7 days reported in earlier trials. In addition, ketamine had significant efficacy in patients resistant to electroconvulsive therapy (ECT) and produced more rapid antidepressant effects compared to ECT (Ibrahim et al., 2011). Unlike the almost immediate alleviation of depressive symptomatology associated with ketamine infusion, similar reductions in symptoms were observed approximately 1–2 weeks following the first of the thrice-weekly ECT exposures. Furthermore, the use of ketamine as the anesthetic prior to ECT has been suggested to improve outcome and response to ECT (Hoyer et al., 2013). Indeed, the administration of ketamine/propofol (ketofol) improved the severity of seizure duration, induced an earlier onset of the antidepressant effect and significantly improved cognitive performances compared to propofol (Wang et al., 2012). Recently, it was reported that sub-anesthetic doses of S-ketamine with propofol actually worsened the post-treatment disorientation in some patients (Jarventausta et al., 2013). Further research is ongoing to determine the benefit of the S-enantiomer over the commonly used racemic mixture of ketamine. One group suggested that S-ketamine did not induce the transient psychotomimetic effects evident in the initial phase of infusion (Segmiller et al., 2013).

An extensive clinical trial involving 67 patients at two sites with documented TRD established the most definitive antidepressant efficacy of ketamine, in comparison with the benzodiazepine, midazolam, used as an active placebo control (Murrough et al., 2013). The response rates to ketamine vs. midazolam were 64 and 28%, respectively, with ketamine significantly reducing scores in the MADRS by 7.95 points. Ketamine-treated patients continued to exhibit improved scores over the 7-day period post-infusion compared to midazolam, however, the reduction of depressive scores on day 7 was no longer significant. Although most studies of ketamine have involved only a small number of patients, this is the best-designed and most extensive clinical trial to confirm the efficacy of ketamine in rapidly and persistently alleviating depressive symptomatology.

Because the clinical effects of ketamine are transient, studies have assessed the efficacy of ketamine administration when given chronically. Significant improvement of symptoms persisted following six infusions of ketamine over 11 days, although the 9 patients treated in this trial eventually relapsed 19 days after the final infusion (aan het Rot et al., 2010). In addition, the effects of oral administration of ketamine given over a long-term period yielded positive findings, with patients exhibiting improved mood over the 28-day treatment period. Interestingly, although the level of symptom alleviation was the same as that achieved by I.V. infusion of ketamine, oral ketamine did not elicit a significant effect on depressive symptoms until day 14 of treatment but fortunately did relieve anxiety symptoms within 3 days of treatment (Irwin et al., 2013). Psychotomimetic effects were not observed in these patients; however, there were some reports of sleep disturbances and diarrhea. Moreover, another study conducted in bipolar patients using sublingual ketamine indicated significant (70%) numbers of individuals exhibiting improved mood with limited side effects with rapid onset of action. These data indicate that further evaluation of the administration route of ketamine and their side effect profiles may be beneficial.

Although there is a clear consensus on the rapidity of the antidepressant effect of ketamine in TRD, with most patients experiencing elevated mood starting approximately 120 min post-infusion, not all patients respond to ketamine treatment. Response rates across studies have ranged between 25 and 85% at 24 h and 14–70% at 72 h (Aan Het Rot et al., 2012). In addition, the duration of the antidepressant effect has varied across studies. In most of the trials conducted so far, only approximately half of the patients exhibited relief of depressive symptoms from ketamine lasting past 72 h. The reasons underlying variability in the response to ketamine are unknown. Given the heterogeneous nature of depression, a number of genetic, environmental and patient characteristics may be associated with treatment response. For example, patients with a family history of alcohol use disorder (AUD) exhibit better outcomes in response to ketamine administration, reporting less psychotomimetic disturbances and greater reductions of depression symptoms, compared to MDD patients without a history of AUD (Phelps et al., 2009). In addition, potential biomarkers or genetic variants will likely be found to augment or prevent responsiveness to ketamine.

Some clinical studies have tried to identify the critical pharmacological characteristics of ketamine associated with treatment response. Modification of the NMDA receptor subunit NR2B may confer an increased treatment response; indeed, NR2B antagonists, CP-1016060 and MK-0657 have shown good efficacy in treating TRD patients (Preskorn et al., 2008; Ibrahim et al., 2012a). AZD6765, a NMDA channel blocker, was assessed for its antidepressant-like qualities in a double blind crossover study involving 22 subjects. Although no psychotomimetic effects of this compound were reported, depressive symptoms were alleviated only for the first 2 h following infusion (Zarate et al., 2013). Similarly, administration of riluzole, (a sodium channel blocker, which indirectly inhibits glutamate release) for 4 weeks following ketamine infusion did not potentiate symptom improvement compared to placebo (Ibrahim et al., 2012b). These reports and a growing literature indicate that the mechanisms of

action mediating ketamine's antidepressant effects have not yet been identified and are not elicited simply by the blockade of NMDA receptors.

ANTIDEPRESSANT-LIKE BEHAVIORAL EFFECTS OF KETAMINE IN RODENTS

The ability of ketamine to affect depressive-like behavior in a number of preclinical behavioral paradigms and models of depression has been widely studied in the past few years. Many reports indicate that acute administration of ketamine produces antidepressant-like effects in rodents (**Table 1**). However, some of the findings have not been replicated consistently by other laboratories. The literature concerning the antidepressant-like effects of ketamine is reviewed here, focusing on the effects of varying test conditions on behavioral outcomes. In addition, many studies have now reported that the effects of a single dose of ketamine can be measured over a protracted period of time lasting between days to weeks (**Table 2**). The time course of these protracted effects resembles the time course for ketamine's clinical effects (Yilmaz et al., 2002; Maeng et al., 2008), and may represent a new animal behavioral paradigm that correlates with the clinical effects of rapidly acting antidepressants.

FORCED SWIM TEST (FST)

The FST is the most frequently used behavioral test for measuring depressive-like behavior in rodents. It has also been a frequently used test within the preclinical ketamine literature. Mice and rats placed in cylinders containing water rapidly become immobile, demonstrated by floating passively or making only movements necessary to remain afloat. Based on an immobility response induced by inescapable exposure to stress, the FST also has strong predictive validity because short-term administration of antidepressant compounds from a variety of pharmacological classes reduces immobility time in the FST. These drugs include tricyclic compounds, MAO inhibitors, atypical antidepressants, and SSRIs (Cryan et al., 2005). Furthermore, the behavioral effects of tricyclics and SSRIs do not last beyond a few hours following their acute administration (Hoshaw et al., 2008).

Several groups have reported that a single administration of ketamine produced acute reductions of immobility in the FST shortly after injection (**Table 1**). Although the majority of these studies utilized a 10 mg/kg dose administered intraperitoneally (i.p.), subanesthetic doses of ketamine ranging from 10–50 mg/kg have produced antidepressant-like effects in the FST. However, some studies failed to detect acute effects of ketamine using the FST in mice (Bechtholt-Gompf et al., 2011) or in rats (Popik et al., 2008).

A feature of ketamine's pharmacology distinct from conventional antidepressants is that it produces protracted behavioral effects persisting between one to several days after administration (**Table 2**). The majority of studies indicate that the FST remains sensitive to the protracted effects of ketamine up to 1 week after a single injection (**Table 1**). These protracted effects were reported to persist for 8 days (Ma et al., 2013), 10 days (Yilmaz et al., 2002), 12 days (Garcia et al., 2008a), and 2 weeks (Maeng et al., 2008). Interestingly, antidepressant-like effects of ketamine were observed in the FST 2 months following the cessation of a 15-day

treatment of rats during adolescence (Parise et al., 2013). This result is in line with other studies that have used a 10 or 12-day dosing regimen to establish longer-lasting effects of chronic ketamine on depressive-like activity in the FST (Tizabi et al., 2012; Akinfiresoye and Tizabi, 2013). Only one study examining the protracted effects of ketamine failed to report this finding (Lindholm et al., 2012).

The presence of chronic stress has been shown to facilitate the detection of antidepressant-like effects of ketamine in the FST (Koike et al., 2013a). There are also significant strain differences in the sensitivity to ketamine. For example, Wistar rats are insensitive to the antidepressant-like effects of low dose ketamine (2.5 and 5 mg/kg) following chronic treatment. In contrast, WKY rats were extremely sensitive to ketamine-induced reductions in FST immobility (Tizabi et al., 2012). WKY rats have a high baseline immobility level in the FST, which may allow for a greater sensitivity to compounds. Moreover, WKY rats are a genetic model of pathological depression and anxiety (Will et al., 2003; Solberg et al., 2004), which could provide them greater sensitivity to the effects of ketamine. Finally, the WKY strain is insensitive to SSRIs (Lopez-Rubalcava and Lucki, 2000; Tejani-Butt et al., 2003; Will et al., 2003) showing that ketamine is active under conditions where current antidepressants are ineffective. This feature makes WKY rats a useful strain in which to assess novel compounds resembling ketamine, which may be screened for efficacy in TRD.

TAIL SUSPENSION TEST (TST)

The TST is widely used in the preclinical ketamine literature as a less stressful test of behavioral despair when mice are suspended from their tail (Steru et al., 1985; Cryan et al., 2005). TST has predictive validity because it measures antidepressant-like responses from various classes of drugs. Ketamine reduces immobility levels in mice acutely, with studies reporting reductions in immobility time at 30 min (Mantovani et al., 2003; Rosa et al., 2003; Cruz et al., 2009; Koike et al., 2011a) and 24 h (Koike et al., 2011b) following a single injection of ketamine.

The most effective dose in the TST was 30 mg/kg. ICR mice were particularly sensitive to ketamine and continued to exhibit decreased immobility 72 h after treatment (Koike et al., 2011b). Furthermore, a lower dose of ketamine (10 mg/kg) was effective in reducing TST immobility increased by chronic mild stress (CMS) 48 h after ketamine injection (Ma et al., 2013). In contrast, two studies indicated that the acute reduction in immobility by high dose ketamine (50 and 160 mg/kg) was not maintained 1 week following treatment in mice (Popik et al., 2008; Bechtholt-Gompf et al., 2011). These data suggest that the TST is most valuable in the assessment of the more immediate antidepressant effects of ketamine. However, exposure to stress could increase the sensitivity to ketamine in the TST. To date there are no studies that have investigated whether the TST is sensitive to a chronic dosing regimen of ketamine.

NOVELTY SUPPRESSED FEEDING (NSF)

Exposure to a novel environment produces an anxiety-like phenotype in rodents known as hyponeophagia. In the NSF and novelty-induced hypophagia (NIH) tests, the latency to feed is increased and the amount of food consumption is reduced in a

Table 1 | Acute effects of ketamine.

References	Species and strain	Ketamine—supplier and dose	Behavioral alterations	Molecular alterations
ACUTE EFFECTS OF KETAMINE				
Burgdorf et al., 2013	Male adult (2–3 months) Sprague-Dawley rats	Fort Dodge (Butler, USA), I.V., I.P., and S.C. 10 mg/kg	Reduced immobility in FST 20–60 min and 24 h post i.p. Injection (10 mg/kg). Reduced latency to feed in the NIH 1 h post 10 mg/kg i.v.	Increased NR2B and GluR1 expression in the mPFC and HC 24 h post-injection
Carrier and Kabbaj, 2013	Male (250–270 g) and female (200–225 g) Sprague-Dawley rats	Fort Dodge (Butler Schein), Inc. 2.5–0 mg/kg	Latency to feed was reduced in the NSF 24 h post-injection (5 and 10 mg/kg). Increased sucrose consumption of males 48 h post-injection in the SPT. Reduced immobility in FST in males & females 30 min post-injection	Increased mTOR phosphorylation in males and females, reduced eEF2 phosphorylation in males (5 mg/kg)
Gigliucci et al., 2013	Male (280–320 g) Sprague- Dawley rats	Vetoquinol Ltd., UK (1.0 mg/ml). 10–25 mg/kg i.p.	Rats exhibited antidepressant-like effects in the FST at 1 or 24 h after a single injection of ketamine. Ketamine was ineffective following 3 injections (24, 5 and 1 h prior to testing). Ketamine (25 mg/kg) reversed stress-induced immobility; this was prevented by pCPA treatment at 24 h but not at 1 h post-injection	Depletion of cortical serotonin levels by pCPA (1.0 mg/kg once daily for 3 days) attenuated the antidepressant-like effect of ketamine in the FST
Koike et al., 2013a	Male Sprague-Dawley rats (185–325 g at testing)	Ketalar® Sankyo Yell Pharmaceutical Co., Ltd., 1–10 mg/kg i.p.	Ketamine (10 mg/kg) decreased immobility 30 min post-treatment in rats exposed to 21 days of corticosterone administration	N/A
Koike et al., 2013b	Male ICR (5 weeks) and male C57BL/6J (9 weeks)	Ketalar® Sankyo Yell Pharmaceutical Co., Ltd. 30 mg/kg i.p.	Ketamine decreased immobility in the FST & latency to feed in the NSF at 30 min and 24 h post-injection. K252a prevented ketamine's effects at 24 h.	N/A
Muller et al., 2013	Male Sprague Dawley rats (330–400 g)	Fort-Dodge (Pfizer CT), USA. 15 mg/kg (i.p.)	Reduced immobility in FST 2 h post-injection	Increased p- α CamKII and decreased SNARE complex expression 1– 4 h post-injection. No effect on GSK-3 activity. Protracted increased in synapsin expression1 h to 7 days post-injection
Walker et al., 2013	CD-1 mice (6 wks. old) and C57BL/6J mice (12 weeks old)	Fort Dodge Animal Health 6 mg/kg (i.p.)	Ketamine co-administered with LPS but not pretreatment 24 h prior blocked LPS-induced immobility in FST and anhedonia in the SPT. 10 h post LPS, ketamine administration reversed the anhedonia in SPT, this was blocked by NBQX	Ketamine did not block the LPS-induced increases in kynurenone metabolites, cytokines or BDNF expression at 6–28 h
Iijima et al., 2012	C57Bl/6J mice (9 weeks)	Sigma-Aldrich 30 mg/kg (i.p.)	Latency to feed in the NSF was reduced at 30 min and 24 h post-injection. Rapamycin reversed the 24 h reduction in NSF latency	N/A

(Continued)

Table 1 | Continued

References	Species and strain	Ketamine—supplier and dose	Behavioral alterations	Molecular alterations
Liu et al., 2012	BDNF knockin mice, (Val66Met SNP) Val/Met, Met/Met and Val/Val (WT) 6–8 months	Hospira Inc. 10 mg/kg (i.p.)	24 h post-injection the AD effects of ketamine in the FST were blocked in Met/Met mice	Met/Met knockin mice are insensitive to the molecular effects of ketamine on spine head diameter and spine length modulated in WT mice
Yang et al., 2012	Male Wistar rats (180–220 g)	Gutian Pharmaceutical CO. Ltd., Fujian, China 10 mg/kg (i.p.)	Reduced immobility in FST 30 min post-injection	Increased mTOR phosphorylation in HC and PFC
Yang et al., 2013b	Male Wistar rats (200–300 g)	Gutian Pharmaceutical CO. Ltd., Fujian, China 5–15 mg/kg (i.p.)	Dose-dependent reduction in immobility in the FST 30 min post-injection	Increased BDNF levels in the HC following 10 and 15 mg/kg. Dose dependent increase in phosphorylated mTOR levels in HC
Wang et al., 2011	Male Wistar rats (60 days old)	Sigma-Aldrich 15 mg/kg (i.p.)	Decreased immobility in the FST 60 min post-injection	Increased BDNF expression and decreased phosphorylation of GluR1 (Ser845) in HC 60 min post-injection
Beurel et al., 2011	WT and GSK-3 Knock in mice	10 mg/kg (i.p.)	AD effects in LH in WT but not GSK-3 knock-in mice	Increased pGSK-3β (CTX and HC) 30 and 60 min post-injection
Koike et al., 2011a	Male ICR mice (25–35 g)	Sigma-Aldrich 3–30 mg/kg (i.p.)	Ketamine reduced immobility in the TST 24 h post 30 mg/kg injection. Rapamycin reversed the ketamine-induced reduction in TST immobility	N/A
Reus et al., 2011	Male Wistar rats (60 days old)	Fort Dodge Animal Health—0.1 g/ml injectable solution, 5–10 mg/kg	Immobility in the FST was reduced at 60 min postinjection by 10 mg/kg only	Ketamine 5 mg/kg increased the expression of BDNF, CREB, and PKC phosphorylation in the PFC. 5mg/kg increased BDNF in the HC and Amg. 10 mg/kg decreased BDNF in the PFC, HC, and Amg. 10 mg/kg increased CREB expression and PKC phosphorylation in the PFC
Li et al., 2010	Male Sprague Dawley rats (150–250 g)	Sigma-Aldrich 10 mg/kg (i.p.)	Ketamine produced AD effects in the FST, LH and NSF test 24 h post-injection, blocked by rapamycin	Ketamine 10 mg/kg activated mTOR, ERK, and PKB/Akt signaling, blocked by NBQX, Ketamine 10 mg/kg increased expression of certain synaptic proteins at 2, 6, and 72 h post-injection, blocked by rapamycin
Ghasemi et al., 2010	Male NMRI mice (23–30 g)	Sigma-Aldrich 0.5–5 mg/kg (i.p.)	Ketamine reduced immobility in the FST 45 min post-injection (2 and 5 mg/kg)	N/A
Cruz et al., 2009	Male Swiss mice (25–35 g)	Sigma-Aldrich 6.35–50 mg/kg (i.p.)	12.5, 25, and 50 mg/kg ketamine reduced immobility in the FST 30 min	N/A

(Continued)

Table 1 | Continued

References	Species and strain	Ketamine—supplier and dose	Behavioral alterations	Molecular alterations
			post-injection. Only 50 mg/kg ketamine reduced immobility in the TST	
Engin et al., 2009	Male Sprague-Dawley rats (180–360 g)	10–50 mg/kg (i.p.)	Ketamine (50 mg/kg) increased the % of open arm entries in the EPM. Both doses decreased immobility in the FST 30 min post-injection	N/A
Rezin et al., 2009	Male Wistar rats (300 g)	Fort Dodge Animal Health 15 mg/kg (i.p.)	Ketamine did not reverse the CMS-induced reduction in consumption of sweet food	Ketamine reversed the CMS-induced reductions in mitochondrial respiratory chain enzymes
Garcia et al., 2008a	Male Wistar rats (60 days old)	Fort Dodge (Brazil) 5, 10, and 15 mg/kg (i.p.)	1 h post-injection ketamine (5 & 10 mg/kg) significantly reduced immobility in the FST	BDNF increased in the HC following ketamine injection (15 mg/kg)
Hayase et al., 2006	Male ICR mice (60–90 days old)	Sankyo Co., Ltd. Tokyo, Japan 30–1.0 mg/kg (i.p.)	Ketamine increased the latency to immobility in the FST and was anxiolytic in the EPM at both doses 60 and 120 min post-injection	N/A
Rosa et al., 2003	Swiss mice male and female (30–40 g)	Sigma-Aldrich 5 mg/kg (i.p.)	Ketamine reduced immobility in the TST 30 min post-injection	N/A
Mantovani et al., 2003	Male Swiss mice 35–45 g	0.1 mg/kg (i.p.)	Ketamine reduced immobility in the TST 30 min post-injection	N/A

This table outlines studies that have assessed the antidepressant-like effects of ketamine at 30 min to 24 h post-administration in commonly used behavioral tests. Molecular alterations of relevance to ketamine's molecular mechanism of action are also reported. FST, forced swim test; TST, tail suspension test; LH, learned helplessness; NSF, novelty suppressed feeding; SPT, sucrose preference test; EPM, elevated plus maze; AD, antidepressant; CMS, chronic mild stress; LPS, lipopolysaccharide; HC, hippocampus; CTX, cortex; Amg, amygdala; mPFC, medial prefrontal cortex; WT, wild type.

novel environment. These tests, based on a similar principle, differ in methodology; NSF requires acute food deprivation 24 h prior to testing whereas the NIH utilizes an 8–10-day training period without deprivation. These tests have considerable face validity, although interpretation of results with the NSF may be limited by the use of food deprivation. Hyponeophagia is one of the few anxiety-related tests that are reliably attenuated following chronic, but not acute, administration of antidepressant drugs (Bodnoff et al., 1988; Dulawa and Hen, 2005). In contrast, ketamine reduced the latency to eat within hours of treatment. The effective dose range for ketamine in this task varied across studies: 30 min and 24 h following 5–10 mg/kg (Li et al., 2010; Carrier and Kabbaj, 2013) and 30 mg/kg (Iijima et al., 2012), but all tests resulted in a significant reduction in the latency to feed in the novel environment. Moreover, ketamine (10 mg/kg) successfully reduced the latency to eat in the NIH 1 h post-injection (Burgdorf et al., 2013). More protracted effects of acute ketamine treatment (3 mg/kg) were observed 48 h following treatment in mice exposed to chronic stress, although ketamine did not reduce feeding latency in stress naïve mice in this study (Autry et al., 2011).

Overall, these data suggest that hyponeophagia is highly sensitive to a single dose of ketamine, although additional parameters

of these tests remain to be examined more systematically. The fact that ketamine produced anxiolytic effects rapidly whereas conventional antidepressants require chronic treatment for weeks agrees with a more rapid onset of clinical effects. As TRD patients exhibit increased comorbid anxiety compared to treatment responsive MDD patients, the usefulness of assessing ketamine in anxiety tests should not be overlooked.

SUCROSE PREFERENCE TEST (SPT)

Sucrose consumption is widely accepted as a measure of anhedonia in rodents and has significant face validity in terms of its sensitivity to chronic stress and antidepressant treatment. Repeated administration of ketamine (7 days) reversed the decrease in sucrose consumption in rats exposed to chronic stress. Although it should be noted that this dosing regimen with ketamine also increased sweet food consumption in both stressed and non-stressed rats (Garcia et al., 2009). Furthermore, administration of a low dose of ketamine (0.5 mg/kg) for 10 days significantly increased sucrose consumption in WKY rats (Akinfesoye and Tizabi, 2013). Marked increases in sucrose consumption in rats persisted at 1, 3, 5, and 7 days after a single treatment with ketamine (10 mg/kg) (Li et al., 2011), indicating significant protracted effects of ketamine on this behavior.

Table 2 | Protracted effects of ketamine.

References	Species and strain	Ketamine—supplier and dose	Behavioral alterations	Molecular alterations
PROTRACTED EFFECTS OF KETAMINE				
Akinfiresoye and Tizabi, 2013	Male WKY rats	Fort-Dodge (Henry Schein), 0.25 and 0.5 mg/kg (i.p.), administered daily for 10 days	Only chronic administration of 0.5 mg/kg reduced immobility in the FST and increased sucrose intake in the SPT	0.25 mg/kg ketamine did not alter mTOR phosphorylation or synapsin 1 and BDNF expression
Liu et al., 2013	Male Sprague-Dawley rats (150–250 g)	Hospira Inc., 1 and 10 mg/kg (i.p.)	Ketamine reduced immobility in the FST 24 h and 1 week following a single 10 mg/kg injection. This effect was not observed 2 weeks post-injection	Ketamine increased p-S6K, p-ERK, p-Akt but not p-mTOR or GSK-3b 1 h post-injection (10 mg/kg). These changes were not detected 24 h post-injection. 5-HT and hypocretin induced EPSCs were increased 24 h following ketamine treatment (10 mg/kg). Ketamine 1 and 10 mg/kg increased spine head diameter and spine density
Ma et al., 2013	C57BL/6J mice (7 wks. old 20 g)	Gutian Pharmaceutical CO. Ltd., Fujian, China. 10 mg/kg (i.p.)	Ketamine reversed CMS-induced increases in immobility in the FST and TST 48 h post-treatment. Ketamine reversed CMS-induced reductions in sucrose intake in the SPT, 24 h, 4, 6, and 8 days post-treatment. In non-stressed animals ketamine reduced immobility in the TST and FST at 3 and 24 h post-injection	N/A
Parise et al., 2013	Male adolescent Sprague-Dawley rats (post-natal day 35–49)	Fort-Dodge (Schein), 5, 10, and 20 mg/kg (i.p.). Administered twice a day for either 1 or 15 days	Ketamine (10 and 20 mg/kg) reduced immobility in the FST 24 h after the 2nd injection. CMS-induced immobility was reversed by ketamine (20 mg/kg). No effect of ketamine on SPT was observed. Two months after chronic ketamine treatment rats exhibited an anxiolytic phenotype on the EPM and AD effects in the FST	N/A
Lindholm et al., 2012	Adult male C57BL/6J and WT & BDNF ± mice	Sigma-Aldrich 20 and 50 mg/kg (i.p.)	Decreased immobility in FST in WT mice at 45 min but not 7 days post-injection	No alterations in TrkB phosphorylation at 60 min or 7 days post-injection
Tizabi et al., 2012	Male and Female WKY and Wistar rats	Fort-Dodge (Schein), 0.25–5 mg/kg (i.p.), administered once or daily for 10 days	No acute/chronic effect of ketamine on Wistar immobility levels in the FST. 2.5 and 5 mg/kg reduced immobility of WKY rats in the FST, the 5 mg/kg dose had protracted effects 1 week post-injection. Chronic administration of 2.5 and	Ketamine (chronic 0.5 mg/kg paradigm) increased AMPA receptor binding & the AMPA/NMDA ratio in WKY rats

(Continued)

Table 2 | Continued

References	Species and strain	Ketamine—supplier and dose	Behavioral alterations	Molecular alterations
Autry et al., 2011	Adult male C57BL/6 WT and inducible BDNF KO mutants	Fort Dodge Animal Health 3 mg/kg (i.p.)	5 mg/kg reduced immobility of WKY but not Wistar. The effect of the 2.5 mg/kg dose were evident 1 week following the cessation of treatment	
Bechtholt-Gompf et al., 2011	CD-1 and BALB/c mice	Sigma-Aldrich, dose range 0.5–3.0 mg/kg	No effect in EPM or fear conditioning 24 h post-injection. Reduced FST immobility at 30 min, 3 h, 24 h, and 1 week, blocked by NBQX. Reduced latency to feed in NSF, increased sucrose intake & decreased immobility in CMS mice 30 min post-injection. Rapamycin did not block ketamine-induced reductions in FST immobility 30 min post-injection. Anisomycin prevented the effects of ketamine in the NSF & FST. TrkB KO mice did not respond to ketamine	Increased TrkB activation. Increased BDNF protein but not mRNA at 30 min and 1 h post-injection. Decreased phosphorylation of eEF2 in HC. Blocked spontaneous activity of NMDARs in HC cultures
Koike et al., 2011b	Male ICR mice (25–35 g) and male Sprague-Dawley rats (230–350 g)	Sigma-Aldrich 3–30 mg/kg (i.p.)	Reduced immobility in TST 1 h post-injection (1.0 mg/kg), not observed at day 7. No effect on FST immobility at any dose, or time point	N/A
Li et al., 2011	Male Sprague Dawley rats (150–250 g)	Sigma-Aldrich 10 mg/kg (i.p.)	Ketamine reduced the number of failures to escape in the LH test 30 min post 10 mg/kg injection. Reduced immobility in the TST 30 min & 72 h post 30 mg/kg injection	N/A
Yilmaz et al., 2002	Male Wistar rats (280–310 g)	Parke-Davis 50 mg/ml stock 1.0 mg/kg (i.p.)	Ketamine reversed CMS-induced anhedonia in the NSF test 2 days post-injection. Sucrose consumption was increased 1, 3, 5, and 7 days following the single ketamine injection	Ketamine reversed CMS-induced deficits in synaptic EPSCs, spine density and synaptic protein expression. At 7 days post-treatment these effects were still apparent
Garcia et al., 2009	Wistar rats (300–350 g)	Fort Dodge Animal Health 15 mg/kg once on day 7 or daily for 7 days	Ketamine reduced FST at 3, 7, and 10 days post-injection, (this was only in the second test of each day).	N/A
			CMS-induced reductions in sucrose intake, weight loss, adrenal hypertrophy, and	No differences in HC BDNF concentrations

(Continued)

Table 2 | Continued

References	Species and strain	Ketamine—supplier and dose	Behavioral alterations	Molecular alterations
Garcia et al., 2008b	Wistar rats (300–350 g)	Fort Dodge Animal Health, 5, 10, and 15 mg/kg—daily i.p. injections for 12 days	increased ACTH and corticosterone levels were reversed by acute and chronic ketamine administration. Chronic ketamine increased sucrose intake in controls	All doses reduced immobility in the FST
Popik et al., 2008	Male Wistar rats (270 g) and male Sprague Dawley rats (275 g), C57/B1/Han male mice (24 g) and Male Swiss mice (28 g)	Biowet, Pulawy, Poland, FST rats, 1.0 mg/kg. TST mice, 50–1.0 mg/kg. FST mice, 1.25–10 mg/kg	Reduction of immobility in the FST in mice but not in rats at 30 min post-injection only (50 mg/kg). Ketamine reduced immobility in the TST at 40 min but not at 1 week post-injection	N/A
Maeng et al., 2008	Mice	Sigma-Aldrich 0.5–10 mg/kg (i.p.)	Ketamine reduced the number of escape failures in LH 24 h post-injection. Ketamine (2.5 mg/kg) reduced immobility in the FST at 30 min and 2 weeks post-injection	Ketamine reduced phosphorylation of HC GluR1 (S845), rescued by NBQX

This table outlines studies that have assessed the antidepressant-like effects of ketamine from day 2, or, 48 h post-administration onwards in commonly used behavioral tests. In some of these studies earlier time points have been assessed, the results are also included in this table. Molecular alterations of relevance to ketamine's molecular mechanism of action are also reported. FST, forced swim test; TST, tail suspension test; LH, learned helplessness; NSF, novelty suppressed feeding; SPT, sucrose preference test; EPM, elevated plus maze; AD, antidepressant; CMS, chronic mild stress; LPS, lipopolysaccharide; HC, hippocampus; CTX, cortex; Amg, amygdala; mPFC, medial prefrontal cortex; WTm, wild type.

Decreases in sucrose consumption induced by exposure to LPS (Walker et al., 2013) and CMS (Ma et al., 2013) were reversed following a single ketamine treatment. Protracted effects of acute ketamine treatment were evident in CMS exposed mice tested at 4, 6, and 8 days after a single ketamine treatment (Ma et al., 2013). In contrast, the consumption of sugar pellets in CMS exposed rats was not altered by ketamine treatment (Rezin et al., 2009), although this particular test is not directly comparable to the traditional SPT. It should be noted that there is a lack of consensus on the most appropriate SPT protocol to model an anhedonic state in rats. Nevertheless, these data support the use of the SPT as a sensitive screening test for rapid-acting antidepressant-like drugs such as ketamine.

ELEVATED PLUS MAZE (EPM)

The EPM is frequently used to measure anxiety behavior in rodents (Bourin, 1997; Rodgers et al., 1997) and has strong predictive validity for screening anxiolytics. However, it is generally not sensitive to antidepressant treatments. Ketamine induced an anxiolytic phenotype in rats during exposure to the EPM 30 min after a single ketamine injection (Engin et al., 2009). A similar effect was observed in mice 1 and 2 h following treatment (Hayase

et al., 2006). These studies indicate that the EPM was not sensitive to low doses of ketamine; only higher doses (30 mg/kg) induced a significant anxiolytic effect. Moreover, lower doses of ketamine did not induce an anxiolytic response in the EPM in stress naïve mice (Autry et al., 2011). The lack of effect of low doses of ketamine is also characteristic of the TST. Parise and colleagues described significant anxiolytic effects in the EPM in rats 2 months after the completion of a 15-day dosing regimen of 20 mg/kg per day during adolescence (Parise et al., 2013). Although the presence of drug effects after such a long interval could indicate sensitivity to the protracted effects of ketamine, developmental factors may have played a greater role. At present the EPM can only be proposed as a tool for assessing the more immediate anxiolytic effects of ketamine.

LOCOMOTOR ACTIVITY

Antidepressant-like effects of ketamine are usually evaluated in conjunction with spontaneous activity, because increased motor activity can produce false positive effects in the aforementioned behavioral tasks. Ketamine produces significant hyperactivity immediately following injection; 10 min post i.p. injection of low dose ketamine (5–15 mg/kg), rats displayed hyperactivity in

spontaneous activity (da Silva et al., 2010). In addition, repeated administration of ketamine (50 mg/kg) sensitized rats to its hyperactive effects (Popik et al., 2008).

However, most studies have reported either no change or a reduction of locomotor activity after ketamine. A reduction of open field behavior was produced by ketamine in rats at 30 min post 50 mg/kg (Engin et al., 2009) and 1 h post 10 and 25 mg/kg (Gigliucci et al., 2013). In addition, a single injection of ketamine did not alter locomotor activity beyond 30 min post-injection in rats (Reus et al., 2011; Tizabi et al., 2012; Yang et al., 2012; Akinfiresoye and Tizabi, 2013) or in mice (Lindholm et al., 2012). At 24 h post-injection, there was no effect on locomotor activity in mice by ketamine or by the NMDA antagonists CPP and MK-801 (Autry et al., 2011).

Furthermore, chronic administration of low dose ketamine did not affect spontaneous activity in adult rats (Garcia et al., 2008b; Ma et al., 2013). Interestingly, it was shown recently that hyperactivity was displayed in adolescent but not adult rats following chronic ketamine administration (Parise et al., 2013). Many of the experiments assessed in this review did not measure the effects of ketamine on locomotor activity at the dose and time point used. However, taken together, the data suggest it is important practice to assess changes in activity measures post-treatment to identify and eliminate the involvement of any potential locomotor effect in the behavioral responses to ketamine.

LEARNED HELPLESSNESS (LH)

The LH model of depression produces escape deficits in rodents exposed to unpredictable and uncontrollable stress (Seligman et al., 1980). LH is a popular model of depression as it has good face validity and induces a number of endophenotypes that can be measured in other behavioral tasks, including the FST and NSF. Repeated treatment with antidepressants reversed the coping behavior deficits in rats and mice (Shanks and Anisman, 1988; Caldarone et al., 2000). A single administration of ketamine (10 mg/kg) has been reported to reverse the deficits in coping behavior induced by learned helplessness 30–60 min (Beurel et al., 2011; Koike et al., 2011a) and 24 h after treatment (Maeng et al., 2008; Li et al., 2010). Furthermore, ketamine is effective in producing antidepressant-like effects in the LH in CMS-treated mice at even a lower dose (3 mg/kg) (Autry et al., 2011). Currently, there is no information regarding the protracted effects of ketamine in LH.

CHRONIC MILD STRESS (CMS)

Exposure to the CMS model induces depressive behavior in rodents following the presentation of a series of stressors in an unpredictable sequence over a prolonged period of time. CMS produces a number of behavioral changes in rodents thought to resemble features of depressed patients, such as anhedonia or loss of grooming (Willner, 1997, 2005). CMS satisfies most of the criteria of validity for an animal model of depression; it is etiologically relevant with good design, resulting in similar pathological alterations observed in humans that are sensitive to chronic antidepressant treatment. The behavioral and molecular changes induced by CMS are reversed by treatment with antidepressant drugs, but only after administration for several weeks.

In contrast, ketamine reversed the behavioral and physiological alterations induced by CMS in rats following acute administration and the effects were maintained following chronic treatment. Acute and chronic treatment with ketamine reversed the increase in adrenal gland weight, promoted regain of body weight, and normalized circulating corticosterone and ACTH levels (Garcia et al., 2009). Physiological alterations induced by CMS were reversed by acute ketamine treatment in a similar study but failed to reverse CMS-induced anhedonia in the SPT (Rezin et al., 2009). In addition, CMS-exposed adolescent rats exhibited decreased immobility, increased sucrose consumption and latency to feed immediately following acute ketamine treatment (Parise et al., 2013).

Because the CMS is accepted as a rodent model of depression, CMS is an ideal paradigm with which to screen the antidepressant-like effects of novel therapeutics like ketamine. Reversal of CMS-induced depressive-like phenotypes measured using the mouse FST, NSF, and SPT has been reported by ketamine in the absence of any drug effect in stress naïve mice (Autry et al., 2011). Furthermore, the effect of ketamine in the NSF test was observed to persist in CMS mice 48 h post-injection. In line with these findings, two similar studies have indicated an increased sensitivity of CMS-exposed mice to ketamine (Li et al., 2011; Ma et al., 2013). Taken together, the CMS data is the most consistent and possibly the most valid method of examining the antidepressant-like effects of ketamine in preclinical studies.

KETAMINE—MOLECULAR MECHANISMS OF ACTION

In order to develop novel and more effective antidepressants, the molecular mechanisms underlying the protracted behavioral improvement associated with ketamine treatment need to be understood fully. The majority of this information has been garnered from preclinical animal studies and the principle findings are detailed in the following section.

NMDA AND AMPA RECEPTORS

Currently the hypothesis for ketamine's mechanism of action focuses on a cascade of neurochemical events that are initiated shortly after administration of ketamine. The events then persist in a protracted manner for days following its metabolism and elimination.

Reductions in neurogenesis and synaptic plasticity play a key role in the pathophysiology of MDD. Synaptic plasticity refers to the dynamic capability of synapses to form and retract processes, thereby modifying synaptic strength and communication. The most well studied mechanisms mediating changes in plasticity are long-term potentiation (LTP) and long-term depression (LTD). These processes involve significant alterations in pre and post-synaptic scaffolding proteins and glutamate receptors, primarily the glutamatergic receptor, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). The AMPA receptor containing the subunits GluR1, GluR4, and GluR2 are involved in LTP, whereas GluR2, GluR3, and GluR4 are required for the AMPA receptor internalization needed to facilitate LTD (Kessels and Malinow, 2009). N-methyl-D-aspartate (NMDA) receptors at excitatory synapses are also subject to trafficking and significantly decrease in synaptic density during LTD (Peng et al., 2010). In the

pyramidal cells of the hippocampus, LTP and LTD bidirectionally regulate dendritic spine growth and retraction, whereas AMPA expression is positively related to the size of the spine head. These dynamic processes are stabilized by concurrent alterations in the expression of synaptic proteins and signaling pathways.

Ketamine blocks NMDA receptors (NMDARs) at concentrations of 2–50 μ M. The subsequent suppression of tonic glutamate input to GABAergic interneurons, results in disinhibition of glutamate signaling. This disinhibition and increase in glutamate neurotransmission is mediated by a decrease in GABAergic inhibitory feedback of the pyramidal neurons in layer V of the PFC, a region widely implicated in the development of psychiatric disorders (Homayoun and Moghaddam, 2007). Interestingly, post-mortem studies report reductions in pyramidal cells and GABAergic interneurons in the PFC of depressed individuals (Choudary et al., 2005; Rajkowska et al., 2007). Increases in glutamate will activate ionotropic AMPARs resulting in Na^{2+} influx and subsequent membrane depolarization, induction of signaling cascades and protein synthesis. Certain AMPARs that lack the GluR2 subunit actually result in Ca^{2+} influx (Kessels and Malinow, 2009). Upregulation of AMPA receptor expression following ketamine administration mediates the increased sensitivity to glutamate. It has been suggested that this increased sensitivity or “synaptic scaling” is necessary to maintain stability in synaptic plasticity and increased protein synthesis in the presence of chronic NMDAR blockade (Kavalali and Monteggia, 2012).

Pharmacological inhibition of ketamine’s behavioral effects has been achieved using the AMPA receptor antagonist, 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2, 3-dione (NBQX), reversing the antidepressant effects of ketamine in the LH paradigm (Maeng et al., 2008; Koike et al., 2011b). Furthermore, co-administration of AMPAR antagonists blocked the effects of ketamine in the FST (Autry et al., 2011). As AMPARs have a clear role in mediating ketamine’s effects, a recent study showed the antidepressant-like effect of AMPA administration in the depressive-like WKY rats (Akintiresoye and Tizabi, 2013). This data indicate that AMPA receptors and indeed the AMPA/NMDA ratio is an important consideration and target in the development of potential therapeutics.

mTOR SIGNALING

Data suggests that the protracted antidepressant-like effects of ketamine are mediated by molecular alterations to the signaling pathway for the mammalian target of rapamycin (mTOR) (see **Figure 1**), a serine/threonine kinase and key component of the insulin-signaling pathway (Li et al., 2010). Two functional mTOR complexes regulate the initiation of protein translation in mammalian cells, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Rosner and Hengstschlager, 2011). A recent post-mortem study implicated decreases in cortical mTOR-signaling kinases in the pathophysiology of MDD (Jernigan et al., 2011). Additionally, rats exposed to CMS exhibit significant reductions in the phosphorylation of several kinases in the mTOR pathway in the amygdala of stressed rats (Chandran et al., 2013).

There is an inverted U-shape associated with ketamine-induced mTOR activation, with higher doses having no effect.

In rodents, ketamine administration induced mTOR signaling approximately 30 min after injection. Li and colleagues elucidated some core features of ketamine’s mechanism of action, primarily focusing on the alterations in mTOR dependent synapse formation in the PFC of rats (Li et al., 2010). In addition, they reported increased phosphorylation of mTOR, p70 KD ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). P70S6K is required to inhibit suppression of eEF2, which prevents protein translation. Simultaneously, the phosphorylation of 4E-BP1 results in the release of eukaryotic translation initiation factor 4E (eIF-4E), thereby triggering the initiation of translation of synaptic proteins. These changes were accompanied by antidepressant-like behavior in the FST and NSF test (Li et al., 2010).

mTOR is ubiquitously expressed and has been found to localize in the cytoplasm of dendrites, where it can initiate the translation of synaptic proteins essential for the induction of LTP (Duman et al., 2012). PSD95, GluR1 and synapsin are upregulated approximately 2 h post-ketamine; this increase is observed for up to 72 h. Similarly, upregulation of Arc, a cytoskeletal protein is observed approximately 1 h post-injection and sustained for up to 6 h (Li et al., 2010). Arc is linked to the induction of early and late phase LTP and memory formation (Panja et al., 2009). A recent study confirmed that ketamine and MK-801 induced increases in immediate early genes, such as Arc, C-fos and Homer1a. Homer1a/Homer1b/PSD-95 signaling is implicated in glutamate induced synaptic plasticity (de Bartolomeis et al., 2013) and may be an interesting marker of plasticity for ketamine-like compounds. Similarly, reductions in the expression of eukaryotic elongation factor 2 (eEF2) is consistently observed in rodents following ketamine administration both in the PFC (Carrier and Kabbaj, 2013) and hippocampus (Autry et al., 2011). Interestingly, females are more sensitive to the behavioral effects of low dose ketamine compared to males; however, females do not exhibit decreases in eEF2 (Carrier and Kabbaj, 2013). Nevertheless, phosphorylation and inhibition of eEF2 may be a useful marker for rapid antidepressants, as increased phosphorylation of eEF2 in the PFC is also reported following chronic fluoxetine treatment in rats (Dagestad et al., 2006).

Pharmacological modulation of different components of the mTOR-signaling pathway (**Figure 1**) has been used to investigate mechanisms underlying the acute and protracted behavioral actions of ketamine. Inhibition of Akt, following blockade of phosphatidylinositol-3-kinase (PI3K) by LY294002, and inhibition of ERK using U0126, prevented ketamine reversal of CMS-induced deficits (Li et al., 2010). The TrkB inhibitor K252a blocked the effects of ketamine in the TST and the NSF when tested 24 h, but not at 1 h (Koike et al., 2013b). The rapamycin-FKBP12 complex inhibits mTOR signaling when directly bound to mTORC1 (Hoeffer and Klann, 2010). Rapamycin pretreatment inhibited both the molecular and behavioral effects of ketamine on FST, NSF and the LH 24 h post-injection (Li et al., 2010). Furthermore, rapamycin administration did not inhibit the effects of ketamine in the NSF test at 30 min post-injection, but ketamine’s effects were completely blocked at 24 h post-injection (Iijima et al., 2012). Thus, it appears that mTOR signaling is clearly associated with the protracted behavioral effects

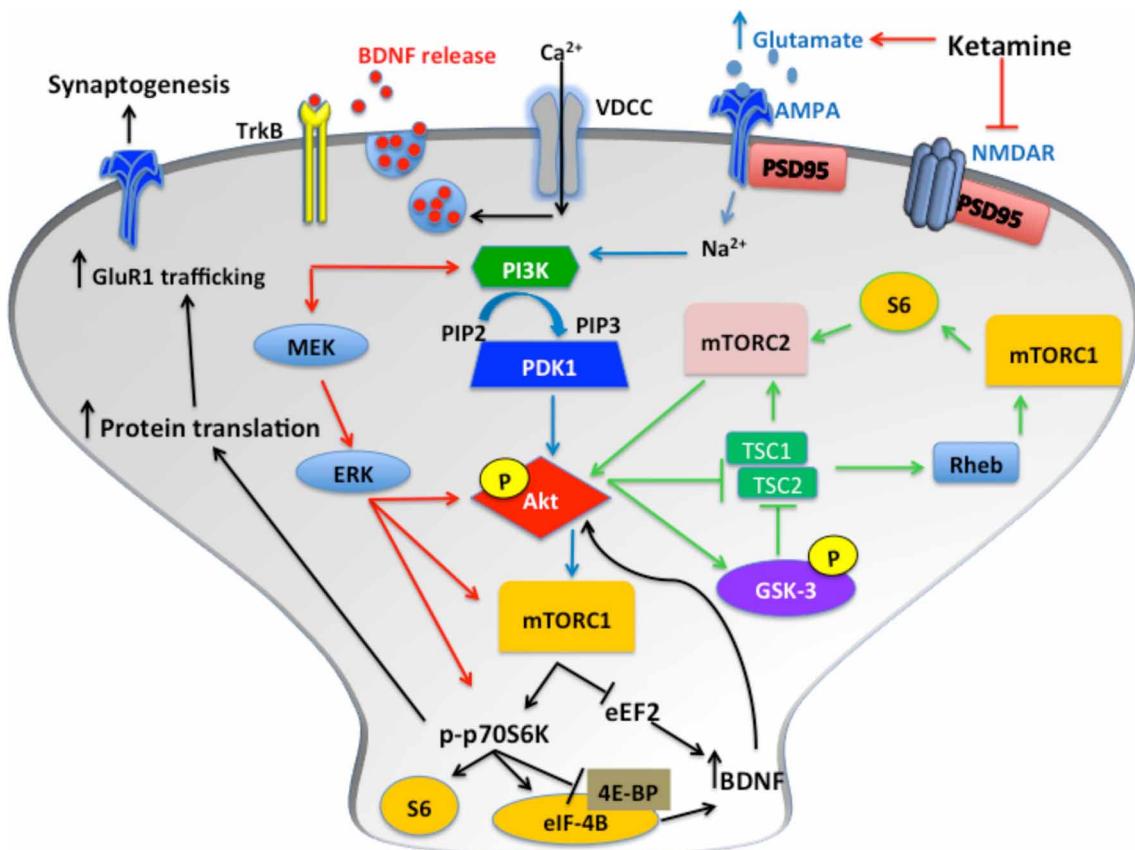


FIGURE 1 | Following blockade of NMDARs, phosphorylation of Akt activates mTOR complex 1 (mTORC1), which results in increased p70S6K phosphorylation and increased protein translation via inhibition of 4E-BP and release of eIF-4B. Glutamate binds AMPARs, which induces depolarization of the membrane, enabling Ca^{2+} influx through VDCCs. This results in BDNF release from synaptic vesicles. The subsequent binding of

TrkB receptors induces ERK and Akt signaling. These pathways all converge to increase synaptic protein translation and receptor trafficking to the cell membrane. Additionally, activation of mTORC2 by S6, and inhibition of GSK-3, induces mTORC1 activation via increased Akt phosphorylation. Furthermore, mTORC2 activation induces protein kinase C (PKC) signaling transduction, which regulates actin and other cytoskeletal proteins.

of ketamine measured 24 h later or longer, but other mechanisms may be involved in the immediate effects of ketamine, such as increased AMPAR activation. It is of interest to note that other antidepressants, including 5-HT_{2C} receptor antagonists, citalopram and electro-convulsive seizures (ECS, the equivalent to ECT in rodents) all increase mTORC1 levels (Elfving et al., 2013; Opal et al., 2013). However, the SSRI, sertraline, and the TCA, imipramine, actually have anti-proliferative effects that are mediated by inhibition of mTOR (Lin et al., 2010; Jeon et al., 2011). Furthermore, there is evidence that suggests rapamycin administration alone and the subsequent inhibition of mTOR signaling is capable of inducing antidepressant-like effects in the rat FST (Cleary et al., 2008). Moreover, the effects of long-term modulation of mTOR have yet to be assessed. These data indicate the role of mTOR signaling may be more complex than originally anticipated.

Other drugs have been used to identify neural mechanisms that might account for the antidepressant-like behavioral effects of ketamine. NMDA receptor blockade using MK-801 or CPP reduced immobility in the FST for up to 3 and 24 h, respectively,

but neither compound reproduced the protracted effects of ketamine at longer intervals (Autry et al., 2011). The NR2B antagonist RO-25-6981 was suggested to induce mTOR signaling, resulting in similar molecular and behavioral effects as those observed following ketamine administration (Maeng et al., 2008; Li et al., 2010). In addition, the mGlu2/3 receptor antagonists LY341495 and MGS0039 decreased immobility time in the TST. NBQX had a limited effect on these antagonists, whereas rapamycin reversed the behavioral effects of these compounds at 24 h post-treatment, suggesting a role for mTOR signaling but not AMPA in mediating the antidepressant-like effects of mGluR2/3 antagonists (Koike et al., 2011a). The mGluR5 antagonist MPEP induced antidepressant-like effects in the NSF at 30 min and 24 h post-injection (Iijima et al., 2012). The effects at 24 h were blocked by rapamycin and the protein synthesis inhibitor anisomycin but not by the TrkB inhibitor K252a. In addition, the mGluR7 agonist AMN082 produced an antidepressant like effect in the TST 40 min post-injection which was reversed by NBQX pretreatment, suggesting that AMPA mediates the antidepressant effects of this compound (Bradley et al., 2012). Finally, the glycine

functional partial agonist GLYX-13 produced an antidepressant-like effect in the FST, NIH and LH tests that extended for 24 h after injection, similar to the effects of ketamine (Burgdorf et al., 2013). These data suggest that when investigating the potential of novel compounds targeting glutamate, both mTOR and AMPA mediation should be assessed. Furthermore, it is important to choose an appropriate rodent strain in which to conduct these assays. For example, CD-1 mice are insensitive to modulation of the glutamatergic system and the subsequent antidepressant-like effects of AMNO82 and the mGluR 7 negative modulator MMPIP (O'Connor and Cryan, 2013).

BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

Chronic administration of antidepressant drugs increases neurotrophins including BDNF (Duman and Monteggia, 2006). BDNF has high affinity for tyrosine kinase receptor B (TrkB), activating a number of signaling pathways that regulate neuronal growth and survival. This pathway also regulates the phosphorylation of cyclic-amp response element binding protein (CREB), which is integral to affective behavior, in addition to learning and memory (Autry and Monteggia, 2012). Post-mortem studies have reported reductions in BDNF and TrkB expression in the hippocampus and PFC of MDD patients and depressed suicides (Krishnan et al., 2007; Castren and Rantamaki, 2010; Yu and Chen, 2011). Rodent models of chronic stress and depression have recapitulated these region-specific changes of BDNF (Duman and Monteggia, 2006; Autry and Monteggia, 2012). At a behavioral level, BDNF administration reduces immobility in the FST (Shirayama et al., 2002; Hoshaw et al., 2005; Deltheil et al., 2008). Additionally, the over-expression of TrkB receptors leads to an antidepressant-like behavioral phenotype in mice (Koponen et al., 2004). BDNF deficient mice are depressive-like in some behavioral tests and fail to respond to conventional antidepressants in the CMS and FST compared to wild type mice (Saarelainen et al., 2003; Monteggia et al., 2007; Ibarguen-Vargas et al., 2009).

Activation of the mTOR pathway by ketamine enhances translation of BDNF in the hippocampus (Garcia et al., 2008a; Autry et al., 2011; Yang et al., 2012). The inhibition of eEF2 and subsequent increase in BDNF translation is proposed to mediate the rapid antidepressant-like effects of ketamine (Monteggia et al., 2013). Equally ketamine is capable of inducing a rapid release of glutamate. Following NMDA receptor blockade, AMPAR activation results in calcium influx via L-type voltage gated calcium channels (VDCC) inducing the release of BDNF from synaptic vesicles (see **Figure 1**). Furthermore, BDNF regulates neuronal mTOR function via Akt and PI3K, creating a positive feedback loop of BDNF production following the activation of mTOR by ketamine (Hay and Sonnenberg, 2004; Hoeffer and Klann, 2010).

A single nucleotide polymorphism Val66Met (rs6265) in the BDNF gene has been proposed as a potential impediment to the antidepressant response to ketamine in TRD patients. Val/Val carriers are more sensitive to the antidepressant-effects of ketamine compared to the Val/Met carriers (Laje et al., 2012). However, not all studies have reported a positive correlation of improvement in depressive symptoms with increased BDNF (Machado-Vieira et al., 2009; Rybakowski et al., 2013). It is worth noting that BDNF

serum concentrations were significantly lower in bipolar patients that did not respond to ketamine treatment compared to responders at baseline (Rybakowski et al., 2013). Mice that possess this polymorphism did not respond to ketamine and displayed significant impairments in synaptogenesis (Lindholm et al., 2012; Liu et al., 2012). However, at higher doses, repeated dosing or continuous infusion of ketamine, BDNF levels were increased, although this increase was correlated with neurodegeneration and cognitive deficits (Ibla et al., 2009; Goulart et al., 2010). Similarly, humans who chronically abuse ketamine exhibit higher BDNF concentrations compared to healthy controls (Ricci et al., 2011).

As a downstream product of multiple signaling cascades induced by ketamine, the production of BDNF occurs rapidly and may underlie the protracted behavioral response to ketamine. Indeed, acute i.c.v infusion of both BDNF and insulin-like growth factor (IGF-1) are capable of mediating protracted antidepressant like effects in the FST lasting up to 6 days following the infusion (Hoshaw et al., 2008). These data not only indicate that alterations in BDNF levels are most likely involved in the protracted effects of ketamine, but also confirms that rapid and persistent increases in neurotrophins are useful markers of novel rapid-acting antidepressants.

GLYCOGEN SYNTHASE KINASE-3 (GSK-3)

GSK-3 is a serine/threonine protein kinase and a major target for the mood stabilizer lithium (Klein and Melton, 1996; Stambolic et al., 1996). TRD patients are often given a period of antidepressant augmentation treatment with lithium when they fail to response to SSRIs alone (Carvalho et al., 2007; Bauer et al., 2010). Furthermore, studies have shown that GSK-3 is functionally regulated by serotonin modulation, primarily mediated by 5-HT_{1A} autoreceptors and via iPi3K/Akt signaling (Polter et al., 2012). GSK-3 β^{\pm} heterozygous mice display significant reductions in immobility in the FST (O'Brien et al., 2004). Interestingly mice with a knock-in mutation of GSK-3, which prevents its phosphorylation, do not respond to ketamine treatment in the LH paradigm, suggesting that some of ketamine's potential therapeutic efficacy might be mediated following inhibition of this kinase (Beurel et al., 2011). Furthermore, combination of ketamine and the GSK-3 inhibitor, SB216763, significantly reduced immobility in the FST; at a molecular level, this combination of ketamine and SB216763 amplified the frequency of 5-HT and hypocretin-induced EPSCs and increased spine density in the mPFC. Conversely, it had been shown that ketamine has limited effects on GSK-3 expression in hippocampal synaptosomes (Muller et al., 2013). Moreover, a single dose of ketamine reversed the behavioral effects of CMS, but the GSK-3 inhibitor SB216763 had no effect on CMS-induced behavioral scores (Ma et al., 2013). Further preclinical studies are required to evaluate the role of GSK-3 β in the antidepressant-like response to ketamine. A recent assessment of three depressed patients indicates a significant increase in phosphorylated GSK-3 β in the plasma of ketamine-treated individuals over the 120-min assessment period (Yang et al., 2013a). Although the inhibition of GSK-3 β modulates mTOR signaling (**Figure 1**) and may potentially augment the effects of antidepressants such as ketamine, it is unclear whether GSK-3 directly mediates the effects of ketamine.

CONCLUSION AND FUTURE DIRECTIONS

The development of ketamine as a rapidly acting antidepressant drug has the potential to revolutionize clinical treatment. Nevertheless, the clinical use of ketamine for depression poses a number of challenges. Ketamine is an hallucinogenic drug subject to abuse and must be given in a controlled setting. The effects of ketamine are short-lasting and can only be sustained by its repeated treatment. A desirable research direction would be to develop other drugs with similar antidepressant effects that are devoid of ketamine's liabilities. However, progress in this area is constrained by uncertainty concerning the critical pharmacological mechanisms underlying the antidepressant effects of ketamine.

Animal models have the potential to translate the pharmacological effects of ketamine that are most critical for its clinical antidepressant effects. A substantial body of literature now indicates that ketamine produces antidepressant-like effects in pre-clinical tests for antidepressant activity and in animal models of depression. Acute ketamine produces immediate effects on many behavioral tests that are similar to antidepressants. However, the protracted effects of ketamine measured for days after a single administration are not produced by conventional antidepressants. They define a new paradigm for antidepressant drug discovery that is the best temporal correlate with ketamine's clinical activity. Inconsistent findings across laboratories may arise from a disparity in methodology used across studies. The most pertinent variables are that the efficacious dose is dependent on the behavioral task employed, conditions surrounding administration and the time of testing post-administration of ketamine. For example, evidence suggests that the effects of low and seemingly sub-efficacious doses of ketamine are more effective following stress exposure. Behavioral tests with high predictive validity for antidepressant-like effects, such as the FST, are sensitive to acute and chronic ketamine. They can be utilized in conjunction with other tests sensitive only to chronic antidepressant treatment, such as the NSF/SPT, to measure the protracted benefits that are unique to ketamine. Overall, combination of a stress or genetic model of depression/anxiety with behavioral assessment over a 1–2 week period post-treatment with low doses of ketamine will yield the most valid and useful information.

Among the many barriers to translation of ketamine's clinical antidepressant effects across species stand a number of key pharmacological factors. The route of administration of ketamine in preclinical models is by i.p. injection, whereas intravenous infusion is usually employed in clinical trials. Therefore, it may be beneficial for animal studies to employ intravenous infusion where practical. In addition, plasma levels of ketamine monitored in the first 2 h following administration can determine whether the dose/route of administration of ketamine produces comparable bioavailability across species. Given that the half-life of ketamine is short, differing levels of ketamine may account for some variation in the behavioral tests. However, ketamine is no longer present when protracted behavioral effects are measured days after administration. These protracted changes result from rapid and sustained molecular alterations induced following a single treatment with ketamine. In addition, the preservative benzethonium chloride (BCL) is universally used in ketamine

preparations both for clinical and preclinical use. Although present in low concentrations, BCL can act synergistically with ketamine to inhibit muscarinic and α 7-nicotinic acetylcholine receptors (Durieux and Nietgen, 1997; Coates and Flood, 2001). The extent to which the additive properties of BCL on ketamine-induced modulation of the cholinergic system may affect the antidepressant-like response to ketamine is unknown. In the present review, there was no systematic evidence that positive or negative findings were associated with the source of ketamine in the behavioral studies examined here (Tables 1, 2).

The mechanisms underlying ketamine's effects, the simultaneous blockade of NMDA receptors and activation of AMPA receptors, are integral for the induction of the antidepressant response. The long-term consequences of these molecular alterations are likely to mediate ketamine's protracted antidepressant-like effects mediated via increased synaptic plasticity, neuronal survival and maturation. These changes occur within hours of ketamine administration and occur in parallel with both the rapid and protracted behavioral effects in animal models of depression. The rapid modulation of mTOR, its downstream mediators, such as Akt and ERK, and BDNF represent markers of the molecular correlates of the antidepressant effects of ketamine and its ability to modify synaptic plasticity. Novel therapeutics for TRD are likely to modulate these markers in a similar temporal pattern to that of ketamine and can be used to identify better pharmaceutical agents to treat TRD.

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REFERENCES

- aan het Rot, M., Collins, K. A., Murrough, J. W., Perez, A. M., Reich, D. L., Charney, D. S., et al. (2010). Safety and efficacy of repeated-dose intravenous ketamine for treatment-resistant depression. *Biol. Psychiatry* 67, 139–145. doi: 10.1016/j.biopsych.2009.08.038
- Aan Het Rot, M., Zarate, C. A. Jr., Charney, D. S., and Mathew, S. J. (2012). Ketamine for depression: where do we go from here? *Biol. Psychiatry* 72, 537–547. doi: 10.1016/j.biopsych.2012.05.003
- Akinfiresoye, L., and Tizabi, Y. (2013). Antidepressant effects of AMPA and ketamine combination: role of hippocampal BDNF, synapsin, and mTOR. *Psychopharmacology (Berl.)* 230, 291–298. doi: 10.1007/s00213-013-3153-2
- Autry, A. E., Adachi, M., Nosyreva, E., Na, E. S., Los, M. F., Cheng, P. F., et al. (2011). NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* 475, 91–95. doi: 10.1038/nature10130
- Autry, A. E., and Monteggia, L. M. (2012). Brain-derived neurotrophic factor and neuropsychiatric disorders. *Pharmacol. Rev.* 64, 238–258. doi: 10.1124/pr.111.005108
- Bauer, M., Adli, M., Bschor, T., Pilhatsch, M., Pfennig, A., Sasse, J., et al. (2010). Lithium's emerging role in the treatment of refractory major depressive episodes: augmentation of antidepressants. *Neuropsychobiology* 62, 36–42. doi: 10.1159/000314308
- Bechtholt-Gompf, A. J., Smith, K. L., John, C. S., Kang, H. H., Carlezon, W. A. Jr., Cohen, B. M., et al. (2011). CD-1 and Balb/cJ mice do not show enduring antidepressant-like effects of ketamine in tests of acute antidepressant efficacy. *Psychopharmacology (Berl.)* 215, 689–695. doi: 10.1007/s00213-011-2169-8
- Berman, R. M., Cappiello, A., Anand, A., Oren, D. A., Heninger, G. R., Charney, D. S., et al. (2000). Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry* 47, 351–354. doi: 10.1016/S0006-3223(99)00230-9
- Beurel, E., Song, L., and Jope, R. S. (2011). Inhibition of glycogen synthase kinase-3 is necessary for the rapid antidepressant effect of ketamine in mice. *Mol. Psychiatry* 16, 1068–1070. doi: 10.1038/mp.2011.47

- Bodnoff, S. R., Suranyi-Cadotte, B., Aitken, D. H., Quirion, R., and Meaney, M. J. (1988). The effects of chronic antidepressant treatment in an animal model of anxiety. *Psychopharmacology (Berl.)* 95, 298–302. doi: 10.1007/BF00181937
- Bourin, M. (1997). Animal models of anxiety: are they suitable for predicting drug action in humans? *Pol. J. Pharmacol.* 49, 79–84.
- Bradley, S. R., Uslaner, J. M., Flick, R. B., Lee, A., Groover, K. M., and Hutson, P. H. (2012). The mGluR7 allosteric agonist AMN082 produces antidepressant-like effects by modulating glutamatergic signaling. *Pharmacol. Biochem. Behav.* 101, 35–40. doi: 10.1016/j.pbb.2011.11.006
- Burgdorf, J., Zhang, X. L., Nicholson, K. L., Balster, R. L., Leander, J. D., Stanton, P. K., et al. (2013). GLYX-13, a NMDA receptor glycine-site functional partial agonist, induces antidepressant-like effects without ketamine-like side effects. *Neuropsychopharmacology* 38, 729–742. doi: 10.1038/npp.2012.246
- Caldarone, B. J., George, T. P., Zachariou, V., and Picciotto, M. R. (2000). Gender differences in learned helplessness behavior are influenced by genetic background. *Pharmacol. Biochem. Behav.* 66, 811–817. doi: 10.1016/S0091-3057(00)00271-9
- Carrier, N., and Kabbaj, M. (2013). Sex differences in the antidepressant-like effects of ketamine. *Neuropharmacology* 70, 27–34. doi: 10.1016/j.neuropharm.2012.12.009
- Carvalho, A. F., Cavalcante, J. L., Castelo, M. S., and Lima, M. C. (2007). Augmentation strategies for treatment-resistant depression: a literature review. *J. Clin. Pharm. Ther.* 32, 415–428. doi: 10.1111/j.1365-2710.2007.00846.x
- Castren, E., and Rantamaki, T. (2010). Role of brain-derived neurotrophic factor in the aetiology of depression: implications for pharmacological treatment. *CNS Drugs* 24, 1–7. doi: 10.2165/11530010-00000000-00000
- Chandran, A., Iyo, A. H., Jernigan, C. S., Legutko, B., Austin, M. C., and Karolewicz, B. (2013). Reduced phosphorylation of the mTOR signaling pathway components in the amygdala of rats exposed to chronic stress. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 40, 240–245. doi: 10.1016/j.pnpbp.2012.08.001
- Choudary, P. V., Molnar, M., Evans, S. J., Tomita, H., Li, J. Z., Vawter, M. P., et al. (2005). Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15653–15658. doi: 10.1073/pnas.0507901102
- Cleary, C., Linde, J. A., Hiscock, K. M., Hadas, I., Belmaker, R. H., Agam, G., et al. (2008). Antidepressive-like effects of rapamycin in animal models: Implications for mTOR inhibition as a new target for treatment of affective disorders. *Brain Res. Bull.* 76, 469–473. doi: 10.1016/j.brainresbull.2008.03.005
- Coates, K. M., and Flood, P. (2001). Ketamine and its preservative, benzethonium chloride, both inhibit human recombinant alpha7 and alpha4beta2 neuronal nicotinic acetylcholine receptors in Xenopus oocytes. *Br. J. Pharmacol.* 134, 871–879. doi: 10.1038/sj.bjp.0704315
- Cruz, S. L., Soberanes-Chavez, P., Paez-Martinez, N., and Lopez-Rubalcava, C. (2009). Toluene has antidepressant-like actions in two animal models used for the screening of antidepressant drugs. *Psychopharmacology (Berl.)* 204, 279–286. doi: 10.1007/s00213-009-1462-2
- Cryan, J. F., Mombereau, C., and Vassout, A. (2005). The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci. Biobehav. Rev.* 29, 571–625. doi: 10.1016/j.neubiorev.2005.03.009
- Cryan, J. F., Valentino, R. J., and Lucki, I. (2005). Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. *Neurosci. Biobehav. Rev.* 29, 547–569. doi: 10.1016/j.neubiorev.2005.03.008
- Dagestad, G., Kuipers, S. D., Messaoudi, E., and Bramham, C. R. (2006). Chronic fluoxetine induces region-specific changes in translation factor eIF4E and eEF2 activity in the rat brain. *Eur. J. Neurosci.* 23, 2814–2818. doi: 10.1111/j.1460-9568.2006.04817.x
- da Silva, F. C., do Carmo de Oliveira Cito, M., da Silva, M. I., Moura, B. A., de Aquino Neto, M. R., et al. (2010). Behavioral alterations and pro-oxidant effect of a single ketamine administration to mice. *Brain Res. Bull.* 83, 9–15. doi: 10.1016/j.brainresbull.2010.05.011
- de Bartolomeis, A., Sarappa, C., Buonaguro, E. F., Marmo, F., Eramo, A., Tomasetti, C., et al. (2013). Different effects of the NMDA receptor antagonists ketamine, MK-801, and memantine on postsynaptic density transcripts and their topography: role of Homer signaling, and implications for novel antipsychotic and pro-cognitive targets in psychosis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 46C, 1–12. doi: 10.1016/j.pnpbp.2013.06.010
- Deltheil, T., Guiard, B. P., Guilloux, J. P., Nicolas, L., Delomenie, C., Reperant, C., et al. (2008). Consequences of changes in BDNF levels on serotonin neurotransmission, 5-HT transporter expression and function: studies in adult mice hippocampus. *Pharmacol. Biochem. Behav.* 90, 174–183. doi: 10.1016/j.pbb.2007.09.018
- Diazgranados, N., Ibrahim, L., Brutsche, N. E., Newberg, A., Kronstein, P., Khalife, S., et al. (2010). A randomized add-on trial of an N-methyl-D-aspartate antagonist in treatment-resistant bipolar depression. *Arch. Gen. Psychiatry* 67, 793–802. doi: 10.1001/archgenpsychiatry.2010.90
- Dulawa, S. C., and Hen, R. (2005). Recent advances in animal models of chronic antidepressant effects: the novelty-induced hypophagia test. *Neurosci. Biobehav. Rev.* 29, 771–783. doi: 10.1016/j.neubiorev.2005.03.017
- Duman, R. S., Li, N., Liu, R. J., Duric, V., and Aghajanian, G. (2012). Signaling pathways underlying the rapid antidepressant actions of ketamine. *Neuropharmacology* 62, 35–41. doi: 10.1016/j.neuropharm.2011.08.044
- Duman, R. S., and Monteggia, L. M. (2006). A neurotrophic model for stress-related mood disorders. *Biol. Psychiatry* 59, 1116–1127. doi: 10.1016/j.biopsych.2006.02.013
- Durieux, M. E., and Nietgen, G. W. (1997). Synergistic inhibition of muscarinic signaling by ketamine stereoisomers and the preservative benzethonium chloride. *Anesthesiology* 86, 1326–1333. doi: 10.1097/00000542-199706000-00014
- Elfving, B., Christensen, T., Ratner, C., Wienecke, J., and Klein, A. B. (2013). Transient activation of mTOR following forced treadmill exercise in rats. *Synapse* 67, 620–625. doi: 10.1002/syn.21668
- Engin, E., Treit, D., and Dickson, C. T. (2009). Anxiolytic- and antidepressant-like properties of ketamine in behavioral and neurophysiological animal models. *Neuroscience* 161, 359–369. doi: 10.1016/j.neuroscience.2009.03.038
- Fava, M., Rush, A. J., Alpert, J. E., Balasubramani, G. K., Wisniewski, S. R., Carmin, C. N., et al. (2008). Difference in treatment outcome in outpatients with anxious versus nonanxious depression: a STAR*D report. *Am. J. Psychiatry* 165, 342–351. doi: 10.1176/appi.ajp.2007.06111868
- Garcia, L. S., Comim, C. M., Valvassori, S. S., Reus, G. Z., Barbosa, L. M., Andreazza, A. C., et al. (2008a). Acute administration of ketamine induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32, 140–144. doi: 10.1016/j.pnpbp.2007.07.027
- Garcia, L. S., Comim, C. M., Valvassori, S. S., Reus, G. Z., Andreazza, A. C., Stertz, L., et al. (2008b). Chronic administration of ketamine elicits antidepressant-like effects in rats without affecting hippocampal brain-derived neurotrophic factor protein levels. *Basic Clin. Pharmacol. Toxicol.* 103, 502–506. doi: 10.1111/j.1742-7843.2008.00210.x
- Garcia, L. S., Comim, C. M., Valvassori, S. S., Reus, G. Z., Stertz, L., Kapczinski, F., et al. (2009). Ketamine treatment reverses behavioral and physiological alterations induced by chronic mild stress in rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 450–455. doi: 10.1016/j.pnpbp.2009.01.004
- Ghasemi, M., Raza, M., and Dehpour, A. R. (2010). NMDA receptor antagonists augment antidepressant-like effects of lithium in the mouse forced swimming test. *J. Psychopharmacol.* 24, 585–594. doi: 10.1177/0269881109104845
- Gigliucci, V., O'Dowd, G., Casey, S., Egan, D., Gibney, S., and Harkin, A. (2013). Ketamine elicits sustained antidepressant-like activity via a serotonin-dependent mechanism. *Psychopharmacology (Berl.)* 228, 157–166. doi: 10.1007/s00213-013-3024-x
- Goulart, B. K., de Lima, M. N., de Farias, C. B., Reolon, G. K., Almeida, V. R., Quevedo, J., et al. (2010). Ketamine impairs recognition memory consolidation and prevents learning-induced increase in hippocampal brain-derived neurotrophic factor levels. *Neuroscience* 167, 969–973. doi: 10.1016/j.neuroscience.2010.03.032
- Hay, N., and Sonnenberg, N. (2004). Upstream and downstream of mTOR. *Genes Dev.* 18, 1926–1945. doi: 10.1101/gad.1212704
- Hayase, T., Yamamoto, Y., and Yamamoto, K. (2006). Behavioral effects of ketamine and toxic interactions with psychostimulants. *BMC Neurosci.* 7:25. doi: 10.1186/1471-2202-7-25
- Hoeffer, C. A., and Klann, E. (2010). mTOR signaling: at the crossroads of plasticity, memory and disease. *Trends Neurosci.* 33, 67–75. doi: 10.1016/j.tins.2009.11.003
- Homayoun, H., and Moghaddam, B. (2007). NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J. Neurosci.* 27, 11496–11500. doi: 10.1523/JNEUROSCI.2213-07.2007

- Hoshaw, B. A., Hill, T. I., Crowley, J. J., Malberg, J. E., Khawaja, X., Rosenzweig-Lipson, S., et al. (2008). Antidepressant-like behavioral effects of IGF-I produced by enhanced serotonin transmission. *Eur. J. Pharmacol.* 594, 109–116. doi: 10.1016/j.ejphar.2008.07.023
- Hoshaw, B. A., Malberg, J. E., and Lucki, I. (2005). Central administration of IGF-I and BDNF leads to long-lasting antidepressant-like effects. *Brain Res.* 1037, 204–208. doi: 10.1016/j.brainres.2005.01.007
- Hoyer, C., Kranaster, L., Janke, C., and Sartorius, A. (2013). Impact of the anesthetic agents ketamine, etomidate, thiopental, and propofol on seizure parameters and seizure quality in electroconvulsive therapy: a retrospective study. *Eur. Arch. Psychiatry Clin. Neurosci.* doi: 10.1007/s00406-013-0420-5. [Epub ahead of print].
- Ibarguen-Vargas, Y., Surget, A., Vourc'h, P., Leman, S., Andres, C. R., Gardier, A. M., et al. (2009). Deficit in BDNF does not increase vulnerability to stress but dampens antidepressant-like effects in the unpredictable chronic mild stress. *Behav. Brain Res.* 202, 245–251. doi: 10.1016/j.bbr.2009.03.040
- Ibla, J. C., Hayashi, H., Bajic, D., and Soriano, S. G. (2009). Prolonged exposure to ketamine increases brain derived neurotrophic factor levels in developing rat brains. *Curr. Drug Saf.* 4, 11–16. doi: 10.2174/157488609787354495
- Ibrahim, L., Diaz Granados, N., Jolkovsky, L., Brutsche, N., Luckenbaugh, D. A., Herring, W. J., et al. (2012a). A Randomized, placebo-controlled, crossover pilot trial of the oral selective NR2B antagonist MK-0657 in patients with treatment-resistant major depressive disorder. *J. Clin. Psychopharmacol.* 32, 551–557. doi: 10.1097/JCP.0b013e31825d70d6
- Ibrahim, L., Diazgranados, N., Franco-Chaves, J., Brutsche, N., Henter, I. D., Kronstein, P., et al. (2012b). Course of improvement in depressive symptoms to a single intravenous infusion of ketamine vs add-on riluzole: results from a 4-week, double-blind, placebo-controlled study. *Neuropsychopharmacology* 37, 1526–1533. doi: 10.1038/npp.2011.338
- Ibrahim, L., Diazgranados, N., Luckenbaugh, D. A., Machado-Vieira, R., Baumann, J., Mallinger, A. G., et al. (2011). Rapid decrease in depressive symptoms with an N-methyl-D-aspartate antagonist in ECT-resistant major depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1155–1159. doi: 10.1016/j.pnpbp.2011.03.019
- Iijima, M., Fukumoto, K., and Chaki, S. (2012). Acute and sustained effects of a metabotropic glutamate 5 receptor antagonist in the novelty-suppressed feeding test. *Behav. Brain Res.* 235, 287–292. doi: 10.1016/j.bbr.2012.08.016
- Irwin, S. A., Iglewicz, A., Nelesen, R. A., Lo, J. Y., Carr, C. H., Romero, S. D., et al. (2013). Daily oral ketamine for the treatment of depression and anxiety in patients receiving hospice care: a 28-day open-label proof-of-concept trial. *J. Palliat. Med.* 16, 958–965. doi: 10.1089/jpm.2012.0617
- Jarvenpaa, K., Chrapel, W., Kampman, O., Tuohimaa, K., Bjorkqvist, M., Hakkinen, H., et al. (2013). Effects of S-ketamine as anesthetic adjuvant to propofol on treatment response to electroconvulsive therapy in treatment-resistant depression: a randomized pilot study. *J. ECT* 29, 158–161. doi: 10.1097/YCT.0b013e318283b7e9
- Jeon, S. H., Kim, S. H., Kim, Y., Kim, Y. S., Lim, Y., Lee, Y. H., et al. (2011). The tricyclic antidepressant imipramine induces autophagic cell death in U-87MG glioma cells. *Biochem. Biophys. Res. Commun.* 413, 311–317. doi: 10.1016/j.bbrc.2011.08.093
- Jernigan, C. S., Goswami, D. B., Austin, M. C., Iyo, A. H., Chandran, A., Stockmeier, C. A., et al. (2011). The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1774–1779. doi: 10.1016/j.pnpbp.2011.05.010
- Joffe, R. T., Bagby, R. M., and Levitt, A. (1993). Anxious and nonanxious depression. *Am. J. Psychiatry* 150, 1257–1258.
- Kavalali, E. T., and Monteggia, L. M. (2012). Synaptic mechanisms underlying rapid antidepressant action of ketamine. *Am. J. Psychiatry* 169, 1150–1156. doi: 10.1176/appi.ajp.2012.12040531
- Kessels, H. W., and Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. *Neuron* 61, 340–350. doi: 10.1016/j.neuron.2009.01.015
- Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., and Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry* 62, 617–627. doi: 10.1001/archpsyc.62.6.617
- Klein, P. S., and Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. U.S.A.* 93, 8455–8459. doi: 10.1073/pnas.93.16.8455
- Koike, H., Iijima, M., and Chaki, S. (2011a). Involvement of the mammalian target of rapamycin signaling in the antidepressant-like effect of group II metabotropic glutamate receptor antagonists. *Neuropharmacology* 61, 1419–1423. doi: 10.1016/j.neuropharm.2011.08.034
- Koike, H., Iijima, M., and Chaki, S. (2011b). Involvement of AMPA receptor in both the rapid and sustained antidepressant-like effects of ketamine in animal models of depression. *Behav. Brain Res.* 224, 107–111. doi: 10.1016/j.bbr.2011.05.035
- Koike, H., Iijima, M., and Chaki, S. (2013a). Effects of ketamine and LY341495 on the depressive-like behavior of repeated corticosterone-injected rats. *Pharmacol. Biochem. Behav.* 107, 20–23. doi: 10.1016/j.pbb.2013.03.017
- Koike, H., Fukumoto, K., Iijima, M., and Chaki, S. (2013b). Role of BDNF/TrkB signaling in antidepressant-like effects of a group II metabotropic glutamate receptor antagonist in animal models of depression. *Behav. Brain Res.* 238, 48–52. doi: 10.1016/j.bbr.2012.10.023
- Koponen, E., Lakso, M., and Castren, E. (2004). Overexpression of the full-length neurotrophin receptor TrkB regulates the expression of plasticity-related genes in mouse brain. *Brain Res. Mol. Brain Res.* 130, 81–94. doi: 10.1016/j.molbrainres.2004.07.010
- Krishnan, V., Han, M. H., Graham, D. L., Berton, O., Renthal, W., Russo, S. J., et al. (2007). Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 131, 391–404. doi: 10.1016/j.cell.2007.09.018
- Laje, G., Lally, N., Mathews, D., Brutsche, N., Chemerinski, A., Akula, N., et al. (2012). Brain-derived neurotrophic factor Val66Met polymorphism and antidepressant efficacy of ketamine in depressed patients. *Biol. Psychiatry* 72, e27–e28. doi: 10.1016/j.biopsych.2012.05.031
- Li, N., Lee, B., Liu, R. J., Banasr, M., Dwyer, J. M., Iwata, M., et al. (2010). mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* 329, 959–964. doi: 10.1126/science.1190287
- Li, N., Liu, R. J., Dwyer, J. M., Banasr, M., Lee, B., Son, H., et al. (2011). Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biol. Psychiatry* 69, 754–761. doi: 10.1016/j.biopsych.2010.12.015
- Lin, C. J., Robert, F., Sukarieh, R., Michnick, S., and Pelletier, J. (2010). The antidepressant sertraline inhibits translation initiation by curtailing mammalian target of rapamycin signaling. *Cancer Res.* 70, 3199–3208. doi: 10.1158/0008-5472.CAN-09-4072
- Lindholm, J. S., Autio, H., Vesa, L., Antila, H., Lindemann, L., Hoener, M. C., et al. (2012). The antidepressant-like effects of glutamatergic drugs ketamine and AMPA receptor potentiator LY 451646 are preserved in bdnf(+/-) heterozygous null mice. *Neuropharmacology* 62, 391–397. doi: 10.1016/j.neuropharm.2011.08.015
- Liu, R. J., Fuchikami, M., Dwyer, J. M., Lepack, A. E., Duman, R. S., and Aghajanian, G. K. (2013). GSK-3 inhibition potentiates the synaptic and antidepressant-like effects of subthreshold doses of ketamine. *Neuropsychopharmacology* 38, 2268–2277. doi: 10.1038/npp.2013.128
- Liu, R. J., Lee, F. S., Li, X. Y., Bambico, F., Duman, R. S., and Aghajanian, G. K. (2012). Brain-derived neurotrophic factor Val66Met allele impairs basal and ketamine-stimulated synaptogenesis in prefrontal cortex. *Biol. Psychiatry* 71, 996–1005. doi: 10.1016/j.biopsych.2011.09.030
- Lopez-Rubalcava, C., and Lucki, I. (2000). Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test. *Neuropsychopharmacology* 22, 191–199. doi: 10.1016/S0893-133X(99)00100-1
- Ma, X. C., Tang, Y. H., Jia, M., Ma, R., Wang, F., Wu, J., et al. (2013). Long-lasting antidepressant action of ketamine, but not glycogen synthase kinase-3 inhibitor SB216763, in the chronic mild stress model of mice. *PLoS ONE* 8:e56053. doi: 10.1371/journal.pone.0056053
- Machado-Vieira, R., Yuan, P., Brutsche, N., DiazGranados, N., Luckenbaugh, D., Manji, H. K., et al. (2009). Brain-derived neurotrophic factor and initial antidepressant response to an N-methyl-D-aspartate antagonist. *J. Clin. Psychiatry* 70, 1662–1666. doi: 10.4088/JCP.08m04659
- Maeng, S., Zarate, C. A. Jr., Du, J., Schloesser, R. J., McCammon, J., Chen, G., et al. (2008). Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biol. Psychiatry* 63, 349–352. doi: 10.1016/j.biopsych.2007.05.028
- Mantovani, M., Pertile, R., Calixto, J. B., Santos, A. R., and Rodrigues, A. L. (2003). Melatonin exerts an antidepressant-like effect in the tail suspension test in mice: evidence for involvement of N-methyl-D-aspartate receptors and the L-arginine-nitric oxide pathway. *Neurosci. Lett.* 343, 1–4. doi: 10.1016/S0304-3940(03)00306-9

- Mathew, S. J., Shah, A., Lapidus, K., Clark, C., Jarun, N., Ostermeyer, B., et al. (2012). Ketamine for treatment-resistant unipolar depression: current evidence. *CNS Drugs* 26, 189–204. doi: 10.2165/11599770-000000000-00000
- Monteggia, L. M., Gideons, E., and Kavalali, E. T. (2013). The role of eukaryotic elongation factor 2 kinase in rapid antidepressant action of ketamine. *Biol. Psychiatry* 73, 1199–1203. doi: 10.1016/j.biopsych.2012.09.006
- Monteggia, L. M., Luikart, B., Barrot, M., Theobold, D., Malkovska, I., Nef, S., et al. (2007). Brain-derived neurotrophic factor conditional knockouts show gender differences in depression-related behaviors. *Biol. Psychiatry* 61, 187–197. doi: 10.1016/j.biopsych.2006.03.021
- Muller, H. K., Wegener, G., Liebenberg, N., Zarate, C. A. Jr., Popoli, M., and Elfving, B. (2013). Ketamine regulates the presynaptic release machinery in the hippocampus. *J. Psychiatr. Res.* 47, 892–899. doi: 10.1016/j.jpsychires.2013.03.008
- Murrough, J. W., Iosifescu, D. V., Chang, L. C., Al Jurdi, R. K., Green, C. M., Perez, A. M., et al. (2013). Antidepressant efficacy of ketamine in treatment-resistant major depression: a two-site randomized controlled trial. *Am. J. Psychiatry* 170, 1134–1142. doi: 10.1176/appi.ajp.2013.13030392
- O'Brien, W. T., Harper, A. D., Jove, F., Woodgett, J. R., Maretto, S., Piccolo, S., et al. (2004). Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. *J. Neurosci.* 24, 6791–6798. doi: 10.1523/JNEUROSCI.4753-03.2004
- O'Connor, R. M., and Cryan, J. F. (2013). The effects of mGlu7 receptor modulation in behavioural models sensitive to antidepressant action in two mouse strains. *Behav. Pharmacol.* 24, 105–113. doi: 10.1097/FBP.0b013e32835fc78
- Opal, M. D., Klenotich, S. C., Morais, M., Bessa, J., Winkle, J., Doukas, D., et al. (2013). Serotonin 2C receptor antagonists induce fast-onset antidepressant effects. *Mol. Psychiatry*. doi: 10.1038/mp.2013.144. [Epub ahead of print].
- Panja, D., Dagyte, G., Bidinosti, M., Wibrand, K., Kristiansen, A. M., Sonenberg, N., et al. (2009). Novel translational control in Arc-dependent long term potentiation consolidation *in vivo*. *J. Biol. Chem.* 284, 31498–31511. doi: 10.1074/jbc.M109.056077
- Parise, E. M., Alcantara, L. F., Warren, B. L., Wright, K. N., Hadad, R., Sial, O. K., et al. (2013). Repeated Ketamine Exposure Induces an Enduring Resilient Phenotype in Adolescent and Adult Rats. *Biol. Psychiatry* 74, 750–759. doi: 10.1016/j.biopsych.2013.04.027
- Peng, Y., Zhao, J., Gu, Q. H., Chen, R. Q., Xu, Z., Yan, J. Z., et al. (2010). Distinct trafficking and expression mechanisms underlie LTP and LTD of NMDA receptor-mediated synaptic responses. *Hippocampus* 20, 646–658. doi: 10.1002/hipo.20654
- Phelps, L. E., Brutsche, N., Moral, J. R., Luckenbaugh, D. A., Manji, H. K., and Zarate, C. A. Jr. (2009). Family history of alcohol dependence and initial antidepressant response to an N-methyl-D-aspartate antagonist. *Biol. Psychiatry* 65, 181–184. doi: 10.1016/j.biopsych.2008.09.029
- Polter, A. M., Yang, S., Jope, R. S., and Li, X. (2012). Functional significance of glycogen synthase kinase-3 regulation by serotonin. *Cell. Signal.* 24, 265–271. doi: 10.1016/j.cellsig.2011.09.009
- Popik, P., Kos, T., Sowa-Kucma, M., and Nowak, G. (2008). Lack of persistent effects of ketamine in rodent models of depression. *Psychopharmacology (Berl.)* 198, 421–430. doi: 10.1007/s00213-008-1158-z
- Preskorn, S. H., Baker, B., Kolluri, S., Menniti, F. S., Kramps, M., and Landen, J. W. (2008). An innovative design to establish proof of concept of the antidepressant effects of the NR2B subunit selective N-methyl-D-aspartate antagonist, CP-101,606, in patients with treatment-refractory major depressive disorder. *J. Clin. Psychopharmacol.* 28, 631–637. doi: 10.1089/JCP.0b013e31818a6cea
- Rajkowska, G., O'Dwyer, G., Teleki, Z., Stockmeier, C. A., and Miguel-Hidalgo, J. J. (2007). GABAergic neurons immunoreactive for calcium binding proteins are reduced in the prefrontal cortex in major depression. *Neuropsychopharmacology* 32, 471–482. doi: 10.1038/sj.npp.1301234
- Reus, G. Z., Stringari, R. B., Ribeiro, K. F., Ferraro, A. K., Vitto, M. F., Cesconetto, P., et al. (2011). Ketamine plus imipramine treatment induces antidepressant-like behavior and increases CREB and BDNF protein levels and PKA and PKC phosphorylation in rat brain. *Behav. Brain Res.* 221, 166–171. doi: 10.1016/j.bbr.2011.02.024
- Rezin, G. T., Goncalves, C. L., Daufenbach, J. F., Fraga, D. B., Santos, P. M., Ferreira, G. K., et al. (2009). Acute administration of ketamine reverses the inhibition of mitochondrial respiratory chain induced by chronic mild stress. *Brain Res. Bull.* 79, 418–421. doi: 10.1016/j.brainresbull.2009.03.010
- Ricci, V., Martinotti, G., Gelfo, F., Tonioni, F., Caltagirone, C., Bria, P., et al. (2011). Chronic ketamine use increases serum levels of brain-derived neurotrophic factor. *Psychopharmacology (Berl.)* 215, 143–148. doi: 10.1007/s00213-010-2121-3
- Rodgers, R. J., Cao, B. J., Dalvi, A., and Holmes, A. (1997). Animal models of anxiety: an ethological perspective. *Braz. J. Med. Biol. Res.* 30, 289–304. doi: 10.1590/S0100-879X1997000300002
- Rosa, A. O., Lin, J., Calixto, J. B., Santos, A. R., and Rodrigues, A. L. (2003). Involvement of NMDA receptors and L-arginine-nitric oxide pathway in the antidepressant-like effects of zinc in mice. *Behav. Brain Res.* 144, 87–93. doi: 10.1016/S0166-4328(03)00069-X
- Rosner, M., and Hengstschlager, M. (2011). mTOR protein localization is cell cycle-regulated. *Cell Cycle* 10, 3608–3610. doi: 10.4161/cc.10.20.17855
- Rybakowski, J. K., Permoda-Osip, A., Skibinska, M., Adamski, R., and Bartkowska-Sniatkowska, A. (2013). Single ketamine infusion in bipolar depression resistant to antidepressants: are neurotrophins involved? *Hum. Psychopharmacol.* 28, 87–90. doi: 10.1002/hup.2271
- Saarelainen, T., Hendolin, P., Lucas, G., Koponen, E., Sairanen, M., MacDonald, E., et al. (2003). Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *J. Neurosci.* 23, 349–357.
- Schosser, A., Serretti, A., Souery, D., Mendlewicz, J., Zohar, J., Montgomery, S., et al. (2012). European Group for the Study of Resistant Depression (GSRD)—where have we gone so far: review of clinical and genetic findings. *Eur. Neuropsychopharmacol.* 22, 453–468. doi: 10.1016/j.euroneuro.2012.02.006
- Segmiller, F., Ruther, T., Linhardt, A., Padberg, F., Berger, M., Pogarell, O., et al. (2013). Repeated S-ketamine infusions in therapy resistant depression: a case series. *J. Clin. Pharmacol.* 53, 996–998. doi: 10.1002/jcpb.122
- Seligman, M. E., Weiss, J., Weinraub, M., and Schulman, A. (1980). Coping behavior: learned helplessness, physiological change and learned inactivity. *Behav. Res. Ther.* 18, 459–512. doi: 10.1016/0005-7967(80)90011-X
- Shanks, N., and Anisman, H. (1988). Stressor-provoked behavioral changes in six strains of mice. *Behav. Neurosci.* 102, 894–905. doi: 10.1037/0735-7044.102.6.894
- Shirayama, Y., Chen, A. C., Nakagawa, S., Russell, D. S., and Duman, R. S. (2002). Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *J. Neurosci.* 22, 3251–3261.
- Solberg, L. C., Baum, A. E., Ahmadiyah, N., Shimomura, K., Li, R., Turek, F. W., et al. (2004). Sex- and lineage-specific inheritance of depression-like behavior in the rat. *Mamm. Genome* 15, 648–662. doi: 10.1007/s00335-004-2326-z
- Souery, D., Oswald, P., Massat, I., Boller, J., Demetyteneare, K., et al. (2007). Group for the Study of Resistant, Clinical factors associated with treatment resistance in major depressive disorder: results from a European multicenter study. *J. Clin. Psychiatry* 68, 1062–1070. doi: 10.4088/JCP.v68n0713
- Stambolic, V., Ruel, L., and Woodgett, J. R. (1996). Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* 6, 1664–1668. doi: 10.1016/S0960-9822(02)70790-2
- Steru, L., Chermat, R., Thierry, B., and Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl.)* 85, 367–370. doi: 10.1007/BF00428203
- Tejani-Butt, S., Kluczynski, J., and Pare, W. P. (2003). Strain-dependent modification of behavior following antidepressant treatment. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 7–14. doi: 10.1016/S0278-5846(02)00308-1
- Tizabi, Y., Bhatti, B. H., Manaye, K. F., Das, J. R., and Akinfiresoye, L. (2012). Antidepressant-like effects of low ketamine dose is associated with increased hippocampal AMPA/NMDA receptor density ratio in female Wistar-Kyoto rats. *Neuroscience* 213, 72–80. doi: 10.1016/j.neuroscience.2012.03.052
- Trivedi, M. H., Fava, M., Wisniewski, S. R., Thase, M. E., Quitkin, F., Warden, D., et al. (2006). Medication augmentation after the failure of SSRIs for depression. *N. Engl. J. Med.* 354, 1243–1252. doi: 10.1056/NEJMoa052964
- Walker, A. K., Budac, D. P., Bisulco, S., Lee, A. W., Smith, R. A., Beenders, B., et al. (2013). NMDA receptor blockade by ketamine abrogates lipopolysaccharide-induced depressive-like behavior in C57BL/6J Mice. *Neuropsychopharmacology* 38, 1609–1616. doi: 10.1038/npp.2013.71
- Wang, X., Chen, Y., Zhou, X., Liu, F., Zhang, T., and Zhang, C. (2012). Effects of propofol and ketamine as combined anesthesia for electroconvulsive therapy in patients with depressive disorder. *J. ECT* 28, 128–132. doi: 10.1097/YCT.0b013e31824d1d02

- Wang, X., Yang, Y., Zhou, X., Wu, J., Li, J., Jiang, X., et al. (2011). Propofol pretreatment increases antidepressant-like effects induced by acute administration of ketamine in rats receiving forced swimming test. *Psychiatry Res.* 185, 248–253. doi: 10.1016/j.psychres.2010.04.046
- Will, C. C., Aird, F., and Redei, E. E. (2003). Selectively bred Wistar-Kyoto rats: an animal model of depression and hyper-responsiveness to antidepressants. *Mol. Psychiatry* 8, 925–932. doi: 10.1038/sj.mp.4001345
- Willner, P. (1997). Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl.)* 134, 319–329. doi: 10.1007/s002130050456
- Willner, P. (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology* 52, 90–110. doi: 10.1159/000087097
- Yang, C., Li, X., Wang, N., Xu, S., Yang, J., and Zhou, Z. (2012). Tramadol reinforces antidepressant effects of ketamine with increased levels of brain-derived neurotrophic factor and tropomyosin-related kinase B in rat hippocampus. *Front. Med.* 6, 411–415. doi: 10.1007/s11684-012-0226-2
- Yang, C., Zhou, Z. Q., Gao, Z. Q., Shi, J. Y., and Yang, J. J. (2013a). Acute increases in plasma mammalian target of rapamycin, glycogen synthase kinase-3beta, and eukaryotic elongation factor 2 phosphorylation after ketamine treatment in three depressed patients. *Biol. Psychiatry* 73, e35–e36. doi: 10.1016/j.biopsych.2012.07.022a
- Yang, C., Hu, Y. M., Zhou, Z. Q., Zhang, G. F., and Yang, J. J. (2013b). Acute administration of ketamine in rats increases hippocampal BDNF and mTOR levels during forced swimming test. *Ups. J. Med. Sci.* 118, 3–8. doi: 10.3109/03009734.2012.724118
- Yilmaz, A., Schulz, D., Aksay, A., and Canbeyli, R. (2002). Prolonged effect of anesthetic dose of ketamine on behavioral despair. *Pharmacol. Biochem. Behav.* 71, 341–344. doi: 10.1016/S0091-3057(01)00693-1
- Yu, H., and Chen, Z. Y. (2011). The role of BDNF in depression on the basis of its location in the neural circuitry. *Acta Pharmacol. Sin.* 32, 3–11. doi: 10.1038/aps.2010.184
- Zarate, C. A. Jr., Mathews, D., Ibrahim, L., Chaves, J. F., Marquardt, C., Ukoh, I., et al. (2013). A randomized trial of a low-trapping nonselective N-methyl-D-aspartate channel blocker in major depression. *Biol. Psychiatry* 74, 257–264. doi: 10.1016/j.biopsych.2012.10.019
- Zarate, C. A. Jr., Singh, J. B., Carlson, P. J., Brutsche, N. E., Ameli, R., Luckenbaugh, D. A., et al. (2006). A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch. Gen. Psychiatry* 63, 856–864. doi: 10.1001/archpsyc.63.8.856

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Selective serotonin reuptake inhibitor antidepressant treatment discontinuation syndrome: a review of the clinical evidence and the possible mechanisms involved

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Besides demonstrated efficacy, selective serotonin reuptake inhibitors (SSRIs) hold other advantages over earlier antidepressants such as greater tolerability and a wider range of clinical applications. However, there is a growing body of clinical evidence which suggests that SSRIs could, in some cases, be associated with a withdrawal reaction upon cessation of regular use. In addition to sensory and gastrointestinal-related symptoms, the somatic symptoms of the SSRI discontinuation syndrome include dizziness, lethargy, and sleep disturbances. Psychological symptoms have also been documented, usually developing within 1–7 days following SSRI discontinuation. The characteristics of the discontinuation syndrome have been linked to the half-life of a given SSRI, with a greater number of reports emerging from paroxetine compared to other SSRIs. However, many aspects of the neurobiology of the SSRI discontinuation syndrome (or SSRI withdrawal syndrome) remain unresolved. Following a comprehensive overview of the clinical evidence, we will discuss the underlying pathophysiology of the SSRI discontinuation syndrome and comment on the use of animal models to better understand this condition.

Keywords: antidepressant treatment, selective serotonin reuptake inhibitor, SSRI discontinuation syndrome, SSRI withdrawal syndrome, animal models, clinical evidence, serotonin

INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are widely used in the treatment of depressive disorders (Petersen et al., 2002; Chaudhry et al., 2011) and anxiety disorders (van der Linden et al., 2000; Hedges et al., 2007). Total SSRI prescription volume increased threefold between 1995/1996 and 2006/2007 (Lockhart and Guthrie, 2011). Despite this, the use of SSRIs is not without flaws. Apart from potential adverse side effects arising from long-term antidepressant treatment, as well as issues of relapse, and recurrence following remission of a depressive episode (Rucci et al., 2011; Rush et al., 2012), the existence of a SSRI discontinuation syndrome (also known as SSRI withdrawal syndrome) has been suggested in some cases. A decade ago, Harvey et al. (2003) highlighted that although significant progress had been made to uncover the pathophysiology of depression and the mechanisms of actions of SSRIs, the neurobiology of SSRI discontinuation syndrome had not been comprehensively addressed. Following an overview of the clinical evidence, we will discuss the possible molecular mechanisms implicated in the pathology of SSRI discontinuation syndrome, and comment on the use of animal models to better understand this condition.

CLINICAL EVIDENCE OF SSRI DISCONTINUATION SYNDROME

About 15 years ago, Zajecka et al. (1997) proposed a definition of the SSRI discontinuation syndrome as the onset of a cluster of symptoms following the discontinuation of a SSRI, not attributable to other causes (i.e., concomitant medication, illness). Along

with sensory and gastrointestinal symptoms, the SSRI discontinuation syndrome includes somatic symptoms such as dizziness, lethargy, and sleep disturbances, as well as psychological symptoms such as anxiety/agitation, irritability, and poor concentration (Warner et al., 2006; Haddad and Anderson, 2007). The literature on these symptoms initially consisted mainly of case reports (Coupland et al., 1996; Price et al., 1996; Schatzberg et al., 1997; Bryois et al., 1998; Goldstein et al., 1999). Haddad (1997) reviewed 47 separate reports of SSRI discontinuation syndrome, 30 of which involved paroxetine compared to only seven involving fluoxetine, despite the latter being prescribed more frequently. Black et al. (2000) identified 53 different symptoms within the condition (with dizziness being the most common) and proposed a diagnostic criteria for SSRI discontinuation syndrome that requires two or more of the described symptoms developing within 1–7 days of discontinuation (or reduction in dosage) of an SSRI after at least 1 month of treatment.

These case reports have since been followed up by a number of controlled studies (i.e., prospective studies, with a randomized, double-blind interruption period, which included a systematic method for discontinuation symptoms data collection). For example, using the Discontinuation Emergent Signs and Symptoms (DESS) checklist, Rosenbaum et al. (1998) assessed the effects of a 1-week placebo substitution period in patients diagnosed with unipolar depressive disorder who had been maintained on fluoxetine, sertraline, or paroxetine for similar periods of time (~11 months). Following treatment interruption, there was a significant increase in the number of DESS items observed in the

sertraline- and paroxetine-treated patients. In contrast, this was not detected in fluoxetine-treated patients. Another double-blind trial found that placebo substitution for paroxetine was associated with increased frequency and severity of specific physical and psychological symptoms, which arose as early as after the second missed dose (Michelson et al., 2000). In that trial, patients with a history of depression had been undergoing SSRI treatment for similar periods of time (~12–15 months) and were equivalent on several parameters (e.g., similar baseline symptom severity). The discontinuation symptoms seemed to be specific to paroxetine in this study, since patients treated with sertraline or fluoxetine did not exhibit discontinuation syndrome within the 5-day placebo substitution period. However, since sertraline and fluoxetine (but not paroxetine) have active metabolites with half-lives around 2–3 and 7–15 days respectively (Table 1), the effects of longer withdrawal periods are worth assessing in future studies. In the case of fluoxetine, it is possible that discontinuation symptoms may emerge when the levels of the active metabolite decrease after 15 days and beyond. Consistent with that possibility, Zajecka et al. (1998) found a small increase in reports of dizziness among patients who discontinued fluoxetine 4 and 6 weeks after placebo substitution. Bogetto et al. (2002) investigated 97 outpatients diagnosed with dysthymic disorder who were instructed to stop their medication (paroxetine or fluoxetine) after a successful treatment period of at least 8 weeks. With a mean time of onset of symptoms of 2 days after drug discontinuation, discontinuation syndrome was reported by 27% of patients (of which 85% had been treated with paroxetine compared to only 15% treated with fluoxetine). Analyzing randomized controlled studies of escitalopram and paroxetine for the treatment of anxiety disorders (in which treatments were followed by a prospectively defined discontinuation period), Baldwin et al. (2007) reported that individuals taking either SSRI showed more discontinuation symptoms compared to placebo. Following a 12–24 week treatment period, patients were abruptly switched to placebo and were assessed using the DESS checklist 1 and 2 weeks after drug cessation. Overall, paroxetine withdrawal induced significantly more discontinuation symptoms than escitalopram.

There are a variety of symptoms associated with paroxetine withdrawal as Murata et al. (2010) recently provided based on

observations of a group of Japanese outpatients ($n = 56$). These symptoms included dizziness (50% of patients), increased dreaming/vivid dreams (35%), fatigue (30%), nausea/vomiting (30%), headache (25%), anxiety/agitation (25%), paresthesia (15%), insomnia (10%), diarrhea (10%), visual disturbances (10%), fever (10%), tremor (5%), irritability (5%), and chills (5%). In addition to these symptoms, in an assessment of 87 patients who had their treatment interrupted for 4–7 days in a double-blind placebo study, Hindmarch et al. (2000) also reported greater cognitive deficits, poorer quality of sleep and increased depressive symptoms associated with paroxetine discontinuation, symptoms which were not evident in patients ceasing fluoxetine, sertraline, or citalopram treatment.

To date, there is no evidence to suggest that the length of SSRI treatment is associated with the development of more symptoms or with the severity of those symptoms (Rosenbaum et al., 1998; Michelson et al., 2000; Baldwin et al., 2007). However, several studies suggest that an abrupt interruption of treatment results in more symptoms of the discontinuation syndrome compared to a gradual tapering of the drug (van Geffen et al., 2005; Himei and Okamura, 2006; Murata et al., 2010). This suggests that the incidence, timing, and severity of SSRI discontinuation symptoms may be related to plasma elimination characteristics of each drug. In view of this, Michelson et al. (2000) found a statistically significant relationship between the percentage reduction in plasma concentration of the drug and the appearance of discontinuation symptoms resulting from placebo substitution across all drug groups (fluoxetine, sertraline, and paroxetine). Comparing drug concentrations before and after placebo substitution, Henry et al. (2000) found that lower steady-state brain levels of paroxetine (but not fluoxetine) after placebo substitution were associated with greater risk for discontinuation-related adverse events. Overall, discontinuation symptoms are more frequent after the abrupt cessation of drugs with shorter half-lives (Rosenbaum et al., 1998; Bogetto et al., 2002; Judge et al., 2002; Baldwin et al., 2007). However, that relationship may not be an absolute predictor. Indeed, despite paroxetine having a similar half-life to that of fluvoxamine, the rate of withdrawal reactions for the latter drug is 10 times lower (Price et al., 1996). For example, in the 46 patients with discontinuation symptoms reviewed by Black et al. (2000), paroxetine was stopped in 65%

Table 1 | Pharmacokinetic and pharmacological parameters of SSRIs (adapted from Hiemke and Hartter, 2000; Owens et al., 1997, 2001; Tatsumi et al., 1997).

Compounds	Half-life (mean)	SERT (human)	5-HT _{1A} (rat)	5-HT _{2A} (rat)	5-HT _{2C} (porcine)	NET (human)	DAT (human)	Muscarinic (human)
Fluvoxamine	15 h	$K_D: 2.2, K_i: 2.3$	n.d.	n.d.	$K_i: 5786$	$K_D: 1300, K_i: 1427$	$K_D: 9200, K_i: 16790$	$K_i: 31200$
Paroxetine	20 h	$K_D: 0.13, K_i: 0.10$	$K_i: 21168$	$K_i: 6320$	$K_i: 9034$	$K_D: 40, K_i: 45$	$K_D: 490, K_i: 268$	$K_i: 72$
Sertraline	26 h	$K_D: 0.29, K_i: 0.26$	$K_i: 3663$	$K_i: 2207$	$K_i: 2298$	$K_D: 420, K_i: 714$	$K_D: 25, K_i: 22$	$K_i: 427$
Citalopram	36 h	$K_D: 1.16, K_i: 1.6$	n.d.	n.d.	$K_i: 2051$	$K_D: 4070, K_i: 6190$	$K_D: 28100, K_i: 16540$	$K_i: 1430$
Fluoxetine	1–4 days	$K_D: 0.81, K_i: 1.1$	$K_i: 8313$	$K_i: 141$	$K_i: 72$	$K_D: 240, K_i: 599$	$K_D: 3600, K_i: 3764$	$K_i: 702$
Desmethylsertraline	2–3 days	$K_D: 3.0$	n.d.	n.d.	n.d.	$K_D: 390$	$K_D: 129$	n.d.
Norfluoxetine	7–15 days	$K_D: 1.47$	n.d.	n.d.	n.d.	$K_D: 1426$	$K_D: 420$	n.d.

Equilibrium dissociation (K_D) and inhibition (K_i) constants for binding at serotonin, norepinephrine and dopamine transporters (SERT, NET, and DAT, respectively) and several selected neurotransmitter receptors were determined using radioligand binding assays. n.d.: not determined.

of these cases compared to only 7% of cases with fluvoxamine. To date, there are no double-blind trials with placebo substitution comparing the effects of discontinuation from paroxetine and fluvoxamine, and the higher number of reports of withdrawal reactions with paroxetine is likely to reflect the greater prescription rate of paroxetine over fluvoxamine (Lockhart and Guthrie, 2011). Other factors (i.e., pharmacological) could also contribute to the high frequency of discontinuation syndrome with paroxetine.

Notably, paroxetine has significant off-target effects, including an affinity for cholinergic receptors similar to the tricyclic antidepressants (Owens et al., 1997, 2001), raising the possibility for a cholinergic rebound during discontinuation. Fujishiro et al. (2002) further demonstrated that the ability of paroxetine to induce an anticholinergic effect in mice was similar to that of tricyclic antidepressants, whereas fluvoxamine was much less potent in that regard. Paroxetine also exhibits moderately high affinity for the norepinephrine transporter, unlike other SSRIs (Tatsumi et al., 1997). In comparison, the longer half-lives of the active compounds of fluoxetine and sertraline may be the reason underlying fewer symptoms of the discontinuation syndrome. However, as raised previously, the major limitation of most of the clinical studies examining the discontinuation syndrome is the restriction of treatment interruption to only few days, when longer periods (i.e., weeks) might be more suitable for the study of fluoxetine withdrawal. Finally, whether or not the pharmacodynamic characteristics of a drug could also help to explain the clinical observations remains to be addressed. For instance, fluoxetine is also a 5-HT_{2C} receptor antagonist (Sanchez and Hyttel, 1999) with affinity for 5-HT_{2A/2C} receptors (see Table 1), whereas sertraline is the sole SSRI which also inhibits the dopamine transporter (Tatsumi et al., 1997).

The discontinuation syndrome has been reported in relation to nearly every SSRI, although there are increased reports of occurrence in patients ceasing treatment of paroxetine. However, the full extent of discontinuation syndrome might only emerge if future studies are designed to take into account the pharmacokinetics of the drug and availability of its active metabolite. Double-blind trials with placebo substitution have yet to be undertaken to directly compare the effects of discontinuation from SSRIs with shorter half-lives (i.e., paroxetine and fluvoxamine). Overall, the crucial question of whether the discontinuation syndrome is equally related to the lack of specificity and/or the neurobiology related to the half-life of the SSRI remains unclear. Potential genetic-drug interactions should also be systematically assessed in future studies. Indeed, a recent clinical study suggested a possible involvement of the C(-1019)G polymorphism of the serotonin 5-HT_{1A} receptor gene in the occurrence of paroxetine discontinuation syndrome, as patients with the –1019C allele experienced paroxetine discontinuation syndrome more frequently than patients who were –1019G homozygous (Murata et al., 2010).

ANIMAL STUDIES

MECHANISMS OF ACTION OF SSRIs

Numerous laboratories have studied the effects of SSRIs on the serotonergic system, mainly investigating on the pharmacological characteristics of these drugs (Table 1). For example, using intracerebral microdialysis in conscious rodents, extracellular

concentrations of serotonin have been reported to be increased following acute administration of fluoxetine (Hervas and Artigas, 1998), citalopram (Rea et al., 2010), or sertraline (Kitaichi et al., 2010). Further, Sharp et al. (1997) showed that paroxetine induced a larger increase in extracellular serotonin in the frontal cortex when serotonin autoreceptors on both the somatodendrites (5-HT_{1A}) and nerve terminals (5-HT_{1B}) were blocked. Incidentally, the acute effects of SSRIs on serotonin neurotransmission are controlled by several feedback mechanisms, which include 5-HT_{1A} and 5-HT_{1B/1D} autoreceptors (Malagie et al., 2001; Pullar et al., 2004) as well as postsynaptic 5-HT₂ receptors (Boothman et al., 2003; Cremers et al., 2004; Calcagno et al., 2009). Recent discoveries suggest an unexpected complexity in the mechanisms controlling the activity of serotonin neurons (Sharp et al., 2007).

Notably, and particularly relevant to this review, antidepressant drugs typically require chronic administration (i.e., several weeks) to achieve therapeutic efficacy. This delay is thought to partly reflect the time required for autoreceptors to desensitize so as to facilitate serotonin neurotransmission (Kreiss and Lucki, 1995). Rather than the desensitization *per se*, Le Poul et al. (1995) suggested that the progressive increase in the number of serotonergic neurons with desensitized 5-HT_{1A} autoreceptors may play a critical role in the slow development of the antidepressant actions of SSRIs. Extensive research has been done to study the changes to serotonergic signaling induced by chronic SSRI administration (summarized in Table 2).

Although it was initially thought that the antidepressant effects of SSRIs were solely attributable to an increase in brain serotonin, more recent evidence suggests that a sustained increase in serotonin levels (at least within the hippocampus) does not appear to be required for the beneficial anxiolytic/antidepressant-like effects of chronic fluoxetine (Popa et al., 2010). Studies have now shown that SSRIs modulate other neurochemical signaling systems in the brain such as noradrenaline and dopamine. For instance, paroxetine was reported to increase extracellular levels of noradrenaline in the hippocampus of rats that had received repeated administration of the drug (Hajos-Korcsok et al., 2000). Similarly, chronic (but not acute) treatment with fluoxetine potentiates dopamine D2/D3 receptor function (Collu et al., 1997; Dziedzicka-Wasylewska et al., 2002). Therefore, it has now become evident that in addition to effects at receptors regulating monoamine release (e.g., 5-HT₁, 5-HT₂, and α-2 adrenergic receptors), multiple targets (e.g., glutamate/GABA, peptidergic systems, neurotrophic factors, hypothalamic-pituitary-adrenal axis, etc.) are likely to be involved in the mechanism of action of SSRIs (Schechter et al., 2005; Renoir et al., 2012). With that in mind, we next review the effects of SSRI treatment discontinuation in animal models. Most of these reports have focused on the serotonergic system. However, as mentioned, there is scope for future studies to assess non-serotonin signaling pathways in relation to the SSRI discontinuation syndrome.

EFFECTS OF SSRI TREATMENT DISCONTINUATION IN ANIMAL MODELS

In this section we first focus on the few animal studies which report behavioral changes after cessation of SSRI treatment (versus behavioral changes found during treatment), followed by a discussion of the potential molecular mechanisms involved. Despite

Table 2 | Summary of the main serotonergic adaptive changes during chronic administration versus discontinuation from SSRIs (in animal models).

Drug treatment	Effects of chronic treatment	Effects of treatment cessation	Reference
Fluoxetine (30 mg/kg/day for 3 weeks)	↓5-HT and 5-HIAA levels in HC and FC (↔5-HIAA/5-HT)	7 day washout: ↓5-HT and 5-HIAA 14-day washout: 5-HT back to normal but ↓5-HIAA levels	Trouvin et al. (1993)
Citalopram for 2 weeks (minipump: 50 mg/ml)	no effect on serotonin turnover (↔5-HIAA/5-HT)	↑5-HIAA/5-HT ratio after a 48-h washout period	Bosker et al. (2010)
Fluoxetine (70 μmol/kg/day for 2 weeks)	↓5-HT and 5-HIAA levels in HC and FC	5-HT and 5-HIAA levels still reduced after 7 day washout	Caccia et al. (1993)
Paroxetine (40 μmol/kg) and Sertraline (40 μmol/kg) for 2 weeks	↓5-HT and 5-HIAA levels in HC and FC	5-HT and 5-HIAA levels back to normal 7 day washout	Caccia et al. (1993)
Fluoxetine (6.9 mg/kg/day for 3 weeks)	↑5-HIAA/5-HT ratio	5-HIAA/5-HT still increased after 8 day washout	Stenfors and Ross (2002)
Paroxetine (5–10 mg/kg/day) and Sertraline (7.5 mg/kg/day)	↓SERT binding after 21 day	5-HIAA/5-HT still increased after 8 day washout	Benmansour et al. (1999)
Sertraline (7.5 mg/kg/day)	↓SERT binding after 2–3 weeks of treatment	SERT binding back to basal levels after 10 days of washout	Benmansour et al. (2002)
Citalopram	↓SERT binding after long-term treatment	SERT binding back to basal levels after 48 h withdrawal	Horschitz et al. (2001)
Fluoxetine (3 mg/kg/day)	↓ SERT mRNA in raphe after 7 days of treatment	SERT mRNA in raphe back to control levels after 7 days washout	Neumaier et al. (1996)
Citalopram (for 3 weeks)	↑postsynaptic 5-HT _{1A} binding	The oxytocin response was still reduced 60 days after discontinuation of fluoxetine	Gunther et al. (2008), Klimek et al. (1994)
Fluoxetine (10 mg/kg/day for 2 weeks)	↓8-OH-DPAT-induced oxytocin, ACTH, and CORT responses		Van de Kar et al. (2002), Raap et al. (1999)
Fluoxetine (5–10 mg/kg for 2 weeks)	↓8-OH-DPAT-induced reduction in [5-HT]ext		Dawson et al. (2002), Newman et al. (2004)
Citalopram (20 mg/kg/day) and Escitalopram (10mg/kg/day)	↓8-OH-DPAT-induced reduction in [5-HT]ext after 13 day of treatment		Ceglia et al. (2004)
Fluoxetine (minipump: 1 mg/kg/day for 4 weeks)	↓WAY100635-induced increase in [5-HT]ext	5-HT _{1A} autoreceptor desensitization was sustained after a 2-week washout period	Popa et al. (2010)
Fluoxetine (3 mg/kg/day for 7 day)	↓5-HT _{1B} mRNA in raphe but ↑5-HT _{1B} mRNA in FC and HP	5-HT _{1B} mRNA in raphe back to control levels after 7 day washout	Neumaier et al. (1996)
Paroxetine and Fluoxetine (5 mg/kg/day)	↓5-HT _{1B} mRNA in raphe after 8 weeks of treatment	5-HT _{1B} mRNA back to control levels after 3–14 days of washout	Anthony et al. (2000)

(Continued)

Table 2 | Continued

Drug treatment	Effects of chronic treatment	Effects of treatment cessation	Reference
Fluoxetine (5 mg/kg/day for 12 day)	↓CP-93129-induced reduction in [5-HT]ext		Newman et al. (2004)
Citalopram (minipump: 50 mg/ml for 2 weeks)	No change in 5-HT _{1B} sensitivity		Jongsma et al. (2005)
Citalopram (for 3 weeks)	↓Forebrain 5-HT _{2A} binding		Gunther et al. (2008), Klimek et al. (1994)
Paroxetine (10 mg/kg/day) and Fluvoxamine (90 mg/kg/day)	↓mCPP-induced CORT response after 21 days		Yamauchi et al. (2006)
Fluoxetine	↑5-HT ₂ receptor binding in FC and HC		Hrdina and Vu (1993), Klimek et al. (1994)
Fluoxetine (10 mg/kg/day for 21 day)	↑DOI-induced ACTH and CORT response; ↓DOI-induced oxytocin		Damjanoska et al. (2003)
Fluoxetine (10 mg/kg for 21 day)	↑DOI-induced c-fos gene expression in FC and HC		Tilakaratne et al. (1995)
Fluoxetine (for 3 weeks)	↓5-HT _{2C} mRNA in FC↑5-HT _{2C} mRNA in HC	↓5-HT _{2C} mRNA in FC persisted after 1 week withdrawal 5-HT _{2C} mRNA in HC back to basal levels after withdrawal	Barbon et al. (2011)
Fluoxetine (18 mg/kg/day for 24 day) via drin K _i ng water)	↑5-HT _{2C} pre-mRNA editing in BALB/c (but not C57BL/6) mice		Englandar et al. (2005)
Paroxetine (10 mg/kg/day) and Fluvoxamine (90 mg/kg/day)	↓mCPP-induced hypolocomotion		Yamauchi et al. (2004)
Paroxetine and Fluoxetine (10 mg/kg/day p.o. for 21 day)	↓mCPP-induced hypolocomotion		Kennett et al. (1994)
↑Significant increase; ↓significant decrease; SERT: serotonin transporter; CORT: corticosterone; 8-OH-DPAT: 8-hydroxy-2-(di-N-propylamino) tetralin (5-HT _{1A} receptor agonist); WAY100635: N-[2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt (5-HT _{1A} receptor antagonist); DOI: 2,5-dimethoxy-4-iodoamphetamine (5-HT _{2A} receptor agonist); mCPP: meta-chlorophenylpiperazine; CP-93129: 5-HT _{1B} receptor agonist; FC: frontal cortex; HC: hippocampus; [5-HT]ext: extracellular serotonin levels (measured by microdialysis); 5-HAA: 5-hydroxyindoleacetic acid (the main metabolite of serotonin).			

the relatively consistent cluster of clinical symptoms observed in the SSRI discontinuation syndrome, few animal studies have been conducted to explicitly examine the effects of withdrawal from chronic SSRI treatment. Moreover, most of those studies have used fluoxetine, despite a low incidence of discontinuation syndrome associated with this specific SSRI in the clinic.

In one animal study, contrasting with the reduction of locomotor activity observed during chronic administration of fluoxetine (30 mg/kg/day for 5 days), fluoxetine-treated rats showed a significant increase in locomotor activity upon discontinuation (Bjork et al., 1998). Notably, this “rebound effect” was only observed during the first 4 h following the first “missed” dose, and not during the subsequent washout period. These findings suggest that upon cessation of chronic fluoxetine, rats showed an increase in activity resembling withdrawal behavior, in line with the symptoms of anxiety/agitation reported by some patients who develop the discontinuation syndrome. Hyperactivity in response to abrupt fluoxetine discontinuation may result from fluoxetine’s effects on the dopaminergic system, specifically in the ventral tegmental area and in the nucleus accumbens. In that regard, Gardier et al. (1994) found that chronic fluoxetine treatment caused a persistent decrease in striatal dopamine levels lasting up to 14 days after treatment discontinuation. Other reports have provided further evidence of changes in dopaminergic signaling during SSRI discontinuation. By measuring the spontaneous firing rate of hippocampal CA1 neurons, Bijak and Smialowski (1988) found that the excitatory reaction evoked by the selective D1 receptor agonist SKF-38393 was potentiated following repeated citalopram administration, and was further increased after drug withdrawal. Similarly, the enhancement of dopaminergic synaptic modulation (through hippocampal D1 receptor upregulation) induced by chronic fluoxetine was maintained for 1 month after drug withdrawal (Kobayashi et al., 2012). The mesolimbic dopamine system is thought to be involved in the behavioral effects of antidepressants (Collu et al., 1997), as well as in the withdrawal syndrome following cessation of drugs of abuse (Hu, 2007; D’Souza and Markou, 2010; Radke et al., 2011). However, another animal study found no change in intracranial self-stimulation threshold during withdrawal from chronic fluoxetine treatment (Lin et al., 1999). This finding suggests that there is no change in central reward function after cessation of fluoxetine treatment, and therefore that SSRI discontinuation syndrome and withdrawal symptoms in drug addiction may involve distinct pathways. However, since several clinical observations point to the existence of tolerance phenomena during antidepressant treatment in some cases (Fava and Offidani, 2011), further animal research is required to determine the dependence potential of the various types of antidepressant drugs.

In an attempt to compare and contrast the mechanisms underlying the effects of continuous SSRI administration against abrupt SSRI discontinuation, Bosker and colleagues measured the acoustic startle response of rats that had either been receiving 2 weeks of citalopram or had undergone 48 h of discontinuation from citalopram treatment (Bosker et al., 2010). Behavioral assessments started 2 days after the removal of osmotic mini pumps filled with either saline or 50 mg/ml citalopram. Habituation was significantly diminished in the 48-h discontinuation group, as their

acoustic startle response (a transient motor response to an unexpected, intensive stimulus) was significantly greater compared to the rats receiving ongoing 2 week citalopram treatment. Exaggerated acoustic startle response has been previously linked to anxiety-like behaviors in rodents (Plappert and Pilz, 2002). Therefore, the findings of Bosker et al. (2010) suggest a higher level of anxiety in the citalopram discontinuation group, which is similar to the increased incidence of anxiety in patients with the SSRI discontinuation syndrome. An increased reactivity to acoustic stimuli in rats has been linked to long-term depletion of serotonin in the brain (Tanke et al., 2008). Consistent with that notion, Bosker et al. (2010) found that an index of serotonin turnover [the ratio of 5-hydroxyindoleacetic acid to serotonin (5-HIAA/5-HT)] was increased after the 48-h washout period following chronic citalopram treatment, while chronic treatment exerted no significant effect on serotonin turnover. This finding is in agreement with an earlier study reporting that levels of the serotonin metabolite 5-HIAA are increased during withdrawal from chronic fluoxetine (30 mg/kg/day for 21 days), and are sustained at levels exceeding control levels (by 30–50%) for at least 14 days after cessation of chronic fluoxetine treatment (Trouvin et al., 1993). Similarly, while serotonin turnover rates were significantly decreased during the first 24 h after the last injection of a 3-week treatment with fluoxetine (6.9 mg/kg/day), turnover rates increased significantly after an 8-day washout period (Stenfors and Ross, 2002). Behavioral changes in relation to increased serotonin turnover rates during drug washout were not examined. Similarly, as Bosker et al. (2010) assessed the biochemical consequences and behavioral effects of citalopram discontinuation in separate cohorts of animals, no direct comparisons between increased serotonin turnover and increased acoustic startle response could be made. Further behavioral testing is required to validate the finding of a greater acoustic startle response, using additional tests such as the elevated-plus maze, the light-dark box, etc. Finally, based on the low incidence of the discontinuation syndrome reported in patients treated with citalopram or fluoxetine, it is likely that studying the role of serotonin turnover in the behavioral effects of long-term paroxetine administration versus discontinuation might give different results. Indeed, likely resulting from the accumulation of its main active metabolite norfluoxetine after 14 days of chronic administration, only fluoxetine (but not paroxetine)-treated animals exhibited reduced brain serotonin levels after a 1-week washout period (Caccia et al., 1993).

Despite the fact that adaptive changes in 5-HT_{1A}/1B autoreceptors are critically involved in the mechanistic actions of SSRIs (as discussed in See Mechanisms of Action of SSRIs), many preclinical studies of SSRI withdrawal have focused on serotonin levels. As such, a lack of understanding of any possible adaptive functional changes to serotonin receptors remains. Raap et al. (1999) reported that 5-HT_{1A} receptors located in the hypothalamus of rats are desensitized following a 2-week treatment with fluoxetine, an effect which lasted for at least 60 days. Given the implications of the fronto-cortical regions and the hippocampus in the withdrawal symptoms, and based on altered serotonin metabolism mentioned in the previous section, further work is required to improve our understanding of the extent of 5-HT_{1A} receptor adaptation in the brain following SSRI withdrawal. One study suggested that

molecular changes follow a rapid time course, requiring 3–14 days of washout to reverse the reductive effects of chronic fluoxetine and paroxetine treatment on pre-synaptic 5-HT_{1B} receptor mRNA levels in rat dorsal raphe nucleus (Anthony et al., 2000).

Although not the main focus of the studies, two separate groups reported the behavioral effects of long-term administration of paroxetine compared to the behavioral effects of discontinuation of paroxetine treatment (Gervasoni et al., 2002; Elizalde et al., 2008). It was found that the anxiolytic-like effects of chronic paroxetine (10 mg/kg/day for 3 weeks) were no longer observed after a 2-week washout period (Elizalde et al., 2008). The loss of this drug effect is interesting considering the development of symptoms of anxiety in patients with the discontinuation syndrome. On the other hand, the antidepressant effect of paroxetine and the amelioration of cognitive deficits were maintained even after a 2-week washout period, which does not correspond with increased incidence of depressive symptoms in individuals experiencing the discontinuation syndrome. While this study was not designed to explicitly examine the discontinuation syndrome, to our knowledge this is the sole preclinical study assessing the longer term behavioral effects of halting chronic SSRI treatment in an animal model of depression (i.e., chronic mild stress paradigm). As the eventual development of the discontinuation syndrome was not the focus of the study, other behavioral parameters relevant to the clinical symptoms of the syndrome, such as changes in sleep patterns/architecture, were not examined. Gervasoni et al. (2002) reported that in rats, a reduction in the amount of REM sleep, as well as an increase in the duration of REM sleep episodes, was associated with chronic treatment with paroxetine (5 mg/kg/day for 21 days), rather than discontinuation of treatment, as these sleep changes were not observed after a 24-h washout period. Since sleep disturbances are often reported in patients with discontinuation symptoms, it is of importance for future animal studies examining the discontinuation syndrome to assess sleep patterns/architecture as relevant outcomes.

Overall, the number of preclinical studies looking at the effects of SSRI after a washout period remains very limited. Many such reports were published prior to the recognition of SSRI discontinuation syndrome as a clinical condition, and were mainly intended to study the mechanism of action of the SSRI while having the drug on board. To date, clear discontinuation-like behaviors in animal models have not yet been reported. However, it should be noted that we suggest that a flaw of these previous studies did not use animal models of depression. In addition, it is of importance that future studies consider an appropriate time point for assessment based on the half-life of the drug of interest. It is also of importance for future studies to examine the effects of paroxetine, as this SSRI has been implicated in numerous clinical cases of the discontinuation syndrome (Bogetto et al., 2002; Judge et al., 2002; Baldwin et al., 2007). The animal studies performed to date have focused on measures of tissue serotonin levels; as it is now clear that other pathways are involved in the mechanistic actions of SSRIs, future investigations should be broadened to include an examination of these additional actions. The complex nature of SSRI discontinuation syndrome, in terms of the variety of subtle symptoms that may present at the clinic, poses a challenge for pre-clinical studies, and there is a need for appropriate animal models

to be developed in order to facilitate further study of the behavioral aspects of this condition. As mentioned, the majority of studies to date have been based on “normal” animals, whereas the clinical population who develop the discontinuation syndrome are typically taking SSRIs for treatment of depression, anxiety, etc. As such, future studies of the SSRI discontinuation syndrome in rodents should encompass well-established animal models of depression, chronic SSRI treatment, followed by an appropriate period of drug withdrawal.

DISCUSSION AND CONCLUSION

Although initial observations relied only on case reports, there is now a substantial body of clinical evidence suggesting that, in some cases, SSRIs may be associated with a withdrawal response when halted after a period of regular use. The existence of these symptoms, known as the SSRI discontinuation syndrome, has now been confirmed by a number of well-controlled studies (i.e., double-blind randomized placebo-controlled design, in which treatment is followed by a prospectively defined discontinuation period). These studies suggest that the best route of action for cessation of SSRI treatment is to taper down the dose of the medication rather than abrupt termination, as tapering is likely to decrease the possibility of the occurrence of discontinuation symptoms. Due to the broad range of symptoms which can develop as part of the discontinuation syndrome, prior to drug initiation, patients and caregivers need to be provided with adequate education and realistic, objective appraisals of possible outcomes which can develop during and following antidepressant treatment.

The current evidence suggests that the discontinuation syndrome is dependent on the SSRI half-life, with more reports of symptoms occurring in patients treated with paroxetine compared to other SSRIs. However, studies designed to assess the onset of discontinuation syndrome, and how the syndrome coincides temporally with pharmacokinetic withdrawal, are still lacking. Such studies are critical in order to draw conclusions when comparing SSRIs with short versus long half-lives. It is currently unclear as to whether the discontinuation syndrome is equally related to the pharmacological properties of a given SSRI, and/or its half-life. Future studies could also examine possible associations of the syndrome with clinical characteristics, as one study reported that the discontinuation syndrome was more common in patients with earlier onset of dysthymic disorder, and was also more common in females (Bogetto et al., 2002). On the other hand, Baldwin et al. (2007) reported no difference in discontinuation symptoms in patients with depression compared to patients with anxiety disorders. Future clinical investigations need to have enough statistical power to enable examination of within-group comparisons. Current understanding of the pathophysiology associated with the SSRI discontinuation syndrome remains largely speculative (Blier and Tremblay, 2006; Delgado, 2006). In fact, the sole clinical investigation looking at the possible chemical and molecular mechanisms underlying the SSRI discontinuation syndrome was based on a single subject (Kaufman et al., 2003).

Notably, not all patients treated with SSRIs (which represents a very heterogeneous population) experience discontinuation symptoms. In that regard, a recent clinical study indicated a possible involvement of the C(-1019)G polymorphism of the

serotonin 5-HT_{1A} receptor gene in the occurrence of paroxetine discontinuation syndrome (Murata et al., 2010). Whether the development of discontinuation symptoms has a genetic component requires additional studies to provide more conclusive results. The use of genetic animal models might be able to shed light in that regard. Interestingly, using mice with higher (1A-High) or lower (1A-Low) autoreceptor levels, Richardson-Jones et al. (2010) found a negative relationship between 5-HT_{1A} autoreceptor level and response to antidepressants. Further studies looking at the effects of 5-HT_{1A} receptor function on vulnerability to the discontinuation syndrome are of interest. Most of the animal studies performed thus far have naturally focused on the serotonergic system, and have mainly used fluoxetine, despite

the fact that this specific SSRI has been associated with a lower incidence of discontinuation syndrome in the clinic. However, based on the numerous targets known to be changed adaptively during chronic treatment with SSRIs in animal models, further studies assessing non-serotonergic pathways would be worthwhile. Finally, preclinical studies using appropriate animal models of anxiety/depression are still lacking when it comes to the study of SSRI discontinuation.

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REFERENCES

- Anthony, J. P., Sexton, T. J., and Neu-maijer, J. F. (2000). Antidepressant-induced regulation of 5-HT(1b) mRNA in rat dorsal raphe nucleus reverses rapidly after drug discontinuation. *J. Neurosci. Res.* 61, 82–87.
- Baldwin, D. S., Montgomery, S. A., Nil, R., and Lader, M. (2007). Discontinuation symptoms in depression and anxiety disorders. *Int. J. Neuropsychopharmacol.* 10, 73–84.
- Barbon, A., Orlandi, C., La Via, L., Caracciolo, L., Tardito, D., Musazzi, L., et al. (2011). Antidepressant treatments change 5-HT2C receptor mRNA expression in rat prefrontal/frontal cortex and hippocampus. *Neuropsychobiology* 63, 160–168.
- Benmansour, S., Cecchi, M., Morilak, D. A., Gerhardt, G. A., Javors, M. A., Gould, G. G., et al. (1999). Effects of chronic antidepressant treatments on serotonin transporter function, density, and mRNA level. *J. Neurosci.* 19, 10494–10501.
- Benmansour, S., Owens, W. A., Cecchi, M., Morilak, D. A., and Frazer, A. (2002). Serotonin clearance in vivo is altered to a greater extent by antidepressant-induced down-regulation of the serotonin transporter than by acute blockade of this transporter. *J. Neurosci.* 22, 6766–6772.
- Bijak, M., and Smialowski, A. (1988). The effect of acute and prolonged treatment with citalopram on the action of dopamine and SKF 38393 in rat hippocampal slices. *Eur. J. Pharmacol.* 149, 41–47.
- Bjork, J. M., Gaytan, O., Patt, N., Swann, A. C., and Dafny, N. (1998). Behavioral tolerance to and withdrawal from multiple fluoxetine administration. *Int. J. Neurosci.* 93, 163–179.
- Black, K., Shea, C., Dursun, S., and Kutcher, S. (2000). Selective serotonin reuptake inhibitor discontinuation syndrome: proposed diagnostic criteria. *J. Psychiatry Neurosci.* 25, 255–261.
- Blier, P., and Tremblay, P. (2006). Physiologic mechanisms underlying the antidepressant discontinuation syndrome. *J. Clin. Psychiatry* 67(Suppl. 4), 8–13.
- Bogetto, F., Bellino, S., Revello, R. B., and Patria, L. (2002). Discontinuation syndrome in dysthymic patients treated with selective serotonin reuptake inhibitors: a clinical investigation. *CNS Drugs* 16, 273–283.
- Boothman, L. J., Allers, K. A., Rasmussen, K., and Sharp, T. (2003). Evidence that central 5-HT2A and 5-HT2B/C receptors regulate 5-HT cell firing in the dorsal raphe nucleus of the anaesthetised rat. *Br. J. Pharmacol.* 139, 998–1004.
- Bosker, F. J., Tanke, M. A., Jongasma, M. E., Cremer, T. I., Jagtman, E., Pietersen, C. Y., et al. (2010). Biochemical and behavioral effects of long-term citalopram administration and discontinuation in rats: role of serotonin synthesis. *Neurochem. Int.* 57, 948–957.
- Bryois, C., Rubin, C., Zbinden, J. D., and Baumann, P. (1998). [Withdrawal syndrome caused by selective serotonin reuptake inhibitors: apropos of a case]. *Praxis (Bern 1994)* 87, 345–348.
- Caccia, S., Anelli, M., Codegoni, A. M., Fracasso, C., and Garattini, S. (1993). The effects of single and repeated anorectic doses of 5-hydroxytryptamine uptake inhibitors on indole levels in rat brain. *Br. J. Pharmacol.* 110, 355–359.
- Calcagno, E., Guzzetti, S., Canetta, A., Fracasso, C., Caccia, S., Cervo, L., et al. (2009). Enhancement of cortical extracellular 5-HT by 5-HT1A and 5-HT2C receptor blockade restores the antidepressant-like effect of citalopram in non-responder mice. *Int. J. Neuropsychopharmacol.* 12, 793–803.
- Ceglia, I., Accocia, S., Fracasso, C., Colovic, M., Caccia, S., and Invernizzi, R. W. (2004). Effects of chronic treatment with escitalopram or citalopram on extracellular 5-HT in the prefrontal cortex of rats: role of 5-HT1A receptors. *Br. J. Pharmacol.* 142, 469–478.
- Chaudhry, I. B., Rahman, R., Minhas, H. M., Chaudhry, N., Taylor, D., Ansari, M., et al. (2011). Which antidepressant would psychiatrists and nurses from a developing country choose for themselves? *Int. J. Psychiatry Clin. Pract.* 15, 74–78.
- Collu, M., Poggiu, A. S., Devoto, P., and Serra, G. (1997). Behavioural sensitization of mesolimbic dopamine D2 receptors in chronic fluoxetine-treated rats. *Eur. J. Pharmacol.* 322, 123–127.
- Coupland, N. J., Bell, C. J., and Potokar, J. P. (1996). Serotonin reuptake inhibitor withdrawal. *J. Clin. Psychopharmacol.* 16, 356–362.
- Cremer, T. I., Giorgetti, M., Bosker, F. J., Hogg, S., Arnt, J., Mork, A., et al. (2004). Inactivation of 5-HT(2C) receptors potentiates consequences of serotonin reuptake blockade. *Neuropsychopharmacology* 29, 1782–1789.
- Damjanoska, K. J., Van de Kar, L. D., Kindel, G. H., Zhang, Y., D'Souza, D. N., Garcia, F. (2003). Chronic fluoxetine differentially affects 5-hydroxytryptamine (2A) receptor signaling in frontal cortex, oxytocin- and corticotropin-releasing factor-containing neurons in rat paraventricular nucleus. *J. Pharmacol. Exp. Ther.* 306, 563–571.
- Dawson, L. A., Nguyen, H. Q., Smith, D. L., and Schechter, L. E. (2002). Effect of chronic fluoxetine and WAY-100635 treatment on serotonergic neurotransmission in the frontal cortex. *J. Psychopharmacol.* 16, 145–152.
- Delgado, P. L. (2006). Monoamine depletion studies: implications for antidepressant discontinuation syndrome. *J. Clin. Psychiatry* 67(Suppl. 4), 22–26.
- D'Souza, M. S., and Markou, A. (2010). Neural substrates of psychostimulant withdrawal-induced anhedonia. *Curr. Top. Behav. Neurosci.* 3, 119–178.
- Dziedzicka-Wasylewska, M., Rogoz, Z., Skuza, G., Dlaboga, D., and Maj, J. (2002). Effect of repeated treatment with tianeptine and fluoxetine on central dopamine D(2)/D(3) receptors. *Behav. Pharmacol.* 13, 127–138.
- Elizalde, N., Gil-Bea, F. J., Ramirez, M. J., Aisa, B., Lasherias, B., Del Rio, J., et al. (2008). Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. *Psychopharmacology (Berl.)* 199, 1–14.
- Englander, M. T., Dulawa, S. C., Bhansali, P., and Schmauss, C. (2005). How stress and fluoxetine modulate serotonin 2C receptor pre-mRNA editing. *J. Neurosci.* 25, 648–651.
- Fava, G. A., and Offidani, E. (2011). The mechanisms of tolerance in antidepressant action. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1593–1602.
- Fujishiro, J., Imanishi, T., Onozawa, K., and Tsushima, M. (2002). Comparison of the anticholinergic effects of the serotonergic antidepressants, paroxetine, fluvoxamine and clomipramine. *Eur. J. Pharmacol.* 454, 183–188.
- Gardier, A. M., Lepoul, E., Trouvin, J. H., Chanut, E., Dessalles, M. C., and Jacquot, C. (1994). Changes in dopamine metabolism in rat forebrain regions after cessation of long-term fluoxetine treatment:

- relationship with brain concentrations of fluoxetine and norfluoxetine. *Life Sci.* 54, L51–L56.
- Gervasoni, D., Panconi, E., Henninot, V., Boissard, R., Barbagli, B., Fort, P., et al. (2002). Effect of chronic treatment with milnacipran on sleep architecture in rats compared with paroxetine and imipramine. *Pharmacol. Biochem. Behav.* 73, 557–563.
- Goldstein, T. R., Frye, M. A., Denicoff, K. D., Smith-Jackson, E., Leverich, G. S., Bryan, A. L., et al. (1999). Antidepressant discontinuation-related mania: critical prospective observation and theoretical implications in bipolar disorder. *J. Clin. Psychiatry* 60, 563–567. (quiz 568–569).
- Gunther, L., Liebscher, S., Jahkel, M., and Oehler, J. (2008). Effects of chronic citalopram treatment on 5-HT1A and 5-HT2A receptors in group- and isolation-housed mice. *Eur. J. Pharmacol.* 593, 49–61.
- Haddad, M., and Anderson, M. (2007). Recognising and managing antidepressant discontinuation symptoms. *Adv. Psychiatr. Treat.* 13, 447–457.
- Haddad, P. (1997). Newer antidepressants and the discontinuation syndrome. *J. Clin. Psychiatry* 58(Suppl. 7), 17–21; discussion 22.
- Hajos-Korcsok, E., McTavish, S. F., and Sharp, T. (2000). Effect of a selective 5-hydroxytryptamine reuptake inhibitor on brain extracellular noradrenaline: microdialysis studies using paroxetine. *Eur. J. Pharmacol.* 407, 101–107.
- Harvey, B. H., McEwen, B. S., and Stein, D. J. (2003). Neurobiology of antidepressant withdrawal: implications for the longitudinal outcome of depression. *Biol. Psychiatry* 54, 1105–1117.
- Hedges, D. W., Brown, B. L., Shwalb, D. A., Godfrey, K., and Larcher, A. M. (2007). The efficacy of selective serotonin reuptake inhibitors in adult social anxiety disorder: a meta-analysis of double-blind, placebo-controlled trials. *J. Psychopharmacol. (Oxford)* 21, 102–111.
- Henry, M. E., Moore, C. M., Kaufman, M. J., Michelson, D., Schmidt, M. E., Stoddard, E., et al. (2000). Brain kinetics of paroxetine and fluoxetine on the third day of placebo substitution: a fluorine MRS study. *Am. J. Psychiatry* 157, 1506–1508.
- Hervas, I., and Artigas, F. (1998). Effect of fluoxetine on extracellular 5-hydroxytryptamine in rat brain. Role of 5-HT autoreceptors. *Eur. J. Pharmacol.* 358, 9–18.
- Hiemke, C., and Hartter, S. (2000). Pharmacokinetics of selective serotonin reuptake inhibitors. *Pharmacol. Ther.* 85, 11–28.
- Himei, A., and Okamura, T. (2006). Discontinuation syndrome associated with paroxetine in depressed patients: a retrospective analysis of factors involved in the occurrence of the syndrome. *CNS Drugs* 20, 665–672.
- Hindmarch, I., Kimber, S., and Cockle, S. M. (2000). Abrupt and brief discontinuation of antidepressant treatment: effects on cognitive function and psychomotor performance. *Int. Clin. Psychopharmacol.* 15, 305–318.
- Horschitz, S., Hummerich, R., and Schloss, P. (2001). Structure, function and regulation of the 5-hydroxytryptamine (serotonin) transporter. *Biochem. Soc. Trans.* 29, 728–732.
- Hrdina, P. D. and Vu, T. B. (1993). Chronic fluoxetine treatment upregulates 5-HT uptake sites and 5-HT2 receptors in rat brain: an autoradiographic study. *Synapse* 14, 324–331.
- Hu, X. T. (2007). Cocaine withdrawal and neuro-adaptations in ion channel function. *Mol. Neurobiol.* 35, 95–112.
- Jongsma, M. E., Bosker, F. J., Creemers, T. I., Westerink, B. H., and den Boer, J. A. (2005). The effect of chronic selective serotonin reuptake inhibitor treatment on serotonin 1B receptor sensitivity and HPA axis activity. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 29, 738–744.
- Judge, R., Parry, M. G., Quail, D., and Jacobson, J. G. (2002). Discontinuation symptoms: comparison of brief interruption in fluoxetine and paroxetine treatment. *Int. Clin. Psychopharmacol.* 17, 217–225.
- Kaufman, M. J., Henry, M. E., Frederick, B., Hennen, J., Villafuerte, R. A., Stoddard, E. P., et al. (2003). Selective serotonin reuptake inhibitor discontinuation syndrome is associated with a rostral anterior cingulate choline metabolite decrease: a proton magnetic resonance spectroscopic imaging study. *Biol. Psychiatry* 54, 534–539.
- Kennett, G. A., Lightowler, S., de Biasi, V., Stevens, N. C., Wood, M. D., Tulloch, I. F., et al. (1994). Effect of chronic administration of selective 5-hydroxytryptamine and noradrenaline uptake inhibitors on a putative index of 5-HT2C/2B receptor function. *Neuropharmacology* 33, 1581–1588.
- Kitaichi, Y., Inoue, T., Nakagawa, S., Boku, S., Kakuta, A., Izumi, T., et al. (2010). Sertraline increases extracellular levels not only of serotonin, but also of dopamine in the nucleus accumbens and striatum of rats. *Eur. J. Pharmacol.* 647, 90–96.
- Klimek, V., Zak-Knapik, J., and Mackowiak, M. (1994). Effects of repeated treatment with fluoxetine and citalopram, 5-HT uptake inhibitors, on 5-HT1A and 5-HT2 receptors in the rat brain. *J. Psychiatry Neurosci.* 19, 63–67.
- Kobayashi, K., Haneda, E., Higuchi, M., Suhara, T., and Suzuki, H. (2012). Chronic fluoxetine selectively upregulates dopamine D(1)-like receptors in the hippocampus. *Neuropsychopharmacology* 37, 1500–1508.
- Kreiss, D. S., and Lucki, I. (1995). Effects of acute and repeated administration of antidepressant drugs on extracellular levels of 5-hydroxytryptamine measured in vivo. *J. Pharmacol. Exp. Ther.* 274, 866–876.
- Le Poul, E., Laaris, N., Doucet, E., Laporte, A. M., Hamon, M., and Lanfumey, L. (1995). Early desensitization of somato-dendritic 5-HT1A autoreceptors in rats treated with fluoxetine or paroxetine. *Naunyn Schmiedebergs Arch. Pharmacol.* 352, 141–148.
- Lin, D., Koob, G. F., and Markou, A. (1999). Differential effects of withdrawal from chronic amphetamine or fluoxetine administration on brain stimulation reward in the rat – interactions between the two drugs. *Psychopharmacology (Berl.)* 145, 283–294.
- Lockhart, P., and Guthrie, B. (2011). Trends in primary care antidepressant prescribing 1995–2007: a longitudinal population database analysis. *Br. J. Gen. Pract.* 61, e565–572.
- Malagie, I., Trillat, A. C., Bourin, M., Jacquiot, C., Hen, R., and Gardier, A. M. (2001). 5-HT1B Autoreceptors limit the effects of selective serotonin re-uptake inhibitors in mouse hippocampus and frontal cortex. *J. Neurochem.* 76, 865–871.
- Michelson, D., Fava, M., Amsterdam, J., Aptek, J., Løndborg, P., Tamura, R., et al. (2000). Interruption of selective serotonin reuptake inhibitor treatment. Double-blind, placebo-controlled trial. *Br. J. Psychiatry* 176, 363–368.
- Murata, Y., Kobayashi, D., Imuta, N., Haraguchi, K., Ieiri, I., Nishimura, R., et al. (2010). Effects of the serotonin 1A, 2A, 2C, 3A, and 3B and serotonin transporter gene polymorphisms on the occurrence of paroxetine discontinuation syndrome. *J. Clin. Psychopharmacol.* 30, 11–17.
- Neumaier, J. F., Root, D. C., and Hamblin, M. W. (1996). Chronic fluoxetine reduces serotonin transporter mRNA and 5-HT1B mRNA in a sequential manner in the rat dorsal raphe nucleus. *Neuropsychopharmacology* 15, 515–522.
- Newman, M. E., Shalom, G., Ran, A., Gur, E., and Van de Kar, L. D. (2004). Chronic fluoxetine-induced desensitization of 5-HT1A and 5-HT1B autoreceptors: regional differences and effects of WAY-100635. *Eur. J. Pharmacol.* 486, 25–30.
- Owens, M. J., Knight, D. L., and Nemeroff, C. B. (2001). Second-generation SSRIs: human monoamine transporter binding profile of escitalopram and R-fluoxetine. *Biol. Psychiatry* 50, 345–350.
- Owens, M. J., Morgan, W. N., Plott, S. J., and Nemeroff, C. B. (1997). Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J. Pharmacol. Exp. Ther.* 283, 1305–1322.
- Petersen, T., Dording, C., Neault, N. B., Kornbluh, R., Alpert, J. E., Nierenberg, A. A., et al. (2002). A survey of prescribing practices in the treatment of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 26, 177–187.
- Plappert, C. F., and Pilz, P. K. (2002). Difference in anxiety and sensitization of the acoustic startle response between the two inbred mouse strains BALB/cAN and DBA/2N. *Genes Brain Behav.* 1, 178–186.
- Popa, D., Cerdan, J., Reperant, C., Guiard, B. P., Guilloux, J. P., David, D. J., et al. (2010). A longitudinal study of 5-HT outflow during chronic fluoxetine treatment using a new technique of chronic microdialysis in a highly emotional mouse strain. *Eur. J. Pharmacol.* 628, 83–90.
- Price, J. S., Waller, P. C., Wood, S. M., and MacKay, A. V. (1996). A comparison of the post-marketing safety of four selective serotonin re-uptake inhibitors including the investigation of symptoms occurring on withdrawal. *Br. J. Clin. Pharmacol.* 42, 757–763.
- Pullar, I. A., Boot, J. R., Broadmore, R. J., Eyre, T. A., Cooper, J., Sanger, G. J., et al. (2004). The role of the 5-HT1D receptor as a presynaptic autoreceptor in the guinea pig. *Eur. J. Pharmacol.* 493, 85–93.

- Raap, D. K., Garcia, F., Muma, N. A., Wolf, W. A., Battaglia, G., and van de Kar, L. D. (1999). Sustained desensitization of hypothalamic 5-Hydroxytryptamine1A receptors after discontinuation of fluoxetine: inhibited neuroendocrine responses to 8-hydroxy-2-(Dipropylamino) Tetralin in the absence of changes in Gi/o/z proteins. *J. Pharmacol. Exp. Ther.* 288, 561–567.
- Radke, A. K., Rothwell, P. E., and Gewirtz, J. C. (2011). An anatomical basis for opponent process mechanisms of opiate withdrawal. *J. Neurosci.* 31, 7533–7539.
- Rea, K., Folgering, J., Westerink, B. H., and Cremers, T. I. (2010). Alpha1-adrenoceptors modulate citalopram-induced serotonin release. *Neuropharmacology* 58, 962–971.
- Renoir, T., Pang, T. Y., and Lanfumey, L. (2012). Drug withdrawal-induced depression: serotonergic and plasticity changes in animal models. *Neurosci. Biobehav. Rev.* 36, 696–726.
- Richardson-Jones, J. W., Craige, C. P., Guiard, B. P., Stephen, A., Metzger, K. L., Kung, H. F., et al. (2010). 5-HT1A autoreceptor levels determine vulnerability to stress and response to antidepressants. *Neuron* 65, 40–52.
- Rosenbaum, J. F., Fava, M., Hoog, S. L., Ascroft, R. C., and Krebs, W. B. (1998). Selective serotonin reuptake inhibitor discontinuation syndrome: a randomized clinical trial. *Biol. Psychiatry* 44, 77–87.
- Rucci, P., Frank, E., Calugi, S., Miniati, M., Benvenuti, A., Wallace, M., et al. (2011). Incidence and predictors of relapse during continuation treatment of major depression with SSRI, interpersonal psychotherapy, or their combination. *Depress. Anxiety* 28, 955–962.
- Rush, A. J., Wisniewski, S. R., Zisook, S., Fava, M., Sung, S. C., Haley, C. L., et al. (2012). Is prior course of illness relevant to acute or longer-term outcomes in depressed out-patients? A STAR*D report. *Psychol. Med.* 42, 1131–1149.
- Sanchez, C., and Hyttel, J. (1999). Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and on receptor binding. *Cell. Mol. Neurobiol.* 19, 467–489.
- Schatzberg, A. F., Haddad, P., Kaplan, E. M., Lejoyeux, M., Rosenbaum, J. F., Young, A. H., et al. (1997). Serotonin reuptake inhibitor discontinuation syndrome: a hypothetical definition. Discontinuation Consensus panel. *J. Clin. Psychiatry* 58(Suppl. 7), 5–10.
- Schechter, L. E., Ring, R. H., Beyer, C. E., Hughes, Z. A., Khawaja, X., Malberg, J. E., et al. (2005). Innovative approaches for the development of antidepressant drugs: current and future strategies. *NeuroRx* 2, 590–611.
- Sharp, T., Boothman, L., Raley, J., and Queree, P. (2007). Important messages in the “post”: recent discoveries in 5-HT neurone feedback control. *Trends Pharmacol. Sci.* 28, 629–636.
- Sharp, T., Umbers, V., and Gartside, S. E. (1997). Effect of a selective 5-HT reuptake inhibitor in combination with 5-HT1A and 5-HT1B receptor antagonists on extracellular 5-HT in rat frontal cortex *in vivo*. *Br. J. Pharmacol.* 121, 941–946.
- Stenfors, C., and Ross, S. B. (2002). Evidence for involvement of 5-hydroxytryptamine(1B) autoreceptors in the enhancement of serotonin turnover in the mouse brain following repeated treatment with fluoxetine. *Life Sci.* 71, 2867–2880.
- Tanke, M. A., Alserda, E., Doornbos, B., van der Most, P. J., Goeman, K., Postema, F., et al. (2008). Low tryptophan diet increases stress-sensitivity, but does not affect habituation in rats. *Neurochem. Int.* 52, 272–281.
- Tatsumi, M., Groshan, K., Blakely, R. D., and Richelson, E. (1997). Pharmacological profile of antidepressants and related compounds at human monoamine transporters. *Eur. J. Pharmacol.* 340, 249–258.
- Tilakaratne, N., Yang, Z., and Friedman, E. (1995). Chronic fluoxetine or desmethylimipramine treatment alters 5-HT2 receptor mediated c-fos gene expression. *Eur. J. Pharmacol.* 290, 263–266.
- Trouvin, J. H., Gardier, A. M., Chanut, E., Pages, N., and Jacquot, C. (1993). Time course of brain serotonin metabolism after cessation of long-term fluoxetine treatment in the rat. *Life Sci.* 52, L187–192.
- van der Linden, G. J., Stein, D. J., and van Balkom, A. J. (2000). The efficacy of the selective serotonin reuptake inhibitors for social anxiety disorder (social phobia): a meta-analysis of randomized controlled trials. *Int. Clin. Psychopharmacol.* 15(Suppl. 2), S15–23.
- Van de Kar, L. D., Raap, D. K., Battaglia, G., Muma, N. A., Garcia, F., and DonCarlos, L. L. (2002). Treatment of cycling female rats with fluoxetine induces desensitization of hypothalamic 5-HT(1A) receptors with no change in 5-HT(2A) receptors. *Neuropharmacology* 43, 45–54.
- van Geffen, E. C., Hugtenburg, J. G., Heerdink, E. R., van Hulst, R. P., and Egberts, A. C. (2005). Discontinuation symptoms in users of selective serotonin reuptake inhibitors in clinical practice: tapering versus abrupt discontinuation. *Eur. J. Clin. Pharmacol.* 61, 303–307.
- Warner, C. H., Bobo, W., Warner, C., Reid, S., and Rachal, J. (2006). Antidepressant discontinuation syndrome. *Am. Fam. Physician* 74, 449–456.
- Yamauchi, M., Miyara, T., Matsushima, T., and Imanishi, T. (2006). Desensitization of 5-HT2A receptor function by chronic administration of selective serotonin reuptake inhibitors. *Brain Res.* 1067, 164–169.
- Yamauchi, M., Tatebayashi, T., Nagase, K., Kojima, M., and Imanishi, T. (2004). Chronic treatment with fluvoxamine desensitizes 5-HT2C receptor-mediated hypocomotion in rats. *Pharmacol. Biochem. Behav.* 78, 683–689.
- Zajecka, J., Fawcett, J., Amsterdam, J., Quitkin, F., Reimherr, F., Rosenbaum, J., et al. (1998). Safety of abrupt discontinuation of fluoxetine: a randomized, placebo-controlled study. *J. Clin. Psychopharmacol.* 18, 193–197.
- Zajecka, J., Tracy, K. A., and Mitchell, S. (1997). Discontinuation symptoms after treatment with serotonin reuptake inhibitors: a literature review. *J. Clin. Psychiatry* 58, 291–297.

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Opioid receptor desensitization: mechanisms and its link to tolerance

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Opioid receptors (OR) are part of the class A of G-protein coupled receptors and the target of the opiates, the most powerful analgesic molecules used in clinic. During a protracted use, a tolerance to analgesic effect develops resulting in a reduction of the effectiveness. So understanding mechanisms of tolerance is a great challenge and may help to find new strategies to tackle this side effect. This review will summarize receptor-related mechanisms that could underlie tolerance especially receptor desensitization. We will focus on the latest data obtained on molecular mechanisms involved in opioid receptor desensitization: phosphorylation, receptor uncoupling, internalization, and post-endocytic fate of the receptor.

Keywords: opioid receptors, desensitization, tolerance mechanisms, biased signaling, receptor trafficking

INTRODUCTION

Opioids are the most potent drugs used for pain relief. However, their therapeutic potential could be limited as a protracted use will lead to tolerance to analgesic effects requiring escalating doses that is associated with side effects such as respiratory depression. A huge work has been devoted to decipher molecular mechanisms of tolerance. It is now well-established that opioid receptors (OR) desensitization and its molecular mechanisms are intimately connected to this phenomenon. Since the beginning of the 1980's when the parallel between tolerance and desensitization has been evoked, many studies came out on the molecular mechanisms underlying OR desensitization. The number of publications related to OR desensitization increased dramatically with the cloning of the opioid receptor 10 years later. In this review, we made an effort to summarize a large amount of these data and point out conflicting results by discussing about the initial conditions (cell models, agonist treatments...). We also integrated the latest developments obtained on the role of receptor trafficking in desensitization and tolerance and the concept of biased agonism.

STRUCTURE AND FUNCTION OF OPIOID RECEPTORS

DIFFERENT TYPES OF OPIOID RECEPTOR

The idea that opiate narcotic analgesics must bind to specific sites or opiate receptors, in the central nervous system and elsewhere, in order to elicit pharmacological responses dates back for half a century. It was based on the finding that there are

important structural and steric constraints on most of the actions of opiates. Thus, Beckett and Casy (1954), and Portoghese (1965) postulated the existence of multiple OR based on the relationship between molecular structure of opiate drugs and their analgesic activity. Opioid-binding sites in the central nervous system were demonstrated in mammalian brain tissue in the 1970s by using radioligand-binding assays on isolated brain tissue (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973), followed by the characterization of endogenous opioid peptides (Hughes et al., 1975; Cox et al., 1976; Guillemin et al., 1977; Goldstein et al., 1981). The endogenous opioid system, whose involvement in different physiological functions has been recently reviewed (Bodnar, 2014), consists of four distinct neuronal systems that are widely distributed throughout the CNS and peripheral organs. To date, four OR have been cloned, the mu, kappa, delta and nociceptin/orphanin FQ receptor (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993a,b; Meng et al., 1993; Thompson et al., 1993; Fukuda et al., 1994; Mollereau et al., 1994). This latter, despite its sequence homology with the first three ones, poorly binds peptide and alkaloid opioid ligands (Mollereau et al., 1994; Reinscheid et al., 1995). So, only data on mu (MOR), delta (DOR), and kappa (KOR) OR will be included in this review. The endogenous opioid peptides are generated from four precursors: proopiomelanocortin, proenkephalin, prodynorphin, and pronociceptin/orphanin FQ (Nakanishi et al., 1979; Kakidani et al., 1982; Noda et al., 1982; Meunier et al., 1995; Reinscheid et al., 1995), each generating biologically active peptides that are

released at the synaptic terminals of opioidergic neurons. These peptides exert their physiological actions by interacting with the various classes of OR present on both pre- and post-synaptic membranes of opioid and opioid target neurons (Besse et al., 1990).

Receptor subtypes of mu, delta and kappa OR have been proposed from the pharmacological *in vitro* and *in vivo* studies, but at present there is no molecular evidence to account for a further subclassification. Only one molecular entity for each receptor has been cloned from a given species (Knapp et al., 1995; Dhawan et al., 1996), although functional splice variants of MOR have been discovered (Abbadie et al., 2004; Pasternak et al., 2004; Pan et al., 2005; Pasternak and Pan, 2013). Recent explanations, not mutually exclusives, regarding the diversity of pharmacological responses following activation of a single target, have emerged with the identification of OR heterodimers that appear to have properties different from the monomeric receptors (Fujita et al., 2014; Massotte, 2014; Ong and Cahill, 2014) and the notion of biased agonism (see this review and Violin et al., 2014).

STRUCTURE

Opioid receptors belong to the class A of G protein-coupled receptors (GPCR) which share some common features. They possess seven transmembrane domains linked by three intracellular and three extracellular loops, an extracellular amino-terminus and an intracytoplasmic C-terminus tail. The amino-terminus region has putative glycosylation sites. Whereas O- and N-glycosylation seems to be important for DOR maturation and export to plasma membrane (Petaja-Repo et al., 2000), N-glycosylation of MOR doesn't affect its function (Befort et al., 2001; Rostami et al., 2010). The transmembrane domains are composed of a strong proportion of hydrophobic amino-acids organized in alpha helix and demonstrate the highest sequence homology between the three OR (around 70%) (Mollereau et al., 1994). These domains contain cysteine residues that might be important for ligand binding for MOR (Gioannini et al., 1999) but not for DOR (Ehrlich et al., 1998). The three extracellular loops (most divergent in sequence), including the first two ones linked by a disulfide bond would participate in ligand binding (Metzger and Ferguson, 1995). The three intracellular loops would be more involved in G protein interaction (Metzger and Ferguson, 1995; Georgoussi et al., 1997; Megaritis et al., 2000). The carboxy-terminus tail has a low sequence homology between the three OR. It contains putative phosphorylation sites (Ser, Thr, and Tyr) involved in regulation events after ligand binding and a conserved cysteine residue. This latter could be involved in receptor palmitoylation, a reversible post-translational modification that could regulate DOR surface expression for instance (Petaja-Repo et al., 2006). However, in MOR, mutation of the two Cys residues does not affect palmitoylation (Chen et al., 1998).

In the last few months, an important breakthrough has been made with the crystal structures of MOR (Manglik et al., 2012), DOR (Granier et al., 2012), and KOR (Wu et al., 2012) at high resolution. The results obtained by these studies confirmed some previously discovered important characteristics of OR. Pharmacology of OR has been described with the message/address model: the ligand is composed of two parts, one carrying the activity (agonist or antagonist) at the different

subtypes of OR, the "message" and one part, the "address," conveying selectivity toward a given OR (Portoghesi et al., 1990). For the opioid peptides, enkephalins, dynorphins and endorphins, the N-terminal tyrosine residue may be considered as the common message and the C-terminal domain presents the variable address. The deep binding pocket responsible for the "message" recognition is conserved between the different OR subtype, whereas the distal binding site responsible for the "address" recognition is divergent (Metzger and Ferguson, 1995; Granier et al., 2012; Manglik et al., 2012; Filizola and Devi, 2013). For instance, the indole group of naltrindole, carrying the selectivity toward DOR, interacts with the Leu7.35 residue. In the MOR, this amino-acid is replaced by a Trp, preventing naltrindole binding by steric hindrance (Granier et al., 2012; Manglik et al., 2012). Interestingly, MOR crystallized in two-fold symmetrical dimer (Manglik et al., 2012) whereas KOR (Wu et al., 2012) and DOR (Granier et al., 2012) were also shown to adopt anti-parallel arrangements. While those data reinforce the existence of OR dimers (Massotte, 2014), one should keep in mind that the non-physiological conditions (i.e., detergents and modified receptors) used for such crystallographic studies could generate artifactual interactions.

SIGNALING AND BIASED AGONISM

OR are mainly coupled to pertussis toxin-sensitive heterotrimeric $G_{\alpha i/o}$ proteins and to a lesser extent to G_z (Law et al., 2000). G_{α} and $G_{\beta\gamma}$ dimer activate numerous intracellular effectors. The most studied effector is the adenylyl cyclase (ACase) and investigations on OR coupling demonstrated that stimulation of MOR, DOR, and KOR in cellular models or *ex vivo* inhibited ACase mainly via $G_{i/o}$ proteins (Dhawan et al., 1996; Bian et al., 2012). One of the fastest responses obtained after OR activation is the regulation of certain types of ionic channels such as the inhibition of voltage-dependent Ca^{2+} channels or activation of potassium channels such as GIRK (G protein-coupled inwardly rectifying K^+ channels) (Law et al., 2000). Activation of K^+ channels mediates neuronal membrane hyperpolarization and reduces hyperexcitability. The inhibition of voltage-dependent Ca^{2+} channel blocks neurotransmitters release. These two phenomena participate to reduce nociception mediated by OR. OR also activate phospholipase C and mitogen-activated protein (MAP) kinases pathways (Law et al., 2000).

Recently, a new notion has emerged from pharmacological studies of GPCR, called biased agonism or functional selectivity. The binding of different ligands of a single receptor results in distinct conformational changes of receptor; each conformation preferentially interacts with selective partners producing specific signaling cascades (Kenakin, 2011). One could trace back the first data on biased agonism for OR when some authors demonstrated that different ligands for the same OR activate different subsets of $G_{\alpha i/o}$ proteins (Allouche et al., 1999a). Recently, Morse and colleagues revealed a functional selectivity using a large panel of opioid ligands by the label-free dynamic mass redistribution technology which is based on the detection of refractive index alterations measured by biosensor-coated microplates (Morse et al., 2013); this suggests that opioid ligands are able to promote different conformational changes of OR. Many studies have demonstrated the existence of a biased agonism for OR at

different signaling events including desensitization, phosphorylation, endocytosis, trafficking, and *in vivo* effects (see below) (Raehal et al., 2011; Pradhan et al., 2012; Kelly, 2013).

IN VIVO FUNCTION

The anatomical localization of OR in the brain and peripheral tissues has been clearly established using autoradiographic methods with selective radiolabeled ligands and detection of OR transcripts using *in situ* hybridization (Mansour et al., 1995; Dhawan et al., 1996). The different OR are widely distributed throughout the central nervous system that explains the large pharmacological responses observed following administration of opioid agonists.

The highest density of MOR is found in the caudate and putamen, where they exhibit a typical patchy distribution in the rat. High levels of MOR are observed in the cortex, thalamus, nucleus accumbens, hippocampus, and amygdala. Moderate levels are found in the periaqueductal gray matter and raphe nuclei, and low concentrations are seen in the hypothalamus, preoptic area, and globus pallidus (Quirion et al., 1983). MOR are also present in the superficial layers of the dorsal horn of the spinal cord (Besse et al., 1990). This large distribution in both spinal and supraspinal structures, as well as at periphery, shows that MOR play an important role in the control of nociception, in good agreement with the pharmacological studies demonstrating that mu selective agonists are potent antinociceptive drugs. Numerous other physiological functions appear to be controlled by MOR. These include reward, respiration, cardiovascular functions, bowel transit, feeding, learning and memory, locomotor activity, thermoregulation, hormone secretion, and immune functions (Dhawan et al., 1996; Kieffer, 1999; Bodnar, 2014).

The distribution of KOR demonstrates some of the most striking species differences among the OR types. In the rat, they represent only approximately 10% of the total number of OR, while in most other species (guinea pig, monkey, and human) they represent at least a third of the opioid binding population (Dhawan et al., 1996). KOR have been found to be widely distributed throughout the forebrain, midbrain, and brainstem. They are implicated in the regulation of several functions, including nociception, diuresis, mood, feeding, and neuroendocrine secretions (Tejeda et al., 2012; Bodnar, 2014).

Compared to MOR and KOR, DOR are more restricted in their distribution and are densest in forebrain regions, well-conserved across mammalian species. Dense binding is observed in the caudate, putamen, cerebral cortex, and amygdala, while they are generally sparse to nonexistent in thalamus and hypothalamus. They play a role in different functions: nociception, locomotor activity, gastro-intestinal motility, olfaction, cognitive function, and mood driven behavior (Dhawan et al., 1996; Gaveriaux-Ruff and Kieffer, 2002; Bodnar, 2014).

DESENSITIZATION

Chronic opioid use leads to tolerance, defined as a decrease of the drug response. It's possible to reproduce *in vitro* such phenomenon when cellular models expressing OR are exposed to agonists; in that situation, a decrease of signaling is observed and is designated as OR desensitization. Some reports distinguish

the OR desensitization from the cellular tolerance. When rats are chronically exposed to morphine, examination of MOR activity on the outward potassium current shows a reduction compared to naive animals which is not reversible even after 6 h in free-morphine medium; this is cellular tolerance (Levitt and Williams, 2012). In contrast, desensitization may be defined as a reduction of signal transduction from OR after acute activation by agonists that recovers when cells or tissues are placed in agonist-free medium. The first works studying the molecular mechanisms underlying OR desensitization were reported more than 30 years ago (Gahwiler, 1981; Law et al., 1982).

Initially, studying desensitization was made possible by using experimental models endogenously expressing OR such as brain membranes, rabbit cerebellum or cell lines (NG 108-15, SH-SY5Y, SK-N-SH, SK-N-BE . . .). Since the cloning of the first OR, those models have been superseded by heterologous expression systems (HEK, CHO, COS-7, *Xenopus laevis* oocyte) in which OR are easily expressed in large amount but whose cellular characteristics are far from neurons in which OR are endogenously expressed.

Desensitization of OR is studied on different signaling pathways including ACase inhibition, activation of MAP kinases, inhibition of voltage-gated calcium channels and activation of GIRK channels. Desensitization is sometimes evaluated by measuring the ability of OR to activate G proteins in [³⁵S]GTP γ S binding experiments after opioid agonists exposure. In absence of modification on the downstream signaling pathway, G protein uncoupling is a good marker for desensitization but can't be applied for G protein-independent pathways (i.e., MAP kinases). The comparison between desensitization studies suffers also from the various experimental conditions used. Cellular model, agonist, agonist concentration, time of exposure, level of OR expression or signaling pathway studied are among the different parameters that could influence OR desensitization as previously reviewed (Connor et al., 2004).

DEFINITION

As indicated above, desensitization is defined as a progressive reduction of signal transduction that occurs more or less rapidly after OR activation depending on the agonist and the signaling pathway. The rapid desensitization is mainly observed on the regulation of ion channel conductance from sec to several minutes while a sustained desensitization is rather observed on regulation of enzymes (ACase, MAP kinases) after minutes to several tens of minutes. However, in this latter case, other counter-regulatory mechanisms (internalization, traffic of OR) could participate to desensitization making its description complex. Molecular mechanisms turned out to be complicated for several reasons:

- A single OR can activate simultaneously different signaling pathways such ACase, MAP kinases or ion channels and it is possible to observe different levels of desensitization when considering those cellular responses. For instance, we recently showed that remifentanil, a MOR selective agonist, produces a significant desensitization by 60% on the cAMP pathway after 10 min while at the same time desensitization of the MAP kinases ERK1/2 signaling pathway was not significantly affected (Nowoczyn et al., 2013).

- Two types of desensitization, homologous and heterologous, were described. In homologous desensitization, only agonist-activated receptors are desensitized while in heterologous desensitization, both agonist-activated and non-activated receptors sharing the same signaling pathways are inactivated. Those types of desensitization are related to different mechanisms especially in terms of receptor phosphorylation and kinases (Chu et al., 2010). Cross-desensitization between OR and other GPCRs is not systematically investigated and when it is, the level of desensitization between GPCRs using the same signaling pathway can be different (Namir et al., 1997). Recently, Xu et al. showed a cross-desensitization between the dopamine D1 receptors and DOR. This heterologous desensitization characterized by an uncoupling of G proteins from DOR is neither associated with modifications in receptor number nor in their phosphorylation but involves several kinases [cAMP-dependent protein kinase (PKA), MAP kinases/ERK kinase 1 (MEK1) and phosphoinositide-3 kinase (PI3K)] that could phosphorylate signaling proteins (Xu et al., 2013).
- Desensitization results from several regulatory mechanisms of signal transduction and depends on the number of active receptors at the cell surface, the efficiency of OR/G proteins coupling and the post-endocytic traffic. Recently, desensitization of MOR expressed in the neurons from locus coeruleus was demonstrated to result from a decrease of both number of active receptors and the affinity of residual receptors for the agonist (Williams, 2014).

This part will discuss recent data from literature regarding desensitization of the different OR: the impact of the agonist used through the notion of biased agonism, the role of phosphorylation and consequently the kinases involved, the implication of arrestins and OR internalization and their fate after endocytosis. Regarding MOR, a recent review has been published concerning the molecular mechanisms involved in its regulation (Williams et al., 2013).

EFFECT OF BIASED AGONISM ON OR DESENSITIZATION

The first reports describing a differential desensitization of MOR, DOR, and KOR by various agonists came from Reisine's group (Blake et al., 1997a,b; Bot et al., 1997) suggesting that biased agonism could influence desensitization; but at that time this concept was not established yet. Few studies have been designed to evaluate the impact of biased agonism on OR desensitization. They would require determination of the relationship between agonist concentration and the response from a large panel of ligands. More generally, the comparison of the ability of two ligands to promote OR desensitization is realized using the same concentration regardless their intrinsic efficacy.

Biased agonism at MOR and desensitization

Functional studies revealed that [D-Ala²-MePhe⁴-Gly⁵-ol] enkephalin (DAMGO) induced a stronger desensitization of MOR than morphine in different experimental models and signaling pathways (Yu et al., 1997; Whistler and Von Zastrow, 1998; Koch et al., 2001; Blanchet et al., 2003; Bailey et al., 2009). However, such difference was not reported by others

(Liu and Prather, 2001; Borgland et al., 2003; Schulz et al., 2004). In contrast, morphine was demonstrated to promote a stronger MOR desensitization than DAMGO on the increase of intracellular $[Ca^{2+}]$ (Chu et al., 2010). In another model, the human neuroblastoma SH-SY5Y, it is possible to observe a huge difference in MOR desensitization produced by morphine and remifentanil on the cAMP pathway but not on the MAP kinases ERK1/2 (Nowoczyń et al., 2013). All those discrepancies could be due to the different level of OR expression, the cellular models and the existence of spare receptors as previously mentioned (Connor et al., 2004).

Biased agonism at DOR and desensitization

Evidence for a different DOR regulation by methadone and morphine was also reported; a pretreatment with methadone but not with morphine produced a cross-desensitization with [D-Ala², D-Leu⁵]-enkephalin (DADLE) and morphine (Liu et al., 1999a). Similar data were reported by Bot et al. (1997). In our laboratory, we also showed a differential regulation of human DOR (hDOR) on both the inhibition of ACe and the phosphorylation of ERK1/2 in the SK-N-BE cells. Initially, we suggested that peptidic opioid agonists such as [D-Pen²-D-Pen⁵]-enkephalin (DPDPE) and deltorphin I (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂) induced a stronger and faster desensitization compared to the alkaloid agonist etorphine (Allouche et al., 1999b). However, using other peptidic ([Leu⁵]- and [Met⁵]-enkephalins and UFP-512 ([H-Dmt-Tic-NH-CH(CH₂-COOH)-Bid])) and non-peptidic (SNC-80 ((+)-4-[(alpha R)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethyl-benzamide) and ARM-390) ligands we didn't confirm such assumption but our data rather suggest that DOR selective agonists promote profound desensitization compared to non-selective ligands (Marie et al., 2003a; Lecoq et al., 2004; Aguila et al., 2007).

Biased agonism at KOR and desensitization

Very few studies examined the regulation of KOR by different agonists. The group of Pei showed that desensitization of KOR-mediated extracellular acidification response was greater upon dynorphin A (1-13) stimulation than U69,593 ((+)-(5 α , 7 α ,8 β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide) and etorphine (Ling et al., 1998). On the cAMP pathway, U50,488 (trans-(\pm)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide) and dynorphin A (1-17) produced a greater KOR desensitization than etorphine or levorphanol (Blake et al., 1997b).

With respect to desensitization, all those data support the idea that agonists are able to promote a different regulation of OR as demonstrated for other GPCR such as the histamine H2 receptors (Alonso et al., 2014). Such differential desensitization demonstrated for each OR by different agonists is probably related to the set of different regulatory molecular mechanisms (see above).

MECHANISMS OF OR DESENSITIZATION

OR phosphorylation

Numerous studies have been carried out to demonstrate the role of OR phosphorylation in desensitization by using chemical

inhibitors of kinases, *in vitro* or *in vivo* knock-out (KO) of kinases using siRNA or transgenic mice, over-expression of dominant negative mutants of kinases, amino acid substitution or truncation on OR. While in some studies the phosphorylation state of OR is clearly determined, in most of them and especially those using kinase inhibitors this major information is lacking. All those data are summarized in **Figures 1A–C**.

MOR phosphorylation. Using metabolic labeling with [³²P] and different mutants at the C terminal tail, the group of Law demonstrated that rat MOR (rMOR) displays a basal phosphorylation at S363 and T370 and DAMGO increases phosphorylation at T370 and S375 (El Kouhen et al., 2001). Those results were recently confirmed using specific antibodies directed against the phospho-S363, phospho-T370 and phospho-S375 (Doll et al., 2011). As demonstrated for the DOR (see below), agonist-induced MOR phosphorylation is carried out hierarchically with first of all the S375, considered as the major phosphorylation site, followed by T370 (El Kouhen et al., 2001). Morphine was also shown to increase S375 [or S377 for the human MOR (hMOR)] phosphorylation (Nowoczyn et al., 2013) but failed to phosphorylate T370 (Doll et al., 2011). Recently, Just and collaborators showed that MOR is sequentially phosphorylated at S375, T370, T379, and T376 by DAMGO. Interestingly, low concentrations of this opioid agonist rather promote phosphorylation at S375 and T379 while a strong phosphorylation of T370 and S375 is observed at higher concentrations (Just et al., 2013).

Phosphorylation studies using liquid chromatography-mass spectrometry techniques have led to the characterization of two regions at the C terminal tail of the MOR (Lau et al., 2011): the first region (amino acid 349–365) can be mono- or bi-phosphorylated at S363 and in the cluster 354TSST357. While the basal phosphorylation of S363 is not modified by agonist exposure, morphine or DAMGO can increase phosphorylation at the cluster TSST. The second region 375STANT379 is mono- or bi-phosphorylated upon agonist exposure. Rather than qualitative differences, DAMGO and morphine were shown to induce marked quantitatively different phosphorylation increase in MOR. Using a similar experimental approach, two laboratories showed that rMOR and hMOR were phosphorylated in the absence of agonist at S363 and T370 (Moulédous et al., 2012; Chen et al., 2013). Moulédous et al. showed that DAMGO increases hMOR phosphorylation at S356, T370, S375, and T376 (Moulédous et al., 2012) while Chen et al. compared the phosphorylation mediated by DAMGO and morphine; these latter showed that both agonists increase phosphorylation at S356, T357, T370, and S375 (Chen et al., 2013).

Different kinases are involved in MOR phosphorylation. Using siRNA against various forms of the G protein-coupled receptor kinase (GRK) family, DAMGO was demonstrated to phosphorylate T370 and S375 by GRK2 and 3 while morphine increases S375 phosphorylation by GRK5 (Doll et al., 2012). In SH-SY5Y cells, hMOR phosphorylation at S377 (the equivalent of S375 for the rMOR) upon DAMGO exposure does not rely on GRK2 suggesting the implication of another kinases (Moulédous et al., 2012). *In vivo*, using KO mice for either GRK3 or 5, morphine rather promotes MOR phosphorylation at S375 by both kinases

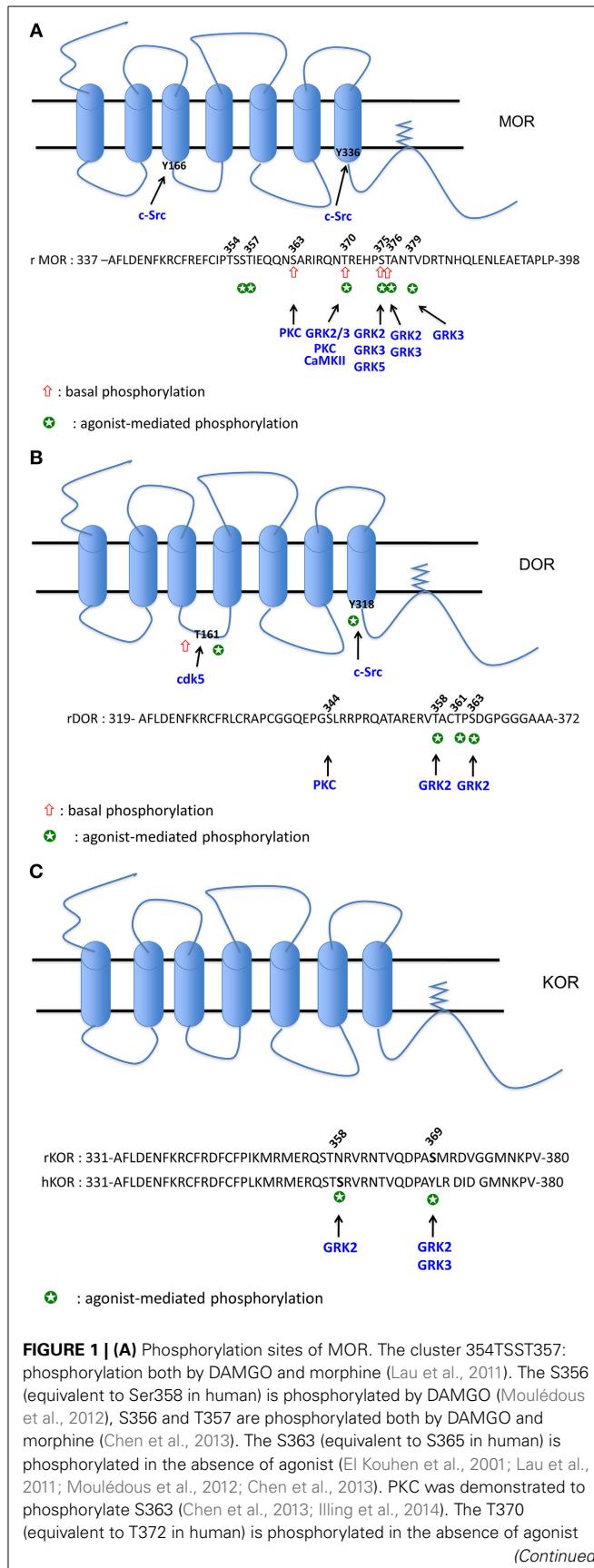


FIGURE 1 | (A) Phosphorylation sites of MOR. The cluster 354TSST357: phosphorylation both by DAMGO and morphine (Lau et al., 2011). The S356 (equivalent to Ser358 in human) is phosphorylated by DAMGO (Moulédous et al., 2012), S356 and T357 are phosphorylated both by DAMGO and morphine (Chen et al., 2013). The S363 (equivalent to S365 in human) is phosphorylated in the absence of agonist (El Kouhen et al., 2001; Lau et al., 2011; Moulédous et al., 2012; Chen et al., 2013). PKC was demonstrated to phosphorylate S363 (Chen et al., 2013; Illing et al., 2014). The T370 (equivalent to T372 in human) is phosphorylated in the absence of agonist (Continued)

FIGURE 1 | Continued

(Moulédous et al., 2012; Chen et al., 2013). A decrease of phosphorylation level is observed upon DAMGO and 1Dme (a neuropeptide FF analog) exposure (Moulédous et al., 2012). PKC (Illing et al., 2014) and CaMKII (Chen et al., 2013) phosphorylate T370. DAMGO, morphine and etonitazene increase phosphorylation at T370 (Doll et al., 2011; Lau et al., 2011). DAMGO-mediated phosphorylation at this residue is ultra-rapid (20 s) (Just et al., 2013) and involves GRK2 and 3 (Doll et al., 2012) but not PKC (Illing et al., 2014). The cluster 375STANT379 displays higher level of phosphorylation upon DAMGO compared to morphine (Lau et al., 2011). S375 or T376 (equivalent to S377 and T378 in human) are phosphorylated upon DAMGO and 1Dme (Moulédous et al., 2012), DAMGO, etonitazene, and morphine (Doll et al., 2011). S375 is considered as the major phosphorylation site as it is rapidly phosphorylated (20 s) upon DAMGO (Just et al., 2013). This agonist-mediated phosphorylation does not implicate PKC (Illing et al., 2014) but rather GRK2 (Chen et al., 2013) or GRK2 and 3 (Doll et al., 2012) upon DAMGO exposure, and GRK5 and to a lesser extent GRK3 upon morphine treatment (Doll et al., 2012). T376 (equivalent to T378 in human) is phosphorylated upon DAMGO and 1Dme (Moulédous et al., 2012), by GRK2 and 3 upon DAMGO exposure but it is considered as a late phosphorylation site (20 min) (Just et al., 2013). T379 is also phosphorylated upon DAMGO exposure after 1 min and required the GRK3 (Just et al., 2013). Y166 (Clayton et al., 2010) and Y336 (Zhang et al., 2009) are phosphorylated by Src. **(B)** Phosphorylation sites of DOR. S344 phosphorylation is mediated by a PKC but is not increased by DPDPE (Xiang et al., 2001). S358 and S363 (Guo et al., 2000; Kouhen et al., 2000) are the two major sites of phosphorylation mediated by GRK2 upon DPDPE exposure. Deltorphin II and morphine are also able to increase phosphorylation at S363 (Navratilova et al., 2005). T361 is phosphorylated by DPDPE but after S358 and S363 phosphorylation (Guo et al., 2000; Kouhen et al., 2000). T161 is phosphorylated by CDK5 in the absence and in the presence of chronic morphine exposure (Xie et al., 2009). Y318 is phosphorylated by Src upon DTLET exposure (Kramer et al., 2000b). **(C)** Phosphorylation sites of KOR. Phosphorylation of S369 (rKOR) is mediated by GRK2 (McLaughlin et al., 2003) and 3 (McLaughlin et al., 2004) upon U50488 exposure. In hKOR, S358 is phosphorylated by GRK2 when activated by U50488 (Li et al., 2002).

while only GRK3 was required for fentanyl-induced MOR phosphorylation (Glück et al., 2014). Using the carboxy-terminal region of MOR fused to glutathione S-transferase and purified kinases, PKC, GRK2, and calmodulin-dependent kinase II (CaMKII) were shown to phosphorylate S363, S375 and T370, respectively (Chen et al., 2013). Various PKC isoforms (PKC α , β II, γ , ϵ) activated by phorbol 12-myristate 13-acetate (PMA) trigger MOR phosphorylation at S363 and T370 but those kinases are not recruited upon DAMGO stimulation (Doll et al., 2011; Feng et al., 2011); those data indicate the role of PKC in the basal and heterologous phosphorylation of MOR (Illing et al., 2014).

The tyrosine kinase Src was also shown to phosphorylate MOR at Y336, located in the NPXXY motif, after sustained morphine treatment followed by naloxone (Zhang et al., 2009). The Y166, located in the DRY motif of the second intracellular loop of MOR, can be phosphorylated by Src but only upon co-activation with DAMGO and epidermal growth factor (EGF) (Clayton et al., 2010).

In summary, those studies revealed that S375 is the main phosphorylation site of MOR but agonists promote a differential and a multi-phosphorylation of this OR as recently reviewed (Mann et al., 2014).

DOR phosphorylation. Pei and colleagues were the first to demonstrate that OR could be phosphorylated upon agonist stimulation (Pei et al., 1995). They showed that DPDPE increases incorporation of [³²P] in a GRK-dependent manner. As shown for MOR, the group of Law showed that DOR was sequentially phosphorylated at S363, T358, and T361 upon DPDPE exposure (Kouhen et al., 2000). Those results were confirmed by another group who also demonstrated the critical role of GRK2 in DPDPE-induced phosphorylation of these residues (Guo et al., 2000; Marie et al., 2008). Deltorphin II is also able to increase S363 phosphorylation at hDOR but to a greater extent than morphine (Navratilova et al., 2005). PKC can phosphorylate DOR at S344 but is not required for DPDPE-induced DOR phosphorylation (Xiang et al., 2001). In a similar way as MOR, DOR phosphorylation of the Y318, located in the NPXXY motif, occurred upon DTLET ([D-Thr²-Leu⁵-Thr⁶]enkephalin) exposure in a Src dependent manner (Kramer et al., 2000a,b). The cyclin-dependent kinase 5 (Cdk5), a proline-directed S/T kinase, was demonstrated to mediate basal and morphine-activated DOR phosphorylation at the T161 located in the second intracellular loop (Xie et al., 2009).

KOR phosphorylation. Concerning KOR phosphorylation, the data from literature are very scarce. The group of Chavkin showed that rKOR is phosphorylated *in vivo* at S369 by GRK3 upon U50,488 exposure (McLaughlin et al., 2004) and *in vitro* by GRK2 (McLaughlin et al., 2003). Upon global evaluation of the hKOR phosphorylation, Li et al. observed that dynorphin A (1-17) and U50,488 promote the highest phosphorylation, etorphine 50% of the maximum and levorphanol failed to induce [³²P] incorporation demonstrating that opioid agonists have different potencies to phosphorylate this receptor (Li et al., 2003). It is noteworthy that human and rodent KOR differ substantially in the amino acid composition in the C-terminal region; such difference could explain the absence of rKOR phosphorylation when activated by U50,488 (Li et al., 2002). In hKOR, the S358, substituted by N in the rKOR, is the major phosphorylation site mediated by the GRK2 upon U50,488 exposure.

In summary, the phosphorylation sites for each OR were mapped and showed that activation of a given receptor by different agonists results in a specific pattern involving different kinases (**Figures 1A–C**). Those data are consistent with the model of barcode established for the β -adrenergic receptor, a prototypic GPCR (Nobles et al., 2011), and could determine the selective interactions between the OR and partners such as arrestins.

Uncoupling between G proteins and OR

Any process interfering with the interaction between G proteins and OR can lead to reduction of signal transduction intensity. G protein uncoupling can be evidenced by binding studies on cellular membranes using the radiolabeled non-hydrolyzable GTP analog [³⁵S]GTP γ S which binds to a G protein activated by the complex receptor-opioid agonist.

In CHO cells over-expressing hDOR, deltorphin II (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂) pretreatment induces desensitization after 30 min on the ACCase inhibition associated with a G protein uncoupling (Navratilova et al., 2007). In the

neuroblastoma×glioma (NG108-15) hybrid cells, morphine pre-treatment failed to promote uncoupling of DOR from G proteins while methadone did (Liu et al., 1999b). Conversely, after 5 days of chronic morphine exposure, it is possible to observe a complete uncoupling between MOR and its cognate G proteins (Bohn et al., 2000). However, upon acute exposure (30 min) morphine failed to promote a reduction of [³⁵S]GTP γ S binding compared to DAMGO indicating a great difference between agonists (Whistler and Von Zastrow, 1998). When expressed in the CHO cell line, the hKOR was demonstrated to undergo a time- and concentration-dependent uncoupling from G proteins but with a moderate impact on the inhibition of ACase (a two-fold increase of the EC₅₀ value of the KOR agonist U50488) (Zhu et al., 1998).

Relationship between OR phosphorylation and desensitization

In most of these studies, the role of OR phosphorylation in desensitization is indirectly demonstrated by using KO mice or kinases chemical inhibitors; in such situations, we cannot rule out the phosphorylation of other signaling proteins involved in regulatory mechanisms of OR. Mutation of putative phosphorylation sites or truncation of the C terminal tail of OR have been extensively used to delineate the role of phosphorylation in desensitization. All those data are summarized in the **Table 1**.

MOR. Comparison between two truncated MOR in the C terminal tail in HEK cells over-expressing a GRK2 peptide known to block G $\beta\gamma$ -mediated recruitment of GRK at the plasma membrane suggest that the amino acids sequence 354TSST357 plays a major role in GRK2-mediated MOR desensitization upon DAMGO exposure (Wang, 2000). In locus coeruleus neurons morphine induced MOR desensitization, measured on K⁺

current, in a PKC-dependent manner while GRK2 was required for DAMGO-induced MOR desensitization (Bailey et al., 2009). Such observations were confirmed by others on Ca²⁺ mobilization; PKC- ϵ was required for morphine-induced MOR desensitization but not upon etorphine, fentanyl and DAMGO (Zheng et al., 2011). Recently, in locus coeruleus neurons and using chemicals activators (phorbol-12,13-dibutyrate and phorbol-12-myristate-13-acetate) or a muscarinic agonist known to activate PKC, acute or sustained desensitization of MOR induced either by morphine or [Met⁵]-enkephalin were demonstrated to differentially required PKC activity but such effects were not inhibited by the potent PKC inhibitor staurosporine (Arttamangkul et al., 2014). Those data suggest that the involvement of PKC in MOR desensitization would be cell-type specific. In the presence of DAMGO or [Met⁵]-enkephalin, the molecular mechanisms involved in MOR desensitization change during brain development. In the locus coeruleus of young rats, those opioid peptides produce heterologous MOR desensitization with $\alpha 2$ adrenoreceptors in a GRK2-dependent manner but independently of its kinase activity; the high GRK2 expression would sequester G $\beta\gamma$ and interfere with K⁺ channels activation while in mature rats, homologous MOR desensitization would be due to receptor phosphorylation by this kinase (Llorente et al., 2012). GRK2 was also shown to mediate heterologous desensitization by promoting MOR transphosphorylation upon neuropeptide FF receptor activation (Moulédous et al., 2012). The role of phosphorylation in MOR desensitization has been challenged: using staurosporine as a broad spectrum kinase inhibitor and a GRK2-mutant mice, Arttamangkul et al. showed no modification of [Met⁵]-enkephalin-induced receptor desensitization on K⁺ channels in locus coeruleus neurons (Arttamangkul et al., 2012).

Table 1 | Role of kinases in OR desensitization/tolerance.

OR	Main results	References
MOR	GRK2-mediated desensitization after DAMGO exposure	Wang, 2000
	DAMGO mediates desensitization in a GRK2-dependent manner while morphine induced-desensitization in a PKC-dependent fashion	Bailey et al., 2009
	Role of PKC ϵ in morphine- but not etorphine-, fentanyl-, and DAMGO-induced desensitization	Zheng et al., 2011
	Role of GRK2 in homologous and heterologous receptor desensitization	Llorente et al., 2012
	Role of GRK2 in heterologous desensitization between MOR and neuropeptide FF receptor	Moulédous et al., 2012
	No evidence for a role of GRK5 in the development of morphine tolerance	Glück et al., 2014
	Staurosporine and GRK inhibitors do not alter desensitization upon [Met ⁵]-enkephalin exposure	Arttamangkul et al., 2012
	Role of PI3K γ in desensitization and tolerance after chronic morphine treatment	Konig et al., 2010
	Role of JNK2 in tolerance and uncoupling after chronic morphine but not fentanyl treatment	Melief et al., 2010
	Role of Src in ACase superactivation after chronic morphine treatment and naloxone addition	Zhang et al., 2009
DOR	GRK2, PKC and a tyrosine kinase are involved in desensitization of hDOR when activated by etorphine	Marie et al., 2008
	Role of GRK6 in DPDPE-mediated desensitization	Willets and Kelly, 2001
	Role of PKC in DOR desensitization upon sustained activation by DADLE and [Leu ⁵]-enkephalin	Yoon et al., 1998; Song and Chueh, 1999
	Role of Src in DPDPE-induced DOR desensitization	Archer-Lahlou et al., 2009; Hong et al., 2009
KOR	Expression of GRK3 or 5 alone is not sufficient to promote desensitization	Appleyard et al., 1999
	Role of GRK3 in development of U50,488 induced tolerance	McLaughlin et al., 2004

The implication of other kinases than GRK and PKC in MOR desensitization was also investigated. The PI3K γ was demonstrated to be involved in MOR desensitization on the inhibition of voltage-gated calcium channels induced by chronic morphine treatment (Konig et al., 2010). Using chemical inhibitor and KO mice, c-Jun amino-terminal kinase 2 (JNK2) was demonstrated to play a major role in morphine- but not fentanyl-induced G protein uncoupling (Mielief et al., 2010).

Some studies were also conducted to identify the amino acids of MOR involved in desensitization. The T180A substitution abolished MOR desensitization compared to wild type but the phosphorylation state of the receptor was not evaluated (Celver et al., 2004). The S375 was shown to play a major role in MOR desensitization on the cAMP and MAP kinase pathways but only when activated by morphine but not DAMGO (Schulz et al., 2004). Activation of PKC by PMA but not DAMGO pretreatment is able to promote MOR uncoupling from G proteins which is attenuated by the S363A mutation (Feng et al., 2011); this indicates that PKC-mediated phosphorylation of S363 as well as T370 upon substance P receptor activation (Illing et al., 2014) are potentially involved in heterologous desensitization. Using the triple mutant (S363A, T370A, and S375A), Zheng et al. showed that MOR desensitization upon etorphine, fentanyl and DAMGO but not morphine was impaired indicating the different role of amino acids phosphorylation in desensitization (Zheng et al., 2011). As they also demonstrated that PKC mediated morphine-induced MOR desensitization, it can be inferred that PKC would phosphorylate MOR at other sites than S363, T370, and S375. MOR desensitization and phosphorylation at S375 produced by morphine can be modulated by other proteins such as the FK binding protein 12 which would compete with kinase at MOR (Yan et al., 2014).

While all those data indicate that MOR phosphorylation would play a crucial role in desensitization, Qiu and collaborators showed that a truncated mutant of MOR from S363 is able to undergo a similar desensitization to the wild type demonstrating that receptor phosphorylation is not an absolute prerequisite for desensitization (Qiu et al., 2003). However, phosphorylation would rather regulate MOR traffic which could indirectly impact receptor desensitization (see Relationship between OR Internalization and Desensitization).

DOR. In SK-N-BE cells, etorphine-induced hDOR desensitization is totally inhibited by using the dominant negative GRK2 mutant K220R but is only reduced when using PKC and tyrosine kinase inhibitors (Marie et al., 2008). In the NG108-15 cell line, rDOR desensitization promoted by a sustained treatment with DPDPE is mediated by GRK6 but not GRK2 as indicated above for hDOR (Willems and Kelly, 2001). The role of PKC in DADLE- and [Leu⁵]-enkephalin-induced DOR desensitization was also demonstrated on the mobilization of Ca²⁺ stores (Yoon et al., 1998; Song and Chueh, 1999). Tyrosine kinases were also suggested to participate in DOR desensitization. Genistein, a broad spectrum tyrosine kinase inhibitor, inhibits hDOR desensitization promoted by DPDPE, deltorphin I, and etorphine (Marie et al., 2008). Hong and collaborators found that DPDPE promotes a tyrosine phosphorylation of DOR which

would recruit and activate Src that in turn could phosphorylate and activate GRK2; this latter would then phosphorylates S363 and triggers desensitization (Hong et al., 2009). So, inhibition of Src by PP2 reduces DPDPE-induced DOR phosphorylation of S363 and desensitization on the cAMP pathway but via an indirect mechanism. The role of Src in DOR regulation was also confirmed by the group of Pineyro (Archer-Lahlou et al., 2009).

The major role of DOR phosphorylation at S363 was confirmed using the mutant receptor S363A. While deltorphin II promotes a rapid receptor phosphorylation at this amino acid and desensitization on the cAMP pathway, this latter is totally abolished in the S363A mutant (Navratilova et al., 2007). The T161 of DOR, located in the second intracellular loop and equivalent to the T180 of MOR, also plays a role in DPDPE-induced desensitization; the substitution T161A severely impairs DOR desensitization measured on GIRK channels (Lowe et al., 2002). However, those authors did not evaluate the phosphorylation at this residue. The importance of phosphorylation in DOR desensitization was challenged by the work of Qiu and colleagues who studies those processes using a DOR mutant in which all Ser/Thr residues in the C-terminus region were mutated to Ala (Qiu et al., 2007). They observed that DPDPE-induced desensitization on the inhibition of ACease was significantly delayed but not abolished. This indicates that other mechanisms than phosphorylation could contribute to receptor desensitization.

KOR. In the Xenopus oocyte expression system, examination of rKOR regulation on the activation of potassium channels revealed that over-expression of GRK3 or 5 alone did not promote a significant desensitization which requires both GRK and arrestin 3 (Appleyard et al., 1999). This was confirmed when rKOR and GRK2 were co-expressed in CHO cells; pretreatment with a high concentration of U50,488 failed to promote KOR uncoupling from G proteins (Li et al., 2002). Truncation of the C terminal tail of the receptor or the substitution S369A severely impaired U69,593-induced desensitization. These data were further confirmed when wild type and mutant rKOR were expressed in the pituitary adenoma cell line aT-20 cells (McLaughlin et al., 2003). As indicated above, S358 is the major phosphorylation site for hKOR and the S358N substitution totally abolished U50,488-induced receptor uncoupling from G proteins (Li et al., 2002).

While most of those studies with either indirect or direct proofs indicate the role of OR phosphorylation in desensitization, some of them clearly ruled out such paradigm. This probably indicates that phosphorylation is not a prerequisite for desensitization but would accelerate such process.

Role of arrestins in OR regulation

From the canonical model of GPCR regulation by Lefkowitz, arrestins (arrestins 2 and 3 also named β -arrestins 1 and 2, respectively) play a pivotal role in receptor regulation by promoting G protein uncoupling and receptor endocytosis (Pierce et al., 2002). As expected, those proteins were also demonstrated to regulate OR functions. Indeed, over-expression of arrestin 2 induces a selective uncoupling of DOR and KOR and reduces inhibition

of ACase (Cheng et al., 1998). However, no significant impact was observed for MOR explaining the lower desensitization rate compared to DOR (Lowe et al., 2002). In recent studies using BRET (Bioluminescence Resonance Energy Transfert) or FRET (Fluorescence Resonance Energy Transfer) techniques, a large panel of opioid ligands were shown to have a different ability to both activate G proteins and recruit arrestins at MOR and DOR (Mcpherson et al., 2010; Molinari et al., 2010; Rivero et al., 2012). For instance, morphine was demonstrated to behave as a partial agonist for DOR and MOR in G protein coupling experiments while almost no interaction with arrestins was detected. This indicates that all opioid ligands do not have the same potency to promote OR desensitization.

Relationship between arrestins and OR desensitization. Genetic ablation of arrestin 3 significantly reduces MOR uncoupling from G proteins upon chronic morphine treatment (Bohn et al., 2000). Using dorsal root ganglion neurons from arrestin 3 KO mice, the role of this protein in mediating inhibitory regulation of MOR by JNK on voltage-dependent calcium channels was evidenced (Mittal et al., 2012). This report suggests that arrestin 3 and not arrestin 2 would promote MOR desensitization by interacting with JNK. However, in dorsal root ganglion neurons obtained from arrestin 3 KO mice, acute MOR desensitization elicited by DAMGO or morphine on the inhibition of voltage-gated calcium channels was not significantly different from wild type mice indicating that arrestin 3 has no major role in those conditions (Walwyn et al., 2007). Similarly, in neurons from locus coeruleus no significant role of arrestin 3 was evidenced in acute MOR desensitization upon [Met⁵]-enkephalin exposure on the activation of K⁺ currents (Dang et al., 2009). Yet, concomitant inhibition of arrestin 3 expression (arrestin 3 KO mice) and ERK1/2 activity by PD98059 results in reduction of MOR desensitization indicating that this process involves two independent pathways. In the Xenopus oocyte, over-expression of arrestin alone is not sufficient to increase DOR (Kovoov et al., 1999) or KOR (Appleyard et al., 1999) desensitization while in HEK cells, this over-expression enables morphine-induced MOR desensitization probably by increasing both G protein uncoupling and receptor internalization (Whistler and Von Zastrow, 1998). However, such potentiation could be obtained either when arrestin and a GRK are co-expressed or when the constitutive active arrestin mutant R169E is present. This suggests that OR phosphorylation is a pre-requisite for arrestin action. This conclusion is in good agreement with the data obtained by Johnson et al. on MOR desensitization (Johnson et al., 2006). The translocation of arrestin-2-GFP from cytosol to plasma membrane is only observed upon DAMGO exposure which promotes MOR phosphorylation by GRK2. In contrast, no such translocation could be detected in morphine-treated cells which produce a PKC-dependent MOR desensitization. The use of mouse embryonic fibroblast (MEF) from single or double KO mice for arrestins 2 and 3 revealed that DOR desensitization induced by DPDPE relies predominantly on arrestin 3 expression suggesting a preferential interaction between DOR and this arrestin isoform (Qiu et al., 2007). In the SK-N-BE cells, DOR desensitization is reduced when arrestin 2 expression is inhibited by shRNA only upon

DPDPE and deltorphin I exposure but not with etorphine (Aguila et al., 2012).

All those data indicate that different mechanisms are responsible for OR desensitization: some are arrestin-dependent and requires GRK while others are arrestin-independent.

OR internalization

The number of active OR at the cell surface is regulated by two processes: endocytosis and export of neosynthesized receptors. Intuitively, when OR internalization is stimulated by agonist exposure, one could expect a reduction in signal transduction. However, the relationship between the number of OR and the cellular response is not linear.

Internalization of OR has been demonstrated in different models with different technical approaches but some discrepancies have been reported. U50,488 and dynorphin A (1-17), but neither etorphine nor levorphanol, promote a time-, and concentration-dependent internalization of hKOR (Li et al., 2003). In several reports, morphine was described as a poor internalizing agonist of MOR in HEK cells (Keith et al., 1998; Whistler and Von Zastrow, 1998; Schulz et al., 2004; Just et al., 2013) but also in enteric neurons (Anselmi et al., 2013) and in brain slice from transgenic mice expressing a FLAG-tagged MOR (Arattamangkul et al., 2008). In few publications, MOR was shown to internalize upon morphine exposure. This was demonstrated for the endogenous MOR in striatal neurons (Haberstock-Debic et al., 2005) and occurred mainly in dendrites (Haberstock-Debic et al., 2003), in the human neuroblastoma cells SH-SY5Y (Nowoczyń et al., 2013) and in double KO MEF for arrestins transfected both with MOR and arrestin 3 (Groer et al., 2011); in those latter publications, morphine-induced receptor internalization was observed for longer time treatment compared to DAMGO. Using a quantitative assay, 30 min morphine exposure promotes half of the MOR internalization induced by DAMGO (Mcpherson et al., 2010). In enteric neurons, morphine promotes a weak internalization of MOR compared to DAMGO as indicated above but chronic morphine exposure results in a significant increase in endocytosis (Patierno et al., 2011).

Role of OR phosphorylation in internalization

The role of OR phosphorylation in endocytosis was mainly investigated using OR mutants defective in phosphorylation. The truncated MOR from S363, which is not phosphorylated by DAMGO treatment, was shown to internalize but with a slower rate than the wild type receptor during the first 30 min (Qiu et al., 2003). The S375A mutation strongly impairs DAMGO-driven MOR endocytosis (Schulz et al., 2004). The T370A substitution has no significant effect on DAMGO-induced MOR internalization while it inhibits endocytosis triggered by PKC activation (Illing et al., 2014). This suggests that PKC is able to phosphorylate MOR at T370 and promotes its internalization. Conversely, the role of PKC in internalization was ruled out using activators of this kinase in the locus coeruleus neurons expressing the FLAG-tagged MOR (Arattamangkul et al., 2014). Herkinorin, a MOR agonist, is unable to promote both phosphorylation and internalization indicating that the two processes could be linked (Groer et al.,

2007). More than a selective phosphorylation on a specific residue of the carboxy-terminal tail of the receptor, the level of MOR internalization would be correlated to the multi-phosphorylation of T370, S375, T376, and T379 (Just et al., 2013).

As demonstrated for MOR, the phosphorylation-deficient DOR mutant (T358A/T361A/S363G) is able to undergo internalization upon DPDPE activation but to a lesser extent than the wild type (Zhang et al., 2005). However, this DOR mutant cannot internalize anymore when arrestin 3 expression is knocked-down suggesting that the non-phosphorylated DOR can internalize but in an arrestin 3-dependent manner. When the major site of phosphorylation of DOR is mutated (S363A), it is possible to observe a deltorphin I-induced endocytosis (Navratilova et al., 2007); however, it is difficult to assume that this mutation has no impact on internalization since no quantitative evaluation was made. This is in contrast with the study of Bradbury et al. who observed a close correlation between the ability of agonists to phosphorylate the S363 and the degree of DOR internalization (Bradbury et al., 2009).

Concerning the rKOR, the phosphorylation-defective mutant S369E is unable to internalize upon U50,488 exposure demonstrating the role of receptor phosphorylation in endocytosis (McLaughlin et al., 2003).

While those data indicate that MOR and DOR phosphorylation would favor their endocytosis, KOR phosphorylation would be essential to promote its internalization. Other proteins involved in internalization could also be phosphorylated as demonstrated for the MOR. Activation of phospholipase D2 would enhance MOR endocytosis by the activation of p38 kinase which in turn phosphorylates the Rab5 effector early endosome antigen 1 required for this process (Yang et al., 2010).

Role of arrestins in OR internalization

The involvement of arrestins in OR internalization was demonstrated by direct (selective knock-down of arrestin expression)

or indirect approaches (visualization of arrestin translocation to plasma membrane) (**Table 2**).

DAMGO-induced MOR internalization in striatal neurons is impaired by over-expression of a dominant negative mutant of arrestin 2 corresponding to the last 100 amino acids (arrestin 2 319–418) (Haberstock-Debic et al., 2005). Etorphine also induces an arrestin-dependent MOR internalization as shown by the reduction of receptor endocytosis when the dominant negative mutant V53D of arrestin is over-expressed (Zhang et al., 1998). While DAMGO triggers MOR internalization by recruiting either arrestin 2 or 3, morphine selectively interacts with arrestin 3 which is recruited at the plasma membrane to promote MOR internalization (Groer et al., 2011). In HEK cells, morphine is a poor inducer of MOR internalization. Whereas over-expression of arrestin 2 alone has not significant impact, over-expression of GRK2 greatly enhances receptor sequestration; such GRK2-mediated MOR internalization is potentiated when both kinase and arrestin 2 are both co- and over-expressed (Zhang et al., 1998). The lack of MOR internalization upon activation with herkinorin would be due to the absence of interaction between receptor and arrestin 3 (Groer et al., 2007). The constitutive MOR internalization is also arrestin 3-dependent (Walwyn et al., 2007). Whereas those reports indicate the crucial role of arrestins in MOR endocytosis, this was recently challenged by Quillinan et al. who still observed a MOR internalization upon [Met⁵]-enkephalin exposure in arrestin 3 KO mice (Quillinan et al., 2011). In a recent work, the group of von Zastrow showed that after being recruited by the phosphorylated MOR, arrestin 3 acts as a scaffold, promoting ubiquitination of two lysyl residues in the first intracellular loop by the ubiquitin ligase Smurf2 (Henry et al., 2012). Epsin 1, through its ubiquitin-interacting motifs, recognizes the ubiquitinated MOR contained in the clathrin-coated pits and triggers scission of the vesicle from the cell surface. Those data revealed new inter-relations between MOR phosphorylation and ubiquitination with internalization.

Table 2 | Role of arrestins in OR trafficking.

OR	Main results	References
MOR	Inhibition of DAMGO-induced MOR internalization by a dominant negative mutant of arrestin 2 in striatal neurons	Haberstock-Debic et al., 2005
	Inhibition of etorphine-induced MOR internalization by a dominant negative mutant of arrestin 2	Zhang et al., 1998
	Morphine promotes MOR internalization by arrestin 3 while upon DAMGO exposure both arrestins 2 and 3 are recruited	Groer et al., 2011
	Morphine induces MOR endocytosis only when GRK2 and arrestin 2 are co-expressed	Zhang et al., 1998
	Herkinorin is unable to promote MOR sequestration	Groer et al., 2007
	MOR is still internalized upon [Met ⁵]-enkephalin exposure in arrestin 3 KO mice	Quillinan et al., 2011
	The arrestin 3 reduces recycling of MOR upon chronic morphine but not methadone exposure	Quillinan et al., 2011
	Role of arrestin 2 in MOR recycling upon sustained activation by DAMGO but not morphine	Groer et al., 2007
DOR	DOR endocytosis promoted by DPDPE involves both arrestins 2 and 3. Only arrestin 3 can mediate sequestration of a non-phosphorylated DOR mutant	Zhang et al., 2005
	Arrestin 2 preferentially interacts with DOR to induce its sequestration	Qiu et al., 2007
	Arrestin 2 is involved in DOR internalization upon etorphine but not DPDPE or deltorphin I exposure	Aguila et al., 2012
	Arrestin 3 targets DOR to lysosome when activated by SNC-80 but not DPDPE	Audet et al., 2012
KOR	Inhibition of U50,488-induced KOR internalization by a dominant negative mutant of arrestin 2	Li et al., 1999

DPDPE also enables arrestin-mediated endocytosis of DOR as shown by the partial reduction of internalization when arrestins 2 or 3 are selectively inhibited (Zhang et al., 2005). The triple DOR mutant T358A/T361A/S363G is still able to internalize but only when arrestin 3 is expressed. This could explain the plasma membrane translocation of arrestin 3-GFP observed in the study of Navratilova and colleagues with the S363A DOR mutant (Navratilova et al., 2007). DOR endocytosis is severely impaired in MEFs obtained from single KO mice for arrestin 2 indicating a preferential interaction between those two proteins (Qiu et al., 2007). It is noteworthy that even when expression of both arrestins 2 and 3 expression is inhibited, a weak proportion of DOR is able to internalize. This is in good agreement with data obtained by Aguila and collaborators who showed that inhibition of arrestin 2 expression reduces etorphine-induced hDOR endocytosis but not upon DPDPE or deltorphin I exposure (Aguila et al., 2012).

As demonstrated for MOR and DOR, KOR also undergoes an arrestin-dependent sequestration when activated by U50,488 as shown by the reduction of internalization when the dominant negative mutant arrestin 2 319–418 is over-expressed (Li et al., 1999).

Together, those data indicate that arrestins are key partners of OR internalization but under specific conditions or agonist exposure, other arrestin-independent mechanisms could occur.

Relationship between OR internalization and desensitization

Arttamangkul and collaborators studied desensitization on potassium currents and internalization in neurons from locus coeruleus of transgenic mice expressing a FLAG-tagged MOR (Arttamangkul et al., 2008). Three kinds of ligands can be identified: those which promote both desensitization and internalization ([Met⁵]-enkephalin, etorphine, and methadone), those which induce a desensitization without internalization (morphine and oxymorphone) and oxycodone which promote neither desensitization nor internalization. This reveals the absence of any strong association between internalization and desensitization.

In the *Xenopus* oocyte expression system, it is possible to observe an acute desensitization of DOR on potassium channels (Kir3) elicited by DPDPE without significant internalization measured by surface biotinylation (Celver et al., 2013). When DOR internalization is significantly inhibited by over-expression of the dominant negative mutant of dynamin (K44E), the desensitization promoted by sustained exposure to DPDPE is not altered (Qiu et al., 2007). This is in good agreement with the observation of Marie et al. who showed that hypertonic sucrose solution totally blocks hDOR endocytosis without any impact on DPDPE- and deltorphin I-induced desensitization (Marie et al., 2003b). Likewise, UFP-512 promotes a strong DOR endocytosis after 15 min exposure without significant desensitization on the cAMP pathway (Aguila et al., 2007). However, upon etorphine exposure a partial reduction of hDOR desensitization is measured when internalization is inhibited.

In contrast, the abolition of rKOR internalization by the S369A substitution also inhibits receptor desensitization on potassium currents (McLaughlin et al., 2003).

Those data demonstrate that desensitization and internalization are usually two independent processes although in some situations a close relationship could be evidenced. Those apparent discrepancies may be related to the different behavior of MOR and DOR in terms of trafficking (see below). For MOR, internalization would rather promotes recycling and resensitization; when blocking endocytosis, desensitization would be increased. In contrast, DOR are preferentially targeted to degradation, and inhibition of endocytosis would reduce their desensitization; however, this assumption assumes that the receptor at the plasma membrane is not uncoupled from G proteins and it's not always the case.

OR trafficking

Once internalized, the OR can follow different routes: sequestration into endosomes, recycling back to the cell surface or targeting to degradation.

The group of Von Zastrow was the first to identify a protein, named GASP for GPCR associated sorting protein, which could actively target DOR to lysosome (Whistler et al., 2002). This protein selectively interacts with the C terminal region of DOR, not MOR, that could explain that under certain circumstances, DOR is degraded while MOR is recycled (Tsao and Von Zastrow, 2000; Whistler et al., 2002). The same group also identified a motif localized at the C terminal region of MOR that enables an active recycling (Tanowitz and Von Zastrow, 2003). This sequence is lacking in DOR but the chimeric DOR containing the last 17 amino acids of MOR recycles after DAMGO activation in contrast to wild type. Arrestin 3, dynamin and GRK2 also participate to MOR resensitization on the activation of potassium channels in neurons from the locus coeruleus of mice treated during 6 days with morphine (Dang et al., 2011). This could suggest that those proteins would be involved in MOR trafficking after its internalization and that internalization itself contributes to resensitization (Dang and Christie, 2012). Using neurons obtained from the locus coeruleus of transgenic mice expressing a FLAG-tagged MOR, chronic morphine but not methadone during 6 days was shown to inhibit resensitization and recycling after an acute [Met⁵]-enkephalin exposure (Quillinan et al., 2011). Such weak resensitization and recycling return to the level observed in naive mice when arrestin 3 was knocked-down indicating that this protein would also play a pivotal role in MOR trafficking. Arrestin 2 could regulate post-endocytic sorting of MOR upon DAMGO exposure but not morphine by enabling receptor ubiquitination, as described for different GPCRs (Marchese and Trejo, 2013), but also dephosphorylation on the S375 (Groer et al., 2011). The first hypothesis is unlikely since the sorting of the MOR either toward recycling or lysosomal degradation does not rely on receptor ubiquitination (Hislop et al., 2011). The recycling process involves protein kinases as shown by staurosporine, which increases recycling and resensitization after [Met⁵]-enkephalin exposure (Arttamangkul et al., 2012). Resensitization of MOR after [Met⁵]-enkephalin- or morphine-induced acute desensitization but not cellular tolerance involves dephosphorylation mediated by protein phosphatases sensitive to calyculin A but not okadaic acid (Levitt and Williams, 2012). Similarly, Doll and colleagues showed that the rapid MOR dephosphorylation at S375

involves the protein phosphatase 1 γ which increases the recycling of receptors contained in endosomes to cell surface (Doll et al., 2012). The role of receptor dephosphorylation was also demonstrated for both recycling and resensitization of DOR after etorphine treatment (Hasbi et al., 2000).

As indicated above, DOR was initially described as a receptor sorted to lysosomal degradation (Tsao and Von Zastrow, 2000). However, etorphine, [Leu⁵]- and [Met⁵]-enkephalins rather promote a recycling of hDOR while DPDPE, Deltorphin I or SNC-80 induce a degradation and a down-regulation (Marie et al., 2003b; Lecoq et al., 2004). This indicates that the differential sorting of DOR either to recycling or degradation pathway depends on the agonist used and refers to the notion of biased agonism. Audet and collaborators found that DOR activated by SNC-80 strongly interacts with arrestin 3 (Audet et al., 2012). Consequently, the receptor is mainly targeted to lysosome while upon DPDPE exposure, interactions between DOR and arrestin 3 are loose allowing receptor recycling. The ability of DOR to recycle also depends on the duration of agonist exposure. For instance, after 30 min of etorphine treatment, DOR recycles while after 4 h this process is severely impaired (Hasbi et al., 2000). Zhang and collaborators showed different mechanisms to explain the differential sorting of DOR (Zhang et al., 2008): when the receptor is phosphorylated by GRK2 and internalized via arrestins it can recycle whereas in a non-phosphorylated form DOR undergoes an arrestin-independent sequestration which is followed by a degradation. As described for MOR, kinases can be involved in OR sorting. Src was shown to inhibit DOR recycling upon DPDPE treatment that would favor desensitization on the cAMP pathway (Archer-Lahlou et al., 2009). Recently, the endothelin converting enzyme-2, localized in endosomes, was shown to modulate recycling of DOR by degrading opioid peptides such as deltorphin II or the opioid peptide bovine adrenal medulla 22 (BAM22), a cleavage product of proenkephalin (Gupta et al., 2014). When this enzyme is inhibited, DOR recycling decreases and consequently, the desensitization increases. It is noteworthy that this enzyme is ineffective when DOR is activated by the endogenous peptide [Met⁵]-enkephalin and has no role on receptor internalization.

MOLECULAR MECHANISMS INVOLVED IN OR DESENSITIZATION: A UNIFIED MECHANISM?

The vast majority of studies on OR desensitization demonstrated that phosphorylation of OR constitutes a rapid and ubiquitous regulatory mechanism. However, as illustrated for MOR, quantitative (Lau et al., 2011) or qualitative (Just et al., 2013) differences in MOR phosphorylation were reported upon DAMGO and morphine exposure and those differences in multi-site phosphorylation would result in differential interactions with partners. Conversely, some studies using phosphorylation-deficient receptor challenged this paradigm (Qiu et al., 2003). OR phosphorylation should rather be viewed as a potentiating mechanism that would increase binding of regulatory proteins such as arrestins to the receptor. Mechanisms of desensitization share common features (phosphorylation, accessory proteins involvement such as arrestin, importance of endocytosis and receptor trafficking) and will depend not only on agonist (biased agonism) but also on

time exposure, cell system and receptor. All those mechanisms are depicted in **Figures 2A,B**.

OPIOID TOLERANCE

DEFINITION

Drug tolerance is the body's ability to protect itself against the presence of a drug. It is generally observed after protracted exposure but also after acute treatment (acute tolerance) and it is not observed for all the pharmacological effects. For opioids, tolerance to analgesia has been primarily studied as it is the main issue in clinical practice. In rodent, the ability of opioid to promote analgesia to different type of stimuli could be measured using numerous behavioral paradigms including hot-plate test and tail-flick for thermal nociception (Barrot, 2012). Different parameters could modulate tolerance such as the opioid agonist used (Enquist et al., 2012), duration of treatment (Soignier et al., 2004), doses (Huidobro et al., 1976) and even the pharmacological effect observed (Mohammed et al., 2013). So, it is now established that tolerance to respiratory depression is lower than the tolerance to analgesia (Mohammed et al., 2013) and might explain fatal overdoses (White and Irvine, 1999).

OPIOID RECEPTOR-RELATED MECHANISMS OF TOLERANCE

Mechanisms of opioid tolerance are complex and multifaceted. We will focus on the mechanisms directly related to receptor regulation such as down-regulation, G protein uncoupling, desensitization, and internalization. Indeed, other mechanisms contribute to tolerance such as activation of anti-opioid systems (NPFF, NMDA) (Ueda and Ueda, 2009) but they are beyond the scope of this review.

Down-regulation

Down-regulation is the reduction of receptor number that may result from receptor internalization followed by their degradation, or decrease in receptor synthesis. So, one could hypothesize that it would contribute to tolerance by diminishing the quantity of available receptor. *In vivo*, chronic treatment with opioids promotes decrease (down-regulation), no change or increase (up-regulation) of OR (Bernstein and Welch, 1998; Stafford et al., 2001; Fabian et al., 2002). When downregulation is observed, tolerance might be measured (Gomes et al., 2002) however in some cases tolerance occurs without receptor downregulation (Polastron et al., 1994). These data suggest that downregulation is not mandatory for tolerance.

Desensitization

Desensitization and tolerance are very similar in their definition as they both include the notion of a reduced response after prolonged treatment. So, it is tempting to speculate that desensitization and its mechanisms would occur in tolerant animals. In chronic morphine-treated animals desensitization of OR was measured on ACase (Noble and Cox, 1996) and associated with tolerance to analgesic effects (Polastron et al., 1994). In cellular model, receptor uncoupling from G proteins was demonstrated to participate in desensitization (see above). Such uncoupling was also evidenced *in vivo* after chronic opioid agonist exposure.

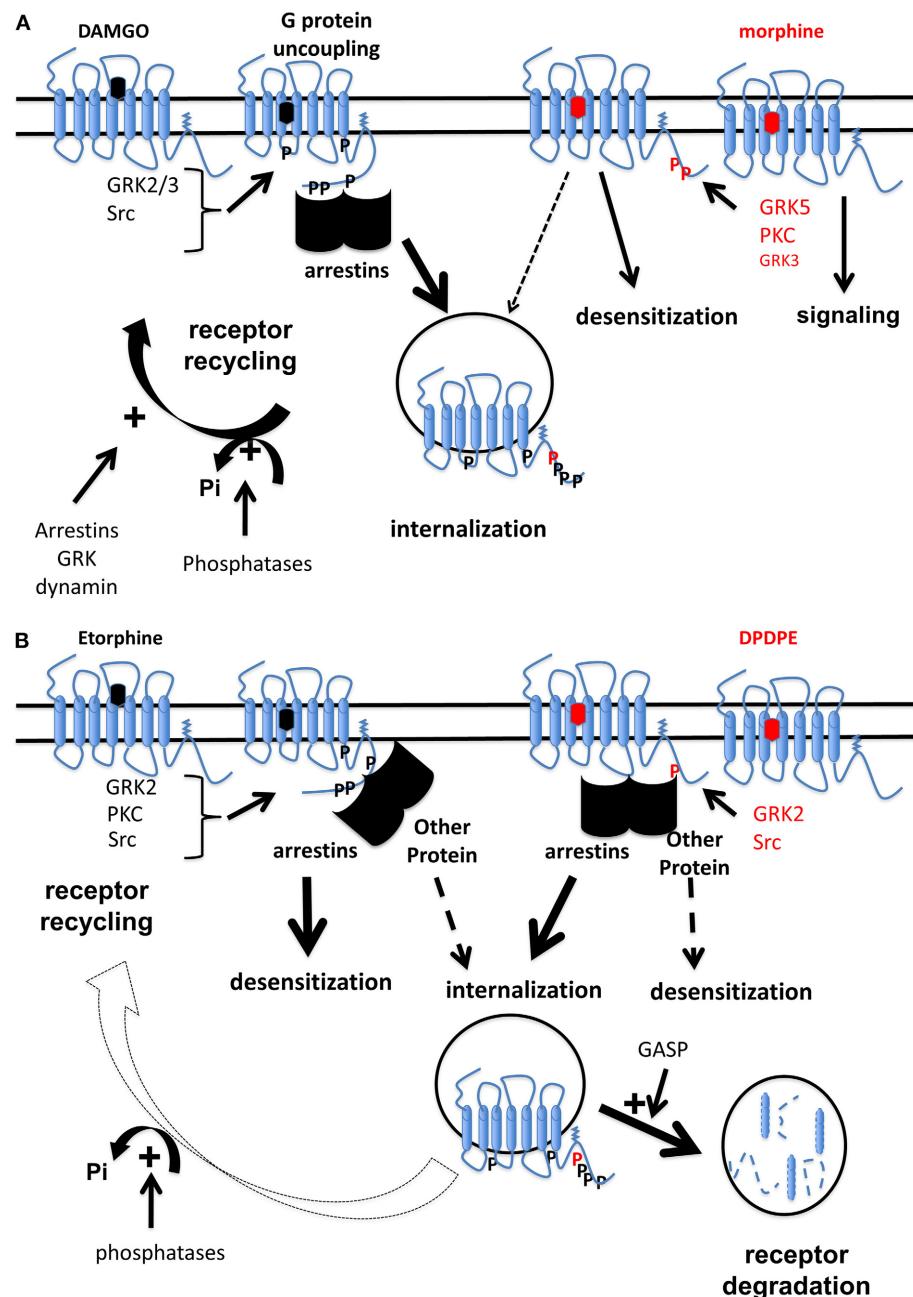


FIGURE 2 | Schematic illustration of mechanisms involved in opioid receptor desensitization by biased agonists. (A) MOR are differentially phosphorylated by different kinases upon either DAMGO or morphine exposure (Doll et al., 2011). This results in binding of arrestins to MOR upon DAMGO while this interaction is weakly detectable for morphine when a GRK is over-expressed (Groer et al., 2007). In such conditions, acute DAMGO exposure promotes G protein uncoupling from MOR while morphine does not (Whistler and Von Zastrow, 1998). However, MOR phosphorylation at S375 induced by morphine is able to promote desensitization but not internalization (Schulz et al., 2004). Some reports rather suggest that under morphine exposure, MOR is not desensitized and this continuous signaling promotes tolerance (Finn and Whistler, 2001). Even if it's now well-admitted that morphine is able to promote MOR internalization (Haberstock-Debic et al., 2005; Nowoczyń et al., 2013), DAMGO induces a stronger internalization compared to morphine (Whistler and Von Zastrow, 1998; Schulz et al., 2004).

MOR is dephosphorylated by phosphatase proteins (Doll et al., 2012) then undergoes an active recycling (Tanowitz and Von Zastrow, 2003). Other proteins such as arrestins, dynamin, or GRK could participate MOR trafficking (Dang et al., 2011). In contrast, as morphine is a poor inducer of MOR internalization, receptor is maintained in a phosphorylation state at S375 for longer time compared to DAMGO. **(B)** Different kinases are involved in the regulation of hDOR (Marie et al., 2008): GRK2 plays a major role in receptor phosphorylation on S363 upon DPDPE and etorphine while other kinases are also implicated. Etorphine-induced desensitization requires arrestins but not receptor internalization. In contrast, an arrestin is involved in hDOR internalization but not desensitization upon DPDPE (Aguila et al., 2012). Once sequestered by etorphine, hDOR is dephosphorylated and recycled back to the cell surface (Hasbi et al., 2000; Marie et al., 2003b) while upon DPDPE exposure, the receptor is mainly targeted to lysosomes for degradation (Marie et al., 2003b) probably by a mechanism involving GASP (Whistler et al., 2002).

In knock-in mice expressing DOR-eGFP, a challenge with SNC-80 but not ARM-390 induces a tolerance to analgesic response in a model of inflammatory pain with a concomitant G protein uncoupling in both brain and spinal cord homogenates (Pradhan et al., 2009). Acute and chronic treatment with morphine or fentanyl promotes a similar regulation of MOR. In parallel with analgesic tolerance, the ability of MOR to enhance [³⁵S]GTP γ S binding was reduced compared to naive animals (Bohn et al., 2000; Melfi et al., 2010). When arrestin 3 was knocked-out, morphine tolerance and MOR uncoupling from G proteins was reduced in chronic treated animals (Bohn et al., 2000). Interestingly, this KO did not affect tolerance induced by 5 days treatment with fentanyl, oxycodone or methadone (Raehal et al., 2011).

Phosphorylation

Anti-nociceptive tolerance induced by morphine, meperidine, and fentanyl was shown to be reduced by PKC inhibitors while DAMGO-induced tolerance and MOR desensitization was shown to rely on GRK (Hull et al., 2010). Whereas *in vitro* experiments showed that S375 is phosphorylated by GRK5 upon morphine exposure (Doll et al., 2012) and S375 phosphorylation plays a major role in MOR desensitization (Schulz et al., 2004), S375A knock-in mice still present anti-nociceptive tolerance upon acute and chronic exposure to morphine (Grecksch et al., 2011). This could indicate that MOR desensitization and tolerance are two unrelated mechanisms. Recently, the role of GRK in morphine tolerance was also questioned: while morphine predominantly promotes S375 phosphorylation by GRK5, chronic morphine treatment induced similar tolerance in wild type and in GRK5 KO mice while dependence was altered (Glück et al., 2014). Similar results were obtained in GRK3 KO mice, when morphine tolerance to analgesia was unchanged whereas tolerance to high efficacy agonists, such as fentanyl or U50,488, was reduced (Terman et al., 2004; Zhang et al., 2013). Rather than inducing desensitization, a chronic morphine treatment could promote a compensatory increase in intracellular cAMP level (also named cAMP overshoot or ACase superactivation) (Avidor-Reiss et al., 1995) and is believed to play a direct role in tolerance (Duman et al., 1988; Javed et al., 2004). In this situation, Src kinase can be recruited at the lipid raft-located MOR and phosphorylates the Y336 leading to ACase superactivation (Zhang et al., 2009). While the mechanism is still unclear, it could implicate Ras/Raf-1 which change the MOR, a GPCR, into a receptor tyrosine kinase like-complex (Zhang et al., 2013).

Endocytosis

Accumulating evidences suggest that OR endocytosis decrease opioid tolerance but by mechanisms not fully understood. The first hypothesis has been built by Whistler's group on the inability of morphine to promote MOR internalization despite its capacity to induce strong tolerance. In this case, during morphine treatment, morphine/MOR complexes would accumulate at the plasma membrane and recruit signaling pathways involved in tolerance such as ACase superactivation and NMDA receptor regulation (Finn and Whistler, 2001; He et al., 2002, 2009). In line with this hypothesis, a knock-in mice, expressing a MOR chimera

where the C-terminus tail was replaced by the C-terminus tail of DOR, demonstrated less tolerance after chronic morphine treatment (Kim et al., 2008), correlated to a decrease of tolerance biomarkers (He et al., 2009). One explanation of this result is the termination of signal transduction because the DOR C-terminus tail will target the chimeric MOR to lysosomes (Finn and Whistler, 2001). Such results were confirmed when comparing other opioid agonist, buprenorphine and etonitazene. Indeed, buprenorphine, like morphine induces tolerance to analgesia without promoting MOR endocytosis, whereas etonitazene promotes less tolerance and has the ability to promote MOR internalization (Grecksch et al., 2006). Interestingly, coadministration of morphine with subactive doses of internalizing opioids, DAMGO or methadone, enables morphine-induced internalization of MOR and blocks tolerance development (He and Whistler, 2005). An alternative hypothesis was proposed by Koch and collaborators. They proposed that morphine promotes an accumulation of desensitized MOR at the plasma membrane that would result in an increase in apparent desensitization by inhibiting resensitization and would promote tolerance (Koch et al., 2001, 2005; Schulz et al., 2004). However, they found that in knock-in mice expressing MOR mutant S375A substitution, proposed by these authors to be the primary site of morphine-induced phosphorylation of MOR responsible for desensitization (Schulz et al., 2004), morphine tolerance was not affected (Grecksch et al., 2011). The RAVE (relative activity vs. endocytosis) concept proposed by Whistler et al. (1999) cannot be extended to DOR. In DOR-eGFP knock-in mice, the internalizing agonist, SNC-80 promotes acute tolerance to analgesia correlated with strong internalization whereas ARM-390 a non-internalizing agonist did not induce acute tolerance (Pradhan et al., 2009, 2010). When SNC-80 and ARM-390 are chronically administrated, tolerance to analgesia develops and is dependent on endocytosis with SNC80 but not for ARM-390. Interestingly, no tolerance for locomotor effects or anxiolysis appears in ARM-390-treated animals underlying the fact that biased agonist could be used at the behavioral level. All those data support the role of internalization and mainly recycling in reducing tolerance by allowing a sufficient quantity of functional receptors at the cell surface to produce the biological response. However, some opioid agonists such as herkinorin can promote a long lasting anti-nociception without internalization due to the absence of arrestin 3 recruitment (Lamb et al., 2012).

CONCLUSIONS

All the data presented in this review demonstrated that mechanisms of OR regulation are consistent with the model proposed by Lefkowitz (Pierce et al., 2002): agonist activation, receptor phosphorylation, arrestin binding, G protein uncoupling, desensitization, endocytosis followed by targeting to lysosomes or recycling. More interestingly, they also showed that many variations around this model exist depending on the initial conditions, revealing the complexity of OR regulation now translated to the concept of biased agonism. It's an exciting challenge to gain insight this complexity because it will offer a great opportunity to design new drugs that will be able to target a particular pharmacological effect with limited side effects.

REFERENCES

- Abbadie, C., Pan, Y. X., and Pasternak, G. W. (2004). Immunohistochemical study of the expression of exon11-containing mu opioid receptor variants in mouse brain. *Neuroscience* 127, 419–430. doi: 10.1016/j.neuroscience.2004.03.033
- Aguila, B., Coulbault, L., Boulouard, M., Leveille, F., Davis, A., Toth, G., et al. (2007). *In vitro* and *in vivo* pharmacological profile of UFP-512, a novel selective delta-opioid receptor agonist; correlations between desensitization and tolerance. *Br. J. Pharmacol.* 152, 1312–1324. doi: 10.1038/sj.bjp.0707497
- Aguila, B., Coulbault, L., Davis, A., Marie, N., Hasbi, A., Le Bras, F., et al. (2012). Betaarrestin1-biased agonism at human delta-opioid receptor by peptidic and alkaloid ligands. *Cell Signal* 24, 699–707. doi: 10.1016/j.cellsig.2011.10.018
- Allouche, S., Polastron, J., Hasbi, A., Homburger, V., and Jauzac, P. (1999a). Differential G-protein activation by alkaloid and peptide opioid agonists in the human neuroblastoma cell line SK-N-BE. *Biochem. J.* 342 (Pt 1), 71–78. doi: 10.1042/0264-6021:3420071
- Allouche, S., Roussel, M., Marie, N., and Jauzac, P. (1999b). Differential desensitization of human delta-opioid receptors by peptide and alkaloid agonists. *Eur. J. Pharmacol.* 371, 235–240. doi: 10.1016/S0014-2999(99)00180-6
- Alonso, N., Monczor, F., Echeverria, E., Davio, C., Shayo, C., and Fernandez, N. (2014). Signal transduction mechanism of biased ligands at histamine H2 receptors. *Biochem. J.* 459, 117–126. doi: 10.1042/BJ20131226
- Anselmi, L., Jaramillo, I., Palacios, M., Huynh, J., and Sternini, C. (2013). Ligand-induced mu opioid receptor internalization in enteric neurons following chronic treatment with the opiate fentanyl. *J. Neurosci. Res.* 91, 854–860. doi: 10.1002/jnr.23214
- Appleyard, S. M., Celver, J., Pineda, V., Kovoov, A., Wayman, G. A., and Chavkin, C. (1999). Agonist-dependent desensitization of the kappa opioid receptor by G protein receptor kinase and beta-arrestin. *J. Biol. Chem.* 274, 23802–23807. doi: 10.1074/jbc.274.34.23802
- Archer-Lahlou, E., Audet, N., Amraei, M. G., Huard, K., Paquin-Gobeil, M., and Pineyro, G. (2009). Src promotes delta opioid receptor (DOR) desensitization by interfering with receptor recycling. *J. Cell. Mol. Med.* 13, 147–163. doi: 10.1111/j.1582-4934.2008.00308.x
- Arattamangkul, S., Birdsong, W., and Williams, J. T. (2014). Does PKC activation increase the homologous desensitization of mu opioid receptors? *Br. J. Pharmacol.* doi: 10.1111/bph.12712
- Arattamangkul, S., Lau, E. K., Lu, H.-W., and Williams, J. T. (2012). Desensitization and trafficking of mu-opioid receptors in locus ceruleus neurons: modulation by kinases. *Mol. Pharmacol.* 81, 348–355. doi: 10.1124/mol.111.076208
- Arattamangkul, S., Quillinan, N., Low, M. J., Von Zastrow, M., Pintar, J., and Williams, J. T. (2008). Differential activation and trafficking of micro-opioid receptors in brain slices. *Mol. Pharmacol.* 74, 972–979. doi: 10.1124/mol.108.048512
- Audet, N., Charfi, I., Mnie-Filali, O., Amraei, M., Chabot-Dore, A. J., Millecamps, M., et al. (2012). Differential association of receptor-Gbetagamma complexes with beta-arrestin2 determines recycling bias and potential for tolerance of delta opioid receptor agonists. *J. Neurosci.* 32, 4827–4840. doi: 10.1523/JNEUROSCI.3734-11.2012
- Avidor-Reiss, T., Bayewitch, M., Levy, R., Matus-Leibovitch, N., Nevo, I., and Vogel, Z. (1995). Adenylcyclase supersensitization in mu-opioid receptor-transfected Chinese hamster ovary cells following chronic opioid treatment. *J. Biol. Chem.* 270, 29732–29738. doi: 10.1074/jbc.270.50.29732
- Bailey, C. P., Oldfield, S., Llorente, J., Caunt, C. J., Teschemacher, A. G., Roberts, L., et al. (2009). Involvement of PKC alpha and G-protein-coupled receptor kinase 2 in agonist-selective desensitization of mu-opioid receptors in mature brain neurons. *Br. J. Pharmacol.* 158, 157–164. doi: 10.1111/j.1476-5381.2009.00140.x
- Barrot, M. (2012). Tests and models of nociception and pain in rodents. *Neuroscience* 211, 39–50. doi: 10.1016/j.neuroscience.2011.12.041
- Beckett, A. H., and Casy, A. F. (1954). Stereochemistry of certain analgesics. *Nature* 173, 1231–1232. doi: 10.1038/1731231a0
- Befort, K., Filliol, D., Decaillot, F. M., Gaveriaux-Ruff, C., Hoehe, M. R., and Kieffer, B. L. (2001). A single nucleotide polymorphic mutation in the human mu-opioid receptor severely impairs receptor signaling. *J. Biol. Chem.* 276, 3130–3137. doi: 10.1074/jbc.M006352200
- Bernstein, M. A., and Welch, S. P. (1998). mu-Opioid receptor down-regulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance. *Brain Res. Mol. Brain Res.* 55, 237–242. doi: 10.1016/S0169-328X(98)00005-9
- Besse, D., Lombard, M. C., Zajac, J. M., Roques, B. P., and Besson, J. M. (1990). Pre- and postsynaptic distribution of mu, delta and kappa opioid receptors in the superficial layers of the cervical dorsal horn of the rat spinal cord. *Brain Res* 521, 15–22. doi: 10.1016/0006-8993(90)91519-M
- Bian, J. M., Wu, N., Su, R. B., and Li, J. (2012). Opioid receptor trafficking and signaling: what happens after opioid receptor activation? *Cell. Mol. Neurobiol.* 32, 167–184. doi: 10.1007/s10571-011-9755-5
- Blake, A. D., Bot, G., Freeman, J. C., and Reisine, T. (1997a). Differential opioid agonist regulation of the mouse mu opioid receptor. *J. Biol. Chem.* 272, 782–790. doi: 10.1074/jbc.272.2.782
- Blake, A. D., Bot, G., Freeman, J. C., and Reisine, T. (1997b). Differential agonist regulation of the human kappa-opioid receptor. *J. Neurochem.* 68, 1846–1852. doi: 10.1046/j.1471-4159.1997.68051846.x
- Blanchet, C., Sollini, M., and Luscher, C. (2003). Two distinct forms of desensitization of G-protein coupled inwardly rectifying potassium currents evoked by alkaloid and peptide mu-opioid receptor agonists. *Mol. Cell. Neurosci.* 24, 517–523. doi: 10.1016/S1044-7431(03)00173-8
- Bodnar, R. J. (2014). Endogenous opiates and behavior: 2013. *Peptides* 62C, 67–136. doi: 10.1016/j.peptides.2014.09.013
- Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, R. J., and Caron, M. G. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408, 720–723. doi: 10.1038/35047086
- Borgland, S. L., Connor, M., Osborne, P. B., Furness, J. B., and Christie, M. J. (2003). Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J. Biol. Chem.* 278, 18776–18784. doi: 10.1074/jbc.M300525200
- Bot, G., Blake, A. D., Li, S., and Reisine, T. (1997). Opioid regulation of the mouse delta-opioid receptor expressed in human embryonic kidney 293 cells. *Mol. Pharmacol.* 52, 272–281.
- Bradbury, F. A., Zelnik, J. C., and Traynor, J. R. (2009). G protein independent phosphorylation and internalization of the delta-opioid receptor. *J. Neurochem.* 109, 1526–1535. doi: 10.1111/j.1471-4159.2009.06082.x
- Celver, J., Sharma, M., Thanawala, V., Christopher Octeau, J., and Kovoov, A. (2013). Arrestin-dependent but G-protein coupled receptor kinase-independent uncoupling of D2-dopamine receptors. *J. Neurochem.* 127, 57–65. doi: 10.1111/jnc.12359
- Celver, J., Xu, M., Jin, W., Lowe, J., and Chavkin, C. (2004). Distinct domains of the mu-opioid receptor control uncoupling and internalization. *Mol. Pharmacol.* 65, 528–537. doi: 10.1124/mol.65.3.528
- Chen, C., Shahabi, V., Xu, W., and Liu-Chen, L. Y. (1998). Palmitoylation of the rat mu opioid receptor. *FEBS Lett.* 441, 148–152. doi: 10.1016/S0014-5793(98)01547-6
- Chen, Y. J., Oldfield, S., Butcher, A. J., Tobin, A. B., Saxena, K., Gurevich, V. V., et al. (2013). Identification of phosphorylation sites in the COOH-terminal tail of the mu-opioid receptor. *J. Neurochem.* 124, 189–199. doi: 10.1111/jnc.12071
- Chen, Y., Mestek, A., Liu, J., Hurley, J. A., and Yu, L. (1993a). Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol. Pharmacol.* 44, 8–12.
- Chen, Y., Mestek, A., Liu, J., and Yu, L. (1993b). Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. *Biochem. J.* 295 (Pt 3), 625–628.
- Cheng, Z. J., Yu, Q. M., Wu, Y. L., Ma, L., and Pei, G. (1998). Selective interference of beta-arrestin 1 with kappa and delta but not mu opioid receptor/G protein coupling. *J. Biol. Chem.* 273, 24328–24333. doi: 10.1074/jbc.273.38.24328
- Chu, J., Zheng, H., Zhang, Y., Loh, H. H., and Law, P. Y. (2010). Agonist-dependent mu-opioid receptor signaling can lead to heterologous desensitization. *Cell. Signal.* 22, 684–696. doi: 10.1016/j.cellsig.2009.12.003
- Clayton, C. C., Bruchas, M. R., Lee, M. L., and Chavkin, C. (2010). Phosphorylation of the mu-opioid receptor at tyrosine 166 (Tyr3.51) in the DRY motif reduces agonist efficacy. *Mol. Pharmacol.* 77, 339–347. doi: 10.1124/mol.109.060558
- Connor, M., Osborne, P. B., and Christie, M. J. (2004). Mu-opioid receptor desensitization: is morphine different? *Br. J. Pharmacol.* 143, 685–696. doi: 10.1038/sj.bjp.0705938
- Cox, B. M., Goldstein, A., and Hi, C. H. (1976). Opioid activity of a peptide, beta-lipotropin-(61–91), derived from beta-lipotropin. *Proc. Natl. Acad. Sci. U.S.A.* 73, 1821–1823. doi: 10.1073/pnas.73.6.1821
- Dang, V. C., Chieng, B., Azriel, Y., and Christie, M. J. (2011). Cellular morphine tolerance produced by betaarrestin-2-dependent impairment

- of mu-opioid receptor resensitization. *J. Neurosci.* 31, 7122–7130. doi: 10.1523/JNEUROSCI.5999-10.2011
- Dang, V. C., and Christie, M. J. (2012). Mechanisms of rapid opioid receptor desensitization, resensitization and tolerance in brain neurons. *Br. J. Pharmacol.* 165, 1704–1716. doi: 10.1111/j.1476-5381.2011.01482.x
- Dang, V. C., Napier, I. A., and Christie, M. J. (2009). Two distinct mechanisms mediate acute mu-opioid receptor desensitization in native neurons. *J. Neurosci.* 29, 3322–3327. doi: 10.1523/JNEUROSCI.4749-08.2009
- Dhawan, B. N., Cesselin, F., Raghuram, R., Reisine, T., Bradley, P. B., Portoghese, P. S., et al. (1996). International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol. Rev.* 48, 567–592.
- Doll, C., Konietzko, J., Poll, F., Koch, T., Hollt, V., and Schulz, S. (2011). Agonist-selective patterns of micro-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *Br. J. Pharmacol.* 164, 298–307. doi: 10.1111/j.1476-5381.2011.01382.x
- Doll, C., Poll, F., Peuker, K., Loktev, A., Gluck, L., and Schulz, S. (2012). Deciphering micro-opioid receptor phosphorylation and dephosphorylation in HEK293 cells. *Br. J. Pharmacol.* 167, 1259–1270. doi: 10.1111/j.1476-5381.2012.02080.x
- Duman, R. S., Tallman, J. F., and Nestler, E. J. (1988). Acute and chronic opiate regulation of adenylate cyclase in brain: specific effects in locus coeruleus. *J. Pharmacol. Exp. Ther.* 246, 1033–1039.
- Ehrlich, G. K., Andria, M. L., Zheng, X., Kieffer, B., Gioannini, T. L., Hiller, J. M., et al. (1998). Functional significance of cysteine residues in the delta opioid receptor studied by site-directed mutagenesis. *Can. J. Physiol. Pharmacol.* 76, 269–277.
- El Kouhen, R., Burd, A. L., Erickson-Herbrandson, L. J., Chang, C. Y., Law, P. Y., and Loh, H. H. (2001). Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. *J. Biol. Chem.* 276, 12774–12780. doi: 10.1074/jbc.M009571200
- Enquist, J., Ferwerda, M., Milan-Lobo, L., and Whistler, J. L. (2012). Chronic methadone treatment shows a better cost/benefit ratio than chronic morphine in mice. *J. Pharmacol. Exp. Ther.* 340, 386–392. doi: 10.1124/jpet.111.187583
- Evans, C. J., Keith, D. E. Jr., Morrison, H., Magendzo, K., and Edwards, R. H. (1992). Cloning of a delta opioid receptor by functional expression. *Science* 258, 1952–1955. doi: 10.1126/science.1335167
- Fabian, G., Bozo, B., Sziksay, M., Horvath, G., Coscia, C. J., and Szucs, M. (2002). Chronic morphine-induced changes in mu-opioid receptors and G proteins of different subcellular loci in rat brain. *J. Pharmacol. Exp. Ther.* 302, 774–780. doi: 10.1124/jpet.102.036152
- Feng, B., Li, Z., and Wang, J. B. (2011). Protein kinase C-mediated phosphorylation of the mu-opioid receptor and its effects on receptor signaling. *Mol. Pharmacol.* 79, 768–775. doi: 10.1124/mol.110.069096
- Filizola, M., and Devi, L. A. (2013). Grand opening of structure-guided design for novel opioids. *Trends Pharmacol. Sci.* 34, 6–12. doi: 10.1016/j.tips.2012.10.002
- Finn, A. K., and Whistler, J. L. (2001). Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* 32, 829–839. doi: 10.1016/S0896-6273(01)00517-7
- Fujita, W., Gomes, I., and Devi, L. A. (2014). Heteromers of μ -delta opioid receptors: new pharmacology and novel therapeutic possibilities. *Br. J. Pharmacol.* doi: 10.1111/bph.12663. [Epub ahead of print].
- Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., et al. (1994). cDNA cloning and regional distribution of a novel member of the opioid receptor family. *FEBS Lett.* 343, 42–46. doi: 10.1016/0014-5793(94)80603-9
- Gahwiler, B. H. (1981). Development of acute tolerance during exposure of hippocampal explants to an opioid peptide. *Brain Res.* 217, 196–200. doi: 10.1016/0006-8993(81)90200-6
- Gaveriaux-Ruff, C., and Kieffer, B. L. (2002). Opioid receptor genes inactivated in mice: the highlights. *Neuropeptides* 36, 62–71. doi: 10.1054/npep.2002.0900
- Georgoussi, Z., Merkouris, M., Mullaney, I., Megaritis, G., Carr, C., Zioudrou, C., et al. (1997). Selective interactions of mu-opioid receptors with pertussis toxin-sensitive G proteins: involvement of the third intracellular loop and the C-terminal tail in coupling. *Biochim. Biophys. Acta* 1359, 263–274. doi: 10.1016/S0167-4889(97)00097-9
- Gioannini, T. L., Onoprishvili, I., Hiller, J. M., and Simon, E. J. (1999). Inactivation of the purified bovine mu opioid receptor by sulfhydryl reagents. *Neurochem. Res.* 24, 37–42. doi: 10.1023/A:1020923928936
- Glück, L., Loktev, A., Moulédous, L., Mollereau, C., Law, P.-Y., and Schulz, S. (2014). Loss of morphine reward and dependence in mice lacking G Protein-coupled receptor kinase 5. *Biol. Psychiatry* 76, 767–774. doi: 10.1016/j.biopsych.2014.01.021
- Goldstein, A., Fischli, W., Lowney, L. I., Hunkapiller, M., and Hood, L. (1981). Porcine pituitary dynorphin: complete amino acid sequence of the biologically active heptadecapeptide. *Proc. Natl. Acad. Sci. U.S.A.* 78, 7219–7223. doi: 10.1073/pnas.78.11.7219
- Gomes, B. A., Shen, J., Stafford, K., Patel, M., and Yoburn, B. C. (2002). Mu-opioid receptor down-regulation and tolerance are not equally dependent upon G-protein signaling. *Pharmacol. Biochem. Behav.* 72, 273–278. doi: 10.1016/S0091-3057(01)00757-2
- Granier, S., Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Weis, W. I., et al. (2012). Structure of the delta-opioid receptor bound to naltrindole. *Nature* 485, 400–404. doi: 10.1038/nature11111
- Grecksch, G., Bartsch, K., Widera, A., Becker, A., Hollt, V., and Koch, T. (2006). Development of tolerance and sensitization to different opioid agonists in rats. *Psychopharmacology (Berl)* 186, 177–184. doi: 10.1007/s00213-006-0365-8
- Grecksch, G., Just, S., Pierstorff, C., Imhof, A. K., Gluck, L., Doll, C., et al. (2011). Analgesic tolerance to high-efficacy agonists but not to morphine is diminished in phosphorylation-deficient S375A mu-opioid receptor knock-in mice. *J. Neurosci.* 31, 13890–13896. doi: 10.1523/JNEUROSCI.2304-11.2011
- Groer, C. E., Schmid, C. L., Jaeger, A. M., and Bohn, L. M. (2011). Agonist-directed interactions with specific beta-arrestins determine mu-opioid receptor trafficking, ubiquitination, and dephosphorylation. *J. Biol. Chem.* 286, 31731–31741. doi: 10.1074/jbc.M111.248310
- Groer, C. E., Tidgewell, K., Moyer, R. A., Harding, W. W., Rothman, R. B., Prisinzano, T. E., et al. (2007). An opioid agonist that does not induce mu-opioid receptor-arrestin interactions or receptor internalization. *Mol. Pharmacol.* 71, 549–557. doi: 10.1124/mol.106.028258
- Guillemin, R., Vargo, T., Rossier, J., Minick, S., Ling, N., Rivier, C., et al. (1977). Beta-Endorphin and adrenocorticotropin are selected concomitantly by the pituitary gland. *Science* 197, 1367–1369. doi: 10.1126/science.197601
- Guo, J., Wu, Y., Zhang, W., Zhao, J., Devi, L. A., Pei, G., et al. (2000). Identification of G protein-coupled receptor kinase 2 phosphorylation sites responsible for agonist-stimulated delta-opioid receptor phosphorylation. *Mol. Pharmacol.* 58, 1050–1056. doi: 10.1124/mol.58.5.1050
- Gupta, A., Fujita, W., Gomes, I., Bobeck, E., and Devi, L. A. (2014). Endothelin converting enzyme-2 differentially regulates opioid receptor activity. *Br. J. Pharmacol.* doi: 10.1111/bph.12833. [Epub ahead of print].
- Haberstock-Debic, H., Kim, K.-A., Yu, Y. J., and Von Zastrow, M. (2005). Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *J. Neurosci.* 25, 7847–7857. doi: 10.1523/JNEUROSCI.5045-04.2005
- Haberstock-Debic, H., Wein, M., Barrot, M., Colago, E. E., Rahman, Z., Neve, R. L., et al. (2003). Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. *J. Neurosci.* 23, 4324–4332.
- Hasbi, A., Allouche, S., Sichel, F., Stanasila, L., Massotte, D., Landemore, G., et al. (2000). Internalization and recycling of delta-opioid receptor are dependent on a phosphorylation-dephosphorylation mechanism. *J. Pharmacol. Exp. Ther.* 293, 237–247.
- He, L., Fong, J., Von Zastrow, M., and Whistler, J. L. (2002). Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* 108, 271–282. doi: 10.1016/S0092-8674(02)00613-X
- He, L., Kim, J. A., and Whistler, J. L. (2009). Biomarkers of morphine tolerance and dependence are prevented by morphine-induced endocytosis of a mutant mu-opioid receptor. *FASEB J.* 23, 4327–4334. doi: 10.1096/fj.09-133223
- He, L., and Whistler, J. L. (2005). An opiate cocktail that reduces morphine tolerance and dependence. *Curr. Biol.* 15, 1028–1033. doi: 10.1016/j.cub.2005.04.052
- Henry, A. G., Hislop, J. N., Grove, J., Thorn, K., Marsh, M., and Von Zastrow, M. (2012). Regulation of endocytic clathrin dynamics by cargo ubiquitination. *Dev. Cell* 23, 519–532. doi: 10.1016/j.devcel.2012.08.003
- Hislop, J. N., Henry, A. G., and Von Zastrow, M. (2011). Ubiquitination in the first cytoplasmic loop of μ -opioid receptors reveals a hierarchical mechanism of lysosomal down-regulation. *J. Biol. Chem.* 286, 40193–40204. doi: 10.1074/jbc.M111.288555
- Hong, M. H., Xu, C., Wang, Y. J., Ji, J. L., Tao, Y. M., Xu, X. J., et al. (2009). Role of Src in ligand-specific regulation of delta-opioid

- receptor desensitization and internalization. *J. Neurochem.* 108, 102–114. doi: 10.1111/j.1471-4159.2008.05740.x
- Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258, 577–580. doi: 10.1038/258577a0
- Huidobro, F., Huidobro-Toro, J. P., and Leong Way, E. (1976). Studies on tolerance development to single doses of morphine in mice. *J. Pharmacol. Exp. Ther.* 198, 318–329.
- Hull, L. C., Llorente, J., Gabra, B. H., Smith, F. L., Kelly, E., Bailey, C., et al. (2010). The effect of protein kinase C and G protein-coupled receptor kinase inhibition on tolerance induced by mu-opioid agonists of different efficacy. *J. Pharmacol. Exp. Ther.* 332, 1127–1135. doi: 10.1124/jpet.109.161455
- Illing, S., Mann, A., and Schulz, S. (2014). Heterologous regulation of agonist-independent mu-opioid receptor phosphorylation by protein kinase C. *Br. J. Pharmacol.* 171, 1330–1340. doi: 10.1111/bph.12546
- Javed, R. R., Dewey, W. L., Smith, P. A., and Smith, F. L. (2004). PKC and PKA inhibitors reverse tolerance to morphine-induced hypothermia and supraspinal analgesia in mice. *Eur. J. Pharmacol.* 492, 149–157. doi: 10.1016/j.ejphar.2004.03.061
- Johnson, E. A., Oldfield, S., Braksator, E., Gonzalez-Cuello, A., Couch, D., Hall, K. J., et al. (2006). Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Mol. Pharmacol.* 70, 676–685. doi: 10.1124/mol.106.022376
- Just, S., Illing, S., Trester-Zedlitz, M., Lau, E. K., Kotowski, S. J., Miess, E., et al. (2013). Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol. Pharmacol.* 83, 633–639. doi: 10.1124/mol.112.082875
- Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., et al. (1982). Cloning and sequence analysis of cDNA for porcine beta-neo-endorphin/dynorphin precursor. *Nature* 298, 245–249. doi: 10.1038/298245a0
- Keith, D. E., Anton, B., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., et al. (1998). mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism *in vitro* and in the mammalian brain. *Mol. Pharmacol.* 53, 377–384.
- Kelly, E. (2013). Ligand bias at the mu-opioid receptor. *Biochem. Soc. Trans.* 41, 218–224. doi: 10.1042/BST20120331
- Kenakin, T. (2011). Functional selectivity and biased receptor signaling. *J. Pharmacol. Exp. Ther.* 336, 296–302. doi: 10.1124/jpet.110.173948
- Kieffer, B. L. (1999). Opioids: first lessons from knockout mice. *Trends Pharmacol. Sci.* 20, 19–26. doi: 10.1016/S0165-6147(98)01279-6
- Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C., and Hirth, C. G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc. Natl. Acad. Sci. U.S.A.* 89, 12048–12052. doi: 10.1073/pnas.89.24.12048
- Kim, J. A., Bartlett, S., He, L., Nielsen, C. K., Chang, A. M., Kharazia, V., et al. (2008). Morphine-induced receptor endocytosis in a novel knockin mouse reduces tolerance and dependence. *Curr. Biol.* 18, 129–135. doi: 10.1016/j.cub.2007.12.057
- Knapp, R. J., Malatyńska, E., Collins, N., Fang, L., Wang, J. Y., Hruby, V. J., et al. (1995). Molecular biology and pharmacology of cloned opioid receptors. *FASEB J.* 9, 516–525.
- Koch, T., Schulz, S., Pfeiffer, M., Klutzny, M., Schroder, H., Kahl, E., et al. (2001). C-terminal splice variants of the mouse mu-opioid receptor differ in morphine-induced internalization and receptor resensitization. *J. Biol. Chem.* 276, 31408–31414. doi: 10.1074/jbc.M100305200
- Koch, T., Widera, A., Bartzsch, K., Schulz, S., Brandenburg, L. O., Wundrack, N., et al. (2005). Receptor endocytosis counteracts the development of opioid tolerance. *Mol. Pharmacol.* 67, 280–287. doi: 10.1124/mol.104.004994
- Konig, C., Gavrilova-Ruch, O., Von Banchet, G. S., Bauer, R., Grun, M., Hirsch, E., et al. (2010). Modulation of mu opioid receptor desensitization in peripheral sensory neurons by phosphoinositide 3-kinase gamma. *Neuroscience* 169, 449–454. doi: 10.1016/j.neuroscience.2010.04.068
- Kouhen, O. M., Wang, G., Solberg, J., Erickson, L. J., Law, P. Y., and Loh, H. H. (2000). Hierarchical phosphorylation of delta-opioid receptor regulates agonist-induced receptor desensitization and internalization. *J. Biol. Chem.* 275, 36659–36664. doi: 10.1074/jbc.M006788200
- Kovoor, A., Celver, J., Abdryashitov, R. I., Chavkin, C., and Gurevich, V. V. (1999). Targeted construction of phosphorylation-independent beta-arrestin mutants with constitutive activity in cells. *J. Biol. Chem.* 274, 6831–6834. doi: 10.1074/jbc.274.11.6831
- Kramer, H. K., Andria, M. L., Esposito, D. H., and Simon, E. J. (2000a). Tyrosine phosphorylation of the delta-opioid receptor. Evidence for its role in mitogen-activated protein kinase activation and receptor internalization*. *Biochem. Pharmacol.* 60, 781–792. doi: 10.1016/S0006-2952(00)00400-7
- Kramer, H. K., Andria, M. L., Kushner, S. A., Esposito, D. H., Hiller, J. M., and Simon, E. J. (2000b). Mutation of tyrosine 318 (Y318F) in the delta-opioid receptor attenuates tyrosine phosphorylation, agonist-dependent receptor internalization, and mitogen-activated protein kinase activation. *Brain Res. Mol. Brain Res.* 79, 55–66. doi: 10.1016/S0169-328X(00)00097-8
- Lamb, K., Tidgewell, K., Simpson, D. S., Bohn, L. M., and Prisinzano, T. E. (2012). Antinociceptive effects of herkinorin, a MOP receptor agonist derived from salvinorin A in the formalin test in rats: new concepts in mu opioid receptor pharmacology: from a symposium on new concepts in mu-opioid pharmacology. *Drug Alcohol Depend.* 121, 181–188. doi: 10.1016/j.drugalcdep.2011.10.026
- Lau, E. K., Trester-Zedlitz, M., Trinidad, J. C., Kotowski, S. J., Krutchinsky, A. N., Burlingame, A. L., et al. (2011). Quantitative encoding of the effect of a partial agonist on individual opioid receptors by multisite phosphorylation and threshold detection. *Sci. Signal.* 4, ra52. doi: 10.1126/scisignal.2001748
- Law, P. Y., Hom, D. S., and Loh, H. H. (1982). Loss of opiate receptor activity in neuroblastoma X glioma NG108-15 hybrid cells after chronic opiate treatment. A multiple-step process. *Mol. Pharmacol.* 22, 1–4.
- Law, P. Y., Wong, Y. H., and Loh, H. H. (2000). Molecular mechanisms and regulation of opioid receptor signaling. *Annu. Rev. Pharmacol. Toxicol.* 40, 389–430. doi: 10.1146/annurev.pharmtox.40.1.389
- Lecoq, I., Marie, N., Jauzac, P., and Allouche, S. (2004). Different regulation of human delta-opioid receptors by SNC-80 [(+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide] and endogenous enkephalins. *J. Pharmacol. Exp. Ther.* 310, 666–677. doi: 10.1124/jpet.103.063958
- Levitt, E. S., and Williams, J. T. (2012). Morphine desensitization and cellular tolerance are distinguished in rat locus ceruleus neurons. *Mol. Pharmacol.* 82, 983–992. doi: 10.1124/mol.112.081547
- Li, J. G., Luo, L. Y., Krupnick, J. G., Benovic, J. L., and Liu-Chen, L. Y. (1999). U50,488H-induced internalization of the human kappa opioid receptor involves a beta-arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation. *J. Biol. Chem.* 274, 12087–12094. doi: 10.1074/jbc.274.17.12087
- Li, J. G., Zhang, F., Jin, X. L., and Liu-Chen, L. Y. (2003). Differential regulation of the human kappa opioid receptor by agonists: etorphine and levorphanol reduced dynorphin A- and U50,488H-induced internalization and phosphorylation. *J. Pharmacol. Exp. Ther.* 305, 531–540. doi: 10.1124/jpet.102.045559
- Li, J., Li, J.-G., Chen, C., Zhang, F., and Liu-Chen, L.-Y. (2002). Molecular basis of differences in (-)(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide-induced desensitization and phosphorylation between human and rat kappa-opioid receptors expressed in Chinese hamster ovary cells. *Mol. Pharmacol.* 61, 73–84. doi: 10.1124/mol.61.1.73
- Ling, K., Ma, L., and Pei, G. (1998). Differential efficacies of kappa agonists to induce homologous desensitization of human kappa opioid receptor. *Neurosci. Lett.* 240, 25–28. doi: 10.1016/S0304-3940(97)00921-X
- Liu, J. G., Liao, X. P., Gong, Z. H., and Qin, B. Y. (1999a). The difference between methadone and morphine in regulation of delta-opioid receptors underlies the antagonistic effect of methadone on morphine-mediated cellular actions. *Eur. J. Pharmacol.* 373, 233–239. doi: 10.1016/S0014-2999(99)00270-8
- Liu, J. G., Liao, X. P., Gong, Z. H., and Qin, B. Y. (1999b). Methadone-induced desensitization of the delta-opioid receptor is mediated by uncoupling of receptor from G protein. *Eur. J. Pharmacol.* 374, 301–308. doi: 10.1016/S0014-2999(99)00322-2
- Liu, J. G., and Prather, P. L. (2001). Chronic exposure to mu-opioid agonists produces constitutive activation of mu-opioid receptors in direct proportion to the efficacy of the agonist used for pretreatment. *Mol. Pharmacol.* 60, 53–62.
- Llorente, J., Lowe, J. D., Sanderson, H. S., Tsisanova, E., Kelly, E., Henderson, G., et al. (2012). mu-Opioid receptor desensitization: homologous or heterologous? *Eur. J. Neurosci.* 36, 3636–3642. doi: 10.1111/ejn.12003
- Lowe, J. D., Celver, J. P., Gurevich, V. V., and Chavkin, C. (2002). mu-Opioid receptors desensitize less rapidly than delta-opioid receptors due to

- less efficient activation of arrestin. *J. Biol. Chem.* 277, 15729–15735. doi: 10.1074/jbc.M200612200
- Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., et al. (2012). Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature* 485, 321–326. doi: 10.1038/nature10954
- Mann, A., Illing, S., Miess, E., and Schulz, S. (2014). Different mechanisms of homologous and heterologous μ -opioid receptor phosphorylation. *Br. J. Pharmacol.* doi: 10.1111/bph.12627. [Epub ahead of print].
- Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 18, 22–29. doi: 10.1016/0166-2236(95)93946-U
- Marchese, A., and Trejo, J. (2013). Ubiquitin-dependent regulation of G protein-coupled receptor trafficking and signaling. *Cell. Signal.* 25, 707–716. doi: 10.1016/j.cellsig.2012.11.024
- Marie, N., Aguila, B., Hasbi, A., Davis, A., Jauzac, P., and Allouche, S. (2008). Different kinases desensitize the human delta-opioid receptor (hDOP-R) in the neuroblastoma cell line SK-N-BE upon peptidic and alkaloid agonists. *Cell. Signal.* 20, 1209–1220. doi: 10.1016/j.cellsig.2008.02.010
- Marie, N., Landemore, G., Debout, C., Jauzac, P., and Allouche, S. (2003a). Pharmacological characterization of AR-M1000390 at human delta opioid receptors. *Life Sci.* 73, 1691–1704. doi: 10.1016/S0024-3205(03)00489-2
- Marie, N., Lecoq, I., Jauzac, P., and Allouche, S. (2003b). Differential sorting of human delta-opioid receptors after internalization by peptide and alkaloid agonists. *J. Biol. Chem.* 278, 22795–22804. doi: 10.1074/jbc.M300084200
- Massotte, D. (2014). *In vivo* opioid receptor heteromerization: where do we stand? *Br. J. Pharmacol.* doi: 10.1111/bph.12702. [Epub ahead of print].
- McLaughlin, J. P., Myers, L. C., Zarek, P. E., Caron, M. G., Lefkowitz, R. J., Czyzyk, T. A., et al. (2004). Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. *J. Biol. Chem.* 279, 1810–1818. doi: 10.1074/jbc.M305796200
- McLaughlin, J. P., Xu, M., Mackie, K., and Chavkin, C. (2003). Phosphorylation of a carboxyl-terminal serine within the kappa-opioid receptor produces desensitization and internalization. *J. Biol. Chem.* 278, 34631–34640. doi: 10.1074/jbc.M304022200
- Mcpherson, J., Rivero, G., Baptist, M., Llorente, J., Al-Sabah, S., Krasel, C., et al. (2010). μ -opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol. Pharmacol.* 78, 756–766. doi: 10.1124/mol.110.066613
- Megaritis, G., Merkouris, M., and Georgoussi, Z. (2000). Functional domains of delta- and mu-opioid receptors responsible for adenylyl cyclase inhibition. *Receptors Channels* 7, 199–212.
- Melief, E. J., Miyatake, M., Bruchas, M. R., and Chavkin, C. (2010). Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11608–11613. doi: 10.1073/pnas.1000751107
- Meng, F., Xie, G. X., Thompson, R. C., Mansour, A., Goldstein, A., Watson, S. J., et al. (1993). Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc. Natl. Acad. Sci. U.S.A.* 90, 9954–9958. doi: 10.1073/pnas.90.21.9954
- Metzger, T. G., and Ferguson, D. M. (1995). On the role of extracellular loops of opioid receptors in conferring ligand selectivity. *FEBS Lett.* 375, 1–4. doi: 10.1016/0014-5793(95)01185-H
- Meunier, J. C., Mollereau, C., Toll, L., Staudeau, C., Moisand, C., Alvinerie, P., et al. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 377, 532–535. doi: 10.1038/377532a0
- Mittal, N., Tan, M., Egbuta, O., Desai, N., Crawford, C., Xie, C. W., et al. (2012). Evidence that behavioral phenotypes of morphine in beta-arr2 $^{-/-}$ mice are due to the unmasking of JNK signaling. *Neuropsychopharmacology* 37, 1953–1962. doi: 10.1038/npp.2012.42
- Mohammed, W., Alhaddad, H., Marie, N., Tardy, F., Lamballais, F., Risede, P., et al. (2013). Comparison of tolerance to morphine-induced respiratory and analgesic effects in mice. *Toxicol. Lett.* 217, 251–259. doi: 10.1016/j.toxlet.2012.12.021
- Molinari, P., Vezzi, V., Sbraccia, M., Grò, C., Riitano, D., Ambrosio, C., et al. (2010). Morphine-like opiates selectively antagonize receptor-arrestin interactions. *J. Biol. Chem.* 285, 12522–12535. doi: 10.1074/jbc.M109.059410
- Mollereau, C., Parmentier, M., Mailleux, P., Butour, J. L., Moisand, C., Chalon, P., et al. (1994). ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett.* 341, 33–38. doi: 10.1016/0014-5793(94)80235-1
- Morse, M., Sun, H., Tran, E., Levenson, R., and Fang, Y. (2013). Label-free integrative pharmacology on-target of opioid ligands at the opioid receptor family. *BMC Pharmacol. Toxicol.* 14:17. doi: 10.1186/2050-6511-14-17
- Moulédous, L., Froment, C., Dauvillier, S., Burlet-Schiltz, O., Zajac, J.-M., and Mollereau, C. (2012). GRK2 protein-mediated transphosphorylation contributes to loss of function of μ -opioid receptors induced by neuropeptide FF (NPFF2) receptors. *J. Biol. Chem.* 287, 12736–12749. doi: 10.1074/jbc.M111.314617
- Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C., Cohen, S. N., et al. (1979). Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 278, 423–427. doi: 10.1038/278423a0
- Namir, N., Polastron, J., Allouche, S., Hasbi, A., and Jauzac, P. (1997). The delta-opioid receptor in SK-N-BE human neuroblastoma cell line undergoes heterologous desensitization. *J. Neurochem.* 68, 1764–1772. doi: 10.1046/j.1471-4159.1997.68041764.x
- Navratilova, E., Eaton, M. C., Stropova, D., Varga, E. V., Vanderah, T. W., Roeske, W. R., et al. (2005). Morphine promotes phosphorylation of the human delta-opioid receptor at serine 363. *Eur. J. Pharmacol.* 519, 212–214. doi: 10.1016/j.ejphar.2005.07.024
- Navratilova, E., Waite, S., Stropova, D., Eaton, M. C., Alves, I. D., Hruby, V. J., et al. (2007). Quantitative evaluation of human delta opioid receptor desensitization using the operational model of drug action. *Mol. Pharmacol.* 71, 1416–1426. doi: 10.1124/mol.106.030023
- Noble, F., and Cox, B. M. (1996). Differential desensitization of mu- and delta-opioid receptors in selected neural pathways following chronic morphine treatment. *Br. J. Pharmacol.* 117, 161–169. doi: 10.1111/j.1476-5381.1996.tb15169.x
- Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., et al. (2011). Distinct phosphorylation sites on the beta(2)-adrenergic receptor establish a barcode that encodes differential functions of beta-arrestin. *Sci. Signal.* 4, ra51. doi: 10.1126/scisignal.2001707
- Noda, M., Teranishi, Y., Takahashi, H., Toyosato, M., Notake, M., Nakanishi, S., et al. (1982). Isolation and structural organization of the human preproenkephalin gene. *Nature* 297, 431–434. doi: 10.1038/297431a0
- Nowocyn, M., Marie, N., Coulbault, L., Hervault, M., Davis, A., Hanouz, J. L., et al. (2013). Remifentanil produces cross-desensitization and tolerance with morphine on the mu-opioid receptor. *Neuropharmacology* 73, 368–379. doi: 10.1016/j.neuropharm.2013.06.010
- Ong, E. W., and Cahill, C. M. (2014). Molecular perspectives for mu/delta opioid receptor heteromers as distinct, functional receptors. *Cells* 3, 152–179. doi: 10.3390/cells3010152
- Pan, L., Xu, J., Yu, R., Xu, M. M., Pan, Y. X., and Pasternak, G. W. (2005). Identification and characterization of six new alternatively spliced variants of the human mu opioid receptor gene, Oprm. *Neuroscience* 133, 209–220. doi: 10.1016/j.neuroscience.2004.12.033
- Pasternak, D. A., Pan, L., Xu, J., Yu, R., Xu, M. M., Pasternak, G. W., et al. (2004). Identification of three new alternatively spliced variants of the rat mu opioid receptor gene: dissociation of affinity and efficacy. *J. Neurochem.* 91, 881–890. doi: 10.1111/j.1471-4159.2004.02767.x
- Pasternak, G. W., and Pan, Y. X. (2013). Mu opioids and their receptors: evolution of a concept. *Pharmacol. Rev.* 65, 1257–1317. doi: 10.1124/pr.112.007138
- Patierno, S., Anselmi, L., Jaramillo, I., Scott, D., Garcia, R., and Sternini, C. (2011). Morphine induces mu opioid receptor endocytosis in guinea pig enteric neurons following prolonged receptor activation. *Gastroenterology* 140, 618–626. doi: 10.1053/j.gastro.2010.11.005
- Pei, G., Kieffer, B. L., Lefkowitz, R. J., and Freedman, N. J. (1995). Agonist-dependent phosphorylation of the mouse delta-opioid receptor: involvement of G protein-coupled receptor kinases but not protein kinase C. *Mol. Pharmacol.* 48, 173–177.
- Pert, C. B., and Snyder, S. H. (1973). Opiate receptor: demonstration in nervous tissue. *Science* 179, 1011–1014. doi: 10.1126/science.179.4077.1011
- Petaja-Repo, U. E., Hogue, M., Laperriere, A., Walker, P., and Bouvier, M. (2000). Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. *J. Biol. Chem.* 275, 13727–13736. doi: 10.1074/jbc.275.18.13727
- Petaja-Repo, U. E., Hogue, M., Leskela, T. T., Markkanen, P. M., Tuusa, J. T., and Bouvier, M. (2006). Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human delta opioid receptor. *J. Biol. Chem.* 281, 15780–15789. doi: 10.1074/jbc.M602267200

- Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002). Seven-transmembrane receptors. *Nat. Rev. Mol. Cell. Biol.* 3, 639–650. doi: 10.1038/nrm908
- Polastron, J., Meunier, J. C., and Jauzac, P. (1994). Chronic morphine induces tolerance and desensitization of mu-opioid receptor but not down-regulation in rabbit. *Eur. J. Pharmacol.* 266, 139–146. doi: 10.1016/0922-4106(94)90103-1
- Portoghese, P. S. (1965). A new concept on the mode of interaction of narcotic analgesics with receptors. *J. Med. Chem.* 8, 609–616. doi: 10.1021/jm00329a013
- Portoghese, P. S., Sultana, M., and Takemori, A. E. (1990). Design of peptidomimetic delta opioid receptor antagonists using the message-address concept. *J. Med. Chem.* 33, 1714–1720. doi: 10.1021/jm00168a028
- Pradhan, A. A., Becker, J. A., Scherrer, G., Tryoen-Toth, P., Filliol, D., Matifas, A., et al. (2009). *In vivo* delta opioid receptor internalization controls behavioral effects of agonists. *PLoS ONE* 4:e5425. doi: 10.1371/journal.pone.0005425
- Pradhan, A. A., Smith, M. L., Kieffer, B. L., and Evans, C. J. (2012). Ligand-directed signalling within the opioid receptor family. *Br. J. Pharmacol.* 167, 960–969. doi: 10.1111/j.1476-5381.2012.20275.x
- Pradhan, A. A., Walwyn, W., Nozaki, C., Filliol, D., Erbs, E., Matifas, A., et al. (2010). Ligand-directed trafficking of the delta-opioid receptor *in vivo*: two paths toward analgesic tolerance. *J. Neurosci.* 30, 16459–16468. doi: 10.1523/JNEUROSCI.3748-10.2010
- Qiu, Y., Law, P. Y., and Loh, H. H. (2003). Mu-opioid receptor desensitization: role of receptor phosphorylation, internalization, and representation. *J. Biol. Chem.* 278, 36733–36739. doi: 10.1074/jbc.M305857200
- Qiu, Y., Loh, H. H., and Law, P. Y. (2007). Phosphorylation of the delta-opioid receptor regulates its beta-arrestins selectivity and subsequent receptor internalization and adenylyl cyclase desensitization. *J. Biol. Chem.* 282, 22315–22323. doi: 10.1074/jbc.M611258200
- Quillinan, N., Lau, E. K., Virk, M., Von Zastrow, M., and Williams, J. T. (2011). Recovery from mu-opioid receptor desensitization after chronic treatment with morphine and methadone. *J. Neurosci.* 31, 4434–4443. doi: 10.1523/JNEUROSCI.4874-10.2011
- Quirion, R., Zajac, J. M., Morgat, J. L., and Roques, B. P. (1983). Autoradiographic distribution of mu and delta opiate receptors in rat brain using highly selective ligands. *Life Sci.* 33 (Suppl. 1), 227–230. doi: 10.1016/0024-3205(83)90484-8
- Raehal, K. M., Schmid, C. L., Groer, C. E., and Bohn, L. M. (2011). Functional selectivity at the mu-opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol. Rev.* 63, 1001–1019. doi: 10.1124/pr.111.004598
- Reinscheid, R. K., Nothacker, H. P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., et al. (1995). Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 270, 792–794. doi: 10.1126/science.270.5237.792
- Rivero, G., Llorente, J., Mcpherson, J., Cooke, A., Mundell, S. J., Mcardle, C. A., et al. (2012). Endomorphin-2: a biased agonist at the mu-opioid receptor. *Mol. Pharmacol.* 82, 178–188. doi: 10.1124/mol.112.078659
- Rostami, A., Rabbani, M., and Mir-Mohammad-Sadeghi, M. (2010). The role of N53Q mutation on the rat mu-opioid receptor function. *J. Biomol. Tech.* 21, 92–96.
- Schulz, S., Mayer, D., Pfeiffer, M., Stumm, R., Koch, T., and Hollt, V. (2004). Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. *EMBO J.* 23, 3282–3289. doi: 10.1038/sj.emboj.7600334
- Simon, E. J., Hiller, J. M., and Edelman, I. (1973). Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate. *Proc. Natl. Acad. Sci. U.S.A.* 70, 1947–1949. doi: 10.1073/pnas.70.7.1947
- Soignier, R. D., Vaccarino, A. L., Fanti, K. A., Wilson, A. M., and Zadina, J. E. (2004). Analgesic tolerance and cross-tolerance to i.c.v. endomorphin-1, endomorphin-2, and morphine in mice. *Neurosci. Lett.* 366, 211–214. doi: 10.1016/j.neulet.2004.05.046
- Song, S. L., and Chueh, S. H. (1999). Phosphorylation promotes the desensitization of the opioid-induced Ca²⁺ increase in NG108-15 cells. *Brain Res.* 818, 316–325. doi: 10.1016/S0006-8993(98)01216-5
- Stafford, K., Gomes, A. B., Shen, J., and Yoburn, B. C. (2001). mu-Opioid receptor downregulation contributes to opioid tolerance *in vivo*. *Pharmacol. Biochem. Behav.* 69, 233–237. doi: 10.1016/S0091-3057(01)00525-1
- Tanowitz, M., and Von Zastrow, M. (2003). A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J. Biol. Chem.* 278, 45978–45986. doi: 10.1074/jbc.M304504200
- Tejeda, H. A., Shippenberg, T. S., and Henriksson, R. (2012). The dynorphin/kappa-opioid receptor system and its role in psychiatric disorders. *Cell. Mol. Life Sci.* 69, 857–896. doi: 10.1007/s00018-011-0844-x
- Terenius, L. (1973). Stereospecific interaction between narcotic analgesics and a synaptic plasm a membrane fraction of rat cerebral cortex. *Acta Pharmacol. Toxicol. (Copenh)* 32, 317–320. doi: 10.1111/j.1600-0773.1973.tb01477.x
- Terman, G. W., Jin, W., Cheong, Y. P., Lowe, J., Caron, M. G., Lefkowitz, R. J., et al. (2004). G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. *Br. J. Pharmacol.* 141, 55–64. doi: 10.1038/sj.bjp.0705595
- Thompson, R. C., Mansour, A., Akil, H., and Watson, S. J. (1993). Cloning and pharmacological characterization of a rat mu opioid receptor. *Neuron* 11, 903–913. doi: 10.1016/0896-6273(93)90120-G
- Tsao, P. I., and Von Zastrow, M. (2000). Type-specific sorting of G protein-coupled receptors after endocytosis. *J. Biol. Chem.* 275, 11130–11140. doi: 10.1074/jbc.275.15.11130
- Ueda, H., and Ueda, M. (2009). Mechanisms underlying morphine analgesic tolerance and dependence. *Front. Biosci. (Landmark Ed.)* 14, 5260–5272. doi: 10.2741/3596
- Violin, J. D., Crombie, A. L., Soergel, D. G., and Lark, M. W. (2014). Biased ligands at G-protein-coupled receptors: promise and progress. *Trends Pharmacol. Sci.* 35, 308–316. doi: 10.1016/j.tips.2014.04.007
- Walwyn, W., Evans, C. J., and Hales, T. G. (2007). Beta-arrestin2 and c-Src regulate the constitutive activity and recycling of mu opioid receptors in dorsal root ganglion neurons. *J. Neurosci.* 27, 5092–5104. doi: 10.1523/JNEUROSCI.1157-07.2007
- Wang, H. L. (2000). A cluster of Ser/Thr residues at the C-terminus of mu-opioid receptor is required for G protein-coupled receptor kinase 2-mediated desensitization. *Neuropharmacology* 39, 353–363. doi: 10.1016/S0028-3908(99)00174-4
- Whistler, J. L., Chuang, H. H., Chu, P., Jan, L. Y., and Von Zastrow, M. (1999). Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 23, 737–746. doi: 10.1016/S0896-6273(01)80032-5
- Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., et al. (2002). Modulation of postendocytic sorting of G protein-coupled receptors. *Science* 297, 615–620. doi: 10.1126/science.1073308
- Whistler, J. L., and Von Zastrow, M. (1998). Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9914–9919. doi: 10.1073/pnas.95.17.9914
- White, J. M., and Irvine, R. J. (1999). Mechanisms of fatal opioid overdose. *Addiction* 94, 961–972. doi: 10.1046/j.1360-0443.1999.9479612.x
- Willets, J., and Kelly, E. (2001). Desensitization of endogenously expressed delta-opioid receptors: no evidence for involvement of G protein-coupled receptor kinase 2. *Eur. J. Pharmacol.* 431, 133–141. doi: 10.1016/S0014-2999(01)01360-7
- Williams, J. T. (2014). Desensitization of functional micro-opioid receptors increases agonist off-rate. *Mol. Pharmacol.* 86, 52–61. doi: 10.1124/mol.114.092098
- Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., Von Zastrow, M., Schulz, S., et al. (2013). Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65, 223–254. doi: 10.1124/pr.112.005942
- Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G. W., Vardy, E., et al. (2012). Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* 485, 327–332. doi: 10.1038/nature10939
- Xiang, B., Yu, G. H., Guo, J., Chen, L., Hu, W., Pei, G., et al. (2001). Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization. *J. Biol. Chem.* 276, 4709–4716. doi: 10.1074/jbc.M006187200
- Xie, W. Y., He, Y., Yang, Y. R., Li, Y. F., Kang, K., Xing, B. M., et al. (2009). Disruption of Cdk5-associated phosphorylation of residue threonine-161 of the delta-opioid receptor: impaired receptor function and attenuated morphine antinociceptive tolerance. *J. Neurosci.* 29, 3551–3564. doi: 10.1523/JNEUROSCI.0415-09.2009
- Xu, W., Chen, C., Li, J. G., Dimattio, K., Wang, Y., Unterwald, E., et al. (2013). PKA and ERK1/2 are involved in dopamine D(1) receptor-induced heterologous

- desensitization of the delta opioid receptor. *Life Sci.* 92, 1101–1109. doi: 10.1016/j.lfs.2013.04.006
- Yan, Y. H., Wang, Y., Zhao, L. X., Jiang, S., Loh, H. H., Law, P. Y., et al. (2014). Role of FK506 binding protein 12 in morphine-induced mu-opioid receptor internalization and desensitization. *Neurosci. Lett.* 566, 231–235. doi: 10.1016/j.neulet.2014.02.059
- Yang, L., Seifert, A., Wu, D., Wang, X., Rankovic, V., Schroder, H., et al. (2010). Role of phospholipase D2/phosphatidic acid signal transduction in micro- and delta-opioid receptor endocytosis. *Mol. Pharmacol.* 78, 105–113. doi: 10.1124/mol.109.063107
- Yoon, S. H., Jin, W., Spencer, R. J., Loh, H. H., and Thayer, S. A. (1998). Desensitization of delta-opioid-induced mobilization of Ca²⁺ stores in NG108-15 cells. *Brain Res.* 802, 9–18. doi: 10.1016/S0006-8993(98)00531-9
- Yu, Y., Zhang, L., Yin, X., Sun, H., Uhl, G. R., and Wang, J. B. (1997). Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J. Biol. Chem.* 272, 28869–28874. doi: 10.1074/jbc.272.46.28869
- Zhang, J., Ferguson, S. S., Barak, L. S., Bodduluri, S. R., Laporte, S. A., Law, P. Y., et al. (1998). Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7157–7162. doi: 10.1073/pnas.95.12.7157
- Zhang, L., Loh, H. H., and Law, P. Y. (2013). A novel noncanonical signaling pathway for the mu-opioid receptor. *Mol. Pharmacol.* 84, 844–853. doi: 10.1124/mol.113.088278
- Zhang, L., Zhao, H., Qiu, Y., Loh, H. H., and Law, P.-Y. (2009). Src phosphorylation of micro-receptor is responsible for the receptor switching from an inhibitory to a stimulatory signal. *J. Biol. Chem.* 284, 1990–2000. doi: 10.1074/jbc.M807971200
- Zhang, X., Wang, F., Chen, X., Chen, Y., and Ma, L. (2008). Post-endocytic fates of delta-opioid receptor are regulated by GRK2-mediated receptor phosphorylation and distinct beta-arrestin isoforms. *J. Neurochem.* 106, 781–792. doi: 10.1111/j.1471-4159.2008.05431.x
- Zhang, X., Wang, F., Chen, X., Li, J., Xiang, B., Zhang, Y.-Q., et al. (2005). Beta-arrestin1 and beta-arrestin2 are differentially required for phosphorylation-dependent and -independent internalization of delta-opioid receptors. *J. Neurochem.* 95, 169–178. doi: 10.1111/j.1471-4159.2005.03352.x
- Zheng, H., Chu, J., Zhang, Y., Loh, H. H., and Law, P. Y. (2011). Modulating micro-opioid receptor phosphorylation switches agonist-dependent signaling as reflected in PKCepsilon activation and dendritic spine stability. *J. Biol. Chem.* 286, 12724–12733. doi: 10.1074/jbc.M110.177089
- Zhu, J., Luo, L. Y., Mao, G. F., Ashby, B., and Liu-Chen, L. Y. (1998). Agonist-induced desensitization and down-regulation of the human kappa opioid receptor expressed in Chinese hamster ovary cells. *J. Pharmacol. Exp. Ther.* 285, 28–36.

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It's MORe exciting than mu: crosstalk between mu opioid receptors and glutamatergic transmission in the mesolimbic dopamine system

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Opioids selective for the G protein-coupled mu opioid receptor (MOR) produce potent analgesia and euphoria. Heroin, a synthetic opioid, is considered one of the most addictive substances, and the recent exponential rise in opioid addiction and overdose deaths has made treatment development a national public health priority. Existing medications (methadone, buprenorphine, and naltrexone), when combined with psychosocial therapies, have proven efficacy in reducing aspects of opioid addiction. Unfortunately, these medications have critical limitations including those associated with opioid agonist therapies (e.g., sustained physiological dependence and opioid withdrawal leading to high relapse rates upon discontinuation), non-adherence to daily dosing, and non-renewal of monthly injection with extended-release naltrexone. Furthermore, current medications fail to ameliorate key aspects of addiction such as powerful conditioned associations that trigger relapse (e.g., cues, stress, the drug itself). Thus, there is a need for developing novel treatments that target neural processes corrupted with chronic opioid use. This requires a basic understanding of molecular and cellular mechanisms underlying effects of opioids on synaptic transmission and plasticity within reward-related neural circuits. The focus of this review is to discuss how crosstalk between MOR-associated G protein signaling and glutamatergic neurotransmission leads to immediate and long-term effects on emotional states (e.g., euphoria, depression) and motivated behavior (e.g., drug-seeking, relapse). Our goal is to integrate findings on how opioids modulate synaptic release of glutamate and postsynaptic transmission via α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and N-methyl-D-aspartate receptors in the nucleus accumbens and ventral tegmental area with the clinical (neurobehavioral) progression of opioid dependence, as well as to identify gaps in knowledge that can be addressed in future studies.

Keywords: morphine, heroin, AMPA, NMDA, GluR1, opioid withdrawal syndrome, plasticity

INTRODUCTION

Opioids comprise a class of endogenous, naturally occurring and synthetic compounds that bind to and activate one of three known opioid receptors: mu, delta, and kappa (MOR, DOR, KOR, respectively). All opioids possess analgesic properties, which humans have taken advantage of for thousands of years. They also have profound effects on physiology and mood that depend on the specific opioid receptor and site of action in the brain. Opiates, a subclass of opioids that are natural derivatives of the opium plant, *papaver somniferum*, include morphine and codeine, which are the two major metabolites of heroin. These compounds primarily activate MORs to produce euphoria that can motivate repeated self-administration, produce tolerance, dependence, and ultimately opioid addiction. One percent of all Americans meet criteria for having an opioid use disorder (OUD); heroin use has doubled since 2007, and 2% of all Americans age 12 and older report misuse of a prescription opioid analgesic within the past 30 days (NSDUH, 2013). In 2008, there were 15,000 accidental overdose deaths related to prescription opioid use alone (Center for Disease Control) and opioid analgesics

are second only to marijuana as the first illicit drug reported taken by 1.9 million youth and older adult Americans (NSDUH, 2013). The partial MOR agonist buprenorphine combined with the diversion-preventing opioid receptor antagonist naloxone has been partially successful in engaging youth and adults with OUD into abstinence-focused treatment (Fudala et al., 2003; Mattick et al., 2008; Woody et al., 2008; Weiss et al., 2011). However, controlled data on longer-term outcomes is lacking and patients taking agonist therapies (e.g. the long-lasting, full MOR agonist methadone and buprenorphine) have high rates of relapse (>75%) upon medication withdrawal (Woody et al., 2008; Weiss et al., 2011).

In fact, the treatment course of OUD is primarily challenged by the experience of the opioid withdrawal syndrome (OWS), which is characterized by both a typical physical syndrome occurring acutely (24–48 h post-withdrawal) and also by an affective/cognitive syndrome of dysphoria, anxiety, irritability, and preoccupation with cravings to use opioids (Kreek and Koob, 1998). These affective withdrawal symptoms occur acutely, but they frequently have a protracted course in humans (Dole et al.,

1966; Martin and Jasinski, 1969; see **Table 1**). Acute and protracted OWS is observed in controlled studies and in clinical practice to precipitate resumed opioid use; this is not only true for those first entering treatment and inexperienced in recovery practices but also true for those in longer-term recovery on agonist therapy who experience OWS during attempts to discontinue agonist therapy (Magura and Rosenblum, 2001; Woody et al., 2008; Weiss et al., 2011). Therefore, there is a great need to develop newer, medical therapies that are not pharmacologically based on opioids themselves to assist people with OUD in tolerating OWS without relapse to opioid use.

The affective/cognitive components of OWS may be the most important target for drug development, since non-opioid medications (e.g., adrenergic antagonists, anti-emetics, sedative-hypnotics) already exist and are widely applied to treat aspects of the physical syndrome. Research in rats demonstrates that naloxone-induced heightened acoustic startle, a pre-clinical proxy for anxiety sensitivity, persists up to 80 days following a single administration of morphine, whereas naloxone-induced conditioned place aversion is not seen after 20 days (Rothwell et al., 2012), suggesting that anxiety may be one of the most persistent protracted symptoms of the OWS. In addition, one clinical study in prescription opioid-dependent individuals suggests that when patients are blinded to buprenorphine taper schedules, their success rates in moving through opioid withdrawal to achieve sustained opioid abstinence may be improved (Sigmon et al., 2013). This could reflect a significant component of anticipatory anxiety about OWS under conditions where individuals are aware of forced reduction.

In order to most successfully treat affective/cognitive components of the OWS, it is imperative to understand how the normal brain processes rewarding and aversive stimuli to modulate behavior, and how opioids subsequently act to change behavior. Excitatory glutamatergic neurotransmission provides a basis for communication between neurons that enables behavior.

Table 1 | Symptoms of unmedicated abstinence in heroin-dependent men*.

Days of abstinence		
Day 3	Day 10	Day 30
• Severe anxiety	• Moderate anxiety	• Mild anxiety
• Moderate depression	• Subclinical depression	• Mild depressive symptoms
• Highest craving	• Moderate craving	• Milder craving
• Nasal discharge	• Nasal discharge	• Nasal discharge
• Mydriasis		
• Abdominal pain		
• Diarrhea		
• Vomiting		

*Based on results of two studies reported by Li et al. (2009) and Shi et al. (2009); anxiety measured by the Hamilton Anxiety Scale (HAM-A), depression by the Beck Depression Inventory (BDI).

Depending on the neural circuits activated, behavior can refer to anticipated stimuli, emotional response, learning (stimulus-response), or action – all of which become dysfunctional with addiction. The goal of this review is to present and synthesize the current state of knowledge on how activation of MORs modulates glutamatergic neurotransmission through α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors. We will focus on MOR–glutamate interactions within the mesolimbic dopamine system, a key neural substrate for the affective consequences of acute and chronic opioids. The basic pharmacology, neuroanatomical localization, and physiology of MORs have been well studied in *in vitro* systems, animal models, and clinical research, and there are numerous comprehensive reviews describing these findings (Law et al., 2000; Williams et al., 2001, 2013; Shalev et al., 2002; Waldhoer et al., 2004; Bailey and Connor, 2005; Pasternak, 2012).

MOR DISTRIBUTION AND ACTIONS

Mu opioid receptors are expressed throughout the brain. Several comprehensive studies have been published in which MOR binding sites are mapped (Mansour et al., 1988, 1994; Le Merrer et al., 2009). MORs are generally perisynaptic: they can be localized postsynaptically on dendrites and cell bodies where they regulate neuronal excitability and transduce receptor activation to downstream signal transduction pathways, and they can also be localized presynaptically on axon terminals where they inhibit neurotransmitter release via activation of K^+ conductance and/or inhibition of Ca^{2+} conductance (Williams et al., 2001). The cellular and neuroanatomical distribution of MORs is critical for understanding the neural circuits involved in the initiation of opiate action and subsequent plasticity with chronic drug use.

In the context of opiate dependence and withdrawal, several key neuroanatomical substrates have been identified, in particular the reciprocal connections within the limbic subcircuit of corticostriatal circuitry: GABAergic neurons of the nucleus accumbens (NAc), dopaminergic neurons of the ventral tegmental area (VTA), and glutamatergic neurons of the prefrontal cortex (PFC). Importantly, these regions contribute to acute opiate reward, dependence, tolerance, somatic and affective signs of withdrawal, and relapse (Wise, 1989; Stinus et al., 1990; Harris and Aston-Jones, 1994; Bonci and Williams, 1997; LaLumiere and Kalivas, 2008; Chartoff et al., 2009; Shen and Kalivas, 2013). Rats will self-administer opiates directly into the VTA (Bozarth and Wise, 1983; Devine and Wise, 1994), which contains dopaminergic cell bodies, and into the ventral striatum NAc (Olds, 1982), which receives dopaminergic input from the VTA. Acute morphine increases dopamine release in the NAc (Di Chiara and Imperato, 1988b; Johnson and North, 1992) by inhibiting GABAergic neurons in the VTA and rostromedial tegmental nucleus (RMTg) that synapse on dopaminergic neurons (Tepper et al., 1995; Jalabert et al., 2011). Morphine dependence – characterized by physical and psychological withdrawal signs – is mediated by several brain regions, with the locus coeruleus and periaqueductal gray (PAG) region most sensitive to naloxone-precipitated somatic withdrawal symptoms (Koob et al., 1992). The mesolimbic system is also important for morphine dependence, with a key role in affective

signs of withdrawal: microinjections of naloxone into the NAc causes conditioned place aversions (Koob et al., 1992), and administration of a dopamine D₂-like, but not a D₁-like, receptor agonist directly into the NAc attenuates somatic withdrawal signs (Harris and Aston-Jones, 1994). Also, dopamine release is decreased in the NAc during morphine withdrawal (Rossetti et al., 1992; Diana et al., 1995; Bonci and Williams, 1997), suggesting that the NAc may mediate certain aspects of morphine dependence. Other key brain regions important for opiate dependence include, but are not limited to, the amygdala, hippocampus, and bed nucleus of the stria terminalis (Mansour et al., 1995b; Gracy et al., 1997).

MOR ACTIVATION AND INTRACELLULAR SIGNALING

The physiological effects of morphine are absent in mice lacking MORs (Matthes et al., 1996; Le Merrer et al., 2009), providing strong support for the idea that MORs are necessary for the clinically relevant effects of opiates. MORs belong to the G protein-coupled receptor (GPCR) superfamily of seven transmembrane receptors and the rhodopsin receptor subfamily and are linked to pertussis toxin-sensitive inhibitory heterotrimeric guanosine triphosphate-binding proteins ($G_{\alpha i}/G_{\alpha o}$). Overall, MORs, DORs, and KORs are approximately 60% identical to each other (Chen et al., 1993).

Upon MOR activation, G protein α and $\beta\gamma$ subunits interact with downstream effector systems to inhibit adenylyl cyclase and voltage-gated Ca^{2+} channels and to stimulate G protein-activated inwardly rectifying K^+ channels (GIRks) and phospholipase C β (Childers, 1991; Waldhoer et al., 2004; Williams et al., 2013; see **Figures 1 and 2**, Naïve condition, for depiction of MOR-dependent signaling). In the presence of chronic morphine, a compensatory increase in adenylyl cyclase activity occurs and cAMP levels return to normal (see **Figures 1 and 2**, GABAergic neurons in Naïve, Acute, and Chronic conditions). When morphine is discontinued or withdrawal is pharmacologically precipitated, cAMP levels dramatically increase (see **Figures 1 and 2**, GABAergic neurons in Withdrawal condition; Nestler and Aghajanian, 1997; Williams et al., 2001). This phenomenon of early inhibition and late positive regulation of adenylyl cyclase by morphine has been demonstrated in several morphine-receptive brain regions (Duman et al., 1988; Nestler and Tallman, 1988; Terwilliger et al., 1991; Van Vliet et al., 1991; Self et al., 1995; Shaw-Lutchman et al., 2002). Upregulation of the cAMP pathway observed during morphine withdrawal activates cAMP-dependent protein kinase A (PKA; Chartoff et al., 2003a,b, 2006). Interestingly, it has been reported that the increase in adenylyl cyclase activity may itself result in a decrease in transcript levels of particular cyclases in the striatum (Spijker et al., 2004).

Protein kinase A phosphorylates and activates numerous substrates, including the transcription factor cAMP response element binding protein (CREB) and the AMPA receptor (AMPAR) subunit GluR1 (**Figure 1**, Withdrawal condition; **Figure 3**; Konradi et al., 1994; Chartoff et al., 2003b, 2006; Mangiavacchi and Wolf, 2004). Optimal PKA-mediated increases in CREB and GluR1 signaling requires NMDA receptor (NMDAR) activation (Konradi et al., 1996; Wolf, 2010), providing early evidence for crosstalk between MORs and glutamatergic transmission. It is through these actions that morphine and heroin may

ultimately modulate fast excitatory transmission via AMPAR and NMDAR.

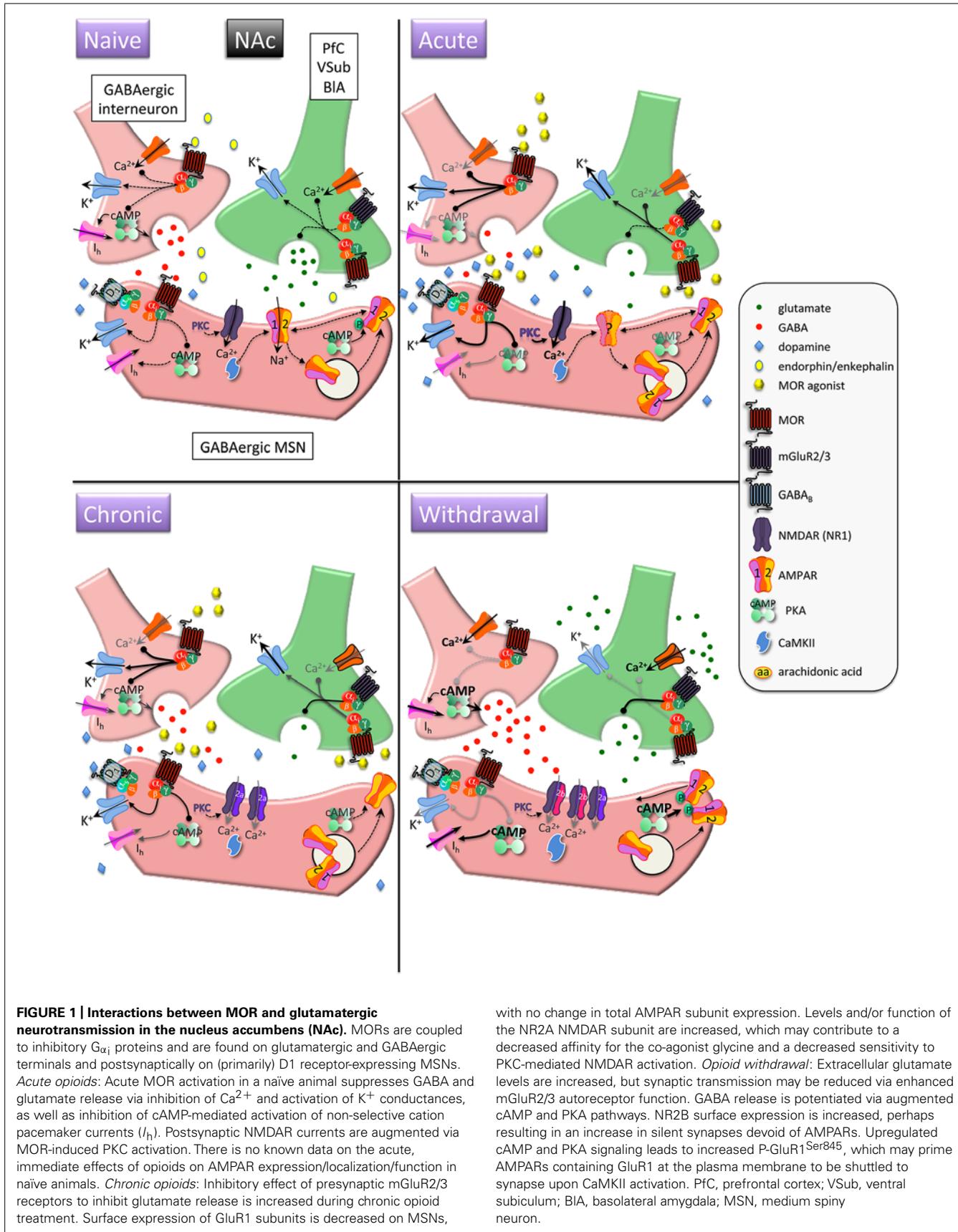
GLUTAMATERGIC NEUROTRANSMISSION

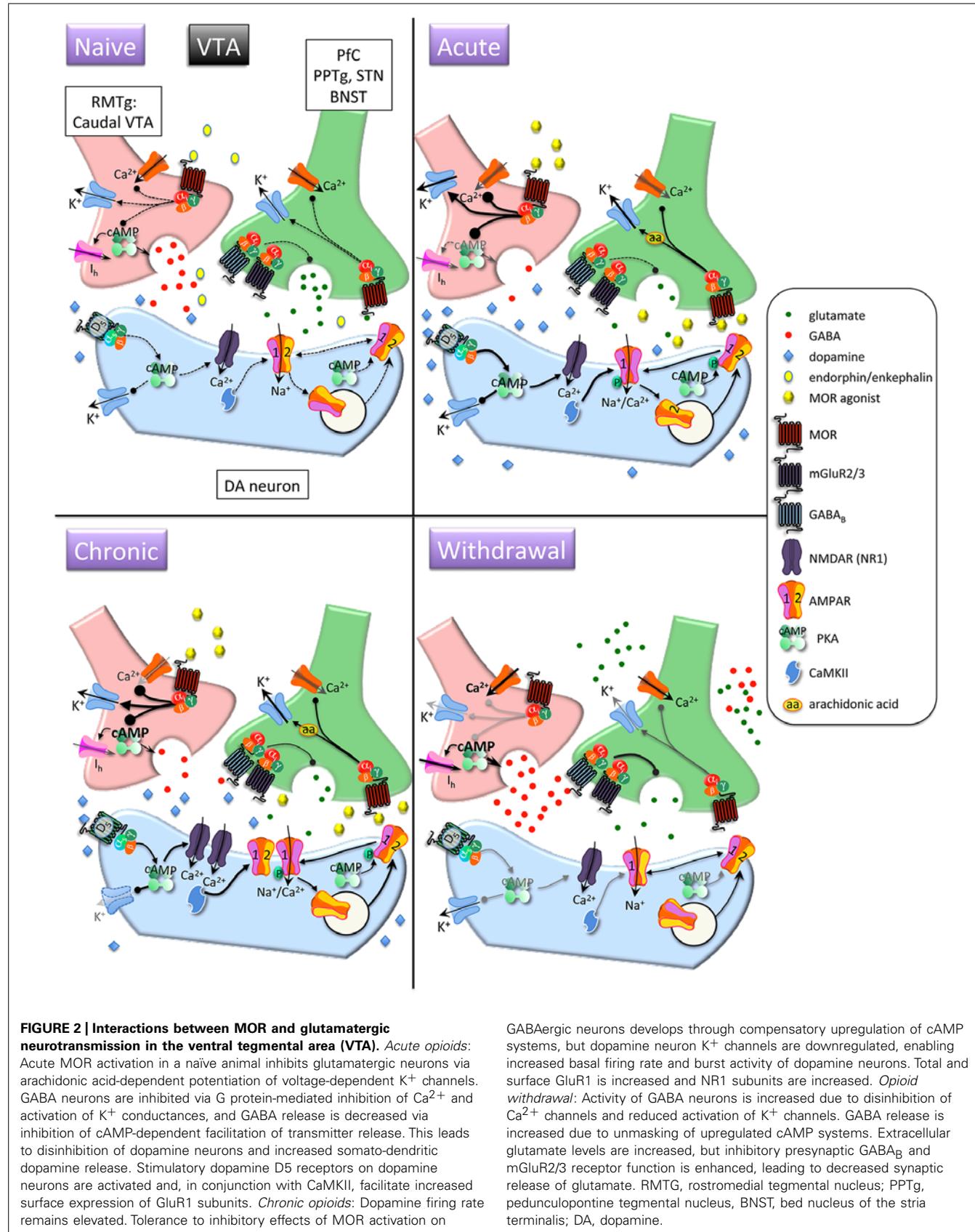
The classic view of glutamate action comprises presynaptic release of glutamate, binding to postsynaptic ionotropic receptors, and clearance of glutamate by Na^+ -dependent glutamate transporters (Anggono and Huganir, 2012). Layered upon this are the more recently discovered influences of glial-derived glutamate release and uptake and extrasynaptic mGluRs on excitatory synaptic transmission and plasticity (Kalivas et al., 2009). Although this review focuses on MOR-mediated modulation of AMPAR and NMDAR-mediated glutamatergic transmission, it is essential to understand that glutamate homeostasis (regulation of synaptic and perisynaptic extracellular glutamate levels) requires ionotropic and metabotropic (mGluR) receptors as well as a delicate balance between glial and synaptic glutamate release and elimination. Comprehensive reviews of glutamate homeostasis in the context of drug addiction are available (Kalivas, 2009; Kalivas et al., 2009).

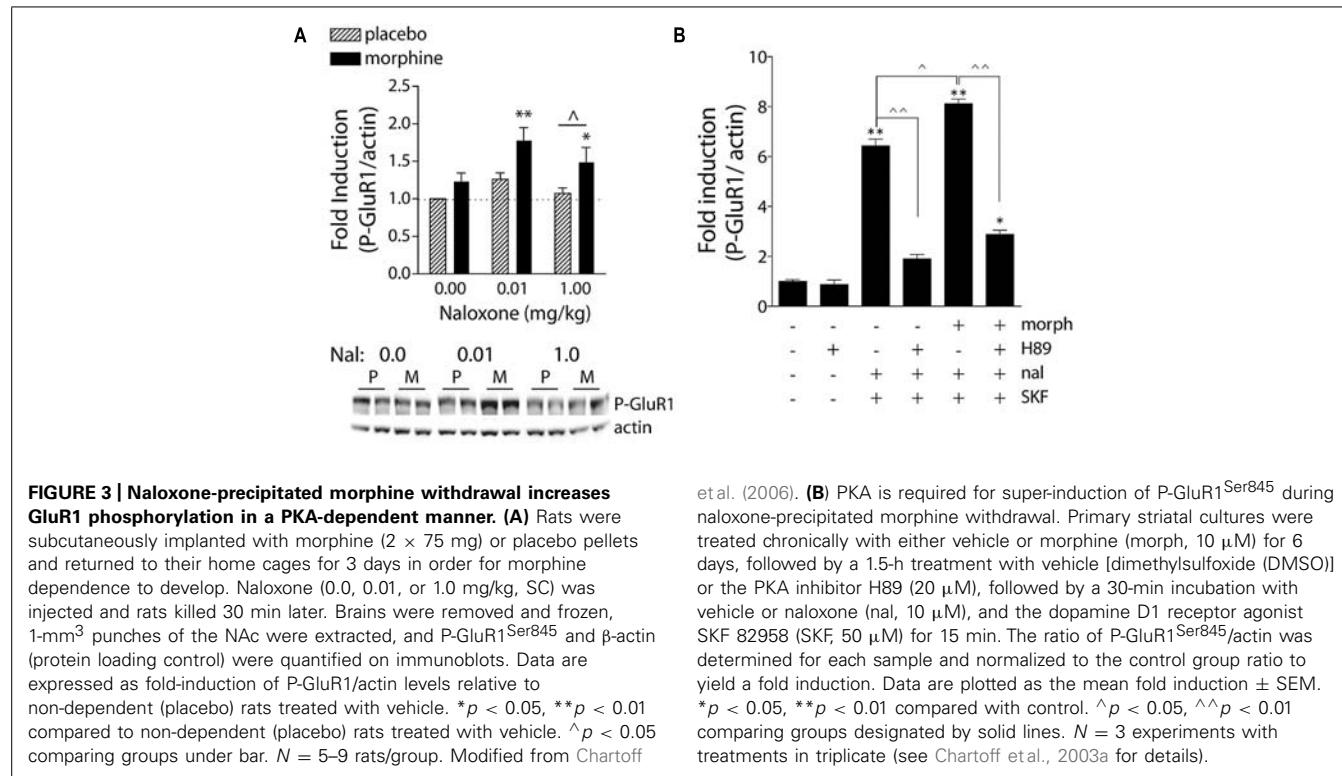
AMPA RECEPTORS

AMPARs are a subgroup of ionotropic glutamate receptors found at most excitatory synapses, are activated at resting membrane potential, and are considered the primary postsynaptic mediators of glutamate transmission in the NAc (Cherubini et al., 1988). AMPARs comprise four subunits (GluR1–4) that assemble in various combinations to form tetramers (Seuberg, 1993; Hollmann and Heinemann, 1994; Dingledine et al., 1999). GluR1–4 share ~70% sequence homology and differ primarily due to post-transcriptional modifications, which confer unique properties to the subunits. For example, the GluR2 transcript undergoes RNA editing such that a glutamine residue in the channel-forming segment of the receptor is converted to an arginine (Sommer et al., 1991). This renders GluR2-containing AMPARs impermeable to Ca^{2+} (Burnashev et al., 1992). Given that AMPARs exist primarily as GluR1–2 and GluR2–3 populations (Wenthold et al., 1996), most AMPARs gate Na^+ but not Ca^{2+} . However, synaptic activity – including *in vivo* experience – can shift the stoichiometry of synaptic AMPAR subunit composition toward GluR2-lacking receptors (Liu and Cull-Candy, 2000; Takahashi et al., 2003; Ju et al., 2004; Clem and Barth, 2006), and increasing GluR1 expression favors formation of GluR1-homomeric AMPARs that allow Ca^{2+} flux (Hollmann et al., 1991).

Trafficking of AMPARs into and out of synapses determines the level of excitatory synaptic strength and is a major mechanism of plasticity underlying learning (Malinow and Malenka, 2002). AMPARs can be endocytosed and exocytosed into perisynaptic regions, and they can also be shuttled laterally along the surface of the neuronal membrane between synaptic and extrasynaptic compartments (Heine et al., 2008). A host of AMPAR auxiliary subunits such as transmembrane AMPAR regulatory proteins (TARPs), Cornichon proteins, Neuropilin, and Tolloid-like proteins (Netos) are necessary for the dynamics of AMPAR subcellular localization (Straub and Tomita, 2012). Heteromers containing GluR2–3 subunits are constitutively recycled and maintain basal AMPAR transmission, whereas heteromers containing GluR1–2







subunits are delivered to synapses in a precisely regulated manner and are critical for experience-dependent plasticity (see Malinow and Malenka, 2002). In the absence of activity, synapses can be devoid of GluR1–2-containing AMPARs. PKA-mediated phosphorylation of GluR1 at Ser⁸⁴⁵ (P-GluR1^{Ser845}) enhances channel conductance and open probability, and in combination with activity-dependent Ca²⁺ signaling (e.g. via NMDARs), phosphorylation can drive GluR1 into synapses, which could allow synaptic strengthening (Esteban et al., 2003). Importantly, P-GluR1^{Ser845} is necessary but not sufficient for trafficking of GluR1 subunits to synapses (e.g. Figure 2, Chronic condition). In the NAc, this type of plasticity might involve convergence of dopamine and glutamate inputs (Wolf et al., 2003): activation of postsynaptic D1 receptors induces P-GluR1^{Ser845} and activation of NMDARs could allow synaptic delivery. Conversely, activation of AMPARs can lead to compensatory dephosphorylation of GluR1 and subsequent removal from synaptic zones to intracellular vesicles (Beattie et al., 2000; Snyder et al., 2003).

Synaptic scaling is a homeostatic form of plasticity in which prolonged activity or lack of activity at AMPARs (~1–3 days) leads to compensatory decreases or increases, respectively, in synaptic AMPAR levels (Turriano, 2008). This phenomenon is thought to stabilize neuronal activity during periods of abnormal or pathological activity, and may be highly relevant to addiction and drug withdrawal.

NMDA RECEPTORS

NMDARs are a subgroup of ionotropic glutamate receptors found throughout the brain that act – in concert with colocalized

AMPARs – as synaptic coincidence detectors to facilitate learning and memory (Tang et al., 1999; Citri and Malenka, 2008). NMDARs exist as heterotetramers composed of two NR1 subunits and two subunits from the NR2 or NR3 family (Seuberg et al., 1995). NR1 subunits are expressed ubiquitously in the brain, whereas NR2 subunits are spatially localized (Dunah et al., 1999). The basal forebrain (includes the NAc) is enriched for NR2A and B, with a predominance of NR2B in NAc medium spiny neurons (MSNs; Chen and Reiner, 1996; Kuppenbender et al., 2000). NMDARs are unique in that they require both ligand (glutamate) binding and membrane depolarization (to release extracellular Mg²⁺ block) in order to be activated. Once activated, NMDARs conduct both Na⁺ and Ca²⁺, which results in excitatory postsynaptic currents (EPSCs) with greater magnitude and longer half-life than those from AMPARs that pass only Na⁺. Perhaps most importantly, NMDAR activation engages Ca²⁺-mediated signal transduction pathways that can have long-lasting effects on gene expression, post-translational modifications of proteins (e.g., phosphorylation), and voltage-gated ion channels (Hyman et al., 2006). In fact, Ca²⁺ influx is required for NMDAR-mediated long-term potentiation (LTP). The NR1 subunit is essential for channel function whereas NR2 subunits control channel gating and Mg²⁺ dependency (Monyer et al., 1992).

Anatomical studies have shown that MORs and NMDARs colocalize on single neurons in many brain regions, including within the dorsal striatum and NAc shell (Trujillo, 2002; Glass et al., 2009). More recently, immunoprecipitation analysis has revealed that MORs can directly interact with NMDA NR1 subunits (Rodriguez-Munoz et al., 2012). This pattern was observed in

the PAG, cerebral cortex, striatum, and dorsal spinal cord, suggesting functional interactions between MOR and NR1 are important for analgesic and affective responses to opioids.

The importance of NMDARs to opioid dependence and the OWS may lie in the well established role of NMDARs in forming associative memories via their ability to detect two coincident synaptic events at the cellular level (i.e., LTP; long-term depression, LTD). This type of learning is thought to be important for phenomena such as conditioned craving and conditioned withdrawal, which are common in abstinent opiate addicts and are major triggers of relapse. There is also evidence that NMDAR-mediated plasticity is necessary for extinction of drug-associated memories. Specifically, the NMDAR partial agonist, D-cycloserine (DCS) facilitates extinction of morphine withdrawal-associated place aversions in morphine-dependent rats (Myers and Carlezon, 2010a) and extinction of cocaine-induced conditioned place preferences (Botreau et al., 2006; Paolone et al., 2009).

Although NMDARs are classically thought of as a major substrate for Hebbian learning, they can also have unconditioned effects on reward and affective states. For example, rats will self-administer competitive and non-competitive NMDAR antagonists directly into the NAc (Carlezon and Wise, 1996b), and NMDAR antagonists potentiate brain stimulation reward (Carlezon and Wise, 1996a). These findings suggest that a reduction in the overall excitability of neurons in the NAc (via NMDAR blockade) and/or a reduction in intracellular Ca^{2+} signaling is sufficient for reward. It is likely that NMDAR-mediated increases in synaptic strength (learning) and changes in affective state are not mutually exclusive processes. One can envision a scenario during drug withdrawal in which the experience of an intense dysphoric state is stamped into memory through NMDAR activation in select brain regions. This idea will be discussed in more detail in the following sections.

NUCLEUS ACCUMBENS

The NAc (ventral striatum) can be subdivided into multiple territories based on functional connectivity and neuronal phenotypes (Zahm and Brog, 1992). The NAc core is a central portion of the ventral striatum that surrounds the anterior commissure and is a functional continuation of the neighboring dorsal striatum. It has been shown to be particularly important for instrumental learning such as cue-induced reinstatement of drug seeking (McFarland and Kalivas, 2001). The shell comprises the most ventral and medial portions of the NAc, and has an important role in drug reward, motivated behavior, behavioral sensitization, and changes in affective state. In addition, subterritories such as the rostral pole, cone and intermediate zone of the NAc shell have been described (Zahm and Brog, 1992). A long-standing conception is that the NAc is a “motivation to movement interface” (Mogenson et al., 1980), and accumulating evidence has confirmed this idea by identifying the neural circuits that loop from limbic and cognitive cortical regions to motor output regions (Haber and Knutson, 2010). Thus, the NAc is a key site for transference of motivational and emotional signals to adaptive behavioral responses. Despite its long tenure as the “reward center” of the brain, increasing evidence supports the idea that the NAc is a bivalent structure that processes positive

and negative emotional stimuli into either approach or avoidance behavior (Becerra et al., 2001; Reynolds and Berridge, 2002; Jensen et al., 2003; Roitman et al., 2005; Carlezon and Thomas, 2009). This has important ramifications for understanding addiction, since drugs of abuse provide hyperbolic positive (drug “high”) and negative (drug withdrawal, “crash”) emotional signals to the NAc.

AFFERENTS

Consistent with the view that the NAc gates rewarding and aversive stimuli and directs subsequent goal-directed behavior, NAc afferents come from brain regions known to be important for processing both positive and negative emotional stimuli, such as the basolateral amygdala (Kelley et al., 1982) and for goal-directed behavior, including the orbitofrontal cortex, insula, cingulate cortex (Berendse et al., 1992), and midline and intra-laminar thalamic nuclei (Berendse and Groenewegen, 1990). In addition, the NAc receives rich innervation from the ventral subiculum of the hippocampus (Kelley and Domesick, 1982; Groenewegen et al., 1987), which likely provides spatial and contextual information about the stimuli (for review of NAc afferents, see Brog et al., 1993; Sesack and Grace, 2010). The vast majority of NAc afferents are glutamatergic and provide the excitatory drive necessary to evoke behavior. The NAc also receives some inhibitory, GABAergic inputs from the ventral pallidum and the VTA, as well as local inhibitory connections from within the striatum (Brog et al., 1993; Sesack and Grace, 2010). Layered on top of fast neurotransmission controlled by glutamate and GABA, the output of the NAc is modulated by robust networks of neuropeptides, both intrinsic and extrinsic to the NAc. These include, but are not limited to, orexin, dynorphin, enkephalin, substance P, and neurotensin (Hokfelt et al., 2000). Finally, dopamine afferents from the VTA provide an essential component of reward processing in the NAc. Dopamine modulates the general excitability of NAc neurons, thus increasing or decreasing behavioral output based on the level of emotional salience coded by the dopamine input (Koob, 1992; Ikemoto and Panksepp, 1999; Wise, 2004).

INTRINSIC SIGNALING

Within the NAc, GABA-containing medium spiny output neurons comprise the majority (~90–95%) of neurons (Wilson and Groves, 1980; Gerfen, 1992), with the remaining cells being either GABAergic or cholinergic interneurons (Kawaguchi et al., 1995). The function of MSNs depends on their particular inputs and outputs, but also on the phenotype of the MSN itself. Only recently have researchers had the tools to begin to dissect the complex microcircuitry of the NAc. As with the dorsal striatum, NAc MSNs can be broadly divided into dopamine D₁-like (includes D₁ and D₅ receptors) or dopamine D₂-like (includes D₂, D₃, and D₄) receptor expressing circuits (Gerfen et al., 1990; Lobo, 2009). MSNs express different constellations of neuropeptides, with dynorphin often co-expressing with dopamine D₁ receptors and enkephalin with dopamine D₂ receptors (Gerfen et al., 1990; Lobo, 2009). In the NAc, MORs are expressed primarily by dynorphin- and D₁ receptor-expressing cells (Georges et al., 1999).

Glutamate neurotransmission is kept under tight control: too much or too little can have devastating effects (Kalivas,

2009), whereas stimulus-dependent changes in glutamatergic transmission are necessary for learning (Kauer and Malenka, 2007). Structurally, synaptic input to MSNs is arranged such that glutamatergic afferents synapse on dendritic spines and modulatory inputs such as dopamine make connections extrasynaptically on dendritic shafts. This triad of spine, glutamate synapse and dopamine synapse allows dopamine to modulate the general excitability of NAc neurons (Surmeier et al., 2007; Sesack and Grace, 2010). Glutamatergic activation of NAc MSNs is mediated primarily by AMPARs (Hu and White, 1996). Approximately 90% of AMPARs in the NAc are made of GluR1 and GluR2 or GluR3 containing tetramers with only about 6% being GluR1–3 complexes (Wolf, 2010; Reimers et al., 2011). There is some evidence for a very small percentage of AMPARs in the NAc to exist as GluR1 homomers. Functionally, this implies that the vast majority of NAc AMPARs conduct Na^+ but not Ca^{2+} , given that GluR2 renders AMPARs impermeable to Ca^{2+} . NMDARs play a critical role in tagging connections that receive convergent glutamate and dopamine inputs. For example, cortical excitation of selected MSNs in the presence of dopamine would lead to an increase in synaptic strength in a two-step process: activation of postsynaptic D₁ receptors induces PKA-dependent P-GluR1^{Ser845} and activation of NMDARs facilitates synaptic delivery of GluR1 (Wolf, 2010).

EFFERENTS

The functional consequences of glutamate transmission in the NAc are being elucidated: in general, NAc neurons are activated in response to aversive stimuli and inhibited in response to rewarding stimuli (Peoples and West, 1996; Carelli, 2002; Roitman et al., 2005). GABAergic MSNs from the NAc project to the ventral pallidum, substantia nigra (SN), VTA, hypothalamus, and brainstem (Haber et al., 1990). There is topographical organization such that a medial (i.e., shell) to lateral (i.e., core/dorsal striatum) series of projection loops allows emotion-based information from limbic-associated structures to transfer to motor-related areas of the basal ganglia (Haber et al., 2000). Within these spiraling loops, some NAc outputs – particularly in the core – are functionally analogous to the direct and indirect pathways described for the dorsal striatum (Sesack and Grace, 2010). Activation of G_{αs}-coupled D₁-like receptors stimulates production of cAMP and tends to excite MSNs that project directly back to the VTA and the ventral pallidum (direct pathway), whereas activation of G_{αi}-coupled D₂-like receptors inhibits cAMP production and tends to inhibit MSNs that selectively project to the ventral pallidum (indirect pathway; Lu et al., 1998; Surmeier et al., 2007). Thus, cortical activation of the direct pathway leads to disinhibition of motor circuits that enable reward acquisition whereas activation of the indirect pathway inhibits motor circuits that are maladaptive (Mink, 1996).

VENTRAL TEGMENTAL AREA

The VTA has been extensively studied for its role in reward and addiction. Opioids are self-administered directly into the VTA (Bozarth and Wise, 1981; Devine and Wise, 1994), while blockade of VTA MORs suppresses heroin self-administration (Britt and

Wise, 1983). Intra-VTA morphine injections produce conditioned place preferences (Bozarth, 1987), enhance the rewarding impact of intracranial self-stimulation (Broekkamp et al., 1976), and reinstate extinguished lever pressing for heroin (Stewart et al., 1984). Dopamine neurons make up about 60–65% of the cells in the VTA, with GABAergic (~25%) and glutamatergic (up to 15%) neurons making up the rest (Swanson, 1982; Nair-Roberts et al., 2008). Most classes of drugs of abuse increase dopamine release in efferent targets of the VTA, including the NAc (Di Chiara and Imperato, 1988a). Comprehensive reviews of the role of VTA dopamine in reward function and addiction have been published (Berridge and Robinson, 1998; Wise, 2004; Fields et al., 2007; Ikemoto, 2007; Wheeler and Carelli, 2009; Salamone and Correa, 2012), with the emerging view that not only does dopamine mediate the positive reinforcing effects of drugs but it is also instrumental in learning how particular behaviors lead to reward or aversion (Volman et al., 2013).

AFFERENTS

The VTA is regulated by an integrated network of excitatory inputs arising from the PFC, the pedunculopontine region (PPTg), the laterodorsal tegmentum (LDTg), and the sub thalamic nucleus (Grace et al., 2007). These connections are organized in the sense that glutamatergic inputs from the medial PFC (mPFC) synapse on dopamine neurons that project back to the mPFC but not on those that project to the NAc (Carr and Sesack, 2000). The VTA and the more caudal “tail” of the VTA (RMTg) receives GABAergic input from the lateral habenula, NAc shell, and ventral pallidum (Zahm and Heimer, 1990; Jhou et al., 2009). Importantly, the RMTg provides tonic GABAergic inhibition of VTA dopamine neurons that keeps them in a pacemaker-type firing pattern in the absence of stimulation (Bourdy and Barrot, 2012). The transition from pacemaker-like firing of dopamine neurons to burst firing, which is thought to represent a phasic dopamine response associated with reward and reward-related cues, requires glutamate input from the PPTg–LDT complex (Floresco et al., 2003; Lodge and Grace, 2006; Grace et al., 2007).

INTRINSIC SIGNALING

GABA neurons of the VTA and RMTg express dense MOR mRNA and immunoreactivity (Mansour et al., 1988, 1995a; Garzon and Pickel, 2001; Svingos et al., 2001; Jhou et al., 2009). Morphine indirectly excites dopamine neurons via inhibition of these GABA neurons that synapse on dopaminergic dendrites in the VTA (Johnson and North, 1992; Jalabert et al., 2011). This disinhibition of dopamine neurons requires NMDAR and AMPAR activation (Jalabert et al., 2011). Taken together, the effects of opioids on VTA function involve a close interaction between postsynaptic MORs and glutamate signaling.

EFFERENTS

There is a topographical organization to the VTA, with dopamine and GABAergic efferents having a medial to lateral projection to output structures such as the NAc, PFC, cingulate cortex, and baso-lateral amygdala (Ikemoto, 2007). In addition, there is a rostral to caudal organization in which the ratio of dopamine to GABA decreases caudally (Bourdy and Barrot, 2012). In broad terms,

there are stronger drug reward associations in the caudal-medial versus anterior VTA (Ikemoto, 2007).

ACUTE OPIOIDS

CLINICAL DESCRIPTION

The National Survey on Drug Use and Health (NSDUH, 2013), the National Monitoring the Future survey study (Johnston et al., 2014), and the Columbia CASA report (The National Center on Addiction and Substance Abuse, Columbia University, 2011) provide consistently alarming trends of early age initiation of prescription opioid misuse (1.5% of children age 12–13 years old used in the prior month) and heroin use, with a national average age of opioid initiation between 22 and 23 years old. Teenagers report high availability of illicit opioids: 20 and 45% of high school seniors report it is easy to get heroin and prescription opioids, respectively (Johnston et al., 2014). Teens report using both to get high and to relieve tension, despite pervasive disapproval and perceived risk (The National Center on Addiction and Substance Abuse, Columbia University, 2011). Teens describe being high on opioids as, “the best feeling ever,” or, “I finally felt happy,” which is not different from the self-reported experiences of adult initiates.

MOR–GLUTAMATE INTERACTIONS IN THE NAc (see Figure 1)

Acute administration of opioids activates MORs and increases extracellular dopamine in the NAc (Figure 1, yellow ovals; Di Chiara and Imperato, 1988a). However, dopamine is not necessary for the acute rewarding effects of opiates in non-dependent animals, as dopamine receptor blockade or 6-hydroxydopamine (6-OHDA)-mediated dopamine denervation of the NAc does not prevent heroin self-administration (Pettit et al., 1984; Gerrits and Van Ree, 1996). Even evidence demonstrating a requirement for the NAc in opiate reward and reinforcement is equivocal. For example, mice can learn to self-administer MOR agonists directly into the NAc (Goeders et al., 1984; David and Cazala, 2000), and yet intra-NAc morphine fails to produce conditioned place preferences in rats (Schildein et al., 1998). Lesions or inactivation of the NAc partially reduce opiate self-administration (Zito et al., 1985; Dworkin et al., 1988; Alderson et al., 2001), but it is difficult to interpret the meaning of these data on their own, since a decrease in the number of drug infusions at a single drug dose can mean either a decrease or an increase in the reinforcing efficacy of a drug (Mello and Negus, 1996). A study showing that NAc lesions reduced progressive ratio (PR) responding for morphine in rats (Suto et al., 2011) supports the idea that the NAc plays a role in the motivation to work for morphine. Yet direct infusions of MOR antagonists into the NAc actually increase heroin self-administration (Vaccarino et al., 1985), which the authors interpret as a decrease in the reinforcing efficacy of heroin driving increased drug-taking. Thus, the NAc can modulate opioid reward and drug-taking behavior, but its precise role is complicated by prior drug experience and method of administration.

Human imaging studies have generally shown that, in drug-experienced people, an immediate (i.e., during the “rush”) effect of opioid administration is an increase in regional cerebral blood flow in the anterior cingulate cortex, thalamus, and amygdala

(Schlaepfer et al., 1998; Kosel et al., 2008). In contrast, after the initial “rush” has subsided and the longer lasting euphoric effects of acute opioids emerge, blood flow tends to be decreased (London et al., 1990; Denier et al., 2013). This is consistent with electrophysiological and neurochemical findings in rats, in which systemic morphine inhibits spontaneous firing of a majority of neurons in the mPFC (Giacchino and Henriksen, 1996). In many neurons, MOR-mediated inhibition of adenylate cyclase results in a decrease in cAMP-dependent activation of voltage-dependent I_h pacemaker currents (Figure 1, GABAergic neurons, Acute condition; Williams et al., 2001). A decrease in cAMP shifts the voltage dependence to more negative potentials, making it harder to depolarize the neuron. MOR activation suppresses basal and evoked increases in extracellular glutamate in the NAc and dorsal striatum (Figure 1, glutamate neurons, Acute and Chronic conditions; Desole et al., 1996; Enrico et al., 1998; Sepulveda et al., 2004). Although the functional consequences of changes in cerebral blood flow and cortical activation are not yet known, the findings suggest that opioid-induced reward is associated with decreased cortical activity and potentially decreased glutamatergic input to downstream NAc MSNs. Despite the evidence for opiate modulation of glutamate release in the NAc, there is relatively little data on the role AMPAR and NMDAR play in mediating the acute rewarding effects of opiates. This is surprising, given the previously discussed findings that acute opiates have profound effects on glutamatergic projections to the NAc and that the activation state of MSNs plays an important role in affect and emotional responses to stimuli (Roitman et al., 2005; Carlezon and Thomas, 2009). One prediction, based on synaptic scaling (Turrigiano, 2008), is that opiate-induced decreases in glutamatergic transmission to the NAc would result in increased surface expression of AMPARs. There are no known studies that address this prediction directly. Rather, there is evidence that expression of NMDAR and AMPAR subunits is decreased in the NAc core 3 days after acute morphine exposure (Jacobs et al., 2005). Similarly, there is one study that reports a decrease in surface levels of NAc GluR1 24 h after an acute morphine injection (Herrold et al., 2013). Unfortunately this time course does not actually reflect the acute rewarding effects of morphine but may rather reflect a state of acute withdrawal (Rothwell et al., 2012).

Using intracellular recordings from NAc slice preparations, it has been reported that acute MOR activation depresses NMDA and non-NMDA (presumably AMPA) excitatory postsynaptic potentials (EPSPs) in the NAc through a presynaptic mechanism involving reductions in spike-generated Ca^{2+} currents (Martin et al., 1997). A general effect of MOR activation that could account for this is inhibition of presynaptic voltage-gated Ca^{2+} channels (L-, N-, P/Q-, R-) through $\text{G}_{\alpha\text{o}}-\beta\gamma$ subunits (see Figures 1 and 2, presynaptic glutamatergic neuron, Acute condition; Law et al., 2000). Although this would predict a decrease in MSN activation, this study also demonstrated that postsynaptic NMDA currents were augmented via a protein kinase C (PKC)-dependent mechanism (Figure 1, postsynaptic GABA neuron, Acute condition). The ultimate consequences of these opposing MOR actions are still not fully understood. A more recent study in awake and behaving rats showed that a non-contingent injection of heroin produced a small

decrease (not significant) in extracellular glutamate in the core in drug-naïve rats (LaLumiere and Kalivas, 2008). Taken together, the available data suggest that acute opiates decrease glutamate release in the NAc in non-dependent animals, which is consistent with the general finding that decreases in NAc MSN activation are associated with reward-like states (Carlezon and Thomas, 2009).

MOR–GLUTAMATE INTERACTIONS IN THE VTA (see Figure 2)

An immediate effect of an acute opiate injection is inhibition of MOR-containing GABAergic neurons (**Figure 2**, GABAergic neuron, Acute condition) in the RMTg that make strong synaptic contacts on the soma and dendrites of dopamine neurons and a subsequent decrease in LTP of these GABAergic synapses (Johnson and North, 1992; Niehaus et al., 2010; Jalabert et al., 2011; Mazei-Robison et al., 2011). A second immediate effect is a presynaptic inhibition of glutamatergic afferents via MOR-mediated, arachidonic acid-dependent, activation of voltage-sensitive K⁺ channels (**Figure 2**, Glutamate neuron, Acute condition; Manzoni and Williams, 1999). This effect on glutamate release is confusing, because one would expect morphine to produce rapid activation of dopamine neurons through both inhibition of GABA inputs and excitation of glutamatergic inputs. In fact, it has been reported that an opiate-dependent increase in AMPAR activation in the VTA is required for disinhibition of dopamine neurons (Gysling and Wang, 1983; Di Chiara and Imperato, 1988a; Jalabert et al., 2011). Although not fully understood, the issue is likely related to timing.

An acute response common to most classes of drugs of abuse, including opiates, is an increase in AMPA transmission in dopamine neurons measured 24 h after acute administration of drug (Ungless et al., 2001; Saal et al., 2003; Brown et al., 2010). This is thought to be due to an increase in surface expression of AMPARs. Since acute morphine inhibits activity of glutamatergic afferent neurons (Giacchino and Henriksen, 1996; Manzoni and Williams, 1999), this observed increase in evoked AMPA transmission may not immediately translate to increased excitation of the VTA. Rather, morphine-induced decreases in glutamate release to the VTA may promote compensatory, postsynaptic increases in AMPA signaling that produce LTP at select synapses. Consistent with this, an increase in surface expression of GluR1 in the VTA (**Figure 2**, Dopamine neuron, Acute condition) has been reported 24 h (Brown et al., 2010), and as early as 1 h (Lane et al., 2008), after morphine injection. The mechanism through which morphine increases GluR1 synaptic insertion and LTP is not fully understood, but is thought to involve stimulation of dopamine D₅ receptors (**Figure 2**, Dopamine neuron, Acute condition), which belong to the Gαs-coupled D1-like receptor family (Schilstrom et al., 2006; Brown et al., 2010). D₅, unlike D₁, receptors are expressed on dopamine neurons of the VTA (Weiner et al., 1991; Khan et al., 2000). Thus, morphine-induced dopamine release can stimulate D₅ receptors in the VTA, which would activate cAMP-dependent processes including PKA-dependent phosphorylation of GluR1. Phosphorylation of GluR1 facilitates synaptic insertion and increases synaptic current (Kessels and Malinow, 2009), providing a potential mechanism for feed-forward enhancement of morphine's actions on dopamine

neurons. Consistent with this, overexpressing GluR1 AMPAR subunits in the VTA sensitizes rats to the locomotor effects of acute morphine and potentiates morphine-induced conditioned place preferences (Carlezon et al., 1997). Although GluR1 trafficking is evident after only one injection of morphine, it is thought that the cumulative effects of repeated morphine treatment are necessary for the plasticity in dopamine neuron excitability that contribute to the development of sensitization (Carlezon and Nestler, 2002).

A critical role for VTA AMPA and NMDARs in acute opiate reward has been demonstrated in behavioral studies. Intra-VTA delivery of an NMDAR or an AMPAR antagonist increased heroin self-administration in the same manner that decreasing the available dose of heroin does (Xi and Stein, 2002). This is consistent with the idea that NMDA and AMPAR activation is necessary for the acute reinforcing effects of opioids. Intra-VTA blockade of either NMDAR or AMPAR decreases both the acquisition and expression of morphine conditioned place preferences (Harris et al., 2004). Place conditioning depends upon an associative memory of the pairing of an affective state (reward or aversion) with a context. Thus, the role of VTA glutamate transmission in opiate effects could be to promote associative learning and/or to promote a rewarding state that has salience as an unconditioned stimulus in the place conditioning paradigm.

CHRONIC OPIOIDS

CLINICAL DESCRIPTION

Most individuals who are recently opioid-dependent are not fully aware they are “hooked.” Getting high is still euphoric, and mild withdrawal symptoms are surprising and manageable. Cognitive appraisal is, “I can stop if I need to.” Seeking a more intensive high may lead the individual to change to a route of administration that produces a more rapid and potent effect (e.g. oral to intranasal, or intranasal to intravenous), to try a more potent formulation (switching analgesics or getting a “good batch” of heroin), or to mixing opioid use with other substances, particularly sedative-hypnotics.

Opioids can be administered chronically in a number of ways (steady dosing of extended release painkillers, repeated intermittent abuse, binge-type self-administration, or any combination of the preceding), which likely influences the resulting neural adaptations. As discussed above, an additional consideration in interpreting data from chronic drug studies is the time point at which molecular or behavioral measures are taken. Effects observed 24 h or more after the end of a chronic drug regimen may more accurately reflect drug withdrawal rather than chronic effects *per se*. Furthermore, accumulating evidence suggests that GPCRs (e.g., MOR) modulate synaptic activity on a timescale that extends well beyond that of initial receptor activation, resulting in a metaplasticity that can either lower or raise the threshold for induction of LTP-like processes (Tenorio et al., 2010).

MOR–GLUTAMATE INTERACTIONS IN THE NAc

Chronic activation of MORs triggers counteradaptations in cAMP signaling such that adenylate cyclase function is enhanced (Nestler and Aghajanian, 1997; Williams et al., 2001). The presence of

chronic opioids masks the effects of increased cAMP, but it alters how other GPCRs signal through adenylate cyclase. As one example, chronic morphine increases the inhibitory efficacy of presynaptic mGluR2/3 receptors on glutamate release in the NAc (**Figure 1**, Glutamatergic neuron, Chronic condition; Martin et al., 1999). This may evolve from an increased functional connectivity between mGluR2/3 receptors and upregulated cAMP signaling.

Numerous studies have examined effects of repeated psychostimulants (contingent and non-contingent administration protocols) on AMPAR-mediated synaptic transmission in the NAc (Wolf et al., 2004; Luscher, 2013; Pierce and Wolf, 2013). Unfortunately, relatively little is known about effects of chronic opioids. These types of studies are important, because available research has shown that cellular and structural consequences of opioids often differ from those of psychostimulants (Badiani et al., 2011). Chronic, steady state levels of morphine achieved with subcutaneous morphine pellet implants do not change total protein levels of AMPAR subunits (Chartoff et al., 2006), although this does not take into account changes in subcellular localization. To address this, Glass et al. (2008) used immunogold ultrastructural analysis to demonstrate that surface expression of GluR1 subunits is decreased after chronic morphine treatment (1 h after 14 days of non-contingent injections; **Figure 1**, postsynaptic GABA neuron, Chronic condition). This effect was localized to dopamine D1 receptor-expressing neurons in the NAc shell and in all MSN types in the core. In contrast, a different opiate regimen (1 day after 1 injection/day for 3 days) produced no change in subcellular distribution of either GluR1 or 2 in the NAc (Mickiewicz and Napier, 2011). The mechanisms by which chronic opioids modulate subcellular distribution of AMPAR subunits are not known. However, given that activation of MORs inhibits adenylate cyclase and cAMP production, it is possible that the resulting brake on PKA function leads to decreased P-GluR1^{Ser845} in the NAc (**Figure 1**, postsynaptic GABA neuron, Chronic condition), which would favor internalization processes (Song and Huganir, 2002; Mangiavacchi and Wolf, 2004). This is consistent with formation of LTD, which requires clathrin-dependent endocytosis of postsynaptic AMPARs (Brebner et al., 2005).

More is known about the effects of chronic morphine on NMDAR-mediated synaptic transmission in the NAc compared to AMPA transmission. As discussed above, acute morphine's actions in the NAc include presynaptic inhibition of glutamate release as well as a postsynaptic potentiation of NMDAR EPSPs via activation of PKC (Martin et al., 1997). Postsynaptically, chronic morphine appears to have several effects on NMDARs, including a decrease in affinity for the co-agonist glycine and a decrease in the sensitivity of PKC-mediated NMDAR activation. Using dissociated primary cultures of NAc neurons, it was shown that these effects may be due, in part, to an increase in expression or function of the NR2A subunit (**Figure 1**, postsynaptic GABA neuron, Chronic condition; Martin et al., 2004). *In vivo* studies have reported increased protein levels of NR1 and NR2A in the NAc after chronic morphine (Inoue et al., 2003; Murray et al., 2007), although a separate study did not detect a change in NR2A (Bajo et al., 2006). An intriguing possibility for how MORs and NMDARs interact is described in the opioid pain literature. It

has been reported that MORs and NR1 subunits physically associate in the periaqueductal gray (Rodriguez-Munoz et al., 2012). Although it is not known if this occurs in the NAc or VTA, it raises the possibility that MORs can have direct effects on glutamate signaling through G protein signaling and/or through direct interaction.

MOR–GLUTAMATE INTERACTIONS IN THE VTA

Both basal firing rate and burst activity of VTA dopamine neurons are increased after acute, and during chronic, morphine treatment, resulting in elevated tonic levels of dopamine in the NAc (Leri et al., 2003; Georges et al., 2006; although see Mazei-Robison et al., 2011). However, an acute morphine challenge fails to further increase dopamine neuron activity (Georges et al., 2006), suggesting tolerance at the level of dopamine neuron activation. Recently it has been shown that chronic morphine increases intrinsic excitability of VTA dopamine neurons through downregulation of K⁺ channels (**Figure 2**, Dopamine neuron, Chronic condition) concomitantly with decreases in dopamine soma size (Mazei-Robison et al., 2011). Thus, dopamine neurons are more likely to fire, but because of their smaller size they release less dopamine. Taken together, these data raise the possibility that not only does chronic morphine maintain its inhibitory influence on GABAergic neurons in the VTA and RMTg, but it also increases the sensitivity of dopamine neurons to excitation (via I_h). Chronic morphine increases total levels of GluR1 and NMDA NR1 subunits in the VTA (Fitzgerald et al., 1996), and ultrastructural analysis showed that surface GluR1 is increased in dopaminergic and non-dopaminergic neurons of the parabrachial and paranigral VTA (**Figure 2**, Dopamine neuron, Chronic condition; Lane et al., 2008). These findings could explain, at least in part, how postsynaptic glutamate transmission is augmented with chronic opioid treatment, and they also demonstrate that normal glutamatergic signaling is fundamentally altered. In the presence of morphine, arachidonic acid-dependent activation of voltage-dependent K⁺ conductances continues to reduce glutamate release from afferent terminals (Manzoni and Williams, 1999). Yet signaling through GluR1 and possibly NR1 subunits on postsynaptic cells is enhanced (LTP-like; Fitzgerald et al., 1996; Lane et al., 2008), with no evidence so far of changes in other AMPAR or NMDAR subunits.

The effects of these seemingly opposite phenomena on behavior are not well understood, and provide an important area of future investigation. Given that AMPARs lacking GluR2 subunits and NMDARs are able to pass Ca²⁺, the increase in VTA GluR1 and NR1 likely results in increased Ca²⁺-mediated signaling (**Figure 2**, Dopamine neuron, Chronic condition), which would be expected to selectively strengthen those synaptic connections that express elevated GluR1 and NR1. Consistent with this, intra-VTA infusion of NMDAR or AMPAR antagonists prior to morphine conditioning sessions or prior to tests for morphine conditioned place preferences blocked the development and expression, respectively, of place preferences (Popik and Kolasiewicz, 1999; Harris et al., 2004). These effects appear to be limited to the rostral VTA (enriched for dopamine neurons), as AMPAR blockade of the caudal VTA (enriched for GABA neurons) had no effect on morphine

conditioned place preferences (Shabat-Simon et al., 2008). Also, mice with a global knockout of GluR1 show reduced naloxone-precipitated withdrawal signs after an escalating dose regimen of morphine (Vekovischeva et al., 2001). This suggests that GluR1 is required for full dependence to develop. Consistent with the idea that increased GluR1 expression in the VTA facilitates dopamine neuron activation, it was shown that transient over-expression of GluR1 using HSV (herpes simplex virus) vectors in the rostral VTA increased the rewarding effects of a morphine challenge, whereas GluR overexpression in the caudal VTA had the opposite effect (Carlezon et al., 2000).

OPIOID WITHDRAWAL

CLINICAL DESCRIPTION

With severe OUD, episodes of opioid withdrawal are more frequent and more aversive, and getting high gives only brief pleasure, mostly attributed to cessation of withdrawal. Individuals now use “to feel normal” and “to be able to function.” Pre-occupation with obtaining a steady source of opioids is now prevalent, and episodes of anxiety, irritability, and dysphoria are more frequent. This is a stage of securing a steady opioid supply through friends, dealers, or a doctor. This is also the stage when an individual who never intended to use intravenously converts to injection use.

Forced abstinence is accompanied by severe withdrawal, anxiety, dysphoria, and intense, recurrent cravings to use opioids. Those who cannot access a source become quite desperate to obtain opioids, and will frequently self-injure in order to receive opioid analgesia in emergency health care settings. The fear of being cut off from opioid supply becomes ever-present and motivation to hoard opioid supplies becomes habitual. This may present as routinely exploring medicine cabinets while visiting friends and family, and finding reasons to visit someone during illness or post-surgical recovery, in hope of surreptitiously taking narcotic analgesics from them. This may also be a time of criminal behavior initiation (stealing, prostitution, running goods, etc.) to support an opioid habit.

Withdrawal from chronic opioids essentially unmasks all the neural adaptations the brain produced in its attempts to equilibrate in the presence of drug. Consequently, neural circuits regulating everything from gastrointestinal function to affective states are instantly unbalanced, and an OWS emerges. A primary cause of psychological withdrawal signs, which include anxiety, dysphoria, depression, and irritability, is thought to be the dramatic reduction in dopamine neuron firing and dopamine release in efferent targets (Diana et al., 1995). Neural circuits other than the mesocorticolimbic system also play critical roles in the OWS – both somatic and psychological. These include norepinephrine (NE; Weinshenker and Schroeder, 2007), corticotropin releasing factor (CRF; Contarino and Papaleo, 2005), orexin (Mahler et al., 2012), dynorphin (Yuferov et al., 2004; Schlosburg et al., 2013), and many more (for review, see Koob, 2009). It is likely that MOR-induced neuroplasticity in glutamate transmission underlies – at least in part – the effects of each of these systems on OWS.

MOR–GLUTAMATE INTERACTIONS IN THE NAc

Spontaneous or naloxone-precipitated withdrawal from chronic opioids leads to a general increase in neuronal activity and

transmitter release due to the removal of inhibitory MOR tone. For example, GABA release is increased in the NAc during withdrawal, particularly after activation of adenylate cyclase (**Figure 1**, presynaptic GABA neuron, Withdrawal condition; Chieng and Williams, 1998). Importantly, the ability of opioids to inhibit GABA release is also enhanced, suggesting that this may be one mechanism underlying the irresistible temptation to fight OWS with opioids themselves. Glutamate release has also been shown to increase, and numerous studies have shown that systemic or intracerebroventricular administration of NMDAR or AMPAR antagonists reduces morphine tolerance and/or withdrawal signs (Trujillo and Akil, 1991; Tokuyama et al., 1996; Gonzalez et al., 1997). Much less is known about the specific role of NAc glutamate transmission in the OWS. Extracellular glutamate levels are significantly increased in the NAc during morphine withdrawal (**Figure 1**, presynaptic Glutamatergic neuron, Withdrawal condition; Aghajanian et al., 1994; Desole et al., 1996; Sepulveda et al., 1998, 2004), although increased extracellular glutamate does not necessarily mean that excitatory synaptic transmission is increased (Kalivas, 2009). For example, it has been shown that presynaptic mGluR2/3 inhibitory autoreceptor function is increased during morphine withdrawal (**Figure 1**, presynaptic Glutamatergic neuron, Withdrawal condition) and mGluR2/3 receptor agonists attenuate behavioral signs of morphine withdrawal (Robbe et al., 2002b) and context-induced reinstatement of heroin seeking (Bossert et al., 2006). These findings support the idea that, although glutamate levels are increased, synaptic transmission may be decreased during withdrawal. Thus, it is not yet clear how the combination of chronic morphine-induced increases in AMPAR and NMDAR subunit expression, withdrawal-induced increases in extracellular glutamate, and increased autoinhibition of cortical afferents is synthesized into behavioral output.

Understanding the molecular mechanisms by which increased extracellular glutamate and AMPAR and NMDAR in the NAc contribute to OWS will be key to understanding and preventing relapse. As one example, the mechanism by which presynaptic mGluR2/3 receptor function is augmented is not known. Under normal conditions, these G α i-coupled receptors inhibit evoked glutamate release by P/Q Ca²⁺ channel inhibition and PKA-dependent mechanisms (Robbe et al., 2002a). Chronic morphine and withdrawal has no effect on these processes in the NAc, raising the possibility of MOR-induced novel signaling mechanisms (Robbe et al., 2002b). In a second example, Shen et al. (2011) showed that 2 weeks of extinction following 2 weeks of daily heroin self-administration resulted in thinner dendritic spines in the NAc concomitant with an increase in surface expression of NR2B subunits (**Figure 1**, postsynaptic GABA neuron, Withdrawal condition). Consequently, overall synaptic strength was unchanged, but the AMPA/NMDA ratio (a proxy for synaptic plasticity) was decreased due to increased NMDA current with no change in AMPA current. What this means for the OWS was not investigated in this study, but Shen et al. (2011) found that the heroin withdrawal-induced increase in surface NR2B was necessary for heroin- and cue-induced reinstatement of heroin seeking. A heroin prime given to rats in which heroin seeking had been extinguished resulted in a rapid increase in spine density and

synaptic strength. This group concluded that increased NR2B-formed silent synapses in PFC to NAc core connections such that a reinstatement trigger enabled synapses to rapidly develop an LTP-like increase in field-potential strength necessary for resumption of heroin seeking. Based on prior studies of how silent synapses are “unsilenced” it is likely that Ca^{2+} -induced Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) facilitates shuttling of AMPARs from extrasynaptic sites on the plasma membrane to synaptic zones (Kerchner and Nicoll, 2008). In a different study, NR2A knockout mice treated chronically with morphine show reduced somatic withdrawal signs (Inoue et al., 2003). Restoration of NR2A expression selectively in the NAc allowed for the expression of somatic withdrawal signs. The NAc is not usually perceived as a substrate for somatic withdrawal, but this and other studies (Harris and Aston-Jones, 1994; Chartoff et al., 2009) indicate that it is a necessary – and perhaps even sufficient – component.

Morphine dependence and withdrawal may lower the threshold for LTP-like processes through a cAMP mechanism. Acute stimulation of G α i-coupled MORs leads to a decrease in cAMP levels (Childers, 1991). In the presence of chronic morphine, however, molecular adaptations occur such that adenylate cyclase activity increases and cAMP levels return to approximately normal; when morphine is discontinued or the opioid receptor antagonist naloxone is administered, cAMP levels dramatically increase (for review, see Nestler and Aghajanian, 1997). Previously we have shown that naloxone-precipitated withdrawal increases levels of phosphorylated CREB (P-CREB) and P-GluR1 $^{\text{Ser}845}$ in the NAc of morphine-dependent rats (**Figure 3A**; Chartoff et al., 2006). Using primary cultures of dissociated striatal neurons, we demonstrated that administration of naloxone to cultures treated chronically with morphine enabled the dopamine D1 receptor agonist SKF 82958 to super-induce P-GluR1 $^{\text{Ser}845}$, an effect blocked by the selective PKA inhibitor, H89 (**Figure 3B**). Together, these data predict that surface expression (although not necessarily synaptic expression) of GluR1 subunits would increase during opioid withdrawal. This has not been directly tested, although it has been shown that targeted overexpression of GluR1 (but not GluR2) in the NAc produces anhedonia in the intracranial self-stimulation paradigm (Todtenkopf et al., 2006). One caveat may be that withdrawal-induced increases in extracellular glutamate trigger internalization/desensitization of AMPARs – reminiscent of synaptic scaling (Turrigiano, 2008). These predictions are not mutually exclusive, as glutamate-triggered desensitization would likely be a pan-NAc effect whereas PKA-mediated P-GluR1 $^{\text{Ser}845}$ and membrane insertion would likely occur only in MOR-expressing neurons.

As discussed in the beginning of this review, acute and protracted OWS has been shown clinically to precipitate relapse. Using an animal model of relapse, several studies have shown that glutamate release and AMPAR activation in the NAc core are necessary for reinstatement of heroin seeking after a period of withdrawal in which operant responding for heroin is extinguished (Bossert et al., 2006, 2011, 2012; LaLumiere and Kalivas, 2008). These studies raise an important issue – namely whether a heroin prime or a heroin-associated context (used as triggers for reinstatement) produces a negative affective state akin

to OWS or a drug-like rewarding state that drives reinstatement. Increasing evidence supports the former: activation of NAc neurons (i.e., via glutamatergic transmission) is associated with aversive states (Carlezon and Thomas, 2009). The relevance of this hypothesis to heroin reinstatement studies remains to be tested.

MOR–GLUTAMATE INTERACTIONS IN THE VTA

During morphine withdrawal GABA release is increased due to MOR-induced upregulation of cAMP signaling (**Figure 2**, GABAergic neuron, Withdrawal condition; Bonci and Williams, 1996, 1997), and glutamate release is decreased due to an increase in the potency of GABA_B receptor and mGluR-mediated presynaptic inhibition (**Figure 2**, Glutamate neuron, Withdrawal condition; Manzoni and Williams, 1999). Combined, these effects lead to a strong suppression of dopamine neuron activation (Diana et al., 1995). Interestingly, it has been found that chronic morphine’s almost ubiquitous upregulation of adenylate cyclase does not play a role in modulation of glutamate release in the VTA during withdrawal (Manzoni and Williams, 1999) leaving the mechanism for augmented inhibition of glutamate release unknown for now.

One confusing aspect of MOR–glutamate interactions in the VTA during opioid withdrawal is that the actual time course of withdrawal-induced effects on glutamatergic neurotransmission is not known. Putting together available data, the immediate effect of withdrawal is relief of MOR-mediated inhibition of glutamatergic and GABAergic afferents to dopamine neurons and an increase in glutamate and GABA release. Subsequently, glutamate and GABA engage the more slowly acting metabotropic mGluR2/3 and GABA_B receptors on glutamatergic terminals resulting in decreased glutamatergic synaptic transmission (**Figure 2**, Glutamate neuron, Withdrawal condition). The complexity of this scenario raises the possibility that plasticity within micro-regions containing MOR-expressing GABA and glutamate terminals that synapse onto dopamine neurons results in fine temporal and spatial control over synaptic communication. How this affects NMDAR and AMPAR function is not known, although one prediction is that AMPARs get promoted to synapses within micro-regions in which glutamate release is decreased and removed from synapses in which glutamate release is increased. This selective strengthening of synapses could provide a mechanism for associative learning that occurs with conditioned withdrawal (Myers and Carlezon, 2010b).

RELAPSE

CLINICAL DESCRIPTION

In humans, the risk for relapse decreases the longer a person remains abstinent. This is thought to be due, in part, to the fact that the most powerful motivation to relapse stems from the desire to alleviate the initial physiological withdrawal. Opioid agonist therapies are extremely successful in treating this phase of the OWS. Unfortunately, withdrawal from these medications also produces withdrawal signs that can trigger relapse. Furthermore, they do not treat other facets of abstinence, including cue reactivity.

Abstinent addicts are at high risk for relapse due to conditioned craving and withdrawal elicited by previously drug-paired cues

(Wikler, 1973; O'Brien et al., 1986). In fact, heroin addicts report that the temptation or urge to use drug is elicited most powerfully by drug-paired cues (Heather et al., 1991). Incentive salience is the term that describes the unconscious and hypervigilant focus on rapid identification of any environmental cues that predict access to opioid using. During early recovery treatment, patients are taught to avoid high-risk “people, places, and things” to prevent cue-conditioned relapse. However, they are often baffled by their inability to reliably detect and avoid such triggers. This is because incentive salience is not a learned association within conscious awareness. A common clinical example would be that of an abstinent opioid addict being drawn to a person who is actively using while not recognizing that behavioral cues associated with that person's drug use, and not his/her personality, are the source of interpersonal interest. On a positive note, cue reactivity wanes with time, and it appears as if the success of abstinence itself begins to provide a protective factor against relapse (see Epstein et al., 2006).

MORPHINE–GLUTAMATE INTERACTIONS IN THE NAc

The majority of studies examining the role of NAc glutamatergic transmission in animal models of opioid relapse utilize the reinstatement model of drug seeking, in which the operant behavior producing contingent opioid administration is extinguished over time and then reinstated with non-contingent drug, cue, or stress presentation (Shalev et al., 2002). There is little data on how glutamatergic transmission regulates negative reinforcement mechanisms stemming from OWS. In a seminal study, LaLumiere and Kalivas (2008) demonstrated in rats that a non-contingent heroin prime or discrete cues previously paired with heroin infusions increased extracellular glutamate in the NAc core via increased synaptic transmission from PFC afferents. Intra-NAc core AMPAR blockade prevented reinstatement of heroin seeking. A separate study showed that microinjections of the mGluR2/3 receptor agonist LY379268 into the NAc shell, which inhibits evoked glutamate release from cortical afferents, reduced context-induced reinstatement of heroin seeking (Bossert et al., 2006). Interestingly, this group proposed that the reduction in heroin seeking was due to decreases in the motivational significance of the heroin context rather than to interference with memory retrieval. This is consistent with the idea that incentive salience underlies the power of a drug-paired cue to evoke drug-seeking behavior, and raises the possibility that heroin-associated cues increase synaptic glutamate release in the NAc thus producing an aversive state (Carlezon and Thomas, 2009; although see Stewart et al., 1984). Finally, chronic heroin self-administration increases NR2B subunits in the NAc (see Chronic Opioids). This is necessary for a heroin prime-induced increase in synaptic strength, dendritic spine enlargement, and reinstatement of heroin seeking after a period of extinction (Shen et al., 2011).

Although the VTA has been implicated in conditioned and unconditioned reinforcing effects of opioids (Stewart et al., 1984), and intra-VTA microinjections of the mGluR2/3 agonist LY379268 partially alleviate context-induced reinstatement of heroin seeking (Bossert et al., 2004), there is relatively little data on VTA glutamatergic transmission and relapse.

IMPLICATIONS FOR MEDICATIONS DEVELOPMENT

Opioid dependence and withdrawal disrupts excitatory neurotransmission in reward-related brain circuits, which contributes to negative affective states associated with OWS and to corruption of motivated behavior away from natural rewards toward obtaining and taking drug. Efforts are underway to develop pharmacotherapies that target these aspects of addiction, but there has not been a major advancement in treatment options. Given what is known about the effects of MOR activation on glutamatergic transmission within the mesolimbic dopamine system, some ideas for targets emerge (see Figures 1 and 2).

- Extracellular glutamate levels are increased in NAc and VTA during opioid withdrawal.
- NMDAR levels/function is increased in both the NAc and VTA with chronic opioids.
- MOR-expressing neurons become hyperexcitable with opioid withdrawal.
- GluR1 AMPAR subunits decrease in NAc and increase in VTA with chronic opioids.

Some compounds that act on these targets and have shown some promise in the treatment of addiction include:

- *Topiramate/Lamotrigine* – Used therapeutically as anticonvulsants and mood stabilizers. Mechanisms of action include inhibition of voltage-gated Na^+ and Ca^{2+} channels and activation of GABA_A receptors (Rogawski and Loscher, 2004). May also block GluR5-containing AMPARs. Showed some promise as an adjunct during detoxification in a small study (Zullino et al., 2004).
- *Lacosamide* – Used therapeutically as an anticonvulsant. Mechanism of action is to enhance slow inactivation of voltage-gated Na^+ channels (Beyreuther et al., 2007). Reduces the reward-related effects of cocaine at doses that do not impact motor capacity (Beguin et al., 2012).
- *Memantine* – Used to treat cognitive decline in Alzheimer's patients. Primary mechanism of action is as a noncompetitive NMDAR antagonist. Reduced expression of naloxone-precipitated physical withdrawal signs in heroin-dependent patients (Bisaga et al., 2001).

None of these compounds have demonstrated remarkable effects, indicating that specifically targeting glutamate transmission will not be a panacea for opioid addiction. Notably none have been tested on protracted withdrawal signs such as anxiety and depression or on conditioned withdrawal or craving.

CONCLUSION

Mu opioid receptor agonists such as morphine and heroin perturb the delicate balance of neurophysiological communication maintained by endogenous opioid peptides in the brain. The fact that a heroin “rush” or naloxone-precipitated withdrawal signs in opiate-dependent individuals can be felt within seconds of intravenous injection is evidence that the onset and offset of MOR activation can have rapid effects on cellular activity. The fact that some people develop a loss of control over opiate intake such that they engage in compulsive drug taking behaviors – despite severe negative consequences – is evidence that activation of MOR-coupled G proteins can have slower effects to alter neural circuits

regulating motivated behavior. And the fact that drug-associated cues or contexts trigger relapse at some point in almost all opiate addicts trying to stay abstinent is evidence that activation of MOR-coupled G proteins facilitates long-lasting synaptic plasticity that maintains drug-related memories. As discussed in this review, this constellation of MOR effects stems in a large part from crosstalk between MOR-associated G protein signaling and glutamatergic neurotransmission.

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REFERENCES

- Aghajanian, G. K., Kogan, J. H., and Moghaddam, B. (1994). Opiate withdrawal increases glutamate and aspartate efflux in the locus coeruleus: an in vivo microdialysis study. *Brain Res.* 636, 126–130. doi: 10.1016/0006-8993(94)90186-4
- Alderson, H. L., Parkinson, J. A., Robbins, T. W., and Everitt, B. J. (2001). The effects of excitotoxic lesions of the nucleus accumbens core or shell regions on intravenous heroin self-administration in rats. *Psychopharmacology (Berl.)* 153, 455–463. doi: 10.1007/s002130000634
- Anggono, V., and Huganir, R. L. (2012). Regulation of AMPA receptor trafficking and synaptic plasticity. *Curr. Opin. Neurobiol.* 22, 461–469. doi: 10.1016/j.conb.2011.12.006
- Badiani, A., Belin, D., Epstein, D., Calu, D., and Shaham, Y. (2011). Opiate versus psychostimulant addiction: the differences do matter. *Nat. Rev. Neurosci.* 12, 685–700. doi: 10.1038/nrn3104
- Bailey, C. P., and Connor, M. (2005). Opioids: cellular mechanisms of tolerance and physical dependence. *Curr. Opin. Pharmacol.* 5, 60–68. doi: 10.1016/j.coph.2004.08.012
- Bajo, M., Crawford, E. F., Roberto, M., Madamba, S. G., and Siggins, G. R. (2006). Chronic morphine treatment alters expression of N-methyl-D-aspartate receptor subunits in the extended amygdala. *J. Neurosci. Res.* 83, 532–537. doi: 10.1002/jnr.20756
- Beattie, E. C., Carroll, R. C., Yu, X., Morishita, W., Yasuda, H., Von Zastrow, M., et al. (2000). Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nat. Neurosci.* 3, 1291–1300. doi: 10.1038/81823
- Becerra, L., Breiter, H. C., Wise, R., Gonzalez, R. G., and Borsook, D. (2001). Reward circuitry activation by noxious thermal stimuli. *Neuron* 32, 927–946. doi: 10.1016/S0896-6273(01)00533-5
- Beguin, C., Potter, D. N., Carlezon, W. A. Jr., Stohr, T., and Cohen, B. M. (2012). Effects of the anticonvulsant lacosamide compared to valproate and lamotrigine on cocaine-enhanced reward in rats. *Brain Res.* 1479, 44–51. doi: 10.1016/j.brainres.2012.08.030
- Berendse, H. W., Galis-De Graaf, Y., and Groenewegen, H. J. (1992). Topographical organization and relationship with ventral striatal compartments of prefrontal corticostriatal projections in the rat. *J. Comp. Neurol.* 316, 314–347. doi: 10.1002/cne.903160305
- Berendse, H. W., and Groenewegen, H. J. (1990). Organization of the thalamostriatal projections in the rat, with special emphasis on the ventral striatum. *J. Comp. Neurol.* 299, 187–228. doi: 10.1002/cne.902990206
- Berridge, K. C., and Robinson, T. E. (1998). What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Res. Brain Res. Rev.* 28, 309–369. doi: 10.1016/S0165-0173(98)00019-8
- Beyreuther, B. K., Freitag, J., Heers, C., Krebsfanger, N., Scharfenecker, U., and Stohr, T. (2007). Lacosamide: a review of preclinical properties. *CNS Drug Rev.* 13, 21–42. doi: 10.1111/j.1527-3458.2007.00001.x
- Bisaga, A., Comer, S. D., Ward, A. S., Popik, P., Kleber, H. D., and Fischman, M. W. (2001). The NMDA antagonist memantine attenuates the expression of opioid physical dependence in humans. *Psychopharmacology (Berl.)* 157, 1–10. doi: 10.1007/s002130100739
- Bonci, A., and Williams, J. T. (1996). A common mechanism mediates long-term changes in synaptic transmission after chronic cocaine and morphine. *Neuron* 16, 631–639. doi: 10.1016/S0896-6273(00)80082-3
- Bonci, A., and Williams, J. T. (1997). Increased probability of GABA release during withdrawal from morphine. *J. Neurosci.* 17, 796–803.
- Bossert, J. M., Gray, S. M., Lu, L., and Shaham, Y. (2006). Activation of group II metabotropic glutamate receptors in the nucleus accumbens shell attenuates context-induced relapse to heroin seeking. *Neuropsychopharmacology* 31, 2197–2209. doi: 10.1038/sj.npp.1300977
- Bossert, J. M., Liu, S. Y., Lu, L., and Shaham, Y. (2004). A role of ventral tegmental area glutamate in contextual cue-induced relapse to heroin seeking. *J. Neurosci.* 24, 10726–10730. doi: 10.1523/JNEUROSCI.3207-04.2004
- Bossert, J. M., Stern, A. L., Theberge, F. R., Cifani, C., Koya, E., Hope, B. T., et al. (2011). Ventral medial prefrontal cortex neuronal ensembles mediate context-induced relapse to heroin. *Nat. Neurosci.* 14, 420–422. doi: 10.1038/nn.2758
- Bossert, J. M., Stern, A. L., Theberge, F. R., Marchant, N. J., Wang, H. L., Morales, M., et al. (2012). Role of projections from ventral medial prefrontal cortex to nucleus accumbens shell in context-induced reinstatement of heroin seeking. *J. Neurosci.* 32, 4982–4991. doi: 10.1523/JNEUROSCI.0005-12.2012
- Botreau, F., Paolone, G., and Stewart, J. (2006). d-Cycloserine facilitates extinction of a cocaine-induced conditioned place preference. *Behav. Brain Res.* 172, 173–178. doi: 10.1016/j.bbr.2006.05.012
- Bourdy, R., and Barrot, M. (2012). A new control center for dopaminergic systems: pulling the VTA by the tail. *Trends Neurosci.* 35, 681–690. doi: 10.1016/j.tins.2012.06.007
- Bozarth, M. A. (1987). Neuroanatomical boundaries of the reward-relevant opiate-receptor field in the ventral tegmental area as mapped by the conditioned place preference method in rats. *Brain Res.* 414, 77–84. doi: 10.1016/0006-8993(87)91327-8
- Bozarth, M. A., and Wise, R. A. (1981). Heroin reward is dependent on a dopaminergic substrate. *Life Sci.* 29, 1881–1886. doi: 10.1016/0024-3205(81)90519-1
- Bozarth, M. A., and Wise, R. A. (1983). Neural substrates of opiate reinforcement. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 7, 569–575. doi: 10.1016/0278-5846(83)90027-1
- Brebner, K., Wong, T. P., Liu, L., Liu, Y., Campsall, P., Gray, S., et al. (2005). Nucleus accumbens long-term depression and the expression of behavioral sensitization. *Science* 310, 1340–1343. doi: 10.1126/science.1116894
- Britt, M. D., and Wise, R. A. (1983). Ventral tegmental site of opiate reward: antagonism by a hydrophilic opiate receptor blocker. *Brain Res.* 258, 105–108. doi: 10.1016/0006-8993(83)91232-5
- Broekkamp, C. L., Van Den Bogaard, J. H., Heijnen, H. J., Rops, R. H., Cools, A. R., and Van Rossum, J. M. (1976). Separation of inhibiting and stimulating effects of morphine on self-stimulation behaviour by intracerebral microinjections. *Eur. J. Pharmacol.* 36, 443–446. doi: 10.1016/0014-2999(76)90099-6
- Brog, J. S., Salyapongse, A., Deutch, A. Y., and Zahm, D. S. (1993). The patterns of afferent innervation of the core and shell in the “accumbens” part of the rat ventral striatum: immunohistochemical detection of retrogradely transported fluoro-gold. *J. Comp. Neurol.* 338, 255–278. doi: 10.1002/cne.903380209
- Brown, M. T., Bellone, C., Mameli, M., Labouebe, G., Bocklisch, C., Balland, B., et al. (2010). Drug-driven AMPA receptor redistribution mimicked by selective dopamine neuron stimulation. *PLoS ONE* 5:e15870. doi: 10.1371/journal.pone.0015870
- Burnashev, N., Monyer, H., Seeburg, P. H., and Sakmann, B. (1992). Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8, 189–198. doi: 10.1016/0896-6273(92)90120-3
- Carelli, R. M. (2002). The nucleus accumbens and reward: neurophysiological investigations in behaving animals. *Behav. Cogn. Neurosci. Rev.* 1, 281–296. doi: 10.1177/1534582302238338
- Carlezon, W. A. Jr., Boundy, V. A., Haile, C. N., Lane, S. B., Kalb, R. G., Neve, R. L., et al. (1997). Sensitization to morphine induced by viral-mediated gene transfer. *Science* 277, 812–814. doi: 10.1126/science.277.5327.812
- Carlezon, W. A. Jr., Haile, C. N., Coppersmith, R., Hayashi, Y., Malinow, R., Neve, R. L., et al. (2000). Distinct sites of opiate reward and aversion within the midbrain identified using a herpes simplex virus vector expressing GluR1. *J. Neurosci.* 20, RC62.
- Carlezon, W. A. Jr., and Nestler, E. J. (2002). Elevated levels of GluR1 in the midbrain: a trigger for sensitization to drugs of abuse? *Trends Neurosci.* 25, 610–615. doi: 10.1016/S0166-2236(02)02289-0

- Carlezon, W. A. Jr., and Thomas, M. J. (2009). Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. *Neuropharmacology* 56(Suppl. 1), 122–132. doi: 10.1016/j.neuropharm.2008.06.075
- Carlezon, W. A. Jr., and Wise, R. A. (1996a). Microinjections of phencyclidine (PCP) and related drugs into nucleus accumbens shell potentiate medial forebrain bundle brain stimulation reward. *Psychopharmacologia* 128, 413–420. doi: 10.1007/s002130050151
- Carlezon, W. A. Jr., and Wise, R. A. (1996b). Rewarding actions of phencyclidine and related drugs in nucleus accumbens shell and frontal cortex. *J. Neurosci.* 16, 3112–3122.
- Carr, D. B., and Sesack, S. R. (2000). Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J. Neurosci.* 20, 3864–3873.
- Chartoff, E. H., Barhight, M. F., Magne, S. D., Sawyer, A. M., and Carlezon, W. A. Jr. (2009). Anatomically dissociable effects of dopamine D1 receptor agonists on reward and relief of withdrawal in morphine-dependent rats. *Psychopharmacology (Berl.)* 204, 227–239. doi: 10.1007/s00213-008-1454-7
- Chartoff, E. H., Magne, S. D., Barhight, M. F., Smith, A. M., and Carlezon, W. A. Jr. (2006). Behavioral and molecular effects of dopamine D1 receptor stimulation during naloxone-precipitated morphine withdrawal. *J. Neurosci.* 26, 6450–6457. doi: 10.1523/JNEUROSCI.0491-06.2006
- Chartoff, E. H., Papadopoulou, M., Konradi, C., and Carlezon, W. A. Jr. (2003a). Dopamine-dependent increases in phosphorylation of cAMP response element binding protein (CREB) during precipitated morphine withdrawal in primary cultures of rat striatum. *J. Neurochem.* 87, 107–118. doi: 10.1046/j.1471-4159.2003.01992.x
- Chartoff, E. H., Papadopoulou, M., Konradi, C., and Carlezon, W. A. Jr. (2003b). Effects of naloxone-precipitated morphine withdrawal on glutamate-mediated signaling in striatal neurons in vitro. *Ann. N. Y. Acad. Sci.* 1003, 368–371. doi: 10.1196/annals.1300.028
- Chen, Q., and Reiner, A. (1996). Cellular distribution of the NMDA receptor NR2A/2B subunits in the rat striatum. *Brain Res.* 743, 346–352. doi: 10.1016/S0006-8993(96)01098-0
- Chen, Y., Mestek, A., Liu, J., and Yu, L. (1993). Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. *Biochem. J.* 295(Pt 3), 625–628.
- Cherubini, E., Herrling, P. L., Lanfumey, L., and Stanzione, P. (1988). Excitatory amino acids in synaptic excitation of rat striatal neurones in vitro. *J. Physiol.* 400, 677–690.
- Chieng, B., and Williams, J. T. (1998). Increased opioid inhibition of GABA release in nucleus accumbens during morphine withdrawal. *J. Neurosci.* 18, 7033–7039.
- Childers, S. R. (1991). Opioid receptor-coupled second messenger systems. *Life Sci.* 48, 1991–2003. doi: 10.1016/0024-3205(91)90154-4
- Citri, A., and Malenka, R. C. (2008). Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 33, 18–41. doi: 10.1038/sj.npp.1301559
- Clem, R. L., and Barth, A. (2006). Pathway-specific trafficking of native AMPARs by in vivo experience. *Neuron* 49, 663–670. doi: 10.1016/j.neuron.2006.01.019
- Contarino, A., and Papaleo, F. (2005). The corticotropin-releasing factor receptor-1 pathway mediates the negative affective states of opiate withdrawal. *Proc. Natl. Acad. Sci. U.S.A.* 102, 18649–18654. doi: 10.1073/pnas.0506999102
- David, V., and Cazala, P. (2000). Anatomical and pharmacological specificity of the rewarding effect elicited by microinjections of morphine into the nucleus accumbens of mice. *Psychopharmacology (Berl.)* 150, 24–34. doi: 10.1007/s002130000425
- Denier, N., Gerber, H., Vogel, M., Klarhofer, M., Riecher-Rossler, A., Wiesbeck, G. A., et al. (2013). Reduction in cerebral perfusion after heroin administration: a resting state arterial spin labeling study. *PLoS ONE* 8:e71461. doi: 10.1371/journal.pone.0071461
- Desole, M. S., Esposito, G., Fresu, L., Micheli, R., Enrico, P., Mura, M. A., et al. (1996). Effects of morphine treatment and withdrawal on striatal and limbic monoaminergic activity and ascorbic acid oxidation in the rat. *Brain Res.* 723, 154–161. doi: 10.1016/0006-8993(96)00235-1
- Devine, D. P., and Wise, R. A. (1994). Self-administration of morphine, DAMGO, and DPDPPE into the ventral tegmental area of rats. *J. Neurosci.* 14, 1978–1984.
- Diana, M., Pistis, M., Muntoni, A., and Gessa, G. (1995). Profound decrease of mesolimbic dopaminergic neuronal activity in morphine withdrawn rats. *J. Pharmacol. Exp. Ther.* 272, 781–785.
- Di Chiara, G., and Imperato, A. (1988a). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U.S.A.* 85, 5274–5278. doi: 10.1073/pnas.85.14.5274
- Di Chiara, G., and Imperato, A. (1988b). Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. *J. Pharmacol. Exp. Ther.* 244, 1067–1080.
- Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999). The glutamate receptor ion channels. *Pharmacol. Rev.* 51, 7–61.
- Dole, V. P., Nyswander, M. E., and Kreek, M. J. (1966). Narcotic blockade. *Arch. Intern. Med.* 118, 304–309. doi: 10.1001/archinte.1966.00290160004002
- Duman, R. S., Tallman, J. F., and Nestler, E. J. (1988). Acute and chronic opiate regulation of adenylate cyclase in brain: specific effects in locus coeruleus. *J. Pharmacol. Exp. Ther.* 246, 1033–1039.
- Dunah, A. W., Yasuda, R. P., Luo, J., Wang, Y., Prybylowski, K. L., and Wolfe, B. B. (1999). Biochemical studies of the structure and function of the N-methyl-D-aspartate subtype of glutamate receptors. *Mol. Neurobiol.* 19, 151–179. doi: 10.1007/BF02743658
- Dworkin, S. I., Guerin, G. F., Goeders, N. E., and Smith, J. E. (1988). Kainic acid lesions of the nucleus accumbens selectively attenuate morphine self-administration. *Pharmacol. Biochem. Behav.* 29, 175–181. doi: 10.1016/0091-3057(88)90292-4
- Enrico, P., Mura, M. A., Esposito, G., Serra, P., Micheli, R., De Natale, G., et al. (1998). Effect of naloxone on morphine-induced changes in striatal dopamine metabolism and glutamate, ascorbic acid and uric acid release in freely moving rats. *Brain Res.* 797, 94–102. doi: 10.1016/S0006-8993(98)00371-0
- Epstein, D. H., Preston, K. L., Stewart, J., and Shaham, Y. (2006). Toward a model of drug relapse: an assessment of the validity of the reinstatement procedure. *Psychopharmacology (Berl.)* 189, 1–16. doi: 10.1007/s00213-006-0529-6
- Esteban, J. A., Shi, S. H., Wilson, C., Nuriya, M., Huganir, R. L., and Malinow, R. (2003). PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nat. Neurosci.* 6, 136–143. doi: 10.1038/nn997
- Fields, H. L., Hjelmstad, G. O., Margolis, E. B., and Nicola, S. M. (2007). Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu. Rev. Neurosci.* 30, 289–316. doi: 10.1146/annurev.neuro.30.051606.094341
- Fitzgerald, L. W., Ortiz, J., Hamedani, A. G., and Nestler, E. J. (1996). Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. *J. Neurosci.* 16, 274–282.
- Floresco, S. B., West, A. R., Ash, B., Moore, H., and Grace, A. A. (2003). Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat. Neurosci.* 6, 968–973. doi: 10.1038/nn1103
- Fudala, P. J., Bridge, T. P., Herbert, S., Williford, W. O., Chiang, C. N., Jones, K., et al. (2003). Office-based treatment of opiate addiction with a sublingual-tablet formulation of buprenorphine and naloxone. *N. Engl. J. Med.* 349, 949–958. doi: 10.1056/NEJMoa022164
- Garzon, M., and Pickel, V. M. (2001). Plasmalemmal mu-opioid receptor distribution mainly in nondopaminergic neurons in the rat ventral tegmental area. *Synapse* 41, 311–328. doi: 10.1002/syn.1088
- Georges, F., Le Moine, C., and Aston-Jones, G. (2006). No effect of morphine on ventral tegmental dopamine neurons during withdrawal. *J. Neurosci.* 26, 5720–5726. doi: 10.1523/JNEUROSCI.5032-05.2006
- Georges, F., Stinus, L., Bloch, B., and Le Moine, C. (1999). Chronic morphine exposure and spontaneous withdrawal are associated with modifications of dopamine receptor and neuropeptide gene expression in the rat striatum. *Eur. J. Neurosci.* 11, 481–490. doi: 10.1046/j.1460-9568.1999.00462.x
- Gerfen, C. R. (1992). The neostriatal mosaic: multiple levels of compartmental organization. *Trends Neurosci.* 15, 133–139. doi: 10.1016/0166-2236(92)90355-C
- Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., et al. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* 250, 1429–1432. doi: 10.1126/science.2147780
- Gerrits, M. A., and Van Ree, J. M. (1996). Effect of nucleus accumbens dopamine depletion on motivational aspects involved in initiation of cocaine and heroin self-administration in rats. *Brain Res.* 713, 114–124. doi: 10.1016/0006-8993(95)01491-8

- Giacchino, J. L., and Henriksen, S. J. (1996). Systemic morphine and local opioid effects on neuronal activity in the medial prefrontal cortex. *Neuroscience* 70, 941–949. doi: 10.1016/0306-4522(95)00409-2
- Glass, M. J., Lane, D. A., Colago, E. E., Chan, J., Schlussman, S. D., Zhou, Y., et al. (2008). Chronic administration of morphine is associated with a decrease in surface AMPA GluR1 receptor subunit in dopamine D1 receptor expressing neurons in the shell and non-D1 receptor expressing neurons in the core of the rat nucleus accumbens. *Exp. Neurol.* 210, 750–761. doi: 10.1016/j.expneurol.2008.01.012
- Glass, M. J., Vanyo, L., Quimson, L., and Pickel, V. M. (2009). Ultrastructural relationship between N-methyl-D-aspartate-NR1 receptor subunit and mu-opioid receptor in the mouse central nucleus of the amygdala. *Neuroscience* 163, 857–867. doi: 10.1016/j.neuroscience.2009.07.020
- Goeders, N. E., Lane, J. D., and Smith, J. E. (1984). Self-administration of methionine enkephalin into the nucleus accumbens. *Pharmacol. Biochem. Behav.* 20, 451–455. doi: 10.1016/0091-3057(84)90284-3
- Gonzalez, P., Cabello, P., Germany, A., Norris, B., and Contreras, E. (1997). Decrease of tolerance to, and physical dependence on morphine by glutamate receptor antagonists. *Eur. J. Pharmacol.* 332, 257–262. doi: 10.1016/S0014-2999(97)01099-6
- Grace, A. A., Floresco, S. B., Goto, Y., and Lodge, D. J. (2007). Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci.* 30, 220–227. doi: 10.1016/j.tins.2007.03.003
- Gracy, K. N., Svingos, A. L., and Pickel, V. M. (1997). Dual ultrastructural localization of mu-opioid receptors and NMDA-type glutamate receptors in the shell of the rat nucleus accumbens. *J. Neurosci.* 17, 4839–4848.
- Groenewegen, H. J., Vermeulen-Van Der Zee, E., Te Kortschot, A., and Witter, M. P. (1987). Organization of the projections from the subiculum to the ventral striatum in the rat. A study using anterograde transport of *Phaseolus vulgaris* leucoagglutinin. *Neuroscience* 23, 103–120. doi: 10.1016/0306-4522(87)90275-2
- Gysling, K., and Wang, R. Y. (1983). Morphine-induced activation of A10 dopamine neurons in the rat. *Brain Res.* 277, 119–127. doi: 10.1016/0006-8993(83)90913-7
- Haber, S. N., Fudge, J. L., and McFarland, N. R. (2000). Striatonigrostriatal pathways in primates form an ascending spiral from the shell to the dorsolateral striatum. *J. Neurosci.* 20, 2369–2382.
- Haber, S. N., and Knutson, B. (2010). The reward circuit: linking primate anatomy and human imaging. *Neuropsychopharmacology* 35, 4–26. doi: 10.1038/npp.2009.129
- Haber, S. N., Lynd, E., Klein, C., and Groenewegen, H. J. (1990). Topographic organization of the ventral striatal efferent projections in the rhesus monkey: an anterograde tracing study. *J. Comp. Neurol.* 293, 282–298. doi: 10.1002/cne.902930210
- Harris, G. C., and Aston-Jones, G. (1994). Involvement of D2 dopamine receptors in the nucleus accumbens in the opiate withdrawal syndrome. *Nature* 371, 155–157. doi: 10.1038/371155a0
- Harris, G. C., Wimmer, M., Byrne, R., and Aston-Jones, G. (2004). Glutamate-associated plasticity in the ventral tegmental area is necessary for conditioning environmental stimuli with morphine. *Neuroscience* 129, 841–847. doi: 10.1016/j.neuroscience.2004.09.018
- Heather, N., Stallard, A., and Tebbutt, J. (1991). Importance of substance cues in relapse among heroin users: comparison of two methods of investigation. *Addict. Behav.* 16, 41–49. doi: 10.1016/0306-4603(91)90038-J
- Heine, M., Groc, L., Frischknecht, R., Beique, J. C., Lounis, B., Rumbaugh, G., et al. (2008). Surface mobility of postsynaptic AMPARs tunes synaptic transmission. *Science* 320, 201–205. doi: 10.1126/science.1152089
- Herrold, A. A., Persons, A. L., and Napier, T. C. (2013). Cellular distribution of AMPA receptor subunits and mGlu5 following acute and repeated administration of morphine or methamphetamine. *J. Neurochem.* 126, 503–517. doi: 10.1111/jnc.12323
- Hokfelt, T., Broberger, C., Xu, Z. Q., Sergeyev, V., Ubink, R., and Diez, M. (2000). Neuropeptides – an overview. *Neuropharmacology* 39, 1337–1356. doi: 10.1016/S0028-3908(00)00010-1
- Hollmann, M., Hartley, M., and Heinemann, S. (1991). Ca^{2+} permeability of KA-AMPA – gated glutamate receptor channels depends on subunit composition. *Science* 252, 851–853. doi: 10.1126/science.1709304
- Hollmann, M., and Heinemann, S. (1994). Cloned glutamate receptors. *Annu. Rev. Neurosci.* 17, 31–108. doi: 10.1146/annurev.ne.17.030194.000335
- Hu, X. T., and White, F. J. (1996). Glutamate receptor regulation of rat nucleus accumbens neurons in vivo. *Synapse* 23, 208–218. doi: 10.1002/(SICI)1098-2396(199607)23:3<208::AID-SYN10>3.0.CO;2-V
- Hyman, S. E., Malenka, R. C., and Nestler, E. J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu. Rev. Neurosci.* 29, 565–598. doi: 10.1146/annurev.neuro.29.051605.113009
- Ikemoto, S. (2007). Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res. Rev.* 56, 27–78. doi: 10.1016/j.brainresrev.2007.05.004
- Ikemoto, S., and Panksepp, J. (1999). The role of nucleus accumbens dopamine in motivated behavior: a unifying interpretation with special reference to reward-seeking. *Brain Res. Brain Res. Rev.* 31, 6–41. doi: 10.1016/S0165-0173(99)00023-5
- Inoue, M., Mishina, M., and Ueda, H. (2003). Locus-specific rescue of GluRepsilon1 NMDA receptors in mutant mice identifies the brain regions important for morphine tolerance and dependence. *J. Neurosci.* 23, 6529–6536.
- Jacobs, E. H., Wardeh, G., Smit, A. B., and Schoffelmeer, A. N. (2005). Morphine causes a delayed increase in glutamate receptor functioning in the nucleus accumbens core. *Eur. J. Pharmacol.* 511, 27–30. doi: 10.1016/j.ejphar.2005.02.009
- Jalabert, M., Bourdy, R., Courtin, J., Veinante, P., Manzoni, O. J., Barrot, M., et al. (2011). Neuronal circuits underlying acute morphine action on dopamine neurons. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16446–16450. doi: 10.1073/pnas.1105418108
- Jensen, J., McIntosh, A. R., Crawley, A. P., Mikulis, D. J., Remington, G., and Kapur, S. (2003). Direct activation of the ventral striatum in anticipation of aversive stimuli. *Neuron* 40, 1251–1257. doi: 10.1016/S0896-6273(03)00724-4
- Jhou, T. C., Geisler, S., Marinelli, M., Degarmo, B. A., and Zahm, D. S. (2009). The mesopontine rostromedial tegmental nucleus: a structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. *J. Comp. Neurol.* 513, 566–596. doi: 10.1002/cne.21891
- Johnson, S. W., and North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J. Neurosci.* 12, 483–488.
- Johnston, L. D., O’Malley, P. M., Miech, R. A., Bachman, J. G., and Schulenberg, J. E. (2014). *Monitoring the Future National Survey Results on Drug Use: 2013 Overview, Key Findings on Adolescent Drug Use*. Ann Arbor: University of Michigan.
- Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T. J., Adams, S. R., et al. (2004). Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat. Neurosci.* 7, 244–253. doi: 10.1038/nn1189
- Kalivas, P. W. (2009). The glutamate homeostasis hypothesis of addiction. *Nat. Rev. Neurosci.* 10, 561–572. doi: 10.1038/nrn2515
- Kalivas, P. W., Lalumiere, R. T., Knackstedt, L., and Shen, H. (2009). Glutamate transmission in addiction. *Neuropharmacology* 56(Suppl. 1), 169–173. doi: 10.1016/j.neuropharm.2008.07.011
- Kauer, J. A., and Malenka, R. C. (2007). Synaptic plasticity and addiction. *Nat. Rev. Neurosci.* 8, 844–858. doi: 10.1038/nrn2234
- Kawaguchi, Y., Wilson, C. J., Augood, S. J., and Emson, P. C. (1995). Striatal interneurons: chemical, physiological and morphological characterization. *Trends Neurosci.* 18, 527–535. doi: 10.1016/0166-2236(95)98374-8
- Kelley, A. E., and Domescick, V. B. (1982). The distribution of the projection from the hippocampal formation to the nucleus accumbens in the rat: an anterograde and retrograde-horseradish peroxidase study. *Neuroscience* 7, 2321–2335. doi: 10.1016/0306-4522(82)90198-1
- Kelley, A. E., Domescick, V. B., and Nauta, W. J. (1982). The amygdalostriatal projection in the rat – an anatomical study by anterograde and retrograde tracing methods. *Neuroscience* 7, 615–630. doi: 10.1016/0306-4522(82)90067-7
- Kerchner, G. A., and Nicoll, R. A. (2008). Silent synapses and the emergence of a postsynaptic mechanism for LTP. *Nat. Rev. Neurosci.* 9, 813–825. doi: 10.1038/nrn2501
- Kessels, H. W., and Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. *Neuron* 61, 340–350. doi: 10.1016/j.neuron.2009.01.015
- Khan, Z. U., Gutierrez, A., Martin, R., Penafiel, A., Rivera, A., and De La Calle, A. (2000). Dopamine D5 receptors of rat and human brain. *Neuroscience* 100, 689–699. doi: 10.1016/S0306-4522(00)00274-8
- Konradi, C., Cole, R. L., Heckers, S., and Hyman, S. E. (1994). Amphetamine regulates gene expression in rat striatum via transcription factor CREB. *J. Neurosci.* 14, 5623–5634.

- Konradi, C., Leveque, J. C., and Hyman, S. E. (1996). Amphetamine and dopamine-induced immediate early gene expression in striatal neurons depends on postsynaptic NMDA receptors and calcium. *J. Neurosci.* 16, 4231–4239.
- Koob, G. F. (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol. Sci.* 13, 177–184. doi: 10.1016/0165-6147(92)90060-J
- Koob, G. F. (2009). Neurobiological substrates for the dark side of compulsion in addiction. *Neuropharmacology* 56(Suppl. 1), 18–31. doi: 10.1016/j.neuropharm.2008.07.043
- Koob, G. F., Maldonado, R., and Stinus, L. (1992). Neural substrates of opiate withdrawal. *Trends Neurosci.* 15, 186–191. doi: 10.1016/0166-2236(92)90171-4
- Kosel, M., Noss, R. S., Hammig, R., Wielepp, P., Bundeli, P., Heidbreder, R., et al. (2008). Cerebral blood flow effects of acute intravenous heroin administration. *Eur. Neuropsychopharmacol.* 18, 278–285. doi: 10.1016/j.euroneuro.2007.11.007
- Kreek, M. J., and Koob, G. F. (1998). Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend.* 51, 23–47. doi: 10.1016/S0376-8716(98)00064-7
- Kuppenbender, K. D., Standaert, D. G., Feuerstein, T. J., Penney, J. B. Jr., Young, A. B., and Landwehrmeyer, G. B. (2000). Expression of NMDA receptor subunit mRNAs in neurochemically identified projection and interneurons in the human striatum. *J. Comp. Neurol.* 419, 407–421. doi: 10.1002/(SICI)1096-9861(20000417)419:4<407::AID-CNE1>3.0.CO;2-I
- LaLumiere, R. T., and Kalivas, P. W. (2008). Glutamate release in the nucleus accumbens core is necessary for heroin seeking. *J. Neurosci.* 28, 3170–3177. doi: 10.1523/JNEUROSCI.5129-07.2008
- Lane, D. A., Lessard, A. A., Chan, J., Colago, E. E., Zhou, Y., Schlussman, S. D., et al. (2008). Region-specific changes in the subcellular distribution of AMPA receptor GluR1 subunit in the rat ventral tegmental area after acute or chronic morphine administration. *J. Neurosci.* 28, 9670–9681. doi: 10.1523/JNEUROSCI.2151-08.2008
- Law, P. Y., Wong, Y. H., and Loh, H. H. (2000). Molecular mechanisms and regulation of opioid receptor signaling. *Annu. Rev. Pharmacol. Toxicol.* 40, 389–430. doi: 10.1146/annurev.pharmtox.40.1.389
- Le Merrer, J., Becker, J. A., Befort, K., and Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412. doi: 10.1152/physrev.00005.2009
- Leri, F., Flores, J., Rajabi, H., and Stewart, J. (2003). Effects of cocaine in rats exposed to heroin. *Neuropsychopharmacology* 28, 2102–2116. doi: 10.1038/sj.npp.1300284
- Li, S. X., Shi, J., Epstein, D. H., Wang, X., Zhang, X. L., Bao, Y. P., et al. (2009). Circadian alteration in neurobiology during 30 days of abstinence in heroin users. *Biol. Psychiatry* 65, 905–912. doi: 10.1016/j.biopsych.2008.11.025
- Liu, S. Q., and Cull-Candy, S. G. (2000). Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. *Nature* 405, 454–458. doi: 10.1038/35013064
- Lobo, M. K. (2009). Molecular profiling of striatonigral and striatopallidal medium spiny neurons past, present, and future. *Int. Rev. Neurobiol.* 89, 1–35. doi: 10.1016/S0074-7742(09)89001-6
- Lodge, D. J., and Grace, A. A. (2006). The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5167–5172. doi: 10.1073/pnas.0510715103
- London, E. D., Broussolle, E. P., Links, J. M., Wong, D. F., Cascella, N. G., Daniels, R. F., et al. (1990). Morphine-induced metabolic changes in human brain. Studies with positron emission tomography and [fluorine 18]fluorodeoxyglucose. *Arch. Gen. Psychiatry* 47, 73–81. doi: 10.1001/archpsyc.1990.01810130075010
- Lu, X. Y., Ghasemzadeh, M. B., and Kalivas, P. W. (1998). Expression of D1 receptor, D2 receptor, substance P and enkephalin messenger RNAs in the neurons projecting from the nucleus accumbens. *Neuroscience* 82, 767–780. doi: 10.1016/S0306-4522(97)00327-8
- Luscher, C. (2013). Cocaine-evoked synaptic plasticity of excitatory transmission in the ventral tegmental area. *Cold Spring Harb. Perspect. Med.* 3, a012013. doi: 10.1101/cshperspect.a012013
- Magura, S., and Rosenblum, A. (2001). Leaving methadone treatment: lessons learned, lessons forgotten, lessons ignored. *Mt. Sinai J. Med.* 68, 62–74.
- Mahler, S. V., Smith, R. J., Moorman, D. E., Sartor, G. C., and Aston-Jones, G. (2012). Multiple roles for orexin/hypocretin in addiction. *Prog. Brain Res.* 198, 79–121. doi: 10.1016/B978-0-444-59489-1.00007-0
- Malinow, R., and Malenka, R. C. (2002). AMPA receptor trafficking and synaptic plasticity. *Annu. Rev. Neurosci.* 25, 103–126. doi: 10.1146/annurev.neuro.25.112701.142758
- Mangiavacchi, S., and Wolf, M. E. (2004). D1 dopamine receptor stimulation increases the rate of AMPA receptor insertion onto the surface of cultured nucleus accumbens neurons through a pathway dependent on protein kinase A. *J. Neurochem.* 88, 1261–1271. doi: 10.1046/j.1471-4159.2003.02248.x
- Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995a). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 18, 22–29. doi: 10.1016/0166-2236(95)93946-U
- Mansour, A., Fox, C. A., Burke, S., Akil, H., and Watson, S. J. (1995b). Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. *J. Chem. Neuroanat.* 8, 283–305. doi: 10.1016/0891-0618(95)00055-C
- Mansour, A., Fox, C. A., Thompson, R. C., Akil, H., and Watson, S. J. (1994). mu-Opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. *Brain Res.* 643, 245–265. doi: 10.1016/0006-8993(94)90031-0
- Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., and Watson, S. J. (1988). Anatomy of CNS opioid receptors. *Trends Neurosci.* 11, 308–314. doi: 10.1016/0166-2236(88)90093-8
- Manzoni, O. J., and Williams, J. T. (1999). Presynaptic regulation of glutamate release in the ventral tegmental area during morphine withdrawal. *J. Neurosci.* 19, 6629–6636.
- Martin, G., Guadano-Ferraz, A., Morte, B., Ahmed, S., Koob, G. F., De Lecea, L., et al. (2004). Chronic morphine treatment alters N-methyl-D-aspartate receptors in freshly isolated neurons from nucleus accumbens. *J. Pharmacol. Exp. Ther.* 311, 265–273. doi: 10.1124/jpet.104.067504
- Martin, G., Nie, Z., and Siggins, G. R. (1997). mu-Opioid receptors modulate NMDA receptor-mediated responses in nucleus accumbens neurons. *J. Neurosci.* 17, 11–22.
- Martin, G., Przewlocki, R., and Siggins, G. R. (1999). Chronic morphine treatment selectively augments metabotropic glutamate receptor-induced inhibition of N-methyl-D-aspartate receptor-mediated neurotransmission in nucleus accumbens. *J. Pharmacol. Exp. Ther.* 288, 30–35.
- Martin, W. R., and Jasinski, D. R. (1969). Physiological parameters of morphine dependence in man – tolerance, early abstinence, protracted abstinence. *J. Psychiatr. Res.* 7, 9–17. doi: 10.1016/0022-3956(69)90007-7
- Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383, 819–823. doi: 10.1038/38319a0
- Mattick, R. P., Kimber, J., Breen, C., and Davoli, M. (2008). Buprenorphine maintenance versus placebo or methadone maintenance for opioid dependence. *Cochrane Database Syst. Rev.* CD002207. doi: 10.1002/14651858.CD002207.pub3
- Mazei-Robison, M. S., Koo, J. W., Friedman, A. K., Lansink, C. S., Robison, A. J., Vinish, M., et al. (2011). Role for mTOR signaling and neuronal activity in morphine-induced adaptations in ventral tegmental area dopamine neurons. *Neuron* 72, 977–990. doi: 10.1016/j.neuron.2011.10.012
- McFarland, K., and Kalivas, P. W. (2001). The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior. *J. Neurosci.* 21, 8655–8663.
- Mello, N. K., and Negus, S. S. (1996). Preclinical evaluation of pharmacotherapies for treatment of cocaine and opioid abuse using drug self-administration procedures. *Neuropsychopharmacology* 14, 375–424. doi: 10.1016/0893-133X(95)00274-H
- Mickiewicz, A. L., and Napier, T. C. (2011). Repeated exposure to morphine alters surface expression of AMPA receptors in the rat medial prefrontal cortex. *Eur. J. Neurosci.* 33, 259–265. doi: 10.1111/j.1460-9568.2010.07502.x
- Mink, J. W. (1996). The basal ganglia: focused selection and inhibition of competing motor programs. *Prog. Neurobiol.* 50, 381–425. doi: 10.1016/S0301-0082(96)00042-1
- Mogenson, G. J., Jones, D. L., and Yim, C. Y. (1980). From motivation to action – functional interface between the limbic system and the motor system. *Prog. Neurobiol.* 14, 69–97. doi: 10.1016/0301-0082(80)90018-0
- Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., et al. (1992). Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 256, 1217–1221. doi: 10.1126/science.256.5060.1217
- Murray, F., Harrison, N. J., Grimwood, S., Bristow, L. J., and Hutson, P. H. (2007). Nucleus accumbens NMDA receptor subunit expression and function

- is enhanced in morphine-dependent rats. *Eur. J. Pharmacol.* 562, 191–197. doi: 10.1016/j.ejphar.2007.01.027
- Myers, K. M., and Carlezon, W. A. Jr. (2010a). D-Cycloserine facilitates extinction of naloxone-induced conditioned place aversion in morphine-dependent rats. *Biol. Psychiatry* 67, 85–87. doi: 10.1016/j.biopsych.2009.08.015
- Myers, K. M., and Carlezon, W. A. Jr. (2010b). Extinction of drug- and withdrawal-paired cues in animal models: relevance to the treatment of addiction. *Neurosci. Biobehav. Rev.* 35, 285–302. doi: 10.1016/j.neubiorev.2010.01.011
- Nair-Roberts, R. G., Chatelain-Badie, S. D., Benson, E., White-Cooper, H., Bolam, J. P., and Ungless, M. A. (2008). Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. *Neuroscience* 152, 1024–1031. doi: 10.1016/j.neuroscience.2008.01.046
- Nestler, E. J., and Aghajanian, G. K. (1997). Molecular and cellular basis of addiction. *Science* 278, 58–63. doi: 10.1126/science.278.5335.58
- Nestler, E. J., and Tallman, J. F. (1988). Chronic morphine treatment increases cyclic AMP-dependent protein kinase activity in the rat locus coeruleus. *Mol. Pharmacol.* 33, 127–132.
- Niehaus, J. L., Murali, M., and Kauer, J. A. (2010). Drugs of abuse and stress impair LTP at inhibitory synapses in the ventral tegmental area. *Eur. J. Neurosci.* 32, 108–117. doi: 10.1111/j.1460-9568.2010.07256.x
- NSDUH. (2013). *Results from the 2012 National Survey on Drug Use and Health: Summary of National Findings*. Rockville: Substance Abuse and Mental Health Services Administration.
- O'Brien, C. P., Ehrman, R. N., and Ternes, J. W. (1986). “Classical conditioning in human opioid dependence,” in *Behavioral Analysis of Drug Dependence*, ed. S. Goldberg (Orlando: Academic Press), 329–356.
- Olds, M. E. (1982). Reinforcing effects of morphine in the nucleus accumbens. *Brain Res.* 237, 429–440. doi: 10.1016/0006-8993(82)90454-1
- Paolone, G., Botreau, F., and Stewart, J. (2009). The facilitative effects of D-cycloserine on extinction of a cocaine-induced conditioned place preference can be long lasting and resistant to reinstatement. *Psychopharmacology (Berl.)* 202, 403–409. doi: 10.1007/s00213-008-1280-y
- Pasternak, G. W. (2012). Preclinical pharmacology and opioid combinations. *Pain Med.* 13(Suppl. 1), S4–S11. doi: 10.1111/j.1526-4637.2012.01335.x
- Peoples, L. L., and West, M. O. (1996). Phasic firing of single neurons in the rat nucleus accumbens correlated with the timing of intravenous cocaine self-administration. *J. Neurosci.* 16, 3459–3473.
- Pettit, H. O., Ettenberg, A., Bloom, F. E., and Koob, G. F. (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. *Psychopharmacology (Berl.)* 84, 167–173. doi: 10.1007/BF00427441
- Pierce, R. C., and Wolf, M. E. (2013). Psychostimulant-induced neuroadaptations in nucleus accumbens AMPA receptor transmission. *Cold Spring Harb. Perspect. Med.* 3, a012021. doi: 10.1101/cshperspect.a012021
- Popik, P., and Kolasiewicz, W. (1999). Mesolimbic NMDA receptors are implicated in the expression of conditioned morphine reward. *Naunyn Schmiedebergs Arch. Pharmacol.* 359, 288–294. doi: 10.1007/PL00005354
- Reimers, J. M., Milovanovic, M., and Wolf, M. E. (2011). Quantitative analysis of AMPA receptor subunit composition in addiction-related brain regions. *Brain Res.* 1367, 223–233. doi: 10.1016/j.brainres.2010.10.016
- Reynolds, S. M., and Berridge, K. C. (2002). Positive and negative motivation in nucleus accumbens shell: bivalent rostrocaudal gradients for GABA-elicited eating, taste “liking”/“disliking” reactions, place preference/avoidance, and fear. *J. Neurosci.* 22, 7308–7320.
- Robbe, D., Alonso, G., Chaumont, S., Bockaert, J., and Manzoni, O. J. (2002a). Role of p/q-Ca²⁺ channels in metabotropic glutamate receptor 2/3-dependent presynaptic long-term depression at nucleus accumbens synapses. *J. Neurosci.* 22, 4346–4356.
- Robbe, D., Bockaert, J., and Manzoni, O. J. (2002b). Metabotropic glutamate receptor 2/3-dependent long-term depression in the nucleus accumbens is blocked in morphine withdrawn mice. *Eur. J. Neurosci.* 16, 2231–2235. doi: 10.1046/j.1460-9568.2002.02273.x
- Rodríguez-Munoz, M., Sanchez-Blazquez, P., Vicente-Sánchez, A., Berrocoso, E., and Garzon, J. (2012). The mu-opioid receptor and the NMDA receptor associate in PAG neurons: implications in pain control. *Neuropsychopharmacology* 37, 338–349. doi: 10.1038/npp.2011.155
- Rogawski, M. A., and Loscher, W. (2004). The neurobiology of antiepileptic drugs. *Nat. Rev. Neurosci.* 5, 553–564. doi: 10.1038/nrn1430
- Roitman, M. F., Wheeler, R. A., and Carelli, R. M. (2005). Nucleus accumbens neurons are innately tuned for rewarding and aversive taste stimuli, encode their predictors, and are linked to motor output. *Neuron* 45, 587–597. doi: 10.1016/j.neuron.2004.12.055
- Rossetti, Z. L., Hmaidan, Y., and Gessa, G. L. (1992). Marked inhibition of mesolimbic dopamine release: a common feature of ethanol, morphine, cocaine and amphetamine abstinence in rats. *Eur. J. Pharmacol.* 221, 227–234. doi: 10.1016/0014-2999(92)90706-A
- Rothwell, P. E., Thomas, M. J., and Gewirtz, J. C. (2012). Protracted manifestations of acute dependence after a single morphine exposure. *Psychopharmacology (Berl.)* 219, 991–998. doi: 10.1007/s00213-011-2425-y
- Saal, D., Dong, Y., Bonci, A., and Malenka, R. C. (2003). Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. *Neuron* 37, 577–582. doi: 10.1016/S0896-6273(03)00021-7
- Salamone, J. D., and Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. *Neuron* 76, 470–485. doi: 10.1016/j.neuron.2012.10.021
- Schildein, S., Agmo, A., Huston, J. P., and Schwarting, R. K. (1998). Intraaccumbens injections of substance P, morphine and amphetamine: effects on conditioned place preference and behavioral activity. *Brain Res.* 790, 185–194. doi: 10.1016/S0006-8993(98)00062-6
- Schilstrom, B., Yaka, R., Argilli, E., Suvarna, N., Schumann, J., Chen, B. T., et al. (2006). Cocaine enhances NMDA receptor-mediated currents in ventral tegmental area cells via dopamine D5 receptor-dependent redistribution of NMDA receptors. *J. Neurosci.* 26, 8549–8558. doi: 10.1523/JNEUROSCI.5179-05.2006
- Schlaepfer, T. E., Strain, E. C., Greenberg, B. D., Preston, K. L., Lancaster, E., Bigelow, G. E., et al. (1998). Site of opioid action in the human brain: mu and kappa agonists’ subjective and cerebral blood flow effects. *Am. J. Psychiatry* 155, 470–473.
- Schlosburg, J. E., Whitfield, T. W. Jr., Park, P. E., Crawford, E. F., George, O., Vendruscolo, L. F., et al. (2013). Long-term antagonism of kappa opioid receptors prevents escalation of and increased motivation for heroin intake. *J. Neurosci.* 33, 19384–19392. doi: 10.1523/JNEUROSCI.1979-13.2013
- Seeburg, P. H. (1993). The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci.* 16, 359–365. doi: 10.1016/0166-2236(93)90093-2
- Seeburg, P. H., Burnashev, N., Kohr, G., Kuner, T., Sprengel, R., and Monyer, H. (1995). The NMDA receptor channel: molecular design of a coincidence detector. *Recent Prog. Horm. Res.* 50, 19–34.
- Self, D. W., McClenahan, A. W., Beittner-Johnson, D., Terwilliger, R. Z., and Nestler, E. J. (1995). Biochemical adaptations in the mesolimbic dopamine system in response to heroin self-administration. *Synapse* 21, 312–318. doi: 10.1002/syn.890210405
- Sepulveda, J., Oliva, P., and Contreras, E. (2004). Neurochemical changes of the extracellular concentrations of glutamate and aspartate in the nucleus accumbens of rats after chronic administration of morphine. *Eur. J. Pharmacol.* 483, 249–258. doi: 10.1016/j.ejphar.2003.10.037
- Sepulveda, M. J., Hernandez, L., Rada, P., Tucci, S., and Contreras, E. (1998). Effect of precipitated withdrawal on extracellular glutamate and aspartate in the nucleus accumbens of chronically morphine-treated rats: an in vivo microdialysis study. *Pharmacol. Biochem. Behav.* 60, 255–262. doi: 10.1016/S0091-3057(97)00550-9
- Sesack, S. R., and Grace, A. A. (2010). Cortico-basal ganglia reward network: microcircuitry. *Neuropsychopharmacology* 35, 27–47. doi: 10.1038/npp.2009.93
- Shabat-Simon, M., Levy, D., Amir, A., Rehavi, M., and Zangen, A. (2008). Dissociation between rewarding and psychomotor effects of opiates: differential roles for glutamate receptors within anterior and posterior portions of the ventral tegmental area. *J. Neurosci.* 28, 8406–8416. doi: 10.1523/JNEUROSCI.1958-08.2008
- Shalev, U., Grimm, J. W., and Shaham, Y. (2002). Neurobiology of relapse to heroin and cocaine seeking: a review. *Pharmacol. Rev.* 54, 1–42. doi: 10.1124/pr.54.1.1
- Shaw-Lutchman, T. Z., Barrot, M., Wallace, T., Gilden, L., Zachariou, V., Impey, S., et al. (2002). Regional and cellular mapping of cAMP response element-mediated

- transcription during naltrexone-precipitated morphine withdrawal. *J. Neurosci.* 22, 3663–3672.
- Shen, H., and Kalivas, P. W. (2013). Reduced LTP and LTD in prefrontal cortex synapses in the nucleus accumbens after heroin self-administration. *Int. J. Neuropsychopharmacol.* 16, 1165–1167. doi: 10.1017/S1461145712001071
- Shen, H., Moussawi, K., Zhou, W., Toda, S., and Kalivas, P. W. (2011). Heroin relapse requires long-term potentiation-like plasticity mediated by NMDA2b-containing receptors. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19407–19412. doi: 10.1073/pnas.1112052108
- Shi, J., Li, S. X., Zhang, X. L., Wang, X., Le Foll, B., Zhang, X. Y., et al. (2009). Time-dependent neuroendocrine alterations and drug craving during the first month of abstinence in heroin addicts. *Am. J. Drug Alcohol Abuse* 35, 267–272. doi: 10.1080/00952990902933878
- Sigmon, S. C., Dunn, K. E., Saulsgiver, K., Patrick, M. E., Badger, G. J., Heil, S. H., et al. (2013). A randomized, double-blind evaluation of buprenorphine taper duration in primary prescription opioid abusers. *JAMA Psychiatry* 70, 1347–1354. doi: 10.1001/jamapsychiatry.2013.2216
- Snyder, G. L., Galdi, S., Fienberg, A. A., Allen, P., Baird, A. C., and Green-gard, P. (2003). Regulation of AMPA receptor dephosphorylation by glutamate receptor agonists. *Neuropharmacology* 45, 703–713. doi: 10.1016/S0028-3908(03)00319-8
- Sommer, B., Kohler, M., Sprengel, R., and Seeburg, P. H. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* 67, 11–19. doi: 10.1016/0092-8674(91)90568-J
- Song, I., and Huganir, R. L. (2002). Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci.* 25, 578–588. doi: 10.1016/S0166-2236(02)02270-1
- Spijkerman, S., Houtzager, S. W., De Gunst, M. C., De Boer, W. P., Schoffelmeer, A. N., and Smit, A. B. (2004). Morphine exposure and abstinence define specific stages of gene expression in the rat nucleus accumbens. *FASEB J.* 18, 848–850. doi: 10.1096/fj.03-0612fje
- Stewart, J., De Wit, H., and Eikelboom, R. (1984). Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. *Psychol. Rev.* 91, 251–268. doi: 10.1037/0033-295X.91.2.251
- Stinus, L., Le Moal, M., and Koob, G. F. (1990). Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. *Neuroscience* 37, 767–773. doi: 10.1016/0306-4522(90)90106-E
- Straub, C., and Tomita, S. (2012). The regulation of glutamate receptor trafficking and function by TARP s and other transmembrane auxiliary subunits. *Curr. Opin. Neurobiol.* 22, 488–495. doi: 10.1016/j.conb.2011.09.005
- Surmeier, D. J., Ding, J., Day, M., Wang, Z., and Shen, W. (2007). D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. *Trends Neurosci.* 30, 228–235. doi: 10.1016/j.tins.2007.03.008
- Suto, N., Wise, R. A., and Vezina, P. (2011). Dorsal as well as ventral striatal lesions affect levels of intravenous cocaine and morphine self-administration in rats. *Neurosci. Lett.* 493, 29–32. doi: 10.1016/j.neulet.2011.02.011
- Svingos, A. L., Garzon, M., Colago, E. E., and Pickel, V. M. (2001). Mu-opioid receptors in the ventral tegmental area are targeted to presynaptically and directly modulate mesocortical projection neurons. *Synapse* 41, 221–229. doi: 10.1002/syn.1079
- Swanson, L. W. (1982). The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res. Bull.* 9, 321–353. doi: 10.1016/0361-9230(82)90145-9
- Takahashi, T., Svoboda, K., and Malinow, R. (2003). Experience strengthening transmission by driving AMPA receptors into synapses. *Science* 299, 1585–1588. doi: 10.1126/science.1079886
- Tang, Y. P., Shimizu, E., Dube, G. R., Rampon, C., Kerchner, G. A., Zhuo, M., et al. (1999). Genetic enhancement of learning and memory in mice. *Nature* 401, 63–69. doi: 10.1038/43432
- Tenorio, G., Connor, S. A., Guevremont, D., Abraham, W. C., Williams, J., O'Dell, T. J., et al. (2010). 'Silent' priming of translation-dependent LTP by ss-adrenergic receptors involves phosphorylation and recruitment of AMPA receptors. *Learn. Mem.* 17, 627–638. doi: 10.1101/lm.1974510
- Tepper, J. M., Martin, L. P., and Anderson, D. R. (1995). GABA_A receptor-mediated inhibition of rat substantia nigra dopaminergic neurons by pars reticulata projection neurons. *J. Neurosci.* 15, 3092–3103.
- Terwilliger, R. Z., Beitner-Johnson, D., Sevarino, K. A., Crain, S. M., and Nestler, E. J. (1991). A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res.* 548, 100–110. doi: 10.1016/0006-8993(91)91111-D
- The National Center on Addiction and Substance Abuse, Columbia University. (2011). *Adolescent Substance Use: America's #1 Public Health Problem*. New York: Columbia University.
- Todtenkopf, M. S., Parsegian, A., Naydenov, A., Neve, R. L., Konradi, C., and Carlezon, W. A. Jr. (2006). Brain reward regulated by AMPA receptor subunits in nucleus accumbens shell. *J. Neurosci.* 26, 11665–11669. doi: 10.1523/JNEUROSCI.3070-06.2006
- Tokuyama, S., Wakabayashi, H., and Ho, I. K. (1996). Direct evidence for a role of glutamate in the expression of the opioid withdrawal syndrome. *Eur. J. Pharmacol.* 295, 123–129. doi: 10.1016/0014-2999(95)00645-1
- Trujillo, K. A. (2002). The neurobiology of opiate tolerance, dependence and sensitization: mechanisms of NMDA receptor-dependent synaptic plasticity. *Neurotox. Res.* 4, 373–391. doi: 10.1080/10298420290023954
- Trujillo, K. A., and Akil, H. (1991). Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251, 85–87. doi: 10.1126/science.1824728
- Turrigiano, G. G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell* 135, 422–435. doi: 10.1016/j.cell.2008.10.008
- Ungless, M. A., Whistler, J. L., Malenka, R. C., and Bonci, A. (2001). Single cocaine exposure *in vivo* induces long-term potentiation in dopamine neurons. *Nature* 411, 583–587. doi: 10.1038/3507907
- Vaccarino, F. J., Bloom, F. E., and Koob, G. F. (1985). Blockade of nucleus accumbens opiate receptors attenuates intravenous heroin reward in the rat. *Psychopharmacology (Berl.)* 86, 37–42. doi: 10.1007/BF00431681
- Van Vliet, B. J., De Vries, T. J., Wardeh, G., Mulder, A. H., and Schoffelmeer, A. N. (1991). mu-Opioid receptor-regulated adenylate cyclase activity in primary cultures of rat striatal neurons upon chronic morphine exposure. *Eur. J. Pharmacol.* 208, 105–111. doi: 10.1016/0922-4106(91)90060-U
- Vekovischeva, O. Y., Zamanillo, D., Echenko, O., Seppala, T., Uusi-Oukari, M., Honkaniemi, A., et al. (2001). Morphine-induced dependence and sensitization are altered in mice deficient in AMPA-type glutamate receptor-A subunits. *J. Neurosci.* 21, 4451–4459.
- Volman, S. F., Lammel, S., Margolis, E. B., Kim, Y., Richard, J. M., Roitman, M. F., et al. (2013). New insights into the specificity and plasticity of reward and aversion encoding in the mesolimbic system. *J. Neurosci.* 33, 17569–17576. doi: 10.1523/JNEUROSCI.3250-13.2013
- Waldhoer, M., Bartlett, S. E., and Whistler, J. L. (2004). Opioid receptors. *Annu. Rev. Biochem.* 73, 953–990. doi: 10.1146/annurev.biochem.73.011303.073940
- Weiner, D. M., Levey, A. I., Sunahara, R. K., Niznik, H. B., O'Dowd, B. F., Seeman, P., et al. (1991). D1 and D2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. U.S.A.* 88, 1859–1863. doi: 10.1073/pnas.88.5.1859
- Weinshenker, D., and Schroeder, J. P. (2007). There and back again: a tale of norepinephrine and drug addiction. *Neuropharmacology* 52, 1433–1451. doi: 10.1038/sj.npp.1301263
- Weiss, R. D., Potter, J. S., Fiellin, D. A., Byrne, M., Connery, H. S., Dickinson, W., et al. (2011). Adjunctive counseling during brief and extended buprenorphine-naloxone treatment for prescription opioid dependence: a 2-phase randomized controlled trial. *Arch. Gen. Psychiatry* 68, 1238–1246. doi: 10.1001/archgenpsychiatry.2011.121
- Wentholt, R. J., Petralia, R. S., Blahos, J., Li, J., and Niedzielski, A. S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J. Neurosci.* 16, 1982–1989.
- Wheeler, R. A., and Carelli, R. M. (2009). Dissecting motivational circuitry to understand substance abuse. *Neuropharmacology* 56(Suppl. 1), 149–159. doi: 10.1016/j.neuropharm.2008.06.028
- Wikler, A. (1973). Dynamics of drug dependence. Implications of a conditioning theory for research and treatment. *Arch. Gen. Psychiatry* 28, 611–616. doi: 10.1001/archpsyc.1973.01750350005001
- Williams, J. T., Christie, M. J., and Manzoni, O. (2001). Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev.* 81, 299–343.
- Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., Von Zastrow, M., Schulz, S., et al. (2013). Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65, 223–254. doi: 10.1124/pr.112.005942

- Wilson, C. J., and Groves, P. M. (1980). Fine structure and synaptic connections of the common spiny neuron of the rat neostriatum: a study employing intracellular inject of horseradish peroxidase. *J. Comp. Neurol.* 194, 599–615. doi: 10.1002/cne.901940308
- Wise, R. A. (1989). Opiate reward: sites and substrates. *Neurosci. Biobehav. Rev.* 13, 129–133. doi: 10.1016/S0149-7634(89)80021-1
- Wise, R. A. (2004). Dopamine, learning and motivation. *Nat. Rev. Neurosci.* 5, 483–494. doi: 10.1038/nrn1406
- Wolf, M. E. (2010). Regulation of AMPA receptor trafficking in the nucleus accumbens by dopamine and cocaine. *Neurotox. Res.* 18, 393–409. doi: 10.1007/s12640-010-9176-0
- Wolf, M. E., Mangiavacchi, S., and Sun, X. (2003). Mechanisms by which dopamine receptors may influence synaptic plasticity. *Ann. N. Y. Acad. Sci.* 1003, 241–249. doi: 10.1196/annals.1300.015
- Wolf, M. E., Sun, X., Mangiavacchi, S., and Chao, S. Z. (2004). Psychomotor stimulants and neuronal plasticity. *Neuropharmacology* 47(Suppl. 1), 61–79. doi: 10.1016/j.neuropharm.2004.07.006
- Woody, G. E., Poole, S. A., Subramaniam, G., Dugosh, K., Bogenschutz, M., Abbott, P., et al. (2008). Extended vs short-term buprenorphine-naloxone for treatment of opioid-addicted youth: a randomized trial. *JAMA* 300, 2003–2011. doi: 10.1001/jama.2008.574
- Xi, Z. X., and Stein, E. A. (2002). Blockade of ionotropic glutamatergic transmission in the ventral tegmental area reduces heroin reinforcement in rat. *Psychopharmacology (Berl.)* 164, 144–150. doi: 10.1007/s00213-002-1190-3
- Yufarov, V., Fussell, D., LaForge, K. S., Nielsen, D. A., Gordon, D., Ho, A., et al. (2004). Redefinition of the human kappa opioid receptor gene (OPRK1) structure and association of haplotypes with opiate addiction. *Pharmacogenetics* 14, 793–804. doi: 10.1097/00008571-200412000-00002
- Zahm, D. S., and Brog, J. S. (1992). On the significance of subterritories in the “accumbens” part of the rat ventral striatum. *Neuroscience* 50, 751–767. doi: 10.1016/0306-4522(92)90202-D
- Zahm, D. S., and Heimer, L. (1990). Two transpallidal pathways originating in the rat nucleus accumbens. *J. Comp. Neurol.* 302, 437–446. doi: 10.1002/cne.903020302
- Zito, K. A., Vickers, G., and Roberts, D. C. (1985). Disruption of cocaine and heroin self-administration following kainic acid lesions of the nucleus accumbens. *Pharmacol. Biochem. Behav.* 23, 1029–1036. doi: 10.1016/0091-3057(85)90110-8
- Zullino, D. F., Krenz, S., Zimmerman, G., Miozzari, A., Rajeswaran, R., Kolly, S., et al. (2004). Topiramate in opiate withdrawal – comparison with clonidine and with carbamazepine/mianserin. *Subst. Abus.* 25, 27–33. doi: 10.1300/J465v25n04_04

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The role of serotonin 5-HT_{2A} receptors in memory and cognition

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Serotonin 5-HT_{2A} receptors (5-HT_{2A}Rs) are widely distributed in the central nervous system, especially in brain region essential for learning and cognition. In addition to endogenous 5-HT, several hallucinogens, antipsychotics, and antidepressants function by targeting 5-HT_{2A}Rs. Preclinical studies show that 5-HT_{2A}R antagonists have antipsychotic and antidepressant properties, whereas agonist ligands possess cognition-enhancing and hallucinogenic properties. Abnormal 5-HT_{2A}R activity is associated with a number of psychiatric disorders and conditions, including depression, schizophrenia, and drug addiction. In addition to its traditional activity as a G protein-coupled receptor (GPCR), recent studies have defined novel operations of 5-HT_{2A}Rs. Here we review progress in the (1) receptor anatomy and biology: distribution, signaling, polymerization and allosteric modulation; and (2) receptor functions: learning and memory, hallucination and spatial cognition, and mental disorders. Based on the recent progress in basic research on the 5-HT_{2A}R, it appears that post-training 5-HT_{2A}R activation enhances non-spatial memory consolidation, while pre-training 5-HT_{2A}R activation facilitates fear extinction. Further, the potential influence that 5-HT_{2A}R-elicited visual hallucinations may have on visual cue (i.e., landmark) guided spatial cognition is discussed. We conclude that the development of selective 5-HT_{2A}R modulators to target distinct signaling pathways and neural circuits represents a new possibility for treating emotional, neuropsychiatric, and neurodegenerative disorders.

Keywords: serotonin, 5-HT_{2A} receptor, learning, memory, cognition

Introduction

The serotonin (5-HT) 5-HT_{2A} receptor (5-HT_{2A}R) is a GPCR of the type A family. It was defined as the classical D receptor initially by Gaddum and Picarelli (1957), and later referred as the 5-HT₂ receptor by Peroutka and Snyder (1979). The 5-HT_{2A}R gene is located on human chromosome 13q14-q21. *HTR2A* gene codes for a 471-amino acid sequence in rat, mouse, and human

Abbreviations: 5-HT, 5-hydroxytryptamine/serotonin; AD, Alzheimer disease; AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor; BLA, basolateral amygdala; CS, conditioned stimulus; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; ERK, extracellular signal-regulated kinases; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; IP, inositol phosphate; LSD; lysergic acid diethylamide; mGluR2, metabotropic glutamate receptor; MUPP1, multiple PDZ protein-1; MWM, Morris water maze; NAc, nucleus accumbens; NMDAR, N-methyl-D-aspartate receptor; NOR, novel object recognition; OCD, obsessive-compulsive disorder; PDZ, postsynaptic density zone; PFC, prefrontal cortex; PKC, protein kinase C; PLC, phospholipase C; PSD, postsynaptic density; RSK2, ribosomal S6 kinase 2; sIPSC, spontaneous inhibitory postsynaptic current; sIPSP, spontaneous inhibitory postsynaptic potential; US, unconditioned stimulus.

(Sparkes et al., 1991). The rat 5-HT_{2A}R was cloned in 1988 (Pritchett et al., 1988) and the human 5-HT_{2A}R was reported by Julius et al. (1990). Central 5-HT_{2A}Rs exert diverse behavioral, physiological, and psychological influences (Hoyer et al., 2002; Hannon and Hoyer, 2008; Homberg, 2012). Abnormality in the structure and function of the 5-HT_{2A}R is associated with a number of disorders, including schizophrenia, depression/anxiety, and drug addiction. Furthermore, many hallucinogenic drugs exert their psychoactive effects by acting as agonists for 5-HT_{2A}Rs. Preclinical studies show that 5-HT_{2A}R blockade has antipsychotic (Meltzer, 1999), antidepressant (Kroese and Roth, 1998; Roth et al., 1998) and anxiolytic properties (Cohen, 2005). Pharmacological studies indicate that high-affinity antagonists of 5-HT_{2A}Rs are effective atypical antipsychotics, due to their demonstrated efficacy to reduce both positive and negative symptoms of schizophrenia. Results from recent molecular biological and neuropharmacological studies suggest some exciting potential new avenues by which 5-HT_{2A}Rs influence CNS function. Here we review progress in understanding the contribution of 5-HT_{2A}Rs to modulation of learning and memory through an analysis of their (1) anatomy and biology: distribution, signaling, polymerization, and allosteric modulation; and (2) functions: learning and memory, hallucination and spatial cognition, and mental disorders. Based on the recent progress in 5-HT_{2A}R research, we suggest that selective 5-HT_{2A}R modulators targeting distinct signaling pathways may hold significant efficacy as new therapeutic approaches for several neurological disorders that present with cognitive impairment.

5-HT_{2A}R Anatomy and Biology in CNS

Cellular and Subcellular Distribution

Serotonin 5-HT_{2A}Rs are widely distributed in the CNS. In the rat brain, immunohistochemical studies show that 5-HT_{2A}Rs are broadly expressed in the cerebral cortex – especially in layers I and IV–V, the piriform and entorhinal cortex, the claustrum, endopiriform nucleus, and olfactory bulb/anterior olfactory nucleus, brainstem, as well as the limbic system and the basal ganglia; especially in the NAc and caudate nucleus (Xu and Pandey, 2000; Hannon and Hoyer, 2008). Interestingly, 5-HT_{2A}R binding appears to be absent from cerebellum (Xu and Pandey, 2000).

In human brain, autoradiographic analysis using [³H]ketanserin indicates a high density of 5-HT_{2A}R binding in laminae III and V of the frontal, parietal, temporal, occipital, anterogenuel cortices, and entorhinal area. 5-HT_{2A}Rs are also visualized in the mammillary bodies of the hypothalamus, claustrum, and the lateral nucleus of the amygdala. The hippocampus, caudate, putamen, and accumbens nuclei present an intermediate density of binding. Areas such as the thalamus, brain stem, cerebellum and spinal cord contained only low to very low densities of binding (Pazos et al., 1987). *In situ* hybridization studies reveal that 5-HT_{2A}R mRNA is present in all neocortical areas, especially in layer 5 pyramidal neurons, and in putative interneurons. 5-HT_{2A}R mRNA was observed at minimal levels

in the hippocampus and not in the raphe, cerebellum, substantia nigra or striatum (Burnet et al., 1995).

Morphological and double immunofluorescence analyses confirmed the presence of 5-HT_{2A}Rs on pyramidal neurons, interneurons, and glial cells in neocortex, amygdala and hippocampus (Willins et al., 1997; Bombardi, 2012, 2014). Thus, predicting the functional influence of activated cortical 5-HT_{2A}Rs is not straightforward, since these receptors would be capable of direct excitation and modulating feed-forward inhibition. In addition, 5-HT_{2A}Rs are located on cholinergic (Quirion et al., 1985) and glutamatergic neurons (Hasuo et al., 2002). 5-HT_{2A}R immunolabeling was also observed on glial cells in many forebrain regions: astrocytes were identified by double immunolabeling as cells in which 5-HT_{2A}R and GFAP was colocalized (Xu and Pandey, 2000); and on microglia (Glebov et al., 2015). These findings demonstrate that consideration of the serotonin-mediated signaling at 5-HT_{2A}Rs must include pathways that involve neurons and glial cells alike. It will be of interest to determine the degree to which functional influences expressed by the activation of 5-HT_{2A}Rs are dependent upon neurons, astrocytes, and microglial cells, and to determine whether clinically relevant features of 5-HT_{2A}Rs are related to changes in neurons or astrocytes.

At the subcellular level, 5-HT_{2A}R immunolabeling is found on cell bodies and processes of neurons (Cornea-Hebert et al., 1999; Xu and Pandey, 2000); in particular, at both pre- and post-synaptic compartments (Miner et al., 2003). However, the majority of evidence suggests a predominant expression at postsynaptic dendritic spines and shafts of non-5-HT neurons. Our own immuno-electron microscopy data revealed that 5-HT_{2A}R is also distributed in the dendritic spines, shafts, and presynaptic terminals of CA1 neurons in the mouse dorsal hippocampus (Zhang et al., 2015). Consideration should also be given to evidence suggesting that 5-HT_{2A}R subunits are extensively and dynamically trafficked between the cytoplasm and the neuronal membrane, as much 5-HT_{2A}R label has been identified at cytoplasmic rather than membrane bound compartments in adult rat neocortex (Cornea-Hebert et al., 1999). It will be of interest to determine the corresponding function of 5-HT_{2A}R subunit trafficking between the respective neuronal sub-compartments, and the intracellular signaling that promotes trafficking.

Interacting Proteins

Multiple interacting proteins regulate the function of 5-HT_{2A}Rs in the membrane. 5-HT_{2A}Rs interact with multiple PDZ protein-1 (MUPP1) and PSD-95 PDZ proteins (Jones et al., 2009). The 5-HT_{2A}R colocalizes with PSD-95 and with MUPP1 in a subset of dendritic spines of rat cortical pyramidal neurons. PDZ proteins are vital for docking 5-HT_{2A}R to the dendrites in cortical neurons and preventing the internalization of 5-HT_{2A}Rs (Xia et al., 2003). MUPP1 is enriched in dendritic spine PSD domains of pyramidal neurons and enhances the localization of 5-HT_{2A}R to the cell surface. Within cortical pyramidal neurons, PSD-95 regulates the functional activity of 5-HT_{2A}R by promoting apical dendritic targeting and stabilizing receptor turnover. The complex of 5-HT_{2A}R and PSD-95 plays a key

role in 5-HT_{2A}R-mediated head-twitch behavior in mice (Abbas et al., 2009). Binding of calmodulin to the 5-HT_{2A}R C-terminus impedes PKC-mediated phosphorylation of the 5-HT_{2A}R, thus, preventing its desensitization (Turner and Raymond, 2005). Conversely, association of p90-RSK2 with 5-HT_{2A}R (intracellular i3 loop) silences the GPCR's signaling (Sheffler et al., 2006). Caveolin-1 interacts with 5-HT_{2A}R and profoundly modulates its signaling by facilitating the interaction of 5-HT_{2A}R with G_{αq} (Bhatnagar et al., 2004). 5-HT_{2A}R and the light chain 2 domain of the microtubule-associated protein MAP1A are co-localized in the intracellular compartment of pyramidal neuronal dendrites of adult rats and may participate in intraneuronal signaling processes involving cytoskeletal elements (Cornea-Hebert et al., 2002). In consideration of these properties, we suggest that altering 5-HT_{2A}R-coupled proteins and pathways may enable an alternative method to selectively promote distinct modulatory functions of 5-HT_{2A}Rs.

Signaling

Activation of neuronal 5-HT_{2A}Rs can induce pleiotropic effects via G protein-dependent, ligand-dependent, and ligand-independent signaling pathways, including phospholipase signaling, ERK pathway, and tyrosine kinase pathway in neurons (Millan et al., 2008; Masson et al., 2012). In most circumstances, activation of 5-HT_{2A}Rs increases intracellular Ca²⁺ levels via G_{αq}-PLC-IP3 signaling (Hagberg et al., 1998). In PFC, activation of 5-HT_{2A}Rs suppresses membrane Ca_v1.2 L-type Ca²⁺ currents via a G_{αq}-mediated PLCβ/IP₃/calcineurin signaling pathway (Day et al., 2002). 5-HT_{2A}R activation also stimulates the G_{α12/13}-phospholipase A2 signal transduction pathway, which promotes arachidonic acid release (Kurrasch-Orbaugh et al., 2003a,b).

Besides PLC-mediated Ca²⁺ signaling, 5-HT_{2A}R activation also induces ERK phosphorylation via diverse intracellular signaling mechanisms (Gooz et al., 2006). Src and calmodulin promote 5-HT_{2A}R-mediated phosphorylation of ERK. In the PC12 cell model system, ERK phosphorylation by 5-HT_{2A}R may not depend on PLC/PKC signaling, and instead requires an increase in intracellular Ca²⁺, and the activation of CaM and Src (Quinn et al., 2002). The ERK target RSK2 directly acts on the third intracellular (i3) loop of 5-HT_{2A}R protein (Sheffler et al., 2006), leading to direct phosphorylation of the i3 loop at the conserved residue Ser-314 to suppress 5-HT_{2A}R signaling. In addition, RSK2 is required for tyrosine kinases, such as the epidermal growth factor receptor and the platelet-derived growth factor receptor, both of which have been demonstrated to attenuate 5-HT_{2A}R functioning in primary cortical neurons (Strachan et al., 2009, 2010).

Besides the G protein, 5-HT_{2A}Rs are also coupled to β-arrestin2. 5-HT binds 5-HT_{2A}R to stimulate Akt phosphorylation via the β-arrestin2/phosphoinositide 3-kinase/Src/Akt cascade (Schmid and Bohn, 2010). Application of the 5-HT_{2A}R agonist DOI to cultured cortical neurons induced phosphorylation of p21-activated kinase (PAK) via Rac guanine nucleotide exchange factor (RacGEF) kalirin-7 (Jones et al., 2009). The 5-HT_{2A}R also regulates the tyrosine kinase pathway activity (Quinn et al., 2002). Excitation of neuronal 5-HT_{2A}Rs

activates transglutaminase which leads to transamidation of Rac1, a small G protein, resulting in constitutive activation of Rac1 (Dai et al., 2008). Chronic treatment with olanzapine, an atypical antipsychotic drug, causes the desensitization of 5-HT_{2A}R signaling. In rat frontal cortex, stimulation of the JAK-STAT pathway desensitizes the 5-HT_{2A}R-mediated PLC activation induced by olanzapine (Singh et al., 2010). Furthermore, constitutive activation of 5-HT_{2A}Rs induces G_{q/11} phosphorylation and desensitization (uncoupling) (Shi et al., 2007).

As indicated above, 5-HT_{2A}Rs are also expressed in microglia and mediate 5-HT-induced exosome release (Glebov et al., 2015). Activation of 5-HT_{2A}R increases intracellular Ca²⁺ via PLC signaling in astrocytes (Hagberg et al., 1998) and Glu efflux from C6 glioma cells (Meller et al., 2002). Considering the diversity of signaling cascades that can be triggered by 5-HT_{2A}R activation, it is perhaps not surprising that serotonergic activation of 5-HT_{2A}Rs can have diverse influences on neuronal responses and CNS functions.

Oligomerization

The GPCRs can form homomers and heteromers, and thereby present distinct signaling and functional activities (Rios et al., 2001). Consistent with this, 5-HT_{2A}Rs have been shown to form oligomers (Lukasiewicz et al., 2010). Fluorescence resonance energy transfer and immunoprecipitation studies revealed that the human 5-HT_{2A}R homodimerizes in cultured cells (Brea et al., 2009). For 5-HT_{2A}R oligomers, the 5-HT_{2A}R agonist DOI caused an increase in energy transfer efficiency to the level of 12%, and ketanserin caused a decrease of 4.4%. Heterodimers of 5-HT_{2A}R and dopamine D₂ receptors respond to DOI and quinpirole, a DA D₂R agonist, with a decrease in FRET efficiency, while ketanserin and butaclamol increase the transfer efficiency value (Lukasiewicz et al., 2010). Heterodimers of 5-HT_{2A}R and mGluR2 receptor form via the linking domain in transmembrane-4 and -5 segments, and are present in the human brain. Post-mortem studies indicate a reduced density of these functional complexes in brains of schizophrenics (Gonzalez-Maeso et al., 2008). Delta-9-tetrahydrocannabinol (THC), the main psychoactive compound of marijuana, induces memory impairments, anxiety, dependence, and analgesia. Vinals et al. (2015) recently reported that amnesic and anxiolytic effects, but not analgesia, induced by THC were suppressed in 5-HT_{2A}R knockout mice. Molecular studies revealed that cannabinoid CB1 receptors (CB1R) and the 5-HT_{2A}R physically interact with each other to form heteromers, which are distributed extensively in hippocampus, cortex, and dorsal striatum, but not in the NAc. *In vivo* experiments have revealed that stimulation of CB1R and 5-HT_{2A}R reduces cell signaling, and the binding of an antagonist to one receptor blocks signaling of the interacting receptor. Heteromer formation leads to a switch in 5-HT_{2A}R-mediated G-protein coupling from G_{αq} to G_i. Synthetic peptides with the sequence of transmembrane helices 5 and 6 of CB1R disrupt CB1R and 5-HT_{2A}R heteromerization *in vivo*, leading to a selective abrogation of memory impairments, but not the antinociceptive properties caused by THC exposure (Vinals et al., 2015). The anatomy, biology and function of 5-HT_{2A}R

homomers and heteromers, including the dynamic formation and dissociation, distribution, signaling and function, remain elusive. Elucidation of 5-HT_{2A}R oligomers will be interesting for both basic science research and potential clinical applications.

Allosteric Modulation

Recent years have witnessed a tremendous advance in the research and development of novel compounds for GPCRs that bind allosteric sites to regulate receptor structure and function. These ligands provide high specificity, novel modes of efficacy and may open up a novel avenue for therapeutic agents against multiple mental and neurological disorders. Allosteric modulators bind to a site distinct from that of the orthosteric ligand-binding site. Usually the allosteric modulator induces a structure change within the GPCR to enhance or suppress the orthosteric ligand's functional activity (Conn et al., 2009; Melancon et al., 2012). Application of the amidated lipid, oleamide significantly potentiated 5-HT-induced hydrolysis of phosphoinositide in pituitary P11 cells expressing endogenously 5-HT_{2A}Rs (Thomas et al., 1997). Taken together, these results indicate that there are several binding sites present on 5-HT_{2A}Rs, and we suggest that it will be of interest to further characterize the functional significance of the distinct ligand-driven actions at the 5-HT_{2A}R.

Constitutive Activity

As mentioned above, 5-HT_{2A}Rs can also be constitutively active (i.e., via activating the receptor in an agonist-independent activity) *in vivo* (Berg et al., 2008). The inverse 5-HT_{2A}R agonists (e.g., risperidone and ketanserin) produce a great suppression of basal IP production, leading to a reduction of basal activity in the C322K mutant 5-HT_{2A}R (Egan et al., 1998). The "constitutively active" arrestin mutant (Arr2-R169E) induces agonist-independent 5-HT_{2A}R internalization, and a constitutive translocation of the Arr2-R169E mutant to the plasma membrane (Gray et al., 2003). The constitutive activity of 5-HT_{2A}Rs may represent another mechanism of regulating cellular function. The specific relationships of these constitutively active 5-HT_{2A}R-mediated properties to distinct behaviors have not been determined.

Electrophysiological Characteristics

Electrophysiological studies reveal complex effects of 5-HT_{2A}R activation on cortical neurons; however, mainly these receptors appear to mediate depolarizing effects on excitatory and inhibitory neurons. Slice recordings from prefrontal cortical neurons indicate depolarizing effects following 5-HT_{2A}R activation (Aghajanian and Marek, 1999; Zhou and Hablitz, 1999; Avesar and Gulledge, 2012). Local application of DOI, a 5-HT_{2A/2C} receptor agonist, increases the firing rates of cortical neurons (Stein et al., 2000) and facilitates synaptic plasticity through an NMDAR-dependent mechanism in presumptive pyramidal neurons of the rat BLA (Chen et al., 2003). Meanwhile, α -methyl-5-hydroxytryptamine (a 5-HT₂R agonist) and DOI induce activation of GABAergic interneurons of the rat BLA (Stein et al., 2000). Double immunofluorescence labeling demonstrated that the 5-HT_{2A}R is primarily localized

to parvalbumin-containing BLA interneurons. Accordingly, 5-HT primarily acts on 5-HT_{2A}Rs to potentiate GABAergic inhibition. 5-HT_{2A}R activation increases the frequency and amplitude of sIPSCs recorded from the pyramidal neurons in BLA of the juvenile rat (Jiang et al., 2009). DOI potentiates NMDAR-mediated changes in membrane potentials and calcium influx without affecting the neuronal resting membrane potential or input resistance. However, DOI does not affect AMPA/kainate receptor-mediated excitatory synaptic responses (Chen et al., 2003). The relationship of 5-HT_{2A}Rs to NMDARs is consistent with the view that 5-HT_{2A}Rs may be an effective target for modulating experience-dependent synaptic plasticity in the CNS. Globally, 5-HT_{2A}Rs have been shown to influence low-frequency field potential oscillations in rat frontal cortex (Celada et al., 2008). Taken together, these findings demonstrate that the 5-HT_{2A}R mediates 5-HT-induced excitation of cortical neurons. However, much remains to be determined as to the neurophysiological consequences of 5-HT_{2A}R activation, in particular as they relate to the regulation of specific behaviors.

Recent molecular and pharmacological research has made significant advances in the understanding of the functional selectivity of 5-HT_{2A}R. The multiple signaling pathways suggests bias agonism and bias signaling of 5-HT_{2A}Rs, which posit that an agonist can produce a mix of signaling, which is potentially determined by cell type and functional status.

5-HT_{2A}R Functions in CNS

Long-term declarative or episodic memory is supported by a network of brain structures in the medial temporal lobe of the mammalian brain. The medial temporal lobe memory system, which includes the hippocampus, dentate gyrus, and surrounding extrahippocampal cortical regions, influence decision-making processes guided by the PFC, and posterior parietal cortex (Squire et al., 2004, 2007; Preston and Eichenbaum, 2013). Serotonergic fibers originating from the raphe nuclei innervate many of the critical nodes within the medial temporal lobe memory system, including the hippocampus and amygdala, and on to the PFC (Vertes, 1991; Vertes et al., 1999). The modulatory influence of 5-HT on simple and more complex forms of learning and memory has been extensively examined in both invertebrate and vertebrate model systems (Kandel and Squire, 2000). The relevance of 5-HT to memory seems to generalize across mammals; dietary tryptophan increases brain 5-HT levels and improves memory in rodents (Khaliq et al., 2006), the elderly, AD patients, and schizophrenics (Levkovitz et al., 2003; Porter et al., 2003). Further, reductions in brain 5-HT concentrations after acute or chronic tryptophan depletion has been demonstrated to impair contextual fear memory in mice (Uchida et al., 2007), object memory in rats (Jenkins et al., 2009), and declarative memory in humans (Schmitt et al., 2006). Below, we describe some evidence suggesting that the 5-HT_{2A}R may hold special significance as one of the substrates by which 5-HT regulates learning and memory (Meneses, 2007).

Learning and Memory

Polymorphisms in the human *HTR2A* gene are associated with altered memory processes. For example, a *HTR2A* gene polymorphism inducing the substitution of the His452 on the receptor subunit to a Tyr residue is associated with a significant impairment in memory recall amongst adults (de Quervain et al., 2003; Sigmund et al., 2008; Zhu et al., 2013). Carriers of the His452Tyr (rs6314) exhibited poor verbal delayed recall and recognition, but performed equivalent to controls on tests of immediate recall, attentional, and executive function (Wagner et al., 2008). Compared to His homozygotes, Tyr carriers exhibited a diminished hippocampal response to novel stimuli and a higher tendency to judge novel stimuli as familiar during delayed recognition (Schott et al., 2011). Amongst schizophrenics and healthy controls, those carriers of homozygous CC (T102C) and GG (A-1438G), or carriers of the so-called *T*-allele (rs6314), of the *HTR2A* gene polymorphisms exhibited significantly impaired short-term verbal memory (Alfimova et al., 2009), and spatial working memory (Blasi et al., 2013). Another polymorphism in the *HTR2A* gene, referred to as rs4941573 was found to be predictive of increased error rate in a spatial working memory task in an adult Chinese subject population (Gong et al., 2011). These results provide just a brief and incomplete view of a broad literature indicating the impressive degree to which alterations in the *HTR2A* gene relate to disordered cognitive functions in normal and abnormal human subjects.

The regional distribution of 5-HT_{2AR}s can be predictive of the memory capacities that are sensitive to serotonin manipulation. The 5-HT_{2AR}s are widely expressed in the neocortex and hippocampus of rats (Xu and Pandey, 2000; Hannon and Hoyer, 2008), rabbits (Aloyo and Harvey, 2000), primates (Jakab and Goldman-Rakic, 1998; Lopez-Gimenez et al., 1998), and humans (Hoyer et al., 1986; Lopez-Gimenez et al., 1998). **Table 1** summarizes the major findings of studies in which the learning and memory effects were examined after 5-HT_{2AR} pharmacological manipulations across distinct tasks and different species. The inconsistency of experimental results may be attributed to the species, selectivity and dose of drug, behavioral task and other effectors.

Object Memory

The spontaneous NOR task, which relies on rodents' inherent preference for exploring novel over familiar stimuli, has become a popular method for examining the neuropharmacological and neurophysiological mechanisms of object memory (Ennaceur, 2010; Cohen and Stackman, 2015). In the task, rodents are exposed to one or two novel objects in a familiar enclosure during a sample session (i.e., training). The rodent is removed from the enclosure after it has sufficiently explored the objects. After a delay of some length, the rodent is returned to the enclosure for a memory test session, during which the enclosure contains one familiar object and a novel object. If the rodent has successfully encoded and consolidated the memory of the original object from the sample session, then it is expected that the rodent will preferentially explore the novel object during the test session. The NOR task offers advantages for testing rodent memory in that the distinct memory processes of encoding,

consolidation and retrieval are operationally defined as events occurring during the sample session, after the sample session, or during the test session, respectively. Another advantage is that the behavioral responses are spontaneous rather than requiring overt motivation such as electrical shock or food restriction. Our recent studies implicate the hippocampus as a key region in the rodent brain for object memory processes (Cohen et al., 2013; Cohen and Stackman, 2015). In light of the fact that 5-HT_{2AR}s are densely expressed in the hippocampus (Lutgen et al., 2004), we examined the contribution of hippocampal 5-HT_{2AR}s in object memory processes in male mice using an NOR task (see **Figure 1**). Systemic activation of 5-HT_{2AR}s with the selective agonist, TCB-2 after the sample session significantly enhanced the time mice spent exploring the new object presented during the test session 24 h later (Zhang et al., 2013). The memory-enhancing effect of TCB-2, was blocked by pretreatment with the 5-HT_{2AR} antagonist, MDL 11,939, which suggests that 5-HT_{2AR} activation enhances the consolidation of object memory. Interestingly, when TCB-2 was administrated before the sample session, or before the test session, the 5-HT_{2AR} agonist failed to increase novel object preference relative to the respective control group. Together, these data suggest that 5-HT_{2AR} activation selectively potentiates memory consolidation. Furthermore, the selective local microinfusion of TCB-2 into the CA1 region of dorsal hippocampus recapitulated the memory enhancing effect observed after systemic treatment (Zhang et al., 2015). The relevance of the 5-HT_{2AR} for object memory processes was also demonstrated by results of a study showing that the local infusion of the 5-HT_{2AR} antagonist MDL 11,939 into the mPFC impaired retrieval of object-in-context memory in rats (Bekinschtein et al., 2013). Interestingly, the 5-HT_{2AR} agonist DOI was found to impair retrieval of memory for an operant response by adult rats in an autoshring task (Meneses, 2007). Thus, it would appear that the influence of the 5-HT_{2AR} on memory is task- and memory system-dependent, and perhaps by the underlying neural circuitry that supports the respective memory process.

The encoding and consolidation of hippocampal-dependent memory appears, in part, to require fast glutamatergic neurotransmission, ensuing phases of synaptic plasticity, and dynamic replay of experience-dependent neurophysiological oscillatory activity within hippocampal cell populations (Eichenbaum, 1999; Karlsson and Frank, 2009). Our published (Zhang et al., 2013) data show that post-training activation of 5-HT_{2AR}s enhances object memory, likely by affecting consolidation. Prevailing views state that the hippocampus transfers recent to-be-remembered information to the neocortex during sharp wave ripples of the hippocampal local field potential (i.e., 100–200 Hz ripples; Chrobak and Buzsaki, 1996; Carr et al., 2011). During sleep, hippocampal neurons 'replay' patterns of spike trains present during a learning episode. As sharp wave ripples and replay may represent systems consolidation of memory, it would be of interest to examine the influence of 5-HT_{2AR}-sensitive drugs on sharp wave ripples and replay of spiking sequences during sleep episodes after a to-be-remembered experience. Postsynaptic 5-HT_{2AR}s may modulate object memory consolidation by also influencing NMDAR-mediated synaptic plasticity. Consistent

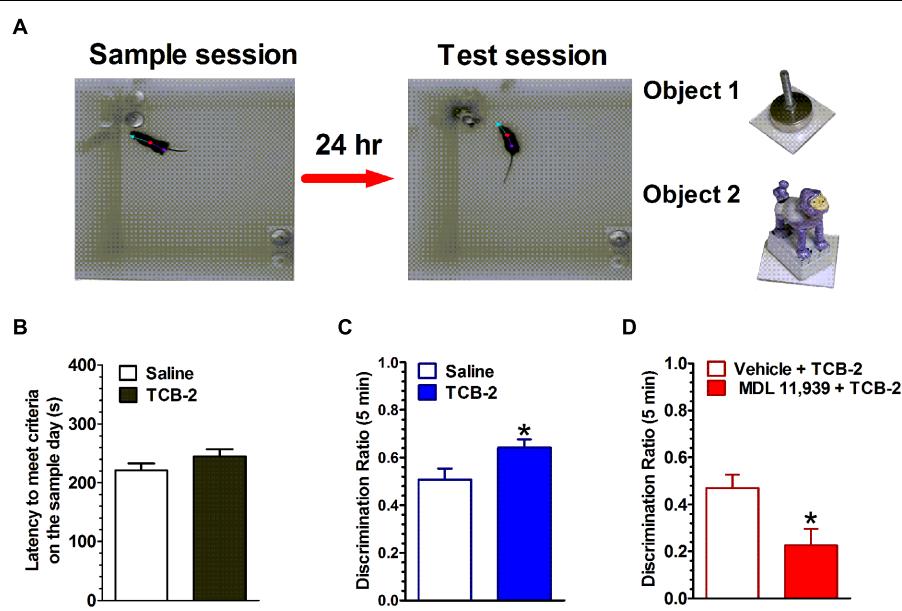
TABLE 1 | Reported effects on learning and memory after pharmacological manipulation of 5-HT_{2A} receptors (5-HT_{2A}Rs).

Drug	Route; dose	Task	Species	Effect	Reference
M100907	0.01–0.1 mg/kg; i.p.	Probabilistic reversal learning	Mice	↑ Acquisition	Amodeo et al., 2014; *BTBR T+tf/J mouse model of autism
M100907	0.01–0.1 mg/kg; i.p.	Serial spatial reversal learning task	Rats	↓ Retrieval	Boulougouris et al., 2008
M100907	0.02–2.0 nmol; olfactory bulb	Reversal-learning task	Rats	↓ Acquisition	Furr et al., 2012
MDL 11,939	0.067–6.7 μmol/kg; s.c.	Nictitating membrane conditioned responses	Rabbits	↓ Acquisition	Welsh et al., 1998
MDL 11,939	300 ng/μl; mPFC	NOR task	Rats	↓ Retrieval	Bekinschtein et al., 2013
Ritanserin, Risperidone	2.5 mg/kg × 11 days; s.c. 1 mg/kg; i.p.	Conditioned olfactory training Reward-dependent operant conditioning task	Rat pup Rats	↑ Acquisition ↓ Acquisition and ↑ extinction	McLean et al., 1996 Frick et al., 2015
Risperidone	0.125 mg; i.p.	Probabilistic reversal learning	B6 mice	↓ Acquisition	Amodeo et al., 2014
Ketanserin Methysergide	1.0–3.0 mg/kg; s.c.; 3.0–15.0 mg/kg; i.p.	Delayed non-matching to position task (working memory)	Rats	↔ Retrieval	Ruotsalainen et al., 1997
Ketanserin	0.1 mg/kg × 14 days; i.p.	Passive avoidance paradigm and MWM	Rats	↓ Acquisition	Fedotova and Ordyan, 2010
DOI Ketanserin	0.01–0.1 mg/kg; i.p. 0.001–0.1 mg/kg, i.p.	Autoshaping learning task	Rats	↑ Consolidation	Meneses et al., 1997
M100907;α-methyl-5-HT	PFC	Oculomotor delayed-response tasks	Monkeys	↓ Acquisition ↑ Acquisition	Williams et al., 2002
TCB-2	1.0 mg/kg; i.p.	NOR task and Trace and delay fear conditioning	Mice	↑ Object memory acquisition; ↑ fear memory extinction	Zhang et al., 2013
DOI	0.1–0.3 mg/kg; i.p.	Autoshaping learning task	Rats	↓ Consolidation	Meneses, 2007
LSD	0.43–12.9 μg/side; hippocampus	Trace eyeblink conditioning.	Rabbits	↑ Acquisition	Romano et al., 2010
LSD	1–300 nmol/kg; i.v.	Nictitating membrane response	Rabbit	↑ Acquisition	Gimpl et al., 1979
LSD	0.13 mg/kg/d × 11 days; s.c.	Bulbectomy-induced deficit in active avoidance learning	Rats	↑ Acquisition	Buchborn et al., 2014
Psilocybin	215 μg/kg; oral	Spatial working memory task	Humans	↔ Retrieval	Carter et al., 2005
Psilocybin	0.1–1.5 mg/kg, i.p.	Trace fear conditioning -	mice	↑ Extinction	Catlow et al., 2013
Psilocin	1.0 mg/kg, i.p. 4.0 mg/kg, i.p.	MWM; Carousel maze (CM)	Rats	↓ Acquisition of CM; ↓ Retrieval of MWM (4 mg/kg); ↔ Consolidation	Rambousek et al., 2014
Quipazine	1.25–10 mg/kg, s.c.	Conditioned avoidance response	Rats	↑ Acquisition	Alhaider et al., 1993

↑, enhance; ↓, suppress; ↔, no effect.

with this possibility, hippocampal 5-HT_{2A}Rs are predominantly expressed at dendritic sites on pyramidal neurons (Cornea-Hebert et al., 1999; Peddie et al., 2008). 5-HT_{2A}R-containing dendritic processes also were immunolabeled for the NMDAR subunit NR1 and GluR2 (Peddie et al., 2008). We have found that 5-HT_{2A}R activation increased the extracellular efflux of glutamate in the dorsal hippocampus, and increased the basal firing rates of CA1 pyramidal neurons in awake behaving mice (Zhang et al., 2015). These results suggest that the 5-HT_{2A}R activation induced facilitation of object memory consolidation, may result from the potentiation of hippocampal glutamate release, and pyramidal neuron temporal dynamics at a critical post-training time period. These data suggest that the 5-HT_{2A}R may serve as a drug target for pharmacological interventions to treat memory impairments. It is conceivable that 5-HT_{2A}R activation promotes an increase in intracellular Ca²⁺, combined

with NMDAR-mediated Ca²⁺ influx, which together would facilitate the behavior-initiated synaptic plasticity. Aghajanian and Marek (1999) reported that activation of 5-HT_{2A}R produces an elevation in the frequency and amplitude of neuronal sEPSP/sEPSC. Consistently, 5-HT_{2A}R activation has been shown to facilitate NMDAR activity and synaptic plasticity in the cortex (Arvanov et al., 1999) and BLA (Chen et al., 2003). Furthermore, 5-HT_{2A}R directly interacts with PSD-95 to regulate receptor trafficking and signaling (Xia et al., 2003). 5-HT_{2A}R activation induces a transient increase in dendritic spinogenesis (Yoshida et al., 2011), phosphorylation of PAK, neuronal Rac guanine nucleotide exchange factor (Jones et al., 2009), BDNF expression (Vaidya et al., 1997), and Erk mitogen-activated protein kinase activity (Florian and Watts, 1998; Watts, 1998). Finally, the 5-HT_{2A}R inverse agonist pimavanserin was shown to reverse NMDAR antagonism-induced object memory impairments in



combination with atypical antipsychotic drugs (Snigdha et al., 2010). These results support the view of a modulatory influence of 5-HT_{2AR} on NMDAR-dependent memory mechanisms. Considering the myriad potential influences of 5-HT_{2AR} on medial temporal lobe memory mechanisms, there would appear to be multiple downstream influences by which 5-HT_{2AR} activation could enhance memory.

Fear Memory

While there is a rich literature on the influence of serotonin on anxiety and an established contribution of serotonergic drugs to the remediation of anxiety disorders in humans, the present review will focus on the influence of 5-HT_{2ARs} on fear memory encoded during Pavlovian conditioning sessions. Pavlovian fear conditioning has become a popular procedure for examining the neurobiological mechanisms of fear memory. As a Pavlovian conditioning procedure, fear conditioning lends itself well to defining processes of encoding, consolidation and retrieval of fear memory. Fear conditioning taxes a well-defined neural circuit within the amygdala, which in turn interacts with the hippocampus, anterior cingulate, or the PFC, depending on the elements of the conditioning session and the stage of memory processing (Zelikowsky et al., 2014). In addition, considerable attention has been given to investigations of the underlying biology of extinction of fear memory. During a delay fear conditioning session, an innocuous stimulus (e.g., a neutral tone or light) becomes a CS when it is repeatedly presented in such a way that it co-terminates with the presentation

of a sufficiently aversive US (e.g., foot shock) (Zhang et al., 2013). The unconditioned response to the foot shock US is typically jumping and running, but the conditioned response to the CS is a defensive freezing response, or the cessation of all movement except for respiration. Thus, the freezing behavior provides a reliable post-conditioning measure of fear memory in rodents (Blanchard and Blanchard, 1969). During fear conditioning, the subject learns to associate the tone CS with the foot shock US, and under certain conditions, learns to associate the foot shock with the environment or context where the conditioning session was presented. Acquisition of both the tone-shock and the context-shock associations requires the amygdala; however, the context-shock associations are also dependent upon the hippocampus (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). There has been considerable debate regarding the involvement of the hippocampus in contextual fear memory since there have been reports that hippocampal lesions impair contextual fear memory (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Anagnostaras et al., 1999; Stiedl et al., 2000), and others reporting that such lesions spare contextual fear memory (Cho et al., 1999; Wiltgen et al., 2006). Consensus seems to be building for the view that if the rodent is permitted sufficient time to acquire a hippocampal-dependent configural representation of the context (the chamber's geometry, olfactory, visual, tactile, and auditory cues) before the US is presented, then the hippocampus is engaged in associating the contextual memory with the foot shock (Rudy et al., 2002, 2004; Matus-Amat et al., 2004; Zelikowsky et al., 2014). In a trace fear conditioning

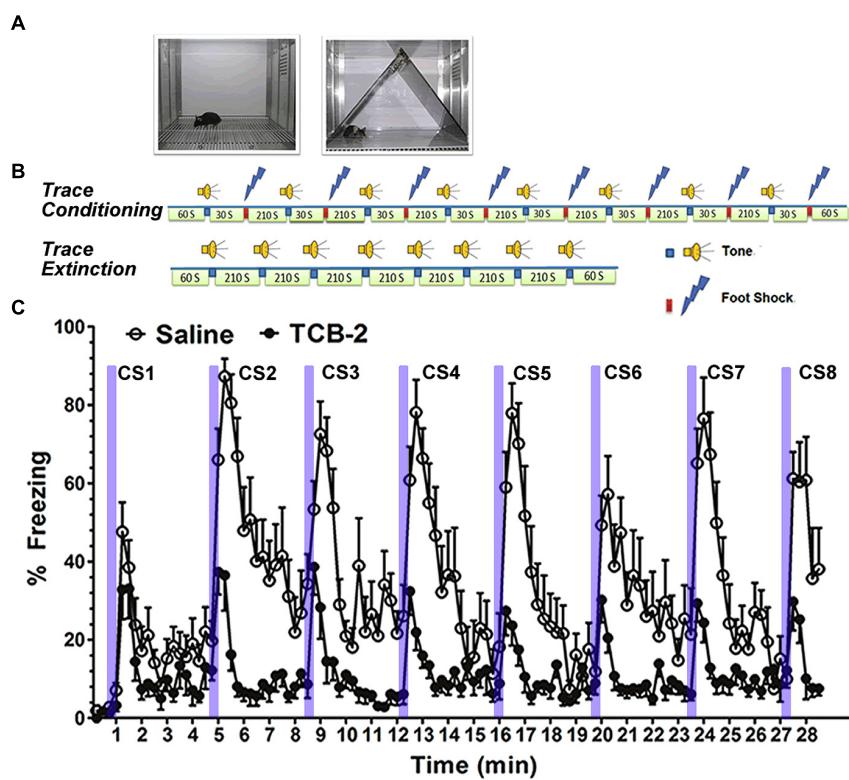


FIGURE 2 | Stimulation of 5-HT_{2A}Rs enhances the acquisition of extinction of trace fear memory. **(A)** Left, a chamber for fear conditioning (Context A) and contextual fear memory test; right, Context B, a modified chamber with different color, context, light density, and odor for cued fear memory test. **(B)** Trace fear conditioning training procedure. After a 60-s habituation to Context A, a tone was presented for 15 s followed by 30 s stimulus-free interval, and then a 0.5-s, 0.75 mA foot shock (US) was presented. The CS-US pairing was repeated eight times with a 210-s intertrial interval (ITI). Mice were removed from the conditioning chamber and returned to their home cages 60 s after the final CS-US pairing. Trace fear conditioning extinction procedure. Sixty seconds after placing the mouse into the modified chamber, eight unpaired 15-s tone CS were presented with a 120-s ITI. **(C)** Mice that received TCB-2 (1.0 mg/kg, i.p.) before a trace fear memory extinction test exhibited accelerated acquisition of extinction as indicated by significantly lower freezing scores earlier in the course of extinction as compared to those of vehicle-treated mice. TCB-2 significantly decreased percent freezing from the second to eighth CS presentation (Zhang et al., 2013).

procedure, a temporal gap is imposed between the offset of the tone CS and the onset of the foot shock US. The acquisition of an appropriately timed (i.e., anticipatory) conditioned freezing response occurs progressively over the course of the repeated CS-US pairings; this temporal fear memory is a form of declarative memory dependent on intact hippocampal function in rodents and humans (Clark and Squire, 1998; McEchron et al., 1998; Chowdhury et al., 2005). It should be clear that in deciphering an influence of 5-HT_{2A}R-sensitive drugs on the distinct processes of memory for contextual and/or cued fear, one must consider the specifics of the conditioning protocol used.

Finally, considerable attention has been directed toward defining the mechanisms of fear memory extinction, in part because of extinction's potential relationship to components of the human disorder post-traumatic stress disorder (Jovanovic and Ressler, 2010). Repeated presentations of the CS alone to the fear-conditioned rodent, promotes the acquisition of a new inhibitory association, which dampens or completely suppresses the expression of conditioned fear responses. Distinct subregions of the rodent PFC contribute differentially to fear extinction;

that is, the prelimbic cortex appears to influence the expression of fear responses, while the infralimbic cortex influences the acquisition of extinction of fear memory (Quirk et al., 2010; Sierra-Mercado et al., 2011). Synaptic plasticity within mPFC-BLA neuronal circuits is induced during fear extinction training, resulting in increased inhibition of CS-elicited activity of BLA extinction neurons (Herry et al., 2008, 2010). Thus, converging evidence implicates the infralimbic and prelimbic cortices of the rodent brain and their differential projections to the amygdala sub-regions and to the hippocampus as contributing significantly to the synaptic plasticity that develops during the acquisition of fear extinction (see Tovote et al., 2015 for a recent review).

We found that systemic administration of the 5-HT_{2A}R agonist TCB-2 (see Figure 2) significantly enhanced the acquisition of fear extinction in mice that had undergone trace fear conditioning or delay fear conditioning (Zhang et al., 2013). Importantly, the 5-HT_{2A}R agonist did not affect locomotor responses or baseline freezing in the mice. Therefore, the effect of TCB-2 on fear extinction appeared to be specific to facilitating the acquisition of the new inhibitory memory that

suppressed fear expression. It is of interest to determine the site of action in the rodent brain at which TCB-2 works to facilitate fear extinction. In light of the plastic changes in neural circuitry that occur during the acquisition of fear extinction, it is possible that TCB-2 influences either the infralimbic cortical neurons or the “extinction neurons” of the BLA to facilitate fear extinction. Izumi and colleagues reported that an amygdala-selective reduction of 5-HT content via site-specific 5,7-DHT injection reduced the expression of conditioned fear responses in rats (Izumi et al., 2012). While this finding is difficult to reconcile with our report that 5-HT_{2A}AR activation enhanced fear extinction, it is possible that the 5-HT denervation may have increased postsynaptic expression of 5-HT_{2A}Rs in the amygdala, which might in turn impair the expression of fear. It is clear that further studies are needed to clarify the neurophysiological influences of 5-HT, and the 5-HT_{2A}AR in particular, on the neural circuitry supporting fear memory encoding, consolidation, retrieval, and extinction.

The influence of the 5-HT_{2A}AR on the extinction and reconsolidation of fear memory may have significant impact on the development of therapeutic approaches for subjects with fear memory invasion, such as phobias and post trauma stress disorder (Quirk et al., 2010). For decades, the pharmacological manipulation of the 5-HT system has been a useful approach to treat emotional and mental disorders, such as depression and anxiety. Recent progress has suggested a promising therapeutic application of hallucinogenic 5-HT₂ agonists to treat depression and anxiety (Grob et al., 2011). These results suggest that despite the historical stigma associated with 5-HT_{2A}AR activators as potential hallucinogens, such compounds may provide important medical potential for treating affective and cognitive symptoms associated with emotional and mental conditions.

Glutamatergic neurons in the amygdala, cortex and hippocampus are essential for memory extinction and reconsolidation. Local infusion of NMDAR antagonists into the BLA or CA1 region of hippocampus before extinction training suppresses fear memory extinction and reconsolidation (Baker and Azorlosa, 1996; Szapiro et al., 2003). The NMDAR partial agonist D-cycloserine facilitates the extinction of fear memory (Walker et al., 2002; Ledgerwood et al., 2003). Knockout of NMDAR in hippocampal CA1 pyramidal cells exclusively impairs the establishment of conditioning between the CS and the US during a trace fear conditioning task. These results suggest that the CS representation and conditioning are entrained within hippocampus cell ensembles, probably via NMDAR-dependent synaptic plasticity (McHugh et al., 1996; Huerta et al., 2000). Recall that 5-HT_{2A}Rs are expressed in the dendrites and dendritic spines of dentate gyrus neurons where NMDARs and AMPARs are assumed to be located (Peddie et al., 2008). 5-HT_{2A}AR activation produces an elevation in the frequency and amplitude of cortical neuronal sEPSP/sEPSCs (Aghajanian and Marek, 1999), facilitates NMDAR activity and synaptic plasticity in the cortex (Arvanov et al., 1999) and BLA (Chen et al., 2003). It is worth while to examine the degree to which NMDARs expressed in the infralimbic and prelimbic cortices contribute to the 5HT_{2A}R-mediated enhancement in fear extinction.

Converging evidence demonstrates that activation of 5-HT_{2A}Rs via systemic injection, or by local microinfusion, appears to enhance two forms of hippocampal-dependent memory in mice: object memory and conditioned fear memory. Administration of a selective 5-HT_{2A}AR antagonist alone was not found to significantly affect object memory or fear memory (Zhang et al., 2013), suggesting that memory consolidation does not require serotonergic activation of 5-HT_{2A}Rs and/or the antagonists do not affect the tonic effect the 5-HT_{2A}AR. Activation of 5-HT_{2A}Rs with TCB-2 was also found to facilitate fear memory extinction in mice. These results offer promising support for the view that the 5-HT_{2A}AR may be an important new target for consideration in the search for mechanisms by which long-term memory can be enhanced in humans.

Hallucination vs. Spatial Cognition

5-HT_{2A}AR and Hallucination

Recent evidence suggests that activation of 5-HT_{2A}Rs may promote experiencing visual hallucinations by increasing neuronal excitability and altering visual-evoked cortical responses (Kometer et al., 2013). Hallucination is a type of misperception defined as the perception of an object without there being an object to perceive. Hallucinations are a significant characteristic found in a diversity of psychiatric and neurological states. Hallucinations can be triggered by at least three categories of drugs: psychedelics, (i.e., DOI, TCB-2, LSD, and psilocybin) via activation of 5-HT_{2A}Rs, psychostimulants (i.e., cocaine or amphetamine) via activation of dopamine D2 receptors and dissociative anesthetics (i.e., phencyclidine or ketamine) via blockade of glutamate NMDARs. The signaling and behavioral responses to each hallucinogen are distinct from each other. Activation of 5-HT_{2A}AR is critical for the psilocybin (found in magic mushroom)-induced α oscillations, N170 visual-evoked potentials, and visual hallucinations (Kometer et al., 2013).

5-hydroxytryptamine/serotonin is an endogenous neurotransmitter and is not considered hallucinogenic. Interesting, N-methyltryptamines, a metabolite of 5-HT, also presents high affinity for 5-HT_{2A}AR and can induce hallucinations in a manner independent of β -arrestin2/phosphoinositide 3-kinase/Src/Akt cascade (Schmid and Bohn, 2010). Signaling for hallucinogens is distinct. Lisuride (an antiparkinsonian agent) and LSD both bind cortical 5-HT_{2A}AR, and thereby regulate PLC activity. LSD signaling involves pertussis toxin-sensitive heterotrimeric G_{i/o} proteins and Src (Gonzalez-Maeso et al., 2007). Non-hallucinogenic agonists, for example lisuride, only stimulate cortical G_q in rats, whereas hallucinogens such as psilocybin (found in magic mushrooms), and LSD stimulate both G_{q/11} and G_i (Gonzalez-Maeso et al., 2007). The β -arrestin pathway is involved in hallucinogen-mediated head shake responses in rodents (Schmid et al., 2008), and 5-HT induces a head shake response in mice via a β -arrestin-2-dependent signaling. However, the DOI invoked head shake behavior is not dependent upon β -arrestin-2 signaling. These findings suggest that the 5-HT_{2A}AR- β -arrestin interaction may be exclusively for endogenous 5-HT action. Further examination of hallucinogen-mediated signaling may have major implications in drug development for treating emotional and mental disorders such

as depression and schizophrenia (Schmid et al., 2008). More research efforts will need to be focused on the hallucination-inducing aspects of 5-HT_{2A}R-sensitive drugs and, relevant to their potential therapeutic potential, it may be important to consider designing novel compounds that yield more of the beneficial effects, without activating those problematic sensory and perceptual effects.

5-HT_{2A}R-mediated Hallucination and Spatial Cognition

5-HT_{2A} receptors may affect spatial cognition. A human population-based study shows that 5-HT_{2A}R TT genotype of rs6313 is associated with better spatial cognitive performance (Gong et al., 2011). Kant et al. (1998) reported that the 5-HT_{2A}R agonist DOI (0.1 and 0.25 mg/kg, 30 min pretreatment) slowed rat performance as assessed by swim time on both a well-learned water maze as well as learning of a new maze, but DOI did not alter error rate on either task. Kant concluded that DOI impaired performance by suppressing motor activity on a water maze (Kant et al., 1998), which was in opposition to another report showing that manipulation of 5-HT_{2A}R did not impair the latency to a visible platform water maze test (Naghdi and Harooni, 2005). The serotonergic hallucinogens may impair the hippocampal-dependent spatial cognition by acting on 5-HT_{2A}Rs (Naghdi and Harooni, 2005). However, the direct evidence of 5-HT_{2A}R on visuospatial cognition and the central target has not been determined.

Serotonergic psychedelics may affect the integrity of visual functioning. Visual-directed spatial cognition and navigation are guided by exteroceptive (e.g., landmarks) and interoceptive (e.g., self-motion information) cues, and their integration. The hippocampus is a pivotal brain region receiving and integrating information for spatial memory and navigation in rodents (Broadbent et al., 2004; Eichenbaum, 2004). The MWM is a classic behavioral task for testing hippocampal-dependent visuospatial cognition, including place learning and memory, orientation and decision-making (Morris et al., 1982; Morris, 1984). Further, hippocampal place cells exhibit location-specific firing, and are considered to be fundamental components of network for spatial problem solving in the mammalian brain (for a review see Moser et al., 2008). The hippocampal neural circuit representing current location, directional heading and its integration is influenced by exteroceptive and interoceptive cues, and is considered to guide spatial cognition and navigation.

We recently found that pre-test activation of 5-HT_{2A}R with TCB-2 significantly delayed the initiation of an accurate search path by well-trained male mice in the hidden platform MWM (Zhang et al., 2015). Importantly, 5-HT_{2A}R activation did not affect swim performance or visual cue-triggered approach behavior in the visible platform water maze task. Taken together, our results suggest that the activation of 5-HT_{2A}R impairs the retrieval of hippocampal spatial memory, but not the accuracy of spatial information retrieval and decision-making. It is conceivable that the delayed initiation of accurate spatial search by TCB-2-treated mice might reflect the possible visual hallucinatory influences of the 5-HT_{2A}R agonist. For example,

perhaps TCB-2-induced a brief aberration of visual input that slowed the perception of current position and local view of the mouse at the start of the water maze probe test. Once, reconciled or reoriented, the mouse was able to swim accurately to the remembered spatial location of the platform. It will be of interest to determine where in the brain TCB-2 is acting to alter spatial memory retrieval. The relatively weak influence of TCB-2-induced visual hallucination on spatial navigation may due to the difference in the visual information passing through the brain and central targets processing the information.

Taken together, the results we have reported here of memory effects after activation of the 5-HT_{2A}R represent a fairly complex picture. The post-training administration of TCB-2 enhanced consolidation of object memory in mice. Pre-test administration of TCB-2 did not affect retrieval of object memory, yet delayed retrieval of spatial memory. Pre-extinction training administration of TCB-2 facilitated the acquisition of extinction of both trace and delay fear memories. The facilitating effect of TCB-2 on fear extinction may have been the result of a combined effect of suppressing fear expression – possibly a consequence of impaired retrieval of fear memory, and enhancing the encoding and consolidation of fear extinction. To characterize the 5-HT_{2A}R agonist as a cognitive enhancer based solely on our object memory results, would be to ignore the other experimental findings. We are interested in conducting a more comprehensive analysis of the impact of TCB-2 on multiple forms of memory. For example, it will be interesting to examine whether post-conditioning TCB-2 might enhance the consolidation of fear memory, in a manner consistent with that observed in the NOR task. Likewise, it will be interesting to test whether post-extinction training TCB-2 facilitates the consolidation of fear extinction. Results of these experiments will help in better appreciating the modulatory influence of the 5-HT_{2A}R on long-term memory processes. This synthesis of recent findings of the influences of 5-HT_{2A}R activation should provide a credible argument that the 5-HT_{2A}R participates significantly to the well-documented contribution of 5-HT to memory (Meneses, 2013).

5-HT_{2A}R and Mental Disorders

A number of psychiatric and neurodegenerative disorders are associated with the variation of structure, expression, and function of 5-HT_{2A}Rs. Positron emission tomography (PET) molecular imaging has the sensitivity to quantify binding of 5-HT_{2A}Rs in CNS disorders. Medication-free depressed subjects presented greater 5-HT_{2A}R binding (Bhagwagar et al., 2006). There was a significant reduction in 5-HT_{2A}R binding in frontal polar, dorsolateral and medial frontal cortex, and parietal and temporal associative cortex of OCD patients and a significant correlation between 5-HT_{2A}R availability in orbitofrontal and dorsolateral frontal cortex and clinical severity (Perani et al., 2008). Schizophrenia patients present with very high 5HT_{2A}R occupancy in the frontal cortex (Talvik-Lotfi et al., 2000). These results suggest that the variation in the number, affinity and/or function of 5-HT_{2A}R participates in the etiology of mental disorders.

Alzheimer's Disease

It is interesting to note that neocortical 5-HT_{2A}R binding is significantly decreased in patients with early stage AD, and in those with mild cognitive impairment; especially in temporal lobe regions associated with long-term memory (Meltzer et al., 1998; Hasselbalch et al., 2008; Santhosh et al., 2009; Marner et al., 2011, 2012). Further, the severity of cognitive impairment in AD patients correlates with the decrease in 5-HT_{2A}R binding (Versijpt et al., 2003). Given the pattern of 5-HT_{2A}R distribution in neocortical regions and their expression on principal excitatory neurons, it is possible that the marked reduction in 5-HT_{2A}R in brains of AD is a direct product of neuron loss in key brain regions. Consistent with evidence from the human studies, the Alzheimer's-like neuropathology and associated memory deficits in rodents, which follow intra-hippocampal injection of β -amyloid(1-42), are associated with a significant reduction in levels of hippocampal 5-HT_{2A}R expression (Christensen et al., 2008). Although we have focused this analysis on the influence of 5-HT_{2A}Rs on long-term, hippocampal-dependent memory, there is clear and compelling evidence to suggest that the 5-HT_{2A}R represents a potential new target by which human long-term memory may be modulated. We assert that it will be of interest in further examine the contribution of 5-HT_{2A}Rs to memory processes, and we are particularly interested in determining neurophysiological influences of 5-HT_{2A}R agonists which promote the enhancement of memory consolidation which we have reported in mice.

Drug Memory

Drug dependence, classified as an impulsive, compulsive, and relapsing psychiatric disorder, represents a devastating societal problem worldwide. The profound symptoms of drug abuse, in particular the cue-elicited relapse to drug use after even long periods of abstinence, are a consequence of robust experience-dependent synaptic plasticity within the brain's reward circuit. Like episodic, semantic, and habit memory, drug-associated memories are persistent and hold a strong influence on current and future behaviors. Of particular interest is the consideration of memory extinction as a psychological tool for remediating the problem of relapse in drug addicts. That is, if the problem of drug abuse is approached as a mental disorder of memory, then pharmacological manipulations that facilitate extinction may hold therapeutic utility for treating drug abuse. Drug exposure alters the expression and function of 5-HT_{2A}R, for example morphine decreases frontocortical 5-HT_{2A}R binding affinity in dogs (Adriaens et al., 2012). 5-HT_{2A}Rs are up-regulated in amygdala, midbrain, pons, and medulla of morphine-tolerant and -dependent rats, but not in morphine-abstinent rats (Gulati and Bhargava, 1989). There is considerable evidence that 5-HT_{2A}Rs modulate the behavioral consequences of repeated exposure to addictive psychomotor stimulants. For example, M100907 suppresses hyperactivity elicited by cocaine (Fletcher et al., 2002), MK-801, amphetamine (O'Neill et al., 1999), and morphine (Auclair et al., 2004). DOM, a 5-HT_{2A}R agonist, attenuates locomotor-stimulating effects of morphine, which could be prevented by M100907 (Li et al., 2013). Furthermore, M100907

attenuated the ability of experimenter-administered cocaine to reinstate lever pressing (Fletcher et al., 2002) and attenuated the drug associated cue-induced reinstatement of cocaine-seeking behavior after extinction (Nic Dhonchadha et al., 2009). M100907 also suppressed reinstatement induced by nicotine prime or nicotine-associated cue (Fletcher et al., 2012) and sensitization (Zaniewska et al., 2010). Intra-NAc infusions of M 100907 blocked the expression of cocaine-induced locomotor sensitization (Zayara et al., 2011). Intra-PFC M100907 decreased cue-elicited reinstatement of cocaine seeking-behavior (Pockros et al., 2011). Together, these results suggest that 5-HT_{2A}Rs modulate drug addiction-dependent behaviors such as craving and drug-seeking and pharmacological blockade of 5-HT_{2A}Rs may represent a therapeutic advance in suppression of cue-evoked craving and/or relapse in drug addicts.

Therapeutic Application of 5-HT_{2A}R

Preclinical and clinical studies have provided support for the use of pharmacological manipulation of 5-HT_{2A}R to treat the symptoms of mental disorders. Activation of 5-HT_{2A}R with TCB-2 in the medial septum-diagonal band of Broca complex enhances neuronal activity and working memory in hemiparkinsonian rats (Li et al., 2015). M100907 had no effect on attentional performance, but abolished the PCP-induced attentional performance deficits in rats (Poyurovsky et al., 2003). M100907 prevents impairment in attentional performance by NMDAR blockade in the rat PFC (Mirjana et al., 2004). There are a number of 5-HT_{2A}R drugs that have been evaluated or are being currently evaluated under clinical trials, for example quetiapine¹ for schizophrenia; M100907² for depression; ACP-103³ for Parkinson's disease; pimavanserin for patients with AD psychosis⁴ or with Parkinson's disease psychosis⁵.

Conclusion

In this review, we have summarized recent progress in the signaling, polymerization and allosteric modulation of 5-HT_{2A}R; and have discussed the critical role of 5-HT_{2A}Rs in a number of cognitive processes. Based on the results of studies from our lab and others, it appears that activation of 5-HT_{2A}Rs may offer a novel approach to treat the impairment of learning and memory associated with several neurodegenerative disorders. Meanwhile, blockade of 5-HT_{2A}R may offer a feasible way to suppress drug craving and/or relapse. It will be very interesting to identify the corresponding signaling pathways by which 5-HT_{2A}Rs modulate these behavioral capacities. Of particular note, we reviewed evidence that 5-HT_{2A}Rs may dimerize with other receptors, and that certain pathways may promote constitutive activation of

¹<https://clinicaltrials.gov/ct2/show/NCT00207064?term=5-HT2A&rank=2>

²<https://clinicaltrials.gov/ct2/show/NCT00070694?term=5-HT2A&rank=5>

³<https://clinicaltrials.gov/ct2/show/NCT00086294?term=5-HT2A&rank=12>

⁴<https://clinicaltrials.gov/ct2/show/NCT02035553?term=5-HT2A&rank=41>

⁵<https://clinicaltrials.gov/ct2/show/NCT00477672?term=5-HT2A&rank=46>

5-HT_{2A}Rs, which likely represent novel receptor signaling influences. Connecting such novel properties of 5-HT_{2A}Rs to distinct functional consequences of 5-HT-, or agonist-, specific activation of the 5-HT_{2A}Rs will be important for improving understanding the myriad influences of 5-HT_{2A}Rs in the CNS. The development of highly selective 5-HT_{2A}AR ligands will be essential for further establishing the critical involvement of the 5-HT_{2A}R for a number of fundamental cognitive behaviors.

References

- Abbas, A. I., Yadav, P. N., Yao, W. D., Arbuckle, M. I., Grant, S. G., Caron, M. G., et al. (2009). PSD-95 is essential for hallucinogen and atypical antipsychotic drug actions at serotonin receptors. *J. Neurosci.* 29, 7124–7136. doi: 10.1523/JNEUROSCI.1090-09.2009
- Adriaens, A. M., Polis, I. E., Vermeire, S. T., Waelbers, T., Duchateau, L., Sys, S. U., et al. (2012). The influence of morphine on cerebral 5-HT_{2A} availability in dogs: a SPECT study. *J. Nucl. Med.* 53, 1969–1973. doi: 10.2967/jnumed.112.103796
- Aghajanian, G. K., and Marek, G. J. (1999). Serotonin, via 5-HT_{2A} receptors, increases EPSCs in layer V pyramidal cells of prefrontal cortex by an asynchronous mode of glutamate release. *Brain Res.* 825, 161–171. doi: 10.1016/S0006-8993(99)01224-X
- Alfimova, M. V., Monakhov, M. V., Abramova, L. I., Golubev, S. A., and Golimbet, V. E. (2009). [Serotonin receptor (5-HTR2A) and dysbindin (DTNBP1) genes and component process variables of short-term verbal memory in schizophrenia]. *Zh. Nevrol. Psichiatr. Im. S.S. Korsakova* 109, 70–75.
- Alhaider, A. A., Ageel, A. M., and Ginawi, O. T. (1993). The quipazine- and TFMPP-increased conditioned avoidance response in rats: role of 5HT1C/5-HT₂ receptors. *Neuropharmacology* 32, 1427–1432. doi: 10.1016/0028-3908(93)90040-A
- Aloyo, V. J., and Harvey, J. A. (2000). Antagonist binding at 5-HT(2A) and 5-HT(2C) receptors in the rabbit: high correlation with the profile for the human receptors. *Eur. J. Pharmacol.* 406, 163–169. doi: 10.1016/S0014-2999(00)00645-2
- Amodeo, D. A., Jones, J. H., Sweeney, J. A., and Ragozzino, M. E. (2014). Risperidone and the 5-HT_{2A} receptor antagonist M100907 improve probabilistic reversal learning in BTBR T + tf/J mice. *Autism Res.* 7, 555–567. doi: 10.1002/aur.1395
- Anagnostaras, S. G., Maren, S., and Fanselow, M. S. (1999). Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. *J. Neurosci.* 19, 1106–1114.
- Arvanov, V. L., Liang, X., Magro, P., Roberts, R., and Wang, R. Y. (1999). A pre- and postsynaptic modulatory action of 5-HT and the 5-HT_{2A}, 2C receptor agonist DOI on NMDA-evoked responses in the rat medial prefrontal cortex. *Eur. J. Neurosci.* 11, 2917–2934. doi: 10.1046/j.1460-9568.1999.00708.x
- Auclair, A., Drouin, C., Cotecchia, S., Glowinski, J., and Tassin, J. P. (2004). 5-HT_{2A} and alpha1b-adrenergic receptors entirely mediate dopamine release, locomotor response and behavioural sensitization to opiates and psychostimulants. *Eur. J. Neurosci.* 20, 3073–3084. doi: 10.1111/j.1460-9568.2004.03805.x
- Avesar, D., and Guldge, A. T. (2012). Selective serotonergic excitation of callosal projection neurons. *Front. Neural Circuits* 6:12. doi: 10.3389/fncir.2012.00012
- Baker, J. D., and Azorlosa, J. L. (1996). The NMDA antagonist MK-801 blocks the extinction of Pavlovian fear conditioning. *Behav. Neurosci.* 110, 618–620. doi: 10.1037/0735-7044.110.3.618
- Bekinschtein, P., Renner, M. C., Gonzalez, M. C., and Weisstaub, N. (2013). Role of medial prefrontal cortex serotonin 2A receptors in the control of retrieval of recognition memory in rats. *J. Neurosci.* 33, 15716–15725. doi: 10.1523/JNEUROSCI.2087-13.2013
- Berg, K. A., Harvey, J. A., Spampinato, U., and Clarke, W. P. (2008). Physiological and therapeutic relevance of constitutive activity of 5-HT 2A and 5-HT 2C receptors for the treatment of depression. *Prog. Brain Res.* 172, 287–305. doi: 10.1016/S0079-6123(08)00914-X
- Bhagwagar, Z., Hinz, R., Taylor, M., Fancy, S., Cowen, P., and Grasby, P. (2006). Increased 5-HT(2A) receptor binding in euthymic, medication-free patients recovered from depression: a positron emission study with [(11)C]MDL 100,907. *Am. J. Psychiatry* 163, 1580–1587.
- Bhatnagar, A., Sheffler, D. J., Kroese, W. K., Compton-Toth, B., and Roth, B. L. (2004). Caveolin-1 interacts with 5-HT_{2A} serotonin receptors and profoundly modulates the signaling of selected Galphaq-coupled protein receptors. *J. Biol. Chem.* 279, 34614–34623. doi: 10.1074/jbc.M404673200
- Blanchard, R. J., and Blanchard, D. C. (1969). Passive and active reactions to fear-eliciting stimuli. *J. Comp. Physiol. Psychol.* 68, 129–135. doi: 10.1037/h0027676
- Blasi, G., De Virgilio, C., Papazacharias, A., Taurisano, P., Gelao, B., Fazio, L., et al. (2013). Converging evidence for the association of functional genetic variation in the serotonin receptor 2a gene with prefrontal function and olanzapine treatment. *JAMA Psychiatry* 70, 921–930. doi: 10.1001/jamapsychiatry.2013.1378
- Bombardi, C. (2012). Neuronal localization of 5-HT_{2A} receptor immunoreactivity in the rat hippocampal region. *Brain Res. Bull.* 87, 259–273. doi: 10.1016/j.brainresbull.2011.11.0068
- Bombardi, C. (2014). Neuronal localization of the 5-HT₂ receptor family in the amygdaloid complex. *Front. Pharmacol.* 5:68. doi: 10.3389/fphar.2014.0006
- Boulougouris, V., Glennon, J. C., and Robbins, T. W. (2008). Dissociable effects of selective 5-HT_{2A} and 5-HT_{2C} receptor antagonists on serial spatial reversal learning in rats. *Neuropsychopharmacology* 33, 2007–2019. doi: 10.1038/sj.npp.1301584
- Brea, J., Castro, M., Giraldo, J., Lopez-Gimenez, J. F., Padin, J. F., Quintian, F., et al. (2009). Evidence for distinct antagonist-revealed functional states of 5-hydroxytryptamine(2A) receptor homodimers. *Mol. Pharmacol.* 75, 1380–1391. doi: 10.1124/mol.108.054395
- Broadbent, N. J., Squire, L. R., and Clark, R. E. (2004). Spatial memory, recognition memory, and the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14515–14520. doi: 10.1073/pnas.0406344101
- Buchborn, T., Schroder, H., Holtt, V., and Grecksch, G. (2014). Repeated lysergic acid diethylamide in an animal model of depression: normalisation of learning behaviour and hippocampal serotonin 5-HT₂ signalling. *J. Psychopharmacol.* 28, 545–552. doi: 10.1177/0269881114531666
- Burnet, P. W., Eastwood, S. L., Lacey, K., and Harrison, P. J. (1995). The distribution of 5-HT_{1A} and 5-HT_{2A} receptor mRNA in human brain. *Brain Res.* 676, 157–168. doi: 10.1016/0006-8993(95)00104-X
- Carr, M. F., Jadhav, S. P., and Frank, L. M. (2011). Hippocampal replay in the awake state: a potential substrate for memory consolidation and retrieval. *Nat. Neurosci.* 14, 147–153. doi: 10.1038/nn.2732
- Carter, O. L., Burr, D. C., Pettigrew, J. D., Wallis, G. M., Hasler, F., and Vollenweider, F. X. (2005). Using psilocybin to investigate the relationship between attention, working memory, and the serotonin 1A and 2A receptors. *J. Cogn. Neurosci.* 17, 1497–1508. doi: 10.1162/089892905774597191
- Catlow, B. J., Song, S., Paredes, D. A., Kirstein, C. L., and Sanchez-Ramos, J. (2013). Effects of psilocybin on hippocampal neurogenesis and extinction of trace fear conditioning. *Exp. Brain Res.* 228, 481–491. doi: 10.1007/s00221-013-3579-0
- Celada, P., Puig, M. V., Diaz-Mataix, L., and Artigas, F. (2008). The hallucinogen DOI reduces low-frequency oscillations in rat prefrontal cortex: reversal by antipsychotic drugs. *Biol. Psychiatry* 64, 392–400. doi: 10.1016/j.biopsych.2008.03.013
- Chen, A., Hough, C. J., and Li, H. (2003). Serotonin type II receptor activation facilitates synaptic plasticity via N-methyl-D-aspartate-mediated mechanism in the rat basolateral amygdala. *Neuroscience* 119, 53–63. doi: 10.1016/S0306-4522(03)00076-9

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- Cho, Y. H., Friedman, E., and Silva, A. J. (1999). Ibotenate lesions of the hippocampus impair spatial learning but not contextual fear conditioning in mice. *Behav. Brain Res.* 98, 77–87. doi: 10.1016/S0166-4328(98)00054-0
- Chowdhury, N., Quinn, J. J., and Fanselow, M. S. (2005). Dorsal hippocampus involvement in trace fear conditioning with long, but not short, trace intervals in mice. *Behav. Neurosci.* 119, 1396–1402. doi: 10.1037/0735-7044.119.5.1396
- Christensen, R., Marcusen, A. B., Wortwein, G., Knudsen, G. M., and Aznar, S. (2008). Abeta(1-42) injection causes memory impairment, lowered cortical and serum BDNF levels, and decreased hippocampal 5-HT(2A) levels. *Exp. Neurol.* 210, 164–171. doi: 10.1016/j.expneurol.2007.10.009
- Chrobak, J. J., and Buzsaki, G. (1996). High-frequency oscillations in the output networks of the hippocampal- entorhinal axis of the freely behaving rat. *J. Neurosci.* 16, 3056–3066.
- Clark, R. E., and Squire, L. R. (1998). Classical conditioning and brain systems: the role of awareness. *Science* 280, 77–81. doi: 10.1126/science.280.5360.77
- Cohen, H. (2005). Anxiolytic effect and memory improvement in rats by antisense oligodeoxynucleotide to 5-hydroxytryptamine-2A precursor protein. *Depress. Anxiety* 22, 84–93. doi: 10.1002/da.20087
- Cohen, S. J., Munchow, A. H., Rios, L. M., Zhang, G., Asgeirsdottir, H. N., and Stackman, R. W. Jr. (2013). The rodent hippocampus is essential for nonspatial object memory. *Curr. Biol.* 23, 1685–1690. doi: 10.1016/j.cub.2013.07.002
- Cohen, S. J., and Stackman, R. W. Jr. (2015). Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behav. Brain Res.* 285, 105–117. doi: 10.1016/j.bbr.2014.08.002
- Conn, P. J., Christopoulos, A., and Lindsley, C. W. (2009). Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discov.* 8, 41–54. doi: 10.1038/nrd2760
- Cornea-Hebert, V., Riad, M., Wu, C., Singh, S. K., and Descarries, L. (1999). Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat. *J. Comp. Neurol.* 409, 187–209. doi: 10.1002/(SICI)1096-9861(19990628)409:2<187::AID-CNE2>3.0.CO;2-P
- Cornea-Hebert, V., Watkins, K. C., Roth, B. L., Kroese, W. K., Gaudreau, P., Leclerc, N., et al. (2002). Similar ultrastructural distribution of the 5-HT(2A) serotonin receptor and microtubule-associated protein MAP1A in cortical dendrites of adult rat. *Neuroscience* 113, 23–35. doi: 10.1016/S0306-4522(02)00146-X
- Dai, Y., Dudek, N. L., Patel, T. B., and Muma, N. A. (2008). Transglutaminase-catalyzed transamidation: a novel mechanism for Rac1 activation by 5-hydroxytryptamine2A receptor stimulation. *J. Pharmacol. Exp. Ther.* 326, 153–162. doi: 10.1124/jpet.107.135046
- Day, M., Olson, P. A., Platzer, J., Striessnig, J., and Surmeier, D. J. (2002). Stimulation of 5-HT(2) receptors in prefrontal pyramidal neurons inhibits Ca(v)1.2 L type Ca(2+) currents via a PLCbeta/IP3/calcineurin signaling cascade. *J. Neurophysiol.* 87, 2490–2504.
- de Quervain, D. J., Henke, K., Aerni, A., Coluccia, D., Wollmer, M. A., Hock, C., et al. (2003). A functional genetic variation of the 5-HT2a receptor affects human memory. *Nat. Neurosci.* 6, 1141–1142. doi: 10.1038/nn1146
- Egan, C., Herrick-Davis, K., and Teitler, M. (1998). Creation of a constitutively activated state of the 5-HT2A receptor by site-directed mutagenesis: revelation of inverse agonist activity of antagonists. *Ann. N. Y. Acad. Sci.* 861, 136–139. doi: 10.1111/j.1749-6632.1998.tb10184.x
- Eichenbaum, H. (1999). The hippocampus and mechanisms of declarative memory. *Behav. Brain Res.* 103, 123–133. doi: 10.1016/S0166-4328(99)00044-3
- Eichenbaum, H. (2004). Hippocampus: cognitive processes and neural representations that underlie declarative memory. *Neuron* 44, 109–120. doi: 10.1016/j.neuron.2004.08.028
- Ennaceur, A. (2010). One-trial object recognition in rats and mice: methodological and theoretical issues. *Behav. Brain Res.* 215, 244–254. doi: 10.1016/j.bbr.2009.12.036
- Fedotova, Y. O., and Ordyan, N. E. (2010). Blockade of 5-HT2A/2C-type receptors impairs learning in female rats in the course of estrous cycle. *Bull. Exp. Biol. Med.* 150, 6–8. doi: 10.1007/s10517-010-1053-6
- Fletcher, P. J., Grottick, A. J., and Higgins, G. A. (2002). Differential effects of the 5-HT(2A) receptor antagonist M100907 and the 5-HT(2C) receptor antagonist SB242084 on cocaine-induced locomotor activity, cocaine self-administration and cocaine-induced reinstatement of responding. *Neuropsychopharmacology* 27, 576–586.
- Fletcher, P. J., Rizos, Z., Noble, K., Soko, A. D., Silenieks, L. B., Le, A. D., et al. (2012). Effects of the 5-HT2C receptor agonist Ro60-0175 and the 5-HT2A receptor antagonist M100907 on nicotine self-administration and reinstatement. *Neuropharmacology* 62, 2288–2298. doi: 10.1016/j.neuropharm.2012.01.023
- Florian, J. A., and Watts, S. W. (1998). Integration of mitogen-activated protein kinase kinase activation in vascular 5-hydroxytryptamine2A receptor signal transduction. *J. Pharmacol. Exp. Ther.* 284, 346–355.
- Frick, L. R., Bernardez-Vidal, M., Hocht, C., Zanutto, B. S., and Rapanelli, M. (2015). Dual role of serotonin in the acquisition and extinction of reward-driven learning: involvement of 5-HT1A, 5-HT2A and 5-HT3 receptors. *Behav. Brain Res.* 277, 193–203. doi: 10.1016/j.bbr.2014.06.025
- Furr, A., Lapiz-Bluhm, M. D., and Morilak, D. A. (2012). 5-HT2A receptors in the orbitofrontal cortex facilitate reversal learning and contribute to the beneficial cognitive effects of chronic citalopram treatment in rats. *Int. J. Neuropsychopharmacol.* 15, 1295–1305. doi: 10.1017/S1461145711001441
- Gaddum, J. H., and Picarelli, Z. P. (1957). Two kinds of tryptamine receptor. *Br. J. Pharmacol. Chemother.* 12, 323–328. doi: 10.1111/j.1476-5381.1957.tb00142.x
- Gimpl, M. P., Gormezano, I., and Harvey, J. A. (1979). Effects of LSD on learning as measured by classical conditioning of the rabbit nictitating membrane response. *J. Pharmacol. Exp. Ther.* 208, 330–334.
- Glebov, K., Lochner, M., Jabs, R., Lau, T., Merkel, O., Schloss, P., et al. (2015). Serotonin stimulates secretion of exosomes from microglia cells. *Glia* 63, 626–634. doi: 10.1002/glia.22772
- Gong, P., Li, J., Wang, J., Lei, X., Chen, D., Zhang, K., et al. (2011). Variations in 5-HT2A influence spatial cognitive abilities and working memory. *Can. J. Neurol. Sci.* 38, 303–308. doi: 10.1017/S0317167100011513
- Gonzalez-Maeso, J., Ang, R. L., Yuen, T., Chan, P., Weisstaub, N. V., Lopez-Gimenez, J. F., et al. (2008). Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452, 93–97. doi: 10.1038/nature06612
- Gonzalez-Maeso, J., Weisstaub, N. V., Zhou, M., Chan, P., Ivic, L., Ang, R., et al. (2007). Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron* 53, 439–452. doi: 10.1016/j.neuron.2007.01.008
- Gooz, M., Gooz, P., Luttrell, L. M., and Raymond, J. R. (2006). 5-HT2A receptor induces ERK phosphorylation and proliferation through ADAM-17 tumor necrosis factor-alpha-converting enzyme (TACE) activation and heparin-bound epidermal growth factor-like growth factor (HB-EGF) shedding in mesangial cells. *J. Biol. Chem.* 281, 21004–21012. doi: 10.1074/jbc.M512096200
- Gray, J. A., Bhatnagar, A., Gurevich, V. V., and Roth, B. L. (2003). The interaction of a constitutively active arrestin with the arrestin-insensitive 5-HT(2A) receptor induces agonist-independent internalization. *Mol. Pharmacol.* 63, 961–972. doi: 10.1124/mol.63.5.961
- Grob, C. S., Danforth, A. L., Chopra, G. S., Hagerty, M., McKay, C. R., Halberstadt, A. L., et al. (2011). Pilot study of psilocybin treatment for anxiety in patients with advanced-stage cancer. *Arch. Gen. Psychiatry* 68, 71–78. doi: 10.1001/archgenpsychiatry.2010.116
- Gulati, A., and Bhargava, H. N. (1989). Brain and spinal cord 5-HT2 receptors of morphine-tolerant-dependent and -abstinent rats. *Eur. J. Pharmacol.* 167, 185–192. doi: 10.1016/0014-2999(89)90578-5
- Hagberg, G. B., Blomstrand, F., Nilsson, M., Tamir, H., and Hansson, E. (1998). Stimulation of 5-HT2A receptors on astrocytes in primary culture opens voltage-independent Ca2+ channels. *Neurochem. Int.* 32, 153–162. doi: 10.1016/S0197-0186(97)00087-9
- Hannon, J., and Hoyer, D. (2008). Molecular biology of 5-HT receptors. *Behav. Brain Res.* 195, 198–213. doi: 10.1016/j.bbr.2008.03.020
- Hasselbalch, S. G., Madsen, K., Svarer, C., Pinborg, L. H., Holm, S., Paulson, O. B., et al. (2008). Reduced 5-HT2A receptor binding in patients with mild cognitive impairment. *Neurobiol. Aging* 29, 1830–1838. doi: 10.1016/j.neurobiolaging.2007.04.011
- Hasuo, H., Matsuoka, T., and Akasu, T. (2002). Activation of presynaptic 5-hydroxytryptamine 2A receptors facilitates excitatory synaptic transmission via protein kinase C in the dorsolateral septal nucleus. *J. Neurosci.* 22, 7509–7517.

- Herry, C., Ciocchi, S., Senn, V., Demmou, L., Muller, C., and Luthi, A. (2008). Switching on and off fear by distinct neuronal circuits. *Nature* 454, 600–606. doi: 10.1038/nature07166
- Herry, C., Ferraguti, F., Singewald, N., Letzkus, J. J., Ehrlich, I., and Luthi, A. (2010). Neuronal circuits of fear extinction. *Eur. J. Neurosci.* 31, 599–612. doi: 10.1111/j.1460-9568.2010.07101.x
- Homberg, J. R. (2012). Serotonin and decision making processes. *Neurosci. Biobehav. Rev.* 36, 218–236. doi: 10.1016/j.neubiorev.2011.06.001
- Hoyer, D., Hannon, J. P., and Martin, G. R. (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol. Biochem. Behav.* 71, 533–554. doi: 10.1016/S0091-3057(01)00746-8
- Hoyer, D., Pazos, A., Probst, A., and Palacios, J. M. (1986). Serotonin receptors in the human brain. II. Characterization and autoradiographic localization of 5-HT1C and 5-HT2 recognition sites. *Brain Res.* 376, 97–107. doi: 10.1016/0006-8993(86)90903-0
- Huerta, P. T., Sun, L. D., Wilson, M. A., and Tonegawa, S. (2000). Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons. *Neuron* 25, 473–480. doi: 10.1016/S0896-6273(00)80909-5
- Izumi, T., Ohmura, Y., Futami, Y., Matsuzaki, H., Kubo, Y., Yoshida, T., et al. (2012). Effects of serotonergic terminal lesion in the amygdala on conditioned fear and innate fear in rats. *Eur. J. Pharmacol.* 696, 89–95. doi: 10.1016/j.ejphar.2012.09.028
- Jakab, R. L., and Goldman-Rakic, P. S. (1998). 5-Hydroxytryptamine2A serotonin receptors in the primate cerebral cortex: possible site of action of hallucinogenic and antipsychotic drugs in pyramidal cell apical dendrites. *Proc. Natl. Acad. Sci. U.S.A.* 95, 735–740. doi: 10.1073/pnas.95.2.735
- Jenkins, T. A., Elliott, J. J., Ardis, T. C., Cahir, M., Reynolds, G. P., Bell, R., et al. (2009). Tryptophan depletion impairs object-recognition memory in the rat: reversal by risperidone. *Behav. Brain Res.* 208, 479–483. doi: 10.1016/j.bbr.2009.12.030
- Jiang, X., Xing, G., Yang, C., Verma, A., Zhang, L., and Li, H. (2009). Stress impairs 5-HT2A receptor-mediated serotonergic facilitation of GABA release in juvenile rat basolateral amygdala. *Neuropsychopharmacology* 34, 410–423. doi: 10.1038/npp.2008.71
- Jones, K. A., Srivastava, D. P., Allen, J. A., Strachan, R. T., Roth, B. L., and Penzes, P. (2009). Rapid modulation of spine morphology by the 5-HT2A serotonin receptor through kalirin-7 signaling. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19575–19580. doi: 10.1073/pnas.0905884106
- Jovanovic, T., and Ressler, K. J. (2010). How the neurocircuitry and genetics of fear inhibition may inform our understanding of PTSD. *Am. J. Psychiatry* 167, 648–662. doi: 10.1176/appi.ajp.2009.09071074
- Julius, D., Huang, K. N., Livelli, T. J., Axel, R., and Jessell, T. M. (1990). The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. *Proc. Natl. Acad. Sci. U.S.A.* 87, 928–932. doi: 10.1073/pnas.87.3.928
- Kandel, E. R., and Squire, L. R. (2000). Neuroscience: breaking down scientific barriers to the study of brain and mind. *Science* 290, 1113–1120. doi: 10.1126/science.290.5494.1113
- Kant, G. J., Wylie, R. M., Chu, K., and Ghosh, S. (1998). Effects of the serotonin agonists 8-OH-DPAT, buspirone, and DOI on water maze performance. *Pharmacol. Biochem. Behav.* 59, 729–735. doi: 10.1016/S0091-3057(97)00553-4
- Karlsson, M. P., and Frank, L. M. (2009). Awake replay of remote experiences in the hippocampus. *Nat. Neurosci.* 12, 913–918. doi: 10.1038/nn.2344
- Khaliq, S., Haider, S., Ahmed, S. P., Perveen, T., and Haleem, D. J. (2006). Relationship of brain tryptophan and serotonin in improving cognitive performance in rats. *Pak. J. Pharm. Sci.* 19, 11–15.
- Kim, J. J., and Fanselow, M. S. (1992). Modality-specific retrograde amnesia of fear. *Science* 256, 675–677. doi: 10.1126/science.1585183
- Kometer, M., Schmidt, A., Jancke, L., and Vollenweider, F. X. (2013). Activation of serotonin 2A receptors underlies the psilocybin-induced effects on alpha oscillations, N170 visual-evoked potentials, and visual hallucinations. *J. Neurosci.* 33, 10544–10551. doi: 10.1523/JNEUROSCI.3007-12.2013
- Kroese, W. K., and Roth, B. L. (1998). The molecular biology of serotonin receptors: therapeutic implications for the interface of mood and psychosis. *Biol. Psychiatry* 44, 1128–1142. doi: 10.1016/S0006-3223(98)00132-2
- Kurrasch-Orbaugh, D. M., Parrish, J. C., Watts, V. J., and Nichols, D. E. (2003a). A complex signaling cascade links the serotonin2A receptor to phospholipase A2 activation: the involvement of MAP kinases. *J. Neurochem.* 86, 980–991. doi: 10.1046/j.1471-4159.2003.01921.x
- Kurrasch-Orbaugh, D. M., Watts, V. J., Barker, E. L., and Nichols, D. E. (2003b). Serotonin 5-hydroxytryptamine 2A receptor-coupled phospholipase C and phospholipase A2 signaling pathways have different receptor reserves. *J. Pharmacol. Exp. Ther.* 304, 229–237. doi: 10.1124/jpet.102.042184
- Ledgerwood, L., Richardson, R., and Cranney, J. (2003). Effects of D-cycloserine on extinction of conditioned freezing. *Behav. Neurosci.* 117, 341–349. doi: 10.1037/0735-7044.117.2.341
- Levkovitz, Y., Ophir-Shaham, O., Bloch, Y., Treves, I., Fennig, S., and Grauer, E. (2003). Effect of L-tryptophan on memory in patients with schizophrenia. *J. Nerv. Ment. Dis.* 191, 568–573. doi: 10.1097/01.nmd.0000087182.29781.e0
- Li, J. X., Shah, A. P., Patel, S. K., Rice, K. C., and France, C. P. (2013). Modification of the behavioral effects of morphine in rats by serotonin 5-HT(1)A and 5-HT(2)A receptor agonists: antinociception, drug discrimination, and locomotor activity. *Psychopharmacology (Berl)* 225, 791–801. doi: 10.1007/s00213-012-2870-2
- Li, L. B., Zhang, L., Sun, Y. N., Han, L. N., Wu, Z. H., Zhang, Q. J., et al. (2015). Activation of serotonin2A receptors in the medial septum-diagonal band of Broca complex enhanced working memory in the hemiparkinsonian rats. *Neuropharmacology* 91, 23–33. doi: 10.1016/j.neuropharm.2014.11.025
- Lopez-Gimenez, J. F., Vilaro, M. T., Palacios, J. M., and Mengod, G. (1998). [³H]MDL 100,907 labels 5-HT2A serotonin receptors selectively in primate brain. *Neuropharmacology* 37, 1147–1158. doi: 10.1016/S0028-3908(98)00102-6
- Lukasiewicz, S., Polit, A., Kedraka-Krok, S., Wedzony, K., Mackowiak, M., and Dziedzicka-Wasylewska, M. (2010). Hetero-dimerization of serotonin 5-HT(2A) and dopamine D(2) receptors. *Biochim. Biophys. Acta* 1803, 1347–1358. doi: 10.1016/j.bbamcr.2010.08.010
- Luttgen, M., Ove Ogren, S., and Meister, B. (2004). Chemical identity of 5-HT2A receptor immunoreactive neurons of the rat septal complex and dorsal hippocampus. *Brain Res.* 1010, 156–165. doi: 10.1016/j.brainres.2004.03.016
- Marner, L., Frokjaer, V. G., Kalbitzer, J., Lehel, S., Madsen, K., Baare, W. F., et al. (2012). Loss of serotonin 2A receptors exceeds loss of serotonergic projections in early Alzheimer's disease: a combined [(11)C]DASB and [(18)F]altanserin-PET study. *Neurobiol. Aging* 33, 479–487. doi: 10.1016/j.neurobiolaging.2010.03.023
- Marner, L., Knudsen, G. M., Madsen, K., Holm, S., Baare, W., and Hasselbalch, S. G. (2011). The reduction of baseline serotonin 2A receptors in mild cognitive impairment is stable at two-year follow-up. *J. Alzheimers Dis.* 23, 453–459.
- Masson, J., Emerit, M. B., Hamon, M., and Darmon, M. (2012). Serotonergic signaling: multiple effectors and pleiotropic effects. *Wiley Interdiscip. Rev. Membr. Transp. Signal.* 1, 685–713. doi: 10.1002/wmrs.50
- Matus-Amat, P., Higgins, E. A., Barrientos, R. M., and Rudy, J. W. (2004). The role of the dorsal hippocampus in the acquisition and retrieval of context memory representations. *J. Neurosci.* 24, 2431–2439. doi: 10.1523/JNEUROSCI.1598-03.2004
- McEchron, M. D., Bouwmeester, H., Tseng, W., Weiss, C., and Disterhoft, J. F. (1998). Hippocampectomy disrupts auditory trace fear conditioning and contextual fear conditioning in the rat. *Hippocampus* 8, 638–646. doi: 10.1002/(SICI)1098-1063(1998)8:6<638::AID-HIPO6>3.0.CO;2-Q
- McHugh, T. J., Blum, K. I., Tsien, J. Z., Tonegawa, S., and Wilson, M. A. (1996). Impaired hippocampal representation of space in CA1-specific NMDAR1 knockout mice. *Cell* 87, 1339–1349. doi: 10.1016/S0092-8674(00)81828-0
- McLean, J. H., Darby-King, A., and Hodge, E. (1996). 5-HT2 receptor involvement in conditioned olfactory learning in the neonate rat pup. *Behav. Neurosci.* 110, 1426–1434. doi: 10.1037/0735-7044.110.6.1426
- Melancon, B. J., Hopkins, C. R., Wood, M. R., Emmite, K. A., Niswender, C. M., Christopoulos, A., et al. (2012). Allosteric modulation of seven transmembrane spanning receptors: theory, practice, and opportunities for central nervous system drug discovery. *J. Med. Chem.* 55, 1445–1464. doi: 10.1021/jm201139r
- Meller, R., Harrison, P. J., Elliott, J. M., and Sharp, T. (2002). In vitro evidence that 5-hydroxytryptamine increases efflux of glial glutamate via 5-HT(2A) receptor activation. *J. Neurosci. Res.* 67, 399–405. doi: 10.1002/jnr.10126
- Meltzer, C. C., Smith, G., Dekosky, S. T., Pollock, B. G., Mathis, C. A., Moore, R. Y., et al. (1998). Serotonin in aging, late-life depression, and Alzheimer's

- disease: the emerging role of functional imaging. *Neuropsychopharmacology* 18, 407–430. doi: 10.1016/S0893-133X(97)00194-2
- Meltzer, H. Y. (1999). The role of serotonin in antipsychotic drug action. *Neuropsychopharmacology* 21, 106S–115S. doi: 10.1016/S0893-133X(99)00046-9
- Meneses, A. (2007). Stimulation of 5-HT1A, 5-HT1B, 5-HT2A/2C, 5-HT3 and 5-HT4 receptors or 5-HT uptake inhibition: short- and long-term memory. *Behav. Brain Res.* 184, 81–90. doi: 10.1016/j.bbr.2007.06.026
- Meneses, A. (2013). 5-HT systems: emergent targets for memory formation and memory alteration. *Rev. Neurosci.* 24, 629–664. doi: 10.1515/revneuro-2013-0026
- Meneses, A., Terron, J. A., and Hong, E. (1997). Effects of the 5-HT receptor antagonists GR127935 (5-HT1B/1D) and MDL100907 (5-HT2A) in the consolidation of learning. *Behav. Brain Res.* 89, 217–223. doi: 10.1016/S0166-4328(97)00055-7
- Millan, M. J., Marin, P., Bockaert, J., and Mannoury La Cour, C. (2008). Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. *Trends Pharmacol. Sci.* 29, 454–464. doi: 10.1016/j.tips.2008.06.007
- Miner, L. A., Backstrom, J. R., Sanders-Bush, E., and Sesack, S. R. (2003). Ultrastructural localization of serotonin2A receptors in the middle layers of the rat prelimbic prefrontal cortex. *Neuroscience* 116, 107–117. doi: 10.1016/S0306-4522(02)00580-8
- Mirjana, C., Baviera, M., Invernizzi, R. W., and Balducci, C. (2004). The serotonin 5-HT2A receptors antagonist M100907 prevents impairment in attentional performance by NMDA receptor blockade in the rat prefrontal cortex. *Neuropsychopharmacology* 29, 1637–1647. doi: 10.1038/sj.npp.1300479
- Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* 11, 47–60. doi: 10.1016/0165-0270(84)90007-4
- Morris, R. G., Garrud, P., Rawlins, J. N., and O'keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature* 297, 681–683. doi: 10.1038/297681a0
- Moser, E. I., Kropff, E., and Moser, M. B. (2008). Place cells, grid cells, and the brain's spatial representation system. *Annu. Rev. Neurosci.* 31, 69–89. doi: 10.1146/annurev.neuro.31.061307.090723
- Naghdi, N., and Harooni, H. E. (2005). The effect of intrahippocampal injections of ritanserin (5HT2A/2C antagonist) and granisetron (5HT3 antagonist) on learning as assessed in the spatial version of the water maze. *Behav. Brain Res.* 157, 205–210. doi: 10.1016/j.bbr.2004.06.024
- Nic Dhonnchadha, B. A., Fox, R. G., Stutz, S. J., Rice, K. C., and Cunningham, K. A. (2009). Blockade of the serotonin 5-HT2A receptor suppresses cue-evoked reinstatement of cocaine-seeking behavior in a rat self-administration model. *Behav. Neurosci.* 123, 382–396. doi: 10.1037/a0014592
- O'Neill, M. F., Heron-Maxwell, C. L., and Shaw, G. (1999). 5-HT2 receptor antagonism reduces hyperactivity induced by amphetamine, cocaine, and MK-801 but not D1 agonist C-APB. *Pharmacol. Biochem. Behav.* 63, 237–243. doi: 10.1016/S0091-3057(98)00240-8
- Pazos, A., Probst, A., and Palacios, J. M. (1987). Serotonin receptors in the human brain-IV. Autoradiographic mapping of serotonin-2 receptors. *Neuroscience* 21, 123–139. doi: 10.1016/0306-4522(87)90327-7
- Peddie, C. J., Davies, H. A., Colyer, F. M., Stewart, M. G., and Rodriguez, J. J. (2008). Colocalisation of serotonin2A receptors with the glutamate receptor subunits NR1 and GluR2 in the dentate gyrus: an ultrastructural study of a modulatory role. *Exp. Neurol.* 211, 561–573. doi: 10.1016/j.expneurol.2008.03.003
- Perani, D., Garibotto, V., Gorini, A., Moresco, R. M., Henin, M., Panzacchi, A., et al. (2008). In vivo PET study of 5HT(2A) serotonin and D2 dopamine dysfunction in drug-naïve obsessive-compulsive disorder. *Neuroimage* 42, 306–314. doi: 10.1016/j.neuroimage.2008.04.233
- Peroutka, S. J., and Snyder, S. H. (1979). Multiple serotonin receptors: differential binding of [³H]5-hydroxytryptamine, [³H]lysergic acid diethylamide and [³H]spiroperidol. *Mol. Pharmacol.* 16, 687–699.
- Phillips, R. G., and LeDoux, J. E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* 106, 274–285. doi: 10.1037/0735-7044.106.2.274
- Pockros, L. A., Pentkowski, N. S., Swinford, S. E., and Neisewander, J. L. (2011). Blockade of 5-HT2A receptors in the medial prefrontal cortex attenuates reinstatement of cue-elicited cocaine-seeking behavior in rats. *Psychopharmacology (Berl)* 213, 307–320. doi: 10.1007/s00213-010-2071-9
- Porter, R. J., Lunn, B. S., and O'brien, J. T. (2003). Effects of acute tryptophan depletion on cognitive function in Alzheimer's disease and in the healthy elderly. *Psychol. Med.* 33, 41–49. doi: 10.1017/s0033291702006906
- Poyurovsky, M., Koren, D., Gonopolsky, I., Schneidman, M., Fuchs, C., Weizman, A., et al. (2003). Effect of the 5-HT2 antagonist mianserin on cognitive dysfunction in chronic schizophrenia patients: an add-on, double-blind placebo-controlled study. *Eur. Neuropsychopharmacol.* 13, 123–128. doi: 10.1016/S0924-977X(02)00155-4
- Preston, A. R., and Eichenbaum, H. (2013). Interplay of hippocampus and prefrontal cortex in memory. *Curr. Biol.* 23, R764–R773. doi: 10.1016/j.cub.2013.05.041
- Pritchett, D. B., Bach, A. W., Wozny, M., Taleb, O., Dal Toso, R., Shih, J. C., et al. (1988). Structure and functional expression of cloned rat serotonin 5HT-2 receptor. *EMBO J.* 7, 4135–4140.
- Quinn, J. C., Johnson-Farley, N. N., Yoon, J., and Cowen, D. S. (2002). Activation of extracellular-regulated kinase by 5-hydroxytryptamine(2A) receptors in PC12 cells is protein kinase C-independent and requires calmodulin and tyrosine kinases. *J. Pharmacol. Exp. Ther.* 303, 746–752. doi: 10.1124/jpet.102.038083
- Quirion, R., Richard, J., and Dam, T. V. (1985). Evidence for the existence of serotonin type-2 receptors on cholinergic terminals in rat cortex. *Brain Res.* 333, 345–349. doi: 10.1016/0006-8993(85)91590-2
- Quirk, G. J., Pare, D., Richardson, R., Herry, C., Monfils, M. H., Schiller, D., et al. (2010). Erasing fear memories with extinction training. *J. Neurosci.* 30, 14993–14997. doi: 10.1523/JNEUROSCI.4268-1.2010
- Rambousek, L., Palenicek, T., Vales, K., and Stuchlik, A. (2014). The effect of psilocin on memory acquisition, retrieval, and consolidation in the rat. *Front. Behav. Neurosci.* 8:180. doi: 10.3389/fnbeh.2014.00180
- Rios, C. D., Jordan, B. A., Gomes, I., and Devi, L. A. (2001). G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol. Ther.* 92, 71–87. doi: 10.1016/S0163-7258(01)00160-7
- Romano, A. G., Quinn, J. L., Li, L., Dave, K. D., Schindler, E. A., Aloyo, V. J., et al. (2010). Intrahippocampal LSD accelerates learning and desensitizes the 5-HT(2A) receptor in the rabbit, Romano et al. *Psychopharmacology (Berl)* 212, 441–448. doi: 10.1007/s00213-010-2004-7
- Roth, B. L., Berry, S. A., Kroese, W. K., Willins, D. L., and Kristiansen, K. (1998). Serotonin 5-HT2A receptors: molecular biology and mechanisms of regulation. *Crit. Rev. Neurobiol.* 12, 319–338. doi: 10.1615/CritRevNeurobiol.v12.i4.30
- Rudy, J. W., Barrientos, R. M., and O'reilly, R. C. (2002). Hippocampal formation supports conditioning to memory of a context. *Behav. Neurosci.* 116, 530–538. doi: 10.1037/0735-7044.116.4.530
- Rudy, J. W., Huff, N. C., and Matus-Amat, P. (2004). Understanding contextual fear conditioning: insights from a two-process model. *Neurosci. Biobehav. Rev.* 28, 675–685. doi: 10.1016/j.neubiorev.2004.09.004
- Ruotsalainen, S., Sirvio, J., Jakala, P., Puunala, T., Macdonald, E., and Riekkinen, P. Sr. (1997). Differential effects of three 5-HT receptor antagonists on the performance of rats in attentional and working memory tasks. *Eur. Neuropsychopharmacol.* 7, 99–108. doi: 10.1016/S0924-977X(96)00389-6
- Santhosh, L., Estok, K. M., Vogel, R. S., Tamagnan, G. D., Baldwin, R. M., Mitsis, E. M., et al. (2009). Regional distribution and behavioral correlates of 5-HT(2A) receptors in Alzheimer's disease with [(18)F]deuteroalantserin and PET. *Psychiatry Res.* 173, 212–217. doi: 10.1016/j.psychresns.2009.03.007
- Schmid, C. L., and Bohn, L. M. (2010). Serotonin, but not N-methyltryptamines, activates the serotonin 2A receptor via a ss-arrestin2/Src/Akt signaling complex in vivo. *J. Neurosci.* 30, 13513–13524. doi: 10.1523/JNEUROSCI.1665-10.2010
- Schmid, C. L., Raehal, K. M., and Bohn, L. M. (2008). Agonist-directed signaling of the serotonin 2A receptor depends on beta-arrestin-2 interactions in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1079–1084. doi: 10.1073/pnas.0708862105
- Schmitt, J. A., Wingen, M., Ramaekers, J. G., Evers, E. A., and Riedel, W. J. (2006). Serotonin and human cognitive performance. *Curr. Pharm. Des.* 12, 2473–2486. doi: 10.2174/138161206777698909
- Schott, B. H., Seidenbecher, C. I., Richter, S., Wustenberg, T., Debska-Vielhaber, G., Schubert, H., et al. (2011). Genetic variation of the serotonin 2a receptor affects hippocampal novelty processing in humans. *PLoS ONE* 6:e15984. doi: 10.1371/journal.pone.0015984

- Sheffler, D. J., Kroeze, W. K., Garcia, B. G., Deutch, A. Y., Hufeisen, S. J., Leahy, P., et al. (2006). p90 ribosomal S6 kinase 2 exerts a tonic brake on G protein-coupled receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* 103, 4717–4722. doi: 10.1073/pnas.0600585103
- Shi, J., Damjanoska, K. J., Singh, R. K., Carrasco, G. A., Garcia, F., Grippo, A. J., et al. (2007). Agonist induced-phosphorylation of Galphai1 protein reduces coupling to 5-HT2A receptors. *J. Pharmacol. Exp. Ther.* 323, 248–256. doi: 10.1124/jpet.107.122317
- Sierra-Mercado, D., Padilla-Coreano, N., and Quirk, G. J. (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropharmacology* 59, 529–538. doi: 10.1038/npp.2010.184
- Sigmund, J. C., Vogler, C., Huynh, K. D., De Quervain, D. J., and Papassotiropoulos, A. (2008). Fine-mapping at the HTR2A locus reveals multiple episodic memory-related variants. *Biol. Psychol.* 79, 239–242. doi: 10.1016/j.biopsych.2008.06.002
- Singh, R. K., Jia, C., Garcia, F., Carrasco, G. A., Battaglia, G., and Muma, N. A. (2010). Activation of the JAK-STAT pathway by olanzapine is necessary for desensitization of serotonin2A receptor-stimulated phospholipase C signaling in rat frontal cortex but not serotonin2A receptor-stimulated hormone release. *J. Psychopharmacol.* 24, 1079–1088. doi: 10.1177/02698811093090
- Snigdha, S., Horiguchi, M., Huang, M., Li, Z., Shahid, M., Neill, J. C., et al. (2010). Attenuation of phencyclidine-induced object recognition deficits by the combination of atypical antipsychotic drugs and pimavanserin (ACP 103), a 5-hydroxytryptamine(2A) receptor inverse agonist. *J. Pharmacol. Exp. Ther.* 332, 622–631. doi: 10.1124/jpet.109.156349
- Sparkes, R. S., Lan, N., Klisak, I., Mohandas, T., Diep, A., Kojis, T., et al. (1991). Assignment of a serotonin 5HT-2 receptor gene (HTR2) to human chromosome 13q14-q21 and mouse chromosome 14. *Genomics* 9, 461–465. doi: 10.1016/0888-7543(91)90411-7
- Squire, L. R., Stark, C. E., and Clark, R. E. (2004). The medial temporal lobe. *Annu. Rev. Neurosci.* 27, 279–306. doi: 10.1146/annurev.neuro.27.070203.144130
- Squire, L. R., Wixted, J. T., and Clark, R. E. (2007). Recognition memory and the medial temporal lobe: a new perspective. *Nat. Rev. Neurosci.* 8, 872–883. doi: 10.1038/nrn2154
- Stein, C., Davidowa, H., and Albrecht, D. (2000). 5-HT(1A) receptor-mediated inhibition and 5-HT(2) as well as 5-HT(3) receptor-mediated excitation in different subdivisions of the rat amygdala. *Synapse* 38, 328–337. doi: 10.1002/1098-2396(20001201)38:3<328::AID-SYN12>3.0.CO;2-T
- Stiedl, O., Birkenfeld, K., Palve, M., and Spiess, J. (2000). Impairment of conditioned contextual fear of C57BL/6J mice by intracerebral injections of the NMDA receptor antagonist APV. *Behav. Brain Res.* 116, 157–168. doi: 10.1016/S0166-4328(00)00269-2
- Strachan, R. T., Allen, J. A., Sheffler, D. J., and Roth, B. L. (2010). p90 Ribosomal S6 kinase 2, a novel GPCR kinase, is required for growth factor-mediated attenuation of GPCR signaling. *Biochemistry* 49, 2657–2671. doi: 10.1021/bi901921k
- Strachan, R. T., Allen, J. A., Sheffler, D. J., Willard, B., Kinter, M., Kiselar, J. G., et al. (2009). Ribosomal S6 kinase 2 directly phosphorylates the 5-hydroxytryptamine 2A (5-HT2A) serotonin receptor, thereby modulating 5-HT2A signaling. *J. Biol. Chem.* 284, 5557–5573. doi: 10.1074/jbc.M805705200
- Szapiro, G., Vianna, M. R., Mcgaugh, J. L., Medina, J. H., and Izquierdo, I. (2003). The role of NMDA glutamate receptors, PKA, MAPK, and CAMKII in the hippocampus in extinction of conditioned fear. *Hippocampus* 13, 53–58. doi: 10.1002/hipo.10043
- Talvik-Lotfi, M., Nyberg, S., Nordstrom, A. L., Ito, H., Halldin, C., Brunner, F., et al. (2000). High 5HT2A receptor occupancy in M100907-treated schizophrenic patients. *Psychopharmacology (Berl.)* 148, 400–403. doi: 10.1007/s002130050069
- Thomas, E. A., Carson, M. J., Neal, M. J., and Sutcliffe, J. G. (1997). Unique allosteric regulation of 5-hydroxytryptamine receptor-mediated signal transduction by oleamide. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14115–14119. doi: 10.1073/pnas.94.25.14115
- Tovote, P., Fadok, J. P., and Luthi, A. (2015). Neuronal circuits for fear and anxiety. *Nat. Rev. Neurosci.* 16, 317–331. doi: 10.1038/nrn3945
- Turner, J. H., and Raymond, J. R. (2005). Interaction of calmodulin with the serotonin 5-hydroxytryptamine2A receptor. A putative regulator of G protein coupling and receptor phosphorylation by protein kinase C. *J. Biol. Chem.* 280, 30741–30750. doi: 10.1074/jbc.M501696200
- Uchida, S., Umeeda, H., Kitamoto, A., Masushige, S., and Kida, S. (2007). Chronic reduction in dietary tryptophan leads to a selective impairment of contextual fear memory in mice. *Brain Res.* 1149, 149–156. doi: 10.1016/j.brainres.2007.02.049
- Vaidya, V. A., Marek, G. J., Aghajanian, G. K., and Duman, R. S. (1997). 5-HT2A receptor-mediated regulation of brain-derived neurotrophic factor mRNA in the hippocampus and the neocortex. *J. Neurosci.* 17, 2785–2795.
- Versijpt, J., Van Laere, K. J., Dumont, F., Decoo, D., Vandecastelle, M., Santens, P., et al. (2003). Imaging of the 5-HT2A system: age-, gender-, and Alzheimer's disease-related findings. *Neurobiol. Aging* 24, 553–561. doi: 10.1016/S0197-4580(02)00137-9
- Vertes, R. P. (1991). A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *J. Comp. Neurol.* 313, 643–668. doi: 10.1002/cne.903130409
- Vertes, R. P., Fortin, W. J., and Crane, A. M. (1999). Projections of the median raphe nucleus in the rat. *J. Comp. Neurol.* 407, 555–582. doi: 10.1002/(SICI)1096-9861(19990517)407:4<555::AID-CNE7>3.0.CO;2-E
- Vinalas, X., Moreno, E., Lanfumey, L., Cordomí, A., Pastor, A., De La Torre, R., et al. (2015). Cognitive impairment induced by delta9-tetrahydrocannabinol occurs through heteromers between cannabinoid CB1 and serotonin 5-HT2A receptors. *PLoS Biol.* 13:e1002194. doi: 10.1371/journal.pbio.1002194
- Wagner, M., Schuhmacher, A., Schwab, S., Zobel, A., and Maier, W. (2008). The His452Tyr variant of the gene encoding the 5-HT2A receptor is specifically associated with consolidation of episodic memory in humans. *Int. J. Psychopharmacol.* 11, 1163–1167. doi: 10.1017/S146114570800905X
- Walker, D. L., Ressler, K. J., Lu, K. T., and Davis, M. (2002). Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. *J. Neurosci.* 22, 2343–2351.
- Watts, S. W. (1998). Activation of the mitogen-activated protein kinase pathway via the 5-HT2A receptor. *Ann. N. Y. Acad. Sci.* 861, 162–168. doi: 10.1111/j.1749-6632.1998.tb10187.x
- Welsh, S. E., Romano, A. G., and Harvey, J. A. (1998). Effects of serotonin 5-HT(2A/C) antagonists on associative learning in the rabbit. *Psychopharmacology (Berl.)* 137, 157–163. doi: 10.1007/s002130050605
- Williams, G. V., Rao, S. G., and Goldman-Rakic, P. S. (2002). The physiological role of 5-HT2A receptors in working memory. *J. Neurosci.* 22, 2843–2854.
- Willins, D. L., Deutch, A. Y., and Roth, B. L. (1997). Serotonin 5-HT2A receptors are expressed on pyramidal cells and interneurons in the rat cortex. *Synapse* 27, 79–82. doi: 10.1002/(SICI)1098-2396(199709)27:1<79::AID-SYN8>3.0.CO;2-A
- Wiltgen, B. J., Sanders, M. J., Anagnostaras, S. G., Sage, J. R., and Fanselow, M. S. (2006). Context fear learning in the absence of the hippocampus. *J. Neurosci.* 26, 5484–5491. doi: 10.1523/JNEUROSCI.2685-05.2006
- Xia, Z., Gray, J. A., Compton-Toth, B. A., and Roth, B. L. (2003). A direct interaction of PSD-95 with 5-HT2A serotonin receptors regulates receptor trafficking and signal transduction. *J. Biol. Chem.* 278, 21901–21908. doi: 10.1074/jbc.M301905200
- Xu, T., and Pandey, S. C. (2000). Cellular localization of serotonin(2A) (5HT(2A)) receptors in the rat brain. *Brain Res. Bull.* 51, 499–505. doi: 10.1016/S0361-9230(99)00278-6
- Yoshida, H., Kanamaru, C., Ohtani, A., Li, F., Senzaki, K., and Shiga, T. (2011). Subtype specific roles of serotonin receptors in the spine formation of cortical neurons in vitro. *Neurosci. Res.* 71, 311–314. doi: 10.1016/j.neures.2011.07.1824
- Zaniewska, M., Mccreary, A. C., Wydra, K., and Filip, M. (2010). Differential effects of serotonin (5-HT)2 receptor-targeting ligands on locomotor responses to nicotine-repeated treatment. *Synapse* 64, 511–519. doi: 10.1002/syn.20756

- Zayara, A. E., Mciver, G., Valdivia, P. N., Lominac, K. D., McCreary, A. C., and Szumlinski, K. K. (2011). Blockade of nucleus accumbens 5-HT2A and 5-HT2C receptors prevents the expression of cocaine-induced behavioral and neurochemical sensitization in rats. *Psychopharmacology (Berl)* 213, 321–335. doi: 10.1007/s00213-010-1996-3
- Zelikowsky, M., Hersman, S., Chawla, M. K., Barnes, C. A., and Fanselow, M. S. (2014). Neuronal ensembles in amygdala, hippocampus, and prefrontal cortex track differential components of contextual fear. *J. Neurosci.* 34, 8462–8466. doi: 10.1523/JNEUROSCI.3624-13.2014
- Zhang, G., Ásgeirsdóttir, H. N., Cohen, S. J., Munchow, A. H., Barrera, M. P., and Stackman, R. W. Jr. (2013). Stimulation of serotonin 2A receptors facilitates consolidation and extinction of fear memory in C57BL/6J mice. *Neuropharmacology* 64, 403–413. doi: 10.1016/j.neuropharm.2012.06.007
- Zhang, G., Cinalli, D., Barrera, M. P., and Stackman, R. W. (2015). “Activation of serotonin 5-HT2A receptor delays the retrieval of spatial memory in a Morris-water maze task,” in *Proceedings of the Society of Neuroscience Conference*, Chicago.
- Zhou, F. M., and Hablitz, J. J. (1999). Activation of serotonin receptors modulates synaptic transmission in rat cerebral cortex. *J. Neurophysiol.* 82, 2989–2999.
- Zhu, B., Chen, C., Loftus, E. F., Moysis, R. K., Dong, Q., and Lin, C. (2013). True but not false memories are associated with the HTR2A gene. *Neurobiol. Learn Mem.* 106, 204–209. doi: 10.1016/j.nlm.2013.09.004

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Interactions of the opioid and cannabinoid systems in reward: Insights from knockout studies

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The opioid system consists of three receptors, mu, delta, and kappa, which are activated by endogenous opioid peptides (enkephalins, endorphins, and dynorphins). The endogenous cannabinoid system comprises lipid neuromodulators (endocannabinoids), enzymes for their synthesis and their degradation and two well-characterized receptors, cannabinoid receptors CB1 and CB2. These systems play a major role in the control of pain as well as in mood regulation, reward processing and the development of addiction. Both opioid and cannabinoid receptors are coupled to G proteins and are expressed throughout the brain reinforcement circuitry. Extending classical pharmacology, research using genetically modified mice has provided important progress in the identification of the specific contribution of each component of these endogenous systems *in vivo* on reward process. This review will summarize available genetic tools and our present knowledge on the consequences of gene knockout on reinforced behaviors in both systems, with a focus on their potential interactions. A better understanding of opioid–cannabinoid interactions may provide novel strategies for therapies in addicted individuals.

Keywords: opioid, cannabinoid, G protein-coupled receptors, reward, genetically modified mice

INTRODUCTION

Drug abuse often leads to a complex pharmaco-dependent state which is defined by the term addiction. Addiction is considered as a neuropsychiatric disease. It develops from an initial recreational drug use, evolves toward compulsive drug-seeking behavior and excessive drug-intake with the appearance of negative emotional states such as anxiety or irritability when the drug is not accessible, and uncontrolled intake reaching a stage where the drug interferes with daily activities, despite the emergence of adverse consequences (Leshner, 1997; Everitt and Robbins, 2005; Robinson and Berridge, 2008; Koob, 2009). This pathological process develops in 15–30% of casual drug users and several factors may explain individual's vulnerability to addiction, including genetic, psychological and environmental factors (Swendsen and Le Moal, 2011; Belin and Deroche-Gamonet, 2012; Pattij and De Vries, 2013; Saunders and Robinson, 2013). Addiction is a major threat to public health and represents a societal problem especially in developed countries and the economic cost it entails (investments in research, treatment and prevention) is considerable (Gustavsson et al., 2011).

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide, N-arachidonoyl-ethanolamide; CB1, type 1 cannabinoid receptor; CB2, type 2 cannabinoid receptor; cKO, conditional knockout mice; CPA, conditioned place aversion; CP 55,940, (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; CPP, conditioned place preference; CPU, caudate putamen; DA, dopamine; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; G protein, guanine nucleotide binding protein; GABA, *c*-aminobutyric acid; GPCR, G protein coupled receptor; KO, knockout; MGL, monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; THC, Delta-9-tetrahydrocannabinol; WIN 55,212-2, 2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoxazin-yl-1-naphthalenylmethanone mesylate; WT, wild-type.

Among illicit drugs, opiate and cannabinoid derivatives are highly abused in Europe. Morphine-like opiates are powerful analgesics and currently represent the major therapeutic remedies for the treatment of severe pain. They are also abused for their recreational euphoric effects. In Europe, 1.3 million people are addicted to heroin, the primary drug for which treatment requests are sought. Cannabis is the most worldwide consumed drug of abuse, with THC being the most abundant active constituent found in the various preparations of the drug. More than 73 million European citizens have used cannabis in the last year and it is estimated that about 7% of cannabis users has become dependent on this drug. There is also a high prevalence of users who seek treatment for dependence on it (<http://www.emcdda.europa.eu/publications/edr/trends-developments/2014>). Interestingly, new derivatives of these abused drugs are invading the market, notably through internet. Fentanyl derivatives as new opioid drugs and synthetic cannabinoids, also known as “spices,” are becoming more and more popular (Fattore and Fratta, 2011). These abusive substances interact with two neuromodulator systems, the opioid and the endocannabinoid systems.

THE OPIOID SYSTEM

The opioid system consists of endogenous opioid peptides (enkephalins, endorphins, and dynorphins) from precursors (Penk, Pdyn, and Pomp) which activate three opioid receptors, namely mu, delta, and kappa (Kieffer, 1995). The three membrane receptors, cloned in the early nineties (Evans et al., 1992; Kieffer et al., 1992; Simonin et al., 1994, 1995; Mestek et al., 1995) are GPCR with coupling to Gi/Go proteins, of which the 3D structure was recently resolved (see Filizola and Devi, 2014).

Opioid receptors and endogenous opioid peptides are largely expressed throughout the nervous system, noticeably within areas of the neurocircuitry of addiction associated with reward, motivation, or learning and stress (Mansour et al., 1995; Le Merrer et al., 2009; Koob and Volkow, 2010; Erbs et al., 2014). Besides its key role in many aspects of addition (Lutz and Kieffer, 2013a), the opioid system also plays a part in a diverse range of physiological functions including nociception, mood control, eating behavior, or cognitive processes (Contet et al., 2004; Pradhan et al., 2011; Stein, 2013; Bodnar, 2014; Nogueiras et al., 2014).

THE ENDOCANNABINOID SYSTEM

The endocannabinoid system is a neuromodulatory system consisting of two well characterized transmembrane receptors coupled to G protein (Gi/Go), CB1, and CB2 cloned in the 1990's (Matsuda et al., 1990; Munro et al., 1993). The endogenous ligands are lipid neuromodulators, the main ones being AEA and 2-AG. Both are synthesized from phospholipid precursors and act locally as retrograde regulators of synaptic transmission throughout the central nervous system. These lipids are released by postsynaptic neurons and mainly activate presynaptic cannabinoid receptors to transiently or persistently suppress transmitter release from both excitatory and inhibitory synapses (recently reviewed in Ohno-Shosaku and Kano, 2014). Multiple pathways are involved in AEA biosynthesis with several still not fully characterized enzymes. AEA can be generated from the membrane phospholipid precursor N-arachidonoyl phosphatidylethanolamine (NAPE) through a two-step process with first a calcium-dependent transacylase followed by a phospholipase D (NAPE-PLD) hydrolysis (Liu et al., 2008). Phospholipase C (PLC) and DAGL are involved in 2-AG synthesis (Ahn et al., 2008). Their degradation is conducted by two specific enzymatic systems, the FAAH (Cravatt et al., 1996) and the MGL (Dinh et al., 2002), for AEA and 2-AG, respectively (Ahn et al., 2008). The endocannabinoid system plays a key role in

energy balance, modulation of pain response, with processing of central and peripheral pain signals, learning and memory, reward and emotions. It has also been shown to be involved in neurogenesis and would play a neuroprotective role in some pathological conditions (for recent reviews see Gardner, 2005; Solinas et al., 2008; Maldonado et al., 2011; Zanettini et al., 2011; Panagis et al., 2014; Piomelli, 2014). Distribution of the two receptors in the central and peripheral system is rather different (Pertwee, 2010). Indeed, CB1 is highly abundant in the central nervous system in areas involved in reward, regulation of appetite and nociception (see **Figure 1**) while CB2 was initially described as a peripheral receptor (Maldonado et al., 2006, 2011; Mackie, 2008). Recent studies have proposed a low but significant expression of this receptor in several brain structures including striatum, hippocampus, and thalamus (Wotherspoon et al., 2005; Gong et al., 2006; Onaivi et al., 2006) and more recently into ventral tegmental area neurons (Zhang et al., 2014). Only few data are therefore available for the CB2 receptor in central function but growing evidence suggest a role in addictive processes, with an implication in cocaine, nicotine, or ethanol effects (Xi et al., 2011; Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013; Ortega-Alvaro et al., 2013). To our knowledge, no data is available thus far concerning a potential role of CB2 in opioid mediated responses. Interestingly, other non-CB1 and non-CB2 receptors have been proposed to interact with endocannabinoids like the orphan GPCR GPR55 or a channel vanilloid TRPV1 recognizing capsaicin. These interactions could potentially explain some pharmacology of cannabis that cannot be accounted for by CB1 and CB2 activation, but further studies using KO approaches may help to provide a better understanding of this pharmacology (De Petrocellis and Di Marzo, 2010).

CROSS TALK BETWEEN THESE NEUROTRANSMITTER SYSTEMS

Many neurotransmitter systems are involved when addiction develops, and both opioid and endocannabinoid systems are

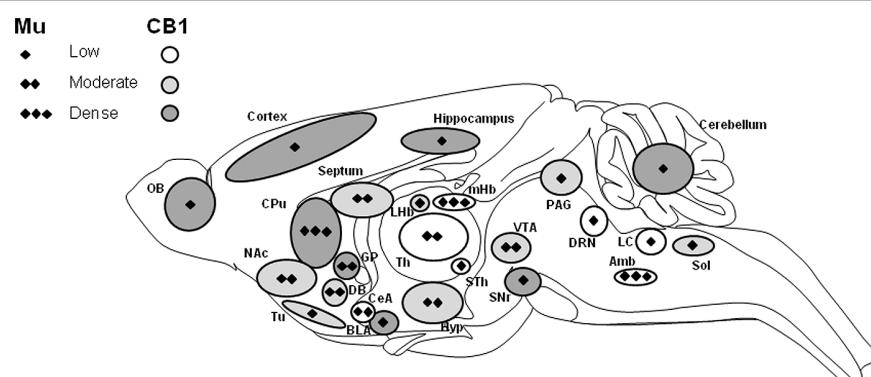


FIGURE 1 | Schematic representation of the distribution of mu opioid and CB1 cannabinoid receptors in the central nervous system. CB1 receptor distribution over the whole central nervous system is indicated by circle shapes with low (white), moderate (gray) and high (dark gray) expression. Major localization of CB1 receptor (mRNA and protein) is in cortical areas, amygdala, striatum, and cerebellum. Moderate and low expression levels are observed in thalamic, hypothalamic, and brainstem regions. Interestingly, the mu opioid receptor is also expressed in these CB1 expressing brain areas but at various levels, indicated by diamonds for low

(one), moderate (two), and high (three) expression levels (adapted from Mackie, 2005; Erbs et al., 2014 and references therein). Amb, ambiguous nucleus; BLA, basolateral amygdala; CeA, central amygdala; CPU, caudate putamen; DB, diagonal band; DRN, dorsal raphe nucleus; GP, globus pallidus; Hyp, hypothalamus; LC, locus coeruleus; LHB, lateral habenular nucleus; mHb, medial habenular nucleus; NAc, nucleus accumbens; OB, olfactory bulb; PAG, periaqueductal gray; SNr, substantia nigra pars reticulata; Sol, nucleus of the solitary tract; STH, subthalamic nucleus (ventral thalamus); Th, dorsal thalamus; Tu, olfactory tubercle; VTA, ventral tegmental area.

major players in addictive disorders. In addition to their specific ligands, both systems have also been implicated in the action mechanism of several other addictive drugs, like ethanol, nicotine, or psychostimulants. Although the endocannabinoid system has been known to interact with other systems like hypocretin, dopaminergic, and adenosinergic systems (Fernandez-Ruiz et al., 2010; Ferre et al., 2010; Tebano et al., 2012), its interaction with the opioid system is now well established (Fattore et al., 2005; Vigano et al., 2005; Robledo et al., 2008; Trigo et al., 2010). These two systems share neuroanatomical, neurochemical, and pharmacological, characteristics, this phenomenon is yet less well documented for the CB2 receptor. **Figure 1** illustrates brain structures expressing CB1 receptors and depicts expression level of mu opioid receptors in these areas. The existence of a specific opioid–cannabinoid interaction in the modulation of neurochemical effects as well as behavioral responses associated with reward and relapse have been demonstrated by pharmacological and genetic approaches but experimental results remain controversial (Manzanares et al., 1999; Fattore et al., 2005; Maldonado et al., 2011). Furthermore, molecular interactions between receptors have been shown with colocalization or heterodimerization data mainly for CB1 and delta or mu opioid receptors within spinal cord, striatum, or locus coeruleus. This phenomenon may also account for specific responses at the cellular level (Scavone et al., 2013; Massotte, 2014). However, the physiological effects of these molecular interactions have had yet to be revealed.

AIM OF THE REVIEW

Pharmacological evidence for cross-talk with the synergistic effect of opioid and cannabinoid ligands in many functions related to addiction (mood, stress, learning process . . .) have been revealed and here we will review the implications of both systems regarding reward aspects. As several reviews have recently reported about these interactions (see above), we will focus our interest only on genetic studies using KO mice. We will first present the available genetic tools for both systems. We will then provide an update of results on reinforced behaviors to highlight insights into the particular role of the opioid system in responses to cannabinoids and the endocannabinoid system in responses to prototypical opiates like morphine. We will summarize the behavioral responses of KO mice to these drugs and propose a role for the potential interaction of these two endogenous systems in addictive processes.

REWARD MEASURES IN MICE

Opioid and cannabinoid derivatives induce dependence. To study rewarding effects mediated by specific brain circuits in preclinical research, several behavioral models have been developed in rodents. The most reliable model to evaluate the reinforcing properties of a psychoactive compound in rodents is the self-administration (SA) paradigm which is based on a voluntary procedure to obtain the drug, coupled with the association of a signal (Panlilio and Goldberg, 2007). This operant system allows measuring both rewarding as well as motivational effects of an abused drug. Several aspect of addictive behaviors can be evaluated with this paradigm, with acquisition (fixed ratio) and motivation (progressive ratio) for the drug as well

as extinction (response rate when drug-delivery has stopped) and reinstatement induced by cues, context or stress (relapse to drug-seeking) which will reflect aspects of excessive consumption (Sanchis-Segura and Spanagel, 2006). Intravenous SA has been extensively developed for opiates but more difficult to establish for cannabinoid compounds. Adaptations including drug priming, low doses, food restriction, animal restraint, or use of various cannabinoid agonists were often necessary (Maldonado, 2002; Panlilio et al., 2010; Panagis et al., 2014). Nevertheless, iv SA of both THC and the synthetic cannabinoid WIN55,212-2 have been successfully described both in rats and mice, and extended to the study of KO mice (Martellotta et al., 1998; Fattore et al., 2001; Mendizabal et al., 2006; Flores et al., 2014). A very recent study demonstrated for the first time that 2-AG is self-administered by rats and stimulates DA transmission (De Luca et al., 2014).

In addition, a well-accepted model to study the reinforcement properties of abused drugs is the CPP which is a non-operant paradigm. The reinforcing properties are associated with environmental stimuli (cues), such as the context in which the drug is administered. If the drug or a combination of drugs is aversive, animals avoid the drug-paired compartment (CPA) (Tzschenk, 2007). These paradigms have been widely used to study opiates or cannabinoids effects in mutant mice. However, data reporting reinforcing properties for THC and other cannabinoids are rather controversial with a critical concern about experimental conditions, with dose or injection schedule as major parameters to reveal either positive CPP or negative CPA properties of cannabinoids (Panagis et al., 2014).

On top of these two main paradigms (SA and CPP) other tasks have been developed like intracranial self-stimulation (ICSS) as a model to measure reward-facilitating effect of an abused substance although it is rather difficult to set up in mice and therefore little data is available (Panagis et al., 2014). Furthermore, withdrawal signs appear after cessation of chronic drug exposure, either spontaneously or precipitated by an antagonist treatment, and these signs can be scored for providing an index of dependence (Maldonado et al., 1996). In order to make a meaningful comparison in the evaluation of the specific involvement of components of opioid or cannabinoid systems in reward process, it is crucial to compare, when possible, the different mutant lines with their WT littermates in the exact same procedure to avoid bias from technical or experimental variations. Interestingly, such direct comparison has been recently performed for the four components of the opioid system (mu, delta, Penk, and Pdyn) to demonstrate differential behavior in the acquisition and relapse of cocaine SA in the four mutant mice (Gutierrez-Cuesta et al., 2014).

GENERATION OF DEFICIENT MICE IN REGARDS TO COMPONENTS OF THE OPIOID OR CANNABINOID SYSTEMS

For each component of the opioid and the cannabinoid systems, various lines of genetically modified mice have been generated. **Table 1** presents a list for conventional KO mouse lines that have been described so far. The original papers describing the development of the constitutive deletion are presented with the targeted area of the suppressed gene.

Table 1 | Knockout mouse lines for the opioid and the cannabinoid systems.

Gene knockout	Targeted exon	Reference
Opioid system		
<i>Oprm</i>	Exon 2	Matthes et al. (1996)
	Exon 1	Sora et al. (2001)
	Exon 1	Tian et al. (1997)
	Exons 2 and 3	Loh et al. (1998)
	Exon 1	Schuller et al. (1999)
	Exons 2 and 3	van Rijn and Whistler (2009)
	Exon 11 (splice variant)	Pan et al. (2009)
<i>Oprd</i>	Exon 2	Zhu et al. (1999)
	Exon 1	Filliol et al. (2000)
	Exon 2	van Rijn and Whistler (2009)
<i>Oprk</i>	Exon 1	Simonin et al. (1998)
	Exon 3	Hough et al. (2000)
	Exon 3	Ansonoff et al. (2006)
	Exon 2	van Rijn and Whistler (2009)
	Exon 3	Van't Veer et al. (2013)
<i>Oprm/oprd</i>		Simonin et al. (2001)
<i>Oprm/oprd/oprk</i>		Simonin et al. (2001)
		Clarke et al. (2002)
<i>Penk</i>	Exon 3	Konig et al. (1996)
	Exon 3	Ragnauth et al. (2001)
<i>Pdyn</i>	Exon 3	Sharifi et al. (2001)
	Exon 3	Zimmer et al. (2001)
	Exon 3	Loacker et al. (2007)
<i>Pomc</i>	Exon 3	Rubinstein et al. (1996)
	Exon 3	Yaswen et al. (1999)
<i>Penk/Pdyn</i>		Clarke et al. (2003)
Cannabinoid system		
<i>Cnr1</i>	Exon 2	Zimmer et al. (1999)
	Exon 2	Ledent et al. (1999)
	Exon 2	Marsicano et al. (2002)
	Exon 2	Robbe et al. (2002)
<i>Cnr2</i>	Exon 2	Jarai et al. (1999), Buckley et al. (2000)
	Exon 2	Wotherspoon et al. (2005)
<i>FAAH</i>	Exon 1	Cravatt et al. (2001)
<i>MGL</i>	Exon 3	Uchigashima et al. (2011)
	Exons 3 and 4	Taschler et al. (2011)
	Intron 3-exon 4 (gene trapping)	Schlosburg et al. (2010)
	Exons 1 and 2	Chanda et al. (2010)
<i>NAPE-PLD</i>	Exon 4	Leung et al. (2006)
	Exon 3	Tsuboi et al. (2011)

(Continued)

Table 1 | Continued

Gene knockout	Targeted exon	Reference
<i>DAGLalpha</i>	Exon 1	Gao et al. (2010)
	Exons 3 and 4	Tanimura et al. (2010)
	Intron 4-Exon 1(gene trapping)	Yoshino et al. (2011)
<i>DAGLbeta</i>	Exon 1	Gao et al. (2010)
	Exons 10 and 11	Tanimura et al. (2010)
	Exon 1 (gene trapping)	Yoshino et al. (2011)
	<i>cnr1/cnr2</i>	Jarai et al. (1999)
<i>FAAH/cnr1</i>		Sun et al. (2009)
<i>FAAH/cnr2</i>		Sun et al. (2009)
<i>FAAH/cnr1</i>		Wise et al. (2007)

This table summarizes the published report of KO mouse lines for the different partners of these two systems and combinatorial lines, with the original papers as reference. The area of the gene that has been targeted is indicated.

THE OPIOID SYSTEM

For components of the opioid system, the mu receptor drew the most attention with the description of six distinct genetically modified lines targeting the coding regions of the *oprm* gene, with either exon 1, exon 2 or both exons 2 and 3 targeted for the deletion (Matthes et al., 1996; Tian et al., 1997; Loh et al., 1998; Schuller et al., 1999; Sora et al., 2001; Pan et al., 2009; van Rijn and Whistler, 2009). Interestingly, the mu opioid receptor KO mice allowed to unambiguously demonstrate that the mu receptor was the molecular target for morphine, the prototype of opiate ligand widely used in clinics for its therapeutic effect in pain relief. Morphine had neither analgesic effects nor rewarding properties in these mutant mice (for reviews, see Contet et al., 2004; Gaveriaux-Ruff, 2013). An additional mutant line was constructed which targeted exon 11, a splice variant for the mu receptor, located upstream of exon 1. In these deficient mice, a 25% decrease of receptor expression was observed (Pan et al., 2009), leading to difficult interpretation of the KO effect on opiate pharmacology (Gaveriaux-Ruff, 2013). For deletion of the delta receptor, either exon 1 or 2 were targeted in the *oprd* gene (Zhu et al., 1999; Filliol et al., 2000; van Rijn and Whistler, 2009). These mice were characterized for behavioral responses related to mood and analgesia, but the contribution of delta receptor in reward processes was less clear (Pradhan et al., 2011; Charbogne et al., 2014). Five distinct constructions have been reported targeting either exon 1, 2, or 3 of the *oprk* gene to obtain KO mice for the kappa opioid receptor (Simonin et al., 1998; Hough et al., 2000; Ansonoff et al., 2006; van Rijn and Whistler, 2009; Van't Veer et al., 2013). The two most recent mutants were strategically obtained in order to generate a parallel conditional KO mice (see below) using a Cre-lox approach, with targeted exons floxed with loxP sites. The mutation impaired pharmacological actions of the selective kappa-agonist U-50,488H, and revealed a tonic implication of kappa receptors in the perception of visceral pain. Morphine-CPP was unchanged, but both morphine withdrawal signs as well as emotional responses during

opiate abstinence were reduced (Simonin et al., 1998; Lutz et al., 2014), suggesting an anti-reward role for kappa receptors.

Mice with deleted opioid peptide precursors were also generated. For proopiomelanocortin (*Pomc*), two lines were produced, one specifically deleting β -endorphin (Rubinstein et al., 1996) while the second was targeting the whole coding region, deleting both opioid and non-opioid active peptides (Yaswen et al., 1999). KO mice for *Penk* gene were generated by two distinct laboratories, both leading to deletion of the 5' part of exon 3 (Konig et al., 1996; Ragnauth et al., 2001). For deleting dynorphin in mutant animals, exons 3 and 4 (Sharifi et al., 2001) or exon 3 with a part of exon 4 (Zimmer et al., 2001) of the *Pdyn* gene were targeted. Data from peptide KO mice in regards to opiate rewarding effect were more complex. The β -endorphin KO mice showed increased (Skoubis et al., 2005) or unchanged (Niikura et al., 2008) morphine-CPP depending on the dose and paradigm used and it was invariable both in mice lacking *Penk* (Skoubis et al., 2005) or *Pdyn* (Zimmer et al., 2001; Mizoguchi et al., 2010).

THE CANNABINOID SYSTEM

Four independent KO lines have been generated for the CB1 receptor, encoded by a single large coding exon in the *cnr1* gene (exon 2). The first three lines were generated with the introduction of a PGK or neomycin resistance cassette in the coding region (Ledent et al., 1999; Zimmer et al., 1999; Robbe et al., 2002). For the fourth line, loxP sites were introduced flanking exon 2 and this floxed line was further crossed with a line constitutively expressing the Cre recombinase enzyme, therefore generating a full CB1 KO by deletion of the sequence between the two lox P sites (Marsicano et al., 2002). These mice were mostly unresponsive to cannabinoid ligands in mediating analgesia, reinforcement, hypothermia, hypolocomotion, and hypotension (Valverde and Torrens, 2012; Nadal et al., 2013). Two mouse lines were described for the deletion of the *cnr2* gene coding for CB2 receptor, one by Zimmer's team (Jarai et al., 1999; Buckley et al., 2000) and the other one by the company Deltagen (Wotherspoon et al., 2005). Both were developed by deleting part of the coding region (in exon 2), leaving the start codon with a portion of the amino terminus sequence and aminoacids coding for some transmembrane domains of the receptor. In these constructions expression of the amino-terminal part of the CB2 receptor could potentially occur, but in both cases, it was shown that the receptor was non-functional in the mutant mice (Dhopeshwarkar and Mackie, 2014). Two mutant lines have been described for the NAPE-PLD enzyme involved in AEA synthesis, targeting exon 3 (Tsuboi et al., 2011) or exon 4 (Leung et al., 2006). These KO mice have highlighted the complexity of AEA synthesis with both calcium-dependent and -independent mechanisms. Two isoforms of DAGL α and DAGL β responsible for the synthesis of 2-AG have been described and KO lines have been generated for each of them with both homologous recombination and gene trapping approaches (Gao et al., 2010; Tanimura et al., 2010; Yoshino et al., 2011). The DAGL α KO animals showed a markedly reduced 2-AG brain content whereas levels were normal in brain regions of KO for the β isoform indicating a much greater contribution of DAGL α to 2-AG biosynthesis in the central nervous system. These mutant mice were particularly useful in the characterization of DAGL involvement in retrograde endocannabinoid signaling

(Frazier, 2011). The endocannabinoid system is characterized by a rapid catabolism of the endogenous ligands. Among the degrading enzymes of endocannabinoids, FAAH is the major enzyme responsible for the degradation of AEA and one KO line was generated targeting exon 1 of the *Faah* gene (Cravatt et al., 2001). These mutant mice exhibited more than 15-fold higher brain levels of AEA than WT animals and displayed reduced pain sensitivity. The major degrading enzyme of the endocannabinoid 2-AG is MGL and four KO lines were generated. Three KO lines targeting *mgl* gene exons 1 and 2 (Chanda et al., 2010), exon 3, or exons 3 and 4 were recently generated with a Cre/lox approach (Taschler et al., 2011; Uchigashima et al., 2011). Another line was obtained by gene trapping technology (Texas Institute of Genomic Medicine) with a gene trap cassette inserted into the *mgl* intron 3, upstream of the catalytic exon 4 (Schlosburg et al., 2010). Genetic deletion of MGL leads to alteration in endocannabinoid signaling with increased brain 2-AG levels by \sim 10-fold. These animals were mainly characterized by behavioral consequences of the gene deletion for pain perception (Schlosburg et al., 2010; Uchigashima et al., 2011; Petrenko et al., 2014).

COMBINATORIAL MOUSE LINES

Interbreeding of mutant mouse lines allowed generating combinatorial mutant mice both within the opioid and the cannabinoid systems (see references in Table 1). These combinatorial lines constituted useful tools to clarify the specific role of particular components of both systems in reward and analgesia, as well as to evaluate *in vivo* selectivity for specific ligands and receptor subtype identification (Kieffer and Gaveriaux-Ruff, 2002; Nadal et al., 2013). Data for reward responses obtained using multiple mutants for cannabinoid or opioid components are detailed below.

COMPENSATORY EFFECTS OF THE NULL MUTATION

Globally, a normal development was described for the various mutant lines, with KO mice being fertile, caring for their offspring, and not showing any major behavioral abnormalities. A higher mortality rate was described for one of the CB1 KO line (Zimmer et al., 1999) but not reported for the two others. Interestingly, among the combinatorial mice, the triple mutant of the opioid receptors present a striking increase in body weight and size, but this obese-like phenotype needs further characterization (Befort and Gaveriaux-Ruff, personal communication). Compensatory mechanisms may have developed in some KO animals, but no systematic studies are available. Deletion of opioid receptors did not markedly modify the expression or activity of the other opioid receptors or the expression of opioid peptides as described by the initial characterizations of the distinct mutants lines (see references in Table 1 and Kitchen et al., 1997; Sloane et al., 1999; Oakley et al., 2003). A complete autoradiographic mapping of the delta KO mice indicated decreased binding levels of mu and kappa ligands in specific brain areas (Goody et al., 2002). Deletion of opioid peptides modified other partners of the opioid system, with a region-dependent increased of both mu and delta receptor expression levels observed in the *Penk* KO line (Brady et al., 1999; Clarke et al., 2003) and for the three opioid receptors in the *Pdyn* and the double *Pdyn/Penk* mutant line with no additive effects (Clarke et al., 2003).

et al., 2003). Interestingly, specific changes of CB1 receptor expression or activity were reported in mu and delta opioid receptor mutant lines (Berrendero et al., 2003). In the mu KO brain, there was no difference in CB1 expression but a decreased efficacy of the classical cannabinoid agonist WIN 55,212-2 was observed specifically in the CPu while both density of CB1 receptor and activation by WIN 55,212-2 increased in substantia nigra of delta KO animals.

Compensatory effects in KO animals concerning the cannabinoid system have been described both for receptor or catabolic enzyme KO mice. The invalidation of the CB1 receptor gene was associated with age-dependent adaptive changes of endocannabinoid metabolism, with increased FAAH and AEA membrane transporter activities in KO hippocampus and cortex, decreased AEA content in hippocampus but no change in 2-AG levels (Di Marzo et al., 2000; Maccarrone et al., 2001, 2002). In the FAAH KO mice, CB1 receptor mRNA decreased in CPu, nucleus accumbens (core), hippocampus (CA1), hypothalamic nucleus (VMN), and amygdala. Its functional activity was also markedly reduced in CPu, the core of nucleus accumbens, and CA3 region of the hippocampus (Vinod et al., 2008). Interestingly, reduction of CB1 receptor density and activity were also observed in MGL KO mouse brain, which may prevent the manifestation of the dramatically enhanced 2-AG behavioral effects in these mice (Chanda et al., 2010; Schlosburg et al., 2010). In DAGL α - and DAGL β -KO, no difference was reported for CB1 mRNA (Gao et al., 2010) or protein (Tanimura et al., 2010) levels in comparison to WT mice. In these KO mice, CB1 brain functional signaling was unaltered (Aaltonen et al., 2014). To our knowledge, no data is available for any compensatory effect on CB2 expression or activity in the distinct cannabinoid KOs. However, some reports indicate modifications of the opioid system in CB1 KO animals. An increase of both enkephalin and dynorphin mRNA expression was observed in the striatum (Steiner et al., 1999; Gerald et al., 2006, 2008) as well as an increase in kappa and delta opioid receptor activities without changes in their binding (Uriguen et al., 2005). No compensatory changes of mRNA levels for the three opioid receptors were reported in dorsal root ganglia or spinal cord of the CB1 KO animals (Pol et al., 2006). In FAAH KO mice, Penk mRNA expression was decreased in both CPu and nucleus accumbens which paralleled a reduced mu opioid receptor functional activity (Vinod et al., 2008). Noteworthy, these compensatory alterations of opioid or cannabinoid components in specific regions of the mutant lines could account for interactions of the two systems which may be relevant for neuroadaptative processes involved in drug dependence.

CONDITIONAL APPROACHES

Knockout mice are very useful tools for understanding the contribution of each component of these systems in various conditions including pain, mood disorders or addiction (Valverde and Torrens, 2012; Gaveriaux-Ruff, 2013; Lutz and Kieffer, 2013b; Nadal et al., 2013; Charbogne et al., 2014). Recent approaches using gene manipulation in mice have been developed with the widely used Cre-loxP recombinase system to generate cKO (Fowler and Kenny, 2012; **Table 2**). It consists of crossing mice whose target genes are floxed (flanked with two loxP sites)

together with mice expressing the Cre-recombinase under a specific promoter. This allows a time-, organ- or site-specific deletion of a target gene. This strategy allowed uncoupling the central and peripheral functions of CB1 receptors (Agarwal et al., 2007) and more recently of mu or delta opioid receptors (Gaveriaux-Ruff et al., 2011; Weibel et al., 2013) using the promoter of the channel Nav1.8 only expressed in DRGs, revealing a key role for these receptors expressed in primary nociceptive neurons in inflammatory pain. To investigate molecular mechanisms at the level of neuronal circuitry, selective deletion of a particular gene can also be achieved in specific neuronal types. For example, deletion of the delta opioid receptors specifically in forebrain GABAergic neurons was obtained by crossing a delta opioid floxed mouse line (Gaveriaux-Ruff et al., 2011) together with a dlx5-6-Cre mouse line, specifically expressing the Cre-recombinase in GABAergic forebrain neurons in order to investigate the role of these specific delta receptors in anxiety (Chu Sin Chung et al., 2014). This latter mouse line was previously crossed with the CB1 floxed mice to successfully obtain a GABA-CB1 conditional mutant (Monory et al., 2006). These mutants were also compared with several other cKO bearing a deletion of CB1 receptor in differing specific neuronal populations: forebrain glutamatergic neurons (CB1CamKIIa-Cre mice or CaMK-CB1KO), cortical glutamatergic neurons (CB1NEX-Cre mice or Glu-CB1KO), both glutamatergic and GABAergic neurons (Glu/GABA-CB1KO) or D1-dopaminergic neurons (CB1Drd1a-Cre mice) (Marsicano et al., 2003; Monory et al., 2006, 2007; Bellocchio et al., 2010) for studying the role of CB1 receptors as well as behavioral and autonomic effects of the agonist THC. For the opioid system, a recent study reported the generation of a conditional mutant for the kappa opioid receptor, selectively deleted in DA-expressing neurons. These kappa cKO mice showed reduced anxiety-like behavior as well as increased sensitivity to cocaine, consistent with a role for kappa receptors in negative regulation of DA function (Van't Veer et al., 2013). For the cannabinoid system, cKO lines were also generated for the CB1 receptor to study its specific implication in neurons (Maresz et al., 2007) or peripheral nerves (Pryce et al., 2014), in serotonergic (Dubreucq et al., 2012b) or paraventricular (Dubreucq et al., 2012a) and ventromedial (Bellocchio et al., 2013) hypothalamic neurons. CB1 was also specifically deleted in astroglial cells to investigate its role in working memory and long-term hippocampal depression (Han et al., 2012). CB1 was deleted in specific cell types like hepatocytes to study its role in ethanol-induced fatty liver (Jeong et al., 2008), lymphocytes (Maresz et al., 2007) or epidermal keratinocytes (Gaffal et al., 2013) to investigate its potential role in regulation of inflammatory responses. Another strategy to generate a cKO mouse is by using viral mediated construct carrying the Cre-recombinase injected directly in the structure of interest of a target gene-floxed mouse. For example, the mu opioid receptor was selectively deleted in the dorsal raphe, the main serotonergic brain area, and this deletion abolished the development of social withdrawal in a model of heroin abstinence (Lutz et al., 2014).

In opposition to the loss of function approach, recent studies used a rescue strategy where the target gene is re-expressed in a null mutant, in only a subset of cells (**Table 2**). This helps to provide information concerning the sufficient role of

Table 2 | Conditional knockout mouse lines for the opioid and the cannabinoid systems.

Target Gene	Targeted neurons or structures for selective deletion “loss of function”	Targeted neurons or structures for selective expression “rescue”	Reference
Opioid system			
<i>Oprm</i>	Primary sensory neurons expressing Nav1.8 channel (Nav1.8-Cre)		Weibel et al. (2013)
		Subpopulation of striatal medium spiny neurons	Cui et al. (2014)
<i>Oprd</i>	Primary sensory neurons expressing Nav1.8 channel (Nav1.8-Cre)		Gaveriaux-Ruff et al. (2011)
	Forebrain GABAergic neurons (Dlx5/6-Cre)		Chu Sin Chung et al. (2014)
<i>Oprk</i>	Dopamine containing neurons (DAT-Cre)		Van’t Veer et al. (2013)
Cannabinoid system			
<i>Cnr1</i>	Principal forebrain neurons (CamKII-Cre)		Marsicano et al. (2003)
	Forebrain GABAergic neurons (Dlx5/6-Cre)		Monory et al. (2006)
	Cortical glutamatergic neurons (NEX-Cre)		Monory et al. (2006)
	Glutamatergic and GABAergic neurons (Glu/GABA)		Bellocchio et al. (2010)
	Primary sensory neurons expressing Nav1.8 channel (Nav1.8-Cre)		Agarwal et al. (2007)
	D1-dopaminergic neurons (Drd1a-Cre)		Monory et al. (2007)
	Serotonergic neurons (TPH2-CreERT ^{T2})		Dubreucq et al. (2012b)
	Paraventricular hypothalamic neurons (Sim1-Cre)		Dubreucq et al. (2012a)
	Ventromedial hypothalamic neurons (SF1-cre)		Bellocchio et al. (2013)
	Neurons Nestin (Nes-Cre)		Maresz et al. (2007)
	Peripheral nerve (peripherin-Cre)		Pryce et al. (2014)
	Astrocytes (GFAP- CreERT ^{T2})		Han et al. (2012)
	Hepatocytes (Alb-Cre)		Jeong et al. (2008)
	Lymphocytes (Ick-Cre)		Maresz et al. (2007)
	Keratinocytes (K14-Cre)		Gaffal et al. (2013)
		Dorsal telencephalic glutamatergic neurons (Glu-CB1-RS)	Ruehle et al. (2013)
<i>FAAH</i>		Nervous system (FAAH-NS)	Cravatt et al. (2004)

This table summarizes the recent published reports of cKO mouse lines for the different partners of opioid and cannabinoid systems using “loss of function” or “rescue” strategies.

the cell type expressing the target gene for a given function or establishing whether other cellular subtypes or circuits are necessary. When mu opioid receptor were re-expressed only in a subpopulation of striatal direct-pathway neurons, in a mu KO background, it restored opiate reward and opiate-induced striatal DA release, partially restored motivation to self-administer an opiate, but the rescued mice lacked opiate analgesia or withdrawal (Cui et al., 2014). In a similar genetic strategy, CB1 receptor expression was restored exclusively in dorsal telencephalic glutamatergic neurons and proved sufficient to control neuronal functions that are in large part hippocampus-dependent, while it was insufficient for proper amygdala functions (Ruehle et al., 2013). A conditional line where the expression of the FAAH enzyme has been restricted to the nervous system (FAAH-NS) was generated by crossing the FAAH KO line with a transgenic mouse,

expressing FAAH under the neural specific enolase promoter (Cravatt et al., 2004). These mice exhibited a discrete subset of the biochemical and behavioral phenotypes observed in FAAH KO mice providing key insights into the distinct functions played by the central and peripheral lipids transmitter signaling systems *in vivo*.

In conclusion, despite potential limits such as developmental effects of the mutation or compensatory mechanisms to overcome consequences of the mutation, the use of mutants wherein a component of either opioid or cannabinoid system is selectively deleted from restricted neuronal populations provides essential tools for a comprehensive understanding of mechanisms underlying cannabinoid or opioid effects in reward circuitry. So far, these conditional lines for opioid and cannabinoid systems were mostly characterized for pain or emotional behavioral responses,

and few data is yet to become available for reward aspects (**Table 2**).

CANNABINOID REINFORCING EFFECTS IN OPIOID KNOCKOUT MICE

For evaluating the effect of cannabinoids in opioid mutant mice, THC-induced CPP was mostly used (**Table 3**). Interestingly, the same protocol was used for all tested opioid KO mice with 1 mg/kg ip dose with a priming injection in the home cage. In these conditions, no differences in place preference induced by THC was observed in delta or kappa KO mice while THC-CPP was abolished in mu KO mutants (Ghozland et al., 2002) as well as in the double mu-delta KO mice (Castane et al., 2003). These data support the hypothesis that mu receptors mediate rewarding properties of

THC. A similar protocol was used to induce aversion, but with a higher dose of THC (5 mg/kg ip) wherein mu KO mice showed a decreased CPA (Ghozland et al., 2002). THC-induced CPA was abolished in similar conditions in both Pdyn (Zimmer et al., 2001) and kappa KO mice (Ghozland et al., 2002). Self-administration of the synthetic cannabinoid agonist WIN55,212-2 was successfully established in freely moving mice with a low priming dose (0.1 mg/kg i.p.) and with this protocol, Pdyn KO mice showed facilitated SA (Mendizabal et al., 2006). Altogether, these data support the idea that the kappa/dynorphin system plays a key role in mediating cannabinoid dysphoric effects and therefore negatively modulates their rewarding effects (Mendizabal et al., 2006). Contribution of delta receptors in reward appears complex (Charbogne et al., 2014; Gutierrez-Cuesta et al., 2014) and it has

Table 3 | Rewarding and dependence responses for cannabinoids and opioids measured in KO mouse lines for both systems.

Gene knockout	Behavioral response	Genotype effect	Reference
Opioid system			
<i>Oprm</i>	CPP, THC (1 mg/kg, i.p.)	Abolished	Ghozland et al. (2002)
	CPA, THC (5 mg/kg, i.p.)	Decreased	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Unchanged	
	WD, THC (10 mg/kg, s.c. 5d)	Unchanged	Lichtman et al. (2001)
	WD, THC (30 or 100 mg/kg, s.c. 5d)	Decreased	
<i>Oprd</i>	CPP, THC (1 mg/kg, i.p.)	Unchanged	Ghozland et al. (2002)
	CPA, THC (5 mg/kg, i.p.)	Unchanged	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Unchanged	
<i>Oprm/Oprd</i>	CPP, THC (1 mg/kg, i.p.)	Decreased	Castane et al. (2003)
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Decreased	
<i>Oprk</i>	CPP, THC (1 mg/kg, i.p.)	Unchanged	Ghozland et al. (2002)
	CPP, THC (1 mg/kg, i.p.) w/o priming	Present, absent in WT	
	CPA, THC (5 mg/kg, i.p.)	Abolished	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Unchanged	
<i>Penk</i>	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Decreased	Valverde et al. (2000)
<i>Pdyn</i>	CPA, THC (5 mg/kg, i.p.)	Abolished	Ghozland et al. (2002)
	SA, WIN 55,212(6.25 mg/kg/inf, i.v.)	Increased	Mendizabal et al. (2006)
	SA, WIN 55,212(12.5 mg/kg/inf, i.v.)	Abolished	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Decreased	Zimmer et al. (2001)
Cannabinoid system			
<i>Cnr1</i>	CPP, morphine (5 mg/kg, s.c.)	Abolished	Martin et al. (2000)
	CPA, morphine + naloxone (20–100 mg/kg i.p. over 6d + 0.1 mg/kg s.c.)	Unchanged	
	CPP, morphine (4–8 mg/kg, i.p.)	Unchanged	Rice et al. (2002)
	CPA, morphine + naloxone (8 mg/kg + 5 mg/kg, i.p.)	Unchanged	
	SA, morphine (2 ug/kg/inf, i.v.)	Abolished	Cossu et al. (2001)
	SA, morphine (1, 2, 4 ug/kg/inf, i.v.)	Decreased	Ledent et al. (1999)
	WD, morphine (20 mg/kg to 100 mg/kg, 5d)	Decreased	
	WD, morphine (75 mg/kg pellet, 5d)	Decreased	Lichtman et al. (2001)
	CPA, U50,488H (1 mg/kg, s.c.)	Abolished	Ledent et al. (1999)

This table summarizes published reports of behavioral responses in reward and precipitated withdrawal for cannabinoids in opioid KO lines and opioids in cannabinoid KO mutants (CPA, conditioned place aversion; CPP, conditioned place preference; d, day; inf, infusion; i.p., intraperitoneal; s.c., subcutaneous; WD, withdrawal; w/o, without).

not yet been established for cannabinoid reward, neither pharmacologically nor genetically. A potential role of this particular receptor in cannabinoid reward awaits further studies investigating either cannabinoid SA (motivation aspects) or delta cKO mutant responses (deletion of specific subpopulation of receptors).

Another aspect that was explored in opioid KO mice is cannabinoid dependence. Upon chronic THC treatment, antagonist-induced withdrawal signs measured in WT animals were unchanged for Pdyn KO (Zimmer et al., 2001) or single mutant mice for mu, delta or kappa opioid receptors (Gholzland et al., 2002). Signs were attenuated in KO animals for Penk (Valverde et al., 2000), for the double mu-delta receptor mutant (Castane et al., 2003) as well as for mu receptor KO, at a high dose (Lichtman et al., 2001) (**Table 3**). No data are yet available for the other opioid peptide KO mice concerning cannabinoid physical dependence. Collectively, available data indicate the involvement of the enkephalinergic system, with a cooperative action of mu and delta receptors, in the expression of cannabinoid dependence.

OPIOID REINFORCING EFFECTS IN CANNABINOID KNOCKOUT MICE

Knockout approaches have greatly improved our knowledge on the role of CB1 receptors in addiction in general, even though contradictory data exist (Maldonado et al., 2006). In particular, for opiate responses (**Table 3**) induced by mu agonists, CB1 KO mice showed no morphine-induced place preference (5 mg/kg, s.c., 3 injections over 6 days) (Martin et al., 2000) and a diminished propensity to self-administer morphine (Ledent et al., 1999; Cossu et al., 2001). A microdialysis study revealed that morphine-induced increase of extracellular DA was not observed in CB1 KO mice (Mascia et al., 1999). Taken together, these data suggest a reduction in morphine's reinforcing activity in the absence of the CB1 receptor. Another study could not reveal any changes in place preference using a slightly more intensive conditioning paradigm and a different set up with two doses of morphine (4 or 8 mg/kg, four injections over 4 days) (Rice et al., 2002). Interestingly, no differences between WT and CB1 KO mice could be observed in a CPA paradigm where the opioid antagonist naloxone was used to induce withdrawal in morphine-treated mice via two distinct paradigms (Martin et al., 2000; Rice et al., 2002). Upon chronic morphine treatment, naloxone-induced withdrawal signs measured in WT animals were attenuated (Ledent et al., 1999; Lichtman et al., 2001). Together, these findings suggest that CB1 receptors are not involved in the dysphoric effects of morphine withdrawal (CPA) but are noticeably required for the development of physical dependence or of somatic signs of opiate withdrawal. Surprisingly, other important effects of morphine, like acute induced analgesia and tolerance to chronic morphine-induced analgesia, were not altered in CB1 KO animals. These findings together with the data on mu opioid KO mice with cannabinoid treatments suggest a bidirectional influence of mu opioid and CB1 cannabinoid receptors on reward processes. Aversive effects of the kappa opioid agonist U50,488H were also blunted in CB1 receptor KO mice (Ledent et al., 1999). Together with the data of kappa opioid and Pdyn KO mice, it indicates that both cannabinoid and opioid systems modulate negative motivational drug effects. To our knowledge, no data concerning the specific effect of delta

selective opioid agonists on reward in CB1 KO mice are available. Interestingly, it has been demonstrated that the absence of CB1 receptor also results in a reduction of the sensitivity to the rewarding properties of sucrose (Sanchis-Segura et al., 2004), as well as other reinforcers (for recent reviews, see (Lopez-Moreno et al., 2010; Maldonado et al., 2013)). Together with pharmacological approaches (Maldonado et al., 2006), KO data therefore provide confirmatory support that CB1 receptor play a modulatory role in the reinforced behaviors maintained by sucrose and some other reinforcers with, in particular, a mutual interaction of opioid and cannabinoid systems.

For the other components of the endocannabinoid system, no specific data for genetically modified animals were reported for the investigation of opioid reward, although pharmacological inhibition of the endocannabinoid catabolic enzymes attenuates both naloxone-induced withdrawal as well as spontaneous withdrawal signs in morphine dependent mice (Ramesh et al., 2011, 2013), indicating a potential role of these enzymes in opioid dependence.

CONCLUSION AND PERSPECTIVES

Globally, despite some compensatory alterations at both opioid and cannabinoid levels in mutant lines, KO studies have provided insights into the mutual role of both opioid and cannabinoid systems on reward. In particular, these studies have highlighted the major role for both mu opioid and CB1 receptors in these processes. Clearly, the mu opioid receptor is a convergent molecular target mediating rewarding properties of opioid compounds but also of other drugs of abuse, including cannabinoids. CB1 receptor also appears as a modulator of opioid reward. On the other hand, KO approaches for endogenous opioid peptides or enzymes for synthesis or degradation of endocannabinoids have been very useful to clarify their specific role in both endogenous systems but less/no data are available for reward mechanisms. These mutants therefore need further investigations to clarify their potential implication in cannabinoid/opioid reward aspects.

Conventional genetically modified animals have strengthened our current knowledge of the interaction between these two systems, but further studies using conditional approaches will be necessary to clarify the potential crosstalk existing specifically in reward processes. Interaction between these two neuromodulator systems may be dependent on the brain area where it occurs, even inside the brain rewarding networks (Parolario et al., 2010). Both mu opioid and CB1 receptors are highly expressed in these networks in similar brain structures and a potential interaction in areas where they are both strongly expressed is probable. Noticeably, opposite expression levels are observed in discrete areas like amygdala (BLA versus central amygdala) as well as habenula (medial versus lateral nuclei) and these differences may also account for a modulatory role of the two systems in reward processes (**Figure 1**). Approaches using double mutants for both receptors would be useful to further understand their mutual role in drug reward. Moreover, in this perspective, conditional approaches will surely provide invaluable insights into opioid and cannabinoid interaction at the circuitry level. The growing number of cKO mutant lines becoming available will help this side of

research. Likewise, the implication of the CB2 receptor in these interactions has not yet been explored and may be particularly relevant in specific brain structures. In fact, demonstration of CB2 expression in several brain structures has opened a field of investigation for a possible role in addiction that should help to reveal potential direct interaction between CB2 and the opioid system.

G protein coupled receptor can associate as heteromers and extended research is now directed toward elucidating the physiological role of such heteromers and finding therapeutic approaches targeting these entities (see recent reviews Fujita et al., 2014; Massotte, 2014). Several lines of evidence have suggested interactions between delta or mu opioid receptors and the CB1 receptors. Close vicinity of CB1 receptors with mu or delta opioid receptors has recently been established at the neuronal level, suggesting heteromeric formation *in vivo* and potential impact on both receptors signaling properties. A recent study demonstrated an important role for the heterodimer CB1-delta in neuropathic pain where cortical functions of delta receptor were altered (Bushlin et al., 2012). CB1 and mu receptors associate as heteromers in cultured cells and a recent study showed that bivalent ligands for both receptors are potent analgesic devoid of tolerance (Le Naour et al., 2013), suggesting potential functional heteromers in pain. Therefore, one can easily predict that similar mechanisms may occur in another pathological state like addiction and this opens up new prospects for pharmacological action of cannabinoid and opioid drugs. In this context, it will be critical to see whether CB2 also plays a role as a potential heteromeric interactor with opioid receptors.

No effective therapeutic approaches for cannabis dependence are currently available and opioid addiction therapies are not fully satisfying for all patients. Further studies are therefore needed to clarify the mechanistic basis of interaction of the two systems, which would aid in the development of drug therapies to reduce dependence and abuse. Antibodies or bivalent ligands as mentioned previously represent interesting therapeutic targets. In addition, dual enkephalinase inhibitors and cannabinoid catabolic enzyme inhibitors have been proposed as attractive therapeutic targets to treat pain (Roques et al., 2012) and such bi-functional compounds may also be relevant as promising strategies for alleviating dependence.

Substantial progress has been made in understanding the cellular and molecular mechanisms of prolonged use of cannabinoid or opioid drugs (Kreek et al., 2012; Fratta and Fattore, 2013). In addition to their direct role in reward, interaction between opioid and cannabinoid neuromodulator systems has been proposed to explain some aspects of vulnerability to addiction and, in this perspective, recent attention has been focused on yet another critical level, epigenetics. These molecular processes, including methylation of DNA, post-translational modifications of histones and regulation by microRNA, regulate gene expression and are crucial in long-term adaptations induced by drugs (Nestler, 2014). Recent studies have shown a direct association between THC-induced Penk upregulation through reduction of histone H3 lysine 9 pattern of methylation and increased heroin SA (Tomasiewicz et al., 2012). Adolescent THC-exposure also resulted in altered heroin SA in the subsequent generation

of rats, an effect associated with changes in mRNA expression of cannabinoid, DA, and glutamatergic receptor genes in the striatum, suggesting adaptations to long-term drug effect and germline transmission, most likely involving epigenetic changes (Szutorisz et al., 2014). How these neuromodulator systems are dependent on various internal and external environmental factors, and therefore are involved in epigenetics and whether one system influences the epigenetic machinery to control the other system, are unresolved questions for upcoming studies (D'Addario et al., 2013). Future investigation in this field will be necessary to better delineate the neurobiological mechanisms underlying these neuroadaptations.

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REFERENCES

- Altonen, N., Riera Ribas, C., Lehtonen, M., Savinainen, J. R., and Laitinen, J. T. (2014). Brain regional cannabinoid CB(1) receptor signalling and alternative enzymatic pathways for 2-arachidonoylglycerol generation in brain sections of diacylglycerol lipase deficient mice. *Eur. J. Pharm. Sci.* 51, 87–95. doi: 10.1016/j.ejps.2013.08.035
- Agarwal, N., Pacher, P., Tegeder, I., Amaya, F., Constantin, C. E., Brenner, G. J., et al. (2007). Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat. Neurosci.* 10, 870–879. doi: 10.1038/nn1916
- Ahn, K., McKinney, M. K., and Cravatt, B. F. (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. *Chem. Rev.* 108, 1687–1707. doi: 10.1021/cr0782067
- Ansonoff, M. A., Zhang, J., Czysyk, T., Rothman, R. B., Stewart, J., Xu, H., et al. (2006). Antinociceptive and hypothermic effects of salvinorin A are abolished in a novel strain of kappa-opioid receptor-1 knockout mice. *J. Pharmacol. Exp. Ther.* 318, 641–648. doi: 10.1124/jpet.106.101998
- Belin, D., and Deroche-Gamonet, V. (2012). Responses to novelty and vulnerability to cocaine addiction: contribution of a multi-symptomatic animal model. *Cold Spring Harb. Perspect. Med.* 2:a011940. doi: 10.1101/cshperspect.a011940
- Belloch, L., Lafenetre, P., Cannich, A., Cota, D., Puente, N., Grandes, P., et al. (2010). Bimodal control of stimulated food intake by the endocannabinoid system. *Nat. Neurosci.* 13, 281–283. doi: 10.1038/nn.2494
- Belloch, L., Soria-Gomez, E., Quarta, C., Metna-Laurent, M., Cardinal, P., Binder, E., et al. (2013). Activation of the sympathetic nervous system mediates hypophagic and anxiety-like effects of CB(1) receptor blockade. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4786–4791. doi: 10.1073/pnas.1218573110
- Berrendero, F., Mendizabal, V., Murtra, P., Kieffer, B. L., and Maldonado, R. (2003). Cannabinoid receptor and WIN 55 212-2-stimulated [35S]-GTPgammaS binding in the brain of mu-, delta-, and kappa-opioid receptor knockout mice. *Eur. J. Neurosci.* 18, 2197–2202. doi: 10.1046/j.1460-9568.2003.02951.x
- Bodnar, R. J. (2014). Endogenous opiates and behavior: 2013. *Peptides* 62C, 67–136. doi: 10.1016/j.peptides.2014.09.013
- Brady, L. S., Herkenham, M., Rothman, R. B., Partilla, J. S., Konig, M., Zimmer, A. M., et al. (1999). Region-specific up-regulation of opioid receptor binding in enkephalin knockout mice. *Brain Res. Mol. Brain Res.* 68, 193–197. doi: 10.1016/S0169-328X(99)00090-X
- Buckley, N. E., McCoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., et al. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur. J. Pharmacol.* 396, 141–149. doi: 10.1016/S0014-2999(00)00211-9

- Bushlin, I., Gupta, A., Stockton, S. D. Jr., Miller, L. K., and Devi, L. A. (2012). Dimerization with cannabinoid receptors allosterically modulates delta opioid receptor activity during neuropathic pain. *PLoS ONE* 7:e49789. doi: 10.1371/journal.pone.0049789
- Castane, A., Robledo, P., Matifas, A., Kieffer, B. L., and Maldonado, R. (2003). Cannabinoid withdrawal syndrome is reduced in double mu and delta opioid receptor knockout mice. *Eur. J. Neurosci.* 17, 155–159. doi: 10.1046/j.1460-9568.2003.02409.x
- Chanda, P. K., Gao, Y., Mark, L., Btesh, J., Strassle, B. W., Lu, P., et al. (2010). Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system. *Mol. Pharmacol.* 78, 996–1003. doi: 10.1124/mol.110.068304
- Charbogne, P., Kieffer, B. L., and Befort, K. (2014). 15 years of genetic approaches in vivo for addiction research: opioid receptor and peptide gene knockout in mouse models of drug abuse. *Neuropharmacology* 76(Pt B), 204–217. doi: 10.1016/j.neuropharm.2013.08.028
- Chu Sin Chung, P., Keyworth, H. L., Martin-Garcia, E., Charbogne, P., Darcq, E., Bailey, A., et al. (2014). A novel anxiogenic role for the delta opioid receptor expressed in GABAergic forebrain neurons. *Biol. Psychiatry* doi: 10.1016/j.biopsych.2014.07.033 [Epub ahead of print].
- Clarke, S., Czzyk, T., Ansonoff, M., Nitsche, J. F., Hsu, M. S., Nilsson, L., et al. (2002). Autoradiography of opioid and ORL1 ligands in opioid receptor triple knockout mice. *Eur. J. Neurosci.* 16, 1705–1712. doi: 10.1046/j.1460-9568.2002.02239.x
- Clarke, S., Zimmer, A., Zimmer, A. M., Hill, R. G., and Kitchen, I. (2003). Region selective up-regulation of micro-, delta- and kappa-opioid receptors but not opioid receptor-like 1 receptors in the brains of enkephalin and dynorphin knockout mice. *Neuroscience* 122, 479–489. doi: 10.1016/j.neuroscience.2003.07.011
- Contet, C., Kieffer, B. L., and Befort, K. (2004). Mu opioid receptor: a gateway to drug addiction. *Curr. Opin. Neurobiol.* 14, 370–378. doi: 10.1016/j.conb.2004.05.005
- Cossu, G., Ledent, C., Fattore, L., Imperato, A., Bohme, G. A., Parmentier, M., et al. (2001). Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. *Behav. Brain Res.* 118, 61–65. doi: 10.1016/S0166-4328(00)00311-9
- Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., et al. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9371–9376. doi: 10.1073/pnas.161191698
- Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384, 83–87. doi: 10.1038/384083a0
- Cravatt, B. F., Saghatelian, A., Hawkins, E. G., Clement, A. B., Bracey, M. H., and Lichtman, A. H. (2004). Functional disassociation of the central and peripheral fatty acid amide signaling systems. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10821–10826. doi: 10.1073/pnas.0401292101
- Cui, Y., Ostlund, S. B., James, A. S., Park, C. S., Ge, W., Roberts, K. W., et al. (2014). Targeted expression of mu-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward. *Nat. Neurosci.* 17, 254–261. doi: 10.1038/nrn.3622
- D'Addario, C., Di Francesco, A., Pucci, M., Finazzi Agro, A., and Maccarrone, M. (2013). Epigenetic mechanisms and endocannabinoid signalling. *FEBS J.* 280, 1905–1917. doi: 10.1111/febs.12125
- De Luca, M. A., Valentini, V., Bimpidis, Z., Cacciapaglia, F., Caboni, P., and Di Chiara, G. (2014). Endocannabinoid 2-arachidonoylglycerol self-administration by sprague-dawley rats and stimulation of in vivo dopamine transmission in the nucleus accumbens shell. *Front. Psychiatry* 5:140. doi: 10.3389/fpsyg.2014.00140
- De Petrocellis, L., and Di Marzo, V. (2010). Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. *J. Neuroimmune Pharmacol.* 5, 103–121. doi: 10.1007/s11481-009-9177-z
- Dhopeshwarkar, A., and Mackie, K. (2014). CB2 cannabinoid receptors as a therapeutic target—what does the future hold? *Mol. Pharmacol.* 86, 430–437. doi: 10.1124/mol.114.094469
- Di Marzo, V., Breivogel, C. S., Tao, Q., Bridgen, D. T., Razdan, R. K., Zimmer, A. M., et al. (2000). Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain. *J. Neurochem.* 75, 2434–2444. doi: 10.1046/j.1471-4159.2000.0752434.x
- Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., et al. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10819–10824. doi: 10.1073/pnas.152334899
- Dubreucq, S., Kambire, S., Conforzi, M., Metna-Laurent, M., Cannich, A., Soria-Gomez, E., et al. (2012a). Cannabinoid type 1 receptors located on single-minded 1-expressing neurons control emotional behaviors. *Neuroscience* 204, 230–244. doi: 10.1016/j.neuroscience.2011.08.049
- Dubreucq, S., Matias, I., Cardinal, P., Haring, M., Lutz, B., Marsicano, G., et al. (2012b). Genetic dissection of the role of cannabinoid type-1 receptors in the emotional consequences of repeated social stress in mice. *Neuropsychopharmacology* 37, 1885–1900. doi: 10.1038/npp.2012.36
- Erbs, E., Faget, L., Scherrer, G., Matifas, A., Filliol, D., Vonesch, J. L., et al. (2014). A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct. Funct.* doi: 10.1007/s00429-014-0717-9 [Epub ahead of print].
- Evans, C. J., Keith, D. E. Jr., Morrison, H., Magendzo, K., and Edwards, R. H. (1992). Cloning of a delta opioid receptor by functional expression. *Science* 258, 1952–1955. doi: 10.1126/science.1335167
- Everitt, B. J., and Robbins, T. W. (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat. Neurosci.* 8, 1481–1489. doi: 10.1038/nn1579
- Fattore, L., Cossu, G., Martellotta, C. M., and Fratta, W. (2001). Intravenous self-administration of the cannabinoid CB1 receptor agonist WIN 55,212-2 in rats. *Psychopharmacology (Berl)* 156, 410–416. doi: 10.1007/s002130100734
- Fattore, L., Deiana, S., Spano, S. M., Cossu, G., Fadda, P., Scherma, M., et al. (2005). Endocannabinoid system and opioid addiction: behavioural aspects. *Pharmacol. Biochem. Behav.* 81, 343–359. doi: 10.1016/j.pbb.2005.01.031
- Fattore, L., and Fratta, W. (2011). Beyond THC: the new generation of cannabinoid designer drugs. *Front. Behav. Neurosci.* 5:60. doi: 10.3389/fnbeh.2011.00060
- Fernandez-Ruiz, J., Hernandez, M., and Ramos, J. A. (2010). Cannabinoid-dopamine interaction in the pathophysiology and treatment of CNS disorders. *CNS Neurosci. Ther.* 16, e72–e91. doi: 10.1111/j.1755-5949.2010.00144.x
- Ferre, S., Lluis, C., Justinova, Z., Quiroz, C., Orru, M., Navarro, G., et al. (2010). Adenosine-cannabinoid receptor interactions. Implications for striatal function. *Br. J. Pharmacol.* 160, 443–453. doi: 10.1111/j.1476-5381.2010.00723.x
- Filizola, M., and Devi, L. A. (2014). Grand opening of structure-guided design for novel opioids. *Trends Pharmacol. Sci.* 34, 6–12. doi: 10.1016/j.tips.2012.10.002
- Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H. W., Simonin, F., et al. (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat. Genet.* 25, 195–200. doi: 10.1038/76061
- Flores, A., Maldonado, R., and Berrendero, F. (2014). The hypocretin/orexin receptor-1 as a novel target to modulate cannabinoid reward. *Biol. Psychiatry* 75, 499–507. doi: 10.1016/j.biopsych.2013.06.012
- Fowler, C. D., and Kenny, P. J. (2012). Utility of genetically modified mice for understanding the neurobiology of substance use disorders. *Hum. Genet.* 131, 941–957. doi: 10.1007/s00439-011-1129-z
- Fratta, W., and Fattore, L. (2013). Molecular mechanisms of cannabinoid addiction. *Curr. Opin. Neurobiol.* 23, 487–492. doi: 10.1016/j.conb.2013.02.002
- Frazier, C. J. (2011). Key questions of endocannabinoid signalling in the CNS: which, where and when? *J. Physiol.* 589, 4807–4808. doi: 10.1113/jphysiol.2011.219493
- Fujita, W., Gomes, I., and Devi, L. A. (2014). Revolution in GPCR signalling: opioid receptor heteromers as novel therapeutic targets: IUPHAR review 10. *Br. J. Pharmacol.* 171, 4155–4176. doi: 10.1111/bph.12798
- Gaffal, E., Cron, M., Glodde, N., Bald, T., Kuner, R., Zimmer, A., et al. (2013). Cannabinoid 1 receptors in keratinocytes modulate proinflammatory chemokine secretion and attenuate contact allergic inflammation. *J. Immunol.* 190, 4929–4936. doi: 10.4049/jimmunol.1201777
- Gao, Y., Vasilyev, D. V., Goncalves, M. B., Howell, F. V., Hobbs, C., Reisenberg, M., et al. (2010). Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. *J. Neurosci.* 30, 2017–2024. doi: 10.1523/JNEUROSCI.5693-09.2010
- Gardner, E. L. (2005). Endocannabinoid signaling system and brain reward: emphasis on dopamine. *Pharmacol. Biochem. Behav.* 81, 263–284. doi: 10.1016/j.pbb.2005.01.032
- Gaveriaux-Ruff, C. (2013). Opiate-induced analgesia: contributions from mu, delta and kappa opioid receptors mouse mutants. *Curr. Pharm. Des.* 19, 7373–7381. doi: 10.2174/138161281942140105163727

- Gaveriaux-Ruff, C., Nozaki, C., Nadal, X., Hever, X. C., Weibel, R., Matifas, A., et al. (2011). Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain* 152, 1238–1248. doi: 10.1016/j.pain.2010.12.031
- Gerald, T. M., Howlett, A. C., Ward, G. R., Ho, C., and Franklin, S. O. (2008). Gene expression of opioid and dopamine systems in mouse striatum: effects of CB1 receptors, age and sex. *Psychopharmacology (Berl.)* 198, 497–508. doi: 10.1007/s00213-008-1141-1148
- Gerald, T. M., Ward, G. R., Howlett, A. C., and Franklin, S. O. (2006). CB1 knockout mice display significant changes in striatal opioid peptide and D4 dopamine receptor gene expression. *Brain Res.* 1093, 20–24. doi: 10.1016/j.brainres.2006.03.088
- Ghozland, S., Matthes, H. W., Simonin, F., Filliol, D., Kieffer, B. L., and Maldonado, R. (2002). Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors. *J. Neurosci.* 22, 1146–1154.
- Gong, J. P., Onaivi, E. S., Ishiguro, H., Liu, Q. R., Tagliaferro, P. A., Brusco, A., et al. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res.* 1071, 10–23. doi: 10.1016/j.brainres.2005.11.035
- Goody, R. J., Oakley, S. M., Filliol, D., Kieffer, B. L., and Kitchen, I. (2002). Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice. *Brain Res.* 945, 9–19. doi: 10.1016/S0006-8993(02)02452-6
- Gustavsson, A., Svensson, M., Jacobi, F., Allgulander, C., Alonso, J., Beghi, E., et al. (2011). Cost of disorders of the brain in Europe 2010. *Eur. Neuropsychopharmacol.* 21, 718–779. doi: 10.1016/j.euroneuro.2011.08.008
- Gutierrez-Cuesta, J., Burokas, A., Mancino, S., Kummer, S., Martin-Garcia, E., and Maldonado, R. (2014). Effects of genetic deletion of endogenous opioid system components on the reinstatement of cocaine-seeking behavior in mice. *Neuropsychopharmacology*. doi: 10.1038/npp.2014.149
- Han, J., Kesner, P., Metna-Laurent, M., Duan, T., Xu, L., Georges, F., et al. (2012). Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. *Cell* 148, 1039–1050. doi: 10.1016/j.cell.2012.01.037
- Hough, L. B., Nalwalk, J. W., Chen, Y., Schuller, A., Zhu, Y., Zhang, J., et al. (2000). Imrogran, a cimetidine analog, induces morphine-like antinociception in opioid receptor-knockout mice. *Brain Res.* 880, 102–108. doi: 10.1016/S0006-8993(00)02776-1
- Ignatowska-Jankowska, B. M., Muldoon, P. P., Lichtman, A. H., and Damaj, M. I. (2013). The cannabinoid CB2 receptor is necessary for nicotine-conditioned place preference, but not other behavioral effects of nicotine in mice. *Psychopharmacology (Berl.)* 229, 591–601. doi: 10.1007/s00213-013-3117-6
- Jarai, Z., Wagner, J. A., Varga, K., Lake, K. D., Compton, D. R., Martin, B. R., et al. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14136–14141. doi: 10.1073/pnas.96.24.14136
- Jeong, W. I., Osei-Hyiaman, D., Park, O., Liu, J., Batkai, S., Mukhopadhyay, P., et al. (2008). Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. *Cell Metab.* 7, 227–235. doi: 10.1016/j.cmet.2007.12.007
- Kieffer, B. L. (1995). Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cell Mol. Neurobiol.* 15, 615–635. doi: 10.1007/BF02071128
- Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C., and Hirth, C. G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc. Natl. Acad. Sci. U.S.A.* 89, 12048–12052. doi: 10.1073/pnas.89.24.12048
- Kieffer, B. L., and Gaveriaux-Ruff, C. (2002). Exploring the opioid system by gene knockout. *Prog. Neurobiol.* 66, 285–306. doi: 10.1016/S0301-0082(02)00008-4
- Kitchen, I., Slowe, S. J., Matthes, H. W., and Kieffer, B. (1997). Quantitative autoradiographic mapping of mu-, delta-, and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene. *Brain Res.* 778, 73–88. doi: 10.1016/S0006-8993(97)00988-8
- Konig, M., Zimmer, A. M., Steiner, H., Holmes, P. V., Crawley, J. N., Brownstein, M. J., et al. (1996). Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature* 383, 535–538. doi: 10.1038/383535a0
- Koob, G. F. (2009). Neurobiological substrates for the dark side of compulsion in addiction. *Neuropharmacology* 56(Suppl. 1), 18–31. doi: 10.1016/j.neuropharm.2008.07.043
- Koob, G. F., and Volkow, N. D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology* 35, 217–238. doi: 10.1038/npp.2009.110
- Kreek, M. J., Levran, O., Reed, B., Schlussman, S. D., Zhou, Y., and Butelman, E. R. (2012). Opiate addiction and cocaine addiction: underlying molecular neurobiology and genetics. *J. Clin. Invest.* 122, 3387–3393. doi: 10.1172/JCI60390
- Le Merrer, J., Becker, J. A., Befort, K., and Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412. doi: 10.1152/physrev.00005.2009
- Le Naour, M., Akgun, E., Yekkirala, A., Lunzer, M. M., Powers, M. D., Kalyuzhny, A. E., et al. (2013). Bivalent ligands that target mu opioid (MOP) and cannabinoid1 (CB1) receptors are potent analgesics devoid of tolerance. *J. Med. Chem.* 56, 5505–5513. doi: 10.1021/jm4005219
- Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J. F., Beslot, F., et al. (1999). Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* 283, 401–404. doi: 10.1126/science.283.540.401
- Leshner, A. I. (1997). Addiction is a brain disease, and it matters. *Science* 278, 45–47. doi: 10.1126/science.278.5335.45
- Leung, D., Saghatelian, A., Simon, G. M., and Cravatt, B. F. (2006). Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry* 45, 4720–4726. doi: 10.1021/bi0601631
- Lichtman, A. H., Sheikh, S. M., Loh, H. H., and Martin, B. R. (2001). Opioid and cannabinoid modulation of precipitated withdrawal in delta(9)-tetrahydrocannabinol and morphine-dependent mice. *J. Pharmacol. Exp. Ther.* 298, 1007–1014.
- Liu, J., Wang, L., Harvey-White, J., Huang, B. X., Kim, H. Y., Luquet, S., et al. (2008). Multiple pathways involved in the biosynthesis of anandamide. *Neuropharmacology* 54, 1–7. doi: 10.1016/j.neuropharm.2007.05.020
- Loacker, S., Sayyah, M., Wittmann, W., Herzog, H., and Schwarzer, C. (2007). Endogenous dynorphin in epileptogenesis and epilepsy: anticonvulsant net effect via kappa opioid receptors. *Brain* 130, 1017–1028. doi: 10.1093/brain/awl384
- Loh, H. H., Liu, H. C., Cavalli, A., Yang, W., Chen, Y. F., and Wei, L. N. (1998). mu Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. *Brain Res. Mol. Brain Res.* 54, 321–326. doi: 10.1016/S0169-328X(97)00353-7
- Lopez-Moreno, J. A., Lopez-Jimenez, A., Gorriti, M. A., and De Fonseca, F. R. (2010). Functional interactions between endogenous cannabinoid and opioid systems: focus on alcohol, genetics and drug-addicted behaviors. *Curr. Drug Targets* 11, 406–428. doi: 10.2174/138945010790980312
- Lutz, P. E., Ayrancı, G., Chu-Sin-Chung, P., Matifas, A., Koebel, P., Filliol, D., et al. (2014). Distinct mu, delta, and kappa opioid receptor mechanisms underlie low sociability and depressive-like behaviors during heroin abstinence. *Neuropsychopharmacology* 39, 2694–2705. doi: 10.1038/npp.2014.126
- Lutz, P. E., and Kieffer, B. L. (2013a). The multiple facets of opioid receptor function: implications for addiction. *Curr. Opin. Neurobiol.* 23, 473–479. doi: 10.1016/j.conb.2013.02.005
- Lutz, P. E., and Kieffer, B. L. (2013b). Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.* 36, 195–206. doi: 10.1016/j.tins.2012.11.002
- Maccarrone, M., Attina, M., Bari, M., Cartoni, A., Ledent, C., and Finazzi-Agro, A. (2001). Anandamide degradation and N-acylethanolamines level in wild-type and CB1 cannabinoid receptor knockout mice of different ages. *J. Neurochem.* 78, 339–348. doi: 10.1046/j.1471-4159.2001.00413.x
- Maccarrone, M., Valverde, O., Barbaccia, M. L., Castane, A., Maldonado, R., Ledent, C., et al. (2002). Age-related changes of anandamide metabolism in CB1 cannabinoid receptor knockout mice: correlation with behaviour. *Eur. J. Neurosci.* 15, 1178–1186. doi: 10.1046/j.1460-9568.2002.01957.x
- Mackie, K. (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb. Exp. Pharmacol.* 299–325.
- Mackie, K. (2008). Cannabinoid receptors: where they are and what they do. *J. Neuroendocrinol.* 20(Suppl. 1), 10–14. doi: 10.1111/j.1365-2826.2008.01671.x
- Maldonado, R. (2002). Study of cannabinoid dependence in animals. *Pharmacol. Ther.* 95, 153–164. doi: 10.1016/S0163-7258(02)00254-1
- Maldonado, R., Berrendero, F., Ozaita, A., and Robledo, P. (2011). Neurochemical basis of cannabis addiction. *Neuroscience* 181, 1–17. doi: 10.1016/j.neuroscience.2011.02.035

- Maldonado, R., Blendy, J. A., Tzavara, E., Gass, P., Roques, B. P., Hanoune, J., et al. (1996). Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science* 273, 657–659. doi: 10.1126/science.273.5275.657
- Maldonado, R., Robledo, P., and Berrendero, F. (2013). Endocannabinoid system and drug addiction: new insights from mutant mice approaches. *Curr. Opin. Neurobiol.* 23, 480–486. doi: 10.1016/j.conb.2013.02.004
- Maldonado, R., Valverde, O., and Berrendero, F. (2006). Involvement of the endocannabinoid system in drug addiction. *Trends Neurosci.* 29, 225–232. doi: 10.1016/j.tins.2006.01.008
- Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 18, 22–29. doi: 10.1016/0166-2236(95)93946-U
- Manzanares, J., Corchero, J., Romero, J., Fernandez-Ruiz, J. J., Ramos, J. A., and Fuentes, J. A. (1999). Pharmacological and biochemical interactions between opioids and cannabinoids. *Trends Pharmacol. Sci.* 20, 287–294. doi: 10.1016/S0165-6147(99)00139-5
- Maresz, K., Pryce, G., Ponomarev, E. D., Marsicano, G., Croxford, J. L., Shriver, L. P., et al. (2007). Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nat. Med.* 13, 492–497. doi: 10.1038/nm1561
- Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., et al. (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* 302, 84–88. doi: 10.1126/science.1088208
- Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 418, 530–534. doi: 10.1038/nature00839
- Martellotta, M. C., Cossu, G., Fattore, L., Gessa, G. L., and Fratta, W. (1998). Self-administration of the cannabinoid receptor agonist WIN 55,212-2 in drug-naïve mice. *Neuroscience* 85, 327–330. doi: 10.1016/S0306-4522(98)00052-9
- Martin, M., Ledent, C., Parmentier, M., Maldonado, R., and Valverde, O. (2000). Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. *Eur. J. Neurosci.* 12, 4038–4046. doi: 10.1046/j.1460-9568.2000.00287.x
- Mascia, M. S., Obinu, M. C., Ledent, C., Parmentier, M., Bohme, G. A., Imperato, A., et al. (1999). Lack of morphine-induced dopamine release in the nucleus accumbens of cannabinoid CB(1) receptor knockout mice. *Eur. J. Pharmacol.* 383, R1–R2. doi: 10.1016/S0014-2999(99)00656-1
- Massotte, D. (2014). In vivo opioid receptor heteromerization: where do we stand? *Br. J. Pharmacol.* 172, 420–434. doi: 10.1111/bph.12702
- Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346, 561–564. doi: 10.1038/346561a0
- Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383, 819–823. doi: 10.1038/383819a0
- Mendizabal, V., Zimmer, A., and Maldonado, R. (2006). Involvement of kappa/dynorphin system in WIN 55,212-2 self-administration in mice. *Neuropharmacology* 31, 1957–1966. doi: 10.1038/sj.npp.1300957
- Mestek, A., Hurley, J. H., Bye, L. S., Campbell, A. D., Chen, Y., Tian, M., et al. (1995). The human mu opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C. *J. Neurosci.* 15, 2396–2406.
- Mizoguchi, H., Watanabe, C., Osada, S., Yoshioka, M., Aoki, Y., Natsui, S., et al. (2010). Lack of a rewarding effect and a locomotor-enhancing effect of the selective mu-opioid receptor agonist amidino-TAPA. *Psychopharmacology (Berl)* 212, 215–225. doi: 10.1007/s00213-010-1946-0
- Monory, K., Blaudzun, H., Massa, F., Kaiser, N., Lemberger, T., Schutz, G., et al. (2007). Genetic dissection of behavioural and autonomic effects of Delta(9)-tetrahydrocannabinol in mice. *PLoS Biol.* 5:e269. doi: 10.1371/journal.pbio.0050269
- Monory, K., Massa, F., Egertova, M., Eder, M., Blaudzun, H., Westenbroek, R., et al. (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* 51, 455–466. doi: 10.1016/j.neuron.2006.07.006
- Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365, 61–65. doi: 10.1038/365061a0
- Nadal, X., La Porta, C., Andreea Bura, S., and Maldonado, R. (2013). Involvement of the opioid and cannabinoid systems in pain control: new insights from knockout studies. *Eur. J. Pharmacol.* 716, 142–157. doi: 10.1016/j.ejphar.2013.01.077
- Navarrete, F., Rodriguez-Arias, M., Martin-Garcia, E., Navarro, D., Garcia-Gutierrez, M. S., Aguilar, M. A., et al. (2013). Role of CB2 cannabinoid receptors in the rewarding, reinforcing, and physical effects of nicotine. *Neuropharmacology* 38, 2515–2524. doi: 10.1038/npp.2013.157
- Nestler, E. J. (2014). Epigenetic mechanisms of drug addiction. *Neuropharmacology* 76 Pt B, 259–268. doi: 10.1016/j.neuropharm.2013.04.004
- Niikura, K., Narita, M., Okutsu, D., Tsurukawa, Y., Nanjo, K., Kurahashi, K., et al. (2008). Implication of endogenous beta-endorphin in the inhibition of the morphine-induced rewarding effect by the direct activation of spinal protein kinase C in mice. *Neurosci. Lett.* 433, 54–58. doi: 10.1016/j.neulet.2007.12.042
- Nogueiras, R., Romero-Pico, A., Vazquez, M. J., Novelle, M. G., Lopez, M., and Dieguez, C. (2014). The opioid system and food intake: homeostatic and hedonic mechanisms. *Obes. Facts* 5, 196–207. doi: 10.1159/000338163
- Oakley, S. M., Toth, G., Borsodi, A., Kieffer, B. L., and Kitchen, I. (2003). G-protein coupling of delta-opioid receptors in brains of mu-opioid receptor knockout mice. *Eur. J. Pharmacol.* 466, 91–98. doi: 10.1016/S0014-2999(03)01531-0
- Ohno-Shosaku, T., and Kano, M. (2014). Endocannabinoid-mediated retrograde modulation of synaptic transmission. *Curr. Opin. Neurobiol.* 29C, 1–8. doi: 10.1016/j.conb.2014.03.017
- Onaivi, E. S., Ishiguro, H., Gong, J. P., Patel, S., Perchuk, A., Meozzi, P. A., et al. (2006). Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Ann. N. Y. Acad. Sci.* 1074, 514–536. doi: 10.1196/annals.1369.052
- Ortega-Alvaro, A., Ternianov, A., Aracil-Fernandez, A., Navarrete, F., Garcia-Gutierrez, M. S., and Manzanares, J. (2013). Role of cannabinoid CB receptor in the reinforcing actions of ethanol. *Addict. Biol.* doi: 10.1111/adb.12076
- Pan, Y. X., Xu, J., Xu, M., Rossi, G. C., Matulonis, J. E., and Pasternak, G. W. (2009). Involvement of exon 11-associated variants of the mu opioid receptor MOR-1 in heroin, but not morphine, actions. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4917–4922. doi: 10.1073/pnas.0811586106
- Panagis, G., Mackey, B., and Vlachou, S. (2014). Cannabinoid regulation of brain reward processing with an emphasis on the role of CB1 receptors: a step back into the future. *Front. Psychiatry* 5:92. doi: 10.3389/fpsyg.2014.00092
- Panlilio, L. V., and Goldberg, S. R. (2007). Self-administration of drugs in animals and humans as a model and an investigative tool. *Addiction* 102, 1863–1870. doi: 10.1111/j.1360-0443.2007.02011.x
- Panlilio, L. V., Justinova, Z., and Goldberg, S. R. (2010). Animal models of cannabinoid reward. *Br. J. Pharmacol.* 160, 499–510. doi: 10.1111/j.1476-5381.2010.00775.x
- Parolario, D., Rubino, T., Vigano, D., Massi, P., Guidali, C., and Realini, N. (2010). Cellular mechanisms underlying the interaction between cannabinoid and opioid system. *Curr. Drug Targets* 11, 393–405. doi: 10.2174/138945010790980367
- Pattij, T., and De Vries, T. J. (2013). The role of impulsivity in relapse vulnerability. *Curr. Opin. Neurobiol.* 23, 700–705. doi: 10.1016/j.conb.2013.01.023
- Pertwee, R. G. (2010). Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. *Curr. Med. Chem.* 17, 1360–1381. doi: 10.2174/092986710790980050
- Petrenko, A. B., Yamazaki, M., Sakimura, K., Kano, M., and Baba, H. (2014). Augmented tonic pain-related behavior in knockout mice lacking monoacylglycerol lipase, a major degrading enzyme for the endocannabinoid 2-arachidonoylglycerol. *Behav. Brain Res.* 271, 51–58. doi: 10.1016/j.bbr.2014.05.063
- Piomelli, D. (2014). More surprises lying ahead. The endocannabinoids keep us guessing. *Neuropharmacology* 76 Pt B, 228–234. doi: 10.1016/j.neuropharm.2013.07.026
- Pol, O., Murtra, P., Caracuel, L., Valverde, O., Puig, M. M., and Maldonado, R. (2006). Expression of opioid receptors and c-fos in CB1 knockout mice exposed to neuropathic pain. *Neuropharmacology* 50, 123–132. doi: 10.1016/j.neuropharm.2005.11.002
- Pradhan, A. A., Befort, K., Nozaki, C., Gaveriaux-Ruff, C., and Kieffer, B. L. (2011). The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol. Sci.* 32, 581–590. doi: 10.1016/j.tips.2011.06.008
- Pryce, G., Visintin, C., Ramagopalan, S. V., Al-Izki, S., De Faveri, L. E., Nuamah, R. A., et al. (2014). Control of spasticity in a multiple sclerosis model using

- central nervous system-excluded CB1 cannabinoid receptor agonists. *FASEB J.* 28, 117–130. doi: 10.1096/fj.13–239442
- Ragnauth, A., Schuller, A., Morgan, M., Chan, J., Ogawa, S., Pintar, J., et al. (2001). Female preproenkephalin-knockout mice display altered emotional responses. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1958–1963. doi: 10.1073/pnas.041598498
- Ramesh, D., Gamage, T. F., Vanuytsel, T., Owens, R. A., Abdullah, R. A., Niphakis, M. J., et al. (2013). Dual inhibition of endocannabinoid catabolic enzymes produces enhanced antiwithdrawal effects in morphine-dependent mice. *Neuropsychopharmacology* 38, 1039–1049. doi: 10.1038/npp.2012.269
- Ramesh, D., Ross, G. R., Schlosburg, J. E., Owens, R. A., Abdullah, R. A., Kinsey, S. G., et al. (2011). Blockade of endocannabinoid hydrolytic enzymes attenuates precipitated opioid withdrawal symptoms in mice. *J. Pharmacol. Exp. Ther.* 339, 173–185. doi: 10.1124/jpet.111.181370
- Rice, O. V., Gordon, N., and Gifford, A. N. (2002). Conditioned place preference to morphine in cannabinoid CB1 receptor knockout mice. *Brain Res.* 945, 135–138. doi: 10.1016/S0006-8993(02)02890-1
- Robbe, D., Kopf, M., Remaury, A., Bockaert, J., and Manzoni, O. J. (2002). Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8384–8388. doi: 10.1073/pnas.122149199
- Robinson, T. E., and Berridge, K. C. (2008). Review. The incentive sensitization theory of addiction: some current issues. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 363, 3137–3146. doi: 10.1098/rstb.2008.0093
- Robledo, P., Berrendero, F., Ozaita, A., and Maldonado, R. (2008). Advances in the field of cannabinoid–opioid cross-talk. *Addict. Biol.* 13, 213–224. doi: 10.1111/j.1369-1600.2008.00107.x
- Roques, B. P., Fournier-Zaluski, M. C., and Wurm, M. (2012). Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. *Nat. Rev. Drug Discov.* 11, 292–310. doi: 10.1038/nrd3673
- Rubinstein, M., Mogil, J. S., Japon, M., Chan, E. C., Allen, R. G., and Low, M. J. (1996). Absence of opioid stress-induced analgesia in mice lacking beta-endorphin by site-directed mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3995–4000. doi: 10.1073/pnas.93.9.3995
- Ruehle, S., Remmers, F., Romo-Parra, H., Massa, F., Wickert, M., Wortge, S., et al. (2013). Cannabinoid CB1 receptor in dorsal telencephalic glutamatergic neurons: distinctive sufficiency for hippocampus-dependent and amygdala-dependent synaptic and behavioral functions. *J. Neurosci.* 33, 10264–10277. doi: 10.1523/JNEUROSCI.4171-12.2013
- Sanchis-Segura, C., Cline, B. H., Marsicano, G., Lutz, B., and Spanagel, R. (2004). Reduced sensitivity to reward in CB1 knockout mice. *Psychopharmacology (Berl)* 176, 223–232. doi: 10.1007/s00213-004-1877-1878
- Sanchis-Segura, C., and Spanagel, R. (2006). Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. *Addict. Biol.* 11, 2–38. doi: 10.1111/j.1369-1600.2006.00012.x
- Saunders, B. T., and Robinson, T. E. (2013). Individual variation in resisting temptation: implications for addiction. *Neurosci. Biobehav. Rev.* 37, 1955–1975. doi: 10.1016/j.neubiorev.2013.02.008
- Scavone, J. L., Sterling, R. C., and Van Bockstaele, E. J. (2013). Cannabinoid and opioid interactions: implications for opiate dependence and withdrawal. *Neuroscience* 248, 637–654. doi: 10.1016/j.neuroscience.2013.04.034
- Schlosburg, J. E., Blankman, J. L., Long, J. Z., Nomura, D. K., Pan, B., Kinsey, S. G., et al. (2010). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat. Neurosci.* 13, 1113–1119. doi: 10.1038/nn.2616
- Schuller, A. G., King, M. A., Zhang, J., Bolan, E., Pan, Y. X., Morgan, D. J., et al. (1999). Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nat. Neurosci.* 2, 151–156. doi: 10.1038/5706
- Sharifi, N., Diehl, N., Yaswen, L., Brennan, M. B., and Hochgeschwender, U. (2001). Generation of dynorphin knockout mice. *Brain Res. Mol. Brain Res.* 86, 70–75. doi: 10.1016/S0169-328X(00)00264-3
- Simonin, F., Befort, K., Gaveriaux-Ruff, C., Matthes, H., Nappey, V., Lannes, B., et al. (1994). The human delta-opioid receptor: genomic organization, cDNA cloning, functional expression, and distribution in human brain. *Mol. Pharmacol.* 46, 1015–1021.
- Simonin, F., Gaveriaux-Ruff, C., Befort, K., Matthes, H., Lannes, B., Micheletti, G., et al. (1995). kappa-Opioid receptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7006–7010. doi: 10.1073/pnas.92.15.7006
- Simonin, F., Slowe, S., Becker, J. A., Matthes, H. W., Filliol, D., Chluba, J., et al. (2001). Analysis of [³H]bremazocine binding in single and combinatorial opioid receptor knockout mice. *Eur. J. Pharmacol.* 414, 189–195. doi: 10.1016/S0014-2999(01)00822-6
- Simonin, F., Valverde, O., Smadja, C., Slowe, S., Kitchen, I., Dierich, A., et al. (1998). Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J.* 17, 886–897. doi: 10.1093/emboj/17.4.886
- Skoubis, P. D., Lam, H. A., Shoblock, J., Narayanan, S., and Maidment, N. T. (2005). Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *Eur. J. Neurosci.* 21, 1379–1384. doi: 10.1111/j.1460-9568.2005.03956.x
- Slowe, S. J., Simonin, F., Kieffer, B., and Kitchen, I. (1999). Quantitative autoradiography of mu-delta- and kappa1 opioid receptors in kappa-opioid receptor knockout mice. *Brain Res.* 818, 335–345. doi: 10.1016/S0006-8993(98)1201-3
- Solinas, M., Goldberg, S. R., and Piomelli, D. (2008). The endocannabinoid system in brain reward processes. *Br. J. Pharmacol.* 154, 369–383. doi: 10.1038/bjp.2008.130
- Sora, I., Elmer, G., Funada, M., Pieper, J., Li, X. F., Hall, F. S., et al. (2001). Mu opiate receptor gene dose effects on different morphine actions: evidence for differential *in vivo* mu receptor reserve. *Neuropsychopharmacology* 25, 41–54. doi: 10.1016/S0893-133X(00)00252-9
- Stein, C. (2013). Opioids, sensory systems and chronic pain. *Eur. J. Pharmacol.* 716, 179–187. doi: 10.1016/j.ejphar.2013.01.076
- Steiner, H., Bonner, T. I., Zimmer, A. M., Kitai, S. T., and Zimmer, A. (1999). Altered gene expression in striatal projection neurons in CB1 cannabinoid receptor knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5786–5790. doi: 10.1073/pnas.96.10.5786
- Sun, X., Wang, H., Okabe, M., Mackie, K., Kingsley, P. J., Marnett, L. J., et al. (2009). Genetic loss of faah compromises male fertility in mice. *Biol. Reprod.* 80, 235–242. doi: 10.1093/biolreprod.108.072736
- Swendsen, J., and Le Moal, M. (2011). Individual vulnerability to addiction. *Ann. N. Y. Acad. Sci.* 1216, 73–85. doi: 10.1111/j.1749-6632.2010.05894.x
- Szutorisz, H., Dinieri, J. A., Sweet, E., Egervari, G., Michaelides, M., Carter, J. M., et al. (2014). Parental THC exposure leads to compulsive heroin-seeking and altered striatal synaptic plasticity in the subsequent generation. *Neuropsychopharmacology* 39, 1315–1323. doi: 10.1038/npp.2013.352
- Tanimura, A., Yamazaki, M., Hashimoto, Y., Uchigashima, M., Kawata, S., Abe, M., et al. (2010). The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. *Neuron* 65, 320–327. doi: 10.1016/j.neuron.2010.01.021
- Taschner, U., Radner, F. P., Heier, C., Schreiber, R., Schweiger, M., Schoiswohl, G., et al. (2011). Monoglyceride lipase deficiency in mice impairs lipolysis and attenuates diet-induced insulin resistance. *J. Biol. Chem.* 286, 17467–17477. doi: 10.1074/jbc.M110.215434
- Tebano, M. T., Martire, A., and Popoli, P. (2012). Adenosine A(2A)-cannabinoid CB(1) receptor interaction: an integrative mechanism in striatal glutamatergic neurotransmission. *Brain Res.* 1476, 108–118. doi: 10.1016/j.brainres.2012.04.051
- Tian, M., Broxmeyer, H. E., Fan, Y., Lai, Z., Zhang, S., Aronica, S., et al. (1997). Altered hematopoiesis, behavior, and sexual function in mu opioid receptor-deficient mice. *J. Exp. Med.* 185, 1517–1522. doi: 10.1084/jem.185.8.1517
- Tomasiewicz, H. C., Jacobs, M. M., Wilkinson, M. B., Wilson, S. P., Nestler, E. J., and Hurd, Y. L. (2012). Proenkephalin mediates the enduring effects of adolescent cannabis exposure associated with adult opiate vulnerability. *Biol. Psychiatry* 72, 803–810. doi: 10.1016/j.biopsych.2012.04.026
- Trigo, J. M., Martin-Garcia, E., Berrendero, F., Robledo, P., and Maldonado, R. (2010). The endogenous opioid system: a common substrate in drug addiction. *Drug Alcohol Depend.* 108, 183–194. doi: 10.1016/j.drugalcdep.2009.10.011
- Tsuboi, K., Okamoto, Y., Ikematsu, N., Inoue, M., Shimizu, Y., Uyama, T., et al. (2011). Enzymatic formation of N-acylethanolamines from N-acylethanolamine plasmalogen through N-acylphosphatidylethanolamine-hydrolyzing phospholipase D-dependent and -independent pathways. *Biochim. Biophys. Acta* 1811, 565–577. doi: 10.1016/j.bbapap.2011.07.009

- Tzschentke, T. M. (2007). Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict. Biol.* 12, 227–462. doi: 10.1111/j.1369-1600.2007.00070.x
- Uchigashima, M., Yamazaki, M., Yamasaki, M., Tanimura, A., Sakimura, K., Kano, M., et al. (2011). Molecular and morphological configuration for 2-arachidonoylglycerol-mediated retrograde signaling at mossy cell-granule cell synapses in the dentate gyrus. *J. Neurosci.* 31, 7700–7714. doi: 10.1523/JNEUROSCI.5665-10.2011
- Uriguen, L., Berrendero, F., Ledent, C., Maldonado, R., and Manzanares, J. (2005). Kappa- and delta-opioid receptor functional activities are increased in the caudate putamen of cannabinoid CB1 receptor knockout mice. *Eur. J. Neurosci.* 22, 2106–2110. doi: 10.1111/j.1460-9568.2005.04372.x
- Valverde, O., Maldonado, R., Valjent, E., Zimmer, A. M., and Zimmer, A. (2000). Cannabinoid withdrawal syndrome is reduced in pre-proenkephalin knock-out mice. *J. Neurosci.* 20, 9284–9289.
- Valverde, O., and Torrens, M. (2012). CB1 receptor-deficient mice as a model for depression. *Neuroscience* 204, 193–206. doi: 10.1016/j.neuroscience.2011.09.031
- Van't Veer, A., Bechtholt, A. J., Onvani, S., Potter, D., Wang, Y., Liu-Chen, L. Y., et al. (2013). Ablation of kappa-opioid receptors from brain dopamine neurons has anxiolytic-like effects and enhances cocaine-induced plasticity. *Neuropsychopharmacology* 38, 1585–1597. doi: 10.1038/npp.2013.58
- van Rijn, R. M., and Whistler, J. L. (2009). The delta(1) opioid receptor is a heterodimer that opposes the actions of the delta(2) receptor on alcohol intake. *Biol. Psychiatry* 66, 777–784. doi: 10.1016/j.biopsych.2009.05.019
- Vigano, D., Rubino, T., and Parolaro, D. (2005). Molecular and cellular basis of cannabinoid and opioid interactions. *Pharmacol. Biochem. Behav.* 81, 360–368. doi: 10.1016/j.pbb.2005.01.021
- Vinod, K. Y., Sanguino, E., Yalamanchili, R., Manzanares, J., and Hungund, B. L. (2008). Manipulation of fatty acid amide hydrolase functional activity alters sensitivity and dependence to ethanol. *J. Neurochem.* 104, 233–243. doi: 10.1111/j.1471-4159.2007.04956.x
- Weibel, R., Reiss, D., Karchewski, L., Gardon, O., Matifas, A., Filliol, D., et al. (2013). Mu opioid receptors on primary afferent nav1.8 neurons contribute to opiate-induced analgesia: insight from conditionally knockout mice. *PLoS ONE* 8:e74706. doi: 10.1371/journal.pone.0074706
- Wise, L. E., Shelton, C. C., Cravatt, B. F., Martin, B. R., and Lichtman, A. H. (2007). Assessment of anandamide's pharmacological effects in mice deficient of both fatty acid amide hydrolase and cannabinoid CB1 receptors. *Eur. J. Pharmacol.* 557, 44–48. doi: 10.1016/j.ejphar.2006.11.002
- Wotherspoon, G., Fox, A., McIntyre, P., Colley, S., Bevan, S., and Winter, J. (2005). Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. *Neuroscience* 135, 235–245. doi: 10.1016/j.neuroscience.2005.06.009
- Xi, Z. X., Peng, X. Q., Li, X., Song, R., Zhang, H. Y., Liu, Q. R., et al. (2011). Brain cannabinoid CB(2) receptors modulate cocaine's actions in mice. *Nat. Neurosci.* 14, 1160–1166. doi: 10.1038/nn.2874
- Yaswen, L., Diehl, N., Brennan, M. B., and Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat. Med.* 5, 1066–1070. doi: 10.1038/12506
- Yoshino, H., Miyamae, T., Hansen, G., Zambrowicz, B., Flynn, M., Pedicord, D., et al. (2011). Postsynaptic diacylglycerol lipase mediates retrograde endocannabinoid suppression of inhibition in mouse prefrontal cortex. *J. Physiol.* 589, 4857–4884. doi: 10.1113/jphysiol.2011.212225
- Zanettini, C., Panlilio, L. V., Alicki, M., Goldberg, S. R., Haller, J., and Yasar, S. (2011). Effects of endocannabinoid system modulation on cognitive and emotional behavior. *Front. Behav. Neurosci.* 5:57. doi: 10.3389/fnbeh.2011.00057
- Zhang, H. Y., Gao, M., Liu, Q. R., Bi, G. H., Li, X., Yang, H. J., et al. (2014). Cannabinoid CB2 receptors modulate midbrain dopamine neuronal activity and dopamine-related behavior in mice. *Proc. Natl. Acad. Sci. U.S.A.* doi: 10.1073/pnas.1413210111
- Zhu, Y., King, M. A., Schuller, A. G., Nitsche, J. F., Reidl, M., Elde, R. P., et al. (1999). Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron* 24, 243–252. doi: 10.1016/S0896-6273(00)8036-3
- Zimmer, A., Valjent, E., Konig, M., Zimmer, A. M., Robledo, P., Hahn, H., et al. (2001). Absence of delta-9-tetrahydrocannabinol dysphoric effects in dynorphin-deficient mice. *J. Neurosci.* 21, 9499–9505.
- Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M., and Bonner, T. I. (1999). Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5780–5785. doi: 10.1073/pnas.96.10.5780
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Inhibitors of MAO-A and MAO-B in Psychiatry and Neurology

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Inhibitors of MAO-A and MAO-B are in clinical use for the treatment of psychiatric and neurological disorders respectively. Elucidation of the molecular structure of the active sites of the enzymes has enabled a precise determination of the way in which substrates and inhibitor molecules are metabolized, or inhibit metabolism of substrates, respectively. Despite the knowledge of the strong antidepressant efficacy of irreversible MAO inhibitors, their clinical use has been limited by their side effect of potentiation of the cardiovascular effects of dietary amines ("cheese effect"). A number of reversible MAO-A inhibitors which are devoid of cheese effect have been described in the literature, but only one, moclobemide, is currently in clinical use. The irreversible inhibitors of MAO-B, selegiline and rasagiline, are used clinically in treatment of Parkinson's disease, and a recently introduced reversible MAO-B inhibitor, safinamide, has also been found efficacious. Modification of the pharmacokinetic characteristics of selegiline by transdermal administration has led to the development of a new drug form for treatment of depression. The clinical potential of MAO inhibitors together with detailed knowledge of the enzyme's binding site structure should lead to future developments with these drugs.

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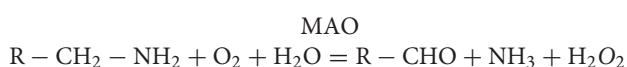
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INTRODUCTION

Monoamine oxidase (MAO; EC 1.4.3.4.) is a widely distributed mitochondrial enzyme with high expression levels in gastro-intestinal and hepatic as well as neuronal tissues. The enzyme catalyzes the oxidative deamination of a variety of monoamines, both endogenous and exogenous, and has major roles in metabolizing released neurotransmitters, and in detoxification of a large variety of endogenous and exogenous amines. Drugs which inhibit MAO are currently in clinical use for treatment of affective disorders and Parkinson's disease (PD). In this chapter we review recent developments in the basic pharmacology of MAO inhibitors (MAOI) and their clinical usage, and discuss the potential for new drug development in this field.

The overall enzyme reaction of MAO can be represented by the following equation:



The aldehydes produced by the action of MAO are metabolized further by aldehyde dehydrogenase and aldehyde reductase leading to the formation of glycals and carboxylic acids (Westfall and Westfall, 2011). The fact that an aldehyde is formed initially together with H_2O_2 which can generate reactive oxygen species (ROS) has drawn attention to the possibility that products of the action of

MAO may be neurotoxic (Jenner, 2003). In this connection it should be realized that ROS and other reactive species are normally metabolized by scavenger enzymes including catalase and superoxide dismutase, and dysfunction of these enzyme systems may be a factor in neurodegenerative disease (Aluf et al., 2011). The dopaminergic neurons of substantia nigra pars compacta (SNpc) are at risk to oxidative stress because of their tonic activity and dense packing. Their degree of oxidative stress increases in early PD when a portion of the neurons have been lost, and the activity of the remaining ones increases in compensation. This situation was modeled recently in a microdialysis study in which a non-diffusible indicator molecule was perfused through a probe placed in the striatum. Following intraventricular injection of 6-hydroxydopamine in a dose adequate to reduce SNpc dopaminergic cell number by 50%, the level of oxidative stress increased markedly (Aluf et al., 2011), and was reduced following systemic injection of an MAO-A or MAO-B inhibitor (Aluf et al., 2013).

MAO ISOFORMS

Two isoenzymes are encoded in the human X-chromosomal gene Xp1 123, MAO-A, and MAO-B. The two forms have over 70% homogeneity. Biochemically, the two forms can be differentiated by their substrate and inhibitor specificities; MAO-A shows greater affinity for hydroxylated amines such as noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5-HT), whereas MAO-B shows greater affinity for non-hydroxylated amines such as benzylamine and beta-phenylethylamine (PEA). The amines dopamine (DA) and tyramine show similar affinity for each enzyme form. Clorgyline is a selective inhibitor of MAO-A while selegiline (*l*-deprenyl) and rasagiline are relatively selective inhibitors of MAO-B. The ratio of selectivity of selegiline and rasagiline for MAO-B is such that in human subjects, doses 2–5 fold higher respectively than the MAO-B selective dose can cause significant inhibition of MAO-A as shown by tyramine pressor responses (Bieck and Antonin, 1994; Goren et al., 2010). Some inhibitors can inhibit both forms of the enzyme (referred to as non-selective inhibitors, although this can cause confusion because the inhibitors are quite selective for MAO as opposed to other enzymes). The precise localization of the two MAO isoforms in brain has not been completely elucidated. Studies using cell cultures (Yu and Hertz, 1983; Carlo et al., 1996) pointed to localization of MAO-A within glial cells, but this is not true for the intact brain, where investigations in both primate and non-primate species have established that the glial enzyme is predominantly type B (Levitt et al., 1982; Denney and Denney, 1985; Westlund et al., 1988). The type A isoform has been localized to several neuronal cell types in primate and rodent species, including NA-ergic neurons of the locus coeruleus, DA-ergic neurons of the substantia nigra pars compacta (SNpc), (Westlund et al., 1993) and striatal medium spiny neurons (Sader-Mazbar et al., 2013). Serotonergic neuronal cell bodies of the raphe nucleus stain positive for MAO-B but the isoform localized to axonal varicosities may be of the A isoform (Denney and Denney, 1985). Selective inhibition of MAO-A leads to increased levels of neurotransmitter within noradrenergic (NA-ergic) and 5-HT-ergic neurons of the CNS,

and clinical antidepressant action, while inhibition of MAO-B leads to increased levels of DA in the Parkinsonian brain with partial depletion of DA-ergic neurons in SNpc, and anti-Parkinsonian action (see Finberg, 2014 for a detailed description of these events at the synaptic level).

Many compounds with MAO inhibitory properties are being prepared by researchers, however the present account is limited to a description of the most important drugs from a therapeutic viewpoint, i.e., affective disorders and Parkinson's disease. The chemical structures of drugs mentioned in this review and a brief description of their major characteristics is shown in **Table 1**.

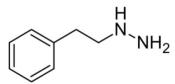
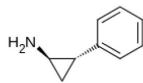
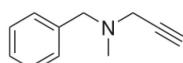
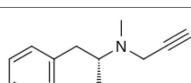
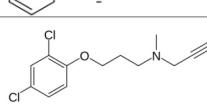
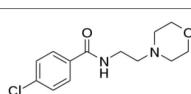
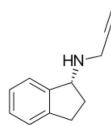
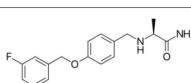
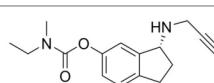
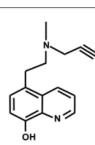
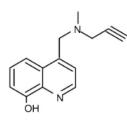
MOLECULAR STRUCTURE OF MAO AND MECHANISM OF ENZYME INHIBITION

For many years, a formula was searched for without success to explain the selectivity of an inhibitor molecule for MAO-A or MAO-B. The problem was solved when the MAO protein molecule was crystallized by the groups of Edmondson and Sukihara, enabling three-dimensional modeling of the protein and its combining site (Binda et al., 2002, 2007; De Colibus et al., 2005; Son et al., 2008). It was then seen that a two-site cavity structure exists for human MAO-B (hMAO-B), with an entry cavity and a reactive site cavity, whereas in human MAO-A (hMAO-A) the active site cavity is not bipartite and is shorter and wider than the longer and narrower substrate cavity in hMAO-B (De Colibus et al., 2005). The reactive site contains a combining moiety in which the N5 atom of FAD is displayed on the inner surface, and tyrosines 398, and 435 guard the entry gate in hMAO-B (Binda et al., 2002). Knowledge of the three-dimensional aspects of these sites, and the associated amino acid positions, can now be utilized in the design of new inhibitors.

Both reversible and irreversible inhibitors of MAO have been developed in previous years, and are currently in use clinically for treatment of affective and neurological disorders. Irreversible inhibitors are of several types: hydrazines, cyclopropylamines, and propargylamines. In all cases, these drugs combine covalently with the N5 atom of the flavin residue, but the rate of dissociation of the drug-enzyme complex is variable. Detailed mechanisms for the drug-enzyme complex formation have been described. Following recognition of the enzyme pharmacophore by the drug, the inhibitor molecule is metabolized leading to a reactive intermediate which combines covalently with the N5 atom of FAD leading to formation of a drug-receptor adduct, which then undergoes aging and irreversible combination. The term "suicide inhibitor" has been used in description of this type of drug action (see Finberg, 2014).

In general, because these inhibitors will irreversibly inactivate the enzyme, their action can only be reversed by generation of new enzyme molecules, a process which can take days or weeks. In clinical use, the drugs are administered daily, using a dose which alone is adequate to cause only partial enzyme inhibition, but when given daily over several days will cause a cumulative inhibition up to 90% or more of the target enzyme in brain. Continued drug administration ensures that newly-formed enzyme molecules are also inhibited, and that the enzyme activity is maintained at a constant low level. The clinical importance of

TABLE 1 | Structures and major characteristics of MAO inhibitors mentioned in the text.

Compound	Activity	Status	Chemical structure
Phenelzine	Irreversible MAO-A + MAO-B	Used as antidepressant Hepatotoxicity Needs dietary control for restriction of tyramine intake	
Tranylcypromine	Irreversible MAO-A + MAO-B	Used as antidepressant with dietary control	
Pargyline	Irreversible MAO-A and MAO-B	Antidepressant and antihypertensive Currently not in clinical use	
Selegiline	Irreversible MAO-B selective (R- enantiomer) Selectivity is dose dependent <i>in vivo</i>	Metabolism to amphetamines	
Clorgyline	Irreversible highly MAO-A selective	Antidepressant effect demonstrated in humans but not in clinical use	
Moclobemide	Reversible highly MAO-A selective	Moderately effective antidepressant drug	
Rasagiline	Irreversible MAO-B selective (R+ enantiomer) Selectivity is dose dependent <i>in vivo</i>	Neuroprotective <i>in vitro</i> , anti-Parkinson drug, metabolism to 1-aminoindan	
Safinamide	Reversible highly MAO-B selective	Anti-Parkinson drug, glutamate receptor antagonistic and Na+ channel blocking properties	
Ladostigil	MAO-A + MAO-B Relative brain selectivity, minimal tyramine potentiation	Cholinesterase and MAO inhibition	
VAR 10303	MAO-A + MAO-B Relative brain selectivity, minimal tyramine potentiation	Fe chelation and MAO inhibition	
M30	MAO-A and MAO-B Relative brain selectivity	Fe chelation and MAO inhibition	

this type of drug use is that a constant high degree of enzyme inhibition can be maintained over time. In addition, however, on stopping treatment enzyme activity will remain at a low level even after the drug itself has been cleared from the body.

TREATMENT OF DEPRESSION WITH MAO INHIBITORS

Non-subtype-Selective Irreversible Inhibitors

The profound antidepressant action of MAOI inhibitors was discovered by chance (Lehmann and Kline, 1983) in

tuberculous patients treated with iproniazid, a derivative of the hydrazine compound isoniazid. Further developments led to the introduction into clinical use of several non-subtype-selective irreversible MAO inhibitors including the hydrazines phenelzine and isocarboxazid, the propargylamine pargyline, and the cyclopropylamine tranylcypromine, but these compounds can all lead to potentiation of the cardiovascular effects of the dietary amine tyramine ("cheese effect"). Following realization that the cheese effect can be avoided by dietary counseling, and that MAO inhibitors are in fact excellent drugs for treatment of drug-resistant and atypical depression, use of certain non-subtype-selective inhibitors, in particular tranylcypromine (Parnate), is now seen with increasing frequency. Tranylcypromine has

pharmacological properties in addition to inhibition of MAO, in particular inhibition of lysine-specific histone demethylation type 1, and interaction with the endogenous cannabinoid system (Lee et al., 2006; Hill et al., 2008). Phenelzine also has an additional pharmacological property which may be involved in its antidepressant actions, namely blockade of GABA and alanine transaminases (Baker et al., 1991; Todd and Baker, 2008).

In a recent review (Heijnen et al., 2015) of a small number of clinical trials, tranylcypromine was found to be an efficacious and safe drug for the treatment of bipolar depression, when administered with correct dietary counseling. Although cheese effect is a potentially serious reaction, the limitations it imposes on treatment of psychiatric patients have been much exaggerated, because the amounts of tyramine occurring in foodstuffs are quite low, and only a gross violation of normal dietary directions would be likely to cause a fatal, or damaging, reaction (Gillman, 2011). The management of such a hypertensive reaction if it does occur has also been well-documented (Gillman, 2011). In addition to cheese effect, another potential danger is serotonin toxicity syndrome (ST), which can occur following the combination of irreversible MAOI with a drug which has the potential to elevate 5-HT synaptic levels, such as a serotonin-selective reuptake inhibitor (SSRI) (Gillman, 2006). In this context, the relatively long period required for return of MAO activity following cessation of therapy with an irreversible inhibitor is important when a change in therapy is required. If therapy with a SSRI is to be used, there is a danger of ST if these drugs are instituted before MAO activity has returned to normal levels. Following cessation of tranylcypromine administration in healthy subjects, a period of 30 days was required for complete normalization of the pressor response to oral tyramine challenge (Bieck and Antonin, 1988). In the case of rasagiline, using platelet activity of MAO-B as the index, enzyme activity returned to baseline levels 2 weeks after cessation of drug administration in healthy subjects (Thebault et al., 2004). The time required for return of enzyme activity in the brain however, is considerably longer than in the periphery. Using (Denney and Denney, 1985) C-labeled selegiline together with positron emission tomography (PET) the half-time for return of MAO-B binding in the brain following complete blockade of binding by an initial injection of selegiline in a baboon was 30 days (Arnett et al., 1987), and using similar technique, following initial MAO-B inactivation by rasagiline in human subjects, was 40 days (Freedman et al., 2005). Recommended periods (by manufacturer) for wash-out after cessation of tranylcypromine range from 7 to 10 days (Gahr et al., 2013).

The antidepressant effect of MAOI has focused interest on the possibility that altered expression levels of the MAO enzyme could be the cause of some forms of depressive disorders. Polymorphisms in the MAO-A gene have been associated with a number of behavioral traits. Reduced enzyme activity is associated with violent behavior and aggression, whereas over-expression may be linked to depression (Alia-Klein et al., 2008). These facts, together with the well-known biogenic amines hypothesis, provide theoretical background in support of the use of MAOI for treatment of affective disorders. Several studies have attempted to link the MAOA-uVNTR polymorphism, which

leads to increased enzyme transcription, with suicidal tendency, but a meta-analysis including 1452 psychiatric patients and 1198 control subjects did not find a significant association (Hung et al., 2012) with this particular trait. In a recent study in which MAO-A expression level (total distribution volume, V_t , of ^{11}C -harmine by PET) was determined in borderline personality disorder (BPD) patients, the MAO-A brain content was correlated with symptom severity (Kolla et al., 2015). Interestingly, MAO-A V_t was increased in prefrontal cortex and anterior cingulate cortex by 43 and 42% respectively in severe BPD subjects in relation to controls.

One of the main restrictions to the clinical use of MAOI for treatment of depression is the cheese effect. In preclinical and clinical studies it was shown that potentiation of the pharmacological effects of tyramine occurs following selective inhibition of MAO-A but not MAO-B (Lader et al., 1970; Finberg and Tenne, 1982; Finberg and Gillman, 2011). This can be attributed to the localization of MAO-A to noradrenergic (as well as serotonergic) neurons (see Finberg, 2014) for detailed review). A corollary to this selective localization of MAO subtypes is that selective inhibitors of MAO-A but not MAO-B are effective antidepressants (Youdim and Finberg, 1983), however no irreversible selective MAO-A inhibitors are in use for treatment of depression.

Reversible Inhibitors of MAO-A

In the 1980s several groups of researchers prepared selective reversible inhibitors of MAO-A (RIMAs) (Tipton et al., 1984) based on the theory that if substrate levels increased as a result of inhibition of the enzyme, the degree of enzyme inhibition would be reversed by increased dissociation of the inhibitor from its combining site, i.e., a reversible inhibitor would possess a built-in safety factor in the case of tyramine ingestion, and therefore reversible inhibitors would not cause cheese effect (Finberg, 2014). The correctness of this notion was confirmed in a number of clinical studies using tyramine challenges in patients (Finberg and Gillman, 2011; Finberg, 2014). Currently, moclobemide is the only RIMA available for clinical use. Although clinical studies carried out in the period following its general release showed an efficacy similar to that of tricyclic antidepressants (TCA) for treatment of depression it was found less effective than irreversible MAOIs (Lotufo-Neto et al., 1999; Shulman et al., 2013). Another reversible selective MAO-A inhibitor with antidepressant properties is methylene blue (Naylor et al., 1987; Ramsay et al., 2007). This interesting drug has several pharmacological actions, including inhibition of nitric oxidase synthase (NOS), and guanylate cyclase (Harvey et al., 2010), and so its antidepressant properties should not be solely ascribed to inhibition of MAO-A.

Selective Inhibitors of MAO-B in Treatment of Depression and Attention Deficit Hyperactivity Disorder (ADHD)

Following introduction of the irreversible selective MAO-B inhibitor selegiline for treatment of PD (see following sections), its efficacy for treatment of depression was examined in several

uncontrolled clinical trials, using the MAO-B-selective dose of 10 mg daily, and as was anticipated following the known involvement of mainly serotonergic and noradrenergic neuronal systems in depression, it was not effective. When examined at the higher doses of 30 or 60 mg daily, however, it did have significant antidepressant effect, especially in treatment-resistant depression (Mann et al., 1989; Sunderland et al., 1994). Based on these positive results a pharmacokinetic strategy was developed (selegiline transdermal system, STS) which permits a greater portion of the administered dose to enter the CNS, and reach tissue levels concomitant with inhibition of both MAO-A and MAO-B while avoiding inactivation of gastro-intestinal and hepatic enzyme (Mawhinney et al., 2003). This technique was developed on the basis of preclinical experiments in guinea-pigs (Mawhinney et al., 2003). In order to understand the mechanism of this relative brain selectivity, it is necessary to understand: (a) that selegiline is only MAO-B selective at low dose, and higher doses will inhibit both MAO-A and MAO-B, and (b) the pharmacokinetics of selegiline (Magyar, 2011). This compound is based on the molecule of R(-)-methamphetamine, and following systemic administration it is metabolized by cytochrome P450 enzymes in the liver, mainly to R(-)-methamphetamine, R(-)-amphetamine, and N-desmethylselegiline (Laine et al., 2000; Azzaro et al., 2007). When administered transdermally the first-pass metabolism is largely avoided, and a larger part of the administered dose directly accesses the brain, and binds irreversibly to both MAO-A and MAO-B. Intact molecules of the drug which leave the brain will be metabolized in the liver, but large scale inhibition of MAO isoforms in gastro-intestinal tract and liver will be avoided (Mawhinney et al., 2003), and the formation of potentially damaging amphetamines is reduced. The success of this strategy has been confirmed in human experiments, in which it was shown: a) that STS is an effective antidepressant, and b) that at antidepressant doses it does not cause cheese effect (Azzaro et al., 2006; Blob et al., 2007). Another dose form of selegiline aimed to produce a similar alteration in pharmacokinetics of the drug is the buccally administered solution (Zydis selegiline) which similarly produces effective antidepressant activity without significant tyramine potentiation (Clarke et al., 2003a,b). Its improved pharmacokinetics permit the use of lower doses which confer greater selectivity for MAO-B over MAO-A inhibition. The MAO-B-selective inhibitor rasagiline has been found effective in treatment of PD depression with a greater response at 2 mg/day than the usual dose of 1 mg/day, possibly because of the greater inhibitory effect on MAO-A at the higher dose (Korchounov et al., 2012).

In preclinical studies in rats, rasagiline administered at selective MAO-B-inhibitory dose did not modify DA, NA or 5-HT levels or induce reserpine reversal (Finberg and Youdim, 2002), however in aged mice, chronic administration of an MAO-B-selective dose (0.2 mg/kg daily for 3 weeks) did increase brain levels of DA and reduce DOPAC, and also showed antidepressant-like effects. Interestingly, the drug returned activity in behavioral paradigms such as learning and forced-swim test, which were reduced in the aged animals, to levels seen in young animals (Weinreb et al., 2015). It is of interest

that these effects of rasagiline in aged mice were produced also by chronic administration of its major metabolite 1-aminoindan, at a dose of 5 mg/kg daily over 3 months (Badinter et al., 2015). The authors of these articles suggested that the effects of 1-aminoindan indicate an action on catecholaminergic systems which is not the result of MAO inhibition, because *ex vivo* brain MAO activity was not inhibited; however since it is a reversible MAO inhibitor (Binda et al., 2005) their *ex vivo* assay of MAO would not be expected to show a change in enzyme activity, because the drug would be washed out or diluted in the brain homogenate used in their assay. On the other hand, the changes in tissue monoamine levels and their metabolites (Mann et al., 1989) are indicative of an inhibition of MAO.

In a placebo-controlled study of 11 children with ADHD selegiline significantly improved attention but not impulsivity (Akhondzadeh et al., 2003; Niederhofer, 2003; Rubinstein et al., 2006). In three studies in which selegiline was compared with methylphenidate in children with ADHD, the two drugs had similar efficacy (Akhondzadeh et al., 2003; Niederhofer, 2003; Mohammadi et al., 2004). Considering the detrimental pharmacology of amphetamine-like drugs used in ADHD, the use of MAO-B inhibitors in this condition warrants further study. Since MAO-B inhibition markedly increases the brain levels of endogenous PEA ("the body's own amphetamine"), this could be an explanation for the selegiline effects observed in the above studies, in addition to inhibition of DA breakdown.

MAOI and Drug Addiction

Use of MAOI in treatment of depression in cocaine-addicted subjects has been proposed, because chronic cocaine administration reduces the activity of monoamine neurotransmitter systems, which are enhanced by MAOI. In addition, by enhancing DA levels MAOI could possibly be used to substitute for the reward-initiating effect of cocaine (Ho et al., 2009). The potential of MAOI to reduce cocaine-induced reward was studied in mice (Ho et al., 2009). The long-term administration of both selegiline (1 mg/kg i.p. daily for 3 weeks) and pargyline (10 mg/kg i.p. daily for 3 weeks) abolished cocaine-supported operant responses whereas long-term treatment with clorgyline (2 mg/kg i.p. daily for 3 weeks) did not. It should be noted that the doses of selegiline and pargyline used were probably adequate to inhibit both MAO-A and MAO-B, as shown by their reduction of dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindole acetic acid (5-HIAA) levels in frontal cortex, while clorgyline enhanced 5-HT but did not reduce 5-HIAA levels in frontal cortex. The authors proposed that the use of MAO-B inhibitors to curb cocaine reward should be further considered. In a pilot study in human subjects, 10 mg p.o. selegiline daily reduced cocaine consumption but in a subsequent larger study (300 subjects) transdermal selegiline did not significantly reduce consumption. An additional study with transdermal selegiline reduced cocaine-related scores of anger and tension as well as craving but also did not reduce subjective reported rewarding effects of a higher dose of cocaine compatible with binge use in humans (Elkashef et al., 2006; Harris et al., 2009). Clinical studies have not found evidence of abuse liability in humans (Yasar et al., 1996), and in addition it should be noted

that selegiline does not induce addictive behavior in monkeys (Winger et al., 1994).

Pharmacotherapy of PD Depression

There are a number of considerations relating to the pharmacotherapy of PD depression, including the stage of the disease, possible interactions with other medications (especially L-dopa, LD), control of the autonomic nervous system, and the disturbed normal balance between the monoamine systems of the brain. In addition, the possibility of cognitive deficits and PD dementia will confuse the understanding of the patient's affective state. In a meta-analysis of 11 controlled clinical trials for pharmacological treatments in PD depression between the years 2004 and 2014 (Sandoval-Rincon et al., 2015), rasagiline was found effective, but at the dose of 2 mg/day, which is higher than the usual dose of 1 mg daily for PD symptomatology.

For advice to clinicians on the ins and outs of treatment of depression with MAOIs, the reader is referred to recent reviews (Cohen and Sclar, 2012; Goldberg and Thase, 2013; Shulman et al., 2013).

NEUROPROTECTIVE ACTIONS OF MAOI

All MAOI possess inherent neuroprotective properties because of their inhibition of H₂O₂ and toxic aldehyde release following oxidative metabolism of amines, however individual inhibitors may possess an intrinsic neuroprotective action in addition. Selegiline was found by Knoll and co-workers to increase the natural life-span of laboratory rats, and subsequently was found to exert an anti-apoptotic effect in a variety of tissues and cells which was independent of MAO inhibition (Tatton and Chalmers-Redman, 1996). It is important to note that the anti-apoptotic properties of desmethylselegiline (the selegiline metabolite which is the active neuroprotective molecule) are superior to those of selegiline, and that R(-)-methamphetamine, the major metabolite of selegiline, antagonizes the neuroprotective property of selegiline and desmethylselegiline (Tatton and Chalmers-Redman, 1996). Subsequently, rasagiline was found to also possess neuroprotective properties both *in vivo* (Aluf et al., 2013) and *in vitro* (Finberg et al., 1998; Weinreb et al., 2011). Both these molecules increase BCl₂ and PKC-epsilon levels, enhance synthesis and release of BDNF and GDNF, and activate additional anti-apoptotic mechanisms (Jenner and Langston, 2011). It is a current unsolved mystery why these small molecules should exert these complex pro-survival effects. One series of studies produced evidence that selegiline binds to GAPDH and prevents the nuclear translocation of this enzyme (Carlile et al., 2000), however the tricyclic selegiline derivative CGP3466 (Omigapil), which does not inhibit MAO, binds GAPDH and prevents its nuclear translocation, possesses anti-apoptotic activity *in vitro* and *in vivo* (Waldmeier et al., 2000) but was not effective in clinical trials for PD and ALS. Rasagiline also prevents the pro-apoptotic nuclear translocation of GAPDH (Maruyama et al., 2001). The rasagiline metabolite 1(R)-aminoindan possesses anti-apoptotic activity (at higher concentrations than the parent molecule), and shows a similar

spectrum of biochemical mechanisms as described for rasagiline (Bar-Am et al., 2010), however the presence of the propargyl moiety seems to be an important factor in neuroprotection, since propargylamine itself also possesses anti-apoptotic activity, albeit at higher concentrations than are needed with rasagiline or selegiline (Weinreb et al., 2005).

Compound Molecules with MAO Inhibitory and Neuroprotective Properties

Ladostigil (Weinstock et al., 2000) is a compound molecule consisting of a molecule of rasagiline with the addition of a propylcarbamate moiety, which confers cholinesterase-inhibiting properties. The combination of these two moieties in the same molecule produced a drug with inhibitory properties on both enzymes *in vivo*, while it is ineffective *in vitro*. An additional fortuitous aspect of this molecule is that its MAO-inhibitory property is brain-selective, so that the likelihood of cheese effect is small. In rats, an oral dose of 75 μmoles/kg daily for 2 weeks inhibits brain cholinesterase by 40% and brain MAO-A and -B by 70% with no significant inhibition of intestinal or hepatic MAO (Weinstock et al., 2000), while higher doses can produce nearly complete inhibition of brain MAO in several species (Youdim et al., 2005).

The active metabolite of ladostigil responsible for the cholinesterase-inhibitory activity is R-MCPAI (6-(N-methyl carbamoyloxy)-1(R)-aminoindan hydrochloride), which is formed from ladostigil by CYP-2C19, while MAO inhibitory activity *in vivo* is due to the metabolite R-HPAI (6-hydroxy-N-propargyl-1(R)-aminoindan mesylate), since ladostigil itself does not inhibit MAO *in vitro*. Recently the self-limiting inhibitory effect of ladostigil on cholinesterases (maximal inhibition level is 50–55%) has been studied in mice, and found to be the result of rapid hydrolysis of the complex between R-MCPAI and the enzyme, not by limitation in formation of R-MCPAI (Moradov et al., 2015). A slow rate of conversion of ladostigil to R-HPAI in the intestine prevents significant inhibition of intestinal MAO, although following absorption of the parent molecule it is converted to R-HPAI in the brain but to a lesser extent in other tissues.

The drug was also found to possess a variety of neuroprotective and cognitive effects in animal models (Weinreb et al., 2012). Mechanistic studies showed that ladostigil binds to the VDAC mitochondrial complex, protecting against reduction in mitochondrial potential, and activates alpha-secretase leading to production of the non-amyloidogenic form of soluble APP by a MAP-Kinase dependent mechanism, in a similar way to rasagiline (Yogev-Falach et al., 2002). Ladostigil possesses anti-apoptotic properties against apoptosis induced by the naturally-occurring neurotoxin N-methyl(R)salsolinol and the peroxynitrite-generating molecule SIN-1 in SH-SY5Y cells (Maruyama et al., 2003). It also possesses anti-inflammatory properties as shown by its ability to reduce TNFα levels in mouse spleen and macrophages, following LPS stimulation (Moradov et al., 2015) and reduces the extent of gliosis and memory deficits following streptozotocin-induced lesions of the CNS in rats (Shoham et al., 2007). Ladostigil was tested

in old rhesus monkeys for cognitive behavioral effects, and was found to improve attention (Buccafusco et al., 2003). Administered to stressed pregnant rats it corrected the depressive behavior of the male offspring (Goelman et al., 2014). The drug is currently in clinical trial for Mild Cognitive Impairment (MCI).

Additional multi-target inhibitors of MAO have been developed with the aim of incorporating iron-chelating activity together with MAO inhibition (Wang et al., 2014). M30 and VAR10303 are both in development for clinical use in neurodegenerative diseases. These compounds are relatively selective inhibitors of brain as opposed to intestinal and liver MAO, with similar degree of inhibitory activity on MAO-A and MAO-B (Gal et al., 2005; Bar-Am et al., 2015). Unlike ladostigil, M30 (Zheng et al., 2005) has potent MAO inhibitory activity *in vitro*. The reason for the brain selectivity of these compounds has not yet been determined.

ANTIPARKINSONIAN FEATURES OF MAO-B INHIBITORS

Parkinson's disease (PD) is a neurodegenerative disorder primarily of the nigrostriatal DA-ergic pathway that affects the motor system and results in symptoms including uncontrollable tremor, muscle rigidity, slowness of movement (bradykinesia) and postural instability (Lang and Lozano, 1998a). It is estimated that it affects more than 4 million people worldwide (Schapira et al., 2005), significantly shortens life, and affects quality of life (Lang and Lozano, 1998a,b).

Much effort is being directed to development of new drugs with neuroprotective properties for the treatment of neurodegenerative diseases. Prior to initiating a new drug development, evidence of target engagement is desirable. In the case of putative neuroprotective therapies for PD this is not readily accomplished. There are few targets in the CNS that are associated with a possible pathogenic mechanism and are accessible to drug treatment, although as described above, MAOI have potential neuroprotective properties. Another problem is that existing assessment scales have a limited range and are particularly insensitive to detecting the modest change in movement that occurs in the early stages of the disease. Identification of a validated biomarker would be a significant advance in diagnosis and objective measurement of disease progression and drug efficacy. Recently Moloshnikov and coworkers (Molochnikov et al., 2012) reported a disease signature using blood RNA that detects idiopathic PD with a sensitivity of 90%. This may help to improve the selection of PD patients for clinical trials. The population of PD patients selected for inclusion in clinical trials is also critically important. Patients in early disease stages are frequently selected because they are likely to have a larger number of remaining neurons that can potentially be protected or rescued than patients in advanced stages of the disease (Fearnley and Lees, 1991). In addition, patients in early stages of the disease are generally still untreated which avoids the complication of confounding drug action. There is, however, a greater

possibility of inaccurate diagnosis in early disease stage and a higher risk of dropout if patients require treatment. Moreover, disease progression is slower in the early stages of the disease, possibly as a result of more efficient functional compensation (Rascol et al., 2011).

Symptoms of PD are improved with DA-replacement therapies such as DA receptor agonists and LD. Over time, however, the benefit of these drugs fluctuates and patients begin to experience loss of benefit with each dose of LD (wearing-off) and involuntary dyskinesia. In addition some parkinsonian symptoms including disturbances of gait and tremor may be resistant to DA-ergic therapy.

The way in which selective inhibition of MAO-A or MAO-B modifies DA release *in vivo* was studied in the rat by microdialysis. Initial studies were made by single dose administration using non-selective doses of the MAOI. The first study to employ MAO subtype-selective doses of clorgyline, selegiline, and rasagiline given chronically was carried out by Lamensdorf and colleagues (Lamensdorf et al., 1996). This study showed clearly that all three MAOI could increase striatal extracellular levels of DA when given over 3 weeks, although clorgyline caused the greatest elevation in DA levels. In a later study by the same group (Lamensdorf et al., 1999), a differential effect of selegiline was found to increase expression of the DA transporter (DAT), whereas rasagiline and clorgyline did not. In a follow-up study (Sader-Mazbar et al., 2013), the effect of rasagiline and clorgyline on LD-induced DA levels was studied in rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion of the substantia nigra. This study showed clearly that clorgyline again was the most effective MAOI in causing an increase in DA extracellular levels, although rasagiline also elevated DA levels, in a dose level which was selective for MAO-B inhibition. The superior effect of clorgyline in elevating striatal extracellular DA levels is thought to be the result of inhibition of the neuronal MAO, whereas the effectiveness of MAO-B inhibitors in enhancement of LD-induced DA output in the brain with a lesion of the DA-ergic neurons of substantia nigra is thought to be the result of inhibition of glial cell MAO-B (Carlo et al., 1996).

Inhibition of MAO-B may conserve the depleted synaptic levels of DA, and delay the need for treatment with LD in patients with early-stage PD. In patients with advanced-stage PD who experience fluctuations in response to LD, MAO-B inhibition potentiates and prolongs the effect of LD and permits use of a lower dose (Riederer and Laux, 2011).

Irreversible MAOI selective for type B of the enzyme are among the earliest drugs used in PD. They can be used with or without LD (Riederer and Laux, 2011). Both selegiline and rasagiline are beneficial in treating motor symptoms in PD as monotherapy as well as in combination with LD and a decarboxylase inhibitor. The main differences between the two drugs are related to their metabolism, interaction with cytochrome P450 enzymes and quantitative properties at the molecular biologic/genetic level. Rasagiline is more potent as shown in the daily dose necessary for a symptomatic effect: selegiline 5–10 mg daily and rasagiline 1 mg daily.

Selegiline

Selegiline is a useful treatment for PD symptoms both in monotherapy and as adjunct therapy to LD (Riederer et al., 2004). However, selegiline undergoes first-pass metabolism to R(–)amphetamine and R(–)methamphetamine, which have the potential to cause cardiovascular and CNS adverse effects (Gal et al., 2005). The contribution of these metabolites to selegiline's clinical symptomatic effects (Elsworth et al., 1982) and the possibility of adverse cardiovascular reactions (Churchyard et al., 1997), has often been discussed. R(–)amphetamine has about one-tenth the activity of S(+)-amphetamine on the sympathetic nervous system but the enantiomers are equivalent in antagonism of DA uptake in the striatum (Coyle and Snyder, 1969). One clinical study in which the effects of 10 mg of selegiline were compared with equivalent doses of R(–)-amphetamine and R(–)-methamphetamine concluded that only selegiline, and not its metabolites, possesses antiakinetic efficacy in Parkinsonian patients (Elsworth et al., 1982). Moreover, from documentation of side effects in the large clinical trials of selegiline there is no evidence for enhanced cardiovascular risk (Parkinson Study Group, 1989, 1993), as is true also when selegiline is compared to treatment based on LD and DA receptor agonists; however these drugs have never been studied in head-to-head comparison. Long term trials have shown that 30–40% of the daily LD dose can be reduced when the drug is combined with selegiline (Birkmayer et al., 1975; Myllyla et al., 1997). One interesting hypothesis about the good beneficial effect of MAO-B inhibitors is that PEA, which increases in brain after selegiline treatment (Reynolds et al., 1978), may have DA release-promoting activity and by this way contribute to the positive effects on motor features and behavior.

In 1989 Tetrud and Langston published a clinical study based on the discovery that selegiline blocked the development of MPTP-induced parkinsonism in laboratory animals, in which they showed that in 22 PD patients medicated with selegiline and 22 who received placebo the necessity to add LD (rescue drug) occurred in the placebo group after 312.1 days and in the selegiline group after 548.9 days (Tetrud and Langston, 1989). In the DATATOP (Deprenyl and Tocopherol Antioxidative Treatment of Parkinsonism) publications (Parkinson Study Group, 1989, 1993) after 12 months of monotherapy with selegiline or placebo (800 patients), 47% in the placebo group had commenced LD therapy while in the selegiline group only 26%. In addition it is important to mention that in the placebo group the median length of time before patients needed LD was 454 days and in the selegiline group 719 days. In selegiline-treated patients the need for addition of LD therapy was postponed by ~9 months.

Shoulson et al. (2002) published interesting data concerning long term follow up (7 years) of patients in the DATATOP group treated with LD and selegiline, as well as those who received placebo after 3–5 years treatment with selegiline. The conclusions revealed that the wearing-off phenomenon did not improve in the selegiline-treated patients but these patients had less on-off phenomenon or freezing of gait and better motor features with lower total UPDRS (Unified Parkinson's Disease Rating Scores) score. Activities of daily living (ADL), motor scores, LD dosages

and use of DA agonists were significantly reduced in the selegiline groups. On the other hand, patients in the placebo group had less dyskinesias than those on selegiline.

It is important to note that, in DATATOP and its extension study it was impossible to distinguish between potential disease-modifying (i.e., neuroprotective) and symptomatic benefits of treatment (Parkinson Study Group, 1996a,b, 1989, 1993; Shoulson et al., 2002). In another study (SELEDO), Przuntek et al. (1999) showed that the length of time in which PD patients treated with selegiline required an increase of 50% in LD dose was 4.9 years, while in placebo-treated patients it was 2.6 years. Myllyla et al. (1992, 1997) reported similar results.

The DATATOP results along with other earlier studies demonstrated a modest symptomatic benefit with selegiline with no significant difference between selegiline and placebo in the occurrence of cardiovascular and other serious adverse events. Long-term post-marketing data, however, have revealed that orthostatic hypotension and hallucinations are seen frequently in selegiline-treated patients, mainly in combination with LD (Perez-Lloret et al., 2013).

A trial in 782 PD patients by the Parkinson's Disease Research Group of the United Kingdom (Lees, 1995; Ben-Shlomo et al., 1998) concluded that the addition of selegiline to ongoing LD therapy provided no additional clinical benefit but was associated with increased motor complications and increased mortality. A follow-up study from the same group examined postural hypotension in a sub-group of patients and found that in 8 out of 22 patients postural hypotension was exacerbated when on selegiline, and this effect was abolished following withdrawal of the drug (Churchyard et al., 1999). When experts who did not participate in the study analyzed the results (Olanow et al., 1998) they found that the conclusions reported were not correct and in fact there was no increase in mortality with selegiline treatment whether administered alone or in combination with LD (Olanow et al., 1998). Considering selegiline treatment in randomized studies it is important to mention a 157-patient, randomized controlled Swedish study of selegiline long-term effects when used in early PD either as monotherapy or in combination with LD. This trial showed that at 7 years, selegiline-treated patients had slower disease progression than their counterparts as measured with UPDRS score. This outcome was observed in the selegiline monotherapy PD patients as well as in those treated in addition with LD (Palhagen et al., 2006), however also in this study, the symptomatic effect of selegiline precludes drawing conclusions about disease modification.

In general, summarizing most of the reports selegiline is well tolerated. Side effects/adverse effects like nausea, vomiting, sleeplessness, dry mouth, orthostatic hypotension and dyskinesias have all been observed in the range of 2–5% of PD patients (Parkinson Study Group, 1993; Reichmann et al., 2000) which is comparable to placebo. Other side effects like headaches, palpitations, dyspneas, confusion, edema, micturition dysfunction, loss of appetite and anxiety have an incidence below 2% (Reichmann et al., 2000).

Waters et al (Waters et al., 2004) examined the effect of Zydis selegiline in a 3 month, randomized, placebo-controlled study in PD patients experiencing motor fluctuations in response to LD

and found that it reduced off time by 2.2 h (compared with 0.6 h in the placebo group), without any increase in drug-related adverse events.

Rasagiline

Rasagiline is a potent, selective, irreversible inhibitor of MAO-B and in contrast to selegiline has no amphetamine-like metabolites (Finberg et al., 1999). Given in disease models relevant to PD (Bar-Am et al., 2010), rasagiline showed good antiparkinsonian and motor restoration activity as well as neuroprotection properties, and its major metabolite 1-aminoindan is also neuroprotective (see above section on Neuroprotection).

In a 10 week, randomized placebo-controlled pilot (phase 2) trial of rasagiline in patients with early, untreated PD, a dose of up to 4 mg/day was well tolerated. There were no cases of hypertension, bradycardia or other cardiovascular adverse experiences (Marek et al., 1997). One of the early randomized clinical studies comparing rasagiline vs. placebo for advanced PD patients was published in 2000 (Rabey et al., 2000). In this study researchers planned to evaluate the safety, tolerability and clinical effect of rasagiline as adjunct therapy with LD, in a multicenter, double blind, placebo-controlled parallel group study (0.5, 1, and 2 mg/day) lasting 12 weeks, in 70 patients with PD (mean age 57.4 years, mean disease duration 5.7 years; 32 patients had motor fluctuations). A beneficial clinical effect was observed in fluctuating patients treated with rasagiline (all doses) and was expressed as a decrease in total UPDRS score (by 23% in rasagiline-, 8.5% in placebo-treated subjects). The anti-Parkinsonian effect of rasagiline was still evident 6 weeks after stopping treatment at all dose levels. The incidence of adverse effects with rasagiline was similar to those on placebo. Determination of platelet MAO activity (MAO-B) showed nearly complete MAO-B inhibition at all rasagiline dose levels. This study showed that rasagiline (up to 2 mg/day) is well-tolerated and has a beneficial clinical effect in fluctuating patients with PD when given together with chronic LD therapy.

In the TEMPO (Rasagiline mesylate in Early Monotherapy for Parkinson's disease Outpatients) study (Parkinson Study Group, 2002), 404 *de novo* (untreated) PD patients received either placebo ($n = 138$), rasagiline 1 mg per day ($n = 134$) or rasagiline 2 mg per day ($n = 132$) for 26 weeks. All the patients receiving rasagiline showed statistically significant improvement compared with placebo in the mean change from baseline of the Unified Parkinson's Disease Rating Scale (UPDRS part I-III) by 4.2 and 3.6 points, in 1 and 2 mg/day groups respectively. Patients on rasagiline showed also a significant and beneficial effect for quality of life as assessed by the Parkinson's Disease Quality of Life (PD-QUALIF) scale.

Considering the results of preclinical studies which suggested that rasagiline may modify the progression of PD (Maruyama et al., 2001; Akao et al., 2002), the TEMPO study was extended into a delayed start trial comparing the effects of early and later initiation of rasagiline on disease progression (Parkinson Study Group, 2004). Three hundred seventy-one subjects from the TEMPO study were included in the 1-year efficacy analysis. Patients who had received rasagiline 1 or 2 mg/day for 6 months received the same dose for a further 6 months, while those

who had been treated with placebo for 6 months were given rasagiline 2 mg/day for a further 6 months. This design was adopted in order to compensate for the symptomatic effect of rasagiline, which prevented concluding that the drug had a disease-modifying action when rasagiline-treated patients were compared with those treated with placebo. In this delayed-start trial, it was possible to compare patients who had received rasagiline 2 mg/day for 12 months with those who had received this dose for only 6 months, but all were receiving the drug at the time of neurological assessment at 12 months from start. The result of this trial was that patients who had received rasagiline 2 mg/day for 12 months had a 2.29-unit smaller increase in UPDRS score than those who were treated with rasagiline 2 mg/day for 6 months ($P = 0.01$). This delayed-start analysis suggested that rasagiline could have a disease-modifying activity.

The ability of rasagiline to improve LD response in more advanced PD patients with motor fluctuations was studied in LARGO (Lasting effect in Adjunct therapy with Rasagiline Given Once daily) (Rascol et al., 2005). In this study 231 individuals, received rasagiline (1 mg daily), 229 received placebo, and 227 received entacapone (200 mg daily). All were treated with LD and a decarboxylase inhibitor. The primary outcome was change in total daily off-time. Other measures included the (CGI) score and the UPDRS scores. Both rasagiline and entacapone reduced mean daily off-time (-1.18 h rasagiline and -1.2 h entacapone vs. placebo -0.4). A significant improvement in clinical global improvement (CGI) scores, and activities of daily living during off-time and motor function during on time was seen with both rasagiline and entacapone. Adverse effects were similar in all groups.

To date the majority of PD clinical studies conducted in patients with motor fluctuations have reported the duration of OFF time during the day with limited evaluation of the severity of PD symptoms during ON time. The irreversible nature of the binding of rasagiline to the essential cofactor of the active site of the MAO-B enzyme means that the duration of its therapeutic action is independent of the drug's half-life and is instead determined by the regeneration rate of MAO-B (Thebault et al., 2004). The Largo study included a subgroup of patients, "UPDRS motor OFF substudy," for which there was a separate informed consent form. Patients included in this sub-study, were receiving optimum LD + decarboxylase inhibitor therapy, were stable for at least 14 days before study start, and experienced motor fluctuations in which they were in OFF state for at least 1 h every day not including morning akinesia. Additional antiparkinsonian therapy was accepted, with the exception of selegiline, tolcapone and previous treatment with entacapone. At the start of the 18 weeks study patients were randomly assigned to receive placebo, rasagiline 1 mg or entacapone 200 mg in addition to LD and decarboxylase inhibitor. The LD dose could be reduced during the first 6 weeks if dyskinesia worsened. Thereafter the LD dose remained the same for the final 12 weeks of the study (Stocchi and Rabey, 2011). Treatment with rasagiline produced a significant improvement over placebo of 5.64 units in UPDRS motor OFF score ($P = 0.013$ vs. placebo). By contrast the effect of adjunct entacapone was not significant ($P = 0.14$ vs. placebo). Retrospective analysis using the Bonferroni correction

of UPDRS motor subdomains further revealed that rasagiline but not entacapone, significantly improved bradykinesia ($p < 0.001$) and showed a trend for improvement in facial expression, speech and axial impairment during OFF time.

In Olanow et al. (2009) conducted a double blind study, with early and delayed start rasagiline (Attenuation of Disease Progression with Rasagiline Given Once-daily, ADAGIO), in order to establish more substantially whether this drug has disease-modifying effect in PD. A total of 1091 untreated PD patients participated in the start of the study; 273 early start rasagiline 1 mg/day, 270 delayed start rasagiline 1 mg/day, 273 early start rasagiline 2 mg/day, and 275 delayed start rasagiline 2 mg/daily. The early start group received the drug for 72 weeks, the delayed start group received placebo for 36 weeks followed by rasagiline for a further 36 weeks.

The putative result required for disease-modification is shown diagrammatically in Figure 1

Three endpoints were required to be met in order to permit a positive result: (a) increased slope of the decline in UPDRS in the placebo group with respect to the rasagiline group in the first phase of the study (i.e., week 12–36); (b) a difference between the early- and delayed-start groups in the change in UPDRS score between weeks 12 and 72, and (c) similarity of the slopes of change in UPDRS score with time in the period between weeks 48 and 72. Finite values of UPDRS were fixed for the three endpoints. The conclusions of the study were: “Early treatment with rasagiline at a dose of 1 mg per day provided benefits that were consistent with a possible disease-modifying effect, but early treatment with rasagiline at a dose of 2 mg per day did not. Because the two doses were associated with different outcomes, the study results must be interpreted with caution” (Churchyard et al., 1999).

Following publication of the Adagio results, there has been much discussion about the significance of the data. Rascol et al. published in 2011 a secondary and *post-hoc* comment on

the ADAGIO study (Rascol et al., 2011). In addition to the criterion of UPDRS score, they included changes in non-motor experiences of daily living (ADL), fatigue scales, the need for additional antiparkinsonian therapy, and (UPDRS) subscores, in the data assessment. In addition to the finding that rasagiline therapy delayed the need for additional antiparkinsonian drugs, and improved ADL scores in the 1 mg/daily early start group, they showed that the rate of deterioration in UPDRS scores correlated with baseline scores, patients with low baseline scores deteriorating slower than those with higher baseline scores. A difference in the distribution of low and high baseline UPDRS scores between the 1 and 2 mg daily groups could have contributed to the lack of difference between early and late start groups at the 2 mg dose level.

In 2014, Jankovic et al. (2014) published another *post-hoc* analysis from the ADAGIO study in which they examined the responses of patients to rasagiline 1 mg/day ($n = 288$) with those to placebo ($n = 588$) on key motor symptoms at 36 weeks. In the rasagiline group, significantly better tremor, bradykinesia, rigidity and postural-instability-gait difficulty scores were seen at week 36 by comparison with placebo. While the placebo group deteriorated from baseline by 2.6 points UPDRS at week 36, patients in the rasagiline group improved initially but then returned to baseline values at week 36. At week 72 patients who had received continuous monotherapy with rasagiline experienced a worsening of only 1.6 points. The conclusions of this analysis were that treatment with rasagiline maintained motor function at baseline values for at least a year with significant benefit observed in all key PD motor symptoms.

Safinamide

Safinamide, an orally active alpha-aminoamide derivative, is a novel reversible and highly selective MAO-B inhibitor which is efficacious as add-on therapy to DA agonists in early-stage PD (Stocchi et al., 2004, 2012) and as adjunct to LD in mid- to late-stage PD (Borgohain et al., 2014). In addition to MAO inhibition, the molecule possesses additional pharmacological properties, including state-dependent blockade of voltage-gated sodium and calcium channels, and inhibition of glutamate release in rat hippocampal synaptosomes (Caccia et al., 2006; Stocchi et al., 2006). These properties may be responsible for its demonstrated neuroprotective effect in laboratory animals.

The use of safinamide as adjunct therapy to LD was investigated in mid- to late-stage PD patients with motor fluctuations (Waters et al., 2004). In an initial 6 month trial, safinamide 50 mg ($n = 197$) or 100 mg ($n = 195$) daily was studied in relation to placebo ($n = 197$) and was found to significantly increase ON time without increasing dyskinesia. The study was then continued in the same patient population for an additional 18 months. In the final evaluation of the 2 year period, both safinamide groups had a significant increase in ON-time compared to placebo, which was maintained over the period between 24 and 102 weeks. A non-significant reduction in Dyskinesia Rating Score (DRS) was found for both safinamide groups, but in considering this result, 74% of the population had no to mild dyskinesia at baseline. In the case of the sub-group with more severe dyskinesia

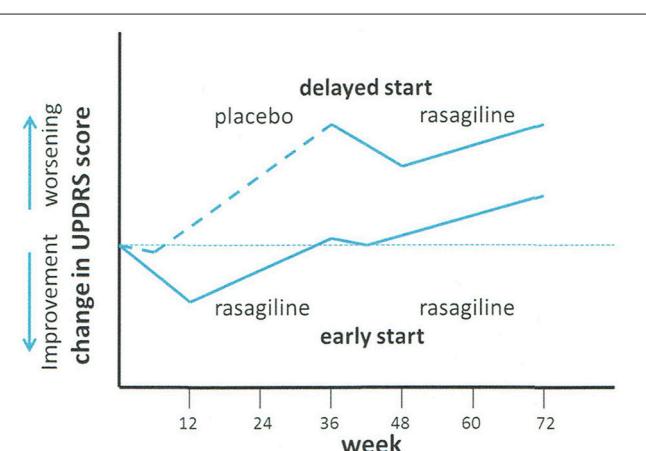


FIGURE 1 | Schematic of ADAGIO delayed start neuroprotection trial of rasagiline. Treatment with placebo (delayed start group) or rasagiline (early start group) was commenced at time 0. At week 36, the placebo group was changed to rasagiline, and the rasagiline group continued with rasagiline. Adapted from Olanow et al. (2009).

(DRS > 4, 36% of the population), safinamide caused a reduction in DRS in relation to placebo, which was significant ($P = 0.0317$) at the 100 mg daily dose level. Other benefits noted by the investigators in the safinamide group included improvements in ADL, depression, clinical status and quality of life (Borgohain et al., 2014). In an efficacy study reported by Schapira et al. (2013) patients received 100 mg, 200 mg safinamide or placebo added to LD, or DA agonists. In their study the conclusions were that the safinamide group did not attain the primary endpoint of increase in time required for additional drug therapy, however *post hoc* analysis showed that safinamide was effective in PD therapy in combination with DA agonists.

A number of research groups are aiming to develop other MAO-A and MAO-B reversible inhibitors. A series of chalcone derivatives with reversible MAO-A and MAO-B selective properties was recently described (Minders et al., 2015). The IC₅₀ of the most potent MAO-B inhibitor in this series was 0.067 μ M, by comparison with 0.098 μ M for safinamide. Further data on *in vivo* metabolism and efficacy will be required to see whether this compound has therapeutic potential.

REFERENCES

- Akao, Y., Maruyama, W., Yi, H., Shamoto-Nagai, M., Youdim, M. B., and Naoi, M. (2002). An anti-Parkinson's disease drug, N-propargyl-1(R)-aminoindan (rasagiline), enhances expression of anti-apoptotic bcl-2 in human dopaminergic SH-SY5Y cells. *Neurosci. Lett.* 326, 105–108. doi: 10.1016/S0304-3940(02)00332-4
- Akhondzadeh, S., Tavakolian, R., Davari-Ashtiani, R., Arabgol, F., and Amini, H. (2003). Selegiline in the treatment of attention deficit hyperactivity disorder in children: a double blind and randomized trial. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 841–845. doi: 10.1016/S0278-5846(03)00117-9
- Alia-Klein, N., Goldstein, R. Z., Kriplani, A., Logan, J., Tomasi, D., Williams, B., et al. (2008). Brain monoamine oxidase A activity predicts trait aggression. *J. Neurosci.* 28, 5099–5104. doi: 10.1523/JNEUROSCI.0925-08.2008
- Aluf, Y., Vaya, J., Khatib, S., and Finberg, J. P. (2011). Alterations in striatal oxidative stress level produced by pharmacological manipulation of dopamine as shown by a novel synthetic marker molecule. *Neuropharmacology* 61, 87–94. doi: 10.1016/j.neuropharm.2011.03.006
- Aluf, Y., Vaya, J., Khatib, S., Loboda, Y., and Finberg, J. P. (2013). Selective inhibition of monoamine oxidase A or B reduces striatal oxidative stress in rats with partial depletion of the nigro-striatal dopaminergic pathway. *Neuropharmacology* 65, 48–57. doi: 10.1016/j.neuropharm.2012.08.023
- Arnett, C. D., Fowler, J. S., MacGregor, R. R., Schlyer, D. J., Wolf, A. P., Langstrom, B., et al. (1987). Turnover of brain monoamine oxidase measured *in vivo* by positron emission tomography using L-[11C]depronyl. *J. Neurochem.* 49, 522–527. doi: 10.1111/j.1471-4159.1987.tb02895.x
- Azzaro, A. J., Vandenberg, C. M., Blob, L. F., Kemper, E. M., Sharoky, M., Oren, D. A., et al. (2006). Tyramine pressor sensitivity during treatment with the selegiline transdermal system 6 mg/24 h in healthy subjects. *J. Clin. Pharmacol.* 46, 933–944. doi: 10.1177/0091270006289852
- Azzaro, A. J., Ziemiak, J., Kemper, E., Campbell, B. J., and VanDenBerg, C. (2007). Pharmacokinetics and absolute bioavailability of selegiline following treatment of healthy subjects with the selegiline transdermal system (6 mg/24 h): a comparison with oral selegiline capsules. *J. Clin. Pharmacol.* 47, 1256–1267. doi: 10.1177/0091270007304779
- Badinter, F., Amit, T., Bar-Am, O., Youdim, M. B., and Weinreb, O. (2015). Beneficial behavioral, neurochemical and molecular effects of 1-(R)-aminoindan in aged mice. *Neuropharmacology* 99, 264–272. doi: 10.1016/j.neuropharm.2015.05.041
- Baker, G. B., Wong, J. T., Yeung, J. M., and Coutts, R. T. (1991). Effects of the antidepressant phenelzine on brain levels of gamma-aminobutyric acid (GABA). *J. Affect. Disord.* 21, 207–211. doi: 10.1016/0165-0327(91)90041-P
- Bar-Am, O., Amit, T., Kupershmidt, L., Aluf, Y., Mechlovich, D., Kabha, H., et al. (2015). Neuroprotective and neurorestorative activities of a novel iron chelator-brain selective monoamine oxidase-A/monoamine oxidase-B inhibitor in animal models of Parkinson's disease and aging. *Neurobiol. Aging* 36, 1529–1542. doi: 10.1016/j.neurobiolaging.2014.10.026
- Bar-Am, O., Weinreb, O., Amit, T., and Youdim, M. B. (2010). The neuroprotective mechanism of 1-(R)-aminoindan, the major metabolite of the anti-parkinsonian drug rasagiline. *J. Neurochem.* 112, 1131–1137. doi: 10.1111/j.1471-4159.2009.06542.x
- Ben-Shlomo, Y., Churchyard, A., Head, J., Hurwitz, B., Overstall, P., Ockelford, J., et al. (1998). Investigation by Parkinson's disease research group of United Kingdom into excess mortality seen with combined levodopa and selegiline treatment in patients with early, mild Parkinson's disease: further results of randomised trial and confidential inquiry. *BMJ* 316:1191–1196. doi: 10.1136/bmj.316.7139.1191
- Bieck, P. R., and Antonin, K. H. (1988). Oral tyramine pressor test and the safety of monoamine oxidase inhibitor drugs: comparison of brofaromine and tranylcypromine in healthy subjects. *J. Clin. Psychopharmacol.* 8, 237–245. doi: 10.1097/00004714-198808000-00002
- Bieck, P. R., and Antonin, K. H. (1994). "Tyramine potentiation during treatment with MAOIs," in *Clinical Advances in Monoamine Oxidase Inhibitor Therapies*, ed S. H. Kennedy (Washington, DC: American Psychiatric Press), 83–110.
- Binda, C., Hubalek, F., Li, M., Herzig, Y., Sterling, J., Edmondson, D. E., et al. (2005). Binding of rasagiline-related inhibitors to human monoamine oxidases: a kinetic and crystallographic analysis. *J. Med. Chem.* 48, 8148–8154. doi: 10.1021/jm0506266
- Binda, C., Newton-Vinson, P., Hubalek, F., Edmondson, D. E., and Mattevi, A. (2002). Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders. *Nat. Struct. Biol.* 9, 22–26. doi: 10.1038/nsb732
- Binda, C., Wang, J., Pisani, L., Caccia, C., Carotti, A., Salvati, P., et al. (2007). Structures of human monoamine oxidase B complexes with selective noncovalent inhibitors: safinamide and coumarin analogs. *J. Med. Chem.* 50, 5848–5852. doi: 10.1021/jm070677y
- Birkmayer, W., Riederer, P., Youdim, M. B., and Linauer, W. (1975). The potentiation of the anti akinetic effect after L-dopa treatment by an

CONCLUSIONS

Today there is a broad spectrum of therapeutic possibilities for the utilization of MAO-A and -B inhibitors, for the management of PD, and also for the treatment of depression. Novel routes of administration, as well as pro-drugs which are converted to active inhibitors by brain enzymes, are promising directions for development of MAOI with selective action in the brain in order to avoid cheese effect. New drug developments which combine different types of activity in the same molecule, and can possibly be effective in more than one disease condition, may be useful in treatment of both neuropsychiatric and neurological disorders, and mechanism-based drug combinations may improve efficacy in PD and other diseases

AUTHOR CONTRIBUTIONS

JF: Initiated this review, contributed introductory section, mechanisms and section on psychiatric uses, reviewed and submitted article; JR: Contributed section on Parkinson's Disease.

- inhibitor of MAO-B, Deprenil. *J. Neural. Transm.* 36, 303–326. doi: 10.1007/BF01253131
- Blob, L. F., Sharoky, M., Campbell, B. J., Kemper, E. M., Gilmor, M. G., VanDenberg, C. M., et al. (2007). Effects of a tyramine-enriched meal on blood pressure response in healthy male volunteers treated with selegiline transdermal system 6 mg/24 hour. *CNS Spectr.* 12, 25–34. doi: 10.1017/S1092852900020496
- Borgohain, R., Szasz, J., Stanzione, P., Meshram, C., Bhatt, M. H., Chirilaine, D., et al. (2014). Two-year, randomized, controlled study of safinamide as add-on to levodopa in mid to late Parkinson's disease. *Mov. Disord.* 29, 1273–1280. doi: 10.1002/mds.25961
- Buccafusco, J. J., Terry, A. V. Jr., Goren, T., and Blaugrun, E. (2003). Potential cognitive actions of (n-propargyl-(3r)-aminoindan-5-yl)-ethyl, methyl carbamate (tv3326), a novel neuroprotective agent, as assessed in old rhesus monkeys in their performance of versions of a delayed matching task. *Neuroscience* 119, 669–678. doi: 10.1016/S0306-4522(02)00937-5
- Caccia, C., Maj, R., Calabresi, M., Maestroni, S., Faravelli, L., Curatolo, L., et al. (2006). Safinamide: from molecular targets to a new anti-Parkinson drug. *Neurology* 67, S18–23. doi: 10.1212/WNL.67.7_suppl_2.S18
- Carlile, G. W., Chalmers-Redman, R. M., Tatton, N. A., Pong, A., Borden, K. E., and Tatton, W. G. (2000). Reduced apoptosis after nerve growth factor and serum withdrawal: conversion of tetrameric glyceraldehyde-3-phosphate dehydrogenase to a dimer. *Mol. Pharmacol.* 57:2–12.
- Carlo, P., Del Rio, M., Violani, E., Sciaiba, L., and Picotti, G. B. (1996). Influence of culture conditions on monoamine oxidase A and B activity in rat astrocytes. *Cell Biochem. Funct.* 14, 19–25. doi: 10.1002/cbf.645
- Churchyard, A., Mathias, C. J., Boonkongchuen, P., and Lees, A. J. (1997). Autonomic effects of selegiline: possible cardiovascular toxicity in Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* 63, 228–234. doi: 10.1136/jnnp.63.2.228
- Churchyard, A., Mathias, C. J., and Lees, A. J. (1999). Selegiline-induced postural hypotension in Parkinson's disease: a longitudinal study on the effects of drug withdrawal. *Mov. Disord.* 14, 246–251. doi: 10.1002/1531-8257(199903)14:2<246::AID-MDS1008>3.0.CO;2-P
- Clarke, A., Brewer, F., Johnson, E. S., Mallard, N., Hartig, F., Taylor, S., et al. (2003b). A new formulation of selegiline: improved bioavailability and selectivity for MAO-B inhibition. *J. Neural. Transm. (Vienna)* 110, 1241–1255. doi: 10.1007/s00702-003-0036-4
- Clarke, A., Johnson, E. S., Mallard, N., Corn, T. H., Johnston, A., Boyce, M., et al. (2003a). A new low-dose formulation of selegiline: clinical efficacy, patient preference and selectivity for MAO-B inhibition. *J. Neural. Transm. (Vienna)* 110, 1257–1271. doi: 10.1007/s00702-003-0042-6
- Cohen, L. J., and Sclar, D. A. (2012). Issues in adherence to treatment with monoamine oxidase inhibitors and the rate of treatment failure. *J. Clin. Psychiatry* 73(Suppl. 1), 31–36. doi: 10.4088/JCP.11096su1c.05
- Coyle, J. T., and Snyder, S. H. (1969). Antiparkinsonian drugs: inhibition of dopamine uptake in the corpus striatum as a possible mechanism of action. *Science* 166, 899–901. doi: 10.1126/science.166.3907.899
- De Colibus, L., Li, M., Binda, C., Lustig, A., Edmondson, D. E., and Mattevi, A. (2005). Three-dimensional structure of human monoamine oxidase A (MAO A): relation to the structures of rat MAO A and human MAO B. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12684–12689. doi: 10.1073/pnas.0505975102
- Denney, R. M., and Denney, C. B. (1985). An update on the identity crisis of monoamine oxidase: new and old evidence for the independence of MAO A and B. *Pharmacol. Ther.* 30, 227–258. doi: 10.1016/0163-7258(85)90050-6
- Elkashef, A., Fudala, P. J., Gorgon, L., Li, S. H., Kahn, R., Chiang, N., et al. (2006). Double-blind, placebo-controlled trial of selegiline transdermal system (STS) for the treatment of cocaine dependence. *Drug. Alcohol. Depend.* 85, 191–197. doi: 10.1016/j.drugalcdep.2006.04.010
- Elsworth, J. D., Sandler, M., Lees, A. J., Ward, C., and Stern, G. M. (1982). The contribution of amphetamine metabolites of (−)-depreranyl to its antiparkinsonian properties. *J. Neural. Transm.* 54, 105–110. doi: 10.1007/BF01249283
- Fearnley, J. M., and Lees, A. J. (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain* 114(Pt 5), 2283–2301. doi: 10.1093/brain/114.5.2283
- Finberg, J. P. (2014). Update on the pharmacology of selective inhibitors of MAO A and MAO-B: focus on modulation of CNS monoamine neurotransmitter release. *Pharmacol. Ther.* 143, 133–152. doi: 10.1016/j.pharmthera.2014.02.010
- Finberg, J. P., and Gillman, P. K. (2011). "Selective inhibitors of monoamine oxidase type b and the cheese effect," in *International Review of Neurobiology*, eds M. B. Youdim and P. Riederer, (Burlington, VT: Academic Press), 169–190.
- Finberg, J. P., Lamensdorf, I., Weinstock, M., Schwartz, M., and Youdim, M. B. (1999). Pharmacology of rasagiline (N-propargyl-1R-aminoindan). *Adv. Neurol.* 80, 495–499.
- Finberg, J. P., Takeshima, T., Johnston, J. M., and Commissiong, J. W. (1998). Increased survival of dopaminergic neurons by rasagiline, a monoamine oxidase B inhibitor. *Neuroreport* 9, 703–707. doi: 10.1097/00001756-199803090-00026
- Finberg, J. P., and Tenne, M. (1982). Relationship between tyramine potentiation and selective inhibition of monoamine oxidase types A and B in the rat vas deferens. *Br. J. Pharmacol.* 77, 13–21. doi: 10.1111/j.1476-5381.1982.tb09263.x
- Finberg, J. P., and Youdim, M. B. (2002). Pharmacological properties of the anti-Parkinson drug rasagiline; modification of endogenous brain amines, reserpine reversal, serotonergic and dopaminergic behaviours. *Neuropharmacology* 43, 1110–1118. doi: 10.1016/S0028-3908(02)00216-2
- Freedman, N. M., Mishani, E., Krausz, Y., Weininger, J., Lester, H., Blaugrund, E., et al. (2005). *In vivo* measurement of brain monoamine oxidase B occupancy by rasagiline, using (11)C-l-depreranyl and PET. *J. Nucl. Med.* 46, 1618–1624.
- Gahr, M., Schonfeldt-Lecuona, C., Kolle, M. A., and Freudenmann, R. W. (2013). Withdrawal and discontinuation phenomena associated with tranylcypromine: a systematic review. *Pharmacopsychiatry* 46:123–129. doi: 10.1055/s-0032-1333265
- Gal, S., Zheng, H., Fridkin, M., and Youdim, M. B. (2005). Novel multifunctional neuroprotective iron chelator-monoamine oxidase inhibitor drugs for neurodegenerative diseases. *In vivo selective brain monoamine oxidase inhibition and prevention of MPTP-induced striatal dopamine depletion*. *J. Neurochem.* 95, 79–88. doi: 10.1111/j.1471-4159.2005.03341.x
- Gillman, P. K. (2006). A review of serotonin toxicity data: implications for the mechanisms of antidepressant drug action. *Biol. Psychiatry* 59, 1046–1051. doi: 10.1016/j.biopsych.2005.11.016
- Gillman, P. K. (2011). Advances pertaining to the pharmacology and interactions of irreversible nonselective monoamine oxidase inhibitors. *J. Clin. Psychopharmacol.* 31, 66–74. doi: 10.1097/JCP.0b013e31820469ea
- Goelman, G., Ilinca, R., Zohar, I., and Weinstock, M. (2014). Functional connectivity in prenatally stressed rats with and without maternal treatment with ladostigil, a brain-selective monoamine oxidase inhibitor. *Eur. J. Neurosci.* 40, 2734–2743. doi: 10.1111/ejn.12621
- Goldberg, J. F., and Thase, M. E. (2013). Monoamine oxidase inhibitors revisited: what you should know. *J. Clin. Psychiatry* 74, 189–191. doi: 10.4088/JCP.12ac08299
- Goren, T., Adar, L., Sasson, N., and Weiss, Y. M. (2010). Clinical pharmacology tyramine challenge study to determine the selectivity of the monoamine oxidase type B (MAO-B) inhibitor rasagiline. *J. Clin. Pharmacol.* 50, 1420–1428. doi: 10.1177/0091270010369674
- Harris, D. S., Everhart, T., Jacob, P. IIrd, Lin, E., Mendelson, J. E., and Jones, R. T. (2009). A phase 1 trial of pharmacologic interactions between transdermal selegiline and a 4-hour cocaine infusion. *BMC Clin. Pharmacol.* 9:13. doi: 10.1186/1472-6904-9-13
- Harvey, B. H., Duvenhage, I., Viljoen, F., Scheepers, N., Malan, S. F., Wegener, G., et al. (2010). Role of monoamine oxidase, nitric oxide synthase and regional brain monoamines in the antidepressant-like effects of methylene blue and selected structural analogues. *Biochem. Pharmacol.* 80, 1580–1591. doi: 10.1016/j.bcp.2010.07.037
- Heijnen, W. T., De Fruyt, J., Wierdsma, A. I., Sienaert, P., and Birkenhager, T. K. (2015). Efficacy of tranylcypromine in bipolar depression: a systematic review. *J. Clin. Psychopharmacol.* 35, 700–705. doi: 10.1097/JCP.0000000000000409
- Hill, M. N., Ho, W. S., Hillard, C. J., and Gorzalka, B. B. (2008). Differential effects of the antidepressants tranylcypromine and fluoxetine on limbic cannabinoid receptor binding and endocannabinoid contents. *J. Neural. Transm.* 115, 1673–1679. doi: 10.1007/s00702-008-0131-7
- Ho, M. C., Cherng, C. G., Tsai, Y. P., Chiang, C. Y., Chuang, J. Y., Kao, S. F., et al. (2009). Chronic treatment with monoamine oxidase-B inhibitors decreases cocaine reward in mice. *Psychopharmacology (Berl.)* 205, 141–149. doi: 10.1007/s00213-009-1524-5

- Hung, C. F., Lung, F. W., Hung, T. H., Chong, M. Y., Wu, C. K., Wen, J. K., et al. (2012). Monoamine oxidase A gene polymorphism and suicide, an association study and meta-analysis. *J. Affect. Disord.* 136, 643–649. doi: 10.1016/j.jad.2011.10.013
- Jankovic, J., Berkovich, E., Eyal, E., and Tolosa, E. (2014). Symptomatic efficacy of rasagiline monotherapy in early Parkinson's disease: *post-hoc* analyses from the ADAGIO trial. *Parkinsonism Relat. Disord.* 20, 640–643. doi: 10.1016/j.parkreldis.2014.02.024
- Jenner, P. (2003). Oxidative stress in Parkinson's disease. *Ann. Neurol.* 53(Suppl. 3), S26–36 discussion S36–28. doi: 10.1002/ana.10483
- Jenner, P., and Langston, J. W. (2011). Explaining ADAGIO: a critical review of the biological basis for the clinical effects of rasagiline. *Mov. Disord.* 26, 2316–2323. doi: 10.1002/mds.23926
- Kolla, N. J., Matthews, B., Wilson, A. A., Houle, S., Bagby, R. M., Links, P., et al. (2015). Lower monoamine oxidase-a total distribution volume in impulsive and violent male offenders with antisocial personality disorder and high psychopathic traits: an [(11)C] harmine positron emission tomography study. *Neuropsychopharmacology* 40, 2596–2603. doi: 10.1038/npp.2015.106
- Korchounov, A., Winter, Y., and Rossy, W. (2012). Combined beneficial effect of rasagiline on motor function and depression in *de novo* PD. *Clin. Neuropharmacol.* 35, 121–124. doi: 10.1097/WNF.0b013e31823b1da8
- Lader, M. H., Sakalis, G., and Tansella, M. (1970). Interactions between sympathomimetic amines and a new monoamine oxidase inhibitor. *Psychopharmacologia* 18:118–123. doi: 10.1007/BF00402391
- Laine, K., Anttila, M., Huupponen, R., Maki-Ikola, O., and Heinonen, E. (2000). Multiple-dose pharmacokinetics of selegiline and desmethylselegiline suggest saturable tissue binding. *Clin. Neuropharmacol.* 23, 22–27. doi: 10.1097/00002826-200001000-00005
- Lamensdorf, I., Porat, S., Simantov, R., and Finberg, J. P. (1999). Effect of low-dose treatment with selegiline on dopamine transporter (DAT) expression and amphetamine-induced dopamine release *in vivo*. *Br. J. Pharmacol.* 126, 997–1002. doi: 10.1038/sj.bjp.0702389
- Lamensdorf, I., Youdim, M. B., and Finberg, J. P. (1996). Effect of long-term treatment with selective monoamine oxidase A and B inhibitors on dopamine release from rat striatum *in vivo*. *J. Neurochem.* 67, 1532–1539. doi: 10.1046/j.1471-4159.1996.67041532.x
- Lang, A. E., and Lozano, A. M. (1998a). Parkinson's disease. First of two parts. *N. Engl. J. Med.* 339, 1044–1053. doi: 10.1056/NEJM199810083391506
- Lang, A. E., and Lozano, A. M. (1998b). Parkinson's disease. Second of two parts. *N. Engl. J. Med.* 339, 1130–1143. doi: 10.1056/NEJM199810153391607
- Lee, M. G., Wynder, C., Schmidt, D. M., McCafferty, D. G., and Shiekhatter, R. (2006). Histone H3 lysine 4 demethylation is a target of nonselective antidepressive medicataions. *Chem. Biol.* 13, 563–567. doi: 10.1016/j.chembiol.2006.05.004
- Lees, A. J. (1995). Comparison of therapeutic effects and mortality data of levodopa and levodopa combined with selegiline in patients with early, mild Parkinson's disease. *Parkinson's Disease research group of the United Kingdom. BMJ* 311:1602–1607. doi: 10.1136/bmj.311.7020.1602
- Lehmann, H. E., and Kline, N. S. (1983). "Clinical discoveries with antidepressant drugs," in *Discoveries in Pharmacology*, eds M. J. Parnham and J. Bruunvelds (Amsterdam: Elsevier), 209–221.
- Levitt, P., Pintar, J. E., and Breakefield, X. O. (1982). Immunocytochemical demonstration of monoamine oxidase B in brain astrocytes and serotonergic neurons. *Proc. Natl. Acad. Sci. U.S.A.* 79, 6385–6389. doi: 10.1073/pnas.79.20.6385
- Lotufo-Neto, F., Trivedi, M., and Thase, M. E. (1999). Meta-analysis of the reversible inhibitors of monoamine oxidase type A moclobemide and brofaromine for the treatment of depression. *Neuropsychopharmacology* 20, 226–247. doi: 10.1016/S0893-133X(98)00075-X
- Magyar, K. (2011). The pharmacology of selegiline. *Int. Rev. Neurobiol.* 100, 65–84. doi: 10.1016/B978-0-12-386467-3.00004-2
- Mann, J. J., Aarons, S. F., Wilner, P. J., Keilp, J. G., Sweeney, J. A., Pearlstein, T., et al. (1989). A controlled study of the antidepressant efficacy and side effects of (−)-deprenyl. A selective monoamine oxidase inhibitor. *Arch. Gen. Psychiatry* 46, 45–50. doi: 10.1001/archpsyc.1989.01810010047007
- Marek, K. L., Friedman, J., Hauser, R., Juncos, J., LeWitt, P., Mijawaki, E., et al. (1997). Phase II evaluation of rasagiline mesylate (TVP-1012), a novel anti-parkinsonian drug, in parkinsonian patients not receiving levodopa/carbidopa. *Mov. Disord.* 12, 838.
- Maruyama, W., Akao, Y., Youdim, M. B., Davis, B. A., and Naoi, M. (2001). Transfection-enforced Bcl-2 overexpression and an anti-Parkinson drug, rasagiline, prevent nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase induced by an endogenous dopaminergic neurotoxin, N-methyl(R)salsolinol. *J. Neurochem.* 78, 727–735. doi: 10.1046/j.1471-4159.2001.00448.x
- Maruyama, W., Weinstock, M., Youdim, M. B., Nagai, M., and Naoi, M. (2003). Anti-apoptotic action of anti-Alzheimer drug, TV3326 [(N-propargyl)-(3R)-aminoindan-5-yl]-ethyl methyl carbamate, a novel cholinesterase-monoamine oxidase inhibitor. *Neurosci. Lett.* 341, 233–236. doi: 10.1016/S0304-3940(03)00211-8
- Mawhinney, M., Cole, D., and Azzaro, A. J. (2003). Daily transdermal administration of selegiline to guinea-pigs preferentially inhibits monoamine oxidase activity in brain when compared with intestinal and hepatic tissues. *J. Pharm. Pharmacol.* 55, 27–34. doi: 10.1111/j.2042-7158.2003.tb02430.x
- Minders, C., Petzer, J. P., Petzer, A., and Lourens, A. C. (2015). Monoamine oxidase inhibitory activities of heterocyclic chalcones. *Bioorg. Med. Chem. Lett.* 25, 5270–5276. doi: 10.1016/j.bmcl.2015.09.049
- Mohammadi, M. R., Ghanizadeh, A., Alaghband-Rad, J., Tehranidoost, M., Mesgarpour, B., and Soori, H. (2004). Selegiline in comparison with methylphenidate in attention deficit hyperactivity disorder children and adolescents in a double-blind, randomized clinical trial. *J. Child Adolesc. Psychopharmacol.* 14, 418–425. doi: 10.1089/cap.2004.14.418
- Molochnikov, L., Rabey, J. M., Dobronevsky, E., Bonucelli, U., Ceravolo, R., Frosini, D., et al. (2012). A molecular signature in blood identifies early Parkinson's disease. *Mol. Neurodegener.* 7:26. doi: 10.1186/1750-1326-7-26
- Moradov, D., Finkin-Groner, E., Bejar, C., Sunita, P., Schorer-Apelbaum, D., Barasch, D., et al. (2015). Dose-limiting inhibition of acetylcholinesterase by ladostigil results from the rapid formation and fast hydrolysis of the drug-enzyme complex formed by its major metabolite, R-MCPAI. *Biochem. Pharmacol.* 94, 164–172. doi: 10.1016/j.bcp.2015.01.017
- Myllyla, V. V., Sotaniemi, K. A., Hakulinen, P., Maki-Ikola, O., and Heinonen, E. H. (1997). Selegiline as the primary treatment of Parkinson's disease—a long-term double-blind study. *Acta Neurol. Scand.* 95, 211–218. doi: 10.1111/j.1600-0404.1997.tb00101.x
- Myllyla, V. V., Sotaniemi, K. A., Vuorinen, J. A., and Heinonen, E. H. (1992). Selegiline as initial treatment in *de novo* parkinsonian patients. *Neurology* 42, 339–343. doi: 10.1212/WNL.42.2.339
- Naylor, G. J., Smith, A. H., and Connelly, P. (1987). A controlled trial of methylene blue in severe depressive illness. *Biol. Psychiatry* 22, 657–659. doi: 10.1016/0006-3223(87)90194-6
- Niederhofer, H. (2003). Selegiline and methylphenidate in treatment of ADHD. *Psychiatr. Danub.* 15, 3–6.
- Olanow, C. W., Myllyla, V. V., Sotaniemi, K. A., Larsen, J. P., Palhagen, S., Przuntek, H., et al. (1998). Effect of selegiline on mortality in patients with Parkinson's disease: a meta-analysis. *Neurology* 51, 825–830. doi: 10.1212/WNL.51.3.825
- Olanow, C. W., Rascol, O., Hauser, R., Feigin, P. D., Jankovic, J., Lang, A., et al. (2009). A double-blind, delayed-start trial of rasagiline in Parkinson's disease. *N. Engl. J. Med.* 361, 1268–1278. doi: 10.1056/NEJMoa0809335
- Palhagen, S., Heinonen, E., Hagglund, J., Kaugesaar, T., Maki-Ikola, O., and Palm, R. (2006). Selegiline slows the progression of the symptoms of Parkinson disease. *Neurology* 66, 1200–1206. doi: 10.1212/01.wnl.0000204007.46190.54
- Perez-Lloret, S., Rey, M. V., Montastruc, J. L., and Rascol, O. (2013). Adverse drug reactions with selegiline and rasagiline compared to levodopa and ropinirole: a study in the French Pharmacovigilance Database. *J. Neurol. Sci.* 333, e129. doi: 10.1016/j.jns.2013.07.432
- Parkinson Study Group (1989). Effect of deprenyl on the progression of disability in early Parkinson's disease. *N. Engl. J. Med.* 321, 1364–1371. doi: 10.1056/NEJM198911163212004
- Parkinson Study Group (1993). Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease. *N. Engl. J. Med.* 328, 176–183. doi: 10.1056/NEJM199301213280305
- Parkinson Study Group (1996a). Impact of deprenyl and tocopherol treatment on Parkinson's disease in DATATOP patients requiring levodopa. *Ann. Neurol.* 39, 37–45. doi: 10.1002/ana.410390107

- Parkinson Study Group (1996b). Impact of deprenyl and tocopherol treatment on Parkinson's disease in DATATOP subjects not requiring levodopa. *Ann. Neurol.* 39, 29–36.
- Parkinson Study Group (2002). A controlled trial of rasagiline in early Parkinson disease: the TEMPO Study. *Arch. Neurol.* 59, 1937–1943. doi: 10.1001/archneur.59.12.1937
- Parkinson Study Group (2004). A controlled, randomized, delayed-start study of rasagiline in early Parkinson disease. *Arch. Neurol.* 61, 561–566. doi: 10.1001/archneur.61.4.561
- Przuntek, H., Conrad, B., Dichgans, J., Kraus, P. H., Krauseneck, P., Pergande, G., et al. (1999). SELEDO: a 5-year long-term trial on the effect of selegiline in early Parkinsonian patients treated with levodopa. *Eur. J. Neurol.* 6, 141–150. doi: 10.1111/j.1468-1331.1999.tb00007.x
- Rabey, J. M., Sagi, I., Huberman, M., Melamed, E., Korczyn, A., Giladi, N., et al. (2000). Rasagiline mesylate, a new MAO-B inhibitor for the treatment of Parkinson's disease: a double-blind study as adjunctive therapy to levodopa. *Clin. Neuropharmacol.* 23, 324–330. doi: 10.1097/00002826-200011000-00005
- Ramsay, R. R., Dunford, C., and Gillman, P. K. (2007). Methylene blue and serotonin toxicity: inhibition of monoamine oxidase A (MAO A) confirms a theoretical prediction. *Br. J. Pharmacol.* 152, 946–951. doi: 10.1038/sj.bjp.0707430
- Rascol, O., Brooks, D. J., Melamed, E., Oertel, W., Poewe, W., Stocchi, F., et al. (2005). Rasagiline as an adjunct to levodopa in patients with Parkinson's disease and motor fluctuations (LARGO, Lasting effect in Adjunct therapy with Rasagiline Given Once daily, study): a randomised, double-blind, parallel-group trial. *Lancet* 365, 947–954. doi: 10.1016/S0140-6736(05)71083-7
- Rascol, O., Fitzer-Attas, C. J., Hauser, R., Jankovic, J., Lang, A., Langston, J. W., et al. (2011). A double-blind, delayed-start trial of rasagiline in Parkinson's disease (the ADAGIO study): prespecified and post-hoc analyses of the need for additional therapies, changes in UPDRS scores, and non-motor outcomes. *Lancet Neurol.* 10, 415–423. doi: 10.1016/S1474-4422(11)70073-4
- Reichmann, H., Sommer, U., Fuchs, G., Heftner, H., Mark, G., Muller, T., et al. (2000). Workshop IV: drug treatment guidelines for the long-term management of Parkinson's disease. *J. Neurol.* 247(Suppl. 4), IV/40–41. doi: 10.1007/PL00007776
- Reynolds, G. P., Elsworth, J. D., Blau, K., Sandler, M., Lees, A. J., and Stern, G. M. (1978). Deprenyl is metabolized to methamphetamine and amphetamine in man. *Br. J. Clin. Pharmacol.* 6, 542–544. doi: 10.1111/j.1365-2125.1978.tb00883
- Riederer, P., Lachenmayer, L., and Laux, G. (2004). Clinical applications of MAO-inhibitors. *Curr. Med. Chem.* 11, 2033–2043. doi: 10.2174/0929867043364775
- Riederer, P., and Laux, G. (2011). MAO-inhibitors in Parkinson's Disease. *Exp. Neurobiol.* 20, 1–17. doi: 10.5607/en.2011.20.1.1
- Rubinstein, S., Malone, M. A., Roberts, W., and Logan, W. J. (2006). Placebo-controlled study examining effects of selegiline in children with attention-deficit/hyperactivity disorder. *J. Child Adolesc. Psychopharmacol.* 16, 404–415. doi: 10.1089/cap.2006.16.404
- Sader-Mazbar, O., Loboda, Y., and Finberg, J. P. M. (2013). Increased L-dopa-derived dopamine following selective MAO-A or -B inhibition in rat striatum depleted of dopaminergic and serotonergic innervation. *Br. J. Pharmacol.* 170, 999–1013. doi: 10.1111/bph.12349
- Sandoval-Rincon, M., Saenz-Farret, M., Miguel-Puga, A., Micheli, F., and Arias-Carrion, O. (2015). Rational pharmacological approaches for cognitive dysfunction and depression in Parkinson's disease. *Front. Neurol.* 6:71. doi: 10.3389/fnneur.2015.00071
- Schapira, A., Bate, G., and Kirkpatrick, P. (2005). Rasagiline. *Nat. Rev. Drug Discov.* 4, 625–626. doi: 10.1038/nrd1803
- Schapira, A. H., Stocchi, F., Borgohain, R., Onofrij, M., Bhatt, M., Lorenzana, P., et al. (2013). Long-term efficacy and safety of safinamide as add-on therapy in early Parkinson's disease. *Eur. J. Neurol.* 20, 271–280. doi: 10.1111/j.1468-1331.2012.03840.x
- Shoham, S., Bejar, C., Kovalev, E., Schorer-Apelbaum, D., and Weinstock, M. (2007). Ladostigil prevents gliosis, oxidative-nitrative stress and memory deficits induced by intracerebroventricular injection of streptozotocin in rats. *Neuropharmacology* 52, 836–843. doi: 10.1016/j.neuropharm.2006.10.005
- Shoulson, I., Oakes, D., Fahn, S., Lang, A., Langston, J. W., LeWitt, P., et al. (2002). Impact of sustained deprenyl (selegiline) in levodopa-treated Parkinson's disease: a randomized placebo-controlled extension of the deprenyl and tocopherol antioxidative therapy of parkinsonism trial. *Ann. Neurol.* 51, 604–612. doi: 10.1002/ana.10191
- Shulman, K. I., Herrmann, N., and Walker, S. E. (2013). Current place of monoamine oxidase inhibitors in the treatment of depression. *CNS Drugs* 27, 789–797. doi: 10.1007/s40263-013-0097-3
- Son, S. Y., Ma, J., Kondou, Y., Yoshimura, M., Yamashita, E., and Tsukihara, T. (2008). Structure of human monoamine oxidase A at 2.2-A resolution: the control of opening the entry for substrates/inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5739–5744. doi: 10.1073/pnas.0710626105
- Stocchi, F., Arnold, G., Onofrij, M., Kwiecinski, H., Szczudlik, A., Thomas, A., et al. (2004). Improvement of motor function in early Parkinson disease by safinamide. *Neurology* 63, 746–748. doi: 10.1212/01.WNL.0000134672.44217.F7
- Stocchi, F., Borgohain, R., Onofrij, M., Schapira, A. H., Bhatt, M., Lucini, V., et al. (2012). A randomized, double-blind, placebo-controlled trial of safinamide as add-on therapy in early Parkinson's disease patients. *Mov. Disord.* 27, 106–112. doi: 10.1002/mds.23954
- Stocchi, F., and Rabey, J. M. (2011). Effect of rasagiline as adjunct therapy to levodopa on severity of OFF in Parkinson's disease. *Eur. J. Neurol.* 18, 1373–1378. doi: 10.1111/j.1468-1331.2011.03512.x
- Stocchi, F., Vacca, L., Grassini, P., De Pandis, M. F., Battaglia, G., Cattaneo, C., et al. (2006). Symptom relief in Parkinson disease by safinamide: biochemical and clinical evidence of efficacy beyond MAO-B inhibition. *Neurology* 67, S24–S29. doi: 10.1212/WNL.67.7_suppl_2.S24
- Sunderland, T., Cohen, R. M., Molchan, S., Lawlor, B. A., Mellow, A. M., Newhouse, P. A., et al. (1994). High-dose selegiline in treatment-resistant older depressive patients. *Arch. Gen. Psychiatry* 51, 607–615. doi: 10.1001/archpsyc.1994.03950080019003
- Tatton, W., and G., Chalmers-Redman, R. M. E. (1996). Modulation of gene expression rather than monoamine oxidase inhibition: (−)-Deprenyl-related compounds in controlling neurodegeneration. *Neurology* 47, S171–S183. doi: 10.1212/WNL.47.6_Suppl_3.171S
- Tetrud, J. W., and Langston, J. W. (1989). The effect of deprenyl (selegiline) on the natural history of Parkinson's disease. *Science* 245, 519–522. doi: 10.1126/science.2502843
- Thebault, J. J., Guillaume, M., and Levy, R. (2004). Tolerability, safety, pharmacodynamics, and pharmacokinetics of rasagiline: a potent, selective, and irreversible monoamine oxidase type B inhibitor. *Pharmacotherapy* 24, 1295–1305. doi: 10.1592/phco.24.14.1295.43156
- Tipton, K. F., Dostert, P., and Strobin Benedetti, M. (eds.). (1984). *Monoamine Oxidase and Disease*. London: Academic Press.
- Todd, K. G., and Baker, G. B. (2008). Neurochemical effects of the monoamine oxidase inhibitor phenelzine on brain GABA and alanine: a comparison with vigabatrin. *J. Pharm. Pharm. Sci.* 11, 14s–21s. doi: 10.18433/J34S38
- Waldmeier, P. C., Boulton, A. A., Cools, A. R., Kato, A. C., and Tatton, W. G. (2000). Neurorescuing effects of the GAPDH ligand CGP B. *J. Neural. Transm. Suppl.* 60, 197–214.
- Wang, L., Esteban, G., Ojima, M., Bautista-Aguilera, O. M., Inokuchi, T., Moraleda, I., et al. (2014). Donepezil + propargylamine + 8-hydroxyquinoline hybrids as new multifunctional metal-chelators, ChE and MAO inhibitors for the potential treatment of Alzheimer's disease. *Eur. J. Med. Chem.* 80, 543–561. doi: 10.1016/j.ejmech.2014.04.078
- Waters, C. H., Sethi, K. D., Hauser, R. A., Molho, E., and Bertoni, J. M. (2004). Zydis selegiline reduces off time in Parkinson's disease patients with motor fluctuations: a 3-month, randomized, placebo-controlled study. *Mov. Disord.* 19, 426–432. doi: 10.1002/mds.20036
- Weinreb, O., Amit, T., Bar-Am, O., Chillag-Talmor, O., and Youdim, M. B. (2005). Novel neuroprotective mechanism of action of rasagiline is associated with its propargyl moiety: interaction of Bcl-2 family members with PKC pathway. *Ann. N. Y. Acad. Sci.* 1053, 348–355. doi: 10.1196/annals.1344.030
- Weinreb, O., Amit, T., Bar-Am, O., and Youdim, M. B. (2012). Ladostigil: a novel multimodal neuroprotective drug with cholinesterase and brain-selective monoamine oxidase inhibitory activities for Alzheimer's disease treatment. *Curr. Drug Targets* 13, 483–494. doi: 10.2174/138945012799499794
- Weinreb, O., Amit, T., Riederer, P., Youdim, M. B., and Mandel, S. A. (2011). Neuroprotective profile of the multitarget drug rasagiline in Parkinson's disease. *Int. Rev. Neurobiol.* 100, 127–149. doi: 10.1016/B978-0-12-386467-3.00007-8

- Weinreb, O., Badinter, F., Amit, T., Bar-Am, O., and Youdim, M. B. (2015). Effect of long-term treatment with rasagiline on cognitive deficits and related molecular cascades in aged mice. *Neurobiol. Aging* 36, 2628–2636. doi: 10.1016/j.neurobiolaging.2015.05.009
- Weinstock, M., Bejar, C., Wang, R. H., Poltyrev, T., Gross, A., Finberg, J. P., et al. (2000). TV3326, a novel neuroprotective drug with cholinesterase and monoamine oxidase inhibitory activities for the treatment of Alzheimer's disease. *J. Neural. Transm. Suppl.* 60, 157–169. doi: 10.1007/978-3-7091-6301-6_10
- Westfall, T. C., and Westfall, D. P. (2011). "Neurotransmission: the autonomic and somatic motor nervous systems," in *The Pharmacological Basis of Therapeutics, 12th Edn*, ed L. L. Brunton (New York, NY: McGraw Hill), 171–218.
- Westlund, K. N., Denney, R. M., Rose, R. M., and Abell, C. W. (1988). Localization of distinct monoamine oxidase A and monoamine oxidase B cell populations in human brainstem. *Neuroscience* 25, 439–456. doi: 10.1016/0306-4522(88)90250-3
- Westlund, K. N., Krakower, T. J., Kwan, S. W., and Abell, C. W. (1993). Intracellular distribution of monoamine oxidase A in selected regions of rat and monkey brain and spinal cord. *Brain Res.* 612, 221–230. doi: 10.1016/0006-8993(93)91664-E
- Winger, G. D., Yasar, S., Negus, S. S., and Goldberg, S. R. (1994). Intravenous self-administration studies with l-deprenyl (selegiline) in monkeys. *Clin. Pharmacol. Ther.* 56, 774–780. doi: 10.1038/clpt.1994.208
- Yasar, S., Goldberg, J. P., and Goldberg, S. R. (1996). Are metabolites of l-deprenyl (selegiline) useful or harmful? *Indications from preclinical research*. *J. Neural. Transm. Suppl.* 48, 61–73. doi: 10.1007/978-3-7091-7494-4_6
- Yogev-Falach, M., Amit, T., Bar-Am, O., Weinstock, M., and Youdim, M. B. (2002). Involvement of MAP kinase in the regulation of amyloid precursor protein processing by novel cholinesterase inhibitors derived from rasagiline. *FASEB J.* 16, 1674–1676. doi: 10.1096/fj.02-0198fje
- Youdim, M. B., and Finberg, J. P. (1983). "Monoamine oxidase inhibitor antidepressants," in *Part I: Preclinical Psychopharmacology*, eds D. G. Grahame-Smith and P. J. Cowen (Amsterdam: Excerpta Medica), 38–70.
- Youdim, M. B., Fridkin, M., and Zheng, H. (2005). Bifunctional drug derivatives of MAO-B inhibitor rasagiline and iron chelator VK-28 as a more effective approach to treatment of brain ageing and ageing neurodegenerative diseases. *Mech. Ageing Dev.* 126, 317–326. doi: 10.1016/j.mad.2004.08.023
- Yu, P. H., and Hertz, L. (1983). Type A and B monoamine oxidase in glial cells in long-term culture. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 7, 687–690. doi: 10.1016/0278-5846(83)90046-5
- Zheng, H., Gal, S., Weiner, L. M., Bar-Am, O., Warshawsky, A., Fridkin, M., et al. (2005). Novel multifunctional neuroprotective iron chelator-monoamine oxidase inhibitor drugs for neurodegenerative diseases: *in vitro* studies on antioxidant activity, prevention of lipid peroxide formation and monoamine oxidase inhibition. *J. Neurochem.* 95, 68–78. doi: 10.1111/j.1471-4159.2005.03340.x

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Cannabidiol as a Potential New Type of an Antipsychotic. A Critical Review of the Evidence

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There is urgent need for the development of mechanistically different and less side-effect prone antipsychotic compounds. The endocannabinoid system has been suggested to represent a potential new target in this indication. While the chronic use of cannabis itself has been considered a risk factor contributing to the development of schizophrenia, triggered by the phytocannabinoid delta-9-tetrahydrocannabinol (Δ^9 -THC), cannabidiol, the second most important phytocannabinoid, appears to have no psychotomimetic potential. Although, results from animal studies are inconsistent to a certain extent and seem to depend on behavioral paradigms, treatment duration and experimental conditions applied, cannabidiol has shown antipsychotic properties in both rodents and rhesus monkeys. After some individual treatment attempts, the first randomized, double-blind controlled clinical trial demonstrated that in acute schizophrenia cannabidiol exerts antipsychotic properties comparable to the antipsychotic drug amisulpride while being accompanied by a superior, placebo-like side effect profile. As the clinical improvement by cannabidiol was significantly associated with elevated anandamide levels, it appears likely that its antipsychotic action is based on mechanisms associated with increased anandamide concentrations. Although, a plethora of mechanisms of action has been suggested, their potential relevance for the antipsychotic effects of cannabidiol still needs to be investigated. The clarification of these mechanisms as well as the establishment of cannabidiol's antipsychotic efficacy and its hopefully benign side-effect profile remains the subject of a number of previously started clinical trials.

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INTRODUCTION

Cannabis sativa has been known and used by humans for several 1000 years and the knowledge that it contains an intoxicating principle dates back to 1000 to 1500 B.C. (Adams, 1942). The two major compounds of cannabis – delta-9-tetrahydrocannabinol (Δ^9 -THC) and cannabidiol – have been chemically identified in the 1940th (Adams et al., 1940a,b,c; Adams, 1942; Todd, 1946). Two decades later, remaining uncertainties regarding the exact position of double bonds were eliminated as new imaging techniques like NMR spectroscopy and X-ray structure determination became available (Mechoulam and Shvo, 1963; Gaoni and Mechoulam, 1964; Jones et al., 1977).

Along with its chemical identification, Δ^9 -THC has been identified as the major pro-psychotic compound of *Cannabis sativa* (Adams and Baker, 1940; Adams et al., 1940b; Allentuck and Bowman, 1942; Wollner et al., 1942; Mechoulam et al., 1970). However, the underlying

neurobiological principles remained conjectural until it was observed that cannabinoid drugs inhibit adenylate cyclase activity in neuroblastoma cells (Howlett, 1984), and the subsequent discovery of the G-protein coupled type 1 cannabinoid receptor (CB₁R) (Devane et al., 1988; Matsuda et al., 1990). A few years later, the type 2 cannabinoid receptor (CB₂R) (Munro et al., 1993) as well as the two major endogenous ligands to cannabinoid receptors – anandamide (Devane et al., 1992) and 2-arachidonoyl-*sn*-glycerol (Mechoulam et al., 1995; Stella et al., 1997) – were discovered. The architecture of the endocannabinoid system (ECS) – including endocannabinoids, cannabinoid receptors as well as synthesizing and degrading enzymes – has been vividly summarized and illustrated in recent reviews by Lutz et al. (2015) and Lu and Mackie (2016).

Studies indicating that cannabis abuse might be a stressor for psychotic relapse and exacerbation of schizophrenic symptoms as well as the observation that Δ⁹-THC induced schizophrenia-like neuropsychological and psychopathological alterations in healthy volunteers, led to the hypothesis that a dysfunctional ECS is involved in the etiology of psychoses (Emrich et al., 1997). In the meantime, several studies reported diverse acute Δ⁹-THC effects in healthy participants and schizophrenic patients, thereby confirming the ECS hypothesis (Leweke et al., 1999, 2000; D'Souza et al., 2004, 2005; Koethe et al., 2006; Bhattacharyya et al., 2009; Fusar-Poli et al., 2009; Koethe et al., 2009; Mason et al., 2009; Sherif et al., 2016). In addition, several epidemiological studies (for review see, Gage et al., 2016) substantiated the view that cannabis use has to be considered an important environmental risk factor for the development of schizophrenia in vulnerable individuals. However, the actual lifetime risk seems to be influenced by the dose (Zammit et al., 2002; Moore et al., 2007) and frequency of cannabis consumption (Di Forti et al., 2009), the potency of consumed cannabis preparations (Di Forti et al., 2009, 2014) and age of onset (Arseneault et al., 2002).

While a dysfunctional ECS seems to contribute to the pathophysiology of schizophrenia, the endocannabinoid anandamide is considered to have protective effects by counteracting neurotransmitter imbalances (Leweke, 2012). Therefore, it has been suggested that modulating the ECS might be a new, promising pharmacological target for schizophrenia.

To date, two main approaches targeting the ECS have been systematically studied in humans: first, trials using CB₁R antagonists to treat both psychotic and cognitive symptoms of schizophrenia, and, second, trials using the second most important phytocannabinoid cannabidiol (Leweke et al., 2016). In addition, a single clinical case series on dronabinol (Δ⁹-THC) in treatment-refractory severe chronic schizophrenia has been conducted (Schwarcz et al., 2009).

In contrast to Δ⁹-THC, cannabidiol appears to have no psychotomimetic potential, but shows antipsychotic effects in rodents and humans. Thus, this review focuses on (1) preclinical studies investigating cannabidiol as a potential antipsychotic in animal models of aspects of schizophrenia, (2) clinical evidence for its antipsychotic action, and, (3) potential mechanisms of action and their potential relevance for the antipsychotic effects of cannabidiol.

METHODS

We conducted a PubMed search up to and including June 28, 2016, using the search terms cannabidiol AND (antipsychotic OR schizophrenia OR psychosis). Identified references were scanned for clinical trials with schizophrenic patients as well as studies with animal models of aspects of schizophrenia, analyzing the effects of cannabidiol on negative symptoms and cognitive deficits. Studies restricted to analysis of locomotor activity or anxiety were not considered. In addition, we used the search term cannabidiol AND targets to scan for reports on the possible mode of action.

ANTIPSYCHOTIC POTENTIAL OF CANNABIDIOL: INSIGHTS FROM PRECLINICAL STUDIES

Schizophrenia is characterized by heterogeneous symptoms that can be grouped into three main symptom categories: (1) positive symptoms (delusions, thought disorder, hallucinations), (2) negative symptoms (anhedonia, blunted affect, social withdrawal) and (3) cognitive impairment (sensory information processing, attention, working memory, executive functions) (Freedman, 2003; Wong and Van Tol, 2003). These main symptoms are often accompanied by more unspecific symptoms like anxiety (Freedman, 2003).

Although psychotic symptoms are human specific to a large extent, animal models are able to provide insight into certain aspects of schizophrenia, including negative symptoms [e.g., inadequate social behavior/social withdrawal, sensorimotor gating deficits as measured by prepulse inhibition (PPI)], cognitive impairments (e.g., working memory deficits) or anxiety. Thus, these animal models for aspects of schizophrenia can be used to investigate the antipsychotic potential of new drugs like cannabidiol on negative and cognitive symptoms. As available antipsychotics do not sufficiently ameliorate negative symptoms and cognitive impairments (Hanson et al., 2010), these studies can make an important contribution.

Schizophrenia is not only characterized by a heterogeneous combination of symptoms but also by a heterogeneous etiology (Cannon and Jones, 1996; Brown, 2011; Kahn et al., 2015). This led to the development of animal models based on different etiological factors of schizophrenia. In the following, the concepts of animal models used to study the effects of cannabidiol are summarized in brief.

As mentioned above, cannabis use is regarded as one important risk factor for the development of schizophrenia. Hence, animal models of early cannabis exposure are used to investigate the long-lasting behavioral consequences of cannabis use and to clarify the underlying cellular mechanisms (Rubino and Parolario, 2016). However, based on the observation that cannabis preparations or single cannabinoids like Δ⁹-THC induce psychotic-like symptoms in healthy volunteers (for review see Sherif et al., 2016), acute cannabinoid administration is also used to mimic schizophrenia-like symptoms in rodents.

Furthermore, based on the hypothesis that disrupted glutamatergic neurotransmission contributes to the development of schizophrenia (Kim et al., 1980), pharmacological and genetic glutamatergic models have been used in cannabidiol research. Since this dysfunction is characterized by hypofunctional N-methyl-D-aspartic acid (NMDA) receptors (for review see Snyder and Gao, 2013), for example, NMDA receptor antagonists like MK-801 are administered to mimic schizophrenic-like symptoms in rodents. The genetic model is based on *neuregulin 1*, a susceptibility gene for schizophrenia (Stefansson et al., 2002). *Neuregulin 1* is involved in neuronal migration, influences myelination and regulates expression of NMDA, γ -aminobutyric acid receptor A (GABA_A) as well as acetylcholin receptor subunits (for review see Corfas et al., 2004). Heterozygous transmembrane *Neuregulin 1* mutant mice (*Nrg1* TM HET) seem to have fewer functional NMDA receptors (Stefansson et al., 2002), a region-specific alteration of NMDA receptor expression as well as decreased dopamine D₂ receptor binding in the striatum (Newell et al., 2013). Interestingly, the CB₁ receptor density is comparable to the density in wild type animals except for a slight increase within the striatum of *Nrg1* TM HET (Newell et al., 2013).

The spontaneously hypertensive rat (SHR) strain has also been suggested as a model for aspects of schizophrenia. These rats show impaired social interaction (Calzavara et al., 2011; Almeida et al., 2014) and reduced PPI (Levin et al., 2011, 2014) as compared to Wistar rats. In addition, antipsychotic drugs reduced abnormalities in contextual fear conditioning (Calzavara et al., 2009), social interaction (Calzavara et al., 2011) as well as PPI (Levin et al., 2011). Nevertheless, other studies observed an increased PPI compared to Sprague Dawley rats (van den Buuse, 2004) or an increased social interaction behavior toward Wistar-Kyoto rats (Hopkins et al., 2009).

Effects of Cannabidiol on Social Behavior

Social withdrawal is a key negative symptom of schizophrenia. Thus, several studies investigated the effects of cannabidiol on social behavior in different rodent animal models for schizophrenia (Table 1).

Cannabidiol (dosage range: 1–50 mg/kg) itself seems to have no effect on social interaction of untreated Sprague Dawley (Malone et al., 2009; Gururajan et al., 2012), Wistar rats (van Ree et al., 1984; Deiana et al., 2015), C57BL/6JArc mice (Long et al., 2010; Gomes et al., 2015b), and wild type-like littermates of *Nrg1* TM HET mice (Long et al., 2012). However, in Wistar rats 1 mg/kg cannabidiol increased social interaction behavior, whereas higher dosages (5, 15, 30, 60 mg/kg) had no effect (Almeida et al., 2013). In addition, impaired social memory was observed in Wistar rats (Deiana et al., 2015) after acute cannabidiol administration (12 and 30 mg/kg, but not 5 mg/kg).

The majority of studies reported that cannabidiol was able to attenuate or reverse induced altered social behavior. Pretreatment with 20 mg/kg cannabidiol reversed the effects of 1 mg/kg Δ⁹-THC (Malone et al., 2009), while 3 mg/kg cannabidiol

inhibited the effects on social investigative behavior of acute MK-801 treatment in a modified social interaction task, increasing it significantly beyond control level (Gururajan et al., 2012). This is in line with the previous finding of the group that cannabidiol (3, 10 mg/kg) partially inhibited MK-801-induced social withdrawal in a classical social interaction paradigm (Gururajan et al., 2011). In mice, pretreatment with 60 mg/kg cannabidiol was found to reverse impaired social interaction induced by chronic MK-801 treatment, while a lower cannabidiol dose (30 mg/kg) attenuated the effects of MK-801 only by trend (Gomes et al., 2015b). As the antipsychotic clozapine also inhibited MK-801 effects on social investigative behavior, it has been suggested that cannabidiol might also be effective in schizophrenia patients showing inadequate social behaviors (Gururajan et al., 2012; Gomes et al., 2015b). However, cannabidiol did not reverse the social recognition impairments induced by an acute low-dose injection of MK-801 in Wistar rats. Furthermore, cannabidiol was not able to elevate the decreased social interaction of SHR rats (Almeida et al., 2013).

Although, *Nrg1* TM HET mutant mice showed similar social behavior compared to their wild type-like littermates (Long et al., 2012), chronic cannabidiol treatment (50 mg/kg, 21 days) increased social interaction as well as specific social behaviors like nosing and anogenital sniffing in mutant mice but not in wild type mice. A higher cannabidiol dose (100 mg/kg) solely increased anogenital-sniffing duration in mutant mice, while lower concentrations (1 mg/kg) led only to increasing nosing frequencies.

Taken together, cannabidiol showed antipsychotic properties in glutamatergic animal models as well as in a model targeting the ECS, whereas it has been ineffective in SHR rats.

Effects of Cannabidiol on Prepulse Inhibition

Prepulse inhibition of the acoustic startle response is a neuropsychological process during which a weak sensory stimulus – prepulse – attenuates the motor response to a subsequent strong startling stimulus (Rohleder et al., 2016). Since PPI impairments are observed in schizophrenia patients and PPI can be reliably assessed in both animals and humans, it has been used as behavioral measure of aspects of schizophrenia. Unfortunately, results from rodent studies analyzing the effects of cannabidiol on PPI and acoustic startle response in animal models of schizophrenia and untreated control rodents are inconsistent to a certain extent (Table 2).

The effects of cannabidiol on startle amplitude and PPI of healthy rodents seem to be not only dose- but also strain- and species-dependent. In male Swiss mice, cannabidiol (15, 30, or 60 mg/kg) did not affect PPI or startle amplitude (Pedrazzi et al., 2015). On the other hand, Long et al. (2006) reported that C57BL/6JArc mice acutely treated with cannabidiol (1 and 15, but not 5 mg/kg) showed increased startle amplitudes while PPI remained unaffected. In contrast, in wild type-like littermates of *Nrg1* TM HET mice, acute treatment with low concentrations of cannabidiol (1 and 50 mg/kg) as well as chronic cannabidiol treatment (1, 50, 100 mg/kg; 21 days) had no effect

TABLE 1 | Animal studies evaluating the effects of cannabidiol (CBD) on social behavior.

Animal model	Treatment regimen and test procedure	Effective dose [kg]	Reference
Spontaneously hypertensive rats (SHR)	1, 5, 15, 30, or 60 mg/kg CBD, i.p. injection 30 min prior to social interaction test	–	Almeida et al., 2013
MK-801 (acute, 0.3 or 0.6 mg/kg), male Sprague Dawley rats	1 or 3 mg/kg CBD, i.p. injection 20 min prior to MK-801 administration. Social interaction test started 20 min after the last injection	3, 10 (partially)	Gururajan et al., 2011
MK-801 (acute, 0.3 mg/kg), male Sprague Dawley rats	1 or 3 mg/kg CBD, i.p. injection 20 min prior to MK-801 administration. A modified social interaction test started 20 min after the last injection	3	Gururajan et al., 2012
MK-801 (chronic: 1 mg/kg, 28 days), male C57BL/6J mice	30 or 60 mg/kg CBD, i.p. injection 30 min prior to social interaction test	60	Gomes et al., 2015b
MK-801 (acute, 0.08 mg/kg), male Wistar rats	5, 12, or 30 mg/kg CBD, i.p. injection 30 min prior to MK-801 administration. Social interaction/recognition test started 30 min after the last injection	–	Deiana et al., 2015
Male <i>Nrg1</i> TM HET mice	Chronic treatment with 1, 50, or 100 mg/kg CBD over 3 weeks	50 (partially 1 and 100)	Long et al., 2012
Δ ⁹ -THC (1 mg/kg), male Sprague Dawley rats	5 or 20 mg/kg CBD, i.p. injection 20 min prior to Δ ⁹ -THC administration. Social interaction test started 20 min after the last injection	20	Malone et al., 2009

TABLE 2 | Animal studies evaluating the effects of cannabidiol (CBD) on prepulse inhibition (PPI).

Animal model	Treatment regimen and test procedure	Effective dose [mg/kg]	Reference
Spontaneously hypertensive rats (SHR)	15, 30, or 60 mg/kg CBD, i.p. 30 min prior to PPI paradigm	30	Levin et al., 2014
MK-801 (acute, 0.3 or 0.6 mg/kg), male Sprague Dawley rats	3, 10, or 30 mg/kg CBD, i.p. injection 20 min prior to MK-801 administration. PPI paradigm started 20 min after the last injection	–	Gururajan et al., 2011
MK-801 (chronic: 1 mg/kg, 28 days), male C57BL/6J mice	30 or 60 mg/kg CBD, i.p. treatment began on 6th day of MK-801 administration. PPI paradigm was conducted on day 29	30, 60	Gomes et al., 2015a
MK-801 (acute, 1 mg/kg), male C57BL/6J mice	5 mg/kg CBD, i.p. injection 20 min prior to MK-801 administration. PPI paradigm started 5 min after the last injection	5	Long et al., 2006
Male <i>Nrg1</i> TM HET mice	1, 50, or 100 mg/kg CBD, i.p. over 21 days. PPI paradigm was done 30–45 min after the first injection and on day 21	100 (acute)	Long et al., 2012
Amphetamine (acute, 10 mg/kg) male Swiss mice	15, 30, or 60 mg/kg CBD, i.p. 30 min prior to amphetamine injection. PPI paradigm started 30 min after the last injection	15, 30, 60	Pedrazzi et al., 2015

on startle amplitude and PPI, but acute administration of a high cannabidiol dosage (100 mg/kg) resulted in an increased startle amplitude (Long et al., 2012).

Such heterogeneous results were also observed in healthy rats. In Sprague Dawley rats, cannabidiol reduced startle amplitude (3 and 10 but not 30 mg/kg) and PPI (10 mg/kg only) in a dose-dependent manner (Gururajan et al., 2011), whereas the startle

amplitude of Wistar rats was not influenced by acute cannabidiol (15, 30, 60 mg/kg) treatment, while higher dosages of cannabidiol (30, 60 mg/kg) seemed to increase PPI (Levin et al., 2014).

Interestingly, animals with transmembrane *Neuregulin 1* mutation, representing a genetic glutamatergic schizophrenia model, showed similar startle amplitude and PPI compared to their wild type like littermates. However, acute administration of

100 mg/kg cannabidiol increased not only the startle amplitude as observed in wild type like littermates, but also PPI. On the other hand, chronic cannabidiol treatment (1, 50, 100 mg/kg; 21 days) had no effect on startle amplitude and PPI in *Nrg1* TM HET or their wild type like littermates (Long et al., 2012).

Pharmacological studies mimicking glutamatergic deficits of schizophrenia revealed that pretreatment with cannabidiol (5 mg/kg) reversed PPI disruption in C57BL/6JArc mice (Long et al., 2006). In addition, chronic cannabidiol treatment (30 or 60 mg/kg) attenuated PPI impairments induced by chronic MK-801 administration in C57BL/6J mice (Gomes et al., 2015a). While cannabidiol seems to be efficacious in treating PPI impairments in mice, pretreatment with cannabidiol had no effect on PPI deficits induced by acute MK-801-injection in Sprague Dawley rats (Gururajan et al., 2011).

However, acute cannabidiol treatment also reversed PPI deficits in two other animal models of aspects of schizophrenia. First, cannabidiol (30 mg/kg) reversed PPI deficit of SHR rats but had no effects on their reduced startle amplitude (Levin et al., 2014). Second, cannabidiol (15, 30, or 60 mg/kg) attenuated the amphetamine-disruptive effects on PPI in male Swiss mice (Pedrazzi et al., 2015). Interestingly, the inhibition of anandamide hydrolysis by URB597 [selective fatty acid amide hydrolase (FAAH) inhibitor] had the same effect. Hence, the authors suggested that an increase of anandamide availability might be involved in the beneficial effects of cannabidiol.

In a nutshell, various studies showed that cannabidiol partially affected startle amplitude and PPI in healthy animals, while it had no effects in rats treated with MK-801, but reversed the PPI disruptive effects of MK-801 and amphetamine in mice as well as the PPI deficit of SHR rats.

These discrepancies, observed in both healthy wild type animals and animal models for aspects of schizophrenia, might be related to the different species/strain or experimental conditions applied. Therefore, more studies clarifying the potential antipsychotic effect of cannabidiol with regard to this specific behavioral deficit are desirable.

Effect of Cannabidiol on Working Memory

Various paradigms are available to test cognitive performance in animals. Two studies investigating the effects of cannabidiol on cognitive performance in Δ^9 -THC- or MK-801-treated animals, respectively, used paradigms based on object and/or spatial recognition. In addition, two further studies investigated the effects of cannabidiol-rich cannabis extracts in a spatial recognition task (**Table 3**).

The visuospatial Paired Associates Learning task (vsPAL) and the Self-Ordered Spatial Search (SOSS) task belong to the category of spatial recognition tasks that are frequently used in non-human primates. In rhesus monkeys, acute intramuscular Δ^9 -THC administration (0.2 and 0.5 mg/kg) impaired overall trial completion accuracy and percent completed trials in the vsPAL with increasing trial-difficulty as well as trial completion accuracy in the SOSS task (Wright et al., 2013). Cannabidiol

itself had no effect on cognitive performance. Interestingly, co-treatment with cannabidiol reversed the effects of Δ^9 -THC on vsPAL, but did not affect Δ^9 -THC-induced SOSS deficits.

These results are in line with a study assessing the effects of cannabidiol on novel object recognition (NOR) impairments in mice. The NOR task is often used in rodents and partially resembles vsPAL. While in vsPAL a familiar object and its former position has to be identified, NOR evaluates whether the animal recognizes a new unfamiliar object, as rodents tend to explore new objects more intensively than familiar ones. Cannabidiol (30 or 60 mg/kg) significantly reversed the NOR performance impairments observed in male C57BL/6J mice chronically treated with MK-801, but had no effect *per se* (Gomes et al., 2015b).

Furthermore, Fadda et al. (2004, 2006) investigated the effects of cannabidiol-rich cannabis extracts in a water maze based delayed-matching-to-position task (DMTP). In the first trial, animals were placed onto a platform. In the second trial animals were released into the water and had to find the platform again. Cannabidiol-rich cannabis extracts did not affect the spatial working memory of rats, although these extracts also contained Δ^9 -THC. In particular the highest dose of 50 mg/kg cannabidiol-rich extracts contained nearly 4 mg/kg Δ^9 -THC, a dose that was sufficient to impair the working memory when given alone. Thus, the authors concluded that cannabidiol is able to antagonize the cognitive impairment. However, the cannabidiol-rich extracts did not reverse memory deficits when administered concurrently with the Δ^9 -THC-rich cannabis extracts. Therefore, it has been suggested, that the cannabidiol/ Δ^9 -THC ratio was not high enough to be effective (Fadda et al., 2004).

Interestingly, cannabidiol-rich extracts (5 and 10 mg/kg) were also unable to reverse working memory deficits induced by MK-801 in rats (Fadda et al., 2006). It might be that the cannabidiol dosage had simply been too low, as higher concentrations (30 or 60 mg/kg) had been shown to be effective in mice (Gomes et al., 2015b). However, even higher dosages of cannabidiol may not reverse working memory deficits induced by MK-801 in rats due to interspecies differences.

Overall, the limited data available seem to suggest that cannabidiol has potential in ameliorating not only negative symptoms but also cognitive functions. However, as task-selective differences were observed, its effectiveness might be restricted to certain aspects of cognitive functions. Therefore, further studies analyzing the effects of cannabidiol on various cognitive aspects are called for.

ANTIPSYCHOTIC POTENTIAL OF CANNABIDIOL: EVIDENCE FROM CLINICAL STUDIES

The results of the first individual treatment attempt with cannabidiol were reported in Zuardi et al. (1995). Daily administration of up to 1500 mg/day over 4 weeks resulted in decreased scores on the Brief Psychiatric Rating Scale (BPRS) and Interactive Observation Scale for Psychiatric Inpatients (IOSPI), indicating an overall improvement of psychotic symptoms.

TABLE 3 | Animal studies evaluating the effects of cannabidiol (CBD) on working memory.

Animal model	Treatment regimen and test procedure	Effective dose [mg/kg]	Reference
MK-801 (chronic: 1 mg/kg, 28 days), male C57BL/6J mice	30 or 60 mg/kg CBD, i.p. injection 30 min prior to novel object recognition test	30, 60	Gomes et al., 2015b
Δ^9 -THC (0.2, 0.5 mg/kg i.m.), male adults rhesus monkeys	0.5 mg/kg CBD, i.m. concurrently with Δ^9 -THC administration. Visuospatial Paired Associates Learning task and Self-Ordered Spatial Search started 30 min after the injections	0.5 (task selective)	Wright et al., 2013
Δ^9 -THC-rich and CBD-rich cannabis extracts, male, adult Lister rats	CBD-rich cannabis extracts (0.5, 5, 10, or 50 mg/kg CBD and up to 4 mg/kg Δ^9 -THC), i.p. 30 min prior to Delayed Matching to Sample task. In addition, CBD-rich cannabis extracts were simultaneously injected with Δ^9 -THC-rich cannabis extract injection	50 (as it contained nearly 4 mg/kg Δ^9 -THC)	Fadda et al., 2004
MK-801 (0.1 mg/kg, acute), male, adult Lister rats	CBD-rich cannabis extracts (5 or 10 mg/kg CBD), i.p. concurrently with MK-801 injection, 30 min prior to Delayed Matching to Sample task. In addition, CBD-rich cannabis extracts were simultaneously injected with Δ^9 -THC-rich cannabis extract injection	–	Fadda et al., 2006

Interestingly, the clinical improvement was not increased by additional treatment with haloperidol, a first generation antipsychotic. Nearly 10 years later, Zuardi et al. (2006) published a small case series of three patients. They were treated with increasing doses of cannabidiol (maximum 1280 mg/kg) over 30 days. Importantly, one of these patients showed a slight improvement of both positive and negative symptoms. Interestingly, no side effects were reported in patients treated with cannabidiol. The first controlled, randomized, double-blind clinical trial was conducted by Leweke et al. (2012). During this 4-week trial 42 schizophrenic patients received either cannabidiol (600–800 mg/day) or amisulpride (600–800 mg/day) – a highly effective second generation antipsychotic, selectively antagonizing $D_{2/3}$ receptors (Leucht et al., 2002). Both drugs resulted in significant clinical improvement of both positive and negative symptoms of psychosis. The efficacy of cannabidiol was comparable to that of amisulpride, but, importantly, cannabidiol revealed a superior side effect profile when compared to amisulpride. In particular, cannabidiol did not induce prolactin increase, weight gain, or extrapyramidal symptoms. Interestingly, in patients randomly allocated to cannabidiol treatment, the reduction of psychotic symptoms was significantly associated with an increase of anandamide levels in serum. This was exclusive for the cannabidiol treatment group and is in support for the hypothesis that cannabidiol's antipsychotic effect is at least in part mediated via anandamide and potentially related to a block of its metabolism or uptake.

As reviewed in Leweke et al. (2016), four additional clinical trials with schizophrenic patients have been initiated so far. Although to date data have not been published in a peer reviewed process, the sponsor of one recent clinical trial investigating the antipsychotic effects of cannabidiol (GW42003, 1000 mg/day) as add-on medication in 88 patients suffering from schizophrenia or related disorders (e.g., schizoaffective or schizophrenia-like disorder) over a period of 6 weeks, announced that cannabidiol was consistently superior to placebo with regard to psychopathology while at the same time showing no relevant side-effect profile (GW Pharmaceuticals plc, 2015).

While cannabidiol seems to develop antipsychotic properties during at least 4 weeks of treatment, acute administration of cannabidiol (300 or 600 mg/kg) did not affect selective attention studied in 28 schizophrenic outpatients (regularly treated with antipsychotics) using the Stroop Color Word Test (Hallak et al., 2010). However, there also were no side effects reported.

Although, only few data on the antipsychotic potential in schizophrenic patients are currently available (summarized in Table 4), they consistently indicate a promising efficacy and favorable side-effect profile of cannabidiol. However, large-scale clinical trials are still needed to evaluate the long-term efficacy and safety of this putative new antipsychotic.

CANNABIDIOL: POTENTIAL MECHANISM OF ACTION

The mode of action of cannabidiol is still not fully understood, although a plethora of possible mechanisms have been proposed.

Cannabidiol and Δ^9 -THC are the most important phytocannabinoids in the cannabis plant. Therefore, it has been hypothesized that both compounds might have the same molecular target, the cannabinoid receptors. However, several binding studies showed that cannabidiol has no significant affinity at CB₁R and CB₂R (Devane et al., 1988; Showalter et al., 1996; Thomas et al., 1998; Bisogno et al., 2001; Jones et al., 2010). In addition, most efficacy studies found no explicit receptor response (Matsuda et al., 1990; Petit et al., 1998; Breivogel et al., 2001; Jones et al., 2010). In fact, it has been reported that cannabidiol acts as an antagonist of CB₁R agonists such as WIN-55212 and CP-55940 (Petit et al., 1998; Pertwee et al., 2002; Thomas et al., 2007). As cannabidiol also inhibited internalization of CB₁R (Laprairie et al., 2014), it has been hypothesized that the observed antagonistic activity might be based on negative allosteric modulation of CB₁R rather than on orthosteric binding (Laprairie et al., 2015). Consistent with these findings, Laprairie et al. (2015) provided evidence that *in vitro*

cannabidiol behaves as a non-competitive negative allosteric modulator of CB₁R.

Owing to the observed significant association of the antipsychotic effect of cannabidiol with an increase of anandamide levels in serum (Leweke et al., 2012), it has been hypothesized that cannabidiol exerts its antipsychotic properties by moderately blocking FAAH, resulting in an inhibition of anandamide and of related fatty acid ethanolamide (palmitoylethanolamide and oleoylethanolamide) degradation. *In vitro*, cannabidiol inhibited FAAH in mouse neuroblastoma cell (N18TG2) membrane preparations (Bisogno et al., 2001), mouse brain microsomes (Watanabe et al., 1996), as well as homogenates of rat brain membranes (De Petrocellis et al., 2011; Leweke et al., 2012). Moreover, it has been shown that cannabidiol blocks anandamide transporters, since it inhibited anandamide uptake by rat basophilic leukemia cells (RBL-2H3) (Rakhshan et al., 2000; Bisogno et al., 2001; De Petrocellis et al., 2011). So far, one possible anandamide transporter, termed FAAH-like anandamide transporter (FLAT) has been identified (Fu et al., 2012). As FLAT seems to be a splicing variant of the *Faah-1* gene, it may be speculated that cannabidiol binds to similar binding sites of FAAH and FLAT proteins and is able to inhibit both, anandamide degradation and uptake. However, it has recently been reported that cannabidiol does not inhibit the human FAAH enzyme, but binds to fatty acid-binding proteins (FABPs) (Elmes et al., 2015), which seem to act as intracellular transporters of anandamide and other *N*-acylethanolamines (Kaczocha et al., 2009, 2012). Elmes et al. (2015) concluded that cannabidiol reduces anandamide inactivation in humans by competing with anandamide for FABPs binding. As long as FABPs are occupied by cannabidiol, anandamide cannot be transported to the FAAH enzyme, localized on the endoplasmic reticulum, resulting in elevated anandamide levels.

Although, various other molecular targets have been suggested to contribute to the antipsychotic effects of cannabidiol, their pharmacological relevance still needs to be evaluated in clinical trials.

On the one hand cannabidiol may facilitate 5-HT_{1A} receptor mediated serotonergic neurotransmission. In Chinese hamster

ovary (CHO) cells transfected with the human receptor, cannabidiol displaced the 5-HT_{1A} receptor agonist [³H]8-OH-DPAT and increased [³⁵S]GTPγS binding (Russo et al., 2005). On the other hand, cannabidiol did not displace [³H]8-OH-DPAT or stimulate [³⁵S]GTPγS binding in rat brainstem membrane preparations, while it increased the maximal efficacy of 8-OH-DPAT at 100 nmol/L, but not at 1, 10, 31.6 nmol/L or 1 μmol/L (Rock et al., 2012). Interestingly, in mice, the anticonvulsant effect of cannabidiol was prevented by the 5-HT_{1A} receptor antagonist WAY100635 (Gomes et al., 2013; Sonego et al., 2016). However, it remains unclear, whether the improvement of negative symptoms and cognitive deficits by cannabidiol involves 5-HT_{1A} receptor activation, as studies addressing this mechanism are lacking.

In addition, it has been reported that cannabidiol binds to the peroxisome proliferator-activated receptor gamma (PPAR γ) *in vitro* (O'Sullivan et al., 2009; Granja et al., 2012). PPAR γ regulates the expression of genes related to lipid and glucose homeostasis as well as inflammatory responses. Thus, cannabidiol may ameliorate both observed disturbances of glucose metabolism and inflammatory/immune processes in schizophrenic patients (Holmes et al., 2006; Leza et al., 2015; Rajasekaran et al., 2015) by PPAR γ activation.

The activation of transient receptor potential vanilloid type 1 receptors (TRPV1Rs) has also been suggested as mechanism of action, as cannabidiol stimulated TRPV1R in HEK293-cells transiently expressing human (Bisogno et al., 2001; Ligresti et al., 2006; De Petrocellis et al., 2011) or rat TRPV1R (Iannotti et al., 2014). *In vivo*, it has been shown that pretreatment with TRPV1R antagonist capsazepine blocked the ameliorating effect of cannabidiol on MK-801 induced PPI decrease (Long et al., 2006), indicating at least a partial involvement of TRPV1R activation. However, capsazepine also blocks calcium channels (Docherty et al., 1997) and nicotinic cholinergic receptors (Liu and Simon, 1997), thus other mechanisms might contribute to this effect as well. Since TRPV1Rs also mediate the perception of spiciness, it may be expected that receptor stimulation by systemic administration of cannabidiol result in such subjective perceptions, given that this mechanism is relevant in humans at the dosage used. Yet, this side effect has not been reported

TABLE 4 | Published clinical trials and case series evaluating the effects of cannabidiol in schizophrenic patients.

Design	Primary efficacy endpoint	Outcome	Reference
Single case report, open-label, treatment-resistant schizophrenia, up to 1500 mg/day CBD over 4 weeks	Psychotic symptoms (BPRS; IOSPI)	Improvement in a treatment-resistant patient	Zuardi et al., 1995
Open-label, case series (three patients), treatment-resistant schizophrenia, up to 1280 mg/day CBD over 30 days	Psychotic symptoms (BPRS)	One patient showed mild improvement in positive and negative symptoms	Zuardi et al., 2006
Double-blind, active controlled acute trial, single CBD (300 or 600 mg) or placebo administration	Stroop Color Word Test (SCWT)	No beneficial effects of single CBD administration on cognitive performance of schizophrenic patients	Hallak et al., 2010
Double-blind, active-controlled RCT with 42 acute schizophrenic patients, 600–800 mg/day over 4 weeks	Psychotic symptoms (PANSS/BPRS)	Significant clinical improvement compared to baseline on days 14 and 28 for CBD and amisulpride. Superior side-effect profile for CBD compared to amisulpride	Leweke et al., 2012

BPRS, Brief Psychiatric Rating Scale; CBD, cannabidiol; IOSPI, Interactive Observation Scale for Psychiatric Inpatients; PANSS, Positive and Negative Syndrome Scale; RCT, randomized clinical trial.

or observed in clinical trials so far (e.g., Leweke et al., 2000, 2012). To date, evidence for a role of TRPV1R activation in schizophrenia is lacking, but TRPV1Rs may be indirectly involved in schizophrenia via its influence on dopaminergic (Tzavara et al., 2006) and glutamatergic neurotransmission (Fawley et al., 2014).

Furthermore, it has been suggested that cannabidiol targets GPR55 and GPR18 receptors, further subtypes of transient receptor potential receptors (TRPV2, TRPM8, TRPA1), $\alpha 3$ glycine receptors, adenosine receptors, μ and δ opioid receptors, nicotinic acetylcholine receptors, enzymes of the arachidonic acid cascade, ion channels like voltage-gated calcium channels or mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchange, nitric oxide signaling and inflammatory cytokines (McPartland et al., 2014; Ibeas Bih et al., 2015). To date, the antipsychotic properties of cannabidiol cannot be directly linked to these possible targets. However, this does not mean that these mechanisms are not relevant for other medical conditions, e.g., epilepsy (Devinsky et al., 2014).

CONCLUSION

The antipsychotic potential of cannabidiol has been investigated in various behavioral paradigms and different animal models of aspects of schizophrenia. Although the results were partially inconsistent, they indicate that cannabidiol treatment ameliorates impairments of PPI, social interaction behavior and cognition in

REFERENCES

- Adams, R. (1942). Marihuana: harvey lecture. *Bull. N. Y. Acad. Med.* 18, 705–730.
- Adams, R., and Baker, B. R. (1940). Structure of cannabidiol. VII. A method of synthesis of a tetrahydrocannabinol which possesses marihuana activity. *J. Am. Chem. Soc.* 62, 2405–2408. doi: 10.1021/ja01866a041
- Adams, R., Hunt, M., and Clark, J. H. (1940a). Structure of cannabidiol, a product isolated from the marihuana extract of minnesota wild hemp. I. *J. Am. Chem. Soc.* 62, 196–200. doi: 10.1021/ja01858a058
- Adams, R., Loewe, S., Pease, D. C., Cain, C. K., Wearn, R. B., Baker, R. B., et al. (1940b). Structure of cannabidiol. VIII. Position of the double bonds in cannabidiol. Marihuana activity of tetrahydrocannabinols. *J. Am. Chem. Soc.* 62, 2566–2567. doi: 10.1021/ja01866a051
- Adams, R., Wolff, H., Cain, C. K., and Clark, J. H. (1940c). Structure of cannabidiol. V. Position of the alicyclic double bonds. *J. Am. Chem. Soc.* 62, 2215–2219. doi: 10.1021/ja01865a085
- Allentuck, S., and Bowman, K. M. (1942). The psychiatric aspects of marihuana intoxication. *Am. J. Psychiatry* 99, 248–251. doi: 10.1176/ajp.99.2.248
- Almeida, V., Levin, R., Peres, F. F., Niigaki, S. T., Calzavara, M. B., Zuardi, A. W., et al. (2013). Cannabidiol exhibits anxiolytic but not antipsychotic property evaluated in the social interaction test. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 41, 30–35. doi: 10.1016/j.pnpbp.2012.10.024
- Almeida, V., Peres, F. F., Levin, R., Suiama, M. A., Calzavara, M. B., Zuardi, A. W., et al. (2014). Effects of cannabinoid and vanilloid drugs on positive and negative-like symptoms on an animal model of schizophrenia: the SHR strain. *Schizophr. Res.* 153, 150–159. doi: 10.1016/j.schres.2014.01.039
- Arseneault, L., Cannon, M., Poulton, R., Murray, R., Caspi, A., and Moffitt, T. E. (2002). Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. *BMJ* 325, 1212–1213. doi: 10.1136/bmj.325.7374.1212
- Bhattacharyya, S., Fusar-Poli, P., Borgwardt, S., Martin-Santos, R., Nosarti, C., O’carroll, C., et al. (2009). Modulation of mesiotemporal and ventrostriatal function in humans by Delta9-tetrahydrocannabinol: a neural basis for the effects of *Cannabis sativa* on learning and psychosis. *Arch. Gen. Psychiatry* 66, 442–451. doi: 10.1001/archgenpsychiatry.2009.17
- Bisogno, T., Hanus, L., De Petrocellis, L., Tchilibon, S., Ponde, D. E., Brandi, I., et al. (2001). Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br. J. Pharmacol.* 134, 845–852. doi: 10.1038/sj.bjp.0704327
- Breivogel, C. S., Griffin, G., Di Marzo, V., and Martin, B. R. (2001). Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol. Pharmacol.* 60, 155–163.
- Brown, A. S. (2011). The environment and susceptibility to schizophrenia. *Prog. Neurobiol.* 93, 23–58. doi: 10.1016/j.pneurobio.2010.09.003
- Calzavara, M. B., Levin, R., Medrano, W. A., Almeida, V., Sampaio, A. P., Barone, L. C., et al. (2011). Effects of antipsychotics and amphetamine on social behaviors in spontaneously hypertensive rats. *Behav. Brain Res.* 225, 15–22. doi: 10.1016/j.bbr.2011.06.026
- Calzavara, M. B., Medrano, W. A., Levin, R., Kameda, S. R., Andersen, M. L., Tufik, S., et al. (2009). Neuroleptic drugs revert the contextual fear conditioning deficit presented by spontaneously hypertensive rats: a potential animal model of emotional context processing in schizophrenia? *Schizophr. Bull.* 35, 748–759. doi: 10.1093/schbul/sbn006
- Cannon, M., and Jones, P. (1996). Schizophrenia. *J. Neurol. Neurosurg. Psychiatry* 60, 604–613. doi: 10.1136/jnnp.60.6.604
- Corfas, G., Roy, K., and Buxbaum, J. D. (2004). Neuregulin 1-erbB signaling and the molecular/cellular basis of schizophrenia. *Nat. Neurosci.* 7, 575–580. doi: 10.1038/nn1258
- De Petrocellis, L., Ligresti, A., Moriello, A. S., Allara, M., Bisogno, T., Petrosino, S., et al. (2011). Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *Br. J. Pharmacol.* 163, 1479–1494. doi: 10.1111/j.1476-5381.2010.01166.x
- Deiana, S., Watanabe, A., Yamasaki, Y., Amada, N., Kikuchi, T., Stott, C., et al. (2015). MK-801-induced deficits in social recognition in rats: reversal by aripiprazole, but not olanzapine, risperidone, or cannabidiol. *Behav. Pharmacol.* 26, 748–765. doi: 10.1097/fbp.0000000000000178
- Devane, W. A., Dysarz, F. A., Johnson, M. R., Melvin, L. S., and Howlett, A. C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* 34, 605–613.

rodents and rhesus monkeys. In addition, individual treatment attempts as well as one randomized, double-blind clinical study, demonstrated the antipsychotic potential of cannabidiol and its superior side effect profile compared to conventional antipsychotics. In addition, a recently conducted clinical trial investigating cannabidiol as an add-on medication showed promising results, although these have not yet been published in a peer reviewed process. Obviously more clinical trials are needed to substantiate the current findings, and in particular to investigate long-term efficacy and safety in larger cohorts.

However, cannabidiol seems to represent a mechanistically different and less side-effect prone antipsychotic compound for the treatment of schizophrenia, even though the underlying pharmacological mechanisms are still under debate. Nevertheless, the association between increased anandamide levels and reduced psychotic symptoms in schizophrenic patients treated with cannabidiol, points to a potentially new antipsychotic mechanism of action involving anandamide.

AUTHOR CONTRIBUTIONS

CR, JKM, BL, and FML performed the review of the literature and CR and FML drafted the manuscript with input from JKM and BL.

- Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., et al. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946–1949. doi: 10.1126/science.1470919
- Devincky, O., Cilio, M. R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., et al. (2014). Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia* 55, 791–802. doi: 10.1111/epi.12631
- Di Forti, M., Morgan, C., Dazzan, P., Pariante, C., Mondelli, V., Marques, T. R., et al. (2009). High-potency cannabis and the risk of psychosis. *Br. J. Psychiatry* 195, 488–491. doi: 10.1192/bjp.bp.109.064220
- Di Forti, M., Sallis, H., Allegri, F., Trotta, A., Ferraro, L., Stilo, S. A., et al. (2014). Daily use, especially of high-potency cannabis, drives the earlier onset of psychosis in cannabis users. *Schizophr. Bull.* 40, 1509–1517. doi: 10.1093/schbul/sbt181
- Docherty, R. J., Yeats, J. C., and Piper, A. S. (1997). Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurones in culture. *Br. J. Pharmacol.* 121, 1461–1467. doi: 10.1038/sj.bjp.0701272
- D'Souza, D. C., Abi-Saab, W. M., Madonick, S., Forselius-Bielen, K., Doersch, A., Braley, G., et al. (2005). Delta-9-tetrahydrocannabinol effects in schizophrenia: implications for cognition, psychosis, and addiction. *Biol. Psychiatry* 57, 594–608. doi: 10.1016/j.biopsych.2004.12.006
- D'Souza, D. C., Perry, E., Macdougall, L., Ammerman, Y., Cooper, T., Wu, Y. T., et al. (2004). The psychotomimetic effects of intravenous delta-9-tetrahydrocannabinol in healthy individuals: implications for psychosis. *Neuropsychopharmacology* 29, 1558–1572. doi: 10.1038/sj.npp.1300496
- Elmes, M. W., Kaczocha, M., Berger, W. T., Leung, K., Ralph, B. P., Wang, L., et al. (2015). Fatty acid-binding proteins (FABPs) are intracellular carriers for Delta9-tetrahydrocannabinol (THC) and cannabidiol (CBD). *J. Biol. Chem.* 290, 8711–8721. doi: 10.1074/jbc.M114.618447
- Emrich, H. M., Leweke, F. M., and Schneider, U. (1997). Towards a cannabinoid hypothesis of schizophrenia: cognitive impairments due to dysregulation of the endogenous cannabinoid system. *Pharmacol. Biochem. Behav.* 56, 803–807. doi: 10.1016/S0091-3057(96)00426-1
- Fadda, P., Robinson, L., Fratta, W., Pertwee, R. G., and Riedel, G. (2004). Differential effects of THC- or CBD-rich cannabis extracts on working memory in rats. *Neuropharmacology* 47, 1170–1179. doi: 10.1016/j.neuropharm.2004.08.009
- Fadda, P., Robinson, L., Fratta, W., Pertwee, R. G., and Riedel, G. (2006). Scopolamine and MK801-induced working memory deficits in rats are not reversed by CBD-rich cannabis extracts. *Behav. Brain Res.* 168, 307–311. doi: 10.1016/j.bbr.2005.11.022
- Fawley, J. A., Hofmann, M. E., and Andresen, M. C. (2014). Cannabinoid 1 and transient receptor potential vanilloid 1 receptors discretely modulate evoked glutamate separately from spontaneous glutamate transmission. *J. Neurosci.* 34, 8324–8332. doi: 10.1523/jneurosci.0315-14.2014
- Freedman, R. (2003). Schizophrenia. *N. Engl. J. Med.* 349, 1738–1749. doi: 10.1056/NEJMra035458
- Fu, J., Bottegoni, G., Sasso, O., Bertorelli, R., Rocchia, W., Masetti, M., et al. (2012). A catalytically silent FAAH-1 variant drives anandamide transport in neurons. *Nat. Neurosci.* 15, 64–69. doi: 10.1038/nn.2986
- Fusar-Poli, P., Crippa, J. A., Bhattacharyya, S., Borgwardt, S. J., Allen, P., Martin-Santos, R., et al. (2009). Distinct Effects of {Delta}9-Tetrahydrocannabinol and Cannabidiol on Neural Activation During Emotional Processing. *Arch. Gen. Psychiatry* 66, 95–105. doi: 10.1001/archgenpsychiatry.2008.519
- Gage, S. H., Hickman, M., and Zammit, S. (2016). Association between cannabis and psychosis: epidemiologic evidence. *Biol. Psychiatry* 79, 549–556. doi: 10.1016/j.biopsych.2015.08.001
- Gaoni, Y., and Mechoulam, R. (1964). Isolation structure + partial synthesis of active constituent of hashish. *J. Am. Chem. Soc.* 86, 1646–1647. doi: 10.1021/ja01062a046
- Gomes, F. V., Del Bel, E. A., and Guimaraes, F. S. (2013). Cannabidiol attenuates catalepsy induced by distinct pharmacological mechanisms via 5-HT1A receptor activation in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 46, 43–47. doi: 10.1016/j.pnpbp.2013.06.005
- Gomes, F. V., Issy, A. C., Ferreira, F. R., Viveros, M. P., Del Bel, E. A., and Guimaraes, F. S. (2015a). Cannabidiol attenuates sensorimotor gating disruption and molecular changes induced by chronic antagonism of NMDA receptors in mice. *Int. J. Neuropsychopharmacol.* 18:yu041. doi: 10.1093/ijnp/pyu041
- Gomes, F. V., Llorente, R., Del Bel, E. A., Viveros, M. P., Lopez-Gallardo, M., and Guimaraes, F. S. (2015b). Decreased glial reactivity could be involved in the antipsychotic-like effect of cannabidiol. *Schizophr. Res.* 164, 155–163. doi: 10.1016/j.schres.2015.01.015
- Granja, A. G., Carrillo-Salinas, F., Pagani, A., Gomez-Canas, M., Negri, R., Navarrete, C., et al. (2012). A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis. *J. Neuroimmune Pharmacol.* 7, 1002–1016. doi: 10.1007/s11481-012-9399-3
- Gururajan, A., Taylor, D. A., and Malone, D. T. (2011). Effect of cannabidiol in a MK-801-rodent model of aspects of schizophrenia. *Behav. Brain Res.* 222, 299–308. doi: 10.1016/j.bbr.2011.03.053
- Gururajan, A., Taylor, D. A., and Malone, D. T. (2012). Cannabidiol and clozapine reverse MK-801-induced deficits in social interaction and hyperactivity in Sprague-Dawley rats. *J. Psychopharmacol.* 26, 1317–1332. doi: 10.1177/0269881112441865
- GW Pharmaceuticals plc (2015). *GW Pharmaceuticals Announces Positive Proof of Concept Data in Schizophrenia*. Available at: <http://globenewswire.com/news-release/2015/09/15/768364/10149367/en/GW-Pharmaceuticals-Announces-Positive-Proof-of-Concept-Data-in-Schizophrenia.html?f=22&ftc=3&fvtv=4000> [accessed June 28, 2016].
- Hallak, J. E., Machado-De-Sousa, J. P., Crippa, J. A., Sanches, R. F., Trzesniak, C., Chaves, C., et al. (2010). Performance of schizophrenic patients in the Stroop Color Word Test and electrodermal responsiveness after acute administration of cannabidiol (CBD). *Rev. Bras. Psiquiatr.* 32, 56–61. doi: 10.1590/S1516-44462010000100011
- Hanson, E., Healey, K., Wolf, D., and Kohler, C. (2010). Assessment of pharmacotherapy for negative symptoms of schizophrenia. *Curr. Psychiatry Rep.* 12, 563–571. doi: 10.1007/s11920-010-0148-0
- Holmes, E., Tsang, T. M., Huang, J. T., Leweke, F. M., Koethe, D., Gerth, C. W., et al. (2006). Metabolic profiling of CSF: evidence that early intervention may impact on disease progression and outcome in schizophrenia. *PLoS Med.* 3:e327. doi: 10.1371/journal.pmed.0030327
- Hopkins, M. E., Sharma, M., Evans, G. C., and Bucci, D. J. (2009). Voluntary physical exercise alters attentional orienting and social behavior in a rat model of attention-deficit/hyperactivity disorder. *Behav. Neurosci.* 123, 599–606. doi: 10.1037/a0015632
- Howlett, A. C. (1984). Inhibition of neuroblastoma adenylate cyclase by cannabinoid and nantradol compounds. *Life Sci.* 35, 1803–1810. doi: 10.1016/0024-3205(84)90278-9
- Iannotti, F. A., Hill, C. L., Leo, A., Alhusaini, A., Soubrane, C., Mazzarella, E., et al. (2014). Nonpsychotropic plant cannabinoids, cannabidiavarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. *ACS Chem. Neurosci.* 5, 1131–1141. doi: 10.1021/cn5000524
- Ibeas Bih, C., Chen, T., Nunn, A. V., Bazelon, M., Dallas, M., and Whalley, B. J. (2015). Molecular targets of cannabidiol in neurological disorders. *Neurotherapeutics* 12, 699–730. doi: 10.1007/s13311-015-0377-3
- Jones, N. A., Hill, A. J., Smith, I., Bevan, S. A., Williams, C. M., Whalley, B. J., et al. (2010). Cannabidiol displays antiepileptiform and antiseizure properties in vitro and in vivo. *J. Pharmacol. Exp. Ther.* 332, 569–577. doi: 10.1124/jpet.109.159145
- Jones, P. G., Falvello, L., Kennard, O., Sheldrick, G. M., and Mechoulam, R. (1977). Cannabidiol. *Acta Crystallogr. B* 33, 3211–3214. doi: 10.1107/S0567740877010577
- Kaczocha, M., Glaser, S. T., and Deutsch, D. G. (2009). Identification of intracellular carriers for the endocannabinoid anandamide. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6375–6380. doi: 10.1073/pnas.0901515106
- Kaczocha, M., Vivieca, S., Sun, J., Glaser, S. T., and Deutsch, D. G. (2012). Fatty acid-binding proteins transport N-acylethanolamines to nuclear receptors and are targets of endocannabinoid transport inhibitors. *J. Biol. Chem.* 287, 3415–3424. doi: 10.1074/jbc.M111.304907
- Kahn, R. S., Sommer, I. E., Murray, R. M., Meyer-Lindenberg, A., Weinberger, D. R., Cannon, T. D., et al. (2015). Schizophrenia. *Nat. Rev. Dis. Primers* 1:15067. doi: 10.1038/nrdp.2015.67

- Kim, J. S., Kornhuber, H. H., Schmid-Burgk, W., and Holzmuller, B. (1980). Low cerebrospinal fluid glutamate in schizophrenic patients and a new hypothesis on schizophrenia. *Neurosci. Lett.* 20, 379–382. doi: 10.1016/0304-3940(80)90178-0
- Koethe, D., Gerth, C. W., Neatby, M. A., Haensel, A., Thies, M., Schneider, U., et al. (2006). Disturbances of visual information processing in early states of psychosis and experimental delta-9-tetrahydrocannabinol altered states of consciousness. *Schizophr. Res.* 88, 142–150. doi: 10.1016/j.schres.2006.07.023
- Koethe, D., Hoyer, C., and Leweke, F. M. (2009). The endocannabinoid system as a target for modelling psychosis. *Psychopharmacology (Berl.)* 206, 551–561. doi: 10.1007/s00213-009-1591-7
- Laprairie, R. B., Bagher, A. M., Kelly, M. E., and Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br. J. Pharmacol.* 172, 4790–4805. doi: 10.1111/bph.13250
- Laprairie, R. B., Bagher, A. M., Kelly, M. E., Dupre, D. J., and Denovan-Wright, E. M. (2014). Type 1 cannabinoid receptor ligands display functional selectivity in a cell culture model of striatal medium spiny projection neurons. *J. Biol. Chem.* 289, 24845–24862. doi: 10.1074/jbc.M114.557025
- Leucht, S., Pitschel-Walz, G., Engel, R. R., and Kissling, W. (2002). Amisulpride, an unusual “atypical” antipsychotic: a meta-analysis of randomized controlled trials. *Am. J. Psychiatry* 159, 180–190. doi: 10.1176/appi.ajp.159.2.180
- Levin, R., Calzavara, M. B., Santos, C. M., Medrano, W. A., Niigaki, S. T., and Abilio, V. C. (2011). Spontaneously hypertensive rats (SHR) present deficits in prepulse inhibition of startle specifically reverted by clozapine. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1748–1752. doi: 10.1016/j.pnpbp.2011.06.003
- Levin, R., Peres, F. F., Almeida, V., Calzavara, M. B., Zuardi, A. W., Hallak, J. E., et al. (2014). Effects of cannabinoid drugs on the deficit of prepulse inhibition of startle in an animal model of schizophrenia: the SHR strain. *Front. Pharmacol.* 5:10. doi: 10.3389/fphar.2014.00010
- Leweke, F. M. (2012). Anandamide dysfunction in prodromal and established psychosis. *Curr. Pharm. Des.* 18, 5188–5193. doi: 10.2174/1381612128028843
- Leweke, F. M., Mueller, J. K., Lange, B., and Rohleder, C. (2016). Therapeutic potential of cannabinoids in psychosis. *Biol. Psychiatry* 79, 604–612. doi: 10.1016/j.biopsych.2015.11.018
- Leweke, F. M., Piomelli, D., Pahlisch, F., Muhl, D., Gerth, C. W., Hoyer, C., et al. (2012). Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl. Psychiatry* 2:e94. doi: 10.1038/tp.2012.15
- Leweke, F. M., Schneider, U., Radwan, M., Schmidt, E., and Emrich, H. M. (2000). Different effects of nabilone and cannabidiol on binocular depth inversion in man. *Pharmacol. Biochem. Behav.* 66, 175–181. doi: 10.1016/S0091-3057(00)00201-X
- Leweke, F. M., Schneider, U., Thies, M., Münte, T. F., and Emrich, H. M. (1999). Effects of synthetic Δ9-tetrahydrocannabinol on binocular depth inversion of natural and artificial objects in man. *Psychopharmacology* 142, 230–235. doi: 10.1007/s002130050884
- Leza, J. C., Garcia-Bueno, B., Bioque, M., Arango, C., Parellada, M., Do, K., et al. (2015). Inflammation in schizophrenia: a question of balance. *Neurosci. Biobehav. Rev.* 55, 612–626. doi: 10.1016/j.neubiorev.2015.05.014
- Ligresti, A., Moriello, A. S., Starowicz, K., Matias, I., Pisanti, S., De Petrocellis, L., et al. (2006). Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J. Pharmacol. Exp. Ther.* 318, 1375–1387. doi: 10.1124/jpet.106.105247
- Liu, L., and Simon, S. A. (1997). Capsazepine, a vanilloid receptor antagonist, inhibits nicotinic acetylcholine receptors in rat trigeminal ganglia. *Neurosci. Lett.* 228, 29–32. doi: 10.1016/S0304-3940(97)00358-3
- Long, L. E., Chesworth, R., Huang, X. F., McGregor, I. S., Arnold, J. C., and Karl, T. (2010). A behavioural comparison of acute and chronic Delta9-tetrahydrocannabinol and cannabidiol in C57BL/6JArc mice. *Int. J. Neuropsychopharmacol.* 13, 861–876. doi: 10.1017/S1461145709990605
- Long, L. E., Chesworth, R., Huang, X. F., Wong, A., Spiro, A., McGregor, I. S., et al. (2012). Distinct neurobehavioural effects of cannabidiol in transmembrane domain neuregulin 1 mutant mice. *PLoS ONE* 7:e34129. doi: 10.1371/journal.pone.0034129
- Long, L. E., Malone, D. T., and Taylor, D. A. (2006). Cannabidiol reverses MK-801-induced disruption of prepulse inhibition in mice. *Neuropsychopharmacology* 31, 795–803. doi: 10.1038/sj.npp.1300838
- Lu, H.-C., and Mackie, K. (2016). An introduction to the endogenous cannabinoid system. *Biol. Psychiatry* 79, 516–525. doi: 10.1016/j.biopsych.2015.07.028
- Lutz, B., Marsicano, G., Maldonado, R., and Hillard, C. J. (2015). The endocannabinoid system in guarding against fear, anxiety and stress. *Nat. Rev. Neurosci.* 16, 705–718. doi: 10.1038/nrn4036
- Malone, D. T., Jongejan, D., and Taylor, D. A. (2009). Cannabidiol reverses the reduction in social interaction produced by low dose Delta(9)-tetrahydrocannabinol in rats. *Pharmacol. Biochem. Behav.* 93, 91–96. doi: 10.1016/j.pbb.2009.04.010
- Mason, O., Morgan, C. J., Dhiman, S. K., Patel, A., Parti, N., Patel, A., et al. (2009). Acute cannabis use causes increased psychotomimetic experiences in individuals prone to psychosis. *Psychol. Med.* 39, 951–956. doi: 10.1017/s0033291708004741
- Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346, 561–564. doi: 10.1038/346561a0
- McPartland, J. M., Duncan, M., Di Marzo, V., and Pertwee, R. (2014). Are cannabidiol and Delta- Δ -tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. *Br. J. Pharmacol.* 172, 737–753. doi: 10.1111/bph.12944
- Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., et al. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50, 83–90. doi: 10.1016/0006-2952(95)00109-D
- Mechoulam, R., Shani, A., Edery, H., and Grunfeld, Y. (1970). Chemical basis of hashish activity. *Science* 169, 611–612. doi: 10.1126/science.169.3945.611
- Mechoulam, R., and Shvo, Y. (1963). Hashish—I: the structure of cannabidiol. *Tetrahedron* 19, 2073–2078. doi: 10.1016/0040-4020(63)85022-x
- Moore, T. H., Zammit, S., Lingford-Hughes, A., Barnes, T. R., Jones, P. B., Burke, M., et al. (2007). Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. *Lancet* 370, 319–328. doi: 10.1016/S0140-6736(07)61162-3
- Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365, 61–65. doi: 10.1038/365061a0
- Newell, K. A., Karl, T., and Huang, X. F. (2013). A neuregulin 1 transmembrane domain mutation causes imbalanced glutamatergic and dopaminergic receptor expression in mice. *Neuroscience* 248, 670–680. doi: 10.1016/j.neuroscience.2013.06.037
- O’Sullivan, S. E., Sun, Y., Bennett, A. J., Randall, M. D., and Kendall, D. A. (2009). Time-dependent vascular actions of cannabidiol in the rat aorta. *Eur. J. Pharmacol.* 612, 61–68. doi: 10.1016/j.ejphar.2009.03.010
- Pedrazzi, J. F., Issy, A. C., Gomes, F. V., Guimaraes, F. S., and Del-Bel, E. A. (2015). Cannabidiol effects in the prepulse inhibition disruption induced by amphetamine. *Psychopharmacology (Berl.)* 232, 3057–3065. doi: 10.1007/s00213-015-3945-7
- Pertwee, R. G., Ross, R. A., Craib, S. J., and Thomas, A. (2002). (-)-Cannabidiol antagonizes cannabinoid receptor agonists and noradrenaline in the mouse vas deferens. *Eur. J. Pharmacol.* 456, 99–106. doi: 10.1016/S0014-2999(02)02624-9
- Petitet, F., Jeantaud, B., Rebaud, M., Imperato, A., and Dubroeucq, M. C. (1998). Complex pharmacology of natural cannabinoids: evidence for partial agonist activity of delta9-tetrahydrocannabinol and antagonist activity of cannabidiol on rat brain cannabinoid receptors. *Life Sci.* 63, L1–L6. doi: 10.1016/S0024-3205(98)00238-0
- Rajasekaran, A., Venkatasubramanian, G., Berk, M., and Debnath, M. (2015). Mitochondrial dysfunction in schizophrenia: pathways, mechanisms and implications. *Neurosci. Biobehav. Rev.* 48, 10–21. doi: 10.1016/j.neubiorev.2014.11.005
- Rakhshan, F., Day, T. A., Blakely, R. D., and Barker, E. L. (2000). Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. *J. Pharmacol. Exp. Ther.* 292, 960–967.
- Rock, E. M., Bolognini, D., Limebeer, C. L., Cascio, M. G., Anavi-Goffer, S., Fletcher, P. J., et al. (2012). Cannabidiol, a non-psychotropic component of cannabis, attenuates vomiting and nausea-like behaviour via indirect agonism of 5-HT(1A) somatodendritic autoreceptors in the dorsal raphe nucleus. *Br. J. Pharmacol.* 165, 2620–2634. doi: 10.1111/j.1476-5381.2011.01621.x
- Rohleder, C., Wiedermann, D., Neumaier, B., Drzezga, A., Timmermann, L., Graf, R., et al. (2016). The functional networks of prepulse inhibition: neuronal

- connectivity analysis based on FDG-PET in awake and unrestrained rats. *Front. Behav. Neurosci.* 10:148. doi: 10.3389/fnbeh.2016.00148
- Rubino, T., and Parolaro, D. (2016). The impact of exposure to cannabinoids in adolescence: insights from animal models. *Biological Psychiatry* 79, 578–585. doi: 10.1016/j.biopsych.2015.07.024
- Russo, E. B., Burnett, A., Hall, B., and Parker, K. K. (2005). Agonistic properties of cannabidiol at 5-HT1a receptors. *Neurochem. Res.* 30, 1037–1043. doi: 10.1007/s11064-005-6978-1
- Schwarcz, G., Karajgi, B., and McCarthy, R. (2009). Synthetic Δ -9-tetrahydrocannabinol (dronabinol) can improve the symptoms of schizophrenia. *J. Clin. Psychopharmacol.* 29, 255–258. doi: 10.1097/JCP.0b013e3181a6bc3b
- Sherif, M., Radhakrishnan, R., D'souza, D. C., and Ranganathan, M. (2016). Human laboratory studies on cannabinoids and psychosis. *Biol. Psychiatry* 79, 526–538. doi: 10.1016/j.biopsych.2016.01.011
- Showalter, V. M., Compton, D. R., Martin, B. R., and Abood, M. E. (1996). Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. *J. Pharmacol. Exp. Ther.* 278, 989–999.
- Snyder, M. A., and Gao, W. J. (2013). NMDA hypofunction as a convergence point for progression and symptoms of schizophrenia. *Front. Cell Neurosci.* 7:31. doi: 10.3389/fncel.2013.00031
- Sonego, A. B., Gomes, F. V., Del Bel, E. A., and Guimaraes, F. S. (2016). Cannabidiol attenuates haloperidol-induced catalepsy and c-Fos protein expression in the dorsolateral striatum via 5-HT1A receptors in mice. *Behav. Brain Res.* 309, 22–28. doi: 10.1016/j.bbr.2016.04.042
- Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., et al. (2002). Neuregulin 1 and susceptibility to schizophrenia. *Am. J. Hum. Genet.* 71, 877–892. doi: 10.1086/342734
- Stella, N., Schweitzer, P., and Piomelli, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388, 773–778. doi: 10.1038/42015
- Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., and Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *Br. J. Pharmacol.* 150, 613–623. doi: 10.1038/sj.bjp.0707133
- Thomas, B. F., Gilliam, A. F., Burch, D. F., Roche, M. J., and Seltzman, H. H. (1998). Comparative receptor binding analyses of cannabinoid agonists and antagonists. *J. Pharmacol. Exp. Ther.* 285, 285–292.
- Todd, A. R. (1946). Hashish. *Experientia* 2, 55–60. doi: 10.1007/bf02163886
- Tzavara, E. T., Li, D. L., Moutsimilli, L., Bisogno, T., Di Marzo, V., Phebus, L. A., et al. (2006). Endocannabinoids activate transient receptor potential vanilloid 1 receptors to reduce hyperdopaminergia-related hyperactivity: therapeutic implications. *Biol. Psychiatry* 59, 508–515. doi: 10.1016/j.biopsych.2005.08.019
- van den Buuse, M. (2004). Prepulse inhibition of acoustic startle in spontaneously hypertensive rats. *Behav. Brain Res.* 154, 331–337. doi: 10.1016/j.bbr.2004.02.021
- van Ree, J. M., Niesink, R. J., and Nir, I. (1984). delta 1-Tetrahydrocannabinol but not cannabidiol reduces contact and aggressive behavior of rats tested in dyadic encounters. *Psychopharmacology (Berl.)* 84, 561–565. doi: 10.1007/BF00431467
- Watanabe, K., Kayano, Y., Matsunaga, T., Yamamoto, I., and Yoshimura, H. (1996). Inhibition of anandamide amidase activity in mouse brain microsomes by cannabinoids. *Biol. Pharm. Bull.* 19, 1109–1111. doi: 10.1248/bpb.19.1109
- Wollner, H. J., Matchett, J. R., Levine, J., and Loewe, S. (1942). Isolation of a physiologically active tetrahydrocannabinol from *Cannabis sativa* Resin. *J. Am. Chem. Soc.* 64, 26–29. doi: 10.1021/ja01253a008
- Wong, A. H. C., and Van Tol, H. H. M. (2003). Schizophrenia: from phenomenology to neurobiology. *Neurosci. Biobehav. Rev.* 27, 269–306. doi: 10.1016/S0149-7634(03)00035-6
- Wright, M. J. Jr., Vandewater, S. A., and Taffe, M. A. (2013). Cannabidiol attenuates deficits of visuospatial associative memory induced by Delta(9) tetrahydrocannabinol. *Br. J. Pharmacol.* 170, 1365–1373. doi: 10.1111/bph.12199
- Zammit, S., Allebeck, P., Andreasson, S., Lundberg, I., and Lewis, G. (2002). Self reported cannabis use as a risk factor for schizophrenia in Swedish conscripts of 1969: historical cohort study. *BMJ* 325:1199. doi: 10.1136/bmj.325.7374.1199
- Zuardi, A. W., Hallak, J. E., Dursun, S. M., Morais, S. L., Sanches, R. F., Musty, R. E., et al. (2006). Cannabidiol monotherapy for treatment-resistant schizophrenia. *J. Psychopharmacol.* 20, 683–686. doi: 10.1177/0269881106060967
- Zuardi, A. W., Morais, S. L., Guimarães, F. S., and Mechoulam, R. (1995). Antipsychotic Effect of Cannabidiol. *J. Clin. Psychiatry* 56, 485–486.

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The Emerging Role for Zinc in Depression and Psychosis

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Zinc participation is essential for all physiological systems, including neural functioning, where it participates in a myriad of cellular processes. Converging clinical, molecular, and genetic discoveries illuminate key roles for zinc homeostasis in association with clinical depression and psychosis which are not yet well appreciated at the clinical interface. Intracellular deficiency may arise from low circulating zinc levels due to dietary insufficiency, or impaired absorption from aging or medical conditions, including alcoholism. A host of medications commonly administered to psychiatric patients, including anticonvulsants, oral medications for diabetes, hormones, antacids, anti-inflammatories and others also impact zinc absorption. Furthermore, inefficient genetic variants in zinc transporter molecules that transport the ion across cellular membranes impede its action even when circulating zinc concentrations are in the normal range. Well powered clinical studies have shown beneficial effects of supplemental zinc in depression and it is important to pursue research using zinc as a potential therapeutic option for psychosis as well. Meta-analyses support the adjunctive use of zinc in major depression and a single study now supports zinc for psychotic symptoms. This manuscript reviews the biochemistry and bench top evidence on putative molecular mechanisms of zinc as a psychiatric treatment.

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INTRODUCTION

Zinc is an essential trace element required by all organisms for various biological processes. Its general actions are well reviewed, as briefly described in this paper and several excellent reviews (Marger et al., 2014; Nowak, 2015; Prakash et al., 2015), but a role for zinc homeostasis with respect to clinical depression and psychosis is not well appreciated by psychiatrists. Zinc is the second most abundant divalent cation after calcium and is a component in hundreds of enzymes and proteins. Playing a determinant role in over 300 biological processes, zinc is required for proper cellular function, including DNA replication, transcription, protein synthesis, maintenance of cell membranes, cellular transport, as well as endocrine, immunological and neuronal systems (Prasad, 1995; Nowak et al., 2003b). Dysregulation of zinc is associated with reduced immunological functioning, stunted tissue regeneration, growth retardation, gastrointestinal complaints, and ocular and sensory disturbances. Zinc insufficiency is also associated with neuropsychiatric manifestations that can present as altered behavior and cognition, reduced ability to learn, and depression (Nowak, 2001; Nowak et al., 2003a; Howland and Wang, 2008). The purpose of this review is to increase the appreciation of zinc with respect to psychiatric disorders.

The connection between zinc dysregulation and psychiatric illness is being continually clarified. Within the limbic system, zinc is predominately sequestered within glutamatergic neurons, typically acting as an inhibitory modulator at the NMDA glutamate receptor (Frederickson et al., 2000; Paz et al., 2008; Szewczyk et al., 2010). In addition to antagonism at NMDAR (*N*-methyl-d-aspartate receptor), and beyond the scope of this review, other zinc actions that may contribute to the prevention or presumed amelioration of depression include agonistic properties for AMPAR (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and complex interactions with 5-HT1A receptors (agonism and antagonist and pre and postsynaptic). In addition, zinc is an agonist for GPR39 activation and for mTOR (mammalian target of rapamycin) (Szewczyk et al., 2015). Inhibitory actions at nAChR (nicotinic cholinergic receptor), GSK3 β (glycogen synthase kinase 3beta) and NOS (nitric oxide synthase) are also pertinent to mechanisms of depression (Nowak, 2015).

ZINC HOMEOSTASIS AND REGULATION

Zinc is an essential element obtained from dietary sources, particularly red meat, poultry, fish, and dairy. Tight regulation of zinc concentrations is essential as dietary intake of zinc varies as much as 15-fold. Its concentration is normally maintained by easy absorption in the digestive tract, but insufficiency can be related to dietary habits, aging, medical comorbidities (including alcoholism and H Pylori) and numerous common medications (including antacids, diuretics, anticonvulsants, anti-retrovirals, hormones, steroids, other anti-inflammatories, and numerous cardiovascular medications). Zinc is present in all body tissues, having higher concentrations within muscle and bone. The vast majority of zinc is protein bound and not in its free form with blood levels normally maintained between 9 and 17 μ M.

Zinc is also tightly regulated in the brain, with higher levels in the amygdala, hippocampus and cortex, predominantly located within glutamatergic neurons known as “zinc enriched neurons” (ZEN) (Nowak et al., 2003b; Prakash et al., 2015). About 15% of all zinc in the CNS is in vesicular form within ZENs (Nowak, 2001). All ZENs are glutamatergic, although not all neurons that release glutamate contain zinc (Nowak et al., 2003b). With neuronal stimulation, zinc is released into the synapse and the cytosol concentration of zinc transiently reaches micromolar ranges, a level at which it physiologically regulates many synaptic processes.

ZINC DYSREGULATION AND DEPRESSION

There appears to be a correlation between zinc dysregulation in both neurological and psychiatric illnesses such as Parkinson’s disease, Alzheimer’s, Amyotrophic Lateral Sclerosis, Down Syndrome, attention deficit disorder hyperactivity and depression (Grabrucker et al., 2011). The association to depression may account for its largest psychiatric impact. Major depressive disorder causes significant morbidity and mortality affecting approximately 350 million people worldwide (WHO,

2012; Vashum et al., 2014). The World Health Organization predicts that by 2020 major depressive disorder will be the second leading cause of morbidity and mortality after ischemic heart disease (Mathews et al., 2012; WHO, 2012; Sowa-Kućma et al., 2013). Associated with decreased quality of life, depression results in over one million deaths by suicide per year. Multiple studies demonstrate reduced serum zinc levels in depressed individuals compared to healthy controls with a meta-analysis demonstrating depressive symptomatology at zinc serum levels of 1.8 μ M or less (Swardfager et al., 2013). Consistent with dose effects and causality, an inverse relationship was observed between lower zinc levels and higher Hamilton Depression Rating Scale scores (Maes et al., 1994; Liuzzi and Cousins, 2004). Clinical studies show lower serum zinc levels in groups of cases with major depression (McLoughlin and Hodge, 1990; Maes et al., 1994; Siwek et al., 2010; Swardfager et al., 2013). Several randomized controlled trials support the effectiveness of zinc as adjunctive therapy for improving mood in both depressed and healthy individuals (Nowak et al., 2003a; Siwek et al., 2009; Sawada and Yokoi, 2010; Lai et al., 2012; Ranjbar et al., 2014; Solati et al., 2015). Zinc supplementation also improved mood in cases with treatment-resistant depression in several studies (Nowak et al., 2003a; Siwek et al., 2009). Recent meta-analyses demonstrated lower serum zinc levels in groups of cases with depression compared to controls, with significant inverse associations between depression severity scores and serum zinc levels and also demonstrating larger effect sizes in hospitalized cases (Swardfager et al., 2013, 2015). Studies on zinc deficiency and zinc supplementation in relation to depression are summarized in Table 1.

The relationship between zinc and depression may be linked to its action on brain-derived neurotropic factor (BDNF), a growth factor promoting neurogenesis and differentiation. The hippocampus is a site of life long neurogenesis, with decreased BDNF expression and diminished neuro/synaptogenesis accompanying episodes of major depression. Rodents fed a diet deficient in zinc demonstrated reduced zinc levels in the hippocampal vesicles, an area of the brain that normally has higher concentrations, with accompanying decreases in progenitor cells and immature neurons. The contrary was observed with zinc-enriched diets, with an increase in progenitor cells (Nowak et al., 2003a; Suh et al., 2009). Zinc interacts with BDNF levels and its deficiency thereby decreases neurogenesis and depressive symptoms ensue. An inverse correlation was observed between serum BDNF levels and depression severity in one clinical trial (Ranjbar et al., 2014). The exact relationship between BDNF and zinc is being elucidated, although a possible role for zinc in synaptogenesis includes its role in transactivating TrkB, a crucial neurotrophic factor (Huang et al., 2008). Zinc’s activation of TrkB is independent of BDNF activation and produces hippocampal mossy fiber potentiation. Further studies with mice suggest that not only zinc is required for mossy fiber potentiation, it can also inhibit it postsynaptically. This suggests that zinc may be required as a dual control to maintain homeostasis (Pan et al., 2011). Zinc thus regulates synaptic plasticity, aiding in neurogenesis and preventing pathological states.

TABLE 1 | Studies relating depression to zinc levels.

Author, Year	Model	Subjects	Measure	Results
Doboszewska et al., 2015	Zinc deficiency, depression	Rodent	FST, Western blot	Normalization of behavior, serum zinc level, and hippocampal GluN1, GluN2A, GluN2B, p-CREB after fluoxetine administration in zinc-deficient mice
Mlyniec et al., 2014b	Zinc deficiency, depression	Rodents + suicide victims	FST, Western blot	Increased immobility time; downregulation of GPR39 receptor, CREB/BDNF/TrkB in zinc-deficient rodents and suicide victims
Mlyniec et al., 2014a	Depression	Rodent	FST, TST	Increased immobility time in GPR39 knockout mice and downregulation of hippocampal CREB/BDNF
Mlyniec et al., 2015	Depression	Rodent	FST	Antidepressant effect only with NMDA antagonists (but not monoamine-based antidepressants) in GPR39 knockout
Nowak et al., 2003a	RCT, zinc supplementation	Unipolar depression on TCA or SSRI (<i>n</i> = 14)	HADRS	Reduced depression scores after 6 and 12 weeks of zinc supplementation compared to placebo
Nowak et al., 2003b	Suicide	Suicide victims (<i>n</i> = 10)	Radioligand binding assay	Reduced potency (26% decrease) of zinc to inhibit MK-801 (an NMDA antagonist) binding to NMDA receptors in hippocampal tissue of suicide victims
Ranjbar et al., 2014	RCT, zinc supplementation	Major depression on antidepressant (<i>n</i> = 37)	HDRS; serum IL-6, TNF-alpha, BDNF	Significantly reduced HDRS after 12 weeks zinc supplementation, but no change in inflammatory cytokines or BDNF
Sawada and Yokoi, 2010	RCT, zinc supplementation	Healthy premenopausal women (<i>n</i> = 30)	Anger-hostility and depression-dejection scores in Profile of Mood States (POMS)	Improvement in anger-hostility and depression-dejection scores after 10 weeks zinc supplementation + MV vs. MV alone
Siwek et al., 2009, 2010	RCT, zinc supplementation	Unipolar depression on imipramine therapy (<i>n</i> = 60)	HDRS, BDI, CGI, MADRS	Reduced depression scores only in treatment-resistant patients, but not in antidepressant responders
Solati et al., 2015	RCT, zinc supplementation	Obese or overweight, regardless of depression status	BDI; serum zinc, BDNF	Higher serum zinc and BDNF and greater reduction in BDI score in zinc-supplemented group; BDI change only in depressed subgroup; negative correlation between serum BDNF and depression; positive correlation between serum BDNF and zinc levels at baseline
Sowa-Kućma et al., 2013	Suicide	Suicide victims (<i>n</i> = 17)	Radioligand binding assay	Reduced potency (29% decrease) of zinc to inhibit MK-801 (an NMDA antagonist) binding to NMDA receptors in hippocampal tissue of suicide victims
Swardfager et al., 2013	Meta-analysis, depression	Human, Depressed vs. control	Serum zinc levels	Zinc concentrations approximately 1.85 umol/L lower in depressed subjects than control subjects
Szewczyk et al., 2009	Zinc supplementation	Rodent	FST	Decreased immobility time with zinc; effect diminished by NMDA administration and AMPAR antagonist
Szewczyk et al., 2010	Zinc supplementation	Rodent	FST	Decreased immobility time with combined zinc and citalopram or fluoxetine at sub-effective doses; effect blocked by ritanserin and WAY 1006335
Vashum et al., 2014	Dietary zinc, depression risk	Two prospective Australian cohorts (<i>n</i> = 2092 men and women, <i>n</i> = 9738 women)	Centre for Epidemiological Studies Depression Scale (CESD)	Dietary zinc associated with lower incidence of depression in men and women 50 years and older

Further alluding of zinc's complex role with BDNF, is zinc's interaction with GPR39 receptors. Zinc acts as a natural ligand which stimulates GPR39 resulting activation of G-protein signal

transduction pathways. In rodent models, GPR39 knockout mice resulted in decreased levels of BDNF in the hippocampus along with increased immobility time in both the forced swim test

(FST) and tail suspension test (TST) which is analogous to the depressive phenotype (Mlyniec et al., 2014a). The GPR39 receptor likely serves as a crucial link in the interaction between zinc and the serotonergic system, necessary for the activity of antidepressants targeting the serotonin pathway (Doboszewska et al., 2017).

ZINC DEFICIENCY IN RELATION TO PSYCHOTIC DISORDERS

Schizophrenia is a disabling syndrome of psychosis and impaired functioning with both neurodevelopmental and degenerative pathologies that affects nearly 21 million individuals worldwide (WHO, 2016). The condition has neurodevelopmental underpinnings and prenatal zinc deficiency may also be relevant, whether a consequence of maternal zinc insufficiency or fetal gene variants that impact the movement of zinc across cellular membranes. Prenatal zinc deficiency produces decreased brain volume in rodent models, consistent with impaired cell proliferation and retarded neuronal maturation (Dvergsten et al., 1983, 1984a,b; Takeda and Tamano, 2009). This is relevant to the risk of schizophrenia, as a 30–50% reduction in brain zinc content is demonstrated for early onset cases compared to control samples in postmortem samples (Kimura and Kumura, 1965; McLardy, 1973). The expression of the schizophrenia phenotype may reflect interactions of prenatal zinc deficiency with other risk genes, and/or ongoing deficiency following birth. Studies on zinc deficiency and zinc supplementation in relation to psychosis are summarized in **Table 2** and are discussed in the following sections.

ZIP PROTEINS AND THE SLC39A13 MUTATION

Abnormalities in intracellular zinc may occur even when serum levels are normal if there is dysfunction in the molecules that move zinc across membranes. The two major families of zinc transporters include the SLC family (ZnT) and the SLC39 family (ZIP). The family of ZIP proteins includes 14 mammalian members responsible for transporting zinc into the cytoplasm, whereas the ten mammalian ZnT transport zinc into the extracellular space (Ilouz et al., 2002; Jeong and Eide, 2013).

The SLC39 solute carrier genes are of particular interest, as recent research identified point mutations in association with severe mental illness (Ilouz et al., 2002; Fukada et al., 2008; Kranz et al., 2015). In one study, a disruptive *de novo* mutation was identified in the zinc transport gene SLC39A13 in a sporadic schizophrenia case compared to healthy parents. A subsequent New York Study identified 4 other rare/novel disruptive variants in this same gene from among 48 unrelated cases (Fukada et al., 2008; Kranz et al., 2015). This gene, also known as ZIP13, is located on chromosome 11 (Kranz et al., 2016). It is responsible for the influx of zinc into the cytoplasm from the extracellular space and/or the efflux of zinc from intracellular organelles. The gene is widespread throughout the body and found in high concentrations within the Golgi apparatus (Kranz et al., 2016).

It is relevant that assaultive behavior in humans (Tokdemir et al., 2003; Mlyniec et al., 2014b) and greater aggression in rodents are observed with zinc deficiency. In 1997 Walsh and colleagues demonstrated significantly lower zinc serum values in criminals with schizophrenia vs. non-criminal subjects (Walsh et al., 1997). The neurobiology associated with the SLC39A13 mutation was further characterized through a magnetic resonance imaging (MRI) approach, which demonstrated significantly reduced neuronal concentrations in the rostral anterior cingulate cortex, which was significantly associated in the imaged sample of cases with reduced verbal intelligence and stable (trait) negative symptoms (Malaspina et al., 2016).

As to mechanisms, it is notable that the SLC39A13 gene plays an important role in BMP/TGF- β signaling pathways, which has crucial roles in development and a host of cellular processes. With respect to schizophrenia, the pathway regulates oligodendrocyte maturation and differentiation (McKinnon et al., 1993; Palazuelos et al., 2014). It also participates in the development of connective tissues (Ilouz et al., 2002; Marger et al., 2014). One particular SLC39A13 mutation is linked to spondylocheiro dysplastic-Ehlers-Danlos syndrome, a condition which includes spine and hand dysplasia and can also entail significant psychopathology, including schizophrenia, in association with joint hypermobility (Sinibaldi et al., 2015; Malaspina et al., 2016). Maintaining zinc levels within a narrow range is also essential for glutamatergic function; another important mechanism as the receptor interacts with zinc. Rodents with zinc deficiency demonstrate alterations in glutamatergic receptor function with reduced neuroplasticity and neurogenesis. This interaction is dysregulated in psychiatric disease states such as schizophrenia and depression (Nowak, 2001; Szewczyk et al., 2010; Prakash et al., 2015). A brief review of the relevant NMDA receptor anatomy and physiology is presented below to illustrate zinc's actions.

While this review emphasizes the role of the SLC39A13 gene, recent studies have shed light on additional zinc transport genes linked to schizophrenia. SLC39A12 was previously identified as a candidate (Bly, 2006), and increased cortical expression of this gene and increased zinc uptake have been demonstrated in post-mortem brain tissue (Scarr et al., 2016). Furthermore, these changes are specific to schizophrenia and do not occur in subjects with mood disorders. SLC39A8 is also implicated in schizophrenia, and genome-wide association studies (GWAS) now reveal shared genetic influences of the SLC39A8 gene on both schizophrenia and inflammatory bowel disease (Pickrell et al., 2016). Variants of SLC39A8 are associated with a shift in gut microbiome composition, T cell immunity, lipid levels, blood pressure, and obesity, highlighting the relationship between schizophrenia, inflammation, and metabolic dysregulation (Marger et al., 2014; Li et al., 2016). Lastly, outside of the SLC39 family, alleles of the SLC30A3 gene have also been shown to confer risk of schizophrenia on female but not male individuals (Perez-Becerril et al., 2016). These studies demonstrate the expanding role of zinc homeostasis on schizophrenia and other disorders.

TABLE 2 | Studies relating psychosis to zinc levels.

Author, Year	Model	Subjects	Measure	Results
Czerniak and Haim, 1971	Zinc supplementation	80 mice, 80 rats	Injection of 5 and 15 microcuries of zinc chloride Zn 65	Phenothiazine compounds increase the total brain zinc uptake in all animals
Holcomb et al., 2005	Human drug administration	Normal volunteers ($n = 13$); Schizophrenic volunteers ($n = 10$)	Drug-induced NMDAR antagonist ketamine (0.3 mg/kg)	Schizophrenic volunteers showed greater relative blood flow increases in the anterior cingulate and correlated with changes in psychosis ratings; ketamine-induced inhibition and increased glutamate release may cause the distorted thoughts and diminished cognitive abilities elicited by NMDAR blockade
Kimura and Kumura, 1965	Human zinc levels	Cases with typical schizophrenia ($n = 10$) and cases of various cerebral diseases ($n = 10$)	Polarogram	Zinc level in brain regions is significantly lower in the schizophrenia group than other diseases
Mortazavi et al., 2015	Double-blind randomized placebo-controlled trial	Schizophrenia inpatients ($n = 30$) receiving 6 mg/day risperidone	Capsules of adjunct Zn sulfate (each containing 50 mg elemental Zn) three times a day	Psychotic symptoms and aggression risk decreased for both groups; higher improvement for Zn sulfate receiving group than placebos
McLardy, 1973				30% deficit of brain Zn ²⁺ content in individuals with early onset schizophrenia
Tokdemir et al., 2003	Human zinc levels	88 schizophrenic patients from Elazig Mental Hospital ($n = 44$ w/criminal record, $n = 44$ no criminal record)	5 mL (IV) heparin blood draw for plasma zinc concentration	Mean plasma zinc values significantly lower in criminal subjects when compared to non-criminal subjects; 68 ± 1.55 microg/dL mean in the criminal subjects and 81 ± 2.73 microg/dL mean in the non-criminal subjects ($p = 0.001$)
Walsh et al., 1997	Human zinc levels	All male patients between the ages of 3–20 years who made a first visit to the outpatient Pfeiffer Treatment Center in Naperville, Ill., during a 2-month period ($n = 135$ assaultive young males, $n = 18$ controls w/no history of assaultive behavior	Blood samples using atomic absorption methods	Depressed plasma zinc in blood samples collected from violence-prone individuals; median Cu/Zn ratio for the assaultive subjects was 1.40 compared to 1.02 for controls, a statistically significant difference ($t = 5.94$; $p < 0.001$)

NMDA RECEPTOR ANATOMY AND PHYSIOLOGY

The N-methyl-D-aspartate (NMDA) receptor is a member of the ligand-gated ion channel family of receptors, which includes the AMPA and kainate glutamate receptors. The NMDA receptor is a multi-domained ion channel that is principally permeable to calcium (Ca^{2+}), and to a lesser extent, to sodium (Na^+) and potassium (K^+) (Nowak, 2001). Although there is vast molecular diversity of NMDA receptors across anatomical locations and physiologic conditions, the core anatomy includes a heterotetramer of two glycine-binding (NR1) subunits and two glutamate-binding (NR2) subunits (Figure 1) (Sowa-Kućma et al., 2013). NMDA receptors tend to be post-synaptic and to collaboratively modulate the excitatory post-synaptic transmission stimulated by glutamate, also by

allosteric modulation by glycine, polyamines and zinc (Nowak, 2001).

In order for the NMDA receptor to function, glutamate's co-transmitter glycine must be present (Sowa-Kućma et al., 2013). Glycine is provided by neighboring glia and released into the synapse for readily available binding (Nowak, 2001; Sowa-Kućma et al., 2013). Closely related to glycine and synthesized by serine racemase, D-serine is a potent allosteric modulator that can be used interchangeably at the glycine allosteric site. D-serine co-exists with glycine at the human synapse; its actions at the glycine allosteric site are beyond the scope of this review (Hashimoto, 2014; Balu, 2016). When magnesium ion (Mg^{2+}) is situated at the entrance of the calcium channel it forms a plug (Mathews et al., 2012) which blocks the NMDA receptor. The necessary “perfect storm” for the mechanical changes that open the channel entail glutamate binding and then the binding of the co-transmitter

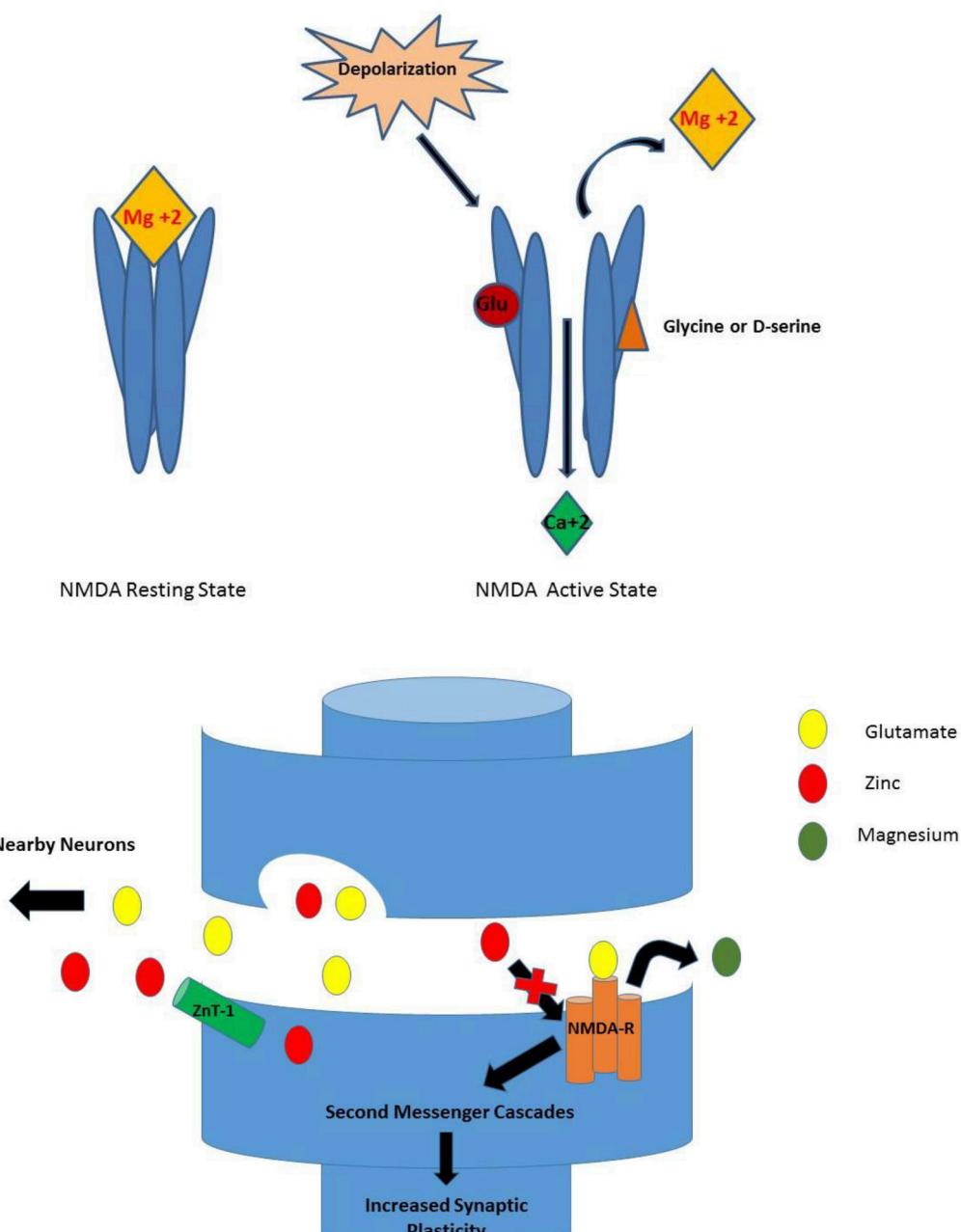


FIGURE 1 | (A) NMDA Receptor Activation. While in the resting state, Mg⁺² blocks the Ca⁺² ionic pore. For a neuron to become depolarized, both glutamate and glycine (or D-serine) must bind to their respective sites for removal of the Mg⁺² ion and permit entry of Ca⁺² through the pore. **(B)** Activation of NMDA-R as Seen in Synapse. When depolarization occurs, glutamate is released from the presynaptic terminal and binds to the NMDA receptor. Once serine binds, the Mg⁺² ion is released from the ionic pore allowing Ca⁺² to enter. The result is a variety of second messenger cascades that result in increased synaptic plasticity. Conversely, when zinc is present in the synapse, the activation of the NMDA receptor is inhibited (Walsh et al., 1997).

glycine (or D-serine). These events displace Mg⁺² so calcium can enter and depolarize the neuron. This leads to signal cascades and in some pathways producing long-term potentiation and increased synaptic plasticity (Figure 1; Sowa-Kućma et al., 2013).

Predominately in the limbic regions, zinc is highly concentrated in hippocampal mossy fiber vesicles and the

axons of dentate granule cells (Marger et al., 2014). While glycine potentiates NMDA receptor's activity leading to calcium entry and subsequent neural depolarization, zinc inhibits this action (Nowak, 2001; Nowak et al., 2003b). It is released concurrently with glutamate (by ZENs) and rapidly reaches micromolar levels which are necessary for synaptic modulation (Marger et al.,

2014). The binding dynamics of zinc to the glutamate-binding NR2 subunit of the NMDA receptor vary across the glutamate binding isoforms. NR2A, which is synaptic, has a high sensitivity to extracellular zinc and requires only nanomolar concentrations to produce inhibition in a voltage-independent fashion (Low et al., 2000; Marger et al., 2014). By contrast, the extrasynaptic NR2B subunit binds zinc at a 100-fold lesser affinity than NR2A to produce voltage dependent inhibition (Sowa-Kućma et al., 2013) consistent with zinc entering the ionic pore. The mechanism of sensitivity to zinc by NR2C and NR2D is less clear, but multiple other processes also entail synaptic modulation by zinc in the micromolar range (Marger et al., 2014).

In addition to modulating NMDA receptor activation via allosteric receptor binding (Howland and Wang, 2008; Marger et al., 2014; Prakash et al., 2015), zinc also stimulates the release of the inhibitory neurotransmitter GABA from interneurons for presynaptic inhibition of glutamate release (Howland and Wang, 2008). With less glutamate in the synapse, glutamate binding at the NMDA receptor is consequently reduced (Salari et al., 2015). Furthermore, the dehydrogenase and decarboxylase enzymes that catabolize glutamate have reduced activity in the presence of zinc (Prakash et al., 2015). Lastly, zinc inhibits the group I metabotropic glutamate receptors (mGluR), diminishing Ca^{+2} release from internal neuron stores. Ca^{+2} enhances the activity of the NMDA receptor, so zinc's role to decrease the Ca^{+2} availability further reduces NMDA receptor functionality (Howland and Wang, 2008; Salari et al., 2015).

The complex interactions between zinc and the glutamatergic system in normal physiological conditions produce a tightly controlled, activity-dependent homeostasis. When these interactions are perturbed, deleterious effects such as excitotoxicity can occur (Howland and Wang, 2008; Marger et al., 2014; Prakash et al., 2015; Salari et al., 2015), which is a risk pathway for a multitude neuropsychiatric disorders. Excitotoxicity describes the pathology of neuronal death or

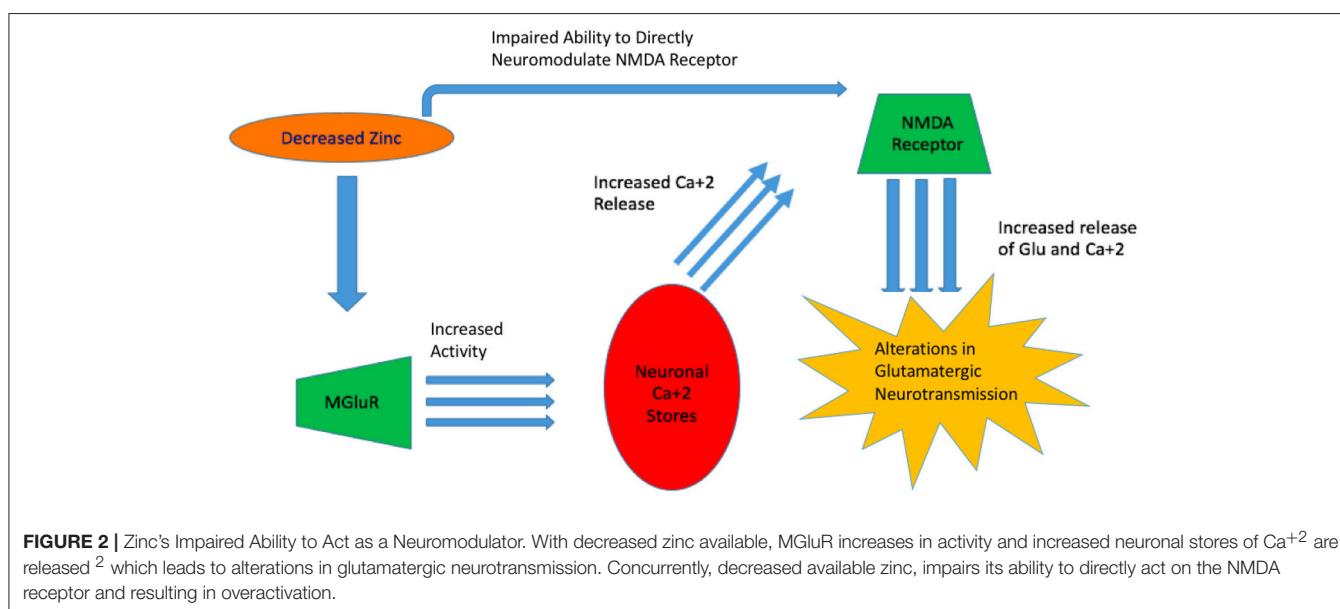
damage resulting from excessive transmission by glutamate and some similar substances. Excitotoxins include NMDA and kainic acid, which bind to the NMDA and AMPA receptors, as well as pathologically high levels of glutamate. These toxins cause excitotoxicity by allowing high levels of Ca^{+2} to enter the cell which in turn activates a number of toxic enzymes, including phospholipases, endonucleases, and proteases such as calpain that damage the cellular cytoskeleton, membranes, and genetic material.

ZINC MODULATES NMDA RECEPTOR ACTIVITY

Glutamate homeostasis and neurotransmission are dysregulated in many mental conditions (Paz et al., 2008; Szewczyk et al., 2010). As zinc is a modulator of NMDA receptor activity together with Mg^{2+} and H^{+} , significant deficiency can increase the propensity of NMDA receptor activation (Low et al., 2000). Notably, the ability of zinc to alter receptor activation sensitivity depends on the NMDA receptor subunit composition. In one study, HEK293 cells were used for the overexpression of NR1/NR2A and NR1/NR2B subunit complexes and electrophysiologically assessed. The experiments revealed that NR1/NR2A mediated current responses were more sensitive to extracellular zinc than those mediated by NR1/NR2B (Chen et al., 1997). This effect is explained by a higher affinity of zinc to its binding site in the NR1/NR2A complex (Paoletti et al., 1997; Rachline et al., 2005) (Figure 2).

GLUTAMATERGIC HYPERACTIVITY AND ZINC DYSREGULATION IN DEPRESSION

Zinc and other antagonists of the NMDA receptor show antidepressant-like effects (Autry et al., 2011), attributed to



the inhibition of NMDA-sensitive glutamate-gated channels, and include preclinical and clinical studies (Szewczyk et al., 2008). Zinc's negation of depressive features was demonstrated in rodents using the forced swim test (Szewczyk et al., 2010), wherein zinc pretreatment was related to longer periods of escape behaviors before immobility (i.e., less depressive-like behavior). Zinc administration in rodents also reduced the number of NMDA receptor complexes, suggesting downregulation (Cichy et al., 2009; Szewczyk et al., 2009, 2010). Conversely, a zinc-deficient diet induces upregulation of NMDA receptor complexes, but these levels normalize following antidepressant treatment, along with reversal of depression-like behavior in mice (Doboszewska et al., 2015).

Depressive-like effects may be consequences of altered NMDA receptor subunit concentrations (Tokita et al., 2012; Sowa-Kućma et al., 2013). In a 2008 post-mortem study of suicide victims, Sowa-Kucma et al. observed a reduced affinity of zinc to interact with hippocampal NMDA receptor subunits compared to controls, even without changes in zinc concentrations. Concurrently, NR2A subunits were significantly elevated while NR2B subunits appeared to be decreased (Sowa-Kućma et al., 2013), consistent with hypersensitivity of the NMDA receptor and compensatory upregulation of NR2A subunits to mitigate zinc actions at the receptor. Victims of suicide diagnosed with depression also showed reduced potency of zinc to inhibit the hippocampal NMDA receptor without zinc deficiency (Nowak et al., 2003b).

Research examining serotonin receptor reuptake inhibitor (SSRI) effects on the NMDA receptor uphold the hypothesis of alterations in the receptor complex during major depressive disorder. Chronic antidepressant treatment decreases the affinity for glycine, reduces glycine-displaceable glutamate sites (Nowak, 2001) and NMDA receptor complex function in the hippocampus (Paul et al., 1994; Nowak, 2001). Chronic antidepressant treatment was similarly observed to change the mRNA expression of genes that encode for the NMDA receptor subunits, resulting in decreased expression and/or decreased functionality of the NMDA receptor, helping to protect against glutamate mediated excitotoxicity (Szewczyk et al., 2010). Not only does chronic antidepressant treatment lead to changes within the human NMDA receptor itself, but levels of zinc, previously decreased, appear to normalize (Wójcik et al., 2006; Siwek et al., 2010; Prakash et al., 2015). A statistically significant 20% increase in hippocampal zinc was observed after 14 days of citalopram administration to rodents (Doboszewska et al., 2015) with similar effects from electroshock in rodents (Nowak and Schlegel-Zawadzka, 1999; Vaidya et al., 1999; Nowak, 2001). As the hippocampus is a major site of synaptogenesis, which is greatly reduced in major depressive disorder (Swardfager et al., 2013; Prakash et al., 2015), the increased hippocampal zinc concentration after successful antidepressant treatment supports its role in neurogenesis as well as in neuroprotection.

The role of serotonergic transmission in depression is well described and has led to the production of many pharmaceuticals for depression treatment. However, its interaction with glutamate and specifically with the NMDA receptor is another putative pathway for treating depression (Szewczyk et al., 2010).

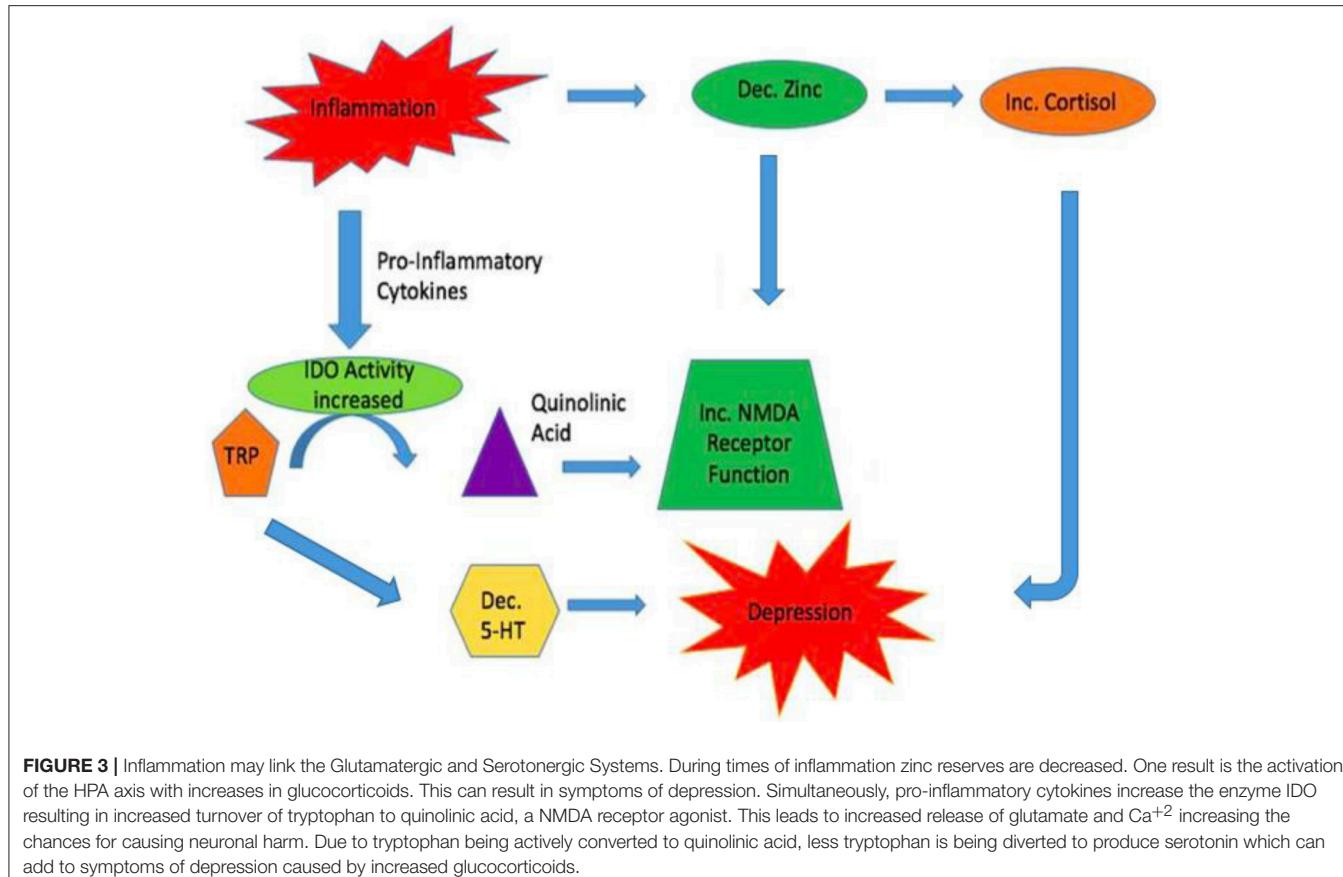
Serotonergic receptors (primarily 5-HT1A) and zinc interact, as observed in the rodent forced swim test (Jeong and Eide, 2013; Nowak, 2015) by complete abatement of the antidepressant effects of zinc in the presence of a 5-HT1A antagonist (Rachline et al., 2005; Satała et al., 2016). The inverse is also exhibited in rodents when zinc was co-administered with SSRIs. The interaction appeared to be synergistic with increases in motility time in the forced swim test. Zinc may serve as a possible allosteric modulator of 5-HT1A receptors, capable of inhibiting both agonist and antagonist binding at relevant concentrations in the synapse (Prakash et al., 2015). Another way that zinc may interact with the serotonergic system is via the previously mentioned GPR39 receptor. In GPR39 knockout mice, only NMDA antagonists, but not monoamine-based antidepressants, have antidepressant effect, thus establishing a connection between zinc, serotonin, and BDNF expression (Młyniec et al., 2015). This research further alludes to serotonin and zinc's interaction.

ZINC AND INFLAMMATION

The inflammatory system is another probable link between the glutamatergic and serotonergic systems in major depressive disorder, as zinc levels are decreased by stress and inflammation (Prakash et al., 2015). Numerous literature has already demonstrated the increase in glucocorticoids from HPA-axis dysregulation resulting in symptoms of depression. Decreased zinc within the hippocampus may activate the HPA-axis (Nowak, 2001). Concurrently, the enzyme indoleamine 2,3-dioxygenase (IDO) is stimulated by pro-inflammatory cytokines. It metabolizes tryptophan into quinolinic acid, a NMDA receptor agonist. It is hypothesized that the increase in IDO activity results in a decrease in available tryptophan for 5-HT synthesis, thus compounding the possible depressive symptoms that have been elicited from HPA-axis dysregulation (Liuzzi and Cousins, 2004). The increase in quinolinic acid results in excess NMDA receptor activity subsequently causing excess glutamate release and neurotoxicity (Prakash et al., 2015). With zinc already decreased in the pro-inflammatory state, it is unable to inhibit the NMDA receptor effectively and hyperactivity ensues, resulting in potential deleterious consequences (**Figure 3**).

GLUTAMATERGIC HYPERACTIVITY IN RELATION TO ZINC DYSREGULATION IN PSYCHOTIC DISORDERS

NMDA hypofunction was first implicated for psychosis when individuals ingesting PCP, an NMDA antagonist, exhibited thought disorganization, auditory hallucinations, emotional blunting, and cognitive disturbances. Psychotic symptoms were also observed in healthy individuals receiving sub-anesthetic doses of ketamine, an NMDA antagonist. Acute administration of ketamine was shown to trigger pyramidal neuronal excitation in the prefrontal cortex and increased metabolic activity (Holcomb et al., 2005; Wójcik et al., 2006). NMDA antagonists may block excitatory inputs to GABAergic interneurons, preventing



the down-regulation of prefrontal glutamatergic neurons. By triggering signal transduction pathways that potentiate glutamatergic excitability (Paz et al., 2008), both psychosis and cognitive deficits may be elicited.

Some literature suggests that psychosis in schizophrenia is related to inflammation of brain tissue (Prakash et al., 2015). As described, inflammation leads to a decrease in zinc levels, resulting in increased NMDA receptor activity and ultimately to excitotoxicity as high levels of calcium enter the cell and activate enzymes that damage subcellular structures. Lower zinc levels in persons with schizophrenia may be a consequence of inflammation and/or genetic defects in molecules that maintain zinc homeostasis or nutritional deficiencies or malabsorption. Each can produce NMDA hyperactivity and possibly psychotic symptomatology. Modulating glutamatergic transmission within the neural networks that converge in the prefrontal cortex using pharmaceuticals that act on the NMDA receptor and/or zinc supplementation may remediate hyperglutamatergic states and possibly reduce psychotic symptoms (Paz et al., 2008).

All typical antipsychotics bind dopamine-2 (D2) receptors at efficacies that are related to antipsychotic symptom reductions. However, it may be D1 receptor antagonists that can diminish glutamatergic hyperactivity by reducing NMDA receptor activity (Paz et al., 2008). Strong D2 antagonists may have minimal effects at modulating glutamatergic activity. Interestingly, the antipsychotic clozapine, which is used for treatment

refractory psychosis, has a higher affinity for the D1 than D2 receptors, possibly exerting a stronger effect on NMDA receptor hyperactivity in the prefrontal cortex (Paz et al., 2008). An inference is that the more robust response from clozapine may entail normalization of prefrontal glutamatergic activity. However, the interaction between D1 blockade and NMDA receptor downregulation is not direct, and not yet well described in terms of signal transduction. Direct modulation of hyperactive NMDA receptors by zinc inhibition may target mircocircuitry that is not accessible to the conventional D2 receptor antagonists (Paz et al., 2008).

Zinc supplementation may be a useful person-specific intervention. A double-blind, placebo-controlled schizophrenia study showed that 220 mg of zinc sulfate TID, used as an adjuvant to 6 mg/day of risperidone, produced a statistically significant improvement of positive and negative symptomatology and reduced aggressive behavior (Mortazavi et al., 2015). These results correlate with the aforementioned hypothesis that increasing zinc through dietary supplementation may be a route to inhibiting NMDA receptors, decreasing glutamate mediated excitotoxicity and thus normalizing glutamatergic transmission in the PFC. Perhaps a further study examining only schizophrenic individuals exhibiting the SLC39A13 mutation or reduced serum zinc levels would reveal a more robust response with zinc supplementation. As the psychoses are heterogeneous, it

is unlikely that zinc supplementation would be useful for all cases.

FUTURE DIRECTIONS

Research from the last two decades suggests a robust amount of evidence connecting zinc dysregulation and deficiency to a multitude of neuropsychiatric conditions. Although there is only a limited amount of data regarding the efficacy of zinc as a treatment modality in psychiatric disorders, it seems quite reasonable, given the amount of substantiated data demonstrating the relevance of zinc dysregulation in psychiatric disorders to further pursue zinc as a potential therapeutic option in psychiatry. The complex relationship between the glutamatergic system and other neurotransmitter systems, including serotonin, dopamine, and inflammatory pathways, demonstrates the multi-faceted and more complex underpinnings of psychopathology exceeding earlier views of the dysregulation of a particular neurotransmitter. Although only one randomized, double blind controlled trial has been completed on zinc supplementation for treatment of schizophrenia, the Mortazavi et al. study remains promising. As the current understanding of schizophrenia becomes more refined, so will treatment modalities.

As pharmacotherapy is costly and comes with increased potential for adverse side-effects, zinc may serve as an adjuvant to help resolve symptoms of mental illness (Vashum et al., 2014; Mortazavi et al., 2015). Zinc is well tolerated with minimal side effects, other than occasional gastrointestinal disturbance. The most common method of zinc administration is oral dietary supplementation and comes in various compounds (i.e., Zinc

oxide, zinc sulfate, zinc acetate, etc.) with various tolerability and absorption profiles. The rate of intestinal absorption of zinc has been observed to be increased when supplemented with vitamin B6 (Grabrucker et al., 2011). However, because zinc uptake into the CNS being an active transport process, it is hard to control the exact zinc level into the brain. There are limited studies which examine potential modalities to deliver zinc to specific brain areas, however certain compounds have shown to aid in increase zinc uptake in rodent models. It has been observed that phenothiazine derivatives which include chlorpromazine, thioridazine and perphenazine increased the absorption of zinc supplemented to rodents, however would possibly be counterintuitive to be adding first generation neuroleptics with high side-effect profiles to aid in CNS transport of zinc (Czerniak and Haim, 1971; Grabrucker et al., 2011).

As current research supports plausible roles for zinc in reducing both depressive and psychotic symptoms, zinc supplementation may reduce the amount of psychotropic medication required, leading to increased adherence, lower costs and more favorable outcomes. Due to the heterogeneity of mental illness, further study of certain subsets that would most benefit from zinc supplementation must be more clearly refined. Clearly more research is needed to elucidate the impact of zinc on neuropsychiatric conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

- Autry, A. E., Adachi, M., Nosyreva, E., Na, E. S., Los, M. F., Peng-fei, C., et al. (2011). NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* 475, 91–95. doi: 10.1038/nature10130
- Balu, D. T. (2016). The NMDA receptor and Schizophrenia: from pathophysiology to treatment. *Adv. Pharmacol.* 76, 351–382. doi: 10.1016/bs.apha.2016.01.006
- Bly, M. (2006). Examination of the zinc transporter gene, SLC39A12. *Schizophr. Res.* 81, 321–322. doi: 10.1016/j.schres.2005.07.039
- Chen, N., Moshaver, A., and Raymond, L. A. (1997). Differential sensitivity of recombinant N-methyl-D-aspartate receptor subtypes to zinc inhibition. *Mol. Pharmacol.* 51, 1015–1023.
- Cichy, A., Sowa-Kućma, M., Legutko, B., Pomierny-Chamioł, L., Siwek, A., Piotrowska, A., et al. (2009). Zinc-induced adaptive changes in NMDA/glutamatergic and serotonergic receptors. *Pharmacol. Rep.* 61, 1184–1191. doi: 10.1016/S1734-1140(09)70182-3
- Czerniak, P., and Haim, D. B. (1971). Phenothiazine derivatives and brain zinc. Turnover radioactive isotope study. *Arch. Neurol.* 24, 555–560. doi: 10.1001/archneur.1971.00480360089012
- Doboszewska, U., Szewczyk, B., Sowa-Kućma, M., Mlyniec, K., Rafalo, A., Ostachowicz, B., et al. (2015). Antidepressant activity of fluoxetine in the zinc deficiency model in rats involves the NMDA receptor complex. *Behav. Brain Res.* 287, 323–330. doi: 10.1016/j.bbr.2015.03.064
- Doboszewska, U., Właź P., Nowak, G., Radziwoń-Zaleska, M., Cui, R., and Mlyniec, K. (2017). Zinc in the monoaminergic theory of depression: its relationship to neural plasticity. *Neural Plast.* 2017:3682752. doi: 10.1155/2017/3682752
- Dvergsten, C. L., Fosmire, G. J., Ollerich, D. A., and Sandstead, H. H. (1983). Alterations in the postnatal development of the cerebellar cortex due to zinc deficiency. I. Impaired acquisition of granule cells. *Brain Res.* 25, 217–226. doi: 10.1016/0006-8993(83)90284-6
- Dvergsten, C. L., Fosmire, G. J., Ollerich, D. A., and Sandstead, H. H. (1984a). Alterations in the postnatal development of the cerebellar cortex due to zinc deficiency. II. Impaired maturation of Purkinje cells. *Brain Res.* 318, 11–20. doi: 10.1016/0165-3806(84)90057-9
- Dvergsten, C. L., Johnson, L. A., and Sandstead, H. H. (1984b). Alterations in the postnatal development of the cerebellar cortex due to zinc deficiency. III. Impaired dendritic differentiation of basket andstellate cells. *Brain Res.* 318, 21–26. doi: 10.1016/0165-3806(84)90058-0
- Frederickson, C. J., Suh, S. W., Silva, D., Frederickson, C. J., and Thompson, R. B. (2000). Importance of zinc in the central nervous system: the zinc-containing neuron. *J. Nutr.* 130(S Suppl), 1471S–1483S.
- Fukada, T., Civic, N., Furuchi, T., Shimoda, S., Mishima, K., Higashiyama, H., et al. (2008). The Zinc Transporter SLC39A13/ZIP13 is required for connective tissue development; its involvement in BMP/TGF- β signaling pathways *PLoS ONE* 3:e3642. doi: 10.1371/journal.pone.0003642
- Grabrucker, A. M., Rowan, M., and Garner, C. C. (2011). Brain-delivery of zinc ions as potential treatment for neurological diseases: mini review. *Drug Deliv. Lett.* 1, 13–23. doi: 10.2174/2210304x11101010013
- Hashimoto, K. (2014). Targeting of NMDA receptors in new treatments for schizophrenia. *Expert Opin. Ther. Targets* 18, 1049–1063. doi: 10.1517/14728222.2014.934225
- Holcomb, H. H., Lahti, A. C., Medoff, D. R., Cullen, T., and Tamminga, C. A. (2005). Effects of noncompetitive NMDA receptor blockade on

- anterior cingulate cerebral blood flow in volunteers with schizophrenia. *Neuropsychopharmacology* 30, 2275–2282. doi: 10.1038/sj.npp.1300824
- Howland, J. G., and Wang, Y. T. (2008). Synaptic plasticity in learning and memory: stress effects in the hippocampus. *Prog. Brain Res.* 169, 145–158. doi: 10.1016/S0079-6123(07)00008-8
- Huang, Y. Z., Pan, E., Xiong, Z. Q., and McNamara, J. O. (2008). Zinc-mediated transactivation of TrkB potentiates the hippocampal mossy fiber-CA3 pyramid synapse. *Neuron* 57, 546–558. doi: 10.1016/j.neuron.2007.11.026
- Ilouz, R., Kaidanovich, O., Gurwitz, D., and Eldar-Finkelman, H. (2002). Inhibition of glycogen synthase kinase-3beta by bivalent zinc ions: insight into the insulin-mimetic action of zinc. *Biochem. Biophys. Res. Commun.* 295, 102–106. doi: 10.1016/S0006-291X(02)00636-8
- Jeong, J., and Eide, D. J. (2013). The SLC39 family of zinc transporters. *Mol. Aspects Med.* 34, 612–619. doi: 10.1016/j.mam.2012.05.011
- Kimura, K., and Kumura, J. (1965). Preliminary reports on the metabolism of trace elements in neuro psychiatric diseases. I. Zinc in schizophrenia. *Proc. Jap. Acad. Sci.* 41, 943–947.
- Kranz, T. M., Berns, A., Shields, J., Rothman, K., Walsh-Messinger, J., Goetz, R. R., et al. (2016). Phenotypically distinct subtypes of psychosis accompany novel or rare variants in four different signaling genes. *EBioMed.* 6, 206–214. doi: 10.1016/j.ebiom.2016.03.008
- Kranz, T. M., Harroch, S., Manor, O., Lichtenberg, P., Friedlander, Y., Seandel, M., et al. (2015). De novo mutations from sporadic schizophrenia cases highlight important signaling genes in an independent sample. *Schizophr. Res.* 166, 119–124. doi: 10.1016/j.schres.2015.05.042
- Lai, J., Moxey, A., Nowak, G., Vashum, K., Bailey, K., and McEvoy, M. (2012). The efficacy of zinc supplementation in depression: systematic review of randomised controlled trials. *J. Affect. Disord.* 136, e31–e39. doi: 10.1016/j.jad.2011.06.022
- Li, D., Achkar, J. P., Haritunians, T., Jacobs, J. P., Hui, K. Y., D'Amato, M., et al. (2016). A pleiotropic missense variant in SLC39A8 is associated with crohn's disease and human gut microbiome composition. *Gastroenterology* 151, 724–732. doi: 10.1053/j.gastro.2016.06.051
- Liuzzi, J. P., and Cousins, R. J. (2004). Mammalian zinc transporters. *Annu. Rev. Nutr.* 24, 151–172. doi: 10.1146/annurev.nutr.24.012003.132402
- Low, C. M., Zheng, F., Lyuboslavsky, P., and Traynelis, S. F. (2000). Molecular determinants of coordinated proton and zinc inhibition of N-methyl-D-aspartate NR1/NR2A receptors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11062–11067. doi: 10.1073/pnas.180307497
- Maes, M., D'Haese, P. C., Scharpé, S., D'Hondt, P., Cosyns, P., and De Broe, M. E. (1994). Hypozincemia in depression. *J. Affect. Disord.* 31, 135–140. doi: 10.1016/0165-0327(94)90117-1
- Malaspina, D., Kranz, T. M., Heguy, A., Harroch, S., Mazgaj, R., Rothman, K., et al. (2016). Prefrontal neuronal integrity predicts symptoms and cognition in schizophrenia and is sensitive to genetic heterogeneity. *Schizophr. Res.* 172, 94–100. doi: 10.1016/j.schres.2016.02.031
- Marger, L., Schubert, C. R., and Bertrand, D. (2014). Zinc: an underappreciated modulatory factor of brain function. *Biochem. Pharmacol.* 91, 426–435. doi: 10.1016/j.bcp.2014.08.002
- Mathews, D. C., Henter, I. D., and Zarate, C. A. (2012). Targeting the glutamatergic system to treat major depressive disorder: rationale and progress to date. *Drugs* 72, 1313–1330. doi: 10.2165/11633130-00000000-00000
- McKinnon, R. D., Piras, G., Ida, J. A. Jr., and Dubois-Dalcq, M. (1993). A role for TGF-beta in oligodendrocyte differentiation. *J. Cell Biol.* 121, 1397–1407. doi: 10.1083/jcb.121.6.1397
- McLardy, T. (1973). Hippocampal zinc in chronic alcoholism and schizophrenia. *IRCS Med. Sci.* 2:1010.
- McLoughlin, I. J., and Hodge, J. S. (1990). Zinc in depressive disorder. *Acta Psychiatr. Scand.* 82, 451–453. doi: 10.1111/j.1600-0447.1990.tb03077.x
- Mlyniec, K., Budziszewska, B., Holst, B., Ostachowicz, B., and Nowak, G. (2014a). GPR39 (zinc receptor) knockout mice exhibit depression-like behavior and CREB/BDNF down-regulation in the hippocampus. *Int. J. Neuropsychopharmacol.* 18:pyu002. doi: 10.1093/ijnp/ypy002
- Mlyniec, K., Doboszewska, U., Szewczyk, B., Sowa-Kućma, M., Misztak, P., Piekoszewski, W., et al. (2014b). The involvement of the GPR39-Zn²⁺-sensing receptor in the pathophysiology of depression. Studies in rodent models and suicide victims. *Neuropharmacology* 79, 290–297. doi: 10.1016/j.neuropharm.2013.12.001
- Mlyniec, K., Gaweł, M., and Nowak, G. (2015). Study of antidepressant drugs in GPR39 (zinc receptor^{-/-}) knockout mice, showing no effect of conventional antidepressants, but effectiveness of NMDA antagonists. *Behav. Brain Res.* 287, 135–138. doi: 10.1016/j.bbrc.2015.03.053
- Mortazavi, M., Farzin, D., Zarhghami, M., Hosseini, S. H., Mansoori, P., Nateghi, G., et al. (2015). Efficacy of zinc sulfate as an add-on therapy to risperidone versus risperidone alone in patients with schizophrenia: a double-blind randomized placebo-controlled trial. *Iran J. Psychiatry Behav. Sci.* 9:e853. doi: 10.17795/ijpbs-853
- Nowak, G. (2001). Does interaction between zinc and glutamate system play a significant role in the mechanism of antidepressant action? *Acta Pol. Pharm.* 8, 73–75.
- Nowak, G. (2015). Zinc, future mono/adjunctive therapy for depression: Mechanisms of antidepressant action. *Pharmacol. Rep.* 67, 659–662. doi: 10.1016/j.pharep.2015.01.015
- Nowak, G., and Schlegel-Zawadzka, M. (1999). Alterations in serum and brain trace element levels after antidepressant treatment: part I. *Zinc. Biol. Trace Elem. Res.* 67, 85–92. doi: 10.1007/BF02784278
- Nowak, G., Siwek, M., Dudek, D., Zieba, A., and Pilc, A. (2003a). Effect of zinc supplementation on antidepressant therapy in unipolar depression: a preliminary placebo-controlled study. *Pol. J. Pharmacol.* 55, 1143–1117.
- Nowak, G., Szewczyk, B., Sadlik, K., Piekoszewski, W., Treła, F., Florek, E., et al. (2003b). Reduced potency of zinc to interact with NMDA receptors in hippocampal tissue of suicide victims. *Pol. J. Pharmacol.* 55, 455–459.
- Palazuelos, J., Klingener, M., and Aguirre, A. (2014). TGFβ signaling regulates the timing of CNS myelination by modulating oligodendrocyte progenitor cell cycle exit through SMAD3/4/FoxO1/Spl. *J. Neurosci.* 34, 7917–7930. doi: 10.1523/JNEUROSCI.0363-14.2014
- Pan, E., et al. (2011). Vesicular zinc promotes presynaptic and inhibits postsynaptic long-term potentiation of mossy fiber-CA3 synapse. *Neuron* 71, 1116–1126. doi: 10.1016/j.neuron.2011.07.019
- Paoletti, P., Ascher, P., and Neyton, J. (1997). High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *J. Neurosci.* 17, 5711–5725.
- Paul, I. A., Nowak, G., Layer, R. T., Popik, P., and Skolnick, P. (1994). Adaptation of the N-methyl-D-aspartate receptor complex following chronic antidepressant treatments. *J. Pharmacol. Exp. Ther.* 269, 95–102.
- Paz, R. D., Tardito, S., Atzori, M., and Tseng, K. Y. (2008). Glutamatergic dysfunction in schizophrenia: from basic neuroscience to clinical psychopharmacology. *Eur. Neuropsychopharmacology* 18, 773–786. doi: 10.1016/j.euroneuro.2008.06.005
- Perez-Becerril, C., Morris, A. G., Mortimer, A., McKenna, P. J., and De belleroche, J. (2016). Common variants in the chromosome 2p23 region containing the SLC30A3 (ZnT3) gene are associated with schizophrenia in female but not male individuals in a large collection of European samples. *Psychiatry Res.* 246, 335–340. doi: 10.1016/j.psychres.2016.09.052
- Pickrell, J. K., Berisa, T., Liu, J. Z., Ségurel, L., Tung, J. Y., and Hinds, D. A. (2016). Detection and interpretation of shared genetic influences on 42 human traits. *Nat. Genet.* 48, 709–717. doi: 10.1038/ng.3570
- Prakash, A., Bharti, K., and Majeed, A. B. (2015). Zinc: indications in brain disorders. *Fundam. Clin. Pharmacol.* 29, 131–149. doi: 10.1111/fcp.12110
- Prasad, A. S. (1995). Zinc: an overview. *Nutrition* 11(1 Suppl), 93–99.
- Rachline, J., Perin-Dureau, F., Le Goff, A., Neyton, J., and Paoletti, P. (2005). The micromolar zinc-binding domain on the NMDA receptor subunit NR2B. *J. Neurosci.* 25, 308–317. doi: 10.1523/jneurosci.3967-04.2005
- Ranjbar, E., Shams, J., Sabetkasaei, M., Shirazi, M., Rashidkhani, B., Mostafavi, A., et al. (2014). Effects of zinc supplementation on efficacy of antidepressant therapy, inflammatory cytokines, and brain-derived neurotrophic factor in patients with major depression. *Nutr. Neurosci.* 17, 65–71. doi: 10.1179/1476830513Y.0000000066
- Salari, S., Khomand, P., Arasteh, M., Yousefzaman, B., and Hassanzadeh, K. (2015). Zinc sulphate: a reasonable choice for depression management in patients with multiple sclerosis: a randomized, doubleblind, placebo controlled clinical trial. *Pharmacol. Rep.* 67, 606–609. doi: 10.1016/j.pharep.2015.01.002

- Satała, G., Duszyńska, B., Stachowicz, K., Rafalo, A., Pochwat, B., Luckhart, C., et al. (2016). Concentration-dependent dual mode of Zn action at serotonin 5HT1A receptors: *in vitro* and *in vivo* studies. *Mol. Neurobiol.* 53, 6869–6881. doi: 10.1007/s12035-015-9586-3
- Sawada, T., and Yokoi, K. (2010). Effect of zinc supplementation on mood states in young women: a pilot study. *Eur. J. Clin. Nutr.* 64, 331–333. doi: 10.1038/ejcn.2009.158
- Scarr, E., Udawela, M., Greenough, M. A., Neo, J., Seo, M. S., Money, T. T., et al. (2016). Increased cortical expression of the zinc transporter SLC39A12 suggests a breakdown in zinc cellular homeostasis as part of the pathophysiology of schizophrenia. *NPJ Schizophr.* 2:16002. doi: 10.1038/npj schz.2016.2
- Sinibaldi, L., Ursini, G., and Castori, M. (2015). Psychopathological manifestations of joint hypermobility and joint hypermobility syndrome/ Ehlers-Danlos syndrome, hypermobility type: the link between connective tissue and psychological distress revised. *Am. J. Med. Genet. C Semin. Med. Genet.* 169C, 97–106. doi: 10.1002/ajmg.c.31430
- Siwek, M., Dudek, D., Paul, I. A., Sowa-Kućma, M., Zięba, A., Popik, P., et al. (2009). Zinc supplementation augments efficacy of imipramine in treatment resistant patients: A double blind, placebo-controlled study. *J. Affect. Disord.* 118, 187–195. doi: 10.1016/j.jad.2009.02.014
- Siwek, M., Dudek, D., Schlegel-Zawadzka, M., Morawska, A., Piekoszewski, W., Opoka, W., et al. (2010). Serum zinc level in depressed patients during zinc supplementation of imipramine treatment. *J. Affect. Disord.* 126, 447–452. doi: 10.1016/j.jad.2010.04.024
- Solati, Z., Jazayeri, S., Tehrani-Doost, M., Mahmoodianfarid, S., and Gohari, M. R. (2015). Zinc monotherapy increases serum brain-derived neurotrophic factor (BDNF) levels and decreases depressive symptoms in overweight or obese subjects: a double-blind, randomized, placebo-controlled trial. *Nutr. Neurosci.* 18, 162–168. doi: 10.1179/1476830513Y.0000000105
- Sowa-Kućma, M., Szewczyk, B., Sadlik, K., Piekoszewski, W., Trela, F., Opoka, W., et al. (2013). Zinc, magnesium and NMDA receptor alterations in the hippocampus of suicide victims. *J. Affect. Disord.* 151, 924–931. doi: 10.1016/j.jad.2013.08.009
- Suh, S. W., Won, S. J., Hamby, A. M., Yoo, B. H., Fan, Y., Sheline, C. T., et al. (2009). Decreased brain zinc availability reduces hippocampal neurogenesis in mice and rats. *J. Cereb. Blood Flow Metab.* 29, 1579–1588. doi: 10.1038/jcbfm.2009.80
- Swardfager, W., Herrmann, N., Mazereeuw, G., Goldberger, K., Harimoto, T., and Lanctôt, K. L. (2013). Zinc in depression: a meta-analysis. *Biol. Psychiatry* 74, 872–878. doi: 10.1016/j.biopsych.2013.05.008
- Swardfager, W., Herrmann, N., Mazereeuw, G., and Lanctôt, K. L. (2015). Reply to: serum zinc and the risk of depression in men: observations from a 20-year follow-up study. *Biol Psychiatry* 77, e13–e14. doi: 10.1016/j.biopsych.2014.06.006
- Szewczyk, B., Pochwat, B., Rafalo, A., Palucha-Poniewiera, A., Domin, H., and Nowaka, G. (2015). Activation of mTOR dependent signaling pathway is a necessary mechanism of antidepressant-like activity of zinc. *Neuropharmacology* 99, 517–526. doi: 10.1016/j.neuropharm.2015.08.026
- Szewczyk, B., Poleszak, E., Sowa-Kućma, M., Siwek, M., Dudek, D., Ryszewska-Pokraśniewicz, B., et al. (2008). Antidepressant activity of zinc and magnesium in view of the current hypotheses of antidepressant action. *Pharmacol. Rep.* 60, 588–589.
- Szewczyk, B., Poleszak, E., Sowa-Kućma, M., Wróbel, A., Slotwiński, S., Listos, J., et al. (2010). The involvement of NMDA and AMPA receptors in the mechanism of antidepressant-like action of zinc in the forced swim test. *Amino Acids* 39, 205–217. doi: 10.1007/s00726-009-0412-y
- Szewczyk, B., Poleszak, E., Właź, P., Wróbel, A., Blicharska, E., Cichy, A., et al. (2009). The involvement of serotonergic system in the antidepressant effect of zinc in the forced swim test. *Progr. Neuro-Psychopharmacol. Biol. Psychiatry* 33, 323–329. doi: 10.1016/j.pnpbp.2008.12.011
- Takeda, A., and Tamano, H. (2009). Insight into zinc signaling from dietary zinc deficiency. *Brain Res. Rev.* 62, 33–44. doi: 10.1016/j.brainresrev.2009.09.003
- Tokdemir, M., Polat, S. A., Acik, Y., Gursu, F., Cikim, G., Deniz, O., et al. (2003). Blood zinc and copper concentrations in criminal and noncriminal schizophrenic men. *Arch. Androl.* 49, 365–368. doi: 10.1080/01485010390219746
- Tokita, K., Yamaji, T., and Hashimoto, K. (2012). Roles of glutamate signaling in preclinical and/or mechanistic models of depression. *Pharmacol. Biochem. Behav.* 100, 688–704. doi: 10.1016/j.pbb.2011.04.016
- Vaidya, V. A., Siuciak, J. A., Du, F., and Duman, R. S. (1999). Hippocampal mossy fiber sprouting induced by chronic electroconvulsive seizures. *Neuroscience* 89, 157–166. doi: 10.1016/S0306-4522(98)00289-9
- Vashum, K. P., McEvoy, M., Milton, A. H., McElduff, P., Hure, A., Byles, J., et al. (2014). Dietary zinc is associated with a lower incidence of depression: findings from two Australian cohorts. *J. Affect. Disord.* 166, 249–257. doi: 10.1016/j.jad.2014.05.016
- Walsh, W. J., Isaacson, H. R., Rehman, F., and Hall, A. (1997). Elevated blood copper/zinc ratios in assaultive youngmales. *Physiol. Behav.* 62, 327–329. doi: 10.1016/S0031-9384(97)88988-3
- WHO (2012). *Depression Fact Sheet no. 369*. WHO (Updated April 2016).
- WHO (2016). *Schizophrenia Fact Sheet no. 397*. WHO (Updated February 2017).
- Wójcik, J., Dudek, D., Schegiel-Zawadzka, M., Grabowska, M., Marcinek, A., Florek, E., et al. (2006). Antepartum/postpartum Depressive Symptoms and Serum Zinc and Magnesium Levels. *Pharmacol. Rep.* 58, 571–576.

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LSD Increases Primary Process Thinking via Serotonin 2A Receptor Activation

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Rationale: Stimulation of serotonin 2A (5-HT2A) receptors by lysergic acid diethylamide (LSD) and related compounds such as psilocybin has previously been shown to increase primary process thinking – an ontologically and evolutionarily early, implicit, associative, and automatic mode of thinking which is typically occurring during altered states of consciousness such as dreaming. However, it is still largely unknown whether LSD induces primary process thinking under placebo-controlled, standardized experimental conditions and whether these effects are related to subjective experience and 5-HT2A receptor activation. Therefore, this study aimed to test the hypotheses that LSD increases primary process thinking and that primary process thinking depends on 5-HT2A receptor activation and is related to subjective drug effects.

Methods: Twenty-five healthy subjects performed an audio-recorded mental imagery task 7 h after drug administration during three drug conditions: placebo, LSD (100 mcg orally) and LSD together with the 5-HT2A receptor antagonist ketanserin (40 mg orally). The main outcome variable in this study was primary index (PI), a formal measure of primary process thinking in the imagery reports. State of consciousness was evaluated using the Altered State of Consciousness (5D-ASC) rating scale.

Results: LSD, compared with placebo, significantly increased primary index ($p < 0.001$, Bonferroni-corrected). The LSD-induced increase in primary index was positively correlated with LSD-induced disembodyment ($p < 0.05$, Bonferroni-corrected), and blissful state ($p < 0.05$, Bonferroni-corrected) on the 5D-ASC. Both LSD-induced increases in primary index and changes in state of consciousness were fully blocked by ketanserin.

Conclusion: LSD induces primary process thinking via activation of 5-HT2A receptors and in relation to disembodyment and blissful state. Primary process thinking appears to crucially organize inner experiences during both dreams and psychedelic states of consciousness.

Keywords: LSD, ketanserin, 5-HT2A receptor, mental imagery, primary and secondary process thinking, primary emotions, cognitive bizarre ness, healthy subjects

INTRODUCTION

There is now accumulating evidence (Sloman and Steinberg, 1996; Evans, 2008; Shanks, 2010) confirming and extending the early meta-psychological theory of Freud (Pribram and Gill, 1976) which posits that there exist two distinct modes of psychic functioning: primary process and secondary process. It is broadly believed that in normal adults, secondary process is a hierarchically higher-level cognitive mode which fulfills an adaptive, reflective, rule-bound function (“reality principle” in Freudian terms) and thus inhibits lower-level, automatic, motivation- and emotion-driven primary process (“pleasure principle” in Freudian terms) (Arminjon, 2011). Under altered psychophysiological conditions such as dreaming, hypnosis, meditation, sensory deprivation, respiratory maneuvers, trance, psychosis, and epilepsy, primary process may become the prevailing cognitive mode (Barr et al., 1972; Vaitl et al., 2005; Hermle and Krahenmann, 2017). Primary process thinking can be operationalized and reliably assessed using formal linguistic measures such as image fusion; unlikely combinations or events; sudden shifts or transformations of images; and contradictory or illogical actions, feelings, or thoughts (Rapaport, 1950; Holt, 1956; Auld et al., 1968; Shevrin, 1996; Sloman and Steinberg, 1996; Brakel et al., 2000).

Previous studies (Landon and Fischer, 1970; Martindale and Fischer, 1977; Natale et al., 1978; Spitzer et al., 1996; Family et al., 2016; Krahenmann et al., 2017) indicate that classical psychedelics such as lysergic acid diethylamide (LSD) and related compounds such as psilocybin activate mental processes which are closely related to primary process, such as vivid, dreamlike imagery, basic emotions, and bizarre thinking. For example, early linguistic studies (Landon and Fischer, 1970; Martindale and Fischer, 1977; Natale et al., 1978) investigated the effects of psychedelics on thought content using primary process dictionaries. They found that psychedelics acutely increase frequency of primary process words in subjective reports of healthy subjects. Moreover, recent studies (Spitzer et al., 1996; Family et al., 2016) showed that psychedelics enhance access to remote and non-obvious associations in tasks where subjects have to rely on automatic, intuitive and uncontrolled thinking. Finally, we (Krahenmann et al., 2017) recently investigated the effects of LSD on imagery reports of healthy subjects. We found that LSD increased cognitive weirdness, a formal measure of dreaming cognition, via activation of the serotonin 2A (5-HT2A) receptor. Taken together, previous research on the effects of psychedelics on thought processes indicate that psychedelics may shift cognition toward primary process thinking.

However, it is still largely unknown whether LSD induces primary process thinking under placebo-controlled, standardized experimental conditions, and whether these effects are related to subjective experience and 5-HT2A receptor activation. A better understanding of the cognitive mechanisms underlying psychedelic states of consciousness is important, especially given that there is accumulating qualitative evidence (Gasser et al., 2015; Belser et al., 2017; Watts et al., 2017) indicating that the therapeutic effects of psychedelics may be mediated by their acute effects on subjective experience. Therefore, in this study,

we compared the post-peak effects of LSD, placebo and LSD after pre-treatment with the 5-HT2A receptor antagonist ketanserin on primary process thinking in mental imagery reports of healthy subjects. Primary index (PI), a formal measure of primary process thinking (Stigler, 2001; Frick et al., 2008), was used as primary endpoint in this study. We hypothesized that LSD would increase PI in verbal imagery reports. We further hypothesized that ketanserin would block the effects of LSD on PI and subjective experience. Finally, given the relative novelty of the primary endpoint variable (PI) in the field of cognitive neuroscience, we performed multiple correlation analyses to quantitatively assess the relationship between PI and other more common measures which had been frequently used to assess psychedelic-induced changes in state of consciousness, using a short version of the Altered State of Consciousness (5D-ASC) self-rating scale; ratings of mental imagery experience, using visual analog scales (VASs); and dreaming cognition, using cognitive weirdness (BD) in the mental imagery reports.

MATERIALS AND METHODS

Study Design

The study followed a double-blind, placebo-controlled, within-subjects, crossover design that involved three experimental sessions in balanced order. The washout periods between sessions were at least 14 days. This study was carried out in accordance with the recommendations of the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice (ICH-GCP). All subjects gave written informed consent. The protocol was approved by the Cantonal Ethics Committee of Zurich. The administration of LSD in healthy subjects was authorized by the Swiss Federal Office for Public Health, Bern, Switzerland.

Participants

Twenty-five healthy subjects (19 men, 6 women; mean age \pm SD: 25 \pm 4 years; range: 20–34 years) participated in the study. Subjects had to be physically and mentally healthy. Exclusion criteria were pregnancy, poor knowledge of the German language, history of alcohol or illicit drug dependence, and previous significant adverse reaction to a psychedelic drug. Nine of the 25 subjects had prior experience with classic psychedelics (number of subjects: psilocybin 6, LSD 3, LSA¹ 1, DMT² 1, 2C-E³ 1).

Study Procedures

The mental imagery task from this study has been described in detail elsewhere (Krahenmann et al., 2017). Briefly, the 30-min task followed the mental imagery method developed by Leuner (1969) and was performed 7 h after drug treatment, during the descending phase of the acute effects of LSD (Dolder et al., 2015). The task was conducted in an esthetic living-room-like room located in a tranquil side wing of the research department. Mental imagery reports from the subjects were audio recorded and transcribed for statistical analysis.

Study Drug

In each of the three experimental sessions, subjects first received pre-treatment, followed by treatment after 1 h. The drug conditions were LSD (placebo + 100 mcg LSD orally), Ket+LSD (40 mg ketanserin + 100 mcg LSD orally), and Pla (placebo + placebo orally).

MEASURES

State of Consciousness and Mental Imagery Experience

Subjective state of consciousness at the time of the mental imagery task (390 min after drug intake) was evaluated using a short version of the Altered State of Consciousness (5D-ASC) rating scale (Studerus et al., 2010) for spiritual experience, blissful state, disembodyment, elementary imagery, and changed meaning of percepts. Mental imagery experience was evaluated using visual analog subscales (VASs) for visual vividness, emotional arousal, positive emotions, negative emotions, insight and relaxation.

To assess the relationship between primary process thinking and dream mentation, we included cognitive bizarre ness (BD) in the multiple correlations analysis of this study (cognitive bizarre ness is a standardized formal measure of dream mentation and had been calculated from the mental imagery reports of this study sample ($N = 25$) elsewhere, see Kraehenmann et al., 2017).

Primary and Secondary Process Thinking

The main outcome measure in this study was primary index (PI), a formal measure of primary process thinking which had been previously used in text-analytical studies on primary process thinking and mental imagery (Stigler, 2001; Frick et al., 2008). PI was calculated by dividing the relative frequency of primary process (PP) scores by the sum of primary process and secondary process (SP) scores in the imagery reports ($PI = 100 \times PP/(PP+SP)$) (Stigler, 2001). The relative frequency of PP and SP scores was calculated by dividing the PP and SP scores by the number of words in the reports.

Primary process was evaluated using the rating scale of Auld et al. (1968), a comprehensive scale for measuring primary process thinking. The scale consists of nine PP categories (condensation, unlikely combinations or events, fluid transformations, visual representation, symbolism, contradiction, magic occurrences, inhibited movement, taboo sexual and aggressive acts) which sum up to the PP score. Examples for PP items from our study subjects: "...a cat is coming from the right side. The cat has huge blue and luminous eyes...the eyes look upward, then down, left, right, always alternating, like a cuckoo clock with moving eyes...now she has turned into a wooden clock hanging on a wall"; "I am part of a metal plate...I am fusing with the metal plate...I am now a part of this plate...it feels like being a liquid...I am only existing in certain parts of my body...The whole room rolls itself and suddenly, everything is dark...I can only see flickering light and two-dimensional faces"; "I see two entangled persons,

like an art painting...when I approach the two persons, they form an ugly bulb and dissolve into bubbles...now I see a huge mouth with yellow teeth...the mouth snaps and draws everything in."

Secondary process was evaluated using a modified version (Natale et al., 1979) of the rating scale of Weintraub and Aronson (1969), a comprehensive scale for measuring secondary process thinking. The scale consists of seven SP categories (non-personal reference, negators, qualifiers, retractors, explaining, expressions of feeling, evaluators) which sum up to the SP score. Examples for SP items from our study subjects: "...the room has got a bed. The bed is covered with a blanket protecting the bed from dust, I suppose. The bed is adorned with two or three carefully arranged pillows - this looks beautiful..."; "It is a little brook with trees along the banks...actually, the water is really cold...it feels good...the water is indeed cold as ice and it is freezing, but because I only dip my feet in the water, it is an extremely good feeling, vitalizing..."; "...it is not such a special house...it has a roof, a balcony, windows, a garden...in front of the house there is scrub, plants...and a green meadow...the meadow is not so beautiful, doesn't look quite as well cared for as it should...".

STATISTICS

The statistical analyses were performed using IBM SPSS Statistics 23 software (IBM, Chicago, IL, United States). Repeated-measures analyses of variance (ANOVAs) were conducted to compare the drug effects in LSD, Ket+LSD, and Pla conditions. Significant main effects or interactions in the ANOVAs were followed by Bonferroni-corrected *post hoc* pairwise comparisons with a significance level of $p < 0.05$ (two-tailed test). Bonferroni-corrected Spearman multiple correlations (Bonferroni-corrected alpha = $0.05/12 = 0.0042$) were used to quantify the relations between the LSD-Pla difference scores for primary index (ΔPI), state of consciousness ($\Delta 5D-ASC$), mental imagery experience (ΔVAS), and cognitive bizarre ness (ΔBD).

RESULTS

State of Consciousness and Mental Imagery Experience

Lysergic acid diethylamide significantly changed state of consciousness, as indicated by a significant main effect of drug [$F(2,48) = 89.42, p < 0.001, \eta_p^2 = 0.79$] in a repeated-measures (drug \times subscale) ANOVA on 5D-ASC score at T3. There was also a significant main effect of subscale [$F(4,96) = 17.63, p < 0.001, \eta_p^2 = 0.42$] and a significant drug \times subscale interaction [$F(8,192) = 16.01, p < 0.001, \eta_p^2 = 0.40$]. Bonferroni-corrected *post hoc* pairwise comparisons revealed a greater score on all five 5D-ASC subscales in the LSD condition than in the Pla and Ket+LSD conditions (all $p < 0.05$). Scores did not differ between the Pla and Ket+LSD conditions for any 5D-ASC subscale (all $p = \text{n.s.}$), indicating that ketanserin pre-treatment

completely blocked all LSD-induced effects (**Supplementary Figure S1**).

Lysergic acid diethylamide significantly changed subjective mental imagery experience, as indicated by a significant main effect of drug [$F(2,48) = 8.57, p < 0.001, \eta_p^2 = 0.26$] in a repeated-measures (drug \times subscale) ANOVA on the retrospectively administered VAS for mental imagery experience. There was also a significant main effect of subscale [$F(2.86,68.71) = 55.23, p < 0.001, \eta_p^2 = 0.70$] and a significant drug \times subscale interaction [$F(5.58, 133.86) = 3.21, p = 0.007, \eta_p^2 = 0.12$]. Bonferroni-corrected *post hoc* pairwise comparisons revealed greater VAS score on the vividness and emotional arousal subscales in the LSD condition than in the Pla condition and on the vividness subscale in the LSD condition than in the Ket+LSD condition (all $p < 0.05$). VAS score did not differ between the Pla and Ket+LSD conditions for any VAS subscale (all $p = \text{n.s.}$), indicating that ketanserin pre-treatment completely blocked all LSD-induced effects (**Supplementary Figure S2**).

Primary and Secondary Process Thinking

Lysergic acid diethylamide significantly increased primary process thinking, as indicated by a significant main effect of drug [$F(1.07,25.70) = 50.63, p < 0.001, \eta_p^2 = 0.6$] in a one-way repeated-measures ANOVA on PI; and Bonferroni-corrected *post hoc* comparisons revealing significantly greater PI in the LSD condition than in the Pla and Ket+LSD conditions (all $p < 0.001$). PI did not differ between the Pla and Ket+LSD conditions ($p = 0.07$), indicating that ketanserin pre-treatment completely blocked the effect of LSD on PI (**Table 1**). Furthermore, the LSD-induced increase in PI was driven by an increase in PP, and not by a decrease in SP, as indicated by a significant drug \times category (PP, SP) interaction [$F(1.54,36.87) = 13.30, p < 0.001, \eta_p^2 = 0.32$] in a separate repeated-measures ANOVA; and Bonferroni-corrected *post hoc* pairwise comparisons revealing greater PP, but unchanged SP, in the LSD condition than in the Pla and Ket+LSD conditions (all $p < 0.001$). PP did not differ between the Pla and Ket+LSD conditions (all $p = \text{n.s.}$), indicating that ketanserin pre-treatment completely blocked the effect of LSD on PP (**Table 1**).

Relations between Outcome Variables

There was a significant positive correlation between LSD-induced change (LSD-Pla difference score) in PI and LSD-induced change in 5D-ASC scores for the disembodyment subscale ($r = 0.61, N = 25, p = 0.012$, Bonferroni-corrected) and for the blissful state subscale ($r = 0.63, N = 25, p = 0.012$, Bonferroni-corrected) (**Figures 1A,B**). Furthermore, there was a highly significant positive correlation between LSD-induced change in PI and LSD-induced change in BD ($r = 0.89, N = 25, p < 0.001$, Bonferroni-corrected) (**Figure 1C**).

DISCUSSION

The main finding of this study was that LSD increased primary process thinking, a lower-level, automatic, motivation- and emotion-driven mode of mental organization which is characterized by image fusion; unlikely combinations or events; sudden shifts or transformations of images; and contradictory or illogical actions, feelings, or thoughts (Rapaport, 1950; Holt, 1956; Auld et al., 1968; Shevrin, 1996; Sloman and Steinberg, 1996; Brakel et al., 2000). Specifically, we show that LSD, in comparison with placebo, increased primary index, a formal linguistic measure of primary process thinking in the imagery reports (Auld et al., 1968; Stigler, 2001) (**Table 1**). Furthermore, we found that the effect of LSD on primary index was completely blocked by ketanserin, a 5-HT2A receptor antagonist (**Table 1**). Finally, we show that the LSD-induced increase in primary index was related to LSD-induced disembodyment and blissful state (**Figures 1A,B**).

Our finding that LSD acutely increased primary process thinking is supported by both direct and indirect evidence: Landon and Fischer (1970), for example, assessed the effects of low-dose (0.08 mg/kg orally) psilocybin on several linguistic parameters. It was found that psilocybin decreased sentence length and syntactic and rhetorical complexity, but increased linguistic concreteness and stereotypy, consistent with primary process thinking. Furthermore, Martindale and Fischer (1977) used content-analytic measures to directly test the hypothesis that psilocybin (0.08–0.2 mg/kg) induces primary process thinking.

TABLE 1 | Relative frequencies and *post hoc* pairwise comparisons for primary process, secondary process, and primary index in the three drug conditions.

Category	Relative frequency ^g			t_{24} value ^h p -value ⁱ		
	Pla ^d	Ket+LSD ^e	LSD ^f	LSD > Pla	LSD > Ket+LSD	Ket+LSD > Pla
PP ^a	0.0007 (0.0013)	0.0012 (0.0022)	0.0080 (0.0063)	6.40 0.000001**	6.42 0.000001**	1.86 0.07
	0.0451 (0.0128)	0.0459 (0.0105)	0.0427 (0.0110)	-0.97 0.34	-1.80 0.08	0.35 0.73
PI ^c	1.3375 (1.9622)	2.3237 (3.2501)	14.7980 (9.9916)	7.26 0.0000002**	7.12 0.0000002**	2.44 0.02

^N = 25. ^aPrimary process. ^bSecondary process. ^cPrimary index, $100 \times PP/(PP+SP)$. ^dPlacebo. ^eKetanserin. ^fLysergic acid diethylamide. ^gMean (SD). ^hPost hoc paired *t* tests, two-tailed. ⁱUncorrected *p*-value. * $p < 0.05$, Bonferroni-corrected; ** $p < 0.001$, Bonferroni-corrected (alpha threshold $0.05/9 = 0.0056$).

¹D-lysergic acid amide.

²N,N-dimethyltryptamine.

³2,5-dimethoxy-4-ethylphenethylamine.

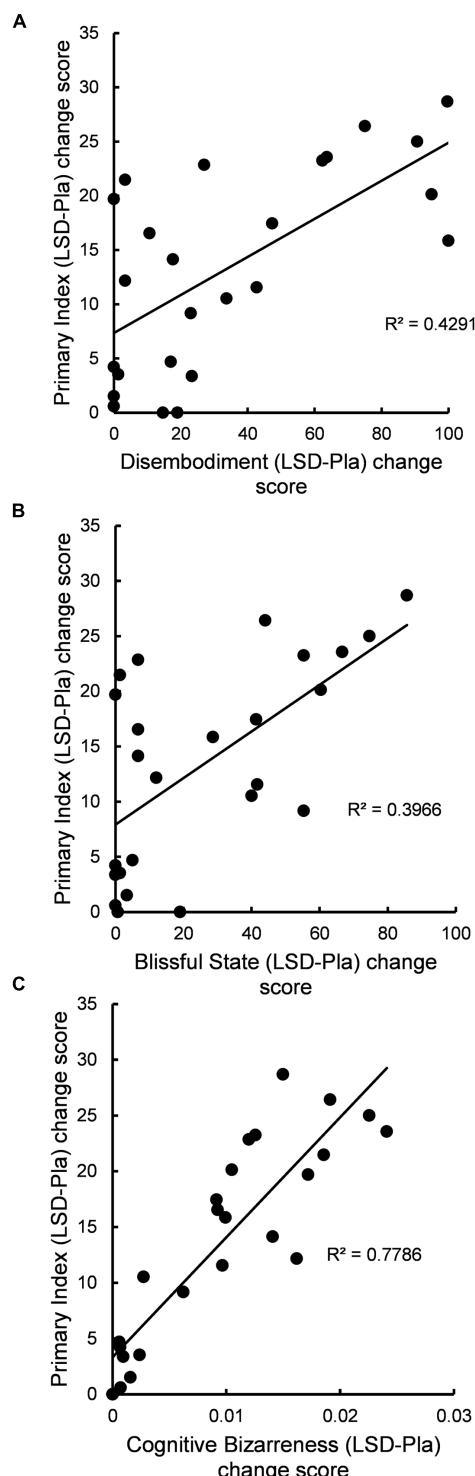


FIGURE 1 | (A) Change in disembodiment was related to change in primary index of mental imagery reports. The scatter plot shows the relation between the LSD-induced increase in score on the disembodiment subscale of the 5D-ASC (difference between LSD and placebo drug conditions, x-axis) and the LSD-induced increase in primary index score of mental imagery reports (difference between LSD and placebo drug conditions, y-axis) ($r = 0.61$, $N = 25$, $p = 0.012$, Bonferroni-corrected); **(B)** Change in blissful state was related to change in primary index of mental imagery reports. The scatter plot shows the relation between the LSD-induced increase in blissful state score (difference between LSD and placebo drug conditions, x-axis) and the LSD-induced increase in primary index score of mental imagery reports (difference between LSD and placebo drug conditions, y-axis) ($r = 0.63$, $N = 25$, $p = 0.012$, Bonferroni-corrected); **(C)** Change in cognitive bizarreness was related to change in primary index of mental imagery reports. The scatter plot shows the relation between the LSD-induced increase in cognitive bizarreness score (difference between LSD and placebo drug conditions, x-axis) and the LSD-induced increase in primary index score of mental imagery reports (difference between LSD and placebo drug conditions, y-axis) ($r = 0.89$, $N = 25$, $p < 0.001$, Bonferroni-corrected). Pla, placebo; LSD, lysergic acid diethylamide.

FIGURE 1 | Continued

related to change in primary index of mental imagery reports. The scatter plot shows the relation between the LSD-induced increase in score on blissful state subscale of the 5D-ASC (difference between LSD and placebo drug conditions, x-axis) and the LSD-induced increase in primary index score of mental imagery reports (difference between LSD and placebo drug conditions, y-axis) ($r = 0.63$, $N = 25$, $p = 0.012$, Bonferroni-corrected); **(C)** Change in cognitive bizarreness was related to change in primary index of mental imagery reports. The scatter plot shows the relation between the LSD-induced increase in cognitive bizarreness score (difference between LSD and placebo drug conditions, x-axis) and the LSD-induced increase in primary index score of mental imagery reports (difference between LSD and placebo drug conditions, y-axis) ($r = 0.89$, $N = 25$, $p < 0.001$, Bonferroni-corrected). Pla, placebo; LSD, lysergic acid diethylamide.

They showed that psilocybin increased primary process words, particularly content related to regressive imagery. Moreover, Natale et al. (1978, 1979) investigated the effects of low to medium dose LSD (15–100 mcg) on speech patterns of depressed patients during psychoanalytic sessions. They found that LSD increased the patients' use of novel figurative language and of primary process-related words, respectively, consistent with an increase in primary process thinking. Furthermore, Barr et al. (1972) and Holt (2002) investigated the effects of LSD on primary process responses to the Rorschach projective test. They found highly significant LSD effects on formal measures of primary process thinking, including features such as image fusion, fluid transformations of percepts, autistic logic, logical contradictions, verbal condensations, loosening of memory, and unlikely combinations. Finally, recent double-blind, placebo-controlled studies lend further support to the notion that psychedelics enhance primary process thinking: Spitzer et al. (1996) for example, used word-pairs of different semantic distance and showed that psilocybin increased indirect semantic priming, i.e., priming for remotely related word-pairs. Interestingly, the authors interpreted their results as evidence that psilocybin "in fact leads to an increased availability of remote associations and thereby may bring cognitive contents to mind that under normal circumstances remain non-activated." Similar effects were found for LSD in a recent double-blind, placebo-controlled study by Family et al. (2016). Taken together, both our results and previous evidence indicate that psychedelics induce an altered state of consciousness which is characterized by primary process cognition. Our findings are also in line with recent neuroimaging data: the entropic brain theory (Carhart-Harris et al., 2014), for example, holds that secondary process (the cognitive mode of the Freudian "ego") is coded by default mode network (DMN) regions and provides top-down predictions to reduce free-energy associated with the primary process (the Freudian "id") within (para)limbic and anti-correlated neural networks, converting free energy into bound energy. According to the entropic brain theory, psychedelics induce an "unconstrained," "high-entropy" cognitive state whereby DMN activity breaks down, leading to broadband alterations in resting-state functional connectivity between regions that show little connectivity in a baseline state.

However, contrary to such cognitive shift models, which posit that psychedelics decrease secondary process thinking,

leading to disinhibition of primary process thinking (“ego regression,” in psychoanalytical terms), our data did not show a statistically significant effect of LSD on SP, while there was a significant increase in PP during LSD compared to placebo (**Table 1**). These findings seem to suggest that there is no simple “shift” or “transition” from secondary toward primary process thinking during psychedelic states: secondary process thinking during psychedelic states appears preserved, while there is an increase in primary process thinking. This may be an important feature distinguishing night dreams from psychedelic experiences. In fact, a recent neuroimaging study (Lewis et al., 2017) showed that psychedelics increase rather than decrease neural activity in cortical areas that are thought to mediate the features of secondary process thinking, including dorsolateral prefrontal cortex (DLPFC) and temporal cortex (Dresler et al., 2014). Therefore, psychedelic states may be best conceptualized as hybrid states of consciousness which share features of both dreaming and waking consciousness. This is supported by a recent neuroimaging study (Voss et al., 2014) which showed that dreaming (and hence unaware) subjects regained self-awareness in their dreams (they became “lucid”) following frontal low current stimulation of gamma activity over DLPFC regions. In fact, the close neurophenomenological similarity between psychedelic states and lucid dreaming (Krahenmann, 2017) may shed some light on the therapeutic potential of psychedelic-induced experiences: they are not just “epiphenomena” of underlying neuronal oscillations, but rather induce conscious learning experiences that promote self-knowledge and psychological insight.

The human brain is a hierarchically organized and evolutionarily layered organ, and this basic structure is reflected in the cognitive organization of the mind (Montag and Panksepp, 2017). Primary process thinking has been related to neuronal activation of ontologically early, subcortical and limbic regions of the brain which code for instinctual drives and primal affective experiences (Solms and Panksepp, 2012; Montag and Panksepp, 2017). In addition, previous neuroimaging studies (Carhart-Harris et al., 2012; Krahenmann et al., 2015, 2016; Lewis et al., 2017) indicate that psychedelics such as psilocybin modulate information processing in both cortical and subcortical memory and emotion circuits of the brain (e.g., cingulate cortex, temporal cortex, insula, amygdala, hippocampus). This is supported by recent receptor binding studies showing a dense and widespread expression of 5-HT2A/5-HT1A receptors in both cortical and subcortical areas of the human brain (Beliveau et al., 2017). This may explain why, in psychedelic states, basic drives and primary emotions are strongly activated and substantially influence cognition and behavior (Hermle and Krahenmann, 2017). In fact, there is consistent evidence that psychedelics, especially during drug peak effects, induce high emotional arousal: “...intense, labile, personally meaningful emotionality is uniformly produced, with periodic episodes of overwhelming feeling” (Pahnke et al., 1971). Even under high-dose drug conditions, most subjects describe their imagery as highly pleasurable and rewarding (“cosmic joy”), coming along with feelings of “boundlessness” and “unity” (“oceanic boundlessness”) (Griffiths et al., 2011; Studerus et al., 2011).

Moreover, previous factor analytical studies (Studerus et al., 2010, 2011; Lebedev et al., 2015) support the view that psychedelics induce altered states of consciousness based on two main factors: visual imagery on the one hand, and emotionally experienced alterations in self-awareness and loss of self-/body-boundaries on the other hand. Taken together, our results are entirely consistent with this view because LSD significantly induced vivid imagery on the VAS subscale, blissful state (positively valenced mood state) and disembodyment on the 5D-ASC subscale (**Supplementary Figure S1**).

Recent behavioral (Kometer et al., 2012, 2013; Krahenmann et al., 2017) and neurobiological studies (Lebedev et al., 2015; Preller et al., 2017) may help explain why psychedelics are such potent modulators of visual imagery, emotions, and self-/body-awareness. For example, it has been shown that 5-HT2A receptor activation in the brain is a central mechanism underlying psychedelic-induced imagery (Kometer et al., 2013), positive mood states (Kometer et al., 2012), and alterations in the sense of self and body (Vollenweider et al., 1998). Therefore, our results are consistent with this view because ketanserin-pretreatment of LSD completely blocked the observed subjective and behavioral effects of LSD (**Table 1** and **Supplementary Figure S1**). Given that 5-HT receptors are widely expressed in the human brain (Beliveau et al., 2017), they have important functions in the regulation of mood states, instinctual drives, sleep, and dreaming (Nichols and Nichols, 2008; Pace-Schott, 2008). In fact, we (Krahenmann et al., 2017) have recently shown that 5-HT2A receptor activation by LSD induces dreamlike imagery, correlating with LSD-induced loss of self-boundaries and cognitive control. Given that there is a broad phenomenological and neurophysiological overlap between psychedelic states and dreaming (Krahenmann, 2017), and given that primary process thinking is the prevalent cognitive mode in dreams (Rapaport, 1950; Holt, 1956; Auld et al., 1968; Shevlin, 1996; Sloman and Steinberg, 1996; Brakel et al., 2000), it is plausible to assume that 5-HT2A receptor activation by psychedelics induces dreamlike imagery which is related to primary process thinking, emotion activation, and alterations in the sense of self and body. This is strongly supported by our results because LSD-induced primary process thinking was positively correlated with LSD-induced cognitive weirdness, a formal measure of dreaming cognition (**Figure 1C**), and was related to both LSD-induced blissful state (**Figure 1B**) and disembodyment (**Figure 1A**) on the 5D-ASC. Finally, this is also supported by previous neuroimaging studies (Maquet et al., 1996; Braun, 1997; Solms, 2000; Lebedev et al., 2015) which found that both psychedelics and rapid-eye movement dreams activate temporal lobe regions, leading to visual imagery and changes in the sense of self and body.

The close relationship between primary process thinking, dream-like cognitive weirdness, imagery intensity and emotionality during LSD in conjunction with guided mental imagery relative to guided imagery during placebo implicates that LSD in combination with mental imagery induces inner experiences which are different from those produced by either LSD alone or guided mental imagery alone. Given that mental imagery and dreams establish privileged access to latent relational and emotional schemes (Grenell, 2008; Kottje-Birnbacher, 2011),

LSD and other classical psychedelics might be beneficially used as add-on pharmacotherapeutics to deepen psychotherapeutic processes (Krahenmann, 2017). In fact, early clinical studies between 1950 and 1970 used LSD in a similar way (Strassman, 1995). Levine and Ludwig (1965), for example, showed that the combination of hypnosis and LSD produced more profound alterations in consciousness than either hypnosis or LSD alone. Future clinical studies could test this hypothesis by using a study design with several treatment arms comparing either psychedelics without psychotherapy versus psychedelics in conjunction with psychotherapy versus psychotherapy alone.

Limitations

We only assessed primary process thinking during the descending phase of the acute effects of LSD (Dolder et al., 2015). Therefore, we didn't measure drug peak-effects, which might have yielded different results. Nonetheless, we are confident that peak-effects of LSD on cognition and subjective experience would turn out to be similar, if not even stronger, than the observed effects, given that during drug peak, the effects of LSD were completely blocked by ketanserin, and given that LSD induced more primary process in subjects which had more intense subjective drug effects (Figures 1A,B). However, we did not assess dose-dependency of the effects of LSD on primary process thinking. Given that a recent neuroimaging study (Lewis et al., 2017) did not find dose-dependent differences between brain activation patterns in the acute psychedelic state, and given that dose-response relationships for psychedelic drug effects are approximately linear (Studerus et al., 2011), we expect the effects of LSD on primary process thinking to linearly increase with increasing dose.

CONCLUSION

We found that LSD, compared with placebo, enhanced primary process thinking in relation to disembodyment and blissful state. Our results confirm previous studies which showed that psychedelics acutely increase primary process thinking. Furthermore, our results indicate that psychedelic-induced primary process thinking is closely related to 5-HT2A receptor activation and the effects on mood state and sense of self and body. Taken together, we show that psychedelics induce transient, but fundamental changes in consciousness which otherwise are only experienced under psychophysiological conditions where primary process is activated such as in dreams. Finally, the results of this study may help extend current understanding of the

cognitive mechanisms underlying psychedelic-induced subjective experience. Future clinical studies may test the hypothesis that therapeutic efficacy is mediated by the psychedelic-induced primary process thinking.

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Each of the authors participated in this research by contributing to the conception and design of the study (RK, DP, KP, and FV), study management (RK, KP, TP, ES, and FV) performance of laboratory experiments (RK, KP, and TP) and statistical analysis and interpretation (RK, DP, HA, OB, ES, and FV).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2017.00814/full#supplementary-material>

FIGURE S1 | State of consciousness at the time of the mental imagery task. The graph shows the score on each 5D-ASC subscale in each drug condition at time point T3 = 390 min after drug intake. LSD increased the score on all five 5D-ASC subscales. Ketanserin pre-treatment completely blocked all LSD-induced effects (all $p = n.s.$). Asterisks indicate significant differences between LSD and placebo conditions (* $p < 0.05$; ** $p < 0.001$, Bonferroni-corrected). 5D-ASC, Altered States of Consciousness; Pla, placebo; Ket, ketanserin; LSD, lysergic acid diethylamide.

FIGURE S2 | Mental imagery experience. The graph shows the score on each visual analog subscale (VAS) in each drug condition. LSD increased VAS score for visual vividness and emotional arousal. Ketanserin pre-treatment completely blocked all LSD-induced effects (all $p = n.s.$). Asterisks indicate significant differences between LSD and placebo conditions (* $p < 0.05$; ** $p < 0.001$, Bonferroni-corrected). Pla, placebo; Ket, ketanserin; LSD, lysergic acid diethylamide.

REFERENCES

- Arminjon, M. (2011). The four postulates of Freudian unconscious neurocognitive convergences. *Front. Psychol.* 2:125. doi: 10.3389/fpsyg.2011.00125
- Auld, F., Goldenberg, G. M., and Weiss, J. V. (1968). Measurement of primary-process thinking in dream reports. *J. Pers. Soc. Psychol.* 8, 418–426. doi: 10.1037/h0025488
- Barr, H. L., Langs, R. J., Holt, R. R., Goldberger, L., and Klein, G. S. (1972). *LSD: Personality and Experience*. New York, NY: Wiley-Interscience.
- Beliveau, V., Ganz, M., Feng, L., Ozanne, B., Højgaard, L., Fisher, P. M., et al. (2017). A high-resolution in vivo atlas of the human brain's serotonin system. *J. Neurosci.* 37, 120–128. doi: 10.1523/JNEUROSCI.2830-16.2016
- Belser, A. B., Agin-Liebes, G., Swift, T. C., Terrana, S., Devenot, N., Friedman, H. L., et al. (2017). Patient experiences of psilocybin-assisted psychotherapy. An interpretative phenomenological analysis. *J. Hum. Psychol.* 57, 354–388. doi: 10.1177/0022167817706884
- Brakel, L. A., Kleinsorge, S., Snodgrass, M., and Shevrin, H. (2000). The primary process and the unconscious: experimental evidence supporting

- two psychoanalytic presuppositions. *Int. J. Psychoanal.* 81(Pt 3), 553–569. doi: 10.1516/0020757001599951
- Braun, A. (1997). Regional cerebral blood flow throughout the sleep-wake cycle. An H₂(15)O PET study. *Brain* 120, 1173–1197. doi: 10.1093/brain/120.7.1173
- Carhart-Harris, R. L., Erritzoe, D., Williams, T., Stone, J. M., Reed, L. J., Colasanti, A., et al. (2012). Neural correlates of the psychedelic state as determined by fMRI studies with psilocybin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2138–2143. doi: 10.1073/pnas.1119598109
- Carhart-Harris, R. L., Leech, R., Hellyer, P. J., Shanahan, M., Feilding, A., Tagliazucchi, E., et al. (2014). The entropic brain: a theory of conscious states informed by neuroimaging research with psychedelic drugs. *Front. Hum. Neurosci.* 8:20. doi: 10.3389/fnhum.2014.00020
- Dolder, P. C., Schmid, Y., Haschke, M., Rentsch, K. M., and Liechti, M. E. (2015). Pharmacokinetics and concentration-effect relationship of oral LSD in humans. *Int. J. Neuropsychopharmacol.* 19:pyv072. doi: 10.1093/ijnp/pyv072
- Dresler, M., Eibl, L., Fischer, C. F. J., Wehrle, R., Spoormaker, V. I., Steiger, A., et al. (2014). Volitional components of consciousness vary across wakefulness, dreaming and lucid dreaming. *Front. Psychol.* 4:987. doi: 10.3389/fpsyg.2013.00987
- Evans, J. S. B. T. (2008). Dual-processing accounts of reasoning, judgment, and social cognition. *Annu. Rev. Psychol.* 59, 255–278. doi: 10.1146/annurev.psych.59.103006.093629
- Family, N., Vinson, D., Vigliocco, G., Kaelen, M., Bolstridge, M., Nutt, D. J., et al. (2016). Semantic activation in LSD: evidence from picture naming. *Lang. Cogn. Neurosci.* 31, 1320–1327. doi: 10.1080/23273798.2016.1217030
- Frick, E., Stigler, M., Georg, H., Fischer, N., Bumeder, I., and Pokorny, D. (2008). Tumor patients in psychodynamic psychotherapy including daydreaming: can imagery enhance primary process and positive emotions? *Psychother. Res.* 18, 444–453. doi: 10.1080/10503300701832433
- Gasser, P., Kirchner, K., and Passie, T. (2015). LSD-assisted psychotherapy for anxiety associated with a life-threatening disease: a qualitative study of acute and sustained subjective effects. *J. Psychopharmacol.* 29, 57–68. doi: 10.1177/0269881114555249
- Grenell, G. (2008). Affect integration in dreams and dreaming. *J. Am. Psychoanal. Assoc.* 56, 223–251. doi: 10.1177/0003065108315694
- Griffiths, R., Johnson, M. W., Richards, W. A., Richards, B. D., McCann, U., and Jesse, R. (2011). Psilocybin occasioned mystical-type experiences: immediate and persisting dose-related effects. *Psychopharmacology* 218, 649–665. doi: 10.1007/s00213-011-2358-5
- Hermle, L., and Krahenmann, R. (2017). Experimental psychosis research and schizophrenia-similarities and dissimilarities in psychopathology. *Curr. Top. Behav. Neurosci.* doi: 10.1007/7854_2016_460 [Epub ahead of print].
- Holt, R. R. (1956). Gauging primary and secondary processes in Rorschach responses. *J. Proj. Tech.* 20, 14–25. doi: 10.1080/08853126.1956.10380666
- Holt, R. R. (2002). Quantitative research on the primary process: method and findings. *J. Am. Psychoanal. Assoc.* 50, 457–482. doi: 10.1177/00030651020500021501
- Kometer, M., Schmidt, A., Bachmann, R., Studerus, E., Seifritz, E., and Vollenweider, F. X. (2012). Psilocybin biases facial recognition, goal-directed behavior, and mood state toward positive relative to negative emotions through different serotonergic subreceptors. *Biol. Psychiatry* 72, 898–906. doi: 10.1016/j.biopsych.2012.04.005
- Kometer, M., Schmidt, A., Jäncke, L., and Vollenweider, F. X. (2013). Activation of serotonin 2A receptors underlies the psilocybin-induced effects on α oscillations, N170 visual-evoked potentials, and visual hallucinations. *J. Neurosci.* 33, 10544–10551. doi: 10.1523/JNEUROSCI.3007-12.2013
- Kottje-Birnbacher, L. (2011). Imaginations in psychodynamic psychotherapy. *Psychotherapeut* 56, 142–152. doi: 10.1007/s00278-010-07814
- Krahenmann, R. (2017). Dreams and psychedelics: neurophenomenological comparison and therapeutic implications. *Curr. Neuropharmacol.* 15, 1032–1042. doi: 10.2174/1573413713666170619092629
- Krahenmann, R., Pokorny, D., Vollenweider, L., Preller, K. H., Pokorny, T., Seifritz, E., et al. (2017). Dreamlike effects of LSD on waking imagery in humans depend on serotonin 2A receptor activation. *Psychopharmacology* 234, 2031–2046. doi: 10.1007/s00213-017-4610-0
- Krahenmann, R., Preller, K. H., Scheidegger, M., Pokorny, T., Bosch, O. G., Seifritz, E., et al. (2015). Psilocybin-induced decrease in amygdala reactivity correlates with enhanced positive mood in healthy volunteers. *Biol. Psychiatry* 78, 572–581. doi: 10.1016/j.biopsych.2014.04.010
- Krahenmann, R., Schmidt, A., Friston, K., Preller, K. H., Seifritz, E., and Vollenweider, F. X. (2016). The mixed serotonin receptor agonist psilocybin reduces threat-induced modulation of amygdala connectivity. *Neuroimage Clin.* 11, 53–60. doi: 10.1016/j.nicl.2015.08.009
- Landon, M., and Fischer, R. (1970). On similar linguistic structures in creative performance and psilocybin-induced experience. *Confin. Psychiatr.* 13, 115–138.
- Lebedev, A. V., Lövdén, M., Rosenthal, G., Feilding, A., Nutt, D. J., and Carhart-Harris, R. L. (2015). Finding the self by losing the self: neural correlates of ego-dissolution under psilocybin. *Hum. Brain Mapp.* 36, 3137–3153. doi: 10.1002/hbm.22833
- Leuner, H. (1969). Guided affective imagery (GAI). A method of intensive psychotherapy. *Am. J. Psychother.* 23, 4–21.
- Levine, J., and Ludwig, A. (1965). Alterations in consciousness produced by combinations of LSD, hypnosis and psychotherapy. *Psychopharmacologia* 7, 123–137. doi: 10.1007/BF00403635
- Lewis, C. R., Preller, K. H., Krahenmann, R., Michels, L., Staempfli, P., and Vollenweider, F. X. (2017). Two dose investigation of the 5-HT-agonist psilocybin on relative and global cerebral blood flow. *Neuroimage* 159, 70–78. doi: 10.1016/j.neuroimage.2017.07.020
- Maquet, P., Péters, J., Aerts, J., Delfiore, G., Degueldre, C., Luxen, A., et al. (1996). Functional neuroanatomy of human rapid-eye-movement sleep and dreaming. *Nature* 383, 163–166. doi: 10.1038/383163a0
- Martindale, C., and Fischer, R. (1977). The effects of psilocybin on primary process content in language. *Confin. Psychiatr.* 20, 195–202.
- Montag, C., and Panksepp, J. (2017). Primary emotional systems and personality: an evolutionary perspective. *Front. Psychol.* 8:464. doi: 10.3389/fpsyg.2017.00464
- Natale, M., Dahlberg, C. C., and Jaffe, J. (1978). Effect of psychotomimetics (LSD and dextroamphetamine) on the use of primary- and secondary-process language. *J. Consult. Clin. Psychol.* 46, 352–353. doi: 10.1037/0022-006X.46.2.352
- Natale, M., Dahlberg, C. C., and Jaffe, J. (1979). The effects of LSD-25 and dextroamphetamine on the use of defensive language. *J. Clin. Psychol.* 35, 250–254. doi: 10.1002/1097-4679(197904)35:2<250::AID-JCLP2270350205>3.0.CO;2-G
- Nichols, D. E., and Nichols, C. D. (2008). Serotonin receptors. *Chem. Rev.* 108, 1614–1641. doi: 10.1021/cr0782240
- Pace-Schott, E. F. (2008). “Serotonin and dreaming,” in *Serotonin and Sleep: Molecular, Functional and Clinical Aspects*, eds J. M. Monti, S. R. Pandi-Perumal, B. L. Jacobs, and D. J. Nutt (Basel: Birkhäuser Verlag), 307–324. doi: 10.1007/978-3-7643-8561-3_12
- Pahnke, W. N., Kurland, A. A., Unger, S., Savage, C., and Grof, S. (1971). The experimental use of psychedelic (LSD) psychotherapy. *Int. J. Clin. Pharmacol. Ther. Toxicol.* 4, 446–454.
- Preller, K. H., Herdener, M., Pokorny, T., Planzer, A., Krahenmann, R., Stämpfli, P., et al. (2017). The fabric of meaning and subjective effects in LSD-induced states depend on serotonin 2A receptor activation. *Curr. Biol.* 27, 451–457. doi: 10.1016/j.cub.2016.12.030
- Pribram, K. H., and Gill, M. M. (1976). *Freud's 'Project' Reassessed*. New York, NY: Basic Books.
- Rapaport, D. (1950). On the psycho-analytic theory of thinking. *Int. J. Psychoanal.* 31, 161–170.
- Shanks, D. R. (2010). Learning: from association to cognition. *Annu. Rev. Psychol.* 61, 273–301. doi: 10.1146/annurev.psych.093008.100519
- Shevrin, H. (1996). *Conscious and Unconscious Processes: Psychodynamic, Cognitive, and Neurophysiological Convergences*. New York, NY: Guilford Press.
- Sloman, S. A., and Steinberg, R. J. (1996). The empirical case for two systems of reasoning. *Psychol. Bull.* 119, 3–22. doi: 10.1037/0033-2959.119.1.3
- Solms, M. (2000). Dreaming and REM sleep are controlled by different brain mechanisms. *Behav. Brain Sci.* 23, 843–850. doi: 10.1017/S0140525X00003988
- Solms, M., and Panksepp, J. (2012). The “Id” knows more than the “Ego” admits: neuropsychoanalytic and primal consciousness perspectives on the interface between affective and cognitive neuroscience. *Brain Sci.* 2, 147–175. doi: 10.3390/brainsci2020147

- Spitzer, M., Thimm, M., Hermle, L., Holzmann, P., Kovar, K.-A., Heimann, H., et al. (1996). Increased activation of indirect semantic associations under psilocybin. *Biol. Psychiatry* 39, 1055–1057. doi: 10.1016/0006-3223(95)00418-1
- Stigler, M. (2001). Emotions and primary process in guided imagery psychotherapy. *Psychother. Res.* 11, 415–431. doi: 10.1093/ptr/11.4.415
- Strassman, R. J. (1995). Hallucinogenic drugs in psychiatric research and treatment. Perspectives and prospects. *J. Nerv. Ment. Dis.* 183, 127–138. doi: 10.1097/00005053-199503000-00002
- Studerus, E., Gamma, A., and Vollenweider, F. X. (2010). Psychometric evaluation of the altered states of consciousness rating scale (OAV). *PLOS ONE* 5:e12412. doi: 10.1371/journal.pone.0012412
- Studerus, E., Kometer, M., Hasler, F., and Vollenweider, F. X. (2011). Acute, subacute and long-term subjective effects of psilocybin in healthy humans: a pooled analysis of experimental studies. *J. Psychopharmacol.* 25, 1434–1452. doi: 10.1177/0269881110382466
- Vaitl, D., Birbaumer, N., Gruzelier, J., Jamieson, G. A., Kotchoubey, B., Kubler, A., et al. (2005). Psychobiology of altered states of consciousness. *Psychol. Bull.* 131, 98–127. doi: 10.1037/0033-2909.131.1.98
- Vollenweider, F. X., Vollenweider-Scherpenhuyzen, M. F., Bäbler, A., Vogel, H., and Hell, D. (1998). Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2 agonist action. *Neuroreport* 9, 3897–3902. doi: 10.1097/00001756-199812010-00024
- Voss, U., Holzmann, R., Hobson, A., Paulus, W., Koppehele-Gossel, J., Klimke, A., et al. (2014). Induction of self awareness in dreams through frontal low current stimulation of gamma activity. *Nat. Neurosci.* 17, 810–812. doi: 10.1038/nn.3719
- Watts, R., Day, C., Krzanowski, J., Nutt, D., and Carhart-Harris, R. (2017). Patients' accounts of increased "connectedness" and "acceptance" after psilocybin for treatment-resistant depression. *J. Hum. Psychol.* 6, 1–45. doi: 10.1177/0022167817709585
- Weintraub, W., and Aronson, H. (1969). Application of verbal behavior analysis to the study of psychological defense mechanisms. V. Speech pattern associated with overeating. *Arch. Gen. Psychiatry* 21, 739–744. doi: 10.1001/archpsyc.1969.01740240099012

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Unifying Theories of Psychedelic Drug Effects

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How do psychedelic drugs produce their characteristic range of acute effects in perception, emotion, cognition, and sense of self? How do these effects relate to the clinical efficacy of psychedelic-assisted therapies? Efforts to understand psychedelic phenomena date back more than a century in Western science. In this article I review theories of psychedelic drug effects and highlight key concepts which have endured over the last 125 years of psychedelic science. First, I describe the subjective phenomenology of acute psychedelic effects using the best available data. Next, I review late 19th-century and early 20th-century theories—*model psychoses theory*, *filtration theory*, and *psychoanalytic theory*—and highlight their shared features. I then briefly review recent findings on the neuropharmacology and neurophysiology of psychedelic drugs in humans. Finally, I describe recent theories of psychedelic drug effects which leverage 21st-century cognitive neuroscience frameworks—*entropic brain theory*, *integrated information theory*, and *predictive processing*—and point out key shared features that link back to earlier theories. I identify an abstract principle which cuts across many theories past and present: psychedelic drugs perturb universal brain processes that normally serve to constrain neural systems central to perception, emotion, cognition, and sense of self. I conclude that making an explicit effort to investigate the principles and mechanisms of psychedelic drug effects is a uniquely powerful way to iteratively develop and test unifying theories of brain function.

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INTRODUCTION

Lysergic acid diethylamide (LSD), N,N-dimethyltryptamine (DMT), psilocybin, and mescaline—the ‘classic’ psychedelic drugs—can produce a broad range of effects in perception, emotion, cognition, and sense of self. How do they do this? Western science began its ‘first wave’ of systematic investigations into the unique effects of mescaline 125 years ago. By the 1950s, rising interest in mescaline research was expanded to include drugs like DMT, LSD, and psilocybin in a ‘second wave’ of psychedelic science. Because of their dramatic effect on the character and contents of subjective awareness, psychedelic drugs magnified the gaps in our scientific understanding of how brain chemistry relates to subjective experience (see Evarts, 1957; Purpura, 1968). Huxley (1991, p. 12) commented that our understanding circa 1954 was “absurdly inadequate” and amounted to a mere “clue” that he hoped would soon develop into a more robust

understanding. “Meanwhile the clue is being systematically followed, the sleuths—biochemists, psychiatrists, psychologists—are on the trail” (Huxley, 1991, p. 12). A ‘third wave’ of psychedelic science has recently emerged with its own set of sleuths on the trail, sleuths who now wield an arsenal of 21st-century scientific methodologies and are uncovering new sets of clues.

Existing theoretical hurdles span five major gaps in understanding. The first gap is that we do not have an account of how psychedelic drugs can produce such a broad diversity of subjective effects. LSD, for example, can produce subtle intensifications in perception—or it can completely dissolve all sense of space, time, and self. What accounts for this atypical diversity?

The second gap is that we do not understand how pharmacological interactions at neuronal receptors and resulting physiological changes in the neuron lead to large-scale changes in the activity of neural populations, or changes in brain network connectivity, or at the systems-level of global brain dynamics. What are the causal links in the multi-level pharmaconeurophysiological chain?

The third gap is that we do not know how psychedelic drug-induced changes in brain activity—at any level of description—map onto the acute subjective phenomenological changes in perception, emotion, cognition, and sense of self. This kind of question is not unique to psychedelic drugs (i.e., Crick and Koch, 1998; Tononi and Edelman, 1998) but our current understanding of psychedelic drug effects clearly magnifies the disconnect between brain science and subjective experience.

Fourth, there is a gap in our understanding of the relationships between psychedelic effects and symptoms of psychoses, such as perceptual distortion, hallucination, or altered self-reference. What is the relationship between psychedelic effects and symptoms of chronic psychotic disorders?

Fifth and finally, there is a gap in our clinical understanding of the process by which psychedelic-assisted therapies improve mental health (Carhart-Harris and Goodwin, 2017). Which psychedelic drug effects (in the brain or in subjective experience) enable clinical improvement? How?

Scientific efforts to understand diverse natural phenomena aim to produce a single theory that can account for many phenomena using a minimal set of principles. Such theories are sometimes called *unifying theories*. Not everyone agrees on the meaning of ‘unification’ or ‘unifying theory’ in science.¹ Morrison (2000) observed that, although theory unification is a messy process which may not have discernible universal characteristics, historically successful unifying scientific theories tend to have two common features: (1) a *formalized framework* (quantitative mathematical descriptions of the phenomena) and (2) *unifying principles* (abstract concepts that unite diverse phenomena). On this conception, then, a unifying theory of psychedelic drug effects would offer a single formalized (mathematical or computational) framework capable of describing diverse psychedelic phenomena using a minimal set of unifying principles. Unfortunately,

the survey of literature in this review does not locate an existing unifying theory of psychedelic drug effects. It does, however, highlight enduring abstract principles that recur across more than a century of theoretical efforts. Furthermore, it reviews recent formalized frameworks which, although currently heterogeneous and divergent, hint at the possibility of a quantitative groundwork for a future unifying theory.

The field of cognitive neuroscience offers formalized frameworks and general principles designed to track and model the neural correlates of perception, emotion, cognition, and consciousness. These broad frameworks span major levels of description in the brain and attempt to map them onto behavioral and phenomenological data. Corlett et al. (2009, p. 516) argue that until this is done “our understanding of how the pharmacology links to the symptoms will remain incomplete.” Montague et al. (2012, p. 1) argue that ‘computational psychiatry’ can remedy the ‘lack of appropriate intermediate levels of description that bind ideas articulated at the molecular level to those expressed at the level of descriptive clinical entities.’ Seth (2009, p. 50) argues that “computational and theoretical approaches can facilitate a transition from correlation to explanation in consciousness science” and explains how a recent LSD, psilocybin, and ketamine study (Schartner et al., 2017) was motivated by a need to elucidate descriptions at intermediate levels somewhere between pharmacology and phenomenology: “We know there’s a pharmacological link, we know there’s a change in experience and we know there’s a clinical impact. But the middle bit if you like, what are these drugs doing to the global activity of the brain, that’s the gap we’re trying to fill with this study” (quoted in Osborne, 2017). Taken together, the above quotations point to an emerging sense that cognitive neuroscience frameworks can address gaps in our understanding of psychedelic drug effects.

In this article I review theories of psychedelic drug effects. First, making an effort to clearly define the target explananda, I review the acute subjective phenomenological properties of psychedelic effects as well as long-term clinical outcomes from psychedelic-assisted therapies. Second, I review theories from first-wave and second-wave psychedelic science—*model psychoses theory*, *filtration theory*, and *psychoanalytic theory*—and identify core features of these theories. Third, I review findings from recent neurophysiological research in humans under psychedelic drugs. Finally, I review select 21st-century theories of psychedelic effects that have been developed within cognitive neuroscience frameworks; namely, *entropic brain theory*, *integrated information theory*, and *predictive processing*. My analysis of recent theoretical efforts highlights certain features, first conceptualized in 19th- and 20th-century theories, which remain relevant in their ability to capture both the phenomenological and neurophysiological dynamics of psychedelic effects. I describe how these enduring theoretical features are now being operationalized into formalized frameworks and could serve as potential unifying principles for describing diverse psychedelic phenomena.

¹For example, see Kitcher (1981, 1989), Friedman (1983), and Morrison (2000).

PSYCHEDELIC DRUG EFFECTS

There are dozens of molecules known to cause psychedelic-like effects (Schultes and Hofmann, 1973; Shulgin and Shulgin, 1991, 1997). This review focuses only on a limited set of drugs dubbed ‘classical hallucinogens’ or ‘classic psychedelics’ which are: LSD, DMT, psilocybin, and mescaline² (Nichols, 2016). Importantly, there are qualitative inter-drug differences between the effects of the four classic psychedelic drugs (Strassman et al., 1994; Hasler et al., 2004; Studerus et al., 2011; Schmid et al., 2015; Liechti et al., 2017). Drug dosage is a primary factor in predicting the types of effects that will occur (Strassman et al., 1994; Riba et al., 2001b; Hasler et al., 2004; Hintzen and Passie, 2010; Studerus et al., 2011, 2012; Liechti et al., 2017). Effects unfold temporally over a drug session; onset effects are distinct from peak effects and some effects have a higher probability of occurring at specific timepoints over the total duration of drug effects (Masters and Houston, 1966; Preller and Vollenweider, 2016). Furthermore, effects are influenced by non-drug factors traditionally referred to as *set and setting*, such as personality, pre-dose mood, drug session environment, and external stimuli (Figure 1) (Leary et al., 1963; Studerus et al., 2012; Hartogsohn, 2016; Carhart-Harris and Nutt, 2017).

The above variables, while crucial, do not completely prohibit meaningful characterization of general psychedelic effects, as numerous regularities, patterns, and structure can still be identified (Masters and Houston, 1966; Grinspoon and Bakalar, 1979; Preller and Vollenweider, 2016). Indeed, common psychedelic effects can be reliably measured using validated psychometric instruments consisting of self-report questionnaires and rating scales (Strassman et al., 1994; Dittrich, 1998; Riba et al., 2001a; Dittrich et al., 2010; Studerus et al., 2010, 2011; Maclean et al., 2012; Turton et al., 2014; Barrett et al., 2015; Nour et al., 2016) though some of these rating scales may be in need of further validation using modern statistical techniques (Bouso et al., 2016). Items from these rating scales are wrapped in ‘scare quotes’ in the following discussion in an effort to characterize the subjective phenomenology of psychedelic effects from a first-person perspective. An example of rating scale results is given in (Figure 2).

Perceptual Effects

Perceptual effects occur along a dose-dependent range from subtle to drastic. The range of different perceptual effects includes perceptual intensification, distortion, illusion, mental imagery, elementary hallucination, and complex hallucination (Klüver, 1928; Kometer and Vollenweider, 2016; Preller and Vollenweider, 2016). Intensifications of color saturation, texture definition, contours, light intensity, sound intensity, timbre variation, and other perceptual characteristics are common (Kometer and Vollenweider, 2016; Kaelen et al., 2018). The external world is experienced as if in higher resolution, seemingly more crisp and detailed, often accompanied by a distinct sense of ‘clarity’ or ‘freshness’ in the environment (Hofmann, 1980; Huxley, 1991;

²Ayahuasca contains DMT but is importantly different from pure DMT (McKenna et al., 1984).

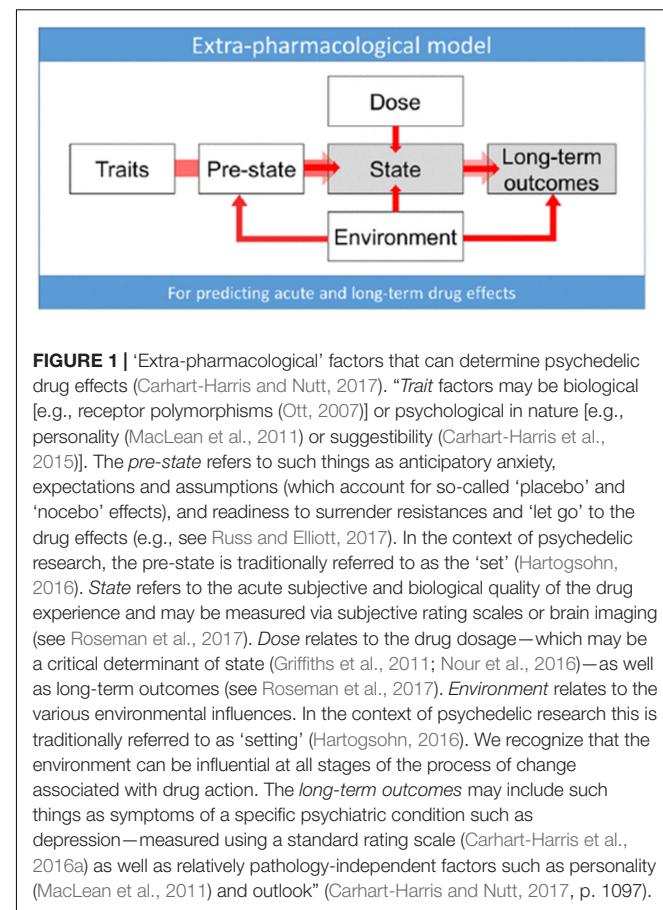


FIGURE 1 | ‘Extra-pharmacological’ factors that can determine psychedelic drug effects (Carhart-Harris and Nutt, 2017). “Trait factors may be biological [e.g., receptor polymorphisms (Ott, 2007)] or psychological in nature [e.g., personality (MacLean et al., 2011) or suggestibility (Carhart-Harris et al., 2015)]. The pre-state refers to such things as anticipatory anxiety, expectations and assumptions (which account for so-called ‘placebo’ and ‘nocebo’ effects), and readiness to surrender resistances and ‘let go’ to the drug effects (e.g., see Russ and Elliott, 2017). In the context of psychedelic research, the pre-state is traditionally referred to as the ‘set’ (Hartogsohn, 2016). State refers to the acute subjective and biological quality of the drug experience and may be measured via subjective rating scales or brain imaging (see Roseman et al., 2017). Dose relates to the drug dosage—which may be a critical determinant of state (Griffiths et al., 2011; Nour et al., 2016)—as well as long-term outcomes (see Roseman et al., 2017). Environment relates to the various environmental influences. In the context of psychedelic research this is traditionally referred to as ‘setting’ (Hartogsohn, 2016). We recognize that the environment can be influential at all stages of the process of change associated with drug action. The long-term outcomes may include such things as symptoms of a specific psychiatric condition such as depression—measured using a standard rating scale (Carhart-Harris et al., 2016a) as well as relatively pathology-independent factors such as personality (MacLean et al., 2011) and outlook” (Carhart-Harris and Nutt, 2017, p. 1097).

Díaz, 2010; Kometer and Vollenweider, 2016). Sense of meaning in percepts is altered, e.g., ‘Things around me had a new strange meaning for me’ or ‘Objects around me engaged me emotionally much more than usual’ (Studerus et al., 2010).

Perceptual distortions and illusions are extremely common, e.g., ‘Things looked strange’ or ‘My sense of size and space was distorted’ or ‘Edges appeared warped’ or ‘I saw movement in things that weren’t actually moving’ (Dittrich, 1998; Muthukumaraswamy et al., 2013). Textures undulate in rhythmic movements, object boundaries warp and pulsate, and the apparent sizes and shapes of objects can shift rapidly (Kometer and Vollenweider, 2016). Controlled psychophysical studies have measured various alterations in motion perception (Carter et al., 2004), object completion (Kometer et al., 2011), and binocular rivalry (Frecska et al., 2004; Carter et al., 2007).

In what are known as *elementary hallucinations*—e.g., ‘I saw geometric patterns’—the visual field can become permeated with intricate tapestries of brightly colored, flowing latticework and other geometric visuospatial ‘form constants’ (Klüver, 1928; Siegel and Jarvik, 1975; Kometer and Vollenweider, 2016). In *complex hallucinations* visual scenes can present elaborate structural motifs, landscapes, cities, galaxies, plants, animals, and human (and non-human) beings (Shanon, 2002; Studerus et al., 2011; Carhart-Harris et al., 2015; Kaelen et al., 2016; Preller and Vollenweider, 2016; Roseman et al., 2016;

Subjective effects of psilocybin

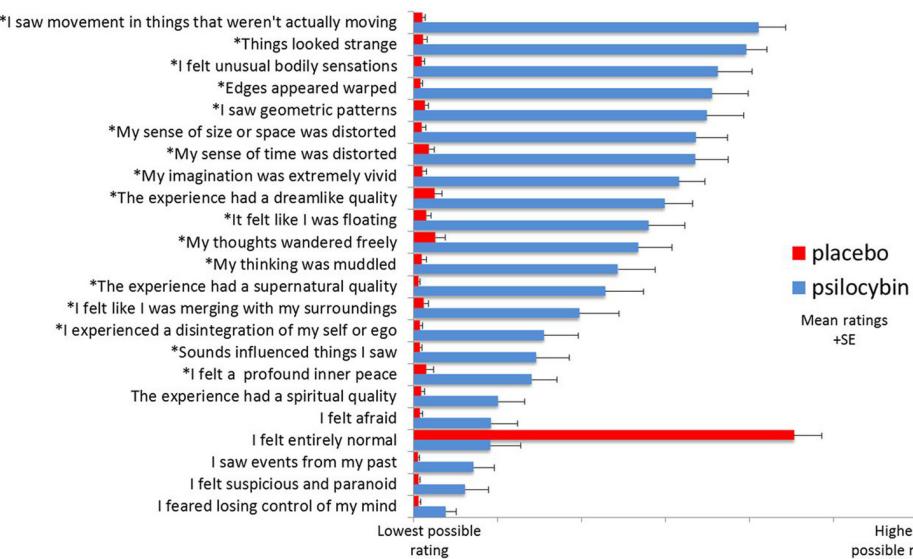


FIGURE 2 | Subjective rating scale items selected after psilocybin (blue) and placebo (red) ($n = 15$) (Muthukumaraswamy et al., 2013). “Items were completed using a visual analog scale format, with a bottom anchor of ‘no, not more than usually’ and a top anchor of ‘yes, much more than usually’ for every item, with the exception of ‘I felt entirely normal,’ which had bottom and top anchors of ‘No, I experienced a different state altogether’ and ‘Yes, I felt just as I normally do,’ respectively. Shown are the mean ratings for 15 participants plus the positive SEMs. All items marked with an asterisk were scored significantly higher after psilocybin infusion at a Bonferroni-corrected significance level of $p < 0.0022$ (0.5/23 items)” (Muthukumaraswamy et al., 2013, p. 15176).

Krahenmann et al., 2017b). Complex hallucinations typically succeed elementary hallucinations and are more likely at higher doses (Komter and Vollenweider, 2016; Liechti et al., 2017) especially under DMT (Strassman et al., 1994; Shanon, 2002). Both elementary and complex hallucinations are more commonly reported behind closed eyelids (“closed eye visuals”; CEVs) but can dose-dependently occur in full light with eyes open (“open eye visuals”; OEVs) (Komter and Vollenweider, 2016). CEVs are often described as vivid mental imagery. Under psychedelic drugs, mental imagery becomes augmented and intensified—e.g., ‘My imagination was extremely vivid’—and is intimately linked with emotional and cognitive effects (Carhart-Harris et al., 2015; Preller and Vollenweider, 2016). “Sometimes sensible film-like scenes appear, but very often the visions consist of scenes quite indescribable in ordinary language, and bearing a close resemblance to the paintings and sculptures of the surrealistic school” (Stockings, 1940, p. 31). Psychedelic mental imagery can be modulated by both verbal (Carhart-Harris et al., 2015) and musical (Kaelen et al., 2016) auditory stimuli. Synesthesia (Ward, 2013) has been reported, especially visual phenomena driven by auditory stimuli—‘Sounds influenced the things I saw’—but classification of these effects as ‘true’ synesthesia is actively debated (Sinke et al., 2012; Brogaard, 2013; Luke and Terhune, 2013; Terhune et al., 2016).

Somatosensory perception can be drastically altered—e.g., ‘I felt unusual bodily sensations’—including body image, size, shape, and location (Savage, 1955; Klee, 1963; Preller and Vollenweider, 2016). Sense of time and causal sequence can lose their usual linear cause-effect structure making it difficult to track

the transitions between moments (Heimann, 1963; Wittmann et al., 2007; Wackermann et al., 2008; Studerus et al., 2011; Schmid et al., 2015).

Overall the perceptual effects of psychedelics are extremely varied, multimodal, and easily modulated by external stimuli. Perceptual effects are tightly linked with emotional and cognitive effects.

Emotional Effects

Emotional psychedelic effects are characterized by a general intensification of feelings, increased (conscious) access to emotions, and a broadening in the overall range of emotions felt over the duration of the drug session. Psychedelics can induce unique states of euphoria characterized by involuntary grinning, uncontrollable laughter, silliness, giddiness, playfulness, and exuberance (Preller and Vollenweider, 2016). Negatively experienced emotions—e.g., ‘I felt afraid’ or ‘I felt suspicious and paranoid’—are often accompanied by a general sense of losing control, e.g., ‘I feared losing control of my mind’ (Strassman, 1984; Johnson et al., 2008; Barrett et al., 2017a). However, the majority of emotional psychedelic effects in supportive contexts are experienced as positive (Studerus et al., 2011; Schmid et al., 2015; Carhart-Harris et al., 2016b; Belser et al., 2017; Watts et al., 2017). Both LSD and psilocybin can bias emotion toward positive responses to social and environmental stimuli (Komter et al., 2012; Carhart-Harris et al., 2016b; Dolder et al., 2016; Pokorny et al., 2017). Spontaneous feelings of awe, wonder, bliss, joy, fun, excitement (and yes, peace and love) are also consistent themes across experimental and anecdotal reports (Huxley, 1991;

Kaelen et al., 2015; Preller and Vollenweider, 2016; Belser et al., 2017). In supportive environments, classic psychedelic drugs can promote feelings of trust, empathy, bonding, closeness, tenderness, forgiveness, acceptance, and connectedness (Dolder et al., 2016; Belser et al., 2017; Carhart-Harris et al., 2017b; Pokorny et al., 2017; Watts et al., 2017). Emotional effects can be modulated by all types of external stimuli, especially music (Bonny and Pahnke, 1972; Shanon, 2002; Kaelen et al., 2015, 2018).

Cognitive Effects

Precise characterization of cognitive psychedelic effects has proven enigmatic and paradoxical (Shanon, 2002; Carhart-Harris et al., 2016b). Acute changes in the normal flow of linear thinking—e.g., ‘My thinking was muddled’ or ‘My thoughts wandered freely’—are extremely common (Hasler et al., 2004; Studerus et al., 2011). This is reflected in reduced performance on standardized measures of working memory and directed attention (Carter et al., 2005; Vollenweider et al., 2007); however, reductions in performance have been shown to occur less often in individuals with extensive past experience with the drug’s effects (Bouso et al., 2013). Crucially, cognitive impairments related to acute psychedelic effects are dose-dependent (Wittmann et al., 2007). Extremely low doses, known as *microdoses*, have been anecdotally associated with improvements in cognitive performance (Waldman, 2017; Wong, 2017) “a claim that urgently requires empirical verification through controlled research” (Carhart-Harris and Nutt, 2017, p. 1103). Theoretical attempts to account for the reported effects of microdosing have yet to emerge in the literature and therefore present an important opportunity to future theoretical endeavors.

Certain cognitive traits associated with creativity can increase under psychedelics (Sessa, 2008; Baggott, 2015) such as divergent thinking (Kuypers et al., 2016), use of unlikely language patterns or word associations (Natale et al., 1978b), expansion of semantic activation (Spitzer et al., 1996; Family et al., 2016), and attribution of meaning to perceptual stimuli (Liechti et al., 2017; Preller et al., 2017) especially musical stimuli (Kaelen et al., 2015, 2018; Atasoy et al., 2017b; Barrett et al., 2017b). Primary-process thinking (Rapaport, 1950)—a widely validated psychological construct (Arminjon, 2011) associated with creativity (Suler, 1980)—is characterized phenomenologically by “image fusion; unlikely combinations or events; sudden shifts or transformations of images; and contradictory or illogical actions, feelings, or thoughts” (Kraehenmann et al., 2017a, p. 2). Psilocybin and LSD have been shown to increase primary-process thinking (Martindale and Fischer, 1977; Natale et al., 1978a; Family et al., 2016; Kraehenmann et al., 2017a) as well as the subjective bizarre and dreamlike nature of mental imagery associated with verbal stimuli (Carhart-Harris et al., 2015; Kraehenmann et al., 2017b). Cognitive flexibility (or ‘loosening’ of cognition) and optimism can remain for up to 2 weeks after the main acute drug effects have dissipated (Carhart-Harris et al., 2016b). Furthermore, long-term increases in creative problem-solving ability (Sweat et al., 2016) and personality trait openness (MacLean et al., 2011; Lebedev et al., 2016).

et al., 2016) have been measured after just one psychedelic experience.

Ego Effects and Ego Dissolution Experiences

Klüver (1926, p. 513) observed that under peyote “the line of demarcation drawn between ‘object’ and ‘subject’ in normal state seemed to be changed. The body, the ego, became ‘objective’ in a certain way, and the objects became ‘subjective.’” Similar observations continued throughout first-wave and second-wave psychedelic science (Beringer, 1927b; Klüver, 1928; Savage, 1955; Eisner and Cohen, 1958; Klee, 1963; Leary et al., 1964; Grof, 1976). Importantly, effects on sense of self and ego occur along a dose-dependent range spanning from subtle to drastic (Letheby and Gerrans, 2017; Millière, 2017). Subtle effects are described as a ‘softening’ of ego with increased insight into one’s own habitual patterns of thought, behavior, personal problems, and past experiences; effects which were utilized in ‘psycholytic’ psychotherapy (Grof, 1980). Drastic ego-effects, known as *ego dissolution*³, are described as “the dissolution of the sense of self and the loss of boundaries between self and world” (Millière, 2017, p. 1)—e.g., ‘I felt like I was merging with my surroundings’ or ‘All notion of self and identity dissolved away’ or ‘I lost all sense of ego’ or ‘I experienced a loss of separation from my environment’ or ‘I felt at one with the universe’ (Dittrich et al., 2010; Nour et al., 2016; Millière, 2017). These descriptions resemble non-drug ‘mystical-type’ experiences (James, 1902; Huxley, 1945; Stace, 1960; Forman, 1998; Baumeister and Exline, 2002); however, the extent of overlap here remains an open question (Hood, 2001; Maclean et al., 2012; Barrett and Griffiths, 2017; Millière, 2017; Winkelman, 2017). Ego dissolution is more likely to occur at higher doses (Griffiths et al., 2011; Studerus et al., 2011, 2012; Liechti et al., 2017). Furthermore, certain psychedelic drugs cause ego dissolution experience more reliably than others; psilocybin, for example, was found to produce full ego dissolution more reliably compared with LSD (Liechti et al., 2017). Ego dissolution experiences can be driven and modulated by external stimuli, most notably music (Carhart-Harris et al., 2016c; Atasoy et al., 2017b; Kaelen et al., 2018). Interestingly, subjects who experienced ‘complete’ ego dissolution in psychedelic-assisted therapy were more likely to evidence positive clinical outcomes (Griffiths et al., 2008, 2016; Majić et al., 2015; Ross et al., 2016; Roseman et al., 2017) as well as long-term changes in life outlook and the personality trait openness (MacLean et al., 2011; Carhart-Harris et al., 2016b; Lebedev et al., 2016).

Clinical Efficacy and Long-Term Effects

Mescaline-assisted therapies showed promising results during first-wave psychedelic science (Beringer, 1927b; Rouhier, 1927) and this trend continued through second-wave psychedelic research on LSD-assisted therapies (Sandison and Whitelaw, 1957; Cohen and Eisner, 1959; Pahnke et al., 1971; Grof, 1976). Recent studies have produced significant evidence for the

³Variously termed ‘ego disintegration,’ ‘ego loss,’ and ‘ego death.’ For a comprehensive review, see Millière (2017).

therapeutic utility of psychedelic drugs in treating a wide range of mental health issues (Tupper et al., 2015; Lieberman and Shalev, 2016; Carhart-Harris and Goodwin, 2017), including anxiety and depression (Grob et al., 2011; Gasser et al., 2014; Carhart-Harris et al., 2016a, 2017a; Dos Santos et al., 2016; Griffiths et al., 2016; Ross et al., 2016), obsessive-compulsive disorder (Moreno et al., 2006), and addiction (Bogenschutz and Johnson, 2016) to alcohol (Bogenschutz et al., 2015) and tobacco (Johnson et al., 2014). In many clinical studies, ego-dissolution experience has correlated with positive clinical outcomes (Griffiths et al., 2008, 2016; Majíć et al., 2015; Ross et al., 2016; Roseman et al., 2017).

Remarkably, as mentioned above, a single psychedelic experience can increase optimism for at least 2 weeks after the session (Carhart-Harris et al., 2016b) and can produce lasting changes in personality trait openness (MacLean et al., 2011; Lebedev et al., 2016). A study of regular (weekly) ayahuasca users showed improved cognitive functioning and increased positive personality traits compared with matched controls (Bouso et al., 2015). Interestingly, these outcomes may expand beyond sanctioned clinical use, as illicit users of classic psychedelic drugs within the general population self-report positive long-term benefits from their psychedelic experiences (Carhart-Harris and Nutt, 2010), are statistically less likely to evidence psychological distress and suicidality (Hendricks et al., 2015; Argento et al., 2017), and show an overall lower occurrence of mental health problems in general (Krebs and Johansen, 2013).

Summary

The above evidence demonstrates the broad diversity of acute subjective effects that classic psychedelic drugs can produce in perceptual, emotional, and cognitive domains. Unique changes in sense of self, ego, body image, and personal meaning are particularly salient themes. How do these molecules produce such dramatic effects? What are the relationships between acute perceptual, emotional, cognitive, and self-related effects? What is the link between acute effects and long-term changes in mental health, personality, and behavior? Theories addressing these questions emerged as soon as Western science recognized the need for a scientific understanding of psychedelic drug effects beginning in the late 19th century.

19th AND 20th CENTURY THEORIES OF PSYCHEDELIC DRUG EFFECTS

The effects described above are what captured the interest of first-wave and second-wave psychedelic scientists, and the theories they developed in their investigations have two central themes. The first theme is the observation that psychedelic effects share descriptive elements with symptoms of psychoses, such as hallucination, altered self-reference, and perceptual distortions. This theme forms the basis of *model psychoses theory* and is what motivated the adoption of the term ‘psychotomimetic’ drugs. The second theme is the observation that psychedelic drugs seem to expand the total range of contents presented subjectively in our perceptual, emotional, cognitive, and self-referential experience. This theme forms the basis of *filtration theory* and is what

motivated the adoption of the term ‘psychedelic’ drugs. A third theoretical account uses *psychoanalytic theory* to address the expanded range of mental phenomena produced by psychedelic drugs as well as the shared descriptive elements with symptoms of psychoses. The next section reviews these themes along with their historically associated theories before tracing their evolution into third-wave (21st-century) psychedelic science.

Model Psychoses Theory

When Lewin (1894, 1927) ‘discovered’⁴ the peyote cactus, his reports caught the attention of adventurous 19th-century scientists like Prentiss and Morgan (1895), Mitchell (1896), and Ellis (1898), who promptly obtained samples and began consuming the cactus and observing its effects on themselves. When Heffter (1898) isolated mescaline from the peyote cactus and Späth (1919) paved the way for laboratory synthesis, scientists began systematically dosing themselves (along with their colleagues and students) with mescaline and publishing their findings in medical journals (Knauer and Maloney, 1913; Klüver, 1926; Beringer, 1927b; Rouhier, 1927; Guttmann, 1936; Stockings, 1940). Klüver (1926, p. 502) argued that systematic investigations into the neural mechanisms of mescaline effects would help neurology “elucidate more general questions of the psychology and pathology of perception.” However, it was the pathology aspect, not the general psychology questions, which became the dominant focus of ensuing mescaline research paradigms.

Model psychoses theory began long before any of the classic psychedelic drugs became known to Western science. Moreau (1845) linked hashish effects with mental illness and Kraepelin (1892) founded “pharmacopsychology” by dosing himself and his students with various psychoactive drugs in the laboratory of Wilhelm Wundt (Müller et al., 2006; Schmied et al., 2006). These scientists hoped to study psychotic symptoms using drugs to induce ‘model psychoses’ (1) in themselves, to gain first-person knowledge of the phenomenology of psychotic symptoms by “administering to one another such substances as will produce in us transitory psychoses” (Knauer and Maloney, 1913, p. 426; see also Guttmann, 1936), and (2) in normal research subjects, allowing for laboratory behavioral observations on how the symptoms emerge and dissipate. Kraepelin and colleagues attempted to model psychoses using many drugs—“tea, alcohol, morphine, trional, bromide, and other drugs”—yet Kraepelin’s pupils Knauer and Maloney (1913, p. 426) argued that these drugs unfortunately “produce mental states which have little similarities to actual insanities” and argued instead that *mescaline* was unique in its ability to truly model psychoses. The dramatic subjective effects of mescaline invigorated the model psychoses paradigm. Growing demand for the ideal chemical agent for

⁴An unnamed JAMA book reviewer critically notes that “it is interesting that attention had not been paid by American scientists to this intoxicant used by the Mexican Indians until a European called attention to it” (Beringer, 1927a).

model psychoses eventually motivated Sandoz Pharmaceuticals to bring LSD to market in the 1940s.⁵

Importantly, model psychoses theory was not initially a theory of drug effects; it was an idealistic paradigm for researching psychoses that was already in use before Western science ‘discovered’ classic psychedelic drugs. Nonetheless, it seeded the idea that psychedelic effects themselves could be explained in terms of psychopathology and motivated a search for common neural correlates. The founding figures of neuropharmacology were driven by questions regarding the relationship between psychoactive drugs and endogenous neurochemicals (see Abramson, 1956). The putative psychoses-mimicking effects of LSD and mescaline inspired the idea that psychotic symptoms might be caused by a “hypothetical endotoxin” (Osmond, 1957, p. 422) or some yet-unknown endogenous neurochemical gone out of balance (Osmond and Smythies, 1952; Abramson, 1956; Himwich, 1959). The discovery that LSD can antagonize serotonin led to the hypothesis that the effects of LSD are serotonergic and simultaneously to the historic hypothesis⁶ that serotonin might play a role in regulating mental function (Gaddum, 1953; Gaddum and Hameed, 1954; Woolley and Shaw, 1954; Shaw and Woolley, 1956; Green, 2008).

At the 1955 *Second Conference on Neuropharmacology* the whole class of drugs was dubbed *psychotomimetic* (Abramson, 1956). Interestingly, the word *mimetic* means to “imitate” “mimic” or “exhibit mimicry” which is the act of *appearing* as something else—for example, when one species mimics the appearance or behavior of another (e.g., the non-venomous bullsnake rattles its tail against dry leaves to *mimic* a venomous rattlesnake). *Psychotomimetic* drug effects, on this literal reading of the term, would merely mimic or imitate—appear as if they are—psychoses. However, to mimic is not to model.⁷ A model intends to capture important structural or functional principles of the entity or phenomena that it models. A mimic, by contrast, merely creates the illusion that it possesses the properties it mimics. Thus, the term *psychotomimetic* implies that the effects of these drugs merely resemble psychoses but do not share functional or structural properties in their underlying biology or phenomenology. Nonetheless, LSD and mescaline were used as *models* to investigate psychotic symptoms. Yet the scientific utility of drug models hinges on our understanding of the mechanisms underpinning the drugs’ effects; we still need a theory of how psychotomimetic drugs work. A subtle explanation-explananda circularity can come into play here, in which psychoses are explained using drug models yet the drug effects are explained using theories of psychoses. Further complicating the matter is the clear difference between *acutely*

⁵A marketing team at Sandoz Pharmaceuticals sent free samples of LSD to physicians around the world and inside each package was a pamphlet which read: “By taking Delysid [LSD] himself, the psychiatrist is able to gain an insight into the world of ideas and sensations of mental patients. Delysid can also be used to induce model psychoses of short duration in normal subjects, thus facilitating studies on the pathogenesis of mental disease” (Hofmann, 1980, p. 47).

⁶In this sense LSD catalyzed the neuroscientific revolution of serotonin neurochemistry (Nichols, 2016) and crystallized the emergence of the field of neuropharmacology.

⁷In fact, in the terminology of biological science, a *model* is “an organism whose appearance a mimic imitates” (Merriam-Webster, 2017).

induced drug effects and the gradual development of a *chronic* mental illness (Osmond and Smythies, 1952). This cluster of conceptual challenges poured fuel on the flaming debates about the merits of drug-induced model psychoses, which in 1957 had already “smoldered for nearly 50 years” (Osmond, 1957, p. 421). An additional conceptual challenge was the fact that mescaline had for years shown promise in *treating* psychopathologies (Beringer, 1927b; Rouhier, 1927) and LSD was gaining popularity for pharmaceutically enhanced psychotherapy (Sandison and Whitelaw, 1957; Eisner and Cohen, 1958; Cohen and Eisner, 1959). Model psychoses theory needed to explain how it was the case that drugs putatively capable of inducing psychotic symptoms could simultaneously be capable of treating them—What Osmond (1957, p. 420) termed the “hair of the dog” problem. In fact, to this day “the apparent paradox by which the same compound can be both a model of, and yet a treatment for, psychopathology has never been properly addressed” (Carhart-Harris et al., 2016b, p. 2). Taken together, the above cluster of conceptual challenges drove Osmond (1957) to doubt his own prior work on model psychoses (Hoffer et al., 1954; i.e., Osmond and Smythies, 1952) and he declared ‘psychotomimetic’ an outmoded term, arguing that the effects of these drugs could not be captured wholly in terms of psychopathology. “If mimicking mental illness were the main characteristic of these agents, ‘psychotomimetics’ would indeed be a suitable generic term. It is true that they do so, but they do much more” (Osmond, 1957, p. 429).

Filtration Theory

Osmond (1957) argued that the ‘psychotomimetic’ class of drugs needed a more appropriate name. “My choice, because it is clear, euphonious, and uncontaminated by other associations, is *psychedelic*, mind-manifesting” (Osmond, 1957, p. 429). But how exactly should we understand psychedelic effects as ‘mind-manifesting?’ Osmond’s nomenclature legacy was directly influenced by his friend Aldous Huxley, who described the core idea to Osmond in the following personal letter dated April 10, 1953 (Huxley, 1953, p. 29):

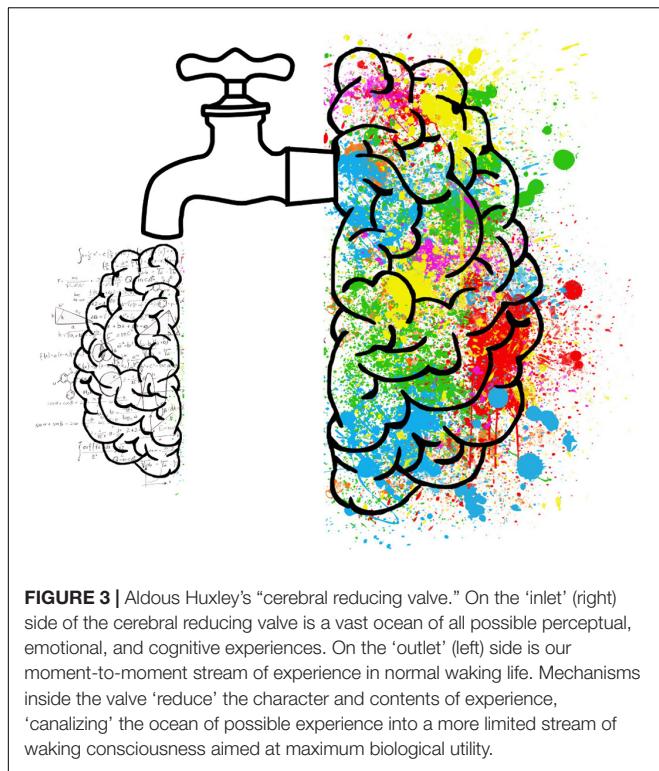
Dear Dr. Osmond,

...

It looks as though the most satisfactory working hypothesis about the human mind must follow, to some extent, the Bergsonian model, in which the brain with its associated normal self, acts as a utilitarian device for limiting, and making selections from, the enormous possible world of consciousness, and for canalizing experience into biologically profitable channels. Disease, mescaline, emotional shock, aesthetic experience and mystical enlightenment have the power, each in its different way and in varying degrees, to inhibit the function of the normal self and its ordinary brain activity, thus permitting the ‘other world’ to rise into consciousness.

Yours sincerely,
Aldous Huxley

Huxley’s letter can help unpack the intended ‘mind-manifesting’ etymology of Osmond’s new term *psychedelic*. Huxley saw the biological function of the brain as a “device” engaged in a continuous process of *elimination and inhibition*



to sustain the "normal self" of everyday waking experience to maximize adaptive fit. Huxley's choice metaphor for visualizing this was the *cerebral reducing valve* (Figure 3).

"What I have called the cerebral reducing valve [is a] normal brain function that limits our mental processes to an awareness, most of the time, of what is biologically useful" (Huxley, 1999, p. 121). Huxley (1961b, p. 193) argued that this "normal brain function" emerges developmentally during the course of psychological maturity, so for a period during childhood, before the cerebral reducing valve has fully developed, "there is this capacity to live in a kind of visionary world." Once the valve is fully developed, however, normal waking life becomes restricted to a "world fabricated by our everyday, biologically useful and socially conditioned perceptions, thoughts and feelings" (Huxley, 1961a, p. 214).

Huxley borrowed the core idea from 19th-century *filtration theory* accounts of various mental phenomena (see Marshall, 2005): "According to filtration theorists, consciousness is ordinarily kept narrow by biological and psychological selection processes that exclude a great deal of subconscious material" (Marshall, 2005, p. 233). Filtration theorists include founding figures of psychopharmacology (Kraepelin, 1892), psychology (James, 1890), and parapsychology (Myers, 1903), along with early 20th-century philosophers Bergson (1911, 1931) and Broad (1923). Bergson (1931) applied his own filtration framework to drug effects in his brief response to James' (1882) glowing descriptions of what it is like to inhale nitrous oxide. James' peculiar state of mind, explained Bergson, should be thought of as a latent potential of the brain/mind, which nitrous oxide simply "brought about materially, by an inhibition of

what inhibited it, by the removing of an obstacle; and this effect was the wholly negative one produced by the drug" (Bergson, 1931). Huxley picked up Bergson's line of thinking and eventually convinced Osmond that it was important to reflect this principle in scientific descriptions of the effects of LSD and mescaline. Smythies (1956, p. 96) also subscribed to this idea, stating that "mescaline may be supposed to inhibit that function in the brain which specifically inhibits the mescaline phenomena from developing in the sensory fields."

Thus, Osmond's (1957) proposed name-change—*psychedelic*—was intended to capture the spirit of filtration theory. In this new descriptive model, *psyche* (mind) *delic* (manifesting) drugs manifest the mind by inhibiting certain brain processes which normally maintain their own inhibitory constraints on our perceptions, emotions, thoughts, and sense of self. Osmond (1957) and Huxley (1991) both found this principle highly applicable to their own direct first-person knowledge of what it is like to experience the effects of mescaline and LSD—the expanded range of feelings, intensification of perceptual stimuli, vivid vision-like mental imagery, unusual thoughts, and expanding (or dissolving) sense of self and identity.

Osmond argued that his 'mind-manifesting' description had further theoretical virtues that could address the conceptual challenges of model psychoses theory and improve our understanding of (1) the diverse range of psychedelic effects, (2) their relationship to psychotic symptoms, and (3) their role in psychedelic-assisted therapies. First, the pharmacological disruption of hypothetical inhibitory brain mechanisms that normally attenuate internal and external stimuli suggested that the kinds of effects produced by the drug would depend on the kinds of stimuli in the system, which is consistent with the diverse range of effects on multiple perceptual modalities, emotional experience, and cognition.

Second, the brain's selective filtration mechanisms, while evolutionarily adaptive and biologically useful, could develop pathological characteristics in two fundamentally distinct ways. First, a chronically *overactive* filter limits too much of the mind, causing a rigid, dull, neurotic life in which mental contents become overly restricted to "those enumerated in the Sears-Roebuck catalog which constitutes the conventionally 'real' world" (Huxley, 1953, p. 30). Second, a chronically *underactive* or 'leaky' filter places too few constraints on the mind and allows too much 'Mind at Large' to enter conscious awareness, potentially resulting in perceptual instability, cognitive confusion, or hallucination. This picture helped Huxley and Osmond understand the relationship between psychedelic phenomena and psychotic phenomena: temporarily opening the cerebral reducing valve with psychedelics could produce mental phenomena that resembled symptoms of chronic natural psychoses precisely because both were the result of (acute or chronic) reductions in brain filtration mechanisms.

Third and finally, filtration theory addressed the paradoxical "hair of the dog" issue—why drugs that 'mimic' psychoses can aid psychotherapy—which, as described in the previous section, was a conceptual challenge for model psychoses theory. The solution to the paradox was in the filtration theory idea that psychedelic

drugs temporarily ‘disable’ brain filtration mechanisms, which could allow patients and therapists to work outside of the patient’s everyday (pathological) inhibitory mechanisms. Thus, filtration theory offered a way to understand psychedelic effects that was consistent with both their psychotomimetic properties and their therapeutic utility.

Osmond and Huxley argued that filtration theory concepts were fully consistent with the subjective phenomenology, psychotomimetic capability, and therapeutic efficacy of psychedelic drugs. However, it remains unclear exactly *what it is* that the brain is filtering and consequently *what it is* that emerges when the filter is pharmacologically perturbed by a psychedelic drug. According to Huxley, LSD and mescaline “inhibit the function of the normal self and its ordinary brain activity, thus permitting the ‘*other world*’ to rise into consciousness” (Huxley, 1953, p. 29; emphasis mine). Huxley (and Bergson) spoke of the brain as a device that filters the *world* and when the filter is removed we experience ‘more’ of reality. Osmond’s ‘mind-manifesting’ (*psyche*) (*delic*) name, by contrast, suggests that these drugs permit latent aspects of *mind* to rise into conscious awareness. So which is it? Do psychedelic drugs manifest latent aspects of *mind* or of *world*? How we answer this question will crucially determine our ontological and epistemological conclusions regarding the nature of psychedelic experience. Huxley and Osmond did not make this clear. Huxley seems to favor the position that psychedelic experience reveals a wider ontological reality and grants epistemic access to greater truth. Osmond’s view, on which these drugs reveal normally hidden aspects of mind, seems less radical, more compatible with materialist science, and less epistemically and ontologically committed. Still, if mind provides us with access to world, then lifting restrictions on mind could in principle expand our access to world. This important point resurfaces in section “Predictive Processing” below.

Psychoanalytic Theory

Freud (1895) developed an elaborate theoretical account of mental phenomena which, like filtration theory, placed great emphasis on inhibition mechanisms in the nervous system.⁸ Freud divided the psyche into two fundamentally distinct modes of activity: *the primary process* and *the secondary process* (Freud, 1895, 1940). In the primary process, the exchange of “neuronal energy” is “freely mobile” and its psychological dynamics are characterized by disorder, vagueness, conceptual paradox, symbolic imagery, intense emotions, and animistic thinking (Freud, 1940, p. 164). In the secondary process, by contrast, the exchange of neuronal energy is “bound” and its psychological dynamics are characterized by order, precision, conceptual consistency, controlled emotions, and rational thinking (Freud, 1895, 1940). Freud (1895) hypothesized that the secondary process is maintained by an organizing neural “mass” called the ego which “contains” and exerts control over the primary

process by binding primary process activity into its own pattern of activity.⁹ Freud hypothesized that secondary process neural organization, sustained by the ego, is required for certain aspects of perceptual processing, directed attention, reality-testing, sense of linear time, and higher cognitive processes (Freud, 1895, 1940). When Freud’s ego is suppressed, such as during dream sleep, wider worlds of experience can emerge, but secondary process functions are lost. The secondary process and its supporting neural organizing pattern—the ego—emerges during ontogenetic development and solidifies with adult maturity: “A unity comparable to the ego cannot exist from the start; the ego has to be developed” (Freud, 1915, p. 77). Furthermore, pathological characteristics can emerge when Freud’s ego restricts either *too much* or *too little* of the primary process.

Freud himself was apparently uninterested in psychedelic drugs and instead emphasized dreams as “the royal road to a knowledge of the unconscious activities of the mind” (Freud, 1900, p. 769). Nonetheless, psychedelic drugs produce dreamlike visions and modes of cognition that feature symbolic imagery, conceptual paradox, and other hallmark characteristics of the primary process (Carhart-Harris and Friston, 2010; Kraehenmann et al., 2017a; Sanz and Tagliazucchi, 2018). How did other psychoanalytic theorists describe psychedelic drug effects? The core idea is that psychedelic drugs interfere with the structural integrity of the ego and thereby reduce its ability to suppress the primary process and support the secondary process (Grof, 1976). This ‘frees’ the primary process which then spills into conscious awareness, resulting in perceptual instability, wildly vivid imagination, emotional intensity, conceptual paradox, and loss of usual self-boundaries. Due in part to the close resemblance between psychedelic effects and primary process phenomena, psychoanalytic theory became the framework of choice during the mid 20th-century boom in psychedelic therapy (Sandison, 1954; Sandison and Whitelaw, 1957; Cohen, 1965; Grof, 1976; Merkur, 1998). Psychedelic ego effects, which range from a subtle loosening to a complete dissolution of ego boundaries, were found to be great tools in psychotherapy because of their capacity to perturb ego and allow primary process phenomena to emerge (Sandison, 1954, p. 509).

But *how* do psychedelic drugs disrupt the structure of the ego? Freud hypothesized that the organizational structure of ego rests upon a basic perceptual schematic of the body and its surrounding environment. Perceptual signals are continuously ‘bound’ and integrated into the somatic boundaries of the ego. Savage (1955) speculated that the LSD’s perceptual effects and ego effects are tightly linked. “LSD acts by altering perception. Continuous correct perception is necessary to maintain ego feeling and ego boundaries. . . . Perception determines our ego boundaries. . . . disturbances in perception caused by LSD make

⁸Huxley was overtly critical of Freud, yet Huxley’s cerebral reducing valve is strikingly similar to Freud’s ego (see Benton, 2016 for a comparison of Freud and Bergson).

⁹“The secondary process is characterized by a bound state in the neurone, which though there is a high cathexis, permits only a small current. . . Now the ego itself is a mass like this of neurones which hold fast to their activity—are, that is in a bound state and this surely can only happen as a result of the effect they have on one another. We can therefore imagine that a perceptual neurone which is active with attention is as a result temporarily, as it were, taken up into the ego and is now subject to the same binding of its energy as are all the other ego neurones. . . This bound state, which combines high activity with small current, would thus characterize processes of thought mechanically” (Freud, 1895, p. 368).

it impossible for the ego to integrate the evidence of the senses and to coordinate its activities ...” (Savage, 1955, p. 14). Klee (1963) expanded Savage’s insights into a set of hypotheses aimed at elucidating the neurobiological mechanisms of a Freudian ‘stimulus barrier’ and its dissolution under LSD:

Such barriers would presumably consist of processes limiting the spread of excitation between different functional areas of the brain. The indications are that LSD, in some manner, breaks down these stimulus barriers of which Freud spoke. *Nor is this merely a figure of speech.* There is some reason to suspect that integrative mechanisms within the central nervous system (CNS) which handle inflowing stimuli are no longer able to limit the spread of excitation in the usual ways. We might speculate that LSD allows greater energy exchanges between certain systems than normally occurs, without necessarily raising the general level of excitation of all cortical and subcortical structures. (Klee, 1963, p. 465; emphasis mine).

Freud hypothesized that ego is sustained by a delicate balance of ‘neuronal energy’ which critically depends on integrative mechanisms to process inflowing sensory stimuli and to ‘bind’ neural excitation into functional structures within the brain. Psychedelic drugs, according to Savage and Klee, perturb integrative mechanisms that normally bind and shape endogenous and exogenous excitation into the structure of the ego. As we will see below, Klee’s ideas strongly anticipate many neurophysiological findings (Alonso et al., 2015; Tagliazucchi et al., 2016; Schartner et al., 2017) and theoretical themes (Carhart-Harris and Friston, 2010; Lethaby and Gerrans, 2017) from 21st-century psychedelic science.

Summary

From the above analysis of first-wave and second-wave theories I have identified four recurring theoretical features which could potentially serve as unifying principles. One feature is the hypothesis that psychedelic drugs inhibit a core brain mechanism that normally functions to ‘reduce’ or ‘filter’ or ‘constrain’ mental phenomena into an evolutionarily adaptive container. A second feature is the hypothesis that this core brain mechanism can behave pathologically, either in the direction of too much, or too little, constraint imposed on perception, emotion, cognition, and sense of self. A third feature is the hypothesis that psychedelic phenomena and symptoms of chronic psychoses share descriptive elements because they both involve situations of relatively *unconstrained* mental processes. A fourth feature is the hypothesis that psychedelic drugs have therapeutic utility via their ability to temporarily inhibit these inhibitory brain mechanisms. But how are these inhibitory mechanisms realized in the brain?

NEUROPHARMACOLOGY AND NEUROPHYSIOLOGICAL CORRELATES OF PSYCHEDELIC DRUG EFFECTS

Klee recognized that his above hypotheses, inspired by psychoanalytic theory and LSD effects, required

neurophysiological evidence. “As far as I am aware, however, adequate neurophysiological evidence is lacking ... The long awaited millennium in which biochemical, physiological, and psychological processes can be freely correlated still seems a great distance off” (Klee, 1963, p. 466, 473). What clues have recent investigations uncovered?

A psychedelic drug molecule impacts a neuron by binding to and altering the conformation of receptors on the surface of the neuron (Nichols, 2016). The receptor interaction most implicated in producing classic psychedelic drug effects is agonist or partial agonist activity at serotonin (5-HT) receptor type 2A (5-HT_{2A}) (Nichols, 2016). A molecule’s propensity for 5-HT_{2A} affinity and agonist activity predicts its potential for (and potency of) subjective psychedelic effects (Glennon et al., 1984; McKenna et al., 1990; Halberstadt, 2015; Nichols, 2016; Rickli et al., 2016). When a psychedelic drug’s 5-HT_{2A} agonist activity is intentionally blocked using 5-HT_{2A} antagonist drugs (e.g., ketanserin), the subjective effects are blocked or attenuated in humans under psilocybin (Vollenweider et al., 1998; Kometer et al., 2013), LSD (Kraehenmann et al., 2017a,b; Preller et al., 2017), and ayahuasca (Valle et al., 2016). Importantly, while the above evidence makes it clear that 5-HT_{2A} activation is a necessary (if not sufficient) mediator of the hallmark subjective effects of classic psychedelic drugs, this does not entail that 5-HT_{2A} activation is the sole neurochemical cause of all subjective effects. For example, 5-HT_{2A} activation might trigger neurochemical modulations ‘downstream’ (e.g., changes in glutamate transmission) which could also play causal roles in producing psychedelic effects (Nichols, 2016). Moreover, most psychedelic drug molecules activate other receptors in addition to 5-HT_{2A} (e.g., 5-HT_{1A}, 5-HT_{2C}, dopamine, sigma, etc.) and these activations may importantly contribute to the overall profile of subjective effects even if 5-HT_{2A} activation is required for their effects to occur (Ray, 2010, 2016).

How does psychedelic drug-induced 5-HT_{2A} receptor agonism change the behavior of the host neuron? Generally, 5-HT_{2A} activation has a depolarizing effect on the neuron, making it more excitable (more likely to fire) (Andrade, 2011; Nichols, 2016). Importantly, this does not necessarily entail that 5-HT_{2A} activation will have an overall excitatory effect throughout the brain, particularly if the excitation occurs in inhibitory neurons (Andrade, 2011). This important consideration (captured by the adage ‘one neuron’s excitation is another neuron’s inhibition’) should be kept in mind when tracing causal links in the pharmaco-neurophysiology of psychedelic drug effects.

In mammalian brains, neurons tend to ‘fire together’ in synchronized rhythms known as *temporal oscillations* (brain waves). MEG and EEG equipment measure the electromagnetic disturbances produced by the temporal oscillations of large neural populations and these measurements can be quantified according to their *amplitude* (power) and *frequency* (timing) (Buzsáki and Draguhn, 2004). Specific combinations of frequency and amplitude can be correlated with distinct brain states, including waking ‘resting’ state, various attentional tasks, anesthesia, REM sleep, and deep sleep (Tononi and Koch, 2008; Atasoy et al., 2017a). In what ways do temporal oscillations change under psychedelic drugs? MEG and EEG

studies consistently show *reductions* in oscillatory power across a broad frequency range under ayahuasca (Riba et al., 2002, 2004; Schenberg et al., 2015; Valle et al., 2016), psilocybin (Muthukumaraswamy et al., 2013; Kometer et al., 2015; Schartner et al., 2017), and LSD (Carhart-Harris et al., 2016c; Schartner et al., 2017). Reductions in the power of alpha-band oscillations, localized mainly to parietal and occipital cortex, have been correlated with intensity of subjective visual effects—e.g., ‘I saw geometric patterns’ or ‘My imagination was extremely vivid’—under psilocybin (Kometer et al., 2013; Muthukumaraswamy et al., 2013; Schartner et al., 2017) and ayahuasca (Riba et al., 2004; Valle et al., 2016). Under LSD, reductions in alpha power still correlated with intensity of subjective visual effects but associated alpha reductions were more widely distributed throughout the brain (Carhart-Harris et al., 2016c). Furthermore, ego-dissolution effects and mystical-type experiences (e.g., ‘I experienced a disintegration of my “self” or “ego”’ or ‘The experience had a supernatural quality’) have been correlated with reductions in alpha power localized to anterior and posterior cingulate cortices and the parahippocampal regions under psilocybin (Muthukumaraswamy et al., 2013; Kometer et al., 2015) and throughout the brain under LSD (Carhart-Harris et al., 2016c).

The concept of *functional connectivity* rests upon fMRI brain imaging observations that reveal temporal correlations of activity occurring in spatially remote regions of the brain which form highly structured patterns (brain networks) (Buckner et al., 2013). Imaging of brains during perceptual or cognitive task performance reveals patterns of functional connectivity known as *functional networks*; e.g., control network, dorsal attention network, ventral attention network, visual network, auditory network, and so on. Imaging brains in taskless resting conditions reveals *resting-state functional connectivity* (RSFC) and structured patterns of RSFC known as resting state networks (RSNs; Deco et al., 2011). One particular RSN, the default mode network (DMN; Buckner et al., 2008), increases activity in the absence of tasks and decreases activity during task performance (Fox and Raichle, 2007). DMN activity is strong during internally directed cognition and a variety of other ‘metacognitive’ functions (Buckner et al., 2008). DMN activation in normal waking states exhibits ‘inverse coupling’ or anticorrelation with the activation of task-positive functional networks, meaning that DMN and functional networks are often mutually exclusive; one deactivates as the other activates and vice versa (Fox and Raichle, 2007).

In what ways does brain network connectivity change under psychedelic drugs? First, functional connectivity between key ‘hub’ areas—mPFC and PCC—is reduced. Second, the ‘strength’ or oscillatory power of the DMN is weakened and its intrinsic functional connectivity becomes disintegrated as its component nodes become decoupled under psilocybin (Carhart-Harris et al., 2012, 2013), ayahuasca (Palhano-Fontes et al., 2015), and LSD (Carhart-Harris et al., 2016c; Speth et al., 2016). Third, brain networks that normally show anticorrelation become active simultaneously under psychedelic drugs. This situation, which can be described as increased *between-network* functional connectivity, occurs under psilocybin (Carhart-Harris et al., 2012,

2013; Roseman et al., 2014; Tagliazucchi et al., 2014), ayahuasca (Palhano-Fontes et al., 2015) and especially LSD (Carhart-Harris et al., 2016c; Tagliazucchi et al., 2016). Fourth and finally, the overall repertoire of explored functional connectivity motifs is substantially expanded and its informational dynamics become more diverse and entropic compared with normal waking states (Tagliazucchi et al., 2014, 2016; Alonso et al., 2015; Lebedev et al., 2016; Viol et al., 2016; Atasoy et al., 2017b; Schartner et al., 2017). Notably, the magnitude of occurrence of the above four neurodynamical themes correlates with subjective intensity of psychedelic effects during the drug session. Furthermore, visual cortex is activated during eyes-closed psychedelic visual imagery (de Araujo et al., 2012; Carhart-Harris et al., 2016c) and under LSD “the early visual system behaves ‘as if’ it were receiving spatially localized visual information” as V1-V3 RSFC is activated in a retinotopic fashion (Roseman et al., 2016, p. 3036).

Taken together, the recently discovered neurophysiological correlates of subjective psychedelic effects present an important puzzle for 21st-century neuroscience. A key clue is that 5-HT_{2A} receptor agonism leads to desynchronization of oscillatory activity, disintegration of intrinsic integrity in the DMN and related brain networks, and an overall brain dynamic characterized by increased between-network global functional connectivity, expanded signal diversity, and a larger repertoire of structured neurophysiological activation patterns. Crucially, these characteristic traits of psychedelic brain activity have been correlated with the phenomenological dynamics and intensity of subjective psychedelic effects.

21st-CENTURY THEORIES OF PSYCHEDELIC DRUG EFFECTS

How should we understand the growing body of clues emerging from investigations into the neurodynamics of psychedelic effects? What are the principles that link these thematic patterns of psychedelic brain activity (or inactivity) to their associated phenomenological effects? Recent theoretical efforts to understand psychedelic drug effects have taken advantage of existing frameworks from cognitive neuroscience designed to track the key neurodynamic principles of human perception, emotion, cognition, and consciousness. The overall picture that emerges from these efforts shares core principles with filtration and psychoanalytic accounts of the late 19th and early 20th century. Briefly, normal waking perception and cognition are hypothesized to rest upon brain mechanisms which serve to suppress entropy and uncertainty by placing various *constraints* on perceptual and cognitive systems. In a ‘selecting’ and ‘limiting’ fashion, neurobiological constraint mechanisms support stability and predictability in the contents of conscious awareness in the interest of adaptability, survival, and evolutionary fitness. The core hypothesis of recent cognitive neuroscience theories of psychedelic effects is that these drugs interfere with the integrity of neurobiological information-processing constraint mechanisms. The net effect of this is that the range of possibilities in perception, emotion, and cognition is dose-dependently expanded. From this core hypothesis, cognitive neuroscience

frameworks are utilized to describe and operationalize the quantitative neurodynamics of key psychedelic phenomena; namely, the diversity of effects across many mental processes, the elements in common with symptoms of psychoses, and the way in which temporarily removing neurobiological constraints is therapeutically beneficial.

This section is organized according to the broad theoretical frameworks informing recent theoretical neuroscience of psychedelic effects: *entropic brain theory*, *integrated information theory*, and *predictive processing*.

Entropic Brain Theory

Entropic Brain Theory (EBT; Carhart-Harris et al., 2014) links the phenomenology and neurophysiology of psychedelic effects by characterizing both in terms of the quantitative notions of entropy and uncertainty. Entropy is a quantitative index of a system's (physical) disorder or randomness which can simultaneously describe its (informational) uncertainty. EBT "proposes that the quality of any conscious state depends on the system's entropy measured via key parameters of brain function" (Carhart-Harris et al., 2014, p. 1). Their hypothesis states that hallmark psychedelic effects (e.g., perceptual destabilization, cognitive flexibility, ego dissolution) can be mapped directly onto elevated levels of entropy/uncertainty measured in brain activity, e.g., widened repertoire of functional connectivity patterns, reduced anticorrelation of brain networks, and desynchronization of RSN activity. More specifically, EBT characterizes the difference between psychedelic states and normal waking states in terms of how the underlying brain dynamics are positioned on a scale between the two extremes of order and disorder—a concept known as 'self-organized criticality' (Beggs and Plenz, 2003). A system with high order (low entropy) exhibits dynamics that resemble 'petrification' and are relatively inflexible but more stable, while a system with low order (high entropy) exhibits dynamics that resemble 'formlessness' and are more flexible but less stable. The notion of 'criticality' describes the transition zone in which the brain remains poised between order and disorder. Physical systems at criticality exhibit increased transient 'metastable' states, increased sensitivity to perturbation, and increased propensity for cascading 'avalanches' of metastable activity. Importantly, EBT points out that these characteristics are consistent with psychedelic phenomenology, e.g., hypersensitivity to external stimuli, broadened range of experiences, or rapidly shifting perceptual and mental contents. Furthermore, EBT uses the notion of criticality to characterize the difference between psychedelic states and normal waking states as it "describes cognition in adult modern humans as 'near critical' but 'sub-critical'—meaning that its dynamics are poised in a position between the two extremes of formlessness and petrification where there is an optimal balance between order and flexibility" (Carhart-Harris et al., 2014, p. 12). EBT hypothesizes that psychedelic drugs interfere with 'entropy-suppression' brain mechanisms which normally sustain sub-critical brain dynamics, thus bringing the brain "closer to criticality in the psychedelic state" (Carhart-Harris et al., 2014, p. 12).

Entropic Brain Theory further characterizes psychedelic neurodynamics using a neo-psychoanalytic framework proposed

in an earlier paper by Carhart-Harris and Friston (2010, p. 1265) where they "recast some central Freudian ideas in a mechanistic and biologically informed fashion." Freud's primary process (renamed "primary consciousness") is hypothesized to be a high-entropy brain dynamic which operates at criticality, while Freud's secondary process (renamed "secondary consciousness") is hypothesized to involve a lower-entropy brain state which sustains a sub-critical dynamic via a key neurobiological entropy-suppression mechanism—the ego—which exerts an organizing influence in order to constrain the criticality-like dynamic of primary consciousness. EBT argues that these ego functions have a signature neural footprint; namely, the DMN's intrinsic functional connectivity and DMN coupling of medial temporal lobes (MTLs) in particular. Furthermore, EBT argues that DMN/ego develops ontogenetically in adult humans and plays an adaptive role in which it sustains secondary consciousness and associated metacognitive abilities (Shimamura, 2000; Fleming et al., 2012) along with an "integrated sense of self" (Carhart-Harris et al., 2014, p. 9).

Importantly, this hypothesis maps onto the subjective phenomenology of psychedelic effects, particularly ego dissolution. As psychedelics weaken the oscillatory power and intrinsic functional connectivity of the DMN, the normally constrained activity of subordinate DMN nodes—MTLs in particular—becomes "freely mobile" allowing the emergence of more uncertain (higher entropy) primary consciousness. This view, based on Freudian metapsychology, is also consistent with filtration accounts, like those of Bergson and Huxley, who hypothesized that psychedelic drug effects are the result of a pharmacological *inhibition* of inhibitory brain mechanisms. EBT recasts these theoretical features using the quantitative terms of physical entropy and informational uncertainty as measured via "the repertoire of functional connectivity motifs that form and fragment across time" (Carhart-Harris et al., 2014, p. 1). In normal waking states, the DMN *constrains* the activity of its cortical and subcortical nodes and prohibits simultaneous co-activation with TPNs. By interfering with DMN integration, psychedelics permit a larger repertoire of brain activity, a wider variety of explored functional connectivity motifs, co-activation of normally mutually exclusive brain networks, increased levels of between-network functional connectivity, and an overall more diverse set of neural interactions.

Carhart-Harris et al. (2014) point out a number of implications of EBT. First, they map the feelings of 'uncertainty' that often accompany psychedelic effects onto the fact that a more entropic brain dynamic is the information-theoretic equivalent to a more 'uncertain' brain dynamic. "Thus, according to the entropic brain hypothesis, just as normally robust principles about the brain lose definition in primary states, so confidence is lost in 'how the world is' and 'who one is' as a personality" (Carhart-Harris et al., 2014, p. 16).

Second, like Huxley's cerebral reducing valve and Freud's ego, EBT argues that the DMN's organizational stronghold over brain activity can be both an evolutionary advantage *and* a source of pathology. "It is argued that this entropy-suppressing function of the human brain serves to promote realism, foresight, careful reflection and an ability to recognize and overcome wishful and

paranoid fantasies. Equally however, it could be seen as exerting a limiting or narrowing influence on consciousness" (Carhart-Harris et al., 2014, p. 7). Carhart-Harris et al. (2014) point out that neuroimaging studies have implicated increased DMN activity and RSFC with various aspects of depressive rumination, trait neuroticism, and depression. "The suggestion is that increased DMN activity and connectivity in mild depression promotes concerted introspection and an especially diligent style of reality-testing. However, what may be gained in mild depression (i.e., accurate reality testing) may be offset by a reciprocal decrease in flexible or divergent thinking (and positive mood)" (Carhart-Harris et al., 2014, p. 10).

Third, consistent with both psychoanalytic and filtration theory, is the notion that psychedelic drugs' capacity to temporarily weaken, collapse, or disintegrate the normal ego/DMN stronghold underpins their therapeutic utility. "Specifically, it is proposed that psychedelics work by dismantling reinforced patterns of negative thought and behavior by breaking down the stable spatiotemporal patterns of brain activity upon which they rest" (Carhart-Harris et al., 2014, p. 1).

Fourth and finally, EBT sheds light on the shared descriptive elements between psychedelic effects and psychotic symptoms by characterizing both in terms of elevated levels of entropy and uncertainty in brain activity which lead to a "regression" into primary consciousness. The collapse of the organizing effect of DMN coupling and anticorrelation patterns, according to EBT, point to "system-level mechanics of the psychedelic state as an exemplar of a regressive style of cognition that can also be observed in REM sleep and early psychosis" (Carhart-Harris et al., 2014, p. 5).

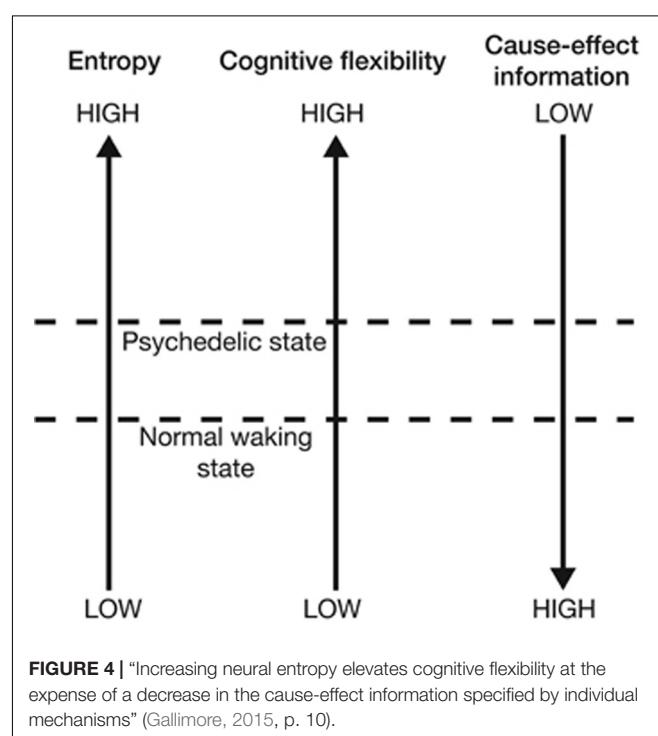
Thus, EBT formulates all four of the theoretical features identified in filtration and psychoanalytic accounts, but does so using 21st-century empirical data plugged into the quantitative concepts of entropy, uncertainty, criticality, and functional connectivity. EBT hints at possible ways to close the gaps in understanding by offering quantitative concepts that link phenomenology to brain activity and pathogenesis to therapeutic mechanisms.

Integrated Information Theory

Integrated Information Theory (IIT) is a general theoretical framework which describes the relationship between consciousness and its physical substrates (Oizumi et al., 2014; Tononi, 2004, 2008). While EBT is already loosely consistent with the core principles of IIT, Gallimore (2015) demonstrates how EBT's hypotheses can be operationalized using the technical concepts of the IIT framework. Using EBT and recent neuroimaging data as a foundation, Gallimore develops an IIT-based model of psychedelic effects. Consistent with EBT, this IIT-based model describes the brain's continual challenge of minimizing entropy while retaining flexibility. Gallimore formally restates this problem using IIT parameters: brains attempt to optimize the give-and-take dynamic between *cause-effect information* and cognitive flexibility. In IIT, a (neural) system generates cause-effect information when the mechanisms which make up its current state constrain the set of states which could casually precede or follow the current state.

In other words, each mechanistic state of the brain: (1) limits the set of past states which could have causally given rise to it, and (2) limits the set of future states which can causally follow from it. Thus, each current state of the mechanisms within a neural system (or subsystem) has an associated *cause-effect repertoire* which specifies a certain amount of cause-effect information as a function of how stringently it constrains the unconstrained state repertoire of all possible system states. Increasing the entropy within a cause-effect repertoire will in effect constrain the system less stringently as the causal possibilities are expanded in both temporal directions as the system moves closer to its unconstrained repertoire of all possible states. Moreover, increasing the entropy within a cause-effect repertoire equivalently increases the uncertainty associated with its past (and future) causal interactions. Using this IIT-based framework, Gallimore (2015) argues that, compared with normal waking states, psychedelic brain states exhibit higher entropy, higher cognitive flexibility, but lower cause-effect information (Figure 4).

Neuroimaging data suggests that human brains exhibit a larger overall repertoire of neurophysiological states under psychedelic drugs, exploring a greater diversity of states in a more random fashion. For example, in normal waking states, DMN activity 'rules out' the activity of TPNs, and vice versa, due to their relatively strict anticorrelation patterns. Brain network anticorrelation generates cause-effect information because it places constraints on the possible causal interactions within and between brain mechanisms; for example, DMN-TPN anticorrelation patterns 'rule out' the DMN activity in the presence of activated TPNs. However, psychedelic drugs 'dissolve' DMN-TPN (and other) network anticorrelation patterns, which



permits simultaneous activation of brain networks which are normally mutually exclusive. The cause-effect repertoire of brain mechanisms thus shifts closer to the unconstrained repertoire of all possible past and future states. This has the effect of “increasing the probability of certain states from zero or, at least, from a very low probability” (Gallimore, 2015, p. 7). Therefore the subjective contents perception and cognition become more diverse, more unusual, and less predictable. This increases flexibility but decreases precision and control as the subjective boundaries which normally demarcate distinct cognitive concepts and perceptual objects dissolve. Gallimore leverages IIT in an attempt unify these phenomena under a formalized framework.

However, as Gallimore notes, “this model does not explain how neural entropy is increased by (psychedelic drugs), but predicts consequences of the entropy increase revealed by functional imaging data” (Gallimore, 2015, p. 7). How do psychedelic drugs increase neural entropy?

Predictive Processing

The first modern brain imaging measurements in humans under psilocybin yielded somewhat unexpected results: *reductions* in oscillatory power (MEG) and cerebral blood flow (fMRI) correlated with the intensity of subjective psychedelic effects (Carhart-Harris et al., 2012; Muthukumaraswamy et al., 2013). In their discussion, the authors suggest that their findings, although surprising through the lens of commonly held beliefs about how brain activity maps to subjective phenomenology, may actually be consistent with a theory of brain function known as the *free energy principle* (FEP; Friston, 2010).

In one model of global brain function based on the free-energy principle (Friston, 2010), activity in deep-layer projection neurons encodes top-down inferences about the world. Speculatively, if deep-layer pyramidal cells were to become hyperexcitable during the psychedelic state, information processing would be biased in the direction of inference—such that implicit models of the world become spontaneously manifest—intruding into consciousness without prior invitation from sensory data. This could explain many of the subjective effects of psychedelics (Muthukumaraswamy et al., 2013, p. 15181).

What is FEP? “In this view, the brain is an inference machine that actively predicts and explains its sensations. Central to this hypothesis is a probabilistic model that can generate predictions, against which sensory samples are tested to update beliefs about their causes” (Friston, 2010). FEP is a formulation of a broader conceptual framework emerging in cognitive neuroscience known as *predictive processing* (PP; Clark, 2013)¹⁰. PP has links to *bayesian brain hypothesis* (Knill and Pouget, 2004), *predictive coding* (Rao and Ballard, 1999), and earlier theories of perception and cognition (MacKay, 1956; Neisser, 1967; Gregory, 1968) dating back to Helmholtz (1925) who was inspired by Kant (1996; see Swanson, 2016). At the turn of the 21st century, the ideas of Helmholtz catalyzed innovations in machine learning (Dayan et al., 1995), new understandings of cortical organization

¹⁰See also Clark (2015) and Wiese and Metzinger (2017) for introductory reviews conceptual overviews.

(Mumford, 1992; Friston, 2005), and theories of how perception works (Kersten and Yuille, 2003; Lee and Mumford, 2003).

PP subsumes key elements from these efforts (see Clark, 2013) to describe a universal principle of brain function captured by the idea of *prediction error minimization* (PEM; Hohwy, 2013). What does it mean to say that the brain works to minimize its own prediction error? Higher-level areas of the nervous system (i.e., higher-order cortical structures) generate top-down synaptic ‘predictions’ aimed at matching the expected bottom-up synaptic activity at lower-level areas, all the way down to ‘input’ activity at sense organs. Top-down signals encode a kind of ‘best guess’ about the most likely (hidden)¹¹ causes of bodily sensations. In this multi-level hierarchical cascade of neural activity, high-level areas attempt to ‘explain’ the states of levels below via synaptic attempts to *inhibit* lower-level activity—“high-level areas tell lower levels to ‘shut up’” (Kersten et al., 2004, p. 297). But lower levels will not ‘shut up’ until they receive top-down feedback (inference) signals that adequately fit (explain) the bottom-up (evidence) signals. Mismatches between synaptic ‘expectation’ and synaptic ‘evidence’ generate *prediction error signals* which ‘carry the news’ by propagating the ‘surprise’ upward to be ‘explained away’ by yet higher levels of hierarchical cortical processing anatomy (see Clark, 2015). This recurrent neural processing scheme approximates (empirical) Bayesian inference (Friston and Stephan, 2007) as the brain continually maps measured bodily effects to different sets of possible causes and attempts to select the set of possible causes that can best ‘explain away’ the measured bodily effects. Crucially, the sets of possible causes must be *narrowed* in order for the system to settle on an explanation (Tenenbaum et al., 2011). Prior constraints which allow the system to narrow the hypothesis space are known as ‘inductive biases’ or *priors* (Kemp et al., 2007; Tenenbaum et al., 2011; Clark, 2013). Efforts in Bayesian statistics and machine learning have demonstrated that improvements in inductive capabilities occur when priors are linked in a multi-level hierarchy, with “not just a single level of hypotheses to explain the data but multiple levels: hypothesis spaces of hypothesis spaces, with priors on priors” (Tenenbaum et al., 2011, p. 1282). Certain priors in the hierarchy, known as ‘hyperpriors’ (Friston et al., 2013) or ‘overhypotheses’ (Goodman, 1983; Kemp et al., 2007) are more abstract and allow the system to ‘rule out’ large swaths of possibilities, drastically narrowing the hypothesis space, making explanation more tractable (Blokpoel et al., 2012). For example, the brute constraints of space and time act as hyperpriors; e.g., prior knowledge “that there is only one object (one cause of sensory input) in one place, at a given scale, at a given moment,” or the fact that “we can only perform one action at a time, choosing the left turn or the right but never both at once” (Clark, 2013, p. 196).

Thus, PP states that brains are neural generative models built from linked hierarchies of priors where higher levels continuously attempt to ‘guess’ and explain activity at lower levels. The entire process can be characterized as the agent’s

¹¹The causes of our bodily sensations cannot be directly observed by the brain: an organism’s brain is ‘skull-bound’ (Hohwy, 2013) and limited to a ‘view from inside the black box’ (Clark, 2013).

attempt to *optimize* its own internal model of the sensorium (and the world) over multiple spatial and temporal scales (Friston, 2010).

Interestingly, PP holds that our perceptions of external objects recruit the same synaptic pathways that enable our capacity for *mental imagery, dreaming, and hallucination*. The brain's ability to 'simulate' its own 'virtual reality' using internal (generative) models of the world's causal structure is thus crucial to its ability to perceive the external world. "[A] fruitful way of looking at the human brain, therefore, is as a system which, even in ordinary waking states, constantly hallucinates at the world, as a system that constantly lets its internal autonomous simulational dynamics collide with the ongoing flow of sensory input, vigorously dreaming at the world and thereby generating the content of phenomenal experience" (Metzinger, 2003).

How do psychedelic molecules perturb predictive processing? If normal perception is a kind of 'controlled hallucination' (see Clark, 2015) where top-down simulation is constrained by bottom-up sensory input colliding with priors upon priors, then, as the above quotation from Muthukumaraswamy et al. (2013) suggests, psychedelic drugs essentially cause perception to be *less controlled* hallucination. The idea is that psychedelic drugs perturb the (learned and innate) prior constraints on internal generative models. Via their 5-HT_{2A} agonism, psychedelic drugs cause hyperexcitation in layer V pyramidal neurons, which might cause endogenous simulations to 'run wild' so that awareness becomes more imaginative, dreamlike, and hallucinatory. This hypothesis could in principle still be consistent with observed *reductions* in brain activity under psychedelics; recall from above that, in PP schemes, the higher-level areas 'explain away' lower-level excitation by *suppressing it with top-down inhibitory signals*. "Here, explaining away just means countering excitatory bottom-up inputs to a prediction error neuron with inhibitory synaptic inputs that are driven by top-down predictions" (Friston, 2010, p. 130).

How does PP tie into filtration theories and psychoanalytic accounts? Carhart-Harris et al. (2012) link Huxley with Friston to interpret their initially surprising fMRI scans of humans under psilocybin (see also Zizzo, 2013). One objection to this linkage might be that Huxley often describes psychedelic opening of the cerebral reducing valve as revealing more of the *world*. At first glance this seems at odds with the above PP account of psychedelic effects, which describes psychedelic drugs causing rampant *internal* simulations of reality, not revealing more of the external world. However, this apparent tension might be resolved in light of *active inference*, a key principle of FEP (Friston, 2010). Active inference shows how internal models do not merely generate top-down (inference) signals but also shape the *sampling* and *accumulation* of bottom-up sensory (evidence) signals. "In short, the agent will selectively sample the sensory inputs that it expects. This is known as active inference. An intuitive example of this process (when it is raised into consciousness) would be feeling our way in darkness: we anticipate what we might touch next and then try to confirm those expectations" (Friston, 2010, p. 129). The principle of active inference hints at a resolution to the apparent tensions between Osmond's 'mind-manifesting' model and Huxley's 'world-manifesting' model.

Psychedelics manifest *mind* by perturbing prior constraints on internal generative models, thereby expanding the possibilities in our inner world of feelings, thoughts, and mental imagery. Importantly, this could also manifest normally ignored aspects of *world* by altering active inference, which would in effect expand the sampling of sensory data to include samples that are normally routinely 'explained away.' Potentially, this understanding goes some way in explaining the perception-hallucination continuum of psychedelic drug effects (reviewed above) as it shows how perceptual *intensifications*, on the one hand, and *distortions and hallucinations*, on the other hand, could both be caused by a synaptic disruption of hierarchically linked priors in internal generative models.

The brief speculative remark by Muthukumaraswamy et al. (2013) is not the only PP-based account of psychedelic drug effects. The PP framework describes a recurrent back-and-forth give-and-take between colliding top-down and bottom-up signals, where internal models serve to shape experience and experience serves to build internal models, so this leaves room for rival PP-based accounts that diverge regarding where exactly the psychedelic drug perturbs the system. For example, increased top-down activity could be the result of pharmacological hyperactivation of top-down synaptic transmission; yet equally plausible is the hypothesis that increased top-down activity is a *compensatory response* to pharmacological attenuations or distortions of bottom-up signal.

For example, Corlett et al. (2009, p. 521) hypothesize that LSD hallucinations result from "noisy, unpredictable bottom-up signaling in the context of preserved and perhaps enhanced top-down processing." In contrast to the PP-based account outlined above, which focuses on changes to top-down signals, the strategy of Corlett et al. (2009) is to map various psychedelic effects to disturbances of top-down *and/or* bottom-up signals. The issue of what is primary and what is compensatory illustrates the vast possibilities in the hypothesis space of PP-based accounts.

While most PP-based accounts point to changes in top-down signaling, even within this hypothesis space there are contrasting conceptions of exactly how psychedelic molecules perturb top-down processing. Briefly, these differing hypotheses include: (1) *hyperactivation* or *heavier weighting* of top-down signaling (Muthukumaraswamy et al., 2013; described above), (2) *reduced* influence of signals from higher cortical areas (Carhart-Harris and Friston, 2010; McKenna and Riba, 2015), (3) interference with *multisensory integration* processes and PP-based binding of sensory signals (Carhart-Harris and Friston, 2010; Lethaby and Gerrans, 2017; Millière, 2017), and (4) changes in the *composition* and *level of detail* specified by top-down signals (Pink-Hashkes et al., 2017).

Carhart-Harris and Friston (2010) argue that the Freudian conception of ego, with its organizing influence over the primary process, is consistent with PP descriptions of higher-level cortical structures predicting and suppressing the excitation in lower levels in the hierarchy (i.e., limbic regions). Freud hypothesized that the secondary process binds, integrates, and organizes the 'lower' and more chaotic neural activity of the primary process into the broader and more cohesive composite structure of the ego. Carhart-Harris and Friston (2010) argue that when

large-scale intrinsic networks become dis-integrated, the activity at lower levels can no longer be ‘explained away’ (suppressed) by certain higher-level systems, causing conscious awareness to take on hallmark characteristics of the primary process. In normal adult waking states, networks based in higher-level areas can successfully predict and explain (suppress and control) the activity of lower level areas. “In non-ordinary states, this function may be perturbed (e.g., in the case of hallucinogenic drugs, through actions at modulatory post-synaptic receptors), compromising the hierarchical organization and suppressive capacity of the intrinsic networks” (Carhart-Harris and Friston, 2010, p. 1274).

Similar PP-based theories of psychedelic ego dissolution have been proposed without invoking Freud (Lethaby and Gerrans, 2017; Millière, 2017). PP posits that the brain explains self-generated stimuli by attributing its causes to a coherent and persisting entity (i.e., the self), much like how it predicts and explains external stimuli by attributing their causes to coherent and persisting external objects (see also Limanowski and Blankenburg, 2013; Allen and Friston, 2016; Lethaby and Gerrans, 2017; Millière, 2017). Lethaby and Gerrans (2017) use the PP framework to recast the psychoanalysis-based theories of LSD ego effects proposed by Savage (1955)¹² and Klee (1963) described in Section “Psychoanalytic Theory.” The core idea is that psychedelic drugs interfere with processes that bind and integrate stimuli according to probabilistic estimates of how relevant the stimuli are to the organism’s (self) goals. Lethaby and Gerrans (2017, p. 7) point out that ego dissolution under psychedelic drugs is correlated with the desynchronization (reductions in intrinsic functional connectivity) of brain networks implicated in “one aspect or another of self-representation”—specifically the salience network (SLN) and the DMN (Tagliazucchi et al., 2016). This causes an ‘unbinding’ of stimuli that are normally processed according to self-binding multisensory integration mechanisms. “Attention is no longer guided exclusively by adaptive and egocentric goals and agendas; salience attribution is no longer bound to personal concern” (Lethaby and Gerrans, 2017, p. 6). This description echoes Huxley’s cerebral reducing valve “in which the brain with its associated *normal self*, acts as a utilitarian device for limiting, and making selections from, the enormous possible world of consciousness, and for canalizing experience into biologically profitable channels” (Huxley, 1999, p. 29; emphasis mine). Lethaby and Gerrans’ PP-based account elucidates how psychedelic drugs could perturb the brain’s “associated normal self” preventing the usual self-binding of internal and external stimuli.

Pink-Hashkes et al. (2017, p. 2907) argue that under psychedelic drugs “top-down predictions in affected brain areas break up and decompose into many more overly detailed predictions due to hyper activation of 5-HT_{2A} receptors in layer V pyramidal neurons.” Pink-Hashkes et al. (2017) state that when internal generative models are described as categorical

probability distributions rather than Gaussian densities (Friston et al., 2015; Kwisthout et al., 2017), “the *state space granularity* (how detailed are the generative models and the predictions that follow from them) is crucial” (Kwisthout et al., 2017, p. 2; see also Kwisthout and van Rooij, 2015). Categorical predictions that are less detailed will ‘explain’ more bottom-up data (because they cover more ground) and thus produce less prediction error. Categorical predictions that are more detailed, by contrast, will carry less precision and thus potentially generate more prediction error (Kwisthout and van Rooij, 2015; Kwisthout et al., 2017). Pink-Hashkes et al. (2017, p. 2908) propose that psychedelic drugs cause brain structures at certain levels of the cortical hierarchy to issue more detailed (less abstract) ‘decomposed’ predictions that “fit less data than the ‘usual’ broad prediction.” They argue that many psychedelic effects stem from the brain’s attempts to *compensate* for these decomposed top-down predictions as it responds to the increase in prediction errors that result from overly detailed predictions.

In summary, the current state of PP-based theories of psychedelic effects reveals a divergent mix of heterogeneous ideas and conflicting hypotheses. Do psychedelic molecules perturb top-down (feedback) signaling, or bottom-up (feedforward) signaling, or both? Do the subjective phenomenological effects result from direct neuropharmacological changes or compensatory mechanisms responding to pharmacological perturbations? Yet there seems to be a core intuition that transcends the conceptual variance here: psychedelic drugs (somehow) interfere with established priors that normally constrain the brain’s internal generative models.

Predictive processing-based accounts, consistent with EBT and IIT (and filtration and psychoanalytic accounts), propose that psychedelic drugs disrupt neural mechanisms (priors on internal generative models) which normally constrain perception and cognition. Perturbing priors causes subjective phenomenology to present a wider range of experiences with increased risk of perceptual instability and excessive cognitive flexibility. As prior constraints on self and world are loosened, the likelihood of psychosis-like phenomena increases. At the same time, novel thinking is increased and the brain becomes more malleable and conducive to therapeutic cognitive and behavioral change.

CONCLUSION

The four key features identified in filtration and psychoanalytic accounts from the late 19th and early 20th century continue to operate in 21st-century cognitive neuroscience: (1) psychedelic drugs produce their characteristic diversity of effects because they perturb adaptive mechanisms which normally constrain perception, emotion, cognition, and self-reference, (2) these adaptive mechanisms can develop pathologies rooted in either too much or too little constraint (3) psychedelic effects appear to share elements with psychotic symptoms because both involve weakened constraints (4) psychedelic drugs are therapeutically useful precisely because they offer a way to temporarily inhibit these adaptive constraints. It is on these four points that EBT,

¹²“Disturbances in perception caused by LSD make it impossible for the ego to integrate the evidence of the senses and to coordinate its activities ...” (Savage, 1955, p. 14).

IIT, and PP seem consistent with each other and with earlier filtration and psychoanalytic accounts. EBT and IIT describe psychedelic brain dynamics and link them to phenomenological dynamics, while PP describes informational principles and plausible neural information exchanges which might underlie the larger-scale dynamics described by EBT and IIT. Certain descriptions of neural entropy-suppression mechanisms (EBT), cause-effect information constraints (IIT), or prediction-error minimization strategies (PP, FEP) are loosely consistent with Freud's ego and Huxley's cerebral reducing valve.

In surveying the literature for this review I can confidently conclude that 21st-century psychedelic science has yet to approach a unifying theory linking the diverse range of phenomenological effects with pharmacology and neurophysiology while tying these to clinical efficacy. However, the historically necessary ingredients for successful theory unification—formalized frameworks and unifying principles (Morrison, 2000)—seem to be taking shape. Formal models are an integral part of 21st-century neuroscience (Forstmann et al., 2011) where they help to describe natural principles in the brain and aid explanation and understanding (Kay, 2017).¹³ Here I have reviewed a handful of formalized frameworks—EBT, IIT, PP—which are just beginning to be used to account for psychedelic effects. I have also highlighted the fact that all of the accounts reviewed here, from the 19th-century to the 21st-century, propose that psychedelic drugs inhibit neurophysiological constraints in order to produce their diverse phenomenological, psychotomimetic, and therapeutic effects.

Why should we pursue a unified theory of psychedelic drug effects at all? To date, theories of brain function and mind in general have resisted the kind of unification that has occurred in other areas of science (Huang, 2008; Edelman, 2012). Because the human brain has evolved disparate and complex layers under diverse environmental circumstances, many doubt the possibility of and debate the merits of seeking 'grand unified theories' (GUTs) of brain function. "There is every reason to think that

¹³ This remains true regardless of the outcome of healthy debates about the nature and proper use of models in science (Frigg and Hartmann, 2017).

REFERENCES

- Abramson, H. A. (1956). *Neuropharmacology. Transactions of the Second Conference May 25, 26, and 27, 1955, Princeton, NJ.* New York, NY: Josiah Macy Jr. Foundation Publication.
- Allen, M., and Friston, K. J. (2016). From cognitivism to autopoiesis: towards a computational framework for the embodied mind. *Synthese* 1–24. doi: 10.1007/s11229-016-1288-5
- Alonso, J. F., Romero, S., Mañanas, M. A., and Riba, J. (2015). Serotonergic psychedelics temporarily modify information transfer in humans. *Int. J. Neuropsychopharmacol.* 18:yv039. doi: 10.1093/ijnp/pyv039
- Anderson, M. L., and Chemero, T. (2013). The problem with brain GUTs: conflation of different senses of "prediction" threatens metaphysical disaster. *Behav. Brain Sci.* 36, 204–205. doi: 10.1017/S0140525X1200221X
- Andrade, R. (2011). Serotonergic regulation of neuronal excitability in the prefrontal cortex. *Neuropharmacology* 61, 382–386. doi: 10.1016/j.neuropharm.2011.01.015
- Argento, E., Strathdee, S. A., Tupper, K., Braschel, M., Wood, E., and Shannon, K. (2017). Does psychedelic drug use reduce risk of suicidality? Evidence from a longitudinal community-based cohort of marginalised women in a Canadian setting. *BMJ Open* 7:e016025. doi: 10.1136/bmjopen-2017-016025
- Arminjon, M. (2011). The four postulates of freudian unconscious neurocognitive convergences. *Front. Psychol.* 2:125. doi: 10.3389/fpsyg.2011.00125
- Atasoy, S., Deco, G., Kringelbach, M. L., and Pearson, J. (2017a). Harmonic brain modes: a unifying framework for linking space and time in brain dynamics. *Neuroscientist* doi: 10.1177/1073858417728032 [Epub ahead of print].
- Atasoy, S., Roseman, L., Kaelen, M., Kringelbach, M. L., Deco, G., and Carhart-Harris, R. L. (2017b). Connectome-harmonic decomposition of human brain activity reveals dynamical repertoire re-organization under LSD. *J. Neurosci.* 37:17661. doi: 10.1523/JNEUROSCI.4598-16.2017
- Baggett, M. J. (2015). Psychedelics and creativity: a review of the quantitative literature. *PeerJ. PrePrints* 3:e1468. doi: 10.7287/peerj.preprints.1202v1
- Barrett, F. S., and Griffiths, R. R. (2017). Classic hallucinogens and mystical experiences: phenomenology and neural correlates. *Curr. Top. Behav. Neurosci.* doi: 10.1007/7854_2017_474 [Epub ahead of print].

there can be no grand unified theory of brain function because there is every reason to think that an organ as complex as the brain functions according to diverse principles" (Anderson and Chemero, 2013, p. 205). Indeed, Anderson and Chemero (2013, p. 205) caution that "we should be skeptical of any GUT of brain function" and charge that PP in particular, when taken as a unified theory as outlined by Clark (2013), "threatens metaphysical disaster."

Given these understandable critical reservations about seeking after GUTs of brain function (and therefore any truly unifying theory of psychedelic drug effects), it is perhaps safer to aspire for theories that feature "broad explanatory frameworks" and offer "conceptual breadth" allowing us to "paint the big picture" (Edelman, 2012). PP and FEP, at the very least, offer a broad explanatory framework that encompasses a large swath of perceptual and cognitive phenomena (Huang, 2008; Friston, 2010; Clark, 2015). Psychedelic drugs offer a unique way to iteratively develop and test such big-picture explanatory frameworks: these molecules can be used to probe the links between neurochemistry and neural computation across multiple layers of neuroanatomy and phenomenology. Meeting the challenge of predicting and explaining psychedelic drug effects is the ultimate acid test for any unified theory of brain function.

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LS researched and wrote the manuscript.

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- Barrett, F. S., Johnson, M. W., and Griffiths, R. R. (2015). Validation of the revised mystical experience questionnaire in experimental sessions with psilocybin. *J. Psychopharmacol.* 29, 1182–1190. doi: 10.1177/0269881115609019
- Barrett, F. S., Johnson, M. W., and Griffiths, R. R. (2017a). Neuroticism is associated with challenging experiences with psilocybin mushrooms. *Pers. Individ. Dif.* 117, 155–160. doi: 10.1016/j.paid.2017.06.004
- Barrett, F. S., Preller, K. H., Herdener, M., Janata, P., and Vollenweider, F. X. (2017b). Serotonin 2A receptor signaling underlies LSD-induced alteration of the neural response to dynamic changes in music. *Cereb. Cortex* doi: 10.1093/cercor/bhw257 [Epub ahead of print].
- Baumeister, R. F., and Exline, J. J. (2002). Mystical self loss: a challenge for psychological theory. *Int. J. Psychol. Relig.* 12, 15–20.
- Beggs, J. M., and Plenz, D. (2003). Neuronal avalanches in neocortical circuits. *J. Neurosci.* 23, 11167–11177.
- Belsler, A. B., Agin-Liebes, G., Swift, T. C., Terrana, S., Devenot, N., Friedman, H. L., et al. (2017). Patient experiences of psilocybin-assisted psychotherapy: an interpretative phenomenological analysis. *J. Humanist. Psychol.* 57, 354–388. doi: 10.1177/0022167817706884
- Benton, A. L. (2016). “Bergson and Freud on aphasia: a comparison,” in *Bergson and Modern Thought: Towards a Unified Science*, eds A. C. Papanicolaou and P. A. Y. Gunter (New York, NY: Harwood Academic Publishers).
- Bergson, H. (1911). *Matter and Memory*. New York, NY: Macmillan.
- Bergson, H. (1931). *The Two Sources of Morality and Religion*. Garden City, NY: Doubleday.
- Beringer, K. (1927a). Der Meskalinrausch. Seine Geschichte und Erscheinungsweise. Berlin: Springer-Verlag.
- Beringer, K. (1927b). *Der Meskalinrausch (Mescaline Intoxication)*. Berlin: Springer.
- Blokpoel, M., Kwisthout, J., and van Rooij, I. (2012). When can predictive brains be truly Bayesian? *Front. Psychol.* 3:406. doi: 10.3389/fpsyg.2012.00406
- Bogenschutz, M. P., Forcehimes, A. A., Pommy, J. A., Wilcox, C. E., Barbosa, P. C. R., and Strassman, R. J. (2015). Psilocybin-assisted treatment for alcohol dependence: a proof-of-concept study. *J. Psychopharmacol.* 29, 289–299. doi: 10.1177/0269881114565144
- Bogenschutz, M. P., and Johnson, M. W. (2016). Classic hallucinogens in the treatment of addictions. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 64, 250–258. doi: 10.1016/j.pnpbp.2015.03.002
- Bonny, H. L., and Pahnke, W. N. (1972). The use of music in psychedelic (LSD) psychotherapy. *J. Music Ther.* 9, 64–87. doi: 10.1093/jmt/9.2.64
- Bouso, J. C., Fábregas, J. M., Antoniоn, R. M., Rodríguez-Fornells, A., and Riba, J. (2013). Acute effects of ayahuasca on neuropsychological performance: differences in executive function between experienced and occasional users. *Psychopharmacology* 230, 415–424. doi: 10.1007/s00213-013-1679
- Bouso, J. C., Palhano-Fontes, F., Rodríguez-Fornells, A., Ribeiro, S., Sanches, R., Crippa, J. A. S., et al. (2015). Long-term use of psychedelic drugs is associated with differences in brain structure and personality in humans. *Eur. Neuropsychopharmacol.* 25, 483–492. doi: 10.1016/j.euroneuro.2015.01.008
- Bouso, J. C., Pedrero-Pérez, E. J., Gandy, S., and Alcázar-Córcoles, M. Á (2016). Measuring the subjective: revisiting the psychometric properties of three rating scales that assess the acute effects of hallucinogens. *Hum. Psychopharmacol.* 31, 356–372. doi: 10.1002/hup.2545
- Broad, C. D. (1923). *The Mind and Its Place in Nature*. London: Routledge & K. Paul.
- Brogaard, B. (2013). Serotonergic hyperactivity as a potential factor in developmental, acquired and drug-induced synesthesia. *Front. Hum. Neurosci.* 7:657. doi: 10.3389/fnhum.2013.00657
- Buckner, R. L., Andrews-Hanna, J. R., and Schacter, D. L. (2008). The brain's default network: anatomy, function, and relevance to disease. *Ann. N. Y. Acad. Sci.* 1124, 1–38. doi: 10.1196/annals.1440.011
- Buckner, R. L., Krienen, F. M., and Yeo, B. T. T. (2013). Opportunities and limitations of intrinsic functional connectivity MRI. *Nat. Neurosci.* 16, 832–837. doi: 10.1038/nn.3423
- Buzsáki, G., and Draguhn, A. (2004). Neuronal oscillations in cortical networks. *Science* 304, 1926–1929. doi: 10.1126/science.1099745
- Carhart-Harris, R. L., Bolstridge, M., Day, C. M. J., Rucker, J., Watts, R., Erritzoe, D. E., et al. (2017a). Psilocybin with psychological support for treatment-resistant depression: six-month follow-up. *Psychopharmacology* 235, 399–408. doi: 10.1007/s00213-017-4771-x
- Carhart-Harris, R. L., Bolstridge, M., Rucker, J., Day, C. M. J., Erritzoe, D., Kaelen, M., et al. (2016a). Psilocybin with psychological support for treatment-resistant depression: an open-label feasibility study. *Lancet Psychiatry* doi: 10.1016/S2215-0366(16)30065-7 [Epub ahead of print].
- Carhart-Harris, R. L., Erritzoe, D., Haijen, E., Kaelen, M., and Watts, R. (2017b). Psychedelics and connectedness. *Psychopharmacology* 235, 547–550. doi: 10.1007/s00213-017-4701-y
- Carhart-Harris, R. L., Kaelen, M., Bolstridge, M., Williams, T. M., Williams, L. T., Underwood, R., et al. (2016b). The paradoxical psychological effects of lysergic acid diethylamide (LSD). *Psychol. Med.* 46, 1379–1390. doi: 10.1017/S0033291715002901
- Carhart-Harris, R. L., Muthukumaraswamy, S., Roseman, L., Kaelen, M., Droog, W., Murphy, K., et al. (2016c). Neural correlates of the LSD experience revealed by multimodal neuroimaging. *Proc. Natl. Acad. Sci. U.S.A.* 113, 4853–4858. doi: 10.1073/pnas.1518377113
- Carhart-Harris, R. L., Erritzoe, D., Williams, T., Stone, J. M., Reed, L. J., Colasanti, A., et al. (2012). Neural correlates of the psychedelic state as determined by fMRI studies with psilocybin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2138–2143. doi: 10.1073/pnas.1119598109
- Carhart-Harris, R. L., and Friston, K. (2010). The default-mode, ego-functions and free-energy: a neurobiological account of Freudian ideas. *Brain* 133, 1265–1283. doi: 10.1093/brain/awq010
- Carhart-Harris, R. L., and Goodwin, G. M. (2017). The therapeutic potential of psychedelic drugs: past, present, and future. *Neuropsychopharmacology* 42, 2105–2113. doi: 10.1038/npp.2017.84
- Carhart-Harris, R. L., Kaelen, M., Whalley, M. G., Bolstridge, M., Feilding, A., and Nutt, D. J. (2015). LSD enhances suggestibility in healthy volunteers. *Psychopharmacology* 232, 785–794. doi: 10.1007/s00213-014-3714-z
- Carhart-Harris, R. L., Leech, R., Erritzoe, D., Williams, T. M., Stone, J. M., Evans, J., et al. (2013). Functional connectivity measures after psilocybin inform a novel hypothesis of early psychosis. *Schizophr. Bull.* 39, 1343–1351. doi: 10.1093/schbul/sbs117
- Carhart-Harris, R. L., Leech, R., Hellyer, P. J., Shanahan, M., Feilding, A., Tagliazucchi, E., et al. (2014). The entropic brain: a theory of conscious states informed by neuroimaging research with psychedelic drugs. *Front. Hum. Neurosci.* 8:20. doi: 10.3389/fnhum.2014.00020
- Carhart-Harris, R. L., and Nutt, D. J. (2010). User perceptions of the benefits and harms of hallucinogenic drug use: a web-based questionnaire study. *J. Subst. Use* 15, 283–300. doi: 10.3109/14659890903271624
- Carhart-Harris, R. L., and Nutt, D. J. (2017). Serotonin and brain function: a tale of two receptors. *J. Psychopharmacol.* 31, 1091–1120. doi: 10.1177/0269881117725915
- Carter, O. L., Burr, D. C., Pettigrew, J. D., Wallis, G. M., Hasler, F., and Vollenweider, F. X. (2005). Using psilocybin to investigate the relationship between attention, working memory, and the serotonin 1A and 2A receptors. *J. Cogn. Neurosci.* 17, 1497–1508. doi: 10.1162/089892905774597191
- Carter, O. L., Hasler, F., Pettigrew, J. D., Wallis, G. M., Liu, G. B., and Vollenweider, F. X. (2007). Psilocybin links binocular rivalry switch rate to attention and subjective arousal levels in humans. *Psychopharmacology* 195, 415–424. doi: 10.1007/s00213-007-0930-9
- Carter, O. L., Pettigrew, J. D., Burr, D. C., Alais, D., Hasler, F., and Vollenweider, F. X. (2004). Psilocybin impairs high-level but not low-level motion perception. *Neuroreport* 15, 1947–1951. doi: 10.1097/00001756-20040826-00023
- Clark, A. (2013). Whatever next? Predictive brains, situated agents, and the future of cognitive science. *Behav. Brain Sci.* 36, 181–204. doi: 10.1017/S0140525X12000477
- Clark, A. (2015). *Surfing Uncertainty: Prediction, Action, and the Embodied Mind*. Oxford: Oxford University Press.
- Cohen, S. (1965). *The Beyond within: The LSD Story*. New York, NY: Atheneum.

- Cohen, S., and Eisner, B. G. (1959). Use of lysergic acid diethylamide in a psychotherapeutic setting. *AMA Arch. Neurol. Psychiatry* 81, 615–619. doi: 10.1001/archneurpsyc.1959.02340170081008
- Corlett, P. R., Frith, C. D., and Fletcher, P. C. (2009). From drugs to deprivation: a Bayesian framework for understanding models of psychosis. *Psychopharmacology* 206, 515–530. doi: 10.1007/s00213-009-1561-0
- Crick, F., and Koch, C. (1998). Feature article consciousness and neuroscience. *Cereb. Cortex* 8, 97–107. doi: 10.1093/cercor/8.2.97
- Dayan, P., Hinton, G. E., Neal, R. M., and Zemel, R. S. (1995). The Helmholtz machine. *Neural Comput.* 7, 889–904. doi: 10.1162/neco.1995.7.5.889
- de Araujo, D. B., Ribeiro, S., Cecchi, G. A., Carvalho, F. M., Sanchez, T. A., Pinto, J. P., et al. (2012). Seeing with the eyes shut: neural basis of enhanced imagery following Ayahuasca ingestion. *Hum. Brain Mapp.* 33, 2550–2560. doi: 10.1002/hbm.21381
- Deco, G., Jirsa, V. K., and McIntosh, A. R. (2011). Emerging concepts for the dynamical organization of resting-state activity in the brain. *Nat. Rev. Neurosci.* 12, 43–56. doi: 10.1038/nrn2961
- Díaz, J. L. (2010). *Sacred Plants and Visionary Consciousness*. Available at: <http://link.springer.com/article/10.1007/s11097-010-9157-z>
- Dittrich, A. (1998). The standardized psychometric assessment of altered states of consciousness (ASCs) in humans. *Pharmacopsychiatry* 31(Suppl. 2), 80–84. doi: 10.1055/s-2007-979351
- Dittrich, A., Lamparter, D., and Maurer, M. (2010). *5D-ASC: Questionnaire for the Assessment of Altered States of Consciousness. A Short Introduction*. Zurich: PSIN PLUS.
- Dolder, P. C., Schmid, Y., Müller, F., Borgwardt, S., and Liechti, M. E. (2016). LSD acutely impairs fear recognition and enhances emotional empathy and sociality. *Neuropsychopharmacology* 41, 2638–2646. doi: 10.1038/npp.2016.82
- Dos Santos, R. G., Osório, F. L., Crippa, J. A. S., Riba, J., Zuardi, A. W., and Hallak, J. E. C. (2016). Antidepressive, anxiolytic, and antiaddictive effects of ayahuasca, psilocybin and lysergic acid diethylamide (LSD): a systematic review of clinical trials published in the last 25 years. *Ther. Adv. Psychopharmacol.* 6, 193–213. doi: 10.1177/2045125316638008
- Edelman, S. (2012). Six challenges to theoretical and philosophical psychology. *Front. Psychol.* 3:219. doi: 10.3389/fpsyg.2012.00219
- Eisner, B. G., and Cohen, S. (1958). Psychotherapy with lysergic acid diethylamide. *J. Nerv. Ment. Dis.* 127, 528–539.
- Ellis, H. (1898). *Mescal: A New Artificial*. Washington, DC: US Government Printing Office.
- Evarts, E. V. (1957). A review of the neurophysiological effects of lysergic acid diethylamide (LSD) and other psychotomimetic agents. *Ann. N. Y. Acad. Sci.* 66, 479–495. doi: 10.1111/j.1749-6632.1957.tb40744.x
- Family, N., Vinson, D., Vigliocco, G., Kaelen, M., Bolstridge, M., Nutt, D. J., et al. (2016). Semantic activation in LSD: evidence from picture naming. *Lang. Cogn. Neurosci.* 31, 1320–1327. doi: 10.1080/23273798.2016.1217030
- Fleming, S. M., Dolan, R. J., and Frith, C. D. (2012). Metacognition: computation, biology and function. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367, 1280–1286. doi: 10.1098/rstb.2012.0021
- Forman, R. K. C. (ed.) (1998). *The Innate Capacity: Mysticism Psychology, and Philosophy*. New York, NY: Oxford University Press.
- Forstmann, B. U., Wagenaars, E.-J., Eichele, T., Brown, S., and Serences, J. T. (2011). Reciprocal relations between cognitive neuroscience and formal cognitive models: opposites attract? *Trends Cogn. Sci.* 15, 272–279. doi: 10.1016/j.tics.2011.04.002
- Fox, M. D., and Raichle, M. E. (2007). Spontaneous fluctuations in brain activity observed with functional magnetic resonance imaging. *Nat. Rev. Neurosci.* 8, 700–711. doi: 10.1038/nrn2201
- Frecska, E., White, K. D., and Luna, L. E. (2004). Effects of ayahuasca on binocular rivalry with dichoptic stimulus alternation. *Psychopharmacology* 173, 79–87. doi: 10.1007/s00213-003-1701-x
- Freud, S. (1895). “Project for a scientific psychology,” in *The Standard Edition of the Complete Psychological Works of Sigmund Freud*, Vol. 1, ed. J. Strachey (London: Vintage).
- Freud, S. (1900). *The Interpretation of Dreams*. London: Penguin.
- Freud, S. (1915). “The unconscious,” in *Standard Edition of Complete Psychological Works of Sigmund Freud*, Vol. 14, ed. J. Strachey (London: Vintage).
- Freud, S. (1940). *An Outline of Psycho-Analysis*. London: Hogarth.
- Friedman, M. (1983). *Foundations of Space-Time Theories: Relativistic Physics and Philosophy of Science*. Princeton, NJ: Princeton University Press.
- Frigg, R., and Hartmann, S. (2017). *Models in Science*. Available at: <https://plato.stanford.edu/archives/spr2017/entries/models-science/>
- Friston, K. (2005). A theory of cortical responses. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360, 815–836. doi: 10.1098/rstb.2005.1622
- Friston, K. (2010). The free-energy principle: a unified brain theory? *Nat. Rev. Neurosci.* 11, 127–138. doi: 10.1038/nrn2787
- Friston, K., Lawson, R., and Frith, C. (2013). On hyperpriors and hypopriors: comment on Pellicano and Burr. *Trends Cogn. Sci.* 17:1. doi: 10.1016/j.tics.2012.11.003
- Friston, K., Rigoli, F., Ognibene, D., Mathys, C., Fitzgerald, T., and Pezzulo, G. (2015). Active inference and epistemic value. *Cogn. Neurosci.* 6, 187–214. doi: 10.1080/17588928.2015.1020053
- Friston, K., and Stephan, K. (2007). Free-energy and the brain. *Synthese* 159, 417–458. doi: 10.1007/s11229-007-9237-y
- Gaddum, J. H. (1953). Antagonism between lysergic acid diethylamide and 5-hydroxytryptamine. *J. Physiol.* 121:15P.
- Gaddum, J. H., and Hameed, K. A. (1954). Drugs which antagonize 5-hydroxytryptamine. *Br. J. Pharmacol. Chemother.* 9, 240–248. doi: 10.1111/j.1476-5381.1954.tb00848.x
- Gallimore, A. R. (2015). Restructuring consciousness—the psychedelic state in light of integrated information theory. *Front. Hum. Neurosci.* 9:346. doi: 10.3389/fnhum.2015.00346
- Gasser, P., Holstein, D., Michel, Y., Doblin, R., Yazar-Klosinski, B., Passie, T., et al. (2014). Safety and efficacy of lysergic acid diethylamide-assisted psychotherapy for anxiety associated with life-threatening diseases. *J. Nerv. Ment. Dis.* 202, 513–520. doi: 10.1097/NMD.0000000000000113
- Glennon, R. A., Titeler, M., and McKenney, J. D. (1984). Evidence for 5-HT₂ involvement in the mechanism of action of hallucinogenic agents. *Life Sci.* 35, 2505–2511. doi: 10.1016/0024-3205(84)90436-3
- Goodman, N. (1983). *Fact, Fiction, and Forecast*. Cambridge, MA: Harvard University Press.
- Green, A. R. (2008). Gaddum and LSD: the birth and growth of experimental and clinical neuropharmacology research on 5-HT in the UK. *Br. J. Pharmacol.* 154, 1583–1599. doi: 10.1038/bjp.2008.207
- Gregory, R. L. (1968). Perceptual illusions and brain models. *Proc. R. Soc. Lond. B Biol. Sci.* 171, 279–296. doi: 10.1098/rspb.1968.0071
- Griffiths, R., Richards, W., Johnson, M., McCann, U., and Jesse, R. (2008). Mystical-type experiences occasioned by psilocybin mediate the attribution of personal meaning and spiritual significance 14 months later. *J. Psychopharmacol.* 22, 621–632. doi: 10.1177/0269881108094300
- Griffiths, R. R., Johnson, M. W., Carducci, M. A., Umbricht, A., Richards, W. A., Richards, B. D., et al. (2016). Psilocybin produces substantial and sustained decreases in depression and anxiety in patients with life-threatening cancer: a randomized double-blind trial. *J. Psychopharmacol.* 30, 1181–1197. doi: 10.1177/0269881116675513
- Griffiths, R. R., Johnson, M. W., Richards, W. A., Richards, B. D., McCann, U., and Jesse, R. (2011). Psilocybin occasioned mystical-type experiences: immediate and persisting dose-related effects. *Psychopharmacology* 218, 649–665. doi: 10.1007/s00213-011-2358-5
- Grinspoon, L., and Bakalar, J. B. (1979). *Psychedelic Drugs Reconsidered*. New York, NY: Basic Books.
- Grob, C. S., Danforth, A. L., Chopra, G. S., Hagerty, M., McKay, C. R., Halberstadt, A. L., et al. (2011). Pilot study of psilocybin treatment for anxiety in patients with advanced-stage cancer. *Arch. Gen. Psychiatry* 68, 71–78. doi: 10.1001/archgenpsychiatry.2010.116
- Grof, S. (1976). *Realms of the Human Unconscious: Observations from LSD Research*. New York, NY: E.P. Dutton.
- Grof, S. (1980). *LSD Psychotherapy*. Pomona, CA: Hunter House.
- Guttmann, E. (1936). Artificial psychoses produced by mescaline. *Br. J. Psychiatry* 82, 203–221. doi: 10.1192/bjp.82.338.203
- Halberstadt, A. L. (2015). Recent advances in the neuropsychopharmacology of serotonergic hallucinogens. *Behav. Brain Res.* 277, 99–120. doi: 10.1016/j.bbr.2014.07.016

- Hartogsohn, I. (2016). Set and setting, psychedelics and the placebo response: an extra-pharmacological perspective on psychopharmacology. *J. Psychopharmacol.* 30, 1259–1267. doi: 10.1177/0269881116677852
- Hasler, F., Grimberg, U., Benz, M. A., Huber, T., and Vollenweider, F. X. (2004). Acute psychological and physiological effects of psilocybin in healthy humans: a double-blind, placebo-controlled dose-effect study. *Psychopharmacology* 172, 145–156. doi: 10.1007/s00213-003-1640-6
- Heffter, A. (1898). Ueber pellote. *Archiv Exp. Pathol. Pharmakol.* 40, 385–429. doi: 10.1007/BF01825267
- Heimann, H. (1963). *Observations on Disturbed Time Perception in Model Psychosis*. Available at: <http://europepmc.org/abstract/med/14112701>
- Helmholtz, H. (1925). *Treatise on Physiological Optics*. Washington, DC: The Optical Society.
- Hendricks, P. S., Thorne, C. B., Clark, C. B., Coombs, D. W., and Johnson, M. W. (2015). Classic psychedelic use is associated with reduced psychological distress and suicidality in the United States adult population. *J. Psychopharmacol.* 29, 280–288. doi: 10.1177/0269881114565653
- Himwich, H. E. (1959). Neuropharmacology: transactions of the second conference. *Am. J. Psychiatry* 116, 88–88. doi: 10.1176/ajp.116.1.88
- Hintzen, A., and Passie, T. (2010). *The Pharmacology of LSD: A Critical Review*. Oxford: Oxford University Press.
- Hoffer, A., Osmond, H., and Smythies, J. (1954). Schizophrenia; a new approach. II. Result of a year's research. *J. Ment. Sci.* 100, 29–45. doi: 10.1192/bjps.100.418.29
- Hofmann, A. (1980). *LSD, My Problem Child*. New York, NY: McGraw-Hill.
- Hohwy, J. (2013). *The Predictive Mind*. Oxford: Oxford University Press. doi: 10.1093/acprof:oso/9780199682737.001.0001
- Hood, R. W. (2001). Review: cleansing the doors of perception: the religious significance of entheogenic plants and chemicals. *Int. J. Psychol. Relig.* 11, 285–286. doi: 10.1207/S15327582IJPR1104_08
- Huang, G. (2008). Is this a unified theory of the brain. *New Sci.* 2658, 30–33. doi: 10.1016/S0262-4079(08)61366-4
- Huxley, A. (1945). *The Perennial Philosophy*. London: Harper Publications.
- Huxley, A. (1953). "Letters to Dr. Humphrey Osmond," in *Moksha: Aldous Huxley's Classic Writings on Psychedelics and the Visionary Experience*, eds M. Horowitz and C. Palmer (Rochester, VT: Park Street Press).
- Huxley, A. (1961a). "Exploring The Borderlands of the Mind," in *Moksha: Aldous Huxley's Classic Writings on Psychedelics and the Visionary Experience*, eds M. Horowitz and C. Palmer (Rochester, VT: Park Street Press).
- Huxley, A. (1961b). "Visionary experience," in *Moksha: Aldous Huxley's Classic Writings on Psychedelics and the Visionary Experience*, eds M. Horowitz and C. Palmer (Rochester, VT: Park Street Press).
- Huxley, A. (1991). *The Doors of Perception, and Heaven and Hell*. New York, NY: HarperPerennial.
- Huxley, A. (1999). *Moksha: Aldous Huxley's Classic Writings on Psychedelics and the Visionary Experience*, eds M. Horowitz and C. Palmer (Rochester, VT: Park Street Press).
- James, W. (1882). *Subjective Effects of Nitrous Oxide*. Available at: <https://philpapers.org/rec/JAMSEO>
- James, W. (1890). *The Principles of Psychology*. New York, NY: Henry Holt and Company.
- James, W. (1902). *The Varieties of Religious Experience: A Study in Human Nature*. New York, NY: Longmans, Green and Co. doi: 10.1037/10004-000
- Johnson, M., Richards, W., and Griffiths, R. (2008). Human hallucinogen research: guidelines for safety. *J. Psychopharmacol.* 22, 603–620. doi: 10.1177/0269881108093587
- Johnson, M. W., Garcia-Romeu, A., Cosimano, M. P., and Griffiths, R. R. (2014). Pilot study of the 5-HT2AR agonist psilocybin in the treatment of tobacco addiction. *J. Psychopharmacol.* 28, 983–992. doi: 10.1177/0269881114548296
- Kaelen, M., Barrett, F. S., Roseman, L., Lorenz, R., Family, N., Bolstridge, M., et al. (2015). LSD enhances the emotional response to music. *Psychopharmacology* 232, 3607–3614. doi: 10.1007/s00213-015-4014-y
- Kaelen, M., Giribaldi, B., Raine, J., Evans, L., Timmerman, C., Rodriguez, N., et al. (2018). The hidden therapist: evidence for a central role of music in psychedelic therapy. *Psychopharmacology* 235, 505–519. doi: 10.1007/s00213-017-4820-5
- Kaelen, M., Roseman, L., Kahan, J., Santos-Ribeiro, A., Orban, C., Lorenz, R., et al. (2016). LSD modulates music-induced imagery via changes in parahippocampal connectivity. *Eur. Neuropsychopharmacol.* 26, 1099–1109. doi: 10.1016/j.euroneuro.2016.03.018
- Kant, I. (1996). *Critique of Pure Reason*, eds W. S. Pluhar and P. Kitcher. Indianapolis, IN: Hackett Publishing Co.
- Kay, K. N. (2017). Principles for models of neural information processing. *Neuroimage* doi: 10.1016/j.neuroimage.2017.08.016 [Epub ahead of print].
- Kemp, C., Perfors, A., and Tenenbaum, J. B. (2007). Learning overhypotheses with hierarchical Bayesian models. *Dev. Sci.* 10, 307–321. doi: 10.1111/j.1467-7687.2007.00585.x
- Kersten, D., Mamassian, P., and Yuille, A. (2004). Object perception as Bayesian inference. *Annu. Rev. Psychol.* 55, 271–304. doi: 10.1146/annurev.psych.55.090902.142005
- Kersten, D., and Yuille, A. (2003). Bayesian models of object perception. *Curr. Opin. Neurobiol.* 13, 150–158. doi: 10.1016/S0959-4388(03)00042-4
- Kitcher, P. (1981). Explanatory unification. *Philos. Sci.* 48, 507–531. doi: 10.1086/289019
- Kitcher, P. (1989). "Explanatory unification and the causal structure of the world," in *Scientific Explanation*, eds P. Kitcher and W. Salmon (Minneapolis, MN: University of Minnesota Press), 410–505.
- Klee, G. D. (1963). Lysergic acid diethylamide (LSD-25) and ego functions. *Arch. Gen. Psychiatry* 8, 461–474. doi: 10.1001/archpsyc.1963.01720110037005
- Klüver, H. (1926). Mescal visions and eidetic vision. *Am. J. Psychol.* 37, 502–515. doi: 10.2307/1414910
- Klüver, H. (1928). *Mescal: The "Divine" Plant and Its Psychological Effects*. London: Kegan Paul, Trench, Trubner & Co.
- Knauer, A., and Maloney, W. J. M. A. (1913). A preliminary note on the psychic action of mescaline, with special reference to the mechanism of visual hallucinations. *J. Nerv. Ment. Dis.* 40, 425–437. doi: 10.1097/00005053-191307000-00001
- Knill, D. C., and Pouget, A. (2004). The Bayesian brain: the role of uncertainty in neural coding and computation. *Trends Neurosci.* 27, 712–719. doi: 10.1016/j.tins.2004.10.007
- Kometer, M., Cahn, B. R., Andel, D., Carter, O. L., and Vollenweider, F. X. (2011). The 5-HT2A/1A agonist psilocybin disrupts modal object completion associated with visual hallucinations. *Biol. Psychiatry* 69, 399–406. doi: 10.1016/j.biopsych.2010.10.002
- Kometer, M., Pokorny, T., Seifritz, E., and Vollenweider, F. X. (2015). Psilocybin-induced spiritual experiences and insightfulness are associated with synchronization of neuronal oscillations. *Psychopharmacology* 232, 3663–3676. doi: 10.1007/s00213-015-4026-7
- Kometer, M., Schmidt, A., Bachmann, R., Studerus, E., Seifritz, E., and Vollenweider, F. X. (2012). Psilocybin biases facial recognition, goal-directed behavior, and mood state toward positive relative to negative emotions through different serotonergic subreceptors. *Biol. Psychiatry* 72, 898–906. doi: 10.1016/j.biopsych.2012.04.005
- Kometer, M., Schmidt, A., Jäncke, L., and Vollenweider, F. X. (2013). Activation of serotonin 2A receptors underlies the psilocybin-induced effects on α oscillations, N170 visual-evoked potentials, and visual hallucinations. *J. Neurosci.* 33, 10544–10551. doi: 10.1523/JNEUROSCI.3007-12.2013
- Kometer, M., and Vollenweider, F. X. (2016). Serotonergic hallucinogen-induced visual perceptual alterations. *Curr. Top. Behav. Neurosci.* doi: 10.1007/7854_2016_461 [Epub ahead of print].
- Kraehenmann, R., Pokorny, D., Aicher, H., Preller, K. H., Pokorny, T., Bosch, O. G., et al. (2017a). LSD increases primary process thinking via serotonin 2A receptor activation. *Front. Pharmacol.* 8:814. doi: 10.3389/fphar.2017.00814
- Kraehenmann, R., Pokorny, D., Vollenweider, L., Preller, K. H., Pokorny, T., Seifritz, E., et al. (2017b). Dreamlike effects of LSD on waking imagery in humans depend on serotonin 2A receptor activation. *Psychopharmacology* 234, 2031–2046. doi: 10.1007/s00213-017-4610-0
- Kraepelin, E. (1892). *Ueber die Beeinflussung Einfacher Psychischer Vorgänge durch Einige Arzneimittel: Experimentelle Untersuchungen*. Jena: G. Fischer.
- Krebs, T. S., and Johansen, P.-Ø. (2013). Psychedelics and mental health: a population study. *PLoS One* 8:e63972. doi: 10.1371/journal.pone.0063972
- Kuypers, K. P. C., Riba, J., de la Fuente Revenga, M., Barker, S., Theunissen, E. L., and Ramaekers, J. G. (2016). Ayahuasca enhances creative divergent thinking

- while decreasing conventional convergent thinking. *Psychopharmacology* 233, 3395–3403. doi: 10.1007/s00213-016-4377-8
- Kwisthout, J., Bekkering, H., and van Rooij, I. (2017). To be precise, the details don't matter: on predictive processing, precision, and level of detail of predictions. *Brain Cogn.* 112, 84–91. doi: 10.1016/j.bandc.2016.2.008
- Kwisthout, J., and van Rooij, I. (2015). Free energy minimization and information gain: the devil is in the details. *Cogn. Neurosci.* 6, 216–218. doi: 10.1080/17588928.2015.1051014
- Leary, T., Litwin, G. H., and Metzner, R. (1963). Reactions to psilocybin administered in a supportive environment. *J. Nerv. Ment. Dis.* 137, 561–573. doi: 10.1097/00005053-196312000-00007
- Leary, T., Metzner, R., and Alpert, R. (1964). *The Psychedelic Experience: A Manual Based on the Tibetan Book of the Dead*. New York, NY: University Books.
- Lebedev, A. V., Kaelen, M., Lövdén, M., Nilsson, J., Feilding, A., Nutt, D. J., et al. (2016). LSD-Induced Entropic Brain Activity Predicts Subsequent Personality Change. Available at: <http://onlinelibrary.wiley.com/doi/10.1002/hbm.23234/full>
- Lee, T. S., and Mumford, D. (2003). Hierarchical Bayesian inference in the visual cortex. *J. Opt. Soc. Am. A Opt. Image Sci. Vis.* 20, 1434–1448. doi: 10.1364/JOSAA.20.001434
- Lethaby, C., and Gerrans, P. (2017). Self unbound: ego dissolution in psychedelic experience. *Neurosci. Conscious.* 3:nix016. doi: 10.1093/nc/nix016
- Lewin, L. (1894). On Anhalonium lewinii and other cacti. *Arch. Exp. Pathol. Pharmakol.* 24, 401–411. doi: 10.1007/BF01923627
- Lewin, L. (1927). *Phantastica, Narcotic and Stimulating Drugs*. London: Rutledge & Kegan Paul Ltd.
- Lieberman, J. A., and Shalev, D. (2016). Back to the future: research renewed on the clinical utility of psychedelic drugs. *J. Psychopharmacol.* 30, 1198–1200. doi: 10.1177/0269881116675755
- Liechti, M. E., Dolder, P. C., and Schmid, Y. (2017). Alterations of consciousness and mystical-type experiences after acute LSD in humans. *Psychopharmacology* 234, 1499–1510. doi: 10.1007/s00213-016-4453-0
- Limanowski, J., and Blankenburg, F. (2013). Minimal self-models and the free energy principle. *Front. Hum. Neurosci.* 7:547. doi: 10.3389/fnhum.2013.00547
- Luke, D. P., and Terhune, D. B. (2013). The induction of synesthesia with chemical agents: a systematic review. *Front. Psychol.* 4:753. doi: 10.3389/fpsyg.2013.00753
- MacKay, D. M. (1956). "The epistemological problem for automata," in *Automata Studies: Annals of Mathematics Studies. Number 34*, eds W. R. Ashby, C. E. Shannon, and J. McCarthy (Princeton, NJ: Princeton University Press).
- MacLean, K. A., Johnson, M. W., and Griffiths, R. R. (2011). Mystical experiences occasioned by the hallucinogen psilocybin lead to increases in the personality domain of openness. *J. Psychopharmacol.* 25, 1453–1461. doi: 10.1177/0269881111420188
- Maclean, K. A., Leoutsakos, J.-M. S., Johnson, M. W., and Griffiths, R. R. (2012). Factor analysis of the mystical experience questionnaire: a study of experiences occasioned by the hallucinogen psilocybin. *J. Sci. Study Relig.* 51, 721–737. doi: 10.1111/j.1468-5906.2012.01685.x
- Majić, T., Schmidt, T. T., and Gallinat, J. (2015). Peak experiences and the afterglow phenomenon: when and how do therapeutic effects of hallucinogens depend on psychedelic experiences? *J. Psychopharmacol.* 29, 241–253. doi: 10.1177/0269881114568040
- Marshall, P. (2005). "Mind beyond the brain: reducing valves and metaphysics," in *Mystical Encounters with the Natural World*, (Oxford: Oxford University Press).
- Martindale, C., and Fischer, R. (1977). The effects of psilocybin on primary process content in language. *Confin. Psychiatr.* 20, 195–202.
- Masters, R. E. L., and Houston, J. (1966). *The Varieties of Psychedelic Experience*. New York, NY: Holt, Rinehart and Winston.
- McKenna, D., and Riba, J. (2015). New world tryptamine hallucinogens and the neuroscience of ayahuasca. *Curr. Top. Behav. Neurosci.* doi: 10.1007/7854_2015_368 [Epub ahead of print].
- McKenna, D. J., Repke, D. B., Lo, L., and Peroutka, S. J. (1990). Differential interactions of indolealkylamines with 5-hydroxytryptamine receptor subtypes. *Neuropharmacology* 29, 193–198. doi: 10.1016/0028-3908(90)90001-8
- McKenna, D. J., Towers, G. H., and Abbott, F. (1984). Monoamine oxidase inhibitors in South American hallucinogenic plants: tryptamine and beta-carboline constituents of ayahuasca. *J. Ethnopharmacol.* 10, 195–223. doi: 10.1016/0378-8741(84)90003-5
- Merkur, D. (1998). *The Ecstatic Imagination: Psychedelic Experiences and the Psychoanalysis of Self-Actualization*. Albany, NY: State University of New York Press.
- Merriam-Webster (2017). *Model*. Springfield, MA: Merriam-Webster.
- Metzinger, T. (2003). *Being no One: the Self-Model Theory of Subjectivity*. London: The MIT Press.
- Millière, R. (2017). Looking for the self: phenomenology, neurophysiology and philosophical significance of drug-induced ego dissolution. *Front. Hum. Neurosci.* 11:245. doi: 10.3389/fnhum.2017.00245
- Mitchell, S. W. (1896). Remarks on the effects of Anhelonium Lewinii (the Mescal Button). *Br. Med. J.* 2, 1625–1629. doi: 10.1136/bmj.2.1875.1625
- Montague, P. R., Dolan, R. J., Friston, K. J., and Dayan, P. (2012). Computational psychiatry. *Trends Cogn. Sci.* 16, 72–80. doi: 10.1016/j.tics.2011.11.018
- Moreau, J. J. (1845). *Du Hashisch et De L'Aliénation Mentale: Études Psychologiques*. Paris: Fortin.
- Moreno, F. A., Wiegand, C. B., Taitano, E. K., and Delgado, P. L. (2006). Safety, tolerability, and efficacy of psilocybin in 9 patients with obsessive-compulsive disorder. *J. Clin. Psychiatry* 67, 1735–1740. doi: 10.4088/JCP.v67n1110
- Morrison, M. (2000). *Unifying Scientific Theories: Physical Concepts and Mathematical Structures*. Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511527333
- Müller, U., Fletcher, P. C., and Steinberg, H. (2006). The origin of pharmacopsychology: Emil Kraepelin's experiments in Leipzig, Dorpat and Heidelberg (1882–1892). *Psychopharmacology* 184, 131–138. doi: 10.1007/s00213-005-0239-5
- Mumford, D. (1992). On the computational architecture of the neocortex. *Biol. Cybern.* 66, 241–251. doi: 10.1007/BF00198477
- Muthukumaraswamy, S., Carhart-Harris, R. L., Moran, R. J., Brookes, M. J., Williams, T. M., Erritzoe, D., et al. (2013). Broadband cortical desynchronization underlies the human psychedelic state. *J. Neurosci.* 33, 15171–15183. doi: 10.1523/JNEUROSCI.2063-13.2013
- Myers, F. W. H. (1903). *Human Personality and its Survival of Bodily Death*. London: Longmans & Co.
- Natale, M., Dahlberg, C. C., and Jaffe, J. (1978a). Effect of psychotomimetics (LSD and dextroamphetamine) on the use of primary- and secondary-process language. *J. Consult. Clin. Psychol.* 46, 352–353. doi: 10.1037/0022-006X.46.2.352
- Natale, M., Kowitz, M., Dahlberg, C. C., and Jaffe, J. (1978b). Effect of psychotomimetics (LSD and dextroamphetamine) on the use of figurative language during psychoanalysis. *J. Consult. Clin. Psychol.* 46, 1579–1580. doi: 10.1037/0022-006X.46.6.1579
- Neisser, U. (1967). *Cognitive Psychology*. Available at: <http://psycnet.apa.org/psycinfo/1967-35031-000> [accessed March 18, 2016].
- Nichols, D. E. (2016). Psychedelics. *Pharmacol. Rev.* 68, 264–355. doi: 10.1124/pr.115.011478
- Nour, M. M., Evans, L., Nutt, D., and Carhart-Harris, R. L. (2016). Ego-dissolution and psychedelics: validation of the Ego-Dissolution Inventory (EDI). *Front. Hum. Neurosci.* 10:269. doi: 10.3389/fnhum.2016.00269
- Oizumi, M., Albantakis, L., and Tononi, G. (2014). From the phenomenology to the mechanisms of consciousness: integrated information theory 3.0. *PLoS Comput. Biol.* 10:e1003588. doi: 10.1371/journal.pcbi.1003588
- Osborne, H. (2017). *Scientists Show LSD and Ketamine Make the Brain Enter a Higher State of Consciousness*. Available at: <http://www.newsweek.com/psychedelic-drugs-lsd-ketamine-brain-higher-consciousness-586076> [accessed July 26, 2017].
- Osmond, H. (1957). A review of the clinical effects of psychotomimetic agents. *Ann. N. Y. Acad. Sci.* 66, 418–434. doi: 10.1111/j.1749-6632.1957.tb40738.x
- Osmond, H., and Smythies, J. (1952). Schizophrenia: a new approach. *J. Ment. Sci.* 98, 309–315. doi: 10.1192/bjps.98.411.309
- Ott, U. (2007). "States of absorption: in search of neurobiological foundations," in *Hypnosis and Conscious States: The Cognitive Neuroscience Perspective*, ed. G. A. Jamieson (New York, NY: Oxford University Press), 257–270.

- Pahnke, W. N., Kurland, A. A., Unger, S., Savage, C., and Grof, S. (1971). The experimental use of psychedelic (LSD) psychotherapy. *Int. Z. Klin. Pharmakol. Ther. Toxikol.* 4, 446–454.
- Palhano-Fontes, F., Andrade, K. C., Tofoli, L. F., Santos, A. C., Crippa, J. A. S., Hallak, J. E. C., et al. (2015). The psychedelic state induced by ayahuasca modulates the activity and connectivity of the default mode network. *PLoS One* 10:e0118143. doi: 10.1371/journal.pone.0118143
- Pink-Hashkes, S., van Rooij, I., and Kwisthout, J. (2017). “Perception is in the details: a predictive coding account of the psychedelic phenomenon,” in *Proceedings of the 39th Annual Meeting of the Cognitive Science Society*, London, 2907–2912.
- Pokorny, T., Preller, K. H., Kometer, M., Dziobek, I., and Vollenweider, F. X. (2017). Effect of psilocybin on empathy and moral decision-making. *Int. J. Neuropsychopharmacol.* 20, 747–757. doi: 10.1093/ijnp/pwx047
- Preller, K. H., Herdener, M., Pokorny, T., Planzer, A., Kraehenmann, R., Stämpfli, P., et al. (2017). The fabric of meaning and subjective effects in LSD-induced states depend on serotonin 2A receptor activation. *Curr. Biol.* 27, 451–457. doi: 10.1016/j.cub.2016.12.030
- Preller, K. H., and Vollenweider, F. X. (2016). Phenomenology, structure, and dynamic of psychedelic states. *Curr. Top. Behav. Neurosci.* doi: 10.1007/7854_2016_459 [Epub ahead of print].
- Prentiss, D. W., and Morgan, T. P. (1895). Anhalonium lewinie (Mescal buttons). *Ther. Gaz.* 9, 577–585.
- Purpura, D. P. (1968). “Neurophysiological actions of LSD,” in *LSD, Man and Society*, eds R. C. DeBold and R. C. Leaf (Middletown, CT: Wesleyan University Press).
- Rao, R. P., and Ballard, D. H. (1999). Predictive coding in the visual cortex: a functional interpretation of some extra-classical receptive-field effects. *Nat. Neurosci.* 2, 79–87. doi: 10.1038/4580
- Rapaport, D. (1950). On the psycho-analytic theory of thinking. *Int. J. Psycho Anal.* 31, 161–170.
- Ray, T. S. (2010). Psychedelics and the human receptorome. *PLoS One* 5:e9019. doi: 10.1371/journal.pone.0009019
- Ray, T. S. (2016). Constructing the ecstasy of MDMA from its component mental organs: proposing the primer/probe method. *Med. Hypotheses* 87, 48–60. doi: 10.1016/j.mehy.2015.12.018
- Riba, J., Anderer, P., Jané, F., Saletu, B., and Barbanjo, M. J. (2004). Effects of the South American psychoactive beverage ayahuasca on regional brain electrical activity in humans: a functional neuroimaging study using low-resolution electromagnetic tomography. *Neuropsychobiology* 50, 89–101. doi: 10.1159/000077946
- Riba, J., Anderer, P., Morte, A., Urbano, G., Jané, F., Saletu, B., et al. (2002). Topographic pharmaco-EEG mapping of the effects of the South American psychoactive beverage ayahuasca in healthy volunteers. *Br. J. Clin. Pharmacol.* 53, 613–628. doi: 10.1046/j.1365-2125.2002.01609.x
- Riba, J., Rodríguez-Fornells, A., Strassman, R. J., and Barbanjo, M. J. (2001a). Psychometric assessment of the Hallucinogen Rating Scale. *Drug Alcohol Depend.* 62, 215–223. doi: 10.1016/S0376-8716(00)00175-7
- Riba, J., Rodríguez-Fornells, A., Urbano, G., Morte, A., Antonijoan, R., Montero, M., et al. (2001b). Subjective effects and tolerability of the South American psychoactive beverage Ayahuasca in healthy volunteers. *Psychopharmacology* 154, 85–95.
- Rickli, A., Moning, O. D., Hoener, M. C., and Liechti, M. E. (2016). Receptor interaction profiles of novel psychoactive tryptamines compared with classic hallucinogens. *Eur. Neuropsychopharmacol.* 26, 1327–1337. doi: 10.1016/j.euroneuro.2016.05.001
- Roseman, L., Leech, R., Feilding, A., Nutt, D. J., and Carhart-Harris, R. L. (2014). The effects of psilocybin and MDMA on between-network resting state functional connectivity in healthy volunteers. *Front. Hum. Neurosci.* 8:204. doi: 10.3389/fnhum.2014.00204
- Roseman, L., Nutt, D., and Carhart-Harris, R. (2017). Quality of acute psychedelic experience predicts therapeutic efficacy of psilocybin for treatment-resistant depression. *Front. Pharmacol.* 8:974. doi: 10.3389/fphar.2017.00974
- Roseman, L., Sereno, M. I., Leech, R., and Kaelen, M. (2016). LSD alters eyes-closed functional connectivity within the early visual cortex in a retinotopic fashion. *Hum. Brain Mapp.* 37, 3031–3040. doi: 10.1002/hbm.23224
- Ross, S., Bossis, A., Guss, J., Agin-Liebes, G., Malone, T., Cohen, B., et al. (2016). Rapid and sustained symptom reduction following psilocybin treatment for anxiety and depression in patients with life-threatening cancer: a randomized controlled trial. *J. Psychopharmacol.* 30, 1165–1180. doi: 10.1177/0269881116675512
- Rouhier, A. (1927). *Le Peyotl*. Paris: G. Doin.
- Russ, S. L., and Elliott, M. S. (2017). Antecedents of mystical experience and dread in intensive meditation. *Psychol. Conscious.* 4, 38–53. doi: 10.1037/cns000119
- Sandison, R. A. (1954). Psychological aspects of the LSD treatment of the neuroses. *J. Ment. Sci.* 100, 508–515. doi: 10.1192/bj.p.100.419.508
- Sandison, R. A., and Whitelaw, J. D. (1957). Further studies in the therapeutic value of lysergic acid diethylamide in mental illness. *J. Ment. Sci.* 103, 332–343. doi: 10.1192/bj.p.103.431.332
- Sanz, C., and Tagliazucchi, E. (2018). The experience elicited by hallucinogens presents the highest similarity to dreaming within a large database of psychoactive substance reports. *Front. Neurosci.* 12:7. doi: 10.3389/fnins.2018.00007
- Savage, C. (1955). Variations in ego feeling induced by D-lysergic acid diethylamide (LSD-25). *Psychoanal. Rev.* 42, 1–16.
- Schartner, M. M., Carhart-Harris, R. L., Barrett, A. B., Seth, A. K., and Muthukumaraswamy, S. (2017). Increased spontaneous MEG signal diversity for psychoactive doses of ketamine, LSD and psilocybin. *Sci. Rep.* 7:46421. doi: 10.1038/srep46421
- Schenberg, E. E., Alexandre, J. F. M., Filev, R., Cravo, A. M., Sato, J. R., Muthukumaraswamy, S. D., et al. (2015). Acute biphasic effects of ayahuasca. *PLoS One* 10:e0137202. doi: 10.1371/journal.pone.0137202
- Schmid, Y., Enzler, F., Gasser, P., Grouzmann, E., Preller, K. H., Vollenweider, F. X., et al. (2015). Acute effects of lysergic acid diethylamide in healthy subjects. *Biol. Psychiatry* 78, 544–553. doi: 10.1016/j.biopsych.2014.11.015
- Schmid, L. A., Steinberg, H., and Sykes, E. A. B. (2006). Psychopharmacology’s debt to experimental psychology. *Hist. Psychol.* 9, 144–157. doi: 10.1037/1093-4510.9.2.144
- Schultes, R. E., and Hofmann, A. (1973). *The Botany and Chemistry of Hallucinogens*. Springfield, IL: Charles C. Thomas.
- Sessa, B. (2008). Is it time to revisit the role of psychedelic drugs in enhancing human creativity? *J. Psychopharmacol.* 22, 821–827. doi: 10.1177/0269881108091597
- Seth, A. (2009). Explanatory correlates of consciousness: theoretical and computational challenges. *Cogn. Comput.* 1, 50–63. doi: 10.1007/s12559-009-9007-x
- Shanon, B. (2002). *The Antipodes of the Mind: Charting the Phenomenology of the Ayahuasca Experience*. New York, NY: Oxford University Press.
- Shaw, E., and Woolley, D. W. (1956). Some serotoninlike activities of lysergic acid diethylamide. *Science* 124, 121–122. doi: 10.1126/science.124.3212.121
- Shimamura, A. P. (2000). Toward a cognitive neuroscience of metacognition. *Conscious. Cogn.* 9, 313–323; discussion 324–326. doi: 10.1006/ccog.2000.0450
- Shulgin, A. T., and Shulgin, A. (1991). *Pihkal: A Chemical Love Story*. Berkeley, CA: Transform Press.
- Shulgin, A. T., and Shulgin, A. (1997). *Tihkal: the Continuation*. Berkeley, CA: Transform Press.
- Siegel, R. K., and Jarvik, M. E. (1975). “Drug-induced hallucinations in animals and man,” in *Hallucinations: Behavior, Experience, and Theory*, eds R. K. Siegel and L. J. West (New York, NY: John Wiley & Sons), 81–161.
- Sinke, C., Halpern, J. H., Zedler, M., Neufeld, J., Emrich, H. M., and Passie, T. (2012). Genuine and drug-induced synesthesia: a comparison. *Conscious. Cogn.* 21, 1419–1434. doi: 10.1016/j.concog.2012.03.009
- Smythies, J. R. (1956). *Analysis of Perception*. London: Routledge and Paul.
- Späth, E. (1919). Über die Anhalonium-Alkalioide. *Monatsh. Chem.* 40, 129–154. doi: 10.1007/BF01524590
- Speth, J., Speth, C., Kaelen, M., Schloerscheidt, A. M., Feilding, A., Nutt, D. J., et al. (2016). Decreased mental time travel to the past correlates with default-mode network disintegration under lysergic acid diethylamide. *J. Psychopharmacol.* 30, 344–353. doi: 10.1177/0269881116628430
- Spitzer, M., Thimm, M., Hermle, L., Holzmann, P., Kovar, K. A., Heimann, H., et al. (1996). Increased activation of indirect semantic associations under psilocybin. *Biol. Psychiatry* 39, 1055–1057. doi: 10.1016/0006-3223(95)00418-1
- Stace, W. T. (1960). *Mysticism and Philosophy*. New York, NY: MacMillan.

- Stockings, G. T. (1940). A clinical study of the mescaline psychosis, with special reference to the mechanism of the genesis of schizophrenic and other psychotic states. *J. Ment. Sci.* 86, 29–47. doi: 10.1192/bjps.86.360.29
- Strassman, R. J. (1984). Adverse reactions to psychedelic drugs. A review of the literature. *J. Nerv. Ment. Dis.* 172, 577–595. doi: 10.1097/00005053-198410000-00001
- Strassman, R. J., Qualls, C. R., Uhlenhuth, E. H., and Kellner, R. (1994). Dose-response study of N,N-dimethyltryptamine in humans. II. Subjective effects and preliminary results of a new rating scale. *Arch. Gen. Psychiatry* 51, 98–108. doi: 10.1001/archpsyc.1994.03950020022002
- Studerus, E., Gamma, A., Kometer, M., and Vollenweider, F. X. (2012). Prediction of psilocybin response in healthy volunteers. *PLoS One* 7:e30800. doi: 10.1371/journal.pone.0030800
- Studerus, E., Gamma, A., and Vollenweider, F. X. (2010). Psychometric evaluation of the altered states of consciousness rating scale (OAV). *PLoS One* 5:e12412. doi: 10.1371/journal.pone.0012412
- Studerus, E., Kometer, M., Hasler, F., and Vollenweider, F. X. (2011). Acute, subacute and long-term subjective effects of psilocybin in healthy humans: a pooled analysis of experimental studies. *J. Psychopharmacol.* 25, 1434–1452. doi: 10.1177/0269881110382466
- Suler, J. R. (1980). Primary process thinking and creativity. *Psychol. Bull.* 88, 144–165. doi: 10.1037/0033-2909.88.1.144
- Swanson, L. R. S. (2016). The predictive processing paradigm has roots in kant. *Front. Syst. Neurosci.* 10:79. doi: 10.3389/fnsys.2016.00079
- Sweat, N. W., Bates, L. W., and Hendricks, P. S. (2016). The associations of naturalistic classic psychedelic use, mystical experience, and creative problem solving. *J. Psychoactive Drugs* 48, 344–350. doi: 10.1080/02791072.2016.1234090
- Tagliazucchi, E., Carhart-Harris, R. L., Leech, R., Nutt, D., and Chialvo, D. R. (2014). Enhanced repertoire of brain dynamical states during the psychedelic experience. *Hum. Brain Mapp.* 35, 5442–5456. doi: 10.1002/hbm.22562
- Tagliazucchi, E., Roseman, L., Kaelen, M., Orban, C., Muthukumaraswamy, S., Murphy, K., et al. (2016). Increased global functional connectivity correlates with LSD-induced Ego dissolution. *Curr. Biol.* 26, 1043–1050. doi: 10.1016/j.cub.2016.02.010
- Tenenbaum, J. B., Kemp, C., Griffiths, T. L., and Goodman, N. D. (2011). How to grow a mind: statistics, structure, and abstraction. *Science* 331, 1279–1285. doi: 10.1126/science.1192788
- Terhune, D. B., Luke, D. P., Kaelen, M., Bolstridge, M., Feilding, A., Nutt, D., et al. (2016). A placebo-controlled investigation of synesthesia-like experiences under LSD. *Neuropsychologia* 88, 28–34. doi: 10.1016/j.neuropsychologia.2016.04.005
- Tononi, G. (2004). An information integration theory of consciousness. *BMC Neurosci.* 5:42. doi: 10.1186/1471-2202-5-42
- Tononi, G. (2008). Consciousness as integrated information: a provisional manifesto. *Biol. Bull.* 215, 216–242. doi: 10.2307/25470707
- Tononi, G., and Edelman, G. M. (1998). Consciousness and complexity. *Science* 282, 1846–1851. doi: 10.1126/science.282.5395.1846
- Tononi, G., and Koch, C. (2008). The neural correlates of consciousness: an update. *Ann. N. Y. Acad. Sci.* 1124, 239–261. doi: 10.1196/annals.1440.004
- Tupper, K. W., Wood, E., Yensen, R., and Johnson, M. W. (2015). Psychedelic medicine: a re-emerging therapeutic paradigm. *CMAJ* 187, 1054–1059. doi: 10.1503/cmaj.141124
- Turton, S., Nutt, D. J., and Carhart-Harris, R. L. (2014). A qualitative report on the subjective experience of intravenous psilocybin administered in an fMRI environment. *Curr. Drug Abuse Rev.* 7, 117–127. doi: 10.2174/1874473708666150107120930
- Valle, M., Maqueda, A. E., Rabella, M., Rodríguez-Pujadas, A., Antonijoen, R. M., Romero, S., et al. (2016). Inhibition of alpha oscillations through serotonin-2A receptor activation underlies the visual effects of ayahuasca in humans. *Eur. Neuropsychopharmacol.* 26, 1161–1175. doi: 10.1016/j.euroneuro.2016.03.012
- Viol, A., Palhano-Fontes, F., Onias, H., de Araujo, D. B., and Viswanathan, G. M. (2016). *Shannon Entropy of Brain Functional Complex Networks under the Influence of the Psychedelic Ayahuasca*. Available at: <http://arxiv.org/abs/1611.00358>
- Vollenweider, F. X., Csorba, P. A., Knappe, B., Geyer, M. A., and Quednow, B. B. (2007). The effects of the preferential 5-HT2A agonist psilocybin on prepulse inhibition of startle in healthy human volunteers depend on interstimulus interval. *Neuropsychopharmacology* 32, 1876–1887. doi: 10.1038/sj.npp.1301324
- Vollenweider, F. X., Vollenweider-Scherpenhuyzen, M. F., Bäbler, A., Vogel, H., and Hell, D. (1998). Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2A agonist action. *Neuroreport* 9, 3897–3902. doi: 10.1097/00001756-199812010-00024
- Wackermann, J., Wittmann, M., Hasler, F., and Vollenweider, F. X. (2008). Effects of varied doses of psilocybin on time interval reproduction in human subjects. *Neurosci. Lett.* 435, 51–55. doi: 10.1016/j.neulet.2008.02.006
- Waldman, A. (2017). *A Really Good Day: How Microdosing Made a Mega Difference in My Mood, My Marriage, and My Life*. New York, NY: Knopf Doubleday Publishing Group.
- Ward, J. (2013). Synesthesia. *Annu. Rev. Psychol.* 64, 49–75. doi: 10.1146/annurev-psych-113011-143840
- Watts, R., Day, C., Krzanowski, J., and Carhart-Harris, R. (2017). Patients' accounts of increased "Connectedness" and "Acceptance" after psilocybin for treatment-resistant depression. *J. Humanist. Psychol.* 57:002216781770958. doi: 10.1177/0022167817709585
- Wiese, W., and Metzinger, T. (2017). "Vanilla PP for philosophers: a primer on predictive processing," in *Philosophy and Predictive Processing*, eds T. Metzinger and W. Wiese (Frankfurt: MIND Group).
- Winkelman, M. J. (2017). The mechanisms of psychedelic visionary experiences: hypotheses from evolutionary psychology. *Front. Neurosci.* 11:539. doi: 10.3389/fnins.2017.00539
- Wittmann, M., Carter, O., Hasler, F., Cahn, B. R., Grimberg, U., Spring, P., et al. (2007). Effects of psilocybin on time perception and temporal control of behaviour in humans. *J. Psychopharmacol.* 21, 50–64. doi: 10.1177/0269881106065859
- Wong, S. (2017). Leading the high life. *New Sci.* 234, 22–23. doi: 10.1016/S0262-4079(17)31161-2
- Woolley, D. W., and Shaw, E. (1954). A biochemical and pharmacological suggestion about certain mental disorders. *Proc. Natl. Acad. Sci. U.S.A.* 40, 228–231. doi: 10.1073/pnas.40.4.228
- Zizo (2013). *How Psilocybin Works: Addition by Subtraction - Psychedelic Frontier*. Available at: <http://psychedelicfrontier.com/how-psilocybin-works-addition-by-subtraction/> [accessed December 14, 2017].

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Quality of Acute Psychedelic Experience Predicts Therapeutic Efficacy of Psilocybin for Treatment-Resistant Depression

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Introduction: It is a basic principle of the “psychedelic” treatment model that the quality of the acute experience mediates long-term improvements in mental health. In the present paper we sought to test this using data from a clinical trial assessing psilocybin for treatment-resistant depression (TRD). In line with previous reports, we hypothesized that the occurrence and magnitude of Oceanic Boundlessness (OBN) (sharing features with mystical-type experience) and Dread of Ego Dissolution (DED) (similar to anxiety) would predict long-term positive outcomes, whereas sensory perceptual effects would have negligible predictive value.

Materials and Methods: Twenty patients with treatment resistant depression underwent treatment with psilocybin (two separate sessions: 10 and 25 mg psilocybin). The Altered States of Consciousness (ASC) questionnaire was used to assess the quality of experiences in the 25 mg psilocybin session. From the ASC, the dimensions OBN and DED were used to measure the mystical-type and challenging experiences, respectively. The Self-Reported Quick Inventory of Depressive Symptoms (QIDS-SR) at 5 weeks served as the endpoint clinical outcome measure, as in later time points some of the subjects had gone on to receive new treatments, thus confounding inferences. In a repeated measure ANOVA, Time was the within-subject factor (independent variable), with QIDS-SR as the within-subject dependent variable in baseline, 1-day, 1-week, 5-weeks. OBN and DED were independent variables. OBN-by-Time and DED-by-Time interactions were the primary outcomes of interest.

Results: For the interaction of OBN and DED with Time (QIDS-SR as dependent variable), the main effect and the effects at each time point compared to baseline were all significant ($p = 0.002$ and $p = 0.003$, respectively, for main effects), confirming our main hypothesis. Furthermore, Pearson’s correlation of OBN with QIDS-SR (5 weeks) was specific compared to perceptual dimensions of the ASC ($p < 0.05$).

Discussion: This report further bolsters the view that the quality of the acute psychedelic experience is a key mediator of long-term changes in mental health.

Future therapeutic work with psychedelics should recognize the essential importance of *quality of experience* in determining treatment efficacy and consider ways of enhancing mystical-type experiences and reducing anxiety.

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INTRODUCTION

Psychedelic therapy may be more appropriately thought of as a distinct form of (drug-assisted) psychotherapy than as a pure pharmacotherapy. Psychedelic therapy involves a small number of high-dose psychedelic dosing sessions that are intended to facilitate a profound, potentially transformative psychological experience (Dyck, 2006; Majić et al., 2015). Psychedelic dosing sessions do not take place in isolation but rather are flanked by psychological preparation and integration. Preparation is intended to facilitate trust and rapport and a mind-set tuned toward emotional openness and “letting go” of psychological resistance (Richards, 2015; Russ and Elliott, 2017). Dosing sessions themselves typically take place in a welcoming environment, with dim lighting, eye-shades, calming and emotionally-directing music, with empathic support provided by trained therapists. The integration sessions subsequent to the dosing session(s) involve the same therapists (usually two) listening to the patient’s narrative of their experience, which may include e.g., details of specific emotional insights.

A guiding principle of psychedelic psychotherapy is that the occurrence of a profound, potentially transformative psychological experience is critical to the treatment’s efficacy. Evidence has shown that high-dose psychedelic sessions can reliably produce profound psychological experiences rated among the most “meaningful” of a person’s life (Griffiths et al., 2006). A number of research teams have referred to these profound experiences and have applied relevant rating scales that have evolved out of studies of spontaneous and drug-induced “mystical,” “spiritual,” “peak” or “religious” experiences (Maslow, 1959; Stace, 1960; Pahnke and Richards, 1966; Maclean et al., 2012). Regardless of the terms chosen to define them, evidence suggests that profound psychological experiences can be predictive of subsequent psychological health, whether induced by psychedelics (O’Reilly and Funk, 1964; Klavetter and Mogar, 1967; Pahnke et al., 1970; Kurland et al., 1972; Richards et al., 1977; Maclean et al., 2011; Garcia-Romeu et al., 2014; Bogenschutz et al., 2015; Griffiths et al., 2016; Johnson et al., 2016; Ross et al., 2016), or other means (James, 1902; Maslow, 1959; Noyes Jr, 1980; Ludwig, 1985; Csikszentmihalyi and Csikszentmihalyi, 1992; Snell and Simmonds, 2015). Furthermore, some recent ketamine for depression studies have also found an association between the quality of acute experience (Sos et al., 2013; Luckenbaugh et al., 2014)—including the occurrence of mystical-type experiences (Dakwar et al., 2014)—subsequent positive clinical outcomes. Given the growing evidence favoring the therapeutic value of psychedelics (dos Santos et al., 2016; Rucker et al., 2016; Carhart-Harris and

Goodwin, 2017), it is timely that we better understand their therapeutic mechanisms.

The so-called “mystical” experience has been a classic problem area for mainstream psychology—if not science more generally. The term “mystical” is particularly problematic, as it suggests associations with the supernatural that may be obstructive or even antithetical to scientific method and progress (Carhart-Harris and Goodwin, 2017). It is important to note that by using the term the mystical-type experience, we are referring only to the phenomenology of the experience and are keen not to endorse any associations between it and supernatural or metaphysical ideas. Readers interested in the phenomenology of mystical-type/peak experiences may wish to explore these classic texts (James, 1902; Stace, 1960; Maslow, 1964; Pahnke and Richards, 1966; Csikszentmihalyi and Csikszentmihalyi, 1992; Hood Jr et al., 2009; Richards, 2015).

In the late 1960s, William Richards and Walter Pahnke (former pupils of Abraham Maslow and Timothy Leary respectively) developed a measure of “peak” or “mystical-type” experience that was much inspired by the work of Stace (1960). Studying reports of “mystical-type” experiences occurring in a variety of different world religions, Stace identified a number of common or “universal” components that are largely independent of religious or cultural context (Stace, 1960). Based on this landmark work, Richards and Pahnke developed the “mystical experience questionnaire” (MEQ) designed to enquire whether related components featured in the psychedelic drug experience. The scale measured six components of experience: (1) sense of unity or oneness, (2) transcendence of time and space, (3) deeply felt positive mood, (4) sense of awesomeness, reverence and wonder, (5) meaningfulness of psychological or philosophical insight, (6) ineffability and paradoxicality (Pahnke and Richards, 1966; Pahnke et al., 1970). A similar questionnaire which is based on Stace (1960) is the “M scale” (Hood Jr, 1975). Both the MEQ and M scale have been found to be predictive of long-term positive therapeutic outcomes in trials of psilocybin for cancer-related distress (Griffiths et al., 2016; Ross et al., 2016), tobacco smoking (Garcia-Romeu et al., 2014; Johnson et al., 2016) and alcohol dependence (Bogenschutz et al., 2015).

Perhaps the most widely used subjective measure of altered states of consciousness, and particularly the psychedelic state, is the altered states of consciousness questionnaire (ASC) (Dittrich, 1998). We chose this scale over the MEQ as it measures a broader range of subjective phenomena, not just the “mystical-type experience.” Crucially, this enabled us to test the *specificity* of the relationship between mystical-type experiences (vs. e.g., perceptual changes) and subsequent therapeutic outcomes. One of the principal ASC factors is named “oceanic boundlessness” (OBN)—a term that has its origins in a conversation between

Sigmund Freud and the French intellectual and “mystic” Romain Rolland (Freud, 1920) and makes reference to an “oceanic feeling” of boundlessness (Freud, 1929). Sharing a common intellectual background in Stace (1960) (Majić et al., 2015), items belonging to the OBN are closely related to those found in the MEQ. Previous factor analyses have parcellated the ASC into either 5 (Dittrich, 1998) or 11 dimensions (Studerus et al., 2010). As one of the original 5 ASC factors, OBN is explicitly linked to Stace’s “mystical experience”, (Studerus et al., 2010) and 4 of the 11 revised ASC factors also relate to OBN. Explicitly, the 4 OBN sub-factors are named “insightfulness,” “blissful state,” “experience of unity” and “spiritual experience” (Studerus et al., 2010).

We recently completed an open-label clinical trial assessing the feasibility of treating 20 patients with treatment-resistant depression (TRD) with psilocybin (Carhart-Harris et al., 2017). Results were encouraging: 47% of patients showed a clinically significant response 5 weeks post treatment ($\geq 50\%$ reduction in depressive symptoms). The present study sought to extend on our previous reports on this trial, by specifically focusing on whether the quality of the acute psychedelic experience was predictive of longer-term clinical outcomes. Specifically, we asked whether psilocybin-induced OBN and Dread of Ego Dissolution (DED) (related to acute anxiety) were predictive of decreases in depression at a key endpoint, whether the relationship between OBN and decreased depression was significantly stronger than between psilocybin’s more generic sensory perceptual effects and depression changes.

MATERIALS AND METHODS

This trial received a favorable opinion from the National Research Ethics Service London—West London, was sponsored and approved by Imperial College London’s Joint Research and Compliance Office (JRCO), and was adopted by the National Institute for Health Research Clinical Research Network. The National Institute for Health Research/Wellcome Trust Imperial Clinical Research Facility gave site-specific approval for the study. The study was reviewed and approved by the Medicines and Healthcare products Regulatory Agency (MHRA) and a Home Office Schedule One license was obtained for drug storage and administration. All participants provided written informed consent after receiving a complete description of the study.

Design

The full study procedure is reported in Carhart-Harris et al. (2016a). The inclusion criteria were major depression of a moderate to severe degree (16+ on the 21-item Hamilton Depression Rating scale [HAM-D]), and no improvement despite two adequate courses of antidepressant treatment. The patients were asked to be antidepressants-free for at least 2 weeks before the study. Twenty patients underwent two psilocybin-assisted therapy sessions, a week apart. The first involved a low-dose of psilocybin (10 mg, p.o.), and the second, a high-dose (25 mg, p.o.). Post capsule ingestion, patients lay with eyes closed and listened to music pre-selected by the research team (Kaelen et al., 2017) (<https://www.mixcloud.com/MendelKa/playlists/psilocybin-v13/>). Two therapists adopted a

non-directive, supportive approach, allowing the patient to experience a mostly uninterrupted introspection. Preparation session occurred 1 week before the 10 mg psilocybin dose and the integration session occurred 1-day and 1-week after the 25 mg psilocybin dose. Out of the initial 20 patients, 19 completed the study (6 females; mean age = 44.7 ± 10.9 ; 27 to 64). Eight more subjects were added to the study since publication of the initial 12 in Carhart-Harris et al. (2016a)—for a full clinical report of the 20 patients see Carhart-Harris et al. (2017).

Clinical Outcomes

Post-treatment ratings of relevant symptomatology were compared against those collected at baseline (before therapy). The main clinical outcome for this analysis was the self-rated 16-item Quick Inventory of Depressive Symptoms (QIDS-SR16 or just “QIDS-SR” for brevity). Five weeks after the 25 mg psilocybin session was chosen as the primary endpoint. The reason for this was that after 5 weeks, the next point of data collection was 3 months, and at this time-point some of the subjects had gone on to receive new treatments, thus confounding potential inferences. The response rate ($\geq 50\%$ reduction in QIDS-SR scores) at the 5 week time point was 47% ($n = 9$). Secondary clinical outcomes were used to further examine the hypothesis that the mystical-type experience relates to positive clinical outcome. These secondary measures were QIDS-SR at 1-day, 1-week, 3-months, and 6-months; Beck Depression Inventory (BDI, original version) at 1-week, 3-months, and 6-months; Clinician rated Hamilton Depression Rating scale (HAM-D) at 1-week; Dysfunctional Attitudes Scale (DAS; measures trait pessimism) at 1-week and 3-months; Spielberger’s Trait Anxiety Inventory (STAI) at 1-day, 1-week, 3-months, and 6-months; Life Orientation Test Revisited (LOT-R; measures optimism) at 1-week and 3-months; and Snaith-Hamilton Pleasure Scale (SHAPS; measures anhedonia) at 1-week and 3-months. Standard criteria for meaningful “response” were calculated for the depression rating scales ($\geq 50\%$ from baseline).

Measures of Acute Psilocybin Session

The altered state of consciousness questionnaire (ASC) (Dittrich, 1998) was used to measure the acute subjective experience. It was completed retrospectively by the patient as the psilocybin session was coming to an end (i.e., $\sim 5\text{--}6$ h post ingestion). As stated above, the ASC can be divided into 5 (Dittrich, 1998) (94 items), or 11 dimensions (Studerus et al., 2010) (42 items). The 5 dimensions are: OBN, DED, *visionary restructuring* (VRS), *auditory alterations* (AUA), and *vigilance reduction* (VIR) (n.b. translation from the German original may explain the slightly peculiar choice of terms e.g., “visionary restructuring”). As noted above, the OBN items were formulated based on six of the nine categories of “mystical experiences” proposed by Stace (1960) (Bodmer et al., 1994; Studerus et al., 2010) in a similar way to the MEQ (Pahnke and Richards, 1966; Maclean et al., 2012). *Dread of ego-dissolution* is considered to probe negative, aversive experiences in which anxiety is a central aspect. *Visionary restructuring* measures altered perception and meaning including visual hallucinations and synesthesia. The 11 sub-dimensions are made only from OBN, DED and VRS. The OBN sub-dimensions are *experience of unity*, *spiritual experience*,

blissful state, insightfulness, and disembodiment. The DED sub-dimensions are *impaired control or cognition*, and *Anxiety*. The VRS sub-dimensions are *complex imagery*, *elementary imagery*, *audio/visual synesthesia*, and *changed meaning of percepts*.

We hypothesized that OBN and DED would predict clinical outcome up to 5 weeks. To test this hypothesis, we used repeated measure ANOVA. (analysis was done in SPSS v24, GLM with repeated measures). Time was the within-subject factor (independent variable), with QIDS-SR as the within-subject dependent variable in baseline, 1-day, 1-week, 5-weeks. OBN and DED were independent variables (covariates in SPSS). OBN-by-Time and DED-by-Time interactions were the primary outcomes of interest. The contrast for the within-subject factor was simple, comparing each level to the 1st one (baseline). Furthermore, we hypothesized specificity in the relationship between OBN and depression changes by comparing the strength of this correlation with that between the perceptual factors from the ASC, namely VRS and AUA, and depression changes (Steiger, 1980; Lee and Preacher, 2013). A threshold of OBN > 0.6 was used to distinguish a “complete” OBN. This threshold is similar to MEQ > 0.6 which was used in other studies to identify “peakers” and “complete mystical-type experience” (Pahnke et al., 1970; Richards et al., 1977; Maclean et al., 2011; Garcia-Romeu et al., 2014; Johnson et al., 2016). In a different study, OBN and MEQ showed a Pearson correlation of 0.93 (Liechti et al., 2017), suggesting that these two questionnaire quantify a similar experience and that a similar threshold can be used. For descriptive purposes, we tested whether those patients who had a “complete” OBN had a better clinical outcome. This analysis was done to expand the initial hypothesis to other time points and questionnaires.

We also issued participants an in-house measure, the 29-item “psychedelic questionnaire” (PQ)—which was completed at the same time as the ASC. The PQ has been previously used in a number of our pharmacological challenge studies due to its brevity relative to the full ASC (Carhart-Harris et al., 2012, 2016b). As a descriptive exploratory analysis, correlation between PQ and clinical outcome at 5 weeks was calculated for all items. The same exploratory analysis was also done on all of the 94 items of the ASC.

RESULTS

Prediction of QIDS-SR up to 5 Weeks

These following are primary results of this study. Table 1 presents the results of the repeated measures ANOVA with Time as the within-subject factor (independent variable), QIDS-SR as the within-subject dependent variable in baseline, 1-day, 1-week, 5-weeks. OBN and DED were independent variables. [Sphericity assumed: Mauchly's $W = 0.71$ ($p = 0.411$)]. For the interactions of Time X OBN, and Time X DED, the within-subjects effect and the within-subjects contrasts at each time point compared to baseline were all significant ($p < 0.05$), confirming our main hypothesis. Regression analysis with Δ QIDS-SR (5-weeks) as a dependent variable and OBN and DED as independent variables found that together they explain 54% of the variance ($r^2 = 0.59$, adjusted $r^2 = 0.54$; standardized beta values of OBN, DED, were 0.605, -0.649, respectively). For descriptive

TABLE 1 | Repeated measures ANOVA; OBN and DED predict changes in QIDS-SR over different time points up to 5 weeks.

Within-subjects effects		F _(3, 48)	p	Partial η ²
Time * OBN	Sphericity Assumed	5.563	0.002	0.258
Time * DED	Sphericity Assumed	15.39	0.003	0.252
Within-subjects contrasts		F _(1, 16)	p	Partial η ²
Time * OBN	1 Day vs. Baseline 1 Week vs. Baseline 5 Weeks vs. Baseline	13.143 7.237 13.29	0.002 0.016 0.002	0.451 0.311 0.454
Time * DED	1 Day vs. Baseline 1 Week vs. Baseline 5 Weeks vs. Baseline	9.941 6.828 15.298	0.006 0.019 0.001	0.383 0.299 0.489

Time is the within-subject factor (independent variable), QIDS-SR is the within-subject dependent variable in baseline, 1-day, 1-week, 5-weeks. OBN and DED are independent variables. Time-by-OBN and Time-by-DED interactions are the primary outcomes of interest (within-subjects effects). Within-subjects simple contrasts are calculated for each time point compared to baseline. A significant within-subject contrast suggests a linear relationship between OBN or DED and the difference between time points in QIDS-SR. Sphericity assumed: Mauchly's $W = 0.71$ ($p = 0.411$). partial $\eta^2 > 0.25$ = large effect size. To inform directionality see Figure 1 for Pearson's correlations: high OBN and low DED predict reductions in QIDS-SR.

purposes, Figure 1 presents plots of Pearson's correlation of the 5 dimensions of the ASC predicting Δ QIDS-SR (5 weeks). Furthermore, based on a standard threshold for defining clinical response ($\geq 50\%$ reduction in QIDS-SR score at 5 weeks vs. baseline), a comparison of responders ($n = 9$) vs. non-responders ($n = 10$) in the 11D ASC scores is presented for descriptive purposes in Figure 2.

As hypothesized, OBN was a significantly better predictor of reductions in depression than both VRS and AUA ($z = 1.64$ and $z = 2.01$, respectively, $p < 0.05$) (Steiger, 1980; Lee and Preacher, 2013).

Prediction of Secondary Clinical Measures

The following are the secondary results of this study. Clinical outcomes for “complete” OBN were compared with those for “non-complete” OBN for secondary clinical outcomes such as measures of trait anxiety, anhedonia, optimism and pessimism (Table 2). Patients that had “complete” OBN ($n = 11$, OBN = 0.83 ± 0.1) had better outcomes than those who did not ($n = 8$, OBN = 0.33 ± 0.16), on a number of different measures and at different time points (1-day, 1-week, 5-weeks, 3-months, and 6-months). Response rates of “complete” OBN are presented in Table 2.

In further exploratory analyses, correlations were calculated between all 94 items of the ASC and Δ QIDS-SR and were ordered by the strength of correlation (Table S1). The same was done for all 29 items of the PQ (Table S2). In both examples, it is apparent that items that best relate to OBN correlate most strongly with positive clinical outcomes, while sensory phenomena correlate less, and anxiety is predictive of worse outcomes.

DISCUSSION

Consistent with our prior hypothesis, psilocybin-induced high OBN (sharing features with mystical-type experience) and low

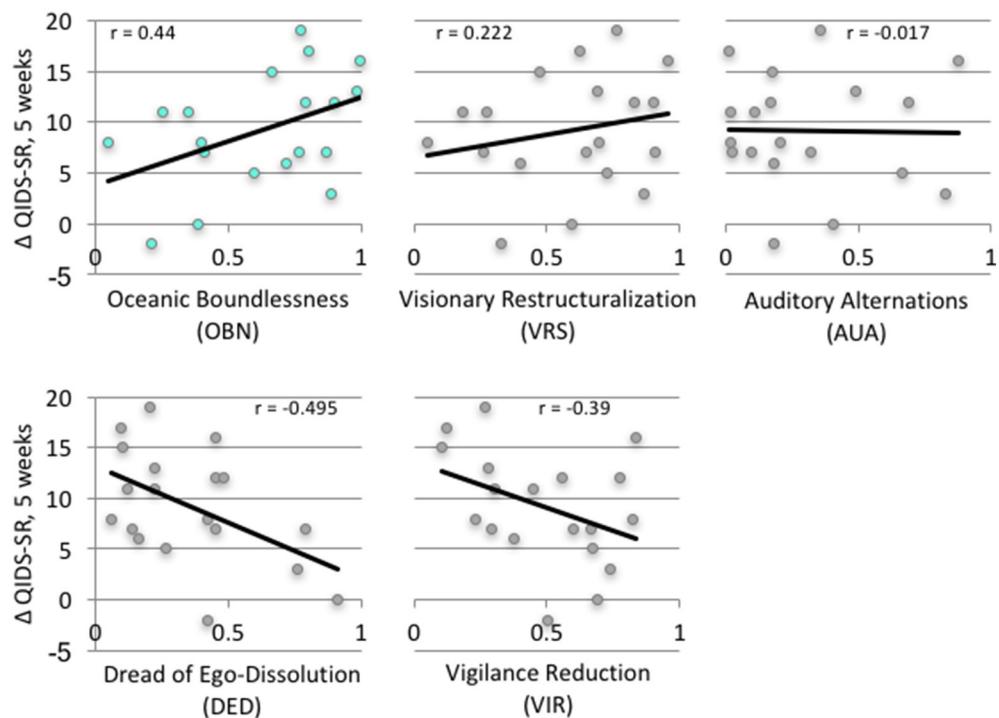


FIGURE 1 | Correlation of ASC (5 dimensions) with change of clinical outcome at 5 weeks (Δ QIDS-SR).

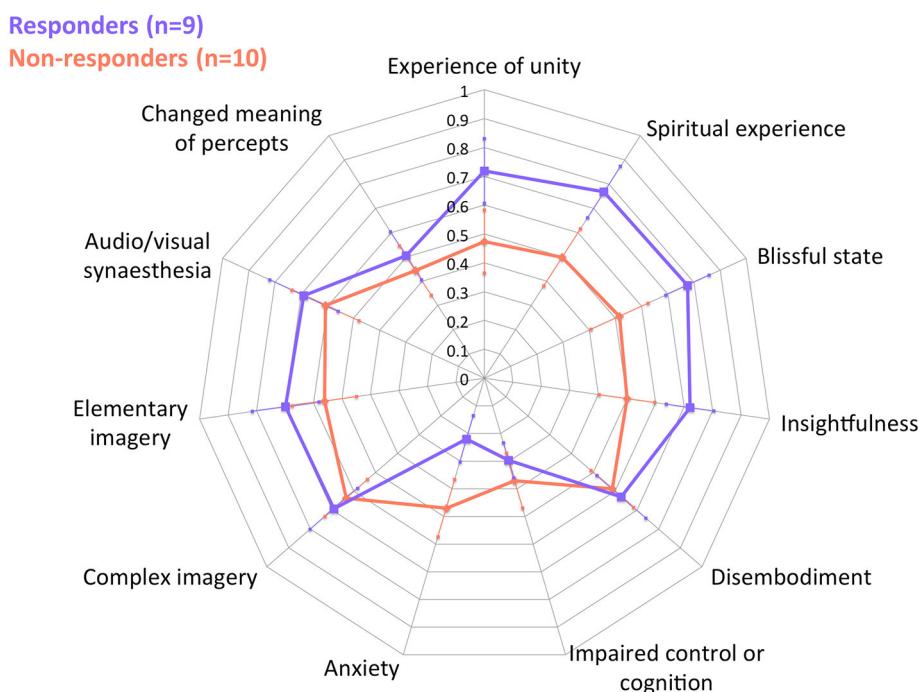


FIGURE 2 | ASC (11 dimensions) of responders and non-responders at 5 weeks. Error Bars = Standard Error.

TABLE 2 | Comparisons of “complete” OBN ($n = 11$) and “non-complete” ($n = 8$) with different clinical measures in different time points.

		Complete vs. Non		Mean Δ		Response rate	
		Cohen's d		Complete ($n = 11$)	Non ($n = 8$)	Complete ($n = 11$)	Non ($n = 8$)
		Complete	Non				
1 Day	Δ QIDS-SR	1.39	13.18 ± 4.4	8.25 ± 5.6	81.8	50	
	Δ STAI	0.94	25.18 ± 12.9	15 ± 17.4			
1 Week	Δ QIDS-SR	1.18	12 ± 4.6	7.75 ± 5.6	81.8	37.5	
	Δ BDI	0.61	24.45 ± 7.3	19.25 ± 15.5	81.8	37.5	
	Δ HAM-D	1.40	17.81 ± 5	10.62 ± 8.9	90.9	25	
	Δ STAI	0.85	27.72 ± 12.3	18.5 ± 18			
	Δ DAS	2.10	44.54 ± 22.9	12.87 ± 19.7			
	Δ SHAPS	-0.07	4.54 ± 3.7	4.75 ± 4.9			
	Δ LOT-R	1.40	7.63 ± 5.2	1.75 ± 6.6			
5 Weeks	Δ QIDS-SR	1.58	11.54 ± 5.1	6 ± 4.8	63.6	25	
3 Months	Δ QIDS-SR	1.17	9.45 ± 5.5	4.12 ± 7.3	54.5	12.5	
	Δ BDI	1.38	20.45 ± 12	8.12 ± 13.3	72.7	12.5	
	Δ STAI	0.79	15.18 ± 11.1	8 ± 14.3			
	Δ DAS	2.22	39.72 ± 24	-1.25 ± 28.1			
	Δ SHAPS	0.81	4.45 ± 3	1.75 ± 6			
	Δ LOT-R	1.13	4.36 ± 3.9	0.75 ± 5			
6 Months	Δ QIDS-SR	0.96	9.18 ± 6.4	4.37 ± 7.7	45.5	25	
	Δ BDI	1.25	19.54 ± 9.7	8.62 ± 14.6	63.6	25	
	Δ STAI	0.15	16.36 ± 12.5	14.87 ± 16.1			

The difference between “complete” OBN and non-complete in effect size (cohen's $d > 0.8$ = large effect); the mean change of each group; and the response rates (%) of each group for depression questionnaires only. BDI, Beck Depression Inventory; DAS, Dysfunctional Attitudes Scale (measures trait pessimism); HAM-D, Clinician rated Hamilton Depression Rating scale; LOT-R, Life Orientation Test Revisited (measures optimism); QIDS-SR, Self-Reported Quick Inventory of Depressive Symptoms; SHAPS, Snaith-Hamilton Pleasure Scale; STAI, Spielberger's Trait Anxiety Inventory.

DED (similar to anxiety) predicted positive long-term clinical outcomes in a clinical trial of psilocybin for TRD. This result replicates those of previous studies showing that psychedelic-induced peak or mystical-type experiences are predictive of positive long-term outcomes (O'Reilly and Funk, 1964; Klavetter and Mogar, 1967; Pahnke et al., 1970; Kurland et al., 1972; Richards et al., 1977; Maclean et al., 2011; Bogenschutz et al., 2015; Griffiths et al., 2016; Johnson et al., 2016; Ross et al., 2016). This relationship appears to be somewhat specific, in that OBN was significantly more predictive of positive clinical outcomes than altered visual and auditory perception—endorsing the moniker “psychedelic” (“mind-revealing”) over “hallucinogen” when referring to this class of drug—at least in the context of psychedelic therapy. It also suggests that the therapeutic effects of psilocybin are not a simple product of isolated pharmacological action but rather are *experience dependent*. We also found that greater DED (anxiety and impaired cognition) experienced during the drug session was predictive of less positive clinical outcomes.

One may naturally infer from these findings that the occurrence of OBN or mystical-type experience mediates long-term positive clinical outcomes (Griffiths et al., 2016; Ross et al., 2016) and while this assumption may be valid, we must exercise caution about ascribing too much to this relationship. It remains

possible that as yet unmeasured and therefore unaccounted for components of psychedelic therapy play important roles in mediating long-term outcomes. There are several candidate factors in this regard, and the following should not be considered an exhaustive list: *emotional insight/breakthrough or catharsis; priming and suggestibility; reliving of trauma/defining life events; insights about the self and relationships; the patients relationship to music heard; his/her success at “letting go”; the quality of therapeutic relationship; and the degree of “closure” attained during post-drug integration work* (Frederking, 1955; Sandison, 1955; Abramson, 1956; Martin, 1957; Eisner and Cohen, 1958; Leuner, 1961; Jensen, 1963; Shagass and Bittle, 1967; Richards, 1978; Loizaga-Velder, 2013; Gasser et al., 2014; Belser et al., 2017; Russ and Elliott, 2017; Watts et al., 2017).

These factors may exert influence before, during and after the acute experience itself and may also be more or less dependent on particular psychological frameworks and their relevant vocabularies. For example, the psychoanalytic models of Freud and Jung were dominant in psychiatry in the mid-twentieth century and thus references to *ego, repression and the unconscious* are commonplace among the psychedelic research literature of this period. While the processes that underlie these constructs may indeed be operative in the context of psychedelics, little effort has been made to define, measure and quantify their

contributions (Shagass and Bittle, 1967; Barr et al., 1972). The development of subjective (Nour et al., 2016), behavioral and biological measures (Carhart-Harris et al., 2012, 2016b; Lebedev et al., 2015; Tagliazucchi et al., 2016) relevant to these constructs, and more importantly, the processes that underlie them, would represent an important advance not just for psychedelic science but for the psychological frameworks themselves (Carhart-Harris et al., 2014). We should be conscious of not being too attached (or averse) to any specific theoretical frameworks however, and approaches that endeavor to access “framework-free” descriptions of phenomena may prove particularly useful in this regard (Varela, 1996; Petitmengin, 2006). Critically, it is our view that it is possible to work toward a secular, biologically-informed account of the mystical-type experience that does not resort to “explaining away” or “reducing down” the core phenomenology and depth psychology may be a useful bedfellow in this regard.

Returning to the present study’s main findings, DED was found to negatively correlate with clinical outcome, yet, none of the patients showed a worsening of clinical symptoms at 5 weeks. Less DED combined with high OBN predicted 54% of the variance of clinical change at 5 weeks—a substantial contribution and one that helps justify the emphasis placed on minimizing anxiety and relinquishing psychological resistance in psychedelic therapy (Eisner and Cohen, 1958; Sherwood et al., 1962; Grof et al., 2008; Richards, 2015), as well as paying careful attention to preparation and “set and setting” (Hartogsohn, 2017; Carhart-Harris et al., in press). That anxiety arises in parallel with psychological struggle is resonant with principles of psychoanalytic theory (Sandison, 1961), as can be seen in the choice of terms for the “DED” and “OBN” factors of the ASC—both of which invoke constructs that can be traced to Freud (1929, 1962).

According to psychoanalytic theory, the overcoming of psychological resistance is required for emotional breakthrough and insight (Freud, 1920) and the occurrence of mystical-type/peak experiences (Jung, 2014). Consistently, writers on the mystical-type/peak experience have reliably identified loss of self or “ego-dissolution” as one of its basic pre-requisites and features (James, 1902; Stace, 1960; Maslow, 1964). Recent work has sought to develop and validate a measure that is sensitive to difficult or challenging psychedelic experiences (Barrett et al., 2016; Carbonaro et al., 2016) and there is some evidence that the intensity of such experiences is predictive of positive long-term outcomes, whereas the duration of struggle is predictive of negative outcomes (Carbonaro et al., 2016). This is presumably because the successful resolution of conflict brings with it, insight and relief, whereas the failure to breakthrough perpetuates suffering. ASC and other questionnaires such as the challenging experience questionnaire (CEQ) (Barrett et al., 2016) may be insensitive to whether or not successful resolution of psychological conflict has occurred. Therefore, the development of new scales specifically designed to focus on *emotional breakthrough* after struggle may add considerable value.

Improving our subjective measures of high-level human experiences such as the mystical-type/peak experience will

enhance our ability to understand their psychology and underlying neural substrates. As touched on in the introduction, psychopharmacology is increasingly acknowledging the importance of “context” and particularly “environment” as a factor mediating the effects of both intrinsic neurobiological features (e.g., genotypes) and exogenous pharmacological inputs—such as drugs (Alexander et al., 1981; Caspi et al., 2010). For example, a recent popular model of the action of SSRIs incorporates “environment” and cognitive (re)appraisal (Harmer et al., 2017) as key determinants of therapeutic efficacy (see also Branchi, 2011; Belsky, 2016). Like SSRIs, classic psychedelic drugs also work on the serotonin system; however, unlike the SSRIs, they are direct agonists at the 5-HT2A receptor (Nichols, 2016). There is compelling evidence that the 5-HT2A receptor is psychedelics’ key site of action (Nichols, 2016). Intriguingly, recent work has found that the phenotypic expression of 5-HT2AR genotypes is significantly dependent on the influence of “environment” (Jokela et al., 2007). These findings may imply that enhanced sensitivity to context is an important function of 5-HT2A receptor signaling (Carhart-Harris and Nutt, 2017).

Ascending from the pharmacological to the whole-brain systems level, increased cortical entropy has been found to be a reliable feature of the psychedelic state (Carhart-Harris et al., 2014), to relate to high-level subjective experiences such as “ego-dissolution” (Nour et al., 2016; Atasoy et al., 2017; Schartner et al., 2017) that are relevant to the mystical-type experience, and to be predictive of longer-term trait changes—such as increased “openness” (Lebedev et al., 2016). Recent work suggests that increased brain entropy under psychedelics is consistent with the brain being more closely tuned to “criticality” (Atasoy et al., 2017). Criticality refers to systems that reside in a functional “sweet spot”, critically poised between order and disorder—in which they can effectively retain information (by being sufficiently ordered) while being appropriately adaptive and sensitive to change (by being sufficiently disordered). Intriguingly, one of the signatures of a critical system is a sensitivity to perturbation (Bak, 1996). It follows that enhanced sensitivity to perturbation in a psychedelically-induced “entropic” and “critical” brain may account for the special sensitivity to “environment” that is characteristic of the psychedelic state (Hartogsohn, 2016; Carhart-Harris et al., in press).

Understanding the neurobiological mechanisms of OBN, mystical-type or peak experiences (Vollenweider, 2001) should enable us to better comprehend, define and study them. This is important, not least because they are proving to be important determinants of treatment success in psychedelic therapy (Richards et al., 1977; Bogenschutz et al., 2015; Griffiths et al., 2016; Johnson et al., 2016; Ross et al., 2016). Crucially, better understanding the biological basis of mystical-type/peak experiences and their longer-term impact on the mind and brain should help to demystify them, facilitating an easier conversation about them with mainstream psychology. Researchers in the mainstream have as much a responsibility as those in “the periphery” to facilitate this. Denying the relevance of these phenomena is as damaging

to scientific progress as denying their physical basis. The prize for successfully integrating mystical-type experience into mainstream science may be their potential to have a substantial positive impact on medicine, education and society—which ironically, may, at least in part, explain why their integration into western society has proved so difficult to achieve (Stevens, 1987).

To summarize, the occurrence of high OBN (sharing features with mystical-type experience) and low DED (relating to anxiety and impaired cognition) under psilocybin predicted positive clinical outcomes in a trial of psilocybin for TRD. This relationship exhibited a degree of specificity, in that psilocybin-induced OBN was significantly more predictive of reduced depressive symptoms than the drug's more generic visual and auditory perceptual effects. Future work, with a larger sample size, is required to more comprehensively and systematically measure the influence of different potential predictive factors on the quality of acute psychedelic experiences (Gasser et al., 2014; Belser et al., 2017; Watts et al., 2017) and subsequent long-term outcomes (Carhart-Harris et al., in press). As psychedelic therapy gains influence and credibility (Carhart-Harris and Goodwin, 2017), it seems vital that appropriate consideration is paid to the importance of promoting a certain kind of experience, as the quality of that experience may be the critical determinant of therapeutic success.

REFERENCES

- Abramson, H. A. (1956). Lysergic acid diethylamide (LSD-25): XXII. Effect on transference. *J. Psychol.* 42, 51–98. doi: 10.1080/00223980.1956.9713025
- Alexander, B. K., Beyerstein, B. L., Hadaway, P. F., and Coombs, R. B. (1981). Effect of early and later colony housing on oral ingestion of morphine in rats. *Pharmacol. Biochem. Behav.* 15, 571–576. doi: 10.1016/0091-3057(81)90211-2
- Atasoy, S., Roseman, L., Kaelen, M., Kringselbach, M. L., Deco, G., and Carhart-Harris, R. L. (2017). Connectome-harmonic decomposition of human brain activity reveals dynamical repertoire re-organization under LSD. *Sci. Rep.* 7:17661. doi: 10.1038/s41598-017-17546-0
- Bak, P. (1996). "Complexity and criticality," in *How Nature Works* (New York, NY: Copernicus), 1–32. doi: 10.1007/978-1-4757-5426-1
- Barr, H. L., Langs, R., Holt, R. R., Goldberger, L., and Klein, G. S. (1972). *LSD: Personality and Experience*. New York, NY: Wiley-Interscience.
- Barrett, F. S., Bradstreet, M. P., Leoutsakos, J.-M. S., Johnson, M. W., and Griffiths, R. R. (2016). The challenging experience questionnaire: characterization of challenging experiences with psilocybin mushrooms. *J. Psychopharmacol.* 30, 1279–1295. doi: 10.1177/0269881116678781
- Belser, A. B., Agin-Liebes, G., Swift, T. C., Terrana, S., Devenot, N., Friedman, H. L., et al. (2017). Patient experiences of psilocybin-assisted psychotherapy: an interpretative phenomenological analysis. *J. Humanist. Psychol.* 57, 354–388. doi: 10.1177/0022167817706884
- Belsky, J. (2016). The differential susceptibility hypothesis: sensitivity to the environment for better and for worse. *JAMA Pediatr.* 170, 321–322. doi: 10.1001/jamapediatrics.2015.4263
- Bodmer, I., Dittrich, A., and Lamparter, D. (1994). Außergewöhnliche Bewußtseinszustände—ihre gemeinsame Struktur und Messung. *Welten Bewußtseins* 3, 45–58.
- Bogenschutz, M. P., Forcehimes, A. A., Pommy, J. A., Wilcox, C. E., Barbosa, P., and Strassman, R. J. (2015). Psilocybin-assisted treatment for alcohol dependence: a proof-of-concept study. *J. Psychopharmacol.* 29, 289–299. doi: 10.1177/0269881114565144
- Branchi, I. (2011). The double edged sword of neural plasticity: increasing serotonin levels leads to both greater vulnerability to depression and improved capacity to recover. *Psychoneuroendocrinology* 36, 339–351. doi: 10.1016/j.psyneuen.2010.08.011
- Carbonaro, T. M., Bradstreet, M. P., Barrett, F. S., Maclean, K. A., Jesse, R., Johnson, M. W., et al. (2016). Survey study of challenging experiences after ingesting psilocybin mushrooms: acute and enduring positive and negative consequences. *J. Psychopharmacol.* 30, 1268–1278. doi: 10.1177/0269881116662634
- Carhart-Harris, R., Bolstridge, M., Day, C., Rucker, J., Watts, R., Erritzoe, D., et al. (2017). Psilocybin with psychological support for treatment-resistant depression: six-month follow-up. *Psychopharmacology* doi: 10.1007/s00213-017-4771-x. [Epub ahead of print].
- Carhart-Harris, R. L., Bolstridge, M., Rucker, J., Day, C. M., Erritzoe, D., Kaelen, M., et al. (2016a). Psilocybin with psychological support for treatment-resistant depression: an open-label feasibility study. *Lancet Psychiatry* 3, 619–662. doi: 10.1016/S2215-0366(16)30065-7
- Carhart-Harris, R. L., Erritzoe, D., Williams, T., Stone, J. M., Reed, L. J., Colasanti, A., et al. (2012). Neural correlates of the psychedelic state as determined by fMRI studies with psilocybin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2138–2143. doi: 10.1073/pnas.1111959109
- Carhart-Harris, R. L., and Goodwin, G. M. (2017). The therapeutic potential of psychedelic drugs: past, present and future. *Neuropsychopharmacology* 42, 2105–2113. doi: 10.1038/npp.2017.84
- Carhart-Harris, R. L., Leech, R., Hellyer, P. J., Shanahan, M., Feilding, A., Tagliazucchi, E., et al. (2014). The entropic brain: a theory of conscious states informed by neuroimaging research with psychedelic drugs. *Front. Hum. Neurosci.* 8:20. doi: 10.3389/fnhum.2014.00020
- Carhart-Harris, R. L., and Nutt, D. J. (2017). Serotonin and brain function: a tale of two receptors. *J. Psychopharmacol.* 31, 1091–1120. doi: 10.1177/0269881117725915
- Carhart-Harris, R., Muthukumaraswamy, S., Roseman, L., Kaelen, M., Droog, W., Murphy, K., et al. (2016b). Neural correlates of the LSD experience revealed by multimodal neuroimaging. *Proc. Natl. Acad. Sci. U.S.A.* 113, 4853–4858. doi: 10.1073/pnas.1518377113
- Carhart-Harris, R., Roseman, L., Haijen, E., Erritzoe, D., Watts, R., Branchi, I., et al. (in press). Psychedelics and the essential importance of context. *J. Psychopharmacol.*

AUTHOR CONTRIBUTIONS

LR analyzed the data and wrote the paper; DN sanctioned the research and approved an earlier draft of the manuscript; RC-H designed and conducted the research, and wrote the paper.

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SUPPLEMENTARY MATERIAL

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- Caspi, A., Hariri, A. R., Holmes, A., Uher, R., and Moffitt, T. E. (2010). Genetic sensitivity to the environment: the case of the serotonin transporter gene and its implications for studying complex diseases and traits. *Focus* 8, 398–416. doi: 10.1176/foc.8.3.foc398
- Csikszentmihalyi, M., and Csikszentmihalyi, I. S. (1992). *Optimal Experience: Psychological Studies of Flow in Consciousness*. Cambridge, UK: Cambridge University Press.
- Dakwar, E., Anerella, C., Hart, C., Levin, F., Mathew, S., and Nunes, E. (2014). Therapeutic infusions of ketamine: do the psychoactive effects matter? *Drug Alcohol Depend.* 136, 153–157. doi: 10.1016/j.drugalcdep.2013.12.019
- Dittrich, A. (1998). The standardized psychometric assessment of altered states of consciousness (ASCs) in humans. *Pharmacopsychiatry* 31, 80–84. doi: 10.1055/s-2007-979351
- dos Santos, R. G., Osório, F. L., Crippa, J. A. S., Riba, J., Zuardi, A. W., and Hallak, J. E. (2016). Antidepressive, anxiolytic, and antiaddictive effects of ayahuasca, psilocybin and lysergic acid diethylamide (LSD): a systematic review of clinical trials published in the last 25 years. *Therapeut. Adv. Psychopharmacol.* 6, 193–213. doi: 10.1177/2045125316638008
- Dyck, E. (2006). Hitting highs at rock bottom: LSD treatment for alcoholism, 1950–1970. *Soc. Hist. Med.* 19, 313–329. doi: 10.1093/shm/hkl039
- Eisner, B. G., and Cohen, S. (1958). Psychotherapy with lysergic acid diethylamide. *J. Nerv. Ment. Dis.* 127, 528–539. doi: 10.1097/00005053-195812000-00006
- Frederking, W. (1955). Intoxicant drugs (mescaline and lysergic acid diethylamide) in psychotherapy. *J. Nerv. Mental Dis.* 121, 262–266. doi: 10.1097/00005053-195503000-00010
- Freud, S. (ed.). (1920). “Resistance and suppression,” in *A General Introduction to Psychoanalysis* (New York, NY: Boni & Liveright).
- Freud, S. (1929). *Civilization and its Discontents*. Peterborough, ON: Broadview Press.
- Freud, S. (1962). *The Ego and the Id*. New York, NY: WW Norton and Company.
- Garcia-Romeu, A. P., Johnson, M. W., and Griffiths, R. R. (2014). Examining the psychological mechanisms of psilocybin-assisted smoking cessation treatment: a pilot study. *Drug Alcohol Depend.* 140:e66. doi: 10.1016/j.drugalcdep.2014.02.200
- Gasser, P., Kirchner, K., and Passie, T. (2014). LSD-assisted psychotherapy for anxiety associated with a life-threatening disease: a qualitative study of acute and sustained subjective effects. *J. Psychopharmacol.* 29, 57–68. doi: 10.1177/0269881114555249
- Griffiths, R. R., Johnson, M. W., Carducci, M. A., Umbricht, A., Richards, W. A., Richards, B. D., et al. (2016). Psilocybin produces substantial and sustained decreases in depression and anxiety in patients with life-threatening cancer: a randomized double-blind trial. *J. Psychopharmacol.* 30, 1181–1197. doi: 10.1177/0269881116675513
- Griffiths, R. R., Richards, W. A., Mccann, U., and Jesse, R. (2006). Psilocybin can occasion mystical-type experiences having substantial and sustained personal meaning and spiritual significance. *Psychopharmacology* 187, 268–283. doi: 10.1007/s00213-006-0457-5
- Grof, S., Hofmann, A., and Weil, A. (2008). *LSD Psychotherapy (The Healing Potential of Psychedelic Medicine)*. Ben Lomond, CA: Multidisciplinary Association for Psychedelic Studies.
- Harmer, C. J., Duman, R. S., and Cowen, P. J. (2017). How do antidepressants work? New perspectives for refining future treatment approaches. *Lancet Psychiatry* 4, 409–418. doi: 10.1016/S2215-0366(17)30015-9
- Hartogsohn, I. (2016). Set and setting, psychedelics and the placebo response: an extra-pharmacological perspective on psychopharmacology. *J. Psychopharmacol.* 30, 1259–1267. doi: 10.1177/0269881116677852
- Hartogsohn, I. (2017). Constructing drug effects: a history of set and setting. *Drug Sci. Policy Law* 3, 1–17. doi: 10.1177/2050324516683325
- Hood Jr, R. W. (1975). The construction and preliminary validation of a measure of reported mystical experience. *J. Sci. Study Religion* 14, 29–41. doi: 10.2307/1384454
- Hood Jr, R. W., Hill, P. C., and Spilka, B. (2009). *The Psychology of Religion: An Empirical Approach*. New York, NY: Guilford Press.
- James, W. (1902). *The Varieties of Religious Experience*. Cambridge, MA: Harvard University Press.
- Jensen, S. (1963). Treatment of chronic alcoholism with lysergic acid diethylamide. *Can. Psychiatr. Assoc. J.* 8, 182–188. doi: 10.1177/070674376300800305
- Johnson, M. W., Garcia-Romeu, A., and Griffiths, R. R. (2016). Long-term follow-up of psilocybin-facilitated smoking cessation. *Am. J. Drug Alcohol Abuse* 43, 55–60. doi: 10.3109/00952990.2016
- Jokela, M., Keltikangas-Järvinen, L., Kivimäki, M., Puttonen, S., Elovaainio, M., Rontu, R., et al. (2007). Serotonin receptor 2A gene and the influence of childhood maternal nurturance on adulthood depressive symptoms. *Arch. Gen. Psychiatry* 64, 356–360. doi: 10.1001/archpsyc.64.3.356
- Jung, C. G. (2014). *On the Nature of the Psyche*. Oxford, UK: Routledge.
- Kaelen, M., Giribaldi, B., Raine, J., Evans, L., Timmerman-Slater, C., Rodriguez, N., et al. (2017). *The Hidden Therapist: Evidence for a Central Role of Music in Psychedelic Therapy*. Open Science Framework. Available online at: <https://osf.io/xkvgd/>
- Klavetter, R. E., and Mogar, R. E. (1967). Peak experiences: investigation of their relationship to psychedelic therapy and self-actualization. *J. Humanist. Psychol.* 7, 171–177. doi: 10.1177/002216786700700206
- Kurland, A. A., Grof, S., Pahnke, W. N., and Goodman, L. E. (1972). Psychedelic drug assisted psychotherapy in patients with terminal cancer. *J. Thanatol.* 2, 644–691.
- Lebedev, A. V., Kaelen, M., Lovden, M., Nilsson, J., Feilding, A., Nutt, D. J., et al. (2016). LSD-induced entropic brain activity predicts subsequent personality change. *Hum. Brain Mapp.* 37, 3203–3213. doi: 10.1002/hbm.23234
- Lebedev, A. V., Lovden, M., Rosenthal, G., Feilding, A., Nutt, D. J., and Carhart-Harris, R. L. (2015). Finding the self by losing the self: neural correlates of ego-dissolution under psilocybin. *Hum. Brain Mapp.* 36, 3137–3153. doi: 10.1002/hbm.22833
- Lee, I. A., and Preacher, K. J. (2013). *Calculation for the Test of the Difference Between Two Dependent Correlations with One Variable in Common [Computer Software]*. Available online at: <http://quantpsy.org>
- Leuner, H. (1961). Psychotherapy with hallucinogens. *Hallucinogenic Drugs* 67, 67–73.
- Liechti, M. E., Dolder, P. C., and Schmid, Y. (2017). Alterations of consciousness and mystical-type experiences after acute LSD in humans. *Psychopharmacology* 234, 1499–1510. doi: 10.1007/s00213-016-4453-0
- Loizaga-Velder, A. (2013). A psychotherapeutic view on therapeutic effects of ritual ayahuasca use in the treatment of addiction. *MAPS Bull.* 23, 36–40.
- Luckenbaugh, D. A., Niciu, M. J., Ionescu, D. F., Nolan, N. M., Richards, E. M., Brutsche, N. E., et al. (2014). Do the dissociative side effects of ketamine mediate its antidepressant effects? *J. Affect. Disord.* 159, 56–61. doi: 10.1016/j.jad.2014.02.017
- Ludwig, A. M. (1985). Cognitive processes associated with “spontaneous” recovery from alcoholism. *J. Stud. Alcohol.* 46, 53–58. doi: 10.15288/jsa.1985.46.53
- Maclean, K. A., Johnson, M. W., and Griffiths, R. R. (2011). Mystical experiences occasioned by the hallucinogen psilocybin lead to increases in the personality domain of openness. *J. Psychopharmacol.* 25, 1453–1461. doi: 10.1177/0269881111420188
- Maclean, K. A., Leoutsakos, J. M. S., Johnson, M. W., and Griffiths, R. R. (2012). Factor analysis of the mystical experience questionnaire: a study of experiences occasioned by the hallucinogen psilocybin. *J. Sci. Study Relig.* 51, 721–737. doi: 10.1111/j.1468-5906.2012.01685.x
- Majić, T., Schmidt, T. T., and Gallinat, J. (2015). Peak experiences and the afterglow phenomenon: when and how do therapeutic effects of hallucinogens depend on psychedelic experiences? *J. Psychopharmacol.* 29, 241–253. doi: 10.1177/0269881114568040
- Martin, A. J. (1957). LSD (lysergic acid diethylamide) treatment of chronic psychoneurotic patients under day-hospital conditions. *Int. J. Soc. Psychiatry* 3, 188–195. doi: 10.1177/002076405700300304
- Maslow, A. H. (1959). Cognition of being in the peak experiences. *J. Genet. Psychol.* 94, 43–66. doi: 10.1080/00221325.1959.10532434
- Maslow, A. H. (1964). *Religions, Values, and Peak-Experiences*. Columbus: Ohio State University Press.
- Nichols, D. E. (2016). Psychedelics. *Pharmacol. Rev.* 68, 264–355. doi: 10.1124/pr.115.011478
- Nour, M. M., Evans, L., Nutt, D., and Carhart-Harris, R. L. (2016). Ego-dissolution and psychedelics: validation of the ego-dissolution inventory (EDI). *Front. Hum. Neurosci.* 10:269. doi: 10.3389/fnhum.2016.00269
- Noyes Jr, R. (1980). Attitude change following near-death experiences. *Psychiatry* 43, 234–242. doi: 10.1080/00332747.1980.11024070

- O'Reilly, P., and Funk, A. (1964). LSD in chronic alcoholism. *Can. Psychiatr. Assoc. J.* 9, 258–261. doi: 10.1177/070674376400900311
- Pahnke, W. N., Kurland, A. A., Unger, S., Savage, C., and Grof, S. (1970). The experimental use of psychedelic (LSD) psychotherapy. *JAMA* 212, 1856–1863. doi: 10.1001/jama.1970.03170240060010
- Pahnke, W. N., and Richards, W. A. (1966). Implications of LSD and experimental mysticism. *J. Relig. Health* 5, 175–208. doi: 10.1007/BF01532646
- Petitmengin, C. (2006). Describing one's subjective experience in the second person: an interview method for the science of consciousness. *Phenomenol. Cogn. Sci.* 5, 229–269. doi: 10.1007/s11097-006-9022-2
- Richards, W. A. (1978). Mystical and archetypal experiences of terminal patients in DPT-assisted psychotherapy. *J. Relig. Health* 17, 117–126. doi: 10.1007/BF01532413
- Richards, W. A. (2015). *Sacred Knowledge: Psychedelics and Religious Experiences*. New York, NY: Columbia University Press.
- Richards, W. A., Rhead, J. C., Dileo, F. B., Yensen, R., and Kurland, A. A. (1977). The peak experience variable in DPT-assisted psychotherapy with cancer patients. *J. Psychedelic Drugs* 9, 1–10. doi: 10.1080/02791072.1977.10472020
- Ross, S., Bossis, A., Guss, J., Agin-Liebes, G., Malone, T., Cohen, B., et al. (2016). Rapid and sustained symptom reduction following psilocybin treatment for anxiety and depression in patients with life-threatening cancer: a randomized controlled trial. *J. Psychopharmacol.* 30, 1165–1180. doi: 10.1177/0269881116675512
- Rucker, J. J., Jelen, L. A., Flynn, S., Frowde, K. D., and Young, A. H. (2016). Psychedelics in the treatment of unipolar mood disorders: a systematic review. *J. Psychopharmacol.* 30, 1220–1229. doi: 10.1177/0269881116679368
- Russ, S. L., and Elliott, M. S. (2017). Antecedents of mystical experience and dread in intensive meditation. *Psychol. Conscious.* 4, 38–53. doi: 10.1037/cns0000119
- Sandison, R. (1955). LSD treatment for psychoneurosis: lysergic acid diethylamide for release of depression. *Nurs. Mirror* 100, 1529–1530.
- Sandison, R. (1961). Certainty and uncertainty in the LSD treatment of psychoneurosis. *Hallucinogenic Drugs* 33, 33–36.
- Schartner, M. M., Carhart-Harris, R. L., Barrett, A. B., Seth, A. K., and Muthukumaraswamy, S. D. (2017). Increased spontaneous MEG signal diversity for psychoactive doses of ketamine, LSD and psilocybin. *Sci. Rep.* 7:46421. doi: 10.1038/srep46421
- Shagass, C., and Bittle, R. M. (1967). Therapeutic effects of LSD: a follow-up study. *J. Nerv. Ment. Dis.* 144, 471–478. doi: 10.1097/00005053-196706000-00004
- Sherwood, J. N., Stolaroff, M. J., and Harman, W. W. (1962). The psychedelic experience—a new concept in psychotherapy. *J. Neuropsychiatr.* 4, 69–80.
- Snell, T. L., and Simmonds, J. G. (2015). Mystical experiences in nature. *Arch. Psychol. Relig.* 37, 169–184. doi: 10.1163/15736121-12341303
- Sos, P., Klirova, M., Novak, T., Kohutova, B., Horacek, J., and Palenicek, T. (2013). Relationship of ketamine's antidepressant and psychotomimetic effects in unipolar depression. *Neuroendocrinol. Lett.* 34, 287–293.
- Stace, W. T. (1960). *Mysticism and Philosophy*. London: Macmillan and Co.
- Steiger, J. H. (1980). Tests for comparing elements of a correlation matrix. *Psychol. Bull.* 87, 245–251. doi: 10.1037/0033-2909.87.2.245
- Stevens, J. (1987). *Storming Heaven: LSD and the American Dream*. New York, NY: Grove Press.
- Studerus, E., Gamma, A., and Vollenweider, F. X. (2010). Psychometric evaluation of the altered states of consciousness rating scale (OAV). *PLoS ONE* 5:e12412. doi: 10.1371/journal.pone.0012412
- Tagliazucchi, E., Roseman, L., Kaelen, M., Orban, C., Muthukumaraswamy, S. D., Murphy, K., et al. (2016). Increased global functional connectivity correlates with LSD-induced ego dissolution. *Curr. Biol.* 26, 1043–1050. doi: 10.1016/j.cub.2016.02.010
- Varela, F. J. (1996). Neurophenomenology: a methodological remedy for the hard problem. *J. Conscious. Stud.* 3, 330–349.
- Vollenweider, F. X. (2001). Brain mechanisms of hallucinogens and entactogens. *Dialogues Clin. Neurosci.* 3, 265–280.
- Watts, R., Day, C., Krzanowski, J., Nutt, D., and Carhart-Harris, R. (2017). Patients' accounts of increased "connectedness" and "acceptance" after psilocybin for treatment-resistant depression. *J. Humanist. Psychol.* 57, 520–564. doi: 10.1177/0022167817709585

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The Grass Might Be Greener: Medical Marijuana Patients Exhibit Altered Brain Activity and Improved Executive Function after 3 Months of Treatment

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The vast majority of states have enacted full or partial medical marijuana (MMJ) programs, causing the number of patients seeking certification for MMJ use to increase dramatically in recent years. Despite increased use of MMJ across the nation, no studies thus far have examined the specific impact of MMJ on cognitive function and related brain activation. In the present study, MMJ patients seeking treatment for a variety of documented medical conditions were assessed prior to initiating MMJ treatment and after 3 months of treatment as part of a larger longitudinal study. In order to examine the effect of MMJ treatment on task-related brain activation, MMJ patients completed the Multi-Source Interference Test (MSIT) while undergoing functional magnetic resonance imaging (fMRI). We also collected data regarding conventional medication use, clinical state, and health-related measures at each visit. Following 3 months of treatment, MMJ patients demonstrated improved task performance accompanied by changes in brain activation patterns within the cingulate cortex and frontal regions. Interestingly, after MMJ treatment, brain activation patterns appeared more similar to those exhibited by healthy controls from previous studies than at pre-treatment, suggestive of a potential normalization of brain function relative to baseline. These findings suggest that MMJ use may result in different effects relative to recreational marijuana (MJ) use, as recreational consumers have been shown to exhibit decrements in task performance accompanied by altered brain activation. Moreover, patients in the current study also reported improvements in clinical state and health-related measures as well as notable decreases in prescription medication use, particularly opioids and benzodiazepines after 3 months of treatment. Further research is needed to clarify the specific neurobiologic impact, clinical efficacy, and unique effects of MMJ for a range of indications and how it compares to recreational MJ use.

Keywords: medical marijuana, cannabis, neuroimaging, fMRI, cognition, executive function, MSIT

INTRODUCTION

Currently, 30 states and the District of Columbia have medical marijuana (MMJ) programs or pending MMJ legislation, an additional 16 states have passed laws to allow limited access to MMJ, and an estimated 2.6 million individuals in the United States are certified for MMJ use (Procon.org). Since societal attitudes toward marijuana (MJ) have generally warmed, an increasing number of individuals are turning to MMJ to help treat a variety of medical conditions, as patients often do not achieve full symptom alleviation with conventional medications and experience unwanted side effects. Data gathered from several US surveys of MMJ patients indicate that the most common indications for MMJ use included pain-related concerns (i.e., chronic pain, headaches), psychiatric disorders (i.e., anxiety, depression), and insomnia (Nunberg et al., 2011; Reinerman et al., 2011; Bonn-Miller et al., 2014; Park and Wu, 2017). Although considerable research efforts have clarified the impact of recreational MJ use, particularly among adolescent and young adult populations, to date, there is a paucity of research focused on examining the impact of MMJ use on neurobiologic measures, including brain function and structure. As the number of MMJ patients continues to grow, research efforts designed to understand potential changes associated with MMJ use are critically important.

A large body of evidence from the past several decades suggests that recreational MJ use is related to cognitive decrements, including deficits in verbal memory (Tait et al., 2011; Auer et al., 2016; Shuster et al., 2016), processing speed (Fried et al., 2005; Lisdahl and Price, 2012; Jacobus et al., 2015), attention (Ehrenreich et al., 1999; Cousijn et al., 2013; Becker et al., 2014) and executive function (Crean et al., 2011; Gruber et al., 2012b; Solowij et al., 2012; Dougherty et al., 2013; Hanson et al., 2014; Jacobus et al., 2015; Dahlgren et al., 2016). While these deficits have been observed in adult MJ users (Nader and Sanchez, 2017), they are most salient among MJ-using adolescents (Lisdahl et al., 2014) who are in the midst of critical neurodevelopment (Giedd et al., 1999). Furthermore, these decrements have also been linked to alterations in brain structure and function. Although the directionality of structural alterations appears to be dependent on the brain region under investigation (Batalla et al., 2013), studies show that gray and white matter alterations are associated with increased executive dysfunction (Medina et al., 2009, 2010; Churchwell et al., 2010; Clark et al., 2012; Price et al., 2015). In addition, functional magnetic resonance imaging (fMRI) studies have reported altered activation patterns within the prefrontal cortex as well as orbitofrontal, cingulate, and subcortical/limbic regions of recreational MJ users compared to non-using control subjects during tasks of executive function (Lisdahl et al., 2014, for review). Further, similar to studies of cognitive performance and brain structure, fMRI studies have revealed that earlier onset of MJ use is related to altered patterns of brain activation during tasks requiring cognitive control and inhibition (Tapert et al., 2007; Gruber et al., 2012a; Sagar et al., 2015).

Although many have posited that MMJ use would be associated with similar deficits, preliminary studies have suggested that this may not be the case. In our own recent

pilot investigation (Gruber et al., 2016), the only study to date to examine the impact of whole plant-derived MMJ products on cognitive performance, we found that MMJ patients did not demonstrate decrements in performance on measures of executive function following 3 months of MMJ treatment. In fact, patients generally demonstrated *improved* performance on a number of measures, particularly those assessing executive function. Improvements were also noted on several measures of quality of life, sleep, and depression relative to pre-MMJ treatment levels. Differences between recreational and MMJ users may be related to a variety of factors, including age of onset of MJ use, duration, magnitude and frequency of use, and choice of actual cannabis products used. Although products used by recreational MJ consumers and MMJ patients are derived from the same plant species, they are generally utilized for different purposes (i.e., to get high/alter one's current state of being vs. symptom alleviation). Accordingly, recreational and medical users often seek different MJ products with various constituent compositions based on the desired effect. Recreational MJ users often seek products high in Δ^9 -tetrahydrocannabinol (THC), the main psychoactive constituent of the cannabis plant, and while medical patients may also choose products with high THC levels they often seek products high in other potentially therapeutic cannabinoids. Research has begun to focus on the beneficial effects of cannabidiol (CBD), the primary non-intoxicating constituent of MJ, which has been touted for its antipsychotic, anxiolytic, anti-seizure, and anti-inflammatory properties (Rong et al., 2017). While studies from recreational MJ users have reported a relationship between higher levels of THC and poorer cognitive performance (Ramaekers et al., 2006; Kowal et al., 2015) the acute administration of CBD prior to THC has been shown to *improve* cognitive function (Morgan et al., 2010; Englund et al., 2013), underscoring the need for further study. Moreover, Yücel et al. (2016) recently found that although MJ users exposed to THC exhibit alterations in hippocampal volume and neurochemistry, those who utilized CBD-containing products did not demonstrate differences relative to healthy controls. Similarly, a recent review of the effects of THC and CBD on neuroanatomy concluded that MJ users are prone to brain alterations in regions with high cannabinoid receptor density, and although THC exacerbates these alterations, CBD appears to protect against these deleterious changes (Lorenzetti et al., 2016). In addition, several researchers have administered pure THC or CBD to healthy control participants to investigate the impact of these constituents on brain activation patterns using fMRI. In general, studies suggest that THC and CBD have opposite effects on cognition-related brain activation (Bhattacharyya et al., 2010, 2015; Winton-Brown et al., 2011). This may be related to the fact that THC is a CB1 agonist with strong binding affinity for CB1 receptors, while CBD appears to exert effects through more indirect mechanisms, which include additional receptor types (Zuardi, 2008; Ashton and Moore, 2011). Despite preliminary work investigating the acute effects of pure THC and CBD on neural networks associated with cognitive domains impacted by MJ use, to our knowledge, no studies thus far have examined the impact of treatment with whole-plant-derived MMJ products on brain activation patterns.

In order to investigate whether pilot observations of improved executive function (Gruber et al., 2016) persist with larger sample sizes and to determine whether these changes co-occur with altered brain activation patterns, MMJ patients from an ongoing longitudinal study underwent fMRI while completing the Multi-Source Interference Test (MSIT). The MSIT is a robust measure of cognitive interference, a core facet of executive functioning, which is related to attentional control and inhibitory processing and requires actively shifting attention by inhibiting automatic responses (Lezak et al., 2004). This task reliably activates frontal brain regions associated with executive functioning, particularly the cingulo-frontal-parietal (CFP) network (Bush and Shin, 2006). Given our previous findings (Gruber et al., 2016), we hypothesized that following 3 months of treatment, MMJ patients would demonstrate improved task performance, and that this improvement would coincide with changes in brain activation patterns measured by fMRI. We have previously utilized the MSIT to better characterize patterns of cingulate and frontal brain activation within clinical and non-clinical cohorts (Gruber et al., 2012a, 2017), and although no studies thus far have examined MMJ patients using neuroimaging techniques, we hypothesized that improved MSIT task performance would be associated with increased activation in these regions following 3 months of MMJ treatment. We also posited that these changes would occur in the context of improved mood and quality of life ratings.

MATERIALS AND METHODS

Participants

To date, of 45 consented participants, 41 MMJ patients were enrolled and data from 22 patients' pre-treatment (Visit 1) and 3-month check-in visits (Visit 2) were available for analyses. In addition, patients who were free of MRI contraindications also completed neuroimaging procedures ($n = 15$). In order to qualify for study entry, patients had to be over the age of 18, and have an estimated IQ of 75 or higher as assessed by the Wechsler Abbreviated Scale of Intelligence (WASI; Wechsler, 1999). In order to minimize the effects of previous MJ exposure on study findings, patients were required to be MJ naïve or be abstinent from MJ use for at least 2 years for their pre-treatment visit. Patients were also required to be certified for MMJ use, or describe a plan to use industrial hemp derived products (which do not currently require certification). All subjects received payment for each study visit, and those who completed MRI procedures were compensated additionally in accordance with Partners IRB-approved protocol procedures.

Study Design

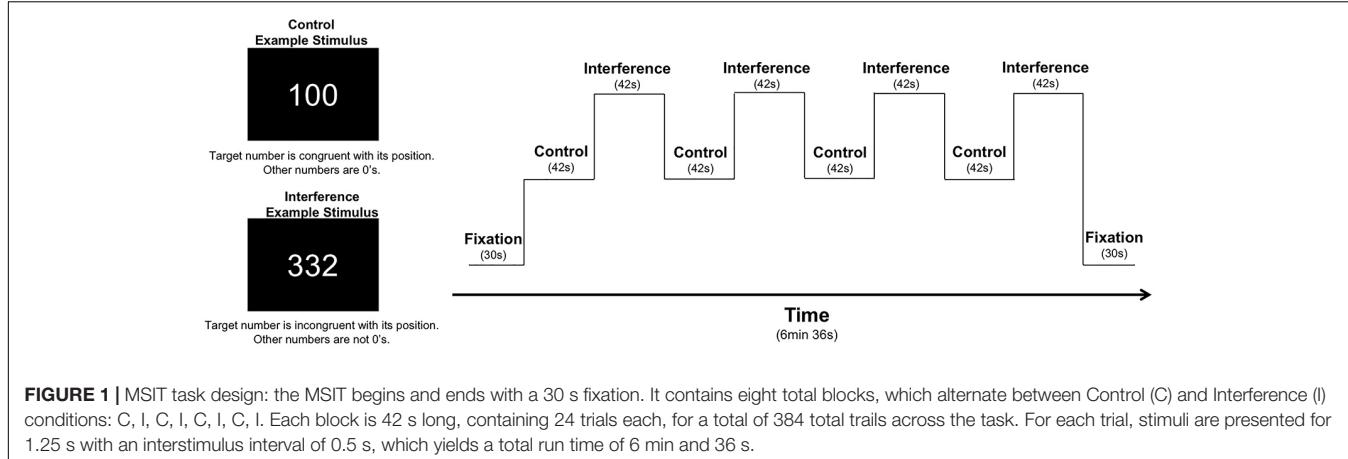
Prior to participation, all study procedures were explained, and each participant was required to provide written informed consent in accordance with the Declaration of Helsinki. This document and all study procedures were approved by the Partners Institutional Review Board. Eligible participants were enrolled in a larger longitudinal study designed to assess the impact of MMJ on cognition and brain function over the course

of 12–24 months. Patients completed all assessments and imaging *prior* to initiation of MMJ treatment and again after 3 months of treatment.

As part of a larger neuroimaging protocol, participants completed the MSIT (Bush et al., 2003; Bush and Shin, 2006) with concurrent fMRI scanning using identical task parameters as reported in our previous studies of recreational MJ users, patients with bipolar disorder, and healthy controls (Gruber et al., 2012a, 2017). Using aspects from well-established measures of cognitive interference (e.g., Stroop, Simon, and Flanker tasks), the MSIT incorporates both spatial and flanker types of interference to measure cognitive control (Bush et al., 2003; Bush and Shin, 2006). During the task, three-digit stimuli sets comprised of the numbers 0, 1, 2, or 3 are presented briefly on a screen. Each set contains two identical distractor numbers and a target number that differs from the distractors. Using a button box, participants report the *identity* of the target number that differs from the two distractor numbers during two conditions: during the Control condition, distractor numbers are always zeros, and the identity of the target number always corresponds to its position on the button box (i.e., 100, 020, 003). During the Interference condition, patients are required to inhibit a prepotent response in favor of a less automatic response (i.e., indicate the identity of the target number rather than its position). Distractor numbers are always numbers other than 0, and the identity of the target number is always incongruent with its position on the button box (e.g., 211, 232, 331, etc.). Performance is measured by reaction time and percent accuracy, which can be further subdivided by error type. Omission errors occur when no response is given and are typically reflective of slower or overloaded cognitive processing while commission errors, or incorrect responses, generally indicate difficulty inhibiting inappropriate responses. The entire task is comprised of four blocks of control trials alternating with four blocks of interference trials; the task begins and ends with a fixation period (30 s), making the total run time 6 min and 36 s (see **Figure 1** for graphic representation of the task design).

Patients also completed a battery of self-report rating scales. Briefly, these included the Profile of Mood States (POMS), Beck Depression Inventory (BDI), Beck Anxiety Inventory (BAI), Pittsburgh Sleep Quality Index (PSQI), Barratt Impulsiveness Scale (BIS-11), and the Short Form-36 Health Survey (SF-36), a measure of functional health and quality of life. During each study visit, participants also provided information regarding dose, frequency, and duration of use for all conventional medications, which were categorized into different classes, including opioids, antidepressants, mood stabilizers, and benzodiazepines. Percent change data was calculated to assess potential changes in medication use from pre- to post-3 months of MMJ treatment.

After completing pre-treatment assessments, patients began MMJ treatment at their discretion. Although patients selected their own products and determined their own treatment regimens, we collected detailed data about MMJ use patterns and products. Between study visits, patients submitted biweekly diaries documenting MMJ use and were contacted by phone on



a monthly basis to acquire information regarding MMJ product type, frequency, magnitude, and modes of use using a modified timeline follow-back procedure (TLFB; Sobell et al., 1998). Following a minimum of 3 months of regular MMJ treatment, patients returned for their first of several check in visits (Visit 2) where they repeated all study measures. In addition, participants were asked to provide a sample of their most frequently used MMJ product(s) to an outside laboratory (ProVerde Laboratories, Inc.) for cannabinoid constituent profiling. These analyses, which quantified the levels of 10 major cannabinoids including THC and CBD, will be used to identify the unique effects of specific cannabinoids in future analyses.

Statistical Analyses

Descriptive statistics were calculated for demographic and MMJ use variables. Repeated-measure analyses of variance (ANOVAs) were used to assess changes in clinical state from Visit 1 to Visit 2. The assumption of homogeneity of variance was confirmed using Levene's F; however, Shapiro-Wilk tests indicated that data for the MSIT were not normally distributed. Accordingly, non-parametric, repeated-measures Wilcoxon Signed Rank Tests were used to assess changes from Visit 1 to Visit 2 for MSIT data. It is of note, however, that the non-parametric tests resulted in similar findings as the ANOVAs; all significant results remained. For the MSIT analyses, alpha was set at 0.05 for the response time and percent accuracy variables. In cases where percent accuracy differed significantly between Visits 1 and 2, comparisons of the two different error types (omission, commission) utilized a Bonferroni correction for multiple comparisons ($\alpha/2 = 0.025$).

fMRI Methods and Analyses

All imaging was performed on a Siemens Trio whole body 3T MRI scanner (Siemens Corporation, Erlangen, Germany) using a 12-channel phased array head coil. For the MSIT, 40 contiguous coronal slices were acquired from each participant, ensuring whole brain coverage (5 mm thick, 0 mm skip), and images were collected with TR = 3000, using a single shot, gradient pulse echo sequence (TE = 30 ms, flip angle = 90°, with a 20 cm field of view and a 64 × 64 acquisition matrix; in plane resolution

3.125 mm × 3.125 mm × 3.125 mm). A total of 132 images per slice were collected.

fMRI images were analyzed using SPM8 (version 4667, Wellcome Department of Imaging Neuroscience, University College, London, United Kingdom) software package running in MATLAB (version R2010b, MathWorks, Natick, MA, United States). First, blood-oxygen-level dependent (BOLD) fMRI data were corrected for slice timing and for motion in SPM8 using a two-step intra-run realignment algorithm that uses the mean image created after the first realignment as a reference. A criterion of 3 mm of head motion in any direction was used as an exclusionary criterion. The realigned images were then normalized to an EPI template in Montreal Neurological Institute stereotactic space using DARTEL. Normalized images were resampled into 3 mm³ voxels and then spatially smoothed using an isotropic Gaussian kernel with 6 mm full width at half maximum. Global scaling was not used, high-pass temporal filtering with a cut-off of 168 s was applied, and serial autocorrelations were modeled with an AR(1) model in SPM8. Using a general linear model, statistical parametric images were calculated individually for each subject showing Interference > Control. These images were subsequently entered into second level model, subjected to a voxel-wise contrast and *t*-test to assess statistical significance. In addition to the realignment during the preprocessing, effects of motion were further corrected by removing motion related components from the data by including the calculated motion parameters from the realignment as regressors in the GLM (e.g., six nuisance regressors corresponding to three directions of translation and three axes of rotation). Regions of interest (ROI) masks were created using the Wake Forest University Pickatlas utility (Maldjian et al., 2003) and included cingulate and frontal regions. Specifically, the cingulate ROI was comprised of both bilateral anterior and mid cingulate regions (22,302 voxels) while the frontal ROI was comprised of bilateral superior frontal, middle frontal and inferior frontal gyri (6,857 voxels; see Supplementary Figure 1). These regions were selected as the cingulate (CC) and frontal cortices are associated with inhibitory processing and are reliably activated during the completion of the MSIT (Bush and Shin, 2006; Gruber et al., 2012a, 2017). Contrast analyses consisted of the subtraction of one map from

the other; for example, the cingulate activity of Visit 1 was subtracted from cingulate activity of Visit 2 to determine which areas showed increased activity over the course of treatment. As in previous studies (Heckers et al., 2004; Harrison et al., 2007; Yucel et al., 2007; Shin et al., 2011; Harding et al., 2012), the fixation point was not included in planned contrast analyses. The statistical threshold was set at $p < 0.05$ for cluster level family-wise-error (FWE), $p < 0.001$ for voxel level FWE with a minimum cluster extent $k = 15$ contiguous voxels in accordance with previously published manuscripts that have utilized the MSIT and have used a k -value of 15 (Gruber et al., 2012a) or lower (Harding et al., 2012; Bush et al., 2013). In addition to utilizing previously published k -values, we also conducted Monte Carlo simulations (Ward, 2000) to determine a more rigorous cluster extent for $p < 0.001$ which yielded $k = 91$. As two ROIs were used for analyses, data was also corrected for multiple comparisons, generating a new statistical threshold ($p < 0.0005$). One patient was excluded from MSIT analyses as they requested early termination of scanning procedures.

RESULTS

Demographics and MMJ Use

All patients (11 male, 11 female) were between the ages of 28–74 ($M = 50.64$, $SD = 13.15$) who reported seeking MMJ treatment for a variety of conditions including pain ($n = 13$), anxiety/PTSD ($n = 10$), sleep ($n = 10$), mood ($n = 8$), and “other” conditions ($n = 8$), which included gastrointestinal issues, difficulty with attention, and additional indications not specified by the state of Massachusetts. Patients in the current sample were generally well-educated; all had earned a high school diploma, many completed advanced education ($M = 15.91$ years, $SD = 1.97$), and all were of at least average intelligence as measured by the WASI ($M = 117.23$, $SD = 7.63$). Upon initiation of MMJ treatment, all patients reported at least weekly use, which ranged from 1.5 times per week to multiple times per day. As noted in Table 1, patients reported using MMJ products an average of 5.34 days per week and 1.83 times per day for an overall average of 10.26 total episodes of MMJ use per week. Patients also indicated various routes of administration, including smoking and vaporizing flower, as well as use of oil and concentrates (vaporized and oral administration), tinctures, edibles, and topicals.

MSIT Behavioral Performance

Relative to pre-treatment, patients demonstrated improved MSIT performance following 3 months of MMJ treatment (Table 2). During the Control condition, patients exhibited improved performance, marked by fewer omission errors; however, qualitative analyses revealed that patients approached near perfect levels of performance pre-and post-treatment for this condition. During the Interference condition, patients performed notably better at Visit 2, demonstrating significantly fewer omission and a trend for fewer commission errors, and thus significantly improved percent accuracy. In addition, MMJ patients also demonstrated faster response times during Visit 2, relative to Visit 1, across both Control and Interference trials.

TABLE 1 | Demographics and MMJ use.

Demographic variable ($n = 22$)	Mean (SD)
Age	50.64 (13.15)
Education (years)	15.91 (1.93)
WASI ^a Full Scale IQ	117.23 (7.63)
MMJ use^b	
Days of MMJ use/week	5.34 (1.99)
Times/day used	1.83 (1.02)
Total MMJ use episodes/week	10.26 (7.71)
Mode of use	
Smoke (flower)	8
Vaporize (flower)	9
Vaporize (oil/concentrates)	6
Oil/concentrates (non-smoked/vaporized)	5
Tincture	6
Edibles	7
Topicals	2

^aWASI, Wechsler Abbreviated Scale of Intelligence; ^breflects average use from the start of regular treatment through Visit 2.

MSIT fMRI Data

Interestingly, in addition to improved task performance, MMJ patients exhibited notable changes in brain activation patterns in terms of both magnitude and location from Visit 1 to Visit 2. Results are provided in Table 3 which includes data for both the *a priori* threshold of $k = 15$ and also indicates which values survived the new threshold of $k = 91$ determined by the Monte Carlo simulations. After initiating MMJ treatment, patients generally exhibited increased activation within both the cingulate and frontal ROIs. Specifically, within the CC ROI, single-sample analyses revealed no significant activation at Visit 1, yet at Visit 2 patients exhibited focal activation within the midcingulate cortex ($k = 165$). Within-subjects contrast analyses between Visit 1 and Visit 2 revealed no significant activation differences for Visit 1 > Visit 2, but the Visit 2 > Visit 1 contrast indicated activation differences within the right anterior cingulate ($k = 43$). Within the frontal ROI, single-sample analyses revealed activation at Visit 1 within the left superior ($k = 65$) and the right inferior frontal gyrus ($k = 19$), and at Visit 2, within the right inferior ($k = 575$), left middle frontal gyrus ($k = 217$), and the left precentral gyrus ($k = 19$). Within-subjects contrast analyses between Visit 1 and Visit 2 yielded no significant activation differences for the Visit 1 > Visit 2 contrast; however, the Visit 2 > Visit 1 contrast revealed significant activation differences within the right middle gyrus ($k = 88$) and superior frontal gyrus ($k = 25$). See Figure 2.

Clinical Ratings and Conventional Medication Use

Following 3 months of MMJ treatment, patients reported some improvement on measures of mood and quality of life (Table 4). Across all rating scales, no significant worsening

of clinical state or quality of life was observed. Moreover, consistent with a previous report (Gruber et al., 2016), patients reported significant *improvements* on measures of depression (BDI), impulsivity (BIS-11), sleep (PSQI), and

quality of life (SF-36). Specifically, on the SF-36, patients indicated significantly improved energy/fatigue and fewer role limitations due to physical health, which reflects how often patients' physical health affects their work and other life

TABLE 2 | Repeated measures Wilcoxon signed rank tests assessing Multi-Source Interference Test (MSIT) performance at pre-treatment and after 3 months of MMJ use (post-treatment).

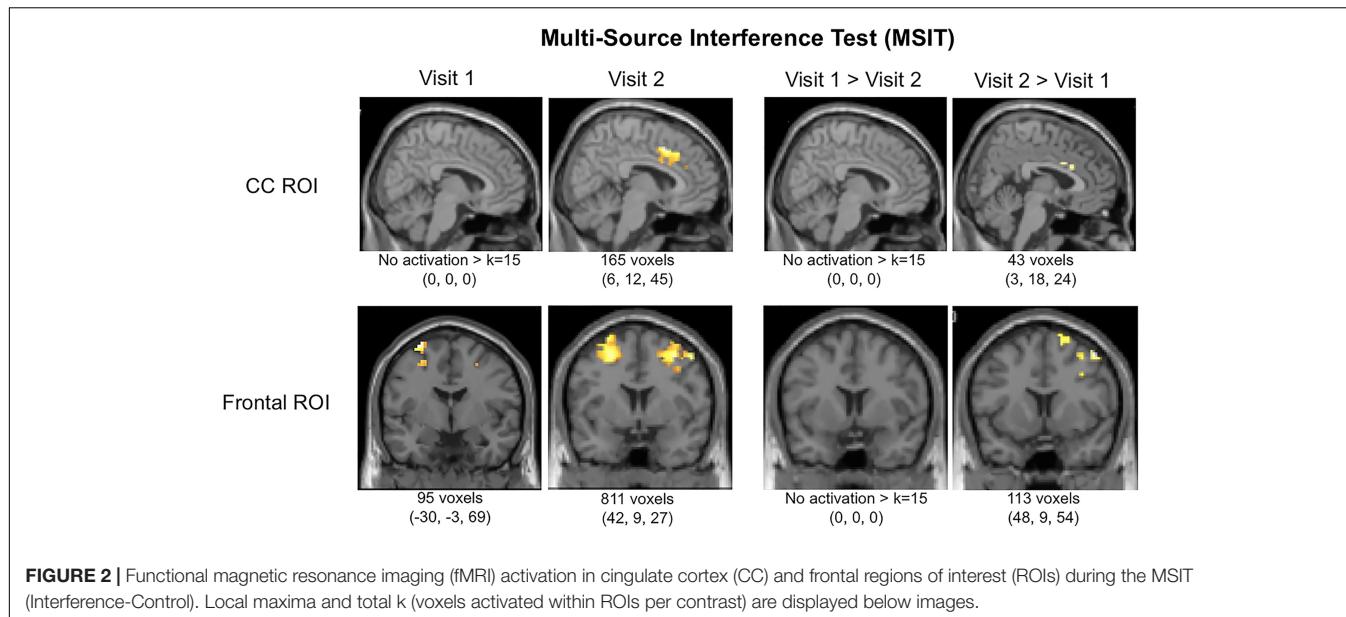
MSIT variable	Visit 1 Pre-treatment Mean (SD)	Visit 2 Post-treatment Mean (SD)	Wilcoxon	
			Z	p (r)
Control condition				
Response time (ms)	608.90 (97.20)	582.62 (64.97)	2.062	0.020 (0.500)*
Percent accuracy	97.40 (2.57)	98.82 (1.74)	2.282	0.011 (0.553)*
Omission errors ^a	1.73 (2.25)	0.68 (1.09)	1.974	0.024 (0.479)*
Commission errors ^a	0.77 (0.97)	0.46 (0.86)	1.461	0.072 (0.354)
Interference condition				
Response time (ms)	914.23 (76.56)	886.62 (82.76)	2.743	0.003 (0.665)*
Percent accuracy	79.03 (18.87)	86.55 (11.88)	2.858	0.002 (0.693)*
Omission errors ^a	11.96 (12.01)	7.27 (7.92)	2.750	0.003 (0.667)*
Commission errors ^a	8.18 (9.11)	5.77 (5.57)	1.718	0.043 (0.417) [†]

df = 1,21; ^acorrected for multiple comparisons using Bonferroni method; *results significant at $p \leq 0.05$ when $\alpha = 0.05$ or, for Bonferroni corrected analyses, at $p \leq 0.025$ when $\alpha = 0.025$; [†]results trending toward significance at $p \leq 0.10$ when $\alpha = 0.05$ or, for Bonferroni corrected analyses, at $p \leq 0.05$ when $\alpha = 0.025$.

TABLE 3 | Multi-Source Interference Task (Interference-Control condition): activation local maxima within cingulate cortex (CC) and frontal cortex regions of interest (ROIs).

ROI	Cluster size (voxels)	x	y	z	SPM {t}	Voxel p (FWE - corrected)
CC						
Visit 1						
No activation $k \geq 15$	–	–	–	–	–	–
Visit 2						
Right middle cingulate cortex	150	6	12	45	8.45	<0.0005
Right anterior cingulate cortex	15	12	36	15	5.18	<0.0005
Visit 1 > Visit 2						
No activation $k \geq 15$	–	–	–	–	–	–
Visit 2 > Visit 1						
Right anterior cingulate cortex	20	3	18	24	5.41	<0.0005
Right anterior cingulate cortex	23	9	30	18	5.21	<0.0005
FRONTAL						
Visit 1						
Left superior frontal gyrus	65	–30	–3	69	9.51	<0.0005
Right inferior frontal gyrus (<i>p. triangularis</i>)	19	45	12	24	8.48	<0.0005
Visit 2						
Right inferior frontal gyrus (<i>p. opercularis</i>)	575	42	9	27	10.75	<0.0005
Left middle frontal gyrus	217	–24	–6	51	10.08	<0.0005
Left precentral gyrus	19	–45	9	36	7.87	<0.0005
Visit 1 > Visit 2						
No activation $k \geq 15$	–	–	–	–	–	–
Visit 2 > Visit 1						
Right middle frontal gyrus	88	48	9	54	6.94	<0.0005
Right superior frontal gyrus	25	24	12	66	6.58	<0.0005

The statistical threshold was initially set at $p < 0.05$ for cluster level family-wise-error (FWE), and $p < 0.0005$ for voxel level FWE (corrected for multiple comparisons) with a minimum cluster extent $k = 15$ contiguous voxels. Bolded results indicate values that survived the Monte Carlo simulation minimum cluster extent ($k = 91$).



activities. A trend also emerged suggesting improved social functioning.

In addition to improvements in clinical state and quality of life, following 3 months of MMJ treatment, patients reported reductions in their use of conventional pharmaceutical products across several drug classes. Specifically, patients taking opioids reported a 47.69% reduction in use and those prescribed benzodiazepines reported a 46.91% reduction in use. Antidepressant use decreased by 22.35% while the use of mood stabilizers decreased by 28.57% between Visit 1 and Visit 2.

DISCUSSION

Following 3 months of MMJ treatment, patients exhibited improved task performance and related alterations in frontal brain activation patterns during the completion of the MSIT, a measure of executive function and cognitive control, relative to pre-MMJ treatment. Within the cingulate cortex (CC), patients did not exhibit any significant pre-treatment activation during the Interference condition of the MSIT; however, after 3 months of treatment, robust activation was noted within this region. In fact, the magnitude of activation significantly increased over the course of treatment such that post-treatment activation patterns appeared more similar to that of healthy controls observed in previous studies (Bush and Shin, 2006; Gruber et al., 2012a). Activation within the frontal ROI was also notably increased following 3 months of MMJ treatment relative to pre-MMJ treatment. Taken together, these changes may be reflective of a potential “normalization” of brain function following 3 months of MMJ use.

Further, changes in brain activation patterns were observed in the context of improved task performance and self-reported

improvements in mood and quality of life as well as reduced sleep disturbance and lower motor impulsivity, consistent with previously published preliminary data (Gruber et al., 2016). It is possible that improvements in symptomatology (i.e., relief of symptoms, improved mood/sleep) are directly related to observed improvements in cognitive function and alterations in brain activation. Patients in the present study most commonly endorsed pain and anxiety as their reasons for MMJ certification; both of these conditions have previously been associated with reduced cognitive performance (Moriarty et al., 2011; Vytal et al., 2013). Symptom improvement may therefore result in improved cognitive performance, and subsequently impact patterns of brain activation during completion of these tasks.

In addition to reduced symptomatology resulting in improved cognitive performance, it is also possible that several other factors may have also contributed to the observed changes. Patients reported notable decreases in their use of opioids, benzodiazepines, antidepressants, and mood stabilizers, and it is possible that reductions in conventional medications influenced changes in brain activation patterns. In fact, several studies have shown that mood stabilizers, benzodiazepines, and antidepressants generally attenuate activation (Bell et al., 2005; Del-Ben et al., 2005; Paulus et al., 2005; Arce et al., 2008; Murphy et al., 2009). In particular, Bell et al. (2005) reported that use of mood stabilizers is related to hypoactivation of the CFP network, a key region implicated in cognitive interference processing. While we are not aware of fMRI research focused on the effects of short-term prescriptive doses of opioids in humans, one study examining opioid dependence reported that normalization of frontal brain activation patterns was related to days since last drug use (Bunce et al., 2015). Reduction or cessation of use of these medications may therefore alter patterns of brain activation. Accordingly, future studies are needed to disentangle the effects

TABLE 4 | Mood and health ratings at pre-treatment and after 3 months of MMJ use (post-treatment).

Rating scale	Visit 1 Pre-treatment Mean (SD)	Visit 2 Post-treatment Mean (SD)	ANOVA			
			F	p (η^2)		
Clinical ratings						
<i>Profile of Mood States (POMS)^a</i>						
Vigor	16.86 (6.19)	16.14 (6.53)	0.543	0.235 (0.025)		
Anger	8.68 (10.33)	8.59 (9.21)	0.003	0.477 (<0.001)		
Confusion	8.00 (6.38)	6.73 (4.92)	2.748	0.056 (0.116)*		
Tension	12.59 (9.96)	12.05 (9.97)	0.153	0.350 (0.007)		
Fatigue	9.91 (7.29)	8.77 (7.24)	1.946	0.089 (0.085)*		
Depression	13.18 (16.78)	14.14 (16.95)	0.217	0.323 (0.010)		
TMD	35.50 (51.08)	34.14 (48.89)	0.055	0.409 (0.003)		
<i>Beck Depression Inventory (BDI)^a</i>						
Total	13.77 (12.60)	9.73 (11.65)	9.559	0.003 (0.313)*		
<i>Beck Anxiety Inventory (BAI)^a</i>						
Total	10.55 (10.32)	9.73 (9.93)	0.227	0.319 (0.011)		
Impulsivity						
<i>Barratt Impulsiveness Scale (BIS-11)^a</i>						
Attention	16.59 (5.77)	16.59 (5.47)	0.000	0.500 (0.000)		
Motor	23.00 (5.43)	21.23 (5.37)	12.531	0.001 (0.374)*		
Non-planning	23.41 (5.53)	23.59 (5.50)	0.077	0.392 (0.004)		
Total	63.00 (15.08)	61.41 (14.64)	1.626	0.108 (0.072)		
Health and quality of life ratings						
<i>Pittsburgh Sleep Quality Index (PSQI)^b</i>						
Total	8.26 (4.46)	6.05 (3.26)	7.167	0.008 (0.285)*		
<i>SF-36^a</i>						
Physical functioning	71.59 (21.40)	71.82 (26.03)	0.002	0.483 (<0.001)		
Role limitations (physical)	44.32 (43.60)	56.82 (44.44)	3.915	0.031 (0.157)*		
Role limitations (emotional)	63.64 (43.53)	60.61 (45.58)	0.096	0.380 (0.005)		
Energy/fatigue	42.73 (25.39)	51.36 (21.67)	10.738	0.002 (0.338)*		
Emotional well-being	67.64 (26.18)	65.82 (27.32)	0.827	0.187 (0.038)		
Social functioning	62.50 (29.12)	68.75 (29.06)	1.819	0.096 (0.080)*		
Pain	52.73 (2.05)	56.59 (26.06)	0.918	0.174 (0.042)		
General health	57.50 (19.75)	60.91 (19.56)	1.668	0.105 (0.074)		

Clinical rating scales (POMS, BDI, BAI), lower scores reflect lower levels of clinical symptoms; BIS-11, lower scores indicate lower levels of self-reported impulsivity; PSQI, lower scores reflect improved sleep quality; SF-36, higher scores indicate higher quality of life. ^adf = 1,21; ^bdf = 1,18; *bolded results are significant at $p \leq 0.05$; ^italicized results approach significance at $p \leq 0.10$.

of MMJ treatment and conventional medication use on brain activation patterns, and may benefit from limiting clinical samples to only those on a single specific class of conventional medication.

Although findings from this study indicate *improvements* in cognitive task performance and more normalized patterns of brain activation after 3 months of MMJ treatment, previous studies, exclusively focused on recreational MJ users, have reported *decrements* in cognitive performance and accompanying atypical neural alterations. A recent review highlighting neuroimaging findings in recreational MJ users found evidence for altered frontal neural function during completion of executive function tasks (Weinstein et al., 2016), a finding observed in our own previous research (e.g., Gruber and Yurgelun-Todd, 2005; Gruber et al., 2012a; Sagar et al., 2015). A number of critical factors may account for the differences between our current findings in MMJ patients relative to findings

from recreational MJ consumers. The majority of studies of recreational MJ use have included adolescent and young adult populations. Overwhelmingly, studies have demonstrated that early/adolescent onset of recreational MJ use is related to poorer task performance and changes in brain structure and function (Lisdahl et al., 2013, 2014; Jacobus and Tapert, 2014; Levine et al., 2017). Given that participants in the current sample are adults (*Mean age* = 50.64) who are well-beyond the critical stages of neurodevelopment (Giedd et al., 1999), they are likely less vulnerable to the adverse neural effects of THC. Interestingly, recent preclinical evidence indicates that THC may have the potential to *improve* cognition in older individuals (Bilkei-Gorzo et al., 2017). Mature and old mice administered low doses of THC demonstrated a reversal of age-related cognitive decline, hypothesized to be related to upregulation of the aging endocannabinoid system via increased signaling secondary to low dose THC exposure. Moreover,

the same exposure resulted in cognitive decrements among young mice. Additional research is needed to more fully understand the mechanisms underlying these improvements and to examine the impact of cannabis and cannabinoids in older adult populations as well as the effects of low doses of THC, as these factors likely influence the impact of MJ use.

It is also important to consider patterns of MJ use, including frequency and duration of use, in order to understand potential reasons for the different outcomes among recreational users and medical patients. In the current study, all patients reported using MMJ at least weekly; on average, they reported using 5 days per week and 1–2 times per day. Traditionally, studies of recreational users have examined chronic, heavy use; although criteria for “heavy use” can vary across investigations, most studies have required participants to use MJ at least 1–4 days per week (Tait et al., 2011; Gruber et al., 2012a; Macher and Earleywine, 2012; Dougherty et al., 2013; Cousijn et al., 2014), similar to the frequency of use among the current sample of medical patients. Given these similarities, it is unlikely that differences between recreational and MMJ patients are solely attributable to frequency of use. Additionally, studies of recreational MJ users typically include consumers with a longer duration of MJ use relative to the current sample of MMJ patients, and differences in cumulative exposure should also be considered. For this reason, our ongoing study is designed to examine MMJ users after increasingly longer periods of use to explore the impact of longer durations of MMJ use on cognitive function.

Further, MMJ patients and recreational MJ users also typically differ in terms of the products they use. Recreational MJ products are often prized for high THC levels, and the goal of the recreational consumer is to change their current state of being or to ‘get high.’ MMJ patients seek symptom alleviation and tend to choose products with rich and varied cannabinoid profiles including constituents other than THC, which may also impact clinical state, cognitive processing and other domains. For example, CBD, which has been touted for its clinical benefits (Rong et al., 2017), has demonstrated efficacy in mitigating the negative cognitive effects of THC (Yücel et al., 2016) and appears to exert opposite effects on task-related brain activation relative to THC (Colizzi and Bhattacharyya, 2017). In addition, although there is a paucity of research in this area, some studies have examined the direct impact of acute CBD administration on cognitive performance. Englund et al. (2013) reported that administration of CBD prior to the administration of THC resulted in better episodic memory relative to placebo pre-treatment in healthy controls. Morgan et al. (2010) examined verbal memory performance in current recreational MJ users and found that those using products without CBD (confirmed by hair sample analysis) performed more poorly on verbal memory measures than those with detectable levels of CBD. While no studies have examined the impact of whole plant-derived MMJ products or assessed the long-term impact of MMJ treatment, some studies have utilized fMRI techniques to examine the acute effects of

individual cannabinoids. Borgwardt et al. (2008) studied the acute impact of THC, CBD, and placebo on executive function in healthy controls using a Go/No go task. Although the authors did not report any performance differences between cannabinoids or placebo, fMRI data demonstrated that THC reduced activation in frontal and anterior cingulate regions, while CBD reduced activation in temporal and insular regions relative to placebo. In addition, Bhattacharyya et al. (2010) found that intravenous administration of THC and CBD resulted in opposite effects on brain activation patterns across multiple regions during the completion of memory, inhibitory function, and affective measures. Given these findings, data from the present study may reflect the direct neurobiologic effects of cannabinoids, as increased endocannabinoid signaling is associated with improved cognition (Egerton et al., 2006), reduced stress response, emotional regulation, and increased endogenous reward signaling (Hill and McEwen, 2010; Befort, 2015), and as previously noted, specific alterations in brain activation patterns (Borgwardt et al., 2008; Bhattacharyya et al., 2010). Results may also reflect the indirect impact of whole plant-derived products, which include an array of cannabinoid constituents, and may exert downstream affects on multiple receptor types and neural systems (i.e., pain and reward circuitry). While THC and CBD are generally the most abundant cannabinoids in patients’ products, and as noted, several studies have begun to explore their impact on cognition and brain activation patterns, a number of other cannabinoids including cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC), and tetrahydrocannabidivarin (THCV), are often present in MMJ products, and may have moderated or indirectly affected the impact typically associated with THC exposure (Englund et al., 2016). Further research is clearly indicated for assessing the specific impact of individual cannabinoids on cognitive and clinical variables in patients using cannabis for medical purposes.

Limitations and Future Directions

Despite the compelling nature of the study findings, several limitations must be noted. First, the current investigation is designed as an observational, longitudinal pre-post study in which patients choose their own products and treatment regimen. The ability to assess the impact of whole plant-derived cannabis-based products is more ecologically valid than studies involving synthetic or non-plant derived products; however, the current legal landscape prohibits the use of dispensary-based products within a clinical trial model and allows only the use of products supplied by the National Institute on Drug Abuse (NIDA). While NIDA’s drug supply program has expanded their portfolio of MJ products available for research, their supply does not currently include the range and scope of products (i.e., product type, potency, constituent profiles, etc.) that patients are seeking and obtaining through dispensaries and caregivers across the nation. Accordingly, as a clinical trial model could not be utilized, the present study collected comprehensive data on product source, selection, dose, frequency, and mode of use. Further, as previously noted, patients also provided a sample of their most frequently

used MMJ products for cannabinoid constituent profiling. Interestingly, 13 of the 22 patients (59%) in the current study were identified as taking products high in CBD which may have contributed to study findings given previous data highlighting its clinical benefits (McGuire et al., 2017; Rong et al., 2017). As initial laboratory analyses revealed a range of cannabinoid constituents from patients' products, additional analyses will be conducted to examine the impact of specific cannabinoids and their relationship with cognitive and clinical variables. Further, future studies will assess potential differences between patients who choose products high in THC compared to those using products high in CBD, as these data may provide critical information regarding efficacy of individual constituents and combinations of constituents for specific indications and conditions to better inform selection of MMJ products.

The current study utilized a pre-post, within-subjects design in which all patients are MJ naïve at Visit 1 and are followed over the course of 12–24 months in order to clarify the impact of prolonged duration of exposure to MMJ treatment. As results from the current study represent only data from baseline and patients' first check-in visit after 3 months of MMJ treatment, data must be considered preliminary. Additional analyses, which are planned for the future, are needed to understand the impact of MMJ over longer treatment periods. Further, as a result of the pre-post study design, repeated administration of cognitive measures was required, and thus practice effects cannot be completely ruled out. Although no studies to date have specifically examined practice effects for the MSIT, given the lengthy duration of time between visits (at least 3 months) and the computerized nature of the task, it is highly unlikely that practice effects would persist. In addition, given the longitudinal nature of the design, each subject's baseline assessment serves as their own control from which to assess change after initiation of MMJ treatment. It could, however, prove beneficial for future investigations to also recruit a control group of patients who report similar symptoms (i.e., pain, insomnia, anxiety, etc.) but who do *not* choose to utilize MMJ. Comparing outcomes of MMJ patients and "treatment as usual" patients over time could strengthen findings if MMJ patients display more positive outcomes relative to those who do not use MMJ but suffer from similar symptoms or conditions.

In addition, statistical thresholds for fMRI analyses were set in accordance with previous investigations (Shin et al., 2011; Gruber et al., 2012a; Harding et al., 2012) in order to aid in the interpretation of findings. While more stringent thresholds were also applied and are noted within the results, it is important to recognize that some findings did not survive the more rigorous thresholds, a common issue in fMRI studies with limited sample sizes.

In order to gain a more thorough understanding of how MMJ impacts cognitive functioning, it will be important for future studies to replicate current study findings using other measures of executive function and to examine additional cognitive domains. As executive functioning has been shown to be impacted by recreational MJ use (for review, Crean et al., 2011), this domain was targeted for the current investigation;

however, it is crucial to examine additional cognitive variables, including verbal memory, which has also been shown to be sensitive to MJ use (for review, Solowij and Battisti, 2008; Broyd et al., 2016).

Finally, this study included MMJ patients using products for a variety of indications, which resulted in a varied clinical sample. While this approach provides a broad assessment of the impact of MMJ, it is likely that individual conditions and symptoms will have unique patterns of responses associated with MMJ treatment. A number of medical and psychiatric conditions have been shown to negatively impact cognitive processing; accordingly, future studies may derive additional power by limiting inclusion to patients with a single condition or indication (i.e., patients using MMJ exclusively for pain) or including only patients taking medications from a single drug class.

CONCLUSION

To our knowledge, this study represents the first neuroimaging investigation of patients using marijuana for medical purposes. Following 3 months of MMJ treatment, brain activation patterns appear more similar to those exhibited by healthy controls from previous studies than at pre-treatment. This finding provides strong evidence that MMJ treatment may normalize brain activity. Importantly, these changes were accompanied by improved task performance as well as positive changes in ratings of clinical state, impulsivity, sleep, and quality of life. Further, patients reported notable decreases in their use of conventional medications, including opioids. In light of the national opioid epidemic, these data clearly underscore the need to expand and extend this study to determine if a reduction in opioid use persists with continued MMJ treatment. Results from the current study raise the possibility that the observed improvements in cognition and related changes in functional activation patterns may be related to direct and/or indirect effects of cannabinoids, specifically within an adult population beyond the stages of critical neuromaturation. Patients utilizing MMJ appear to use products with different cannabinoid profiles (i.e., high CBD) relative to recreational users, which is also likely to impact cognitive function. Observed changes may also be related to secondary or more indirect effects, including the reduction of clinical symptoms, improved sleep, and decreased use of conventional medications. Additional studies using both observational and clinical trial models to examine the impact of actual MMJ products used by patients are needed to clarify the underlying neural mechanisms associated with clinical and behavioral changes that accompany MMJ treatment.

AUTHOR CONTRIBUTIONS

SG conceptualized and designed the current study in consultation with SL. KS assisted SG in manuscript preparation with

additional help provided by the remaining authors. SG, KS, RS, AL, and KC recruited the patients, carried out the study procedures and administered the neuropsychological and clinical assessments. MD completed the statistical analyses and AG completed the neuroimaging analyses.

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REFERENCES

- Arce, E., Simmons, A. N., Lovero, K. L., Stein, M. B., and Paulus, M. P. (2008). Escitalopram effects on insula and amygdala BOLD activation during emotional processing. *Psychopharmacology* 196, 661–672. doi: 10.1007/s00213-007-1004-8
- Ashton, C. H., and Moore, P. B. (2011). Endocannabinoid system dysfunction in mood and related disorders. *Acta Psychiatr. Scand.* 124, 250–261. doi: 10.1111/j.1600-0447.2011.01687.x
- Auer, R., Vittinghoff, E., Yaffe, K., Kunzi, A., Kertesz, S. G., Levine, D. A., et al. (2016). Association between lifetime marijuana use and cognitive function in middle age: the coronary artery risk development in young adults (CARDIA) study. *JAMA Intern. Med.* 176, 352–361. doi: 10.1001/jamainternmed.2015.7841
- Batalla, A., Bhattacharyya, S., Yücel, M., Fusar-Poli, P., Crippa, J. A., Nogué, S., et al. (2013). Structural and functional imaging studies in chronic cannabis users: a systematic review of adolescent and adult findings. *PLOS ONE* 8:e55821. doi: 10.1371/journal.pone.0055821
- Becker, M. P., Collins, P. F., and Luciana, M. (2014). Neurocognition in college-aged daily marijuana users. *J. Clin. Exp. Neuropsychol.* 36, 379–398. doi: 10.1080/13803395.2014.893996
- Befort, K. (2015). Interactions of the opioid and cannabinoid systems in reward: insights from knockout studies. *Front. Pharmacol.* 6:6. doi: 10.3389/fphar.2015.00006
- Bell, E. C., Willson, M. C., Wilman, A. H., Dave, S., and Silverstone, P. H. (2005). Differential effects of chronic lithium and valproate on brain activation in healthy volunteers. *Hum. Psychopharmacol.* 20, 415–424. doi: 10.1002/hup.710
- Bhattacharyya, S., Falkenberg, I., Martin-Santos, R., Atakan, Z., Crippa, J. A., Giampietro, V., et al. (2015). Cannabinoid modulation of functional connectivity within regions processing attentional salience. *Neuropsychopharmacology* 40, 1343–1352. doi: 10.1038/npp.2014.258
- Bhattacharyya, S., Morrison, P. D., Fusar-Poli, P., Martin-Santos, R., Borgwardt, S., Winton-Brown, T., et al. (2010). Opposite effects of delta-9-tetrahydrocannabinol and cannabidiol on human brain function and psychopathology. *Neuropsychopharmacology* 35, 764–774. doi: 10.1038/npp.2009.184
- Bilkei-Gorzo, A., Albayram, O., Draftehn, A., Michel, K., Pianova, A., Oppenheimer, H., et al. (2017). A chronic low dose of Δ9-tetrahydrocannabinol (THC) restores cognitive function in old mice. *Nat. Med.* 23, 782–787. doi: 10.1038/nm.4311
- Bonn-Miller, M. O., Boden, M. T., Bucossi, M. M., and Babson, K. A. (2014). Self-reported cannabis use characteristics, patterns and helpfulness among medical cannabis users. *Am. J. Drug Alcohol Abuse* 40, 23–30. doi: 10.3109/00952990.2013.821477
- Borgwardt, S. J., Allen, P., Bhattacharyya, S., Fusar-Poli, P., Crippa, J. A., Seal, M. L., et al. (2008). Neural basis of delta-9-tetrahydrocannabinol and cannabidiol: effects during response inhibition. *Biol. Psychiatry* 64, 966–973. doi: 10.1016/j.biopsych.2008.05.011
- Broyd, S. J., van Hell, H. H., Beale, C., Yücel, M., and Solowij, N. (2016). Acute and chronic effects of cannabinoids on human cognition – a systematic review. *Biol. Psychiatry* 79, 557–567. doi: 10.1016/j.biopsych.2015.12.002
- Bunce, S. C., Harris, J. D., Bixler, E. O., Taylor, M., Muelly, E., Deneke, E., et al. (2015). Possible evidence for re-regulation of HPA axis and brain reward systems over time in treatment in prescription opioid-dependent patients. *J. Addict. Med.* 9, 53–60. doi: 10.1097/ADM.0000000000000087
- Bush, G., Holmes, J., Shin, L. M., Surman, C., Makris, N., Mick, E., et al. (2013). Atomoxetine increases fronto-parietal functional MRI activation in attention-deficit/hyperactivity disorder: a pilot study. *Psychiatry Res.* 211, 88–91. doi: 10.1016/j.psychresns.2012.09.004
- Bush, G., and Shin, L. M. (2006). The multi-source interference task: an fMRI task that reliably activates the cingulo-frontal-parietal cognitive/attention network. *Nat. Protoc.* 1, 308–313. doi: 10.1038/nprot.2006.48
- Bush, G., Shin, L. M., Holmes, J., Rosen, B. R., and Vogt, B. A. (2003). The multi-source interference task: validation study with fMRI in individual subjects. *Mol. Psychiatry* 8, 60–70. doi: 10.1038/sj.mp.4001217
- Churchwell, J. C., Lopez-Larson, M., and Yurgelun-Todd, D. A. (2010). Altered frontal cortical volume and decision making in adolescent cannabis users. *Front. Psychol.* 1:225. doi: 10.3389/fpsyg.2010.00225
- Clark, D. B., Chung, T., Thatcher, D. L., Pajtek, S., and Long, E. C. (2012). Psychological dysregulation, white matter disorganization and substance use disorders in adolescence. *Addiction* 107, 206–214. doi: 10.1111/j.1360-0443.2011.03566.x
- Colizzi, M., and Bhattacharyya, S. (2017). Does cannabis composition matter? Differential effects of delta-9-tetrahydrocannabinol and cannabidiol on human cognition. *Curr. Addict. Rep.* 4, 62–74. doi: 10.1007/s40429-017-0142-2
- Cousijn, J., Watson, P., Koenders, L., Vingerhoets, W. A., Goudriaan, A. E., and Wiers, R. W. (2013). Cannabis dependence, cognitive control and attentional bias for cannabis words. *Addict. Behav.* 38, 2825–2832. doi: 10.1016/j.addbeh.2013.08.011
- Cousijn, J., Wiers, R. W., Ridderinkhof, K. R., van den Brink, W., Veltman, D. J., and Goudriaan, A. E. (2014). Effect of baseline cannabis use and working-memory network function on changes in cannabis use in heavy cannabis users: a prospective fMRI study. *Hum. Brain Mapp.* 35, 2470–2482. doi: 10.1002/hbm.22342
- Crean, R. D., Crane, N. A., and Mason, B. J. (2011). An evidence based review of acute and long-term effects of cannabis use on executive cognitive functions. *J. Addict. Med.* 5, 1–8. doi: 10.1097/ADM.0b013e31820c23fa
- Dahlgren, M. K., Sagar, K. A., Racine, M. T., Dreiman, M. W., and Gruber, S. A. (2016). Marijuana use predicts cognitive performance on tasks of executive function. *J. Stud. Alcohol Drugs* 77, 298–308. doi: 10.15288/jasad.2016.77.298
- Del-Ben, C. M., Deakin, J. F. W., McKie, S., Delvai, N. A., Williams, S. R., and Anderson, I. M. (2005). The effect of citalopram pretreatment on neuronal responses to neuropsychological tasks in normal volunteers: an fMRI study. *Neuropsychopharmacology* 30, 1724–1734. doi: 10.1038/sj.npp.1300728
- Dougherty, D. M., Mathias, C. W., Dawes, M. A., Furr, R. M., Charles, N. E., Liguori, A., et al. (2013). Impulsivity, attention, memory, and decision-making among adolescent marijuana users. *Psychopharmacology* 226, 307–319. doi: 10.1007/s00213-012-2908-5
- Egerton, A., Allison, C., Brett, R. R., and Pratt, J. A. (2006). Cannabinoids and prefrontal cortical function: insights from preclinical studies. *Neurosci. Biobehav. Rev.* 30, 680–695. doi: 10.1016/j.neubiorev.2005.12.002
- Ehrenreich, H., Rinn, T., Kunert, H. J., Moeller, M. R., Poser, W., Schilling, L., et al. (1999). Specific attentional dysfunction in adults following early start of cannabis use. *Psychopharmacology* 142, 295–301. doi: 10.1007/s002130050892

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2017.00983/full#supplementary-material>

- Englund, A., Atakan, Z., Kralj, A., Tunstall, N., Murray, R., and Morrison, P. (2016). The effect of five day dosing with THCV on THC-induced cognitive, psychological and physiological effects in healthy male human volunteers: a placebo-controlled, double-blind, crossover pilot trial. *J. Psychopharmacol.* 30, 140–151. doi: 10.1177/0269881115615104
- Englund, A., Morrison, P. D., Nottage, J., Hague, D., Kane, F., Bonaccorso, S., et al. (2013). Cannabidiol inhibits THC-elicited paranoid symptoms and hippocampal-dependent memory impairment. *J. Psychopharmacol.* 27, 19–27. doi: 10.1177/0269881112460109
- Fried, P. A., Watson, B., and Gray, R. (2005). Neurocognitive consequences of marihuana—a comparison with pre-drug performance. *Neurotoxicol. Teratol.* 27, 231–239. doi: 10.1016/j.ntt.2004.11.003
- Giedd, J. N., Blumenthal, J., Jeffries, N. O., Castellanos, F. X., Liu, H., Zijdenbos, A., et al. (1999). Brain development during childhood and adolescence: a longitudinal MRI study. *Nat. Neurosci.* 2, 861–863. doi: 10.1038/13158
- Gruber, S. A., Dahlgren, M. K., Sagar, K. A., Gönenc, A., and Killgore, W. D. (2012a). Age of onset of marijuana use impacts inhibitory processing. *Neurosci. Lett.* 511, 89–94. doi: 10.1016/j.neulet.2012.01.039
- Gruber, S. A., Dahlgren, M. K., Sagar, K. A., Gonenc, A., Norris, L., Cohen, B. M., et al. (2017). Decreased Cingulate Cortex activation during cognitive control processing in bipolar disorder. *J. Affect. Disord.* 213, 86–95. doi: 10.1016/j.jad.2017.02.003
- Gruber, S. A., Sagar, K. A., Dahlgren, M. K., Racine, M., and Lukas, S. E. (2012b). Age of onset of marijuana use and executive function. *Psychol. Addict. Behav.* 26, 496–506. doi: 10.1037/a0026269
- Gruber, S. A., Sagar, K. A., Dahlgren, M. K., Racine, M. T., Smith, R. T., and Lukas, S. E. (2016). Splendor in the grass? A pilot study assessing the impact of medical marijuana on executive function. *Front. Pharmacol.* 7:355. doi: 10.3389/fphar.2016.00355
- Gruber, S. A., and Yurgelun-Todd, D. A. (2005). Neuroimaging of marijuana smokers during inhibitory processing: a pilot investigation. *Brain Res. Cogn. Brain Res.* 23, 107–118. doi: 10.1016/j.cogbrainres.2005.02.016
- Hanson, K. L., Thayer, R. E., and Tapert, S. F. (2014). Adolescent marijuana users have elevated risk-taking on the balloon analog risk task. *J. Psychopharmacol.* 28, 1080–1087. doi: 10.1177/0269881114550352
- Harding, I. H., Solowij, N., Harrison, B. J., Takagi, M., Lorenzetti, V., Lubman, D. I., et al. (2012). Functional connectivity in brain networks underlying cognitive control in chronic cannabis users. *Neuropsychopharmacology* 37, 1923–1933. doi: 10.1038/npp.2012.39
- Harrison, B. J., Yucel, M., Fornito, A., Wood, S. J., Seal, M. L., Clarke, K., et al. (2007). Characterizing anterior cingulate activation in chronic schizophrenia: a group and single-subject fMRI study. *Acta Psychiatr. Scand.* 116, 271–279. doi: 10.1111/j.1600-0447.2007.01002.x
- Heckers, S., Weiss, A. P., Deckersbach, T., Goff, D. C., Morecraft, R. J., and Bush, G. (2004). Anterior cingulate cortex activation during cognitive interference in schizophrenia. *Am. J. Psychiatry* 161, 707–715. doi: 10.1176/appi.ajp.161.4.707
- Hill, M. N., and McEwen, B. S. (2010). Involvement of the endocannabinoid system in the neurobehavioural effects of stress and glucocorticoids. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 34, 791–797. doi: 10.1016/j.pnpbp.2009.11.001
- Jacobus, J., Squeglia, L. M., Infante, M. A., Castro, N., Brumback, T., Meruelo, A. D., et al. (2015). Neuropsychological performance in adolescent marijuana users with co-occurring alcohol use: a three-year longitudinal study. *Neuropsychology* 29, 829–843. doi: 10.1037/neu0000203
- Jacobus, J., and Tapert, S. F. (2014). Effects of cannabis on the adolescent brain. *Curr. Pharm. Des.* 20, 2186–2193. doi: 10.2174/13816128113199990426
- Kowal, M. A., Hazekamp, A., Colzato, L. S., van Steenbergen, H., van der Wee, N. J., Durieux, J., et al. (2015). Cannabis and creativity: highly potent cannabis impairs divergent thinking in regular cannabis users. *Psychopharmacology (Berl.)* 232, 1123–1134. doi: 10.1007/s00213-014-3749-1
- Levine, A., Clementza, K., Rynn, M., and Lieberman, J. (2017). Evidence for the risks and consequences of adolescent cannabis exposure. *J. Am. Acad. Child Adolesc. Psychiatry* 56, 214–225. doi: 10.1016/j.jaac.2016.12.014
- Lezak, M. D., Howieson, D. B., Loring, D. W., Hannay, J., and Fischer, J. S. (2004). *Neuropsychological Assessment*, 4th Edn. New York, NY: Oxford University Press, 337–374.
- Lisdahl, K. M., Gilbart, E. R., Wright, N. E., and Shollenbarger, S. (2013). Dare to delay? The impacts of adolescent alcohol and marijuana use on cognition, brain structure, and function. *Front. Psychiatry* 4:53. doi: 10.3389/fpsyg.2013.00053
- Lisdahl, K. M., and Price, J. S. (2012). Increased marijuana use and gender predict poorer cognitive functioning in adolescents and emerging adults. *J. Int. Neuropsychol. Soc.* 18, 678–688. doi: 10.1017/S1355617712000276
- Lisdahl, K. M., Wright, N. E., Kirchner-Medina, C., Maple, K. E., and Shollenbarger, S. (2014). Considering cannabis: the effects of regular cannabis use on neurocognition in adolescents and young adults. *Curr. Addict. Rep.* 1, 144–156. doi: 10.1007/s40429-014-0019-6
- Lorenzetti, V., Solowij, N., and Yucel, M. (2016). The role of cannabinoids in neuroanatomic alterations in cannabis users. *Biol. Psychiatry* 79, e17–e31. doi: 10.1016/j.biopsych.2015.11.013
- Macher, R. B., and Earleywine, M. (2012). Enhancing neuropsychological performance in chronic cannabis users: the role of motivation. *J. Clin. Exp. Neuropsychol.* 34, 405–415. doi: 10.1080/13803395.2011.646957
- Maldjian, J. A., Laurienti, P. J., Kraft, R. A., and Burdette, J. H. (2003). An automated method for neuroanatomic and cytoarchitectonic atlas-based interrogation of fMRI data sets. *Neuroimage* 19, 1233–1239. doi: 10.1016/S1053-8119(03)00169-1
- McGuire, P., Robson, P., Cubala, W. J., Vasile, D., Morrison, P. D., Barron, R., et al. (2017). Cannabidiol (CBD) as an adjunctive therapy in schizophrenia: a multicenter randomized controlled trial. *Am. J. Psychiatry* doi: 10.1176/appi.ajp.2017.17030325 [Epub ahead of print].
- Medina, K. L., McQueeny, T., Nagel, B. J., Hanson, K. L., Yang, T. T., and Tapert, S. F. (2009). Prefrontal cortex morphometry in abstinent adolescent marijuana users: subtle gender effects. *Addict. Biol.* 14, 457–468. doi: 10.1111/j.1369-1600.2009.00166.x
- Medina, K. L., Nagel, B. J., and Tapert, S. F. (2010). Abnormal cerebellar morphometry in abstinent adolescent marijuana users. *Psychiatry Res.* 182, 152–159. doi: 10.1016/j.psychres.2009.12.004
- Morgan, C. J., Schafer, G., Freeman, T. P., and Curran, H. V. (2010). Impact of cannabidiol on the acute memory and psychotomimetic effects of smoked cannabis: naturalistic study. *Br. J. Psychiatry* 197, 285–290. doi: 10.1192/bjp.bp.109.077503
- Moriarty, O., McGuire, B. E., and Finn, D. P. (2011). The effect of pain on cognitive function: a review of clinical and preclinical research. *Prog. Neurobiol.* 93, 385–404. doi: 10.1016/j.pneurobio.2011.01.002
- Murphy, S. E., Norbury, R., O'Sullivan, U., Cowen, P. J., and Harmer, C. J. (2009). Effect of a single dose of citalopram on amygdala response to emotional faces. *Br. J. Psychiatry* 194, 535–540. doi: 10.1192/bjp.bp.108.056093
- Nader, D. A., and Sanchez, Z. M. (2017). Effects of regular cannabis use on neurocognition, brain structure, and function: a systematic review of findings in adults. *Am. J. Drug Alcohol Abuse* doi: 10.1080/00952990.2017.1306746 [Epub ahead of print].
- Nunberg, H., Kilmer, B., Pacula, R. L., and Burgdorf, J. (2011). An analysis of applicants presenting to a medical marijuana specialty practice in California. *J. Drug Policy Anal.* 4:1. doi: 10.2202/1941-2851.1017
- Park, J., and Wu, L. (2017). Prevalence, reasons, perceived effects, and correlates of medical marijuana use: a review. *Drug Alcohol Depend.* 177, 1–13. doi: 10.1016/j.drugalcdep.2017.03.009
- Paulus, M. P., Feinstein, J. S., Castilla, G., Simmons, A. N., and Stein, M. B. (2005). Dose-dependent decrease of activation in bilateral amygdala and insula by lorazepam during emotion processing. *Arch. Gen. Psychiatry* 62, 282–288. doi: 10.1001/archpsyc.62.3.282
- Price, J. S., McQueeny, T., Shollenbarger, S., Browning, E. L., Wieser, J., and Lisdahl, K. M. (2015). Effects of marijuana use on prefrontal and parietal volumes and cognition in emerging adults. *Psychopharmacology* 232, 2939–2950. doi: 10.1007/s00213-015-3931-0
- Ramaekers, J. G., Kauert, G., van Ruitenbeek, P., Theunissen, E. L., Schneider, E., and Moeller, M. R. (2006). High-potency marijuana impairs executive function and inhibitory motor control. *Neuropsychopharmacology* 31, 2296–2303. doi: 10.1038/sj.npp.1301068
- Reinarman, C., Nunberg, H., Lantheir, F., and Heddleston, T. (2011). Who are medical marijuana patients? Population and characteristics from nine California. *J. Psychoact. Drugs* 43, 128–135. doi: 10.1080/02791072.2011.587700
- Rong, C., Lee, Y., Carmona, N. E., Cha, D. S., Raggatt, R. M., Rosenblat, J. D., et al. (2017). Cannabidiol in medical marijuana: research vistas and potential opportunities. *Pharmacol. Res.* 121, 213–218. doi: 10.1016/j.phrs.2017.05.005

- Sagar, K. A., Dahlgren, M. K., Gönenç, A., Racine, M. T., Dreman, M. W., and Gruber, S. A. (2015). The impact of initiation: early onset marijuana smokers demonstrate altered Stroop performance and brain activation. *Dev. Cogn. Neurosci.* 16, 84–92. doi: 10.1016/j.dcn.2015.03.003
- Shin, L. M., Bush, G., Milad, M. R., Lasko, N. B., Brohawn, K. H., Hughes, K. C., et al. (2011). Exaggerated activation of dorsal anterior cingulate cortex during cognitive interference: a monozygotic twin study of posttraumatic stress disorder. *Am. J. Psychiatry* 168, 979–985. doi: 10.1176/appajp.2011.09121812
- Shuster, R. M., Hoeppner, S. S., Ewins, A. E., and Gilman, J. M. (2016). Early onset marijuana use is associated with learning inefficiencies. *Neuropsychology* 30, 405–415. doi: 10.1037/neu0000281
- Sobell, L. C., Sobell, M. B., Leo, G. I., and Cancilla, A. (1998). Reliability of a timeline method: assessing normal drinkers' reports of recent drinking and a comparative evaluation across several populations. *Br. J. Addict.* 83, 393–402. doi: 10.1111/j.1360-0443.1988.tb00485.x
- Solowij, N., and Battisti, R. (2008). The chronic effects of cannabis on memory in humans: a review. *Curr. Drug Abuse Rev.* 1, 81–98. doi: 10.2174/1874473710801010081
- Solowij, N., Jones, K. A., Rozman, M. E., Davis, S. M., Ciarrochi, J., Heaven, P. C., et al. (2012). Reflection impulsivity in adolescent cannabis users: a comparison with alcohol-using and non-substance-using adolescents. *Psychopharmacology* 219, 575–586. doi: 10.1007/s00213-011-2486-y
- Tait, R. J., Mackinnon, A., and Christensen, H. (2011). Cannabis use and cognitive function: 8-year trajectory in a young adult cohort. *Addiction* 106, 2195–2203. doi: 10.1111/j.1360-0443.2011.03574.x
- Tapert, S. F., Schweinsburg, A. D., Drummond, S. P., Paulus, M., Brown, S. A., Yang, T. T., et al. (2007). Functional MRI of inhibitory processing in abstinent adolescent marijuana users. *Psychopharmacology* 194, 173–183. doi: 10.1007/s00213-007-0823-y
- Vytal, K. E., Cornwell, B. R., Letkiewicz, A. M., Arkin, N. E., and Grillon, C. (2013). The complex interaction between anxiety and cognition: insight from spatial and verbal working memory. *Front. Hum. Neurosci.* 7:93. doi: 10.3389/fnhum.2013.00093
- Ward, B. D. (2000). *Simultaneous Inference for fMRI Data. AFNI 3d Deconvolve Documentation*. Milwaukee, WI: Medical College of Wisconsin.
- Wechsler, D. (1999). *Wechsler Abbreviated Scale of Intelligence*. San Antonio, TX: The Psychological Corporation.
- Weinstein, A., Livny, A., and Weizman, A. (2016). Brain imaging studies on the cognitive, pharmacological and neurobiological effects of cannabis in humans. *Curr. Pharm. Des.* 22, 6366–6379. doi: 10.2174/1381612822666160822151323
- Winton-Brown, T. T., Allen, P., Bhattacharyya, S., Borgwardt, S. J., Fusar-Poli, P., Crippa, J. A., et al. (2011). Modulation of auditory and visual processing by delta-9-tetrahydrocannabinol and cannabidiol: an fMRI study. *Neuropsychopharmacology* 36, 1340–1348. doi: 10.1038/npp.2011.17
- Yücel, M., Lorenzetti, V., Suo, C., Zalesky, A., Fornito, A., Takagi, M. J., et al. (2016). Hippocampal harms, protection and recovery following regular cannabis use. *Transl. Psychiatry* 6:e710. doi: 10.1038/tp.2015.201
- Yucel, M., Lubman, D., Harrison, B., Fornito, A., Allen, N., Wellard, R., et al. (2007). A combined spectroscopic and functional MRI investigation of the dorsal anterior cingulate region in opiate addiction. *Mol. Psychiatry* 12, 691–702. doi: 10.1038/sj.mp.4001955
- Zuardi, A. W. (2008). Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action. *Rev. Bras. Psiquiatr.* 30, 271–280. doi: 10.1590/S1516-44462008000300015

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Some Prospective Alternatives for Treating Pain: The Endocannabinoid System and Its Putative Receptors GPR18 and GPR55

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Background: Marijuana extracts (cannabinoids) have been used for several millennia for pain treatment. Regarding the site of action, cannabinoids are highly promiscuous molecules, but only two cannabinoid receptors (CB_1 and CB_2) have been deeply studied and classified. Thus, therapeutic actions, side effects and pharmacological targets for cannabinoids have been explained based on the pharmacology of cannabinoid CB_1/CB_2 receptors. However, the accumulation of confusing and sometimes contradictory results suggests the existence of other cannabinoid receptors. Different orphan proteins (e.g., GPR18, GPR55, GPR119, etc.) have been proposed as putative cannabinoid receptors. According to their expression, GPR18 and GPR55 could be involved in sensory transmission and pain integration.

Methods: This article reviews select relevant information about the potential role of GPR18 and GPR55 in the pathophysiology of pain.

Results: This work summarized novel data supporting that, besides cannabinoid CB_1 and CB_2 receptors, GPR18 and GPR55 may be useful for pain treatment.

Conclusion: There is evidence to support an antinociceptive role for GPR18 and GPR55.

Keywords: GPR18, GPR55, endocannabinoid system, cannabinoid receptors, pain

PHYSIOLOGY OF PAIN

Adaptive Function of Pain

Pain involves unpleasant sensations in response to real or potential tissue damage (Basbaum et al., 2009). Usually, pain unleashes a signal alert to prevent extensive injury by promoting defensive (passive and/or active) actions against the noxious (nociceptive) stimuli. Thus, pain is considered a protective and adaptive mechanism. However, pain may become persistent

and pathological without a recognized protective or adaptive mechanism. When this happens, it affects the quality of life of patients and their social environment. Hence, pathological pain is an important medical problem causing distress and disability that requires prompt clinical investigation and treatment (Julius and Basbaum, 2001; Moffat and Rae, 2011). On the other hand, considering that tissue damage is not always the main origin of pain, cognitive perception and somatic sensation should be considered as related but different phenomena. Cognitive perception involves a psychological component frequently related with emotional experiences. Therefore, pain may be cataloged as a subjective event that requires patient awareness (Basbaum and Woolf, 1999; Julius and Basbaum, 2001; Walker and Hohmann, 2005).

Sensory System: Anatomical and Functional View

The terminal endings of primary afferent neurons whose cell bodies are located in the dorsal root ganglia (DRG) and trigeminal ganglia (TG) are responsible for the transmission of multiple peripheral stimuli (proprioceptive or nociceptive) to the central nervous system (Julius and Basbaum, 2001; Walker and Hohmann, 2005). In the case of nociceptive transmission, two main types of pseudo-unipolar nociceptive neurons are found in those ganglia: (1) non-myelinated small diameter and multimodal C-fibers, which conduct electrical impulses at low speed (~ 1 m/s), sensing and transducing thermal, chemical and mechanical stimuli; and (2) thinly myelinated A δ -fibers that show fast conduction velocity (~ 5 –30 m/s), sensing mechanical and thermal stimuli (Moffat and Rae, 2011). These primary afferent nociceptive fibers sense the peripheral nociceptive environment and send the nociceptive information to the spinal dorsal horn where they make a synapse with second order neurons, which convey neuronal firing to supraspinal sites where the action potentials are decoded and perceived as pain. At the peripheral level, there are several channels and receptors involved in the initiation of nociceptive transmission, such as the transient receptor potential vanilloid type 1 (TRPV1) channel, tetrodotoxin-resistant (Na^+ -TTXr) voltage-gated sodium (Na^+) channels, purinergic P₂X receptors, serotonin (5-HT₃) channel receptor, and calcium (Ca^{2+}) channels, among others.

The nociceptive signal from the peripheral nociceptive fibers is directed toward a second order neuron into the spinal cord, and then the electrical signal is conducted to the brain cortex mainly through the antero-lateral pathway tract where the signal is interpreted as a painful sensation (Snider and McMahon, 1998; Steeds, 2009; Fabbro and Crescentini, 2014). In fact, several sensorial components such as stimuli identification, location, and emotional components are codified in the cortex (Albe-Fessard et al., 1985). The diversity of peripheral and central regions and mechanisms implicated made the control of nociception and pain a complex challenge. Finally, we must keep in mind that nociceptive transmission could be endogenously modulated. For instance, the spinal cord, which is the first relay of nociceptive transmission, could be modulated by diverse neuromodulators (noradrenergic, serotonergic, opioidergic, and oxytocinergic) (for

references see Mason, 2001; Vanegas and Schaible, 2004; Loyd and Murphy, 2009; Condés-Lara et al., 2015; Llorca-Torralba et al., 2016) that may diminish or increase the noxious sensation. Nevertheless, these modulatory systems exist along the noxious pathways, including the cortical station. So, the modulation of nociceptive transmission is complex and involves an array of neurotransmitters, neuromodulators and a wide variety of specific and non-specific receptors, which are dysregulated during pathological pain states (Heinricher, 2016).

Classic Treatments for Pain

Pain treatment can be categorized as pharmacologic and non-pharmacologic. In the first case, there are a variety of druggable targets in both central and peripheral nervous system commonly used for pain treatment. Analgesics are classified as: (i) non-opioid analgesics; (ii) opioid analgesics; and (iii) adjuvant analgesics (Figure 1). The most frequently non-opioid analgesics used are non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen and celecoxib. The primary mechanism of action of NSAIDs is through the inhibition of the cyclooxygenase enzymes (COX) by consequently decreasing the action of prostaglandins and their sensitizing properties. Opioid-like drugs, such as morphine, ameliorate pain by modulating the cellular excitability at the supraspinal, spinal and peripheral level through activation of opioid receptors (μ -, δ -, and κ -opioid receptors). Furthermore, opioids could enhance descending inhibitory pathways and modify the sensory and affective components of pain. In the case of adjuvants, local anesthetics (e.g., lidocaine) stop the electrical impulse by blocking voltage-gated sodium (Na^+) channels. Tricyclic and noradrenaline-reuptake inhibitors act by maintaining and/or augmenting the monoamine levels in descending tracts and anticonvulsants decrease the synaptic transmission affecting neuronal excitability (Basbaum and Woolf, 1999; Sinha et al., 2017).

Non-steroidal Anti-inflammatory Drugs

Non-steroidal anti-inflammatory drugs are substances that inhibit a component of the inflammatory cascade and, thence, are an important therapeutic option for non-steroid-based pain treatment. Briefly, these compounds (with exception of acetaminophen) have anti-inflammatory, antipyretic, and analgesic effects by inhibiting COX activity. At this point, we must keep in mind that the COX enzymes have at least three isoforms (COX-1, COX-2 and COX-3) and the non-selective NSAIDs act to block COX-1 and COX-2 indistinctly, favoring gastrointestinal and renal side effects (mediated by COX-1 inhibition). These side effects are particularly common in the elderly, who are most likely to experience chronic pain (Griffin et al., 1991; Buffum and Buffum, 2000; Horl, 2010). To minimize the side effects, selective COX-2 inhibitors have arrived at clinical practice. Unfortunately, several clinical trials have shown that these inhibitors also increase harmful cardiovascular effects (Bhosale et al., 2015).

Opioid-Based Treatments

Opioid analgesics act in the central nervous system and are typically prescribed to patients suffering chronic pain

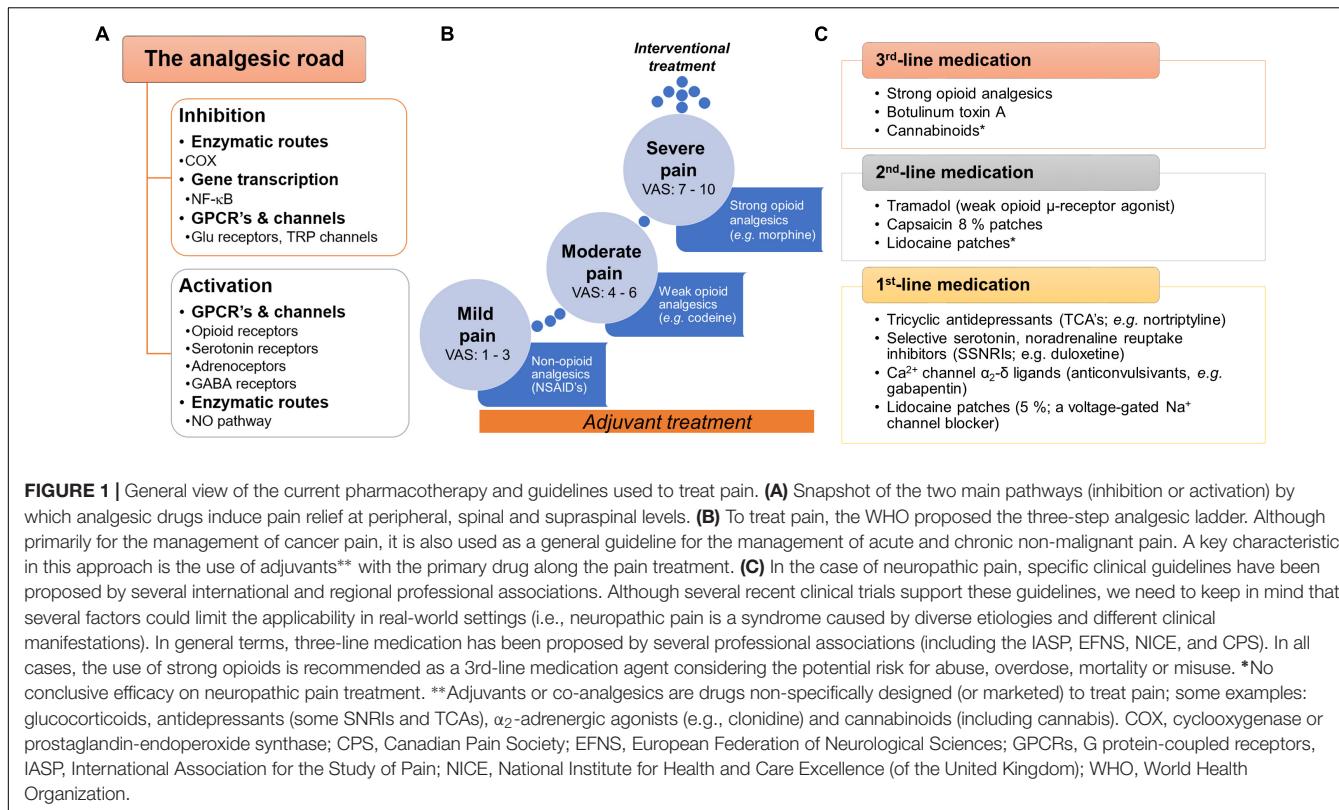


FIGURE 1 | General view of the current pharmacotherapy and guidelines used to treat pain. **(A)** Snapshot of the two main pathways (inhibition or activation) by which analgesic drugs induce pain relief at peripheral, spinal and supraspinal levels. **(B)** To treat pain, the WHO proposed the three-step analgesic ladder. Although primarily for the management of cancer pain, it is also used as a general guideline for the management of acute and chronic non-malignant pain. A key characteristic in this approach is the use of adjuvants** with the primary drug along the pain treatment. **(C)** In the case of neuropathic pain, specific clinical guidelines have been proposed by several international and regional professional associations. Although several recent clinical trials support these guidelines, we need to keep in mind that several factors could limit the applicability in real-world settings (i.e., neuropathic pain is a syndrome caused by diverse etiologies and different clinical manifestations). In general terms, three-line medication has been proposed by several professional associations (including the IASP, EFNS, NICE, and CPS). In all cases, the use of strong opioids is recommended as a 3rd-line medication agent considering the potential risk for abuse, overdose, mortality or misuse. *No conclusive efficacy on neuropathic pain treatment. **Adjuvants or co-analgesics are drugs non-specifically designed (or marketed) to treat pain; some examples: glucocorticoids, antidepressants (some SNRIs and TCAs), α_2 -adrenergic agonists (e.g., clonidine) and cannabinoids (including cannabis). COX, cyclooxygenase or prostaglandin-endoperoxide synthase; CPS, Canadian Pain Society; EFNS, European Federation of Neurological Sciences; GPCRs, G protein-coupled receptors; IASP, International Association for the Study of Pain; NICE, National Institute for Health and Care Excellence (of the United Kingdom); WHO, World Health Organization.

refractory to non-opioid treatment. Despite their well-known side effects (sedation, nausea, vomiting, constipation, pruritus and respiratory depression), opioids are widely accepted as effective for acute pain as well as cancer pain. This group of drugs have high abuse liability and are also toxic in elevated doses. For instance, from 1999 to 2014, more than 165,000 persons died of overdose related to opioids in the United States. In 2013, an estimated of 1.9 million people abused or were dependent on opioid pain medication (Dowell et al., 2016). Moreover, placebo-controlled trials indicate that, on average, opioids do not result in a clinically significant reduction of chronic pain symptoms (Martell et al., 2007), and even in cases where opioid analgesia is adequate for the individual patient, analgesic effects are typically not maintained during the long-term opioid pharmacotherapy due to pharmacokinetic or pharmacodynamic tolerance (Ballantyne and Shin, 2008; Dumas and Pollack, 2008). Eventually, chronic exposure to opioids results in hyperalgesia (Chu et al., 2008).

Antidepressants

Antidepressant drugs have been used as analgesics in chronic pain disorders for decades (Mico et al., 2006). Their pharmacological mechanisms have been associated with the ability to block 5-hydroxytryptamine (serotonin or 5-HT) and noradrenaline re-uptake and consequently with an increase of the activity of the endogenous analgesic system. Tricyclic antidepressants (TCAs) (e.g., amitriptyline and imipramine), tetracyclic antidepressants (TeCAs)

(e.g., amoxapine, maprotiline) and the selective serotonin-norepinephrine reuptake inhibitors (SNRIs) (e.g., duloxetine and venlafaxine) are traditionally used to treat chronic pain (Mika et al., 2013). TCAs have been shown to be effective for different neuropathic pain conditions in randomized controlled trials (Finnerup et al., 2010). TCAs are generally reasonably well-tolerated but high doses are associated with a high risk of sudden cardiac death (Ray et al., 2004). The SNRIs duloxetine and venlafaxine have a well-documented efficacy in painful poly-neuropathy (Finnerup et al., 2010). SNRIs are generally well tolerated. However, the most common side-effects reported are nausea, somnolence, dizziness, constipation, anorexia, dry mouth, hyperhidrosis, and sexual dysfunction (Stahl et al., 2005).

Anticonvulsants

Gabapentin and pregabalin are anticonvulsants with therapeutic activity against neuropathic pain (Rajapakse et al., 2015). Their analgesic mechanism has been associated to their binding to the $\alpha_2\delta_1$ subunit, which in turn blocks voltage-gated calcium (Ca^{2+})-channels at presynaptic sites (Gee et al., 1996) or NMDA receptors at post-synaptic neurons (Chen et al., 2018; Ma et al., 2018). Both drugs are well tolerated but the most common side-effects are somnolence and dizziness, peripheral edema, weight gain, nausea, vertigo, asthenia, dry mouth, and ataxia (Quintero, 2017). Other anticonvulsants used for pain relief are carbamazepine and its analog oxcarbazepine, lamotrigine and valproate. Lamotrigine is effective for central post-stroke pain (Vestergaard et al., 2001) and diabetic neuropathy (Eisenberg et al., 2001), but has failed to relieve pain in patients

with multiple sclerosis (Breuer et al., 2007) and neuropathic pain (Silver et al., 2007). Valproate also has a limited role in the treatment of neuropathic pain (Drewes et al., 1994; Otto et al., 2004; Agrawal et al., 2009).

Cannabinoids

One alternative for pain treatment came from Asia more than 3000 years ago: marijuana extracts (Li, 1974; Touw, 1981; Jensen et al., 2015). The utility of marijuana-based drugs for treating pain is explained by the existence of an ancient system of cellular control named the endocannabinoid system (ECS). Unfortunately, our knowledge about the physiology of the ECS is only partial (see below). In this review, we summarized novel data supporting that, apart from cannabinoid type-1 (CB_1) and cannabinoid type-2 (CB_2) receptors, some putative cannabinoid receptors (i.e., GPR18 and GPR55) may be useful for pain treatment. This should allow researchers to focus their studies on developing endocannabinoid-based options as analgesics and anti-inflammatory drugs.

ENDOCANNABINOID AND PAIN

Endocannabinoid System: Generalities

Despite the ancient and well-known use of cannabis derivatives for pain management, medically recognized use of these compounds has largely subsided due to the lack of knowledge of its molecular pharmacology, its abuse for recreational purposes and additional undesirable effects, such as hypomotility and hypothermia (Crawley et al., 1993), impairments in executive function (Crean et al., 2011) and memory consolidation (Ranganathan and D'Souza, 2006). However, the identification of the major psychoactive component Δ^9 -tetrahydrocannabinol (Δ^9 -THC) (Gaoni and Mechoulam, 1964), and the subsequent isolation of cannabinoid receptors (CB_1 and CB_2 receptors, both G-proteins-coupled receptors linked to $G_{i/o}$ proteins) with high expression levels in the nervous system, led to an explosion of studies exploring the ECS and its regulatory functions in health and disease. Briefly, the ECS consists of endogenous cannabinoids (endocannabinoids, eCBs), cannabinoid receptors, enzymes responsible for synthesis and degradation of eCBs and all genes related to them (Mackie, 2008a,b).

In this context, although several cannabinoids are available, current literature about their potential use for pain treatment remains controversial (Davis, 2014). Indeed, as reviewed by Nurmikko et al. (2007) and Martin-Sánchez et al. (2009), Δ^9 -THC or Δ^9 -THC plus cannabidiol induced relief in only one among six to nine patients (number needed to treat, NNT = 6–9). Moreover, the number needed to harm (NNH) (motor and cognitive dysfunction and altered perception) ranged between five and eight. These data suggest that, apart from its low efficacy, Δ^9 THC could have a narrow therapeutic index. Nevertheless, the above cannabimimetic effects seem to be mainly mediated by CB_1 receptor activation, suggesting that other parts of the ECS could be druggable to treat pain. In addition, one of the physiological functions attributed to the eCBs is to suppress pain (Walker and Huang, 2002).

Endogenous Cannabinoids

The first eCB isolated in the brain was *N*-arachidonoyl ethanolamide (AEA), or anandamide (a name taken from the Sanskrit word Ananda, which means “bliss, joy,” and amide) (Devane et al., 1992; **Figure 2**). AEA is a fatty acid neuromodulator derived from the non-oxidative metabolism of arachidonic acid (AA). The second endocannabinoid identified was 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). As the search for endogenous Δ^9 -THC-like compounds continued, other bioactive lipids were extracted from animal tissues. These include noladin ether (Hanus et al., 2001), virodhamine (Porter et al., 2002) and *N*-arachidonoyl dopamine (NADA) (Huang et al., 2001).

The most widely investigated eCBs are anandamide and 2-AG. Indeed, anandamide is present in about 170-fold lower levels of brain tissue than 2-AG (Stella et al., 1997), and both lipidic derivatives activate cannabinoid CB_1 and CB_2 receptors. Certainly, anandamide shows preferential affinity for CB_1 ($K_i = 89$ nM) compared to CB_2 ($K_i = 371$ nM) receptors (Gauldie et al., 2001), whereas 2-AG is considered a full agonist at both CB_1 and CB_2 receptors (Sugiura and Waku, 2000). Nevertheless, it has been shown that AEA could activate the vanilloid type-1 receptor (TRPV1), which contributes to the many non- CB_1 -mediated effects (Zygmunt et al., 1999; Smart et al., 2000). Furthermore, AEA and other eCBs (palmitoylethanolamide [PEA] and oleylethanolamide [OEA]) also are agonists of the peroxisome proliferator-activated receptor α (PPAR α) (Fu et al., 2003; Bouaboula et al., 2005; Lo Verme et al., 2005). PEA also has a well-established role in pain modulation and inflammation in rodents (Jaggar et al., 1998; Calignano et al., 2001; Lo Verme et al., 2005; D'Agostino et al., 2007; González-Hernández et al., 2015), whereas in humans PEA treatment seems to relieve neuropathic pain (Calabro et al., 2010; Conigliaro et al., 2011; Gatti et al., 2012).

The eCBs are atypical neurotransmitters and/or neuromodulators. They are not stored in synaptic vesicles and are not released from presynaptic terminals via an exocytotic mechanism. In fact, their precursors exist in the cell membrane, are cleaved by specific enzymes “on demand” depending on intracellular calcium increase and are released from cells immediately after their production. The synthesis, release and deactivation of the endogenous cannabinoids are tightly regulated processes. As discussion of these processes is beyond the scope of this review, the interested reader is referred to several reviews on the topic (Howlett, 2002; Piomelli, 2003; Simon and Cravatt, 2006; Okamoto et al., 2007; Ueda et al., 2011; Luchicchi and Pistis, 2012).

Cannabinoid Receptors

To date, there are two known cannabinoid receptors that are part of the ECS, the CB_1 and CB_2 receptors. These receptors belong to the 7-transmembrane G-protein coupled receptors (GPCRs) primarily coupled to $G_{i/o}$ proteins that inhibit adenylyl cyclase (AC) and increase mitogen-activated protein kinase (MAPK) activity downstream of β -arrestin (Howlett, 2002; Vasileiou et al., 2013). Activation of these receptors triggers the inwardly

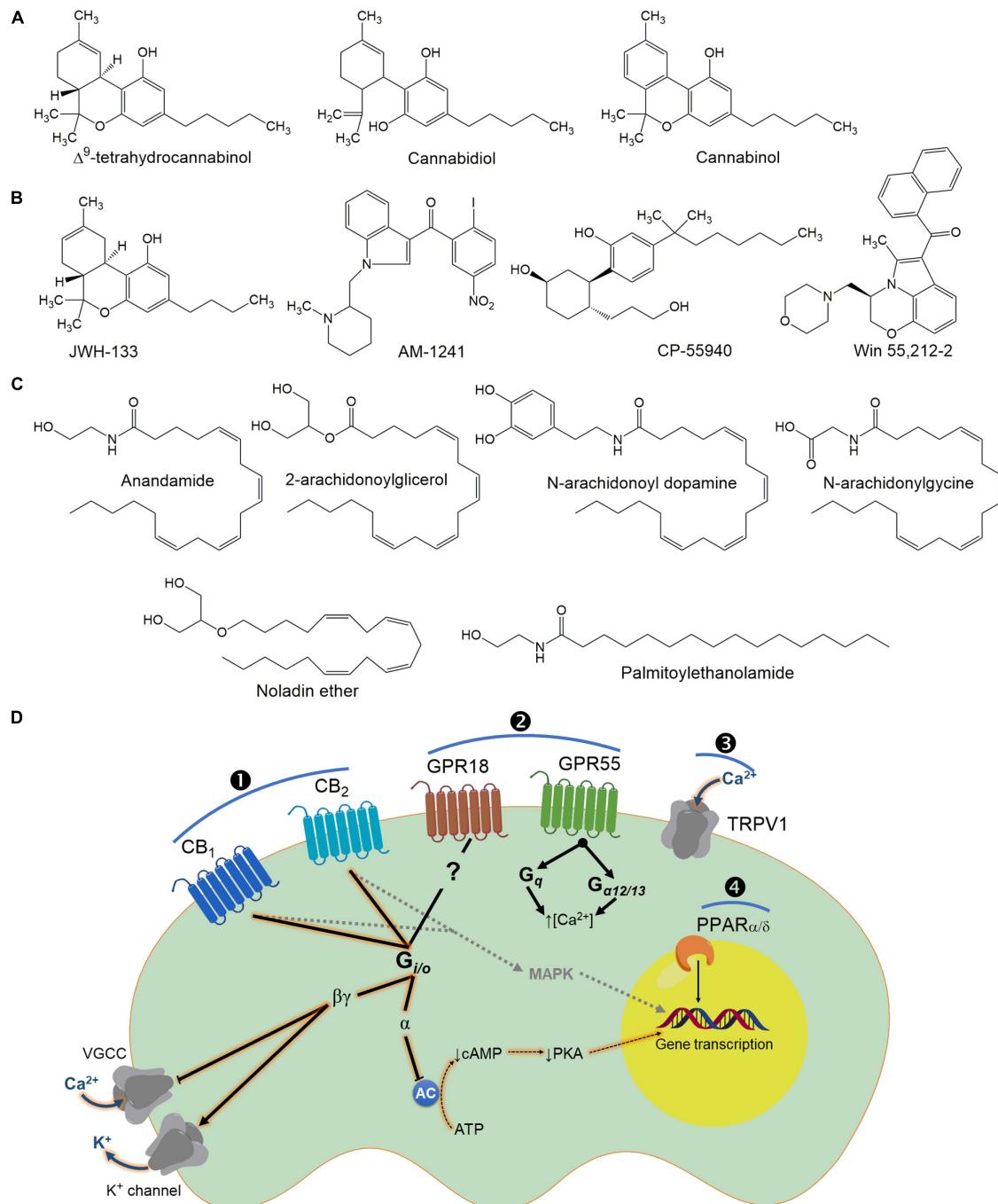


FIGURE 2 | Chemical structures of some plant (**A**), synthetic cannabinoids (**B**) and endocannabinoids (**C**) that bind to cannabinoid receptors (**D**). It is interesting to note that cannabinoids could activate intracellular pathways by direct activation of its receptors (protect **①** and **②**) or modulate other family receptors (protect **③** and **④**), which contribute to the biological effect of these molecules (particularly for the endocannabinoids). In general terms, classic cannabinoid receptors (CB₁ and CB₂) are GPCRs, which are canonically coupled to G_{i/o} proteins. Consequently, under CB_{1/2} receptors: (i) a decrease of adenylyl cyclase (AC) activity; (ii) an inactivation of Ca²⁺ channels; and (iii) activation of inwardly rectifying K⁺ channels are achieved. These are signal transduction systems associated with inhibition of neurotransmitter release. The inhibition of AC occurs via activation of G_{αi}-mediated signaling whereas G_{αo}-activation results in inhibition of voltage-dependent Ca²⁺ channels (VGCCs) through the release of associated βγ subunits (apparently CB₂ receptors are ineffective, compared with CB₁, for shifting ionic currents via βγ subunits). In addition to PKA inhibition, CB_{1/2} receptor signaling also leads to the downstream activation of MAPK which can regulate nuclear transcription factors and consequently expression of several genes. Note that GPR18 seems to be coupled to G_{i/o} proteins, whereas GPR55 has been associated with an increase of intracellular Ca²⁺ via G_{α12/13}. In the case of TRPV1 channels (a non-selective cation channel for Ca²⁺, Mg²⁺, and Na⁺ ions), it is well-known that agonist can be used rationally for the treatment of pain considering that this channel under constant activation desensitizes the nociceptive neuron. Finally, although not fully investigated, cannabinoid compounds could also activate PPAR_{α/δ}, which are involved in pain modulation and transmission.

rectifying potassium (K^+)-channels and A-type potassium (K^+)-channel currents and inhibits N-Type and P/Q type calcium (Ca^{2+})-channel activity (Demuth and Molleman, 2006). The CB₂ receptor is also negatively coupled to adenylyl cyclase but it seems not to be coupled to calcium (Ca^{2+})-channels (Felder et al., 1995). However, CB₁ receptors can also interact with G_s and G_{q/11} under certain conditions and with certain agonists (Mackie, 2005, 2008b). In addition, a pair of orphan-related receptors (GPR18 and GPR55) is also described as cannabinoid putative receptors.

CB₁ receptor expression

The CB₁ receptor is highly expressed in the cortex, cerebellum and associational cortical regions of neocortex (Glass et al., 1997). It is also expressed in the spinal dorsal horn (Sanudo-Pena et al., 1999) and in DRG neurons (Hohmann and Herkenham, 1999; Salio et al., 2002; Walker and Hohmann, 2005). Autonomic nerve terminals express CB₁ receptors (Ishac et al., 1996; Vizi et al., 2001), which negatively modulate the sympathetic tone (Marichal-Cancino et al., 2013). Low levels of these receptors have been reported in the adrenal gland, thymus, heart, bone marrow, tonsils, prostate, uterus, ovary and lung (Galiegue et al., 1995; Rice et al., 1997). A key characteristic of this receptor is the formation of heterodimers, suggesting that intracellular signaling could change under different conditions (Callen et al., 2012; Laprairie et al., 2012; Straiker et al., 2012).

CB₂ receptor expression

The CB₂ receptor is mostly expressed on cells of the immune system and spleen (Munro et al., 1993; Galiegue et al., 1995; Di Marzo et al., 2004). A few studies have found CB₂ immunoreactivity expression in glial and neuronal cells in some areas of the rodent brain (Gong et al., 2006; Onaivi et al., 2006), but this expression remains controversial (Hohmann and Herkenham, 1999; Salio et al., 2002; Walker and Hohmann, 2005). Notably, nerve injury and inflammation upregulate expression of CB₂ receptors in neurons and microglia (Beltramo et al., 2006; Rahn and Hohmann, 2009; Sagar et al., 2009; Hsieh et al., 2011). Furthermore, some studies have demonstrated the presence of CB₂ receptors in the DRG and afferent fibers in the spinal dorsal horn (Ross et al., 2001; Anand et al., 2008).

Role of CB₁ and CB₂ Receptors on Primary Afferent Neurons

DRG neurons express CB₁ receptors (Hohmann and Herkenham, 1999; Ross et al., 2001; Price et al., 2003). This receptor is synthesized in the cell neuronal bodies and inserted on both central and peripheral terminals (Hohmann and Herkenham, 1999; Hohmann et al., 1999). CB₁ receptors are mainly expressed in myelinated fibers of DRG neurons (Hohmann and Herkenham, 1999; Salio et al., 2002; Bridges et al., 2003) and also co-localize with CGRP, TRPV1 and IB4 (Hohmann and Herkenham, 1999; Hohmann et al., 1999; Ahluwalia et al., 2000; Bridges et al., 2003; Veress et al., 2013).

Nerve injury enhances CB₁ receptor expression in the DRG and spinal cord (Lim et al., 2003; Wang et al., 2007; Shiu et al., 2017) and other brain areas related with the emotional

component of pain (Knerlich-Lukoschus et al., 2011). These data give an anatomical basis for the involvement of CB₁ receptors in modulating neuropathic pain. In this regard, it has been shown that systemic and local administration of CB₁ receptor agonists produce anti-nociceptive effects in neuropathic pain models (Herzberg et al., 1997; Fox et al., 2001; Bridges et al., 2003; Yu et al., 2010). Moreover, deletion of CB₁ receptors in peripheral (but not at spinal or supraspinal level) nociceptors reduced analgesia by local or systemic (but no intrathecal) CB₁ receptor agonists (Agarwal et al., 2007). Thus, CB₁ receptors located at primary afferent neurons constitute the prime target for producing cannabinoid analgesia.

Some of the peripheral antinociceptive effects of cannabinoids may occur through interaction with another receptor system. In this regard, an early work in rat nodose ganglion neurons showed that cannabinoid agonists inhibited 5-HT-induced currents in a concentration-dependent manner. The inward current was sensitive to the serotonin (5-HT₃) receptor antagonist MDL72222, suggesting a cannabinoid-mediated inhibition of serotonin (5-HT₃) currents (Fan, 1995). Later, *in vivo* experiments demonstrated that application of CB₁ and CB₂ receptor agonists attenuated the activity of rat peripheral (5-HT₃) receptors on the terminals of cardiopulmonary afferent C-fibers (Godlewski et al., 2003) through an allosteric interaction at a (5-HT₃) modulatory site (Barann et al., 2002). Moreover, the inhibitory effects of cannabinoids may occur through a synergistic action with opioid receptors and their signal transduction pathways (Pugh et al., 1996; Smith et al., 1998; Manzanares et al., 1999; Massi et al., 2003; Scavone et al., 2013) or by a cannabinoid-mediated increase in opioid peptide synthesis and release of endogenous opioids such as enkephalins and dynorphins (Corchero et al., 1997a,b; Valverde et al., 2001).

The use of cannabinoid agonists as analgesic drugs is limited due to adverse effects in the CNS (Clermont-Gnamien et al., 2002; Attal et al., 2004; Turcotte et al., 2010). However, since it has been demonstrated that CB₁ receptors are expressed at primary afferent neurons (Agarwal et al., 2007), the synthesis of CB₁ receptor agonists with limited CNS penetration is under development (Clapper et al., 2010; Yu et al., 2010).

The molecular mechanisms by which the CB₁ receptor has peripheral antinociceptive effects are not completely understood. It is known that CB₁ receptor, coupled to G_{i/o} protein, can modulate several cellular mechanisms, all of which can reduce the excitability of neurons (e.g., opening of inward rectifying potassium (K^+)-channels and A-type potassium (K^+)-channels, and inhibiting N-Type and P/Q type calcium (Ca^{2+})-channels) (Demuth and Molleman, 2006). Moreover, there are several studies showing that cannabinoids can modulate the activity of transient receptor potential (TRP) channels, which are implicated in the modulation of pain processing. For example, multiple studies have shown that activation of the CB₁ receptor suppresses capsaicin-induced hyperalgesia in afferent neurons (Ko and Woods, 1999; Li et al., 1999; Johaneck et al., 2001; Millns et al., 2001; Johaneck and Simone, 2004; Santha et al., 2010). However, there are controversial findings regarding the effects of CB₁ receptor agonists on TRPV1 channels, because the CB₁ receptor agonist anandamide exerts dual effects on afferent

neurons, depending on the concentration used (Ross, 2003; Evans et al., 2004; Sousa-Valente et al., 2014). Specifically, anandamide produces a CB₁-mediated inhibitory effect at nM concentration, while it exerts a TRPV1-mediated stimulatory effect at higher concentrations (μM) in primary afferent neurons (Tognetto et al., 2001; Roberts et al., 2002; Ross, 2003; Fischbach et al., 2007). A recent study using mouse afferent neurons has shown that activation of CB₁ receptors inhibit nerve growth factor (NGF)-induced sensitization of TRPV1 (Wang et al., 2014), possibly through multiple signaling pathways, including ERK1/2 and PI3K (Zhuang et al., 2004; Stein et al., 2006; Zhu and Oxford, 2007).

The analgesic action of cannabinoids may be mediated by the presynaptic inhibition of neurotransmitter release in sensory neurons. For example, presynaptic CB₁ receptors inhibit CGRP and substance P (SP) release from trigeminal sensory nerves (Akerman et al., 2004; Oshita et al., 2005). Moreover, CB₁ receptor agonists reduce voltage-activated Ca^{2+} current in DRG neurons (Ross et al., 2001). On the other hand, it is possible that even more important than peripheral actions, cannabinoids induce analgesia by interfering with circuitry in the rostral ventromedial medulla (RVM) (Meng et al., 1998).

CB₂ receptors have also been found in nociceptive sensory neurons of rodents (Ross et al., 2001; Merriam et al., 2008; Schuelert et al., 2010) and humans (Anand et al., 2008). Like with CB₁ receptors, nerve damage upregulates CB₂ receptors in the superficial laminae of the dorsal horn of the spinal cord and isolated DRG of mice (Wotherspoon et al., 2005) and human beings (Anand et al., 2008).

Although the specific role of the CB₂ receptor in sensory neurons remains unclear, several functional studies in sensory neurons point to an antinociceptive role (Burston and Woodhams, 2014). For instance, the putative CB₂ receptor agonist JWH-133 inhibits capsaicin-induced depolarization of the vagus sensory nerve in guinea pigs and humans (Patel et al., 2003). Moreover, JWH-133 reduces the response of wide dynamic range dorsal horn neurons to both innocuous and noxious intensities of mechanical stimuli (Elmes et al., 2004). This compound also attenuates the capsaicin-evoked Ca^{2+} response in DRG neurons in neuropathic rats (Sagar et al., 2009), while GW818646X (other CB₂ receptor agonist) diminishes capsaicin-induced inward cation currents and elevation of cytoplasmic Ca^{2+} (Anand et al., 2008). Another CB₂ receptor agonist, A-836339, inhibits von Frey-evoked activity of WDR neurons in neuropathic rats (McGaraughty et al., 2009). Local peripheral injection of the selective CB₂ receptor agonist AM1241 into the hind paw produces antinociception to thermal stimulation (Malan et al., 2001). AM1241 also inhibits bradykinin-induced mesenteric afferent nerve activity (Hillsley et al., 2007). This effect was absent in CB₂ knockout mice and blocked by AM630, a CB₂ receptor inverse agonist. Local injection of the PEA analog *N*-(4-methoxy-2-nitrophenyl)hexadecanamide induces CB₁- and CB₂-dependent antinociception in rats (Roa-Coria et al., 2012). Similar results were observed with GW833972A, another putative CB₂ receptor agonist (Belvisi et al., 2008). Interestingly, repeated systemic administration of the CB₂ receptor selective agonist AM1710

suppresses paclitaxel-induced allodynia (Deng et al., 2015). Taken together, the data strongly suggest that CB₁ and CB₂ receptors have an antinociceptive role. Despite this evidence, there are few cannabinoid-based drugs currently available for clinical use (see below).

CB₁ and CB₂-Based Treatment for Pain

A randomized, placebo-controlled, double-blinded crossover design was used to examine the effect of cannabinoids on pain. Low, medium, and high doses of smoked cannabis (respectively 2, 4, and 8% Δ^9 -THC by weight) did not modify capsaicin-induced pain assessed in 15 healthy volunteers 5 min after exposure (Wallace et al., 2007). In contrast, the medium dose of Δ^9 -THC diminished capsaicin-induced pain 45 min after cannabis exposure. Of note, these authors found that a high dose of cannabis increased capsaicin-induced pain (Wallace et al., 2007). Similar results have been reported with a high dose of nabilone (an oral synthetic cannabinoid Δ^9 -THC analog) on 41 patients with postoperative pain (Beaulieu, 2006). Another study evaluated cannabis extract capsules (20 mg of Δ^9 -THC) in 18 healthy female volunteers (Kraft et al., 2008). Treatment with Δ^9 -THC was not able to reduce pain induced by capsaicin, electrical stimulation or sunburn. Taken together, it seems that Δ^9 -THC is not effective for acute pain. A similar conclusion was reached after analyzing a total of 611 patients in seven well-designed studies (Stevens and Higgins, 2017).

Although the effects of cannabinoids in the acute pain setting seem to be disappointing, results of clinical trials evaluating cannabinoids in chronic pain are much more promising (see Table 1). The conditions causing chronic pain varied between studies and included neuropathy (chemotherapy, diabetes, human immunodeficiency virus [HIV]), cancer, fibromyalgia, multiple sclerosis and rheumatoid arthritis (Whiting et al., 2015). Sativex (containing Δ^9 -THC:cannabidiol [CBD] in an approximate 1:1 ratio [oral spray]) reduced neuropathic pain in patients with unilateral neuropathic pain (Berman et al., 2004; Nurmikko et al., 2007; Langford et al., 2013; Serpell et al., 2014). Likewise, treatment with smoked cannabis diminished pain in patients with multiple sclerosis (Rog et al., 2005; Corey-Bloom et al., 2012), neuropathic pain (Wilsey et al., 2013) and diabetic neuropathy (Wallace et al., 2015). In contrast, sativex was ineffective in relieving chemotherapy-induced neuropathic pain (Lynch et al., 2014). Oral administration of dronabinol, a synthetic Δ^9 -THC analog, modestly reduced central pain in patients with multiple sclerosis (Svendsen et al., 2004). Nabilone, another synthetic Δ^9 -THC analog, diminished neuropathic pain in diabetic patients (Toth et al., 2012). Oral administration of Δ^9 -THC (ECP002A) reduced pain in patients with progressive multiple sclerosis. Drug dosage was well tolerated and had a stable pharmacokinetic profile (van Amerongen et al., 2017). Nabilone is also effective in patients with medication overuse headache (Pini et al., 2012). In contrast, nabilone did not reduce pain in patients with fibromyalgia (Skrabek et al., 2008).

A limitation to clinical use of cannabinoids for pain is their unfavorable side-effect profile, such as drowsiness, dizziness, speech impediments, memory impairment and confusion. Results of clinical trials with these agents indicate that high

TABLE 1 | Studies about the antinociceptive effects of CB1 and CB2 receptor agonists in different pain models.

Pain model	Drug treatment and dose	Behavioral readout	Route	Results	Proposed mechanisms of action	Reference
Partial SNL	WIN 55,212-2 0.3–10 mg/kg CP-55,940 0.03–1 mg/kg HU-210 0.001–0.03 mg/kg	Mechanical hyperalgesia Thermal hyperalgesia Tactile allodynia	s.c. or i.t.	They produce complete reversal of mechanical hyperalgesia with catalepsy Only WIN 55,212-2 reversed tactile allodynia and thermal hyperalgesia in this model	Via activation of CB1 receptors in both CNS and in the periphery	Herzberg et al., 1997; Fox et al., 2001; Bridges et al., 2003
SNL or carrageenan model	AZ11713908 0.6–1.2 μmol/kg	Thermal and mechanical hyperalgesia	s.c.	Robust analgesia in both models	Likely via peripheral activation of CB1 receptor	Yu et al., 2010
Mechanical stimulation, formalin or capsaicin models, in mice that lacked CB1 receptor specifically in primary nociceptors	Endocannabinoids (AEA and arachidonic acid)	Thermal and mechanical hyperalgesia		The nociceptor-specific loss of CB1 receptor substantially reduced the analgesia produced by local and systemic but no intrathecal, delivery of cannabinoids	Via CB1 receptors expressed on the peripheral terminals of nociceptors	Agarwal et al., 2007
SNL, carrageenan, LPS or CIA model	URB937 1 mg/kg URB597 10 mg/kg PF-3845 0.1–10 μg/kg	Thermal and mechanical hyperalgesia, tactile allodynia	i.p. or i.t.	Attenuation of hyperalgesia and partial reduction of allodynia	Suppresses FAAH activity and increases AEA levels	Clapper et al., 2010; Kinsey et al., 2011; Booker et al., 2012
FCA, partial SNL, tail flick, hot plate or incision model of postoperative pain	GW405833 0.3–30 mg/kg	Mechanical hyperalgesia and tactile allodynia	i.p.	Elicits potent and efficacious antihyperalgesic effects in rodent models of neuropathic, incisional and chronic inflammatory pain	Via activation of CB2 receptors	Valenzano et al., 2005
FCA, chronic constriction injury, incision model of postoperative pain or knee joint osteoarthritic pain	A796260 11–35 mg/kg	Thermal and tactile allodynia	i.p.	Analgesic activity in all pain models	Via activation of CB2 receptors	Yao et al., 2008
Partial SNL or carrageenan model	JWH133 50–100 nmol/mouse	Tactile allodynia	i.t., i.p. or local	Reverses partial sciatic nerve ligation-induced mechanical allodynia in mice.	Via activation of central CB2 receptors	Patel et al., 2003; Elmes et al., 2004; Yamamoto et al., 2008; Sagar et al., 2009
SNL, Formalin, Carrageenan, FCA or intradermal capsaicin	AM1241 0.03–6 mg/kg	Tactile and thermal allodynia, mechanical hyperalgesia and nocifensive response	i.v., i.p. or i.pl.	Analgesic effects in all pain models	Via activation of peripheral CB2 receptors	Malan et al., 2001, 2002; Ibrahim et al., 2003; Quartilho et al., 2003; LaBuda et al., 2005; Beltramo et al., 2006; Hillsley et al., 2007; Yao et al., 2008
Formalin model or postoperative pain	HU308 30, 50 mg/kg	Nocifensive response and tactile allodynia	i.p.	Reduces blood pressure, blocks defecation, and elicits anti-inflammatory and peripheral analgesic activity	Via activation of CB2 receptors	Hanus et al., 1999; LaBuda et al., 2005
FCA or chronic constriction injury	GW842166X 0.1–0.3, 15 mg/kg	Mechanical hyperalgesia	p.o.	Very potent analgesic in inflammatory and neuropathic pain models	Potent and highly selective full agonist at the CB2 receptor	Clayton et al., 2004; Giblin et al., 2007; Anand et al., 2008
SNL	A836339 1–3 μmol/kg	Tactile allodynia	i.v.	Reduces both spontaneous and von Frey-evoked firing of WDR neurons in neuropathic rats	Via activation of spinal and peripheral CB2 receptors	McGaraughty et al., 2009
Paclitaxel-neuropathic pain	AM1710 0.1–10 mg/kg	Mechanical and thermal allodynia	i.p.	Suppresses allodynia generated by paclitaxel without central side effects	Via activation of CB2 receptors	Rahn et al., 2011; Deng et al., 2015

AEA, anandamide; SNP, spinal nerve ligation; FCA, Freud's complete adjuvant; CIA, collagen-induced arthritis; LPS, lipopolysaccharide; s.c., subcutaneous; i.p., intraperitoneal; i.t., intrathecral; i.v., intravenous; p.o., oral administration; i.pl., intraplantar.

dosages are required to attain therapeutic effects and it is difficult to reach these dosages in clinical practice (Turcotte et al., 2010). At doses that prevent subjective effects, some cannabinoids seem to be ineffective for controlling acute pain (Kalliomäki et al., 2013). Several peripherally restricted CB₁ and CB₂ receptor agonists have been developed to avoid these side effects (Pertwee, 2009; Yu et al., 2010; Rahn et al., 2011; Yrjola et al., 2013). However, additional research is needed to improve study methodologies including the use of standard formulations and/or dosages, the increase in the number of subjects involved, and the general determination of the safe and effective use of cannabis for the treatment of human pain.

Another interesting area of research has recently focused on the evaluation of the possible synergy between cannabinoids and opioids in the management of pain. A combination of Δ⁹-THC and morphine diminished experimental pain in healthy volunteers (Roberts et al., 2006). Furthermore, dronabinol combined with opioids relieved chronic pain in patients (Narang et al., 2008).

In the last years, pain research has focused on the inhibition of the enzymes playing a role in EC metabolism and the elevation of the EC tonus locally. Special emphasis is given on multi-target analgesia compounds, where one of the targets is the EC degrading enzyme. Dual FAAH¹ /TRPV1 blockers, such as *N*-arachidonoyl-serotonin (AA-5-HT) and OMDM198, are effective in animal studies, but this multi-target strategy has not yet reached the clinic (Maione et al., 2007, 2013; Morera et al., 2009; Costa et al., 2010; Malek et al., 2015).

Importantly, cannabinoids interact (apart from CB₁ and CB₂) with several other pharmacological receptors, including the cannabinoid putative receptors GPR18 and GPR55 (which have been even suggested as CB_x and CB₃ receptors). It is likely that the contradictory effects observed in clinical trials using *Cannabis sp.*-based treatments (e.g., Δ⁹-THC) may be due to the high promiscuity of cannabinoids for their receptors. Before achieving a clinical benefit from an EC system-based therapy in pain (and other alterations), it is mandatory to detect and understand the physiological and/or pathophysiological role of the cellular targets involved. In this context, we provide an analysis of the potential participation of the putative cannabinoid receptors GPR18 and GPR55 in pain (see below).

GPR18 AND GPR55: POTENTIAL TARGETS FOR PAIN TREATMENT

GPR55 and GPR18: Generalities

Cannabinoids interact with multiple orphan receptors (Alexander, 2012). Different groups have discussed if G protein-coupled receptor 18 (GPR18) and 55 (GPR55) should be considered as novel cannabinoid receptors (Alexander, 2012; Alexander et al., 2017). Nevertheless, the nomenclature

suggested by the Nomenclature Committee of the Union of Basic and Clinical Pharmacology (NC-IUPHAR) Subcommittee on Cannabinoid Receptors (Pertwee et al., 2010) decided that all criteria to consider these as novel cannabinoid receptors remain incomplete and, accordingly, they were classified again as orphan receptors (Alexander et al., 2017). Independently of the official decision, these receptors clearly interact with cannabinoids directly or indirectly. Expression of GPR18 seems to be rich in the testis, spleen, peripheral blood leucocytes and lymph nodes (Gantz et al., 1997; Vassilatis et al., 2003; Rosenkilde et al., 2006). Its expression suggests a potential role in the control of immune system activity (e.g., leucocytes migration) (Burstein et al., 2011) and accordingly inflammation. Moreover, activation of GPR18 by *N*-arachidonoylglycine leads to apoptosis of inflammatory leukocytes (Burstein et al., 2011; Takenouchi et al., 2012), which in turn reduces local inflammation. There is also evidence that activation of GPR18 lowers intraocular pressure in mice (Miller et al., 2016). All these findings suggest a physiological function of NAGly via GPR18 in different inflammatory processes.

Knowledge about GPR55 physiology in the nervous system has increased recently (Marichal-Cancino et al., 2017). This receptor has been suggested as a potential therapeutic target in Parkinson's disease due to a possible alteration on its expression in the basal nuclei (Celorio et al., 2017), where it is related to procedural memories (Marichal-Cancino et al., 2016). GPR55 is also expressed in the hippocampus, where it has a role in spatial navigation (Marichal-Cancino et al., 2018). Furthermore, it is possible that some antiepileptic actions observed with phytocannabinoids involve the blocking of GPR55 (Kaplan et al., 2017). However, the above is a topic under study and findings are preliminary. Despite all advances in the physiology of GPR55, several actions in different areas of the CNS remain obscure (Marichal-Cancino et al., 2017). Interestingly, PEA (a cannabinoid related compound) is currently used to treat pain and inflammation. Like other cannabinoid related molecules, PEA has a very complex mechanism of action, which includes direct and/or indirect interaction with CB₁, TRPV1, PPAR, GPR55 and GPR18, among other receptors (Keppel Hesselink et al., 2014). Certainly, PEA has high affinity for GPR55 as a full agonist (Ryberg et al., 2007). Thus, it is necessary to investigate whether GPR55 is involved in the analgesic and anti-inflammatory actions of PEA.

Actions of GPR18 and GPR55 and Their Potential Role in the Pharmacology of Pain

GPR18 and GPR55 are differentially expressed in the central and peripheral nociceptive systems of rodents and humans, suggesting a potential role in the modulation of nociceptive pathways (DRG TXome Database)² (Ray et al., 2018). In general, GPR18 is less studied compared to GPR55 (see below). This is partly due to the fact that signaling mechanisms and

¹FAAH, Fatty Acid Amide Hydrolase Enzyme. FAAH is an integral membrane enzyme that hydrolyzes the endocannabinoid anandamide and related amidated signaling lipids. FAAH KO mice display elevated anandamide levels, showing reduced nociceptive transmission in several pain models. Journal of Neurobiology 61: 149–60.

²<http://www.uttdallas.edu/bbs/painneurosciencelab/DRGtranscriptome/search.php>

endogenous ligands are still controversial (Alexander et al., 2017). GPR18 has been suggested to modulate, depending on the ligand, both $G_{\alpha i/o}$ and $G_{\alpha q/11}$ transduction pathways (Console-Bram et al., 2014). In this sense, NAGly is proposed as the endogenous GPR18 ligand (Kohno et al., 2006; McHugh et al., 2010). However, a recent study suggests that NAGly increases Ca^{2+} mobilization and MAPK activity in HAGPR55/CHO cells (Console-Bram et al., 2017). This response is attenuated by ML193 (GPR55 receptor antagonist) suggesting that NAGly-mediated effects depend on GPR55 activation. Moreover, an independent study reported that NAGly does not activate GPR18 receptors (Lu et al., 2013). In support of this, there is a previous observation showing that NAGly does not activate GPR18 (Yin et al., 2009). These discrepancies could be partially explained by the fact that NAGly is also a reversible and non-competitive inhibitor of the glycine transporter type 2 (GlyT2) (Wiles et al., 2006). In line with this, it has been shown that NAGly enhances inhibitory glycinergic transmission synaptic within the superficial dorsal horn by blocking glycine uptake via GlyT2 and decreasing excitatory NMDA-mediated synaptic transmission (Jeong et al., 2010).

It has been proposed that both GPR18 and GPR55 could play a role in the modulation of acute and chronic pain (Table 2). In animal models of inflammatory pain, intraplantar NAGly administration attenuates formalin-induced pain (Huang et al., 2001). Moreover, intrathecal administration of NAGly reduces complete Freund's adjuvant (CFA)-induced mechanical allodynia and thermal hyperalgesia by a CB₁-independent mechanism (Succar et al., 2007). Additionally, NAGly increases the production of 15-deoxy- $\Delta^{13,14}$ -prostaglandin J2 and lipoxin A4, leading to a reduction in the migration of inflammatory cells into the area of acute inflammation (Burstein et al., 2011). GPR18 is expressed on human leukocytes, including polymorphonuclear neutrophils (PMN), monocytes, and macrophages and, furthermore, its activation regulates leukocyte trafficking during acute inflammation (Chiang et al., 2015). GPR18 and TRPV1 are expressed in chondrocytes within the deep zone of cartilage in patients with osteoarthritis (OA) (Dunn et al., 2016), suggesting that GPR18 presence in degenerate tissues could be a target for treatment with cannabinoids.

Nerve injury enhances expression of GPR18 mRNA in spinal cord and/or the DRG of rats, suggesting a potential role of GPR18 in the modulation of neuropathic pain (Malek et al., 2016). Accordingly, intrathecal administration of NAGly reduces mechanical allodynia in rats subjected to spinal nerve ligation and this effect is not prevented by pretreatment with either the CB₁ or CB₂ receptor antagonists AM251 and SR144528, respectively (Vuong et al., 2008). Although NAGly has been proposed as an endogenous GPR18 ligand, recent studies have found that resolvin D2 (RvD2) also activates GPR18 receptors (Chiang et al., 2015; Zhang et al., 2016). RvD2 activates recombinant human GPR18 in a receptor- and ligand-dependent manner and promotes the resolution of bacterial infections and organ protection (Chiang et al., 2015). Moreover, RvD2 enhances

endothelial cell migration in a Rac-dependent manner via GPR18, and GPR18-deficient mice have an endogenous defect in perfusion recovery following hind limb ischemia (Zhang et al., 2016). In rodents, intrathecal administration of RvD2 reverses CFA-induced inflammatory pain, prevents formalin-induced spontaneous pain, and also reverses C-fiber stimulation-evoked long-term potentiation in the spinal cord (Park et al., 2011). However, RvD2 antinociceptive effects seem to be mediated by additional mechanisms involving the inhibition of transient receptor potential (TRPV1 and TRPA1) channels (Park et al., 2011). Undoubtedly, more studies to redefine the signaling pathways, ligands and physiological functions of GPR18 are needed.

GPR55 has been found highly expressed in large-diameter neurons, but present at low levels in small-diameter neurons of the mouse DRG (Lauckner et al., 2008). Indeed, reports suggest that GPR55 plays a role in modulating nociceptor excitability. Activation of GPR55 with lysophosphatidylinositol (LPI) promotes excitability in cultured large DRG neurons by increasing intracellular Ca^{2+} (Lauckner et al., 2008) and also produces mechanical hypersensitivity in mice after local peripheral administration (Gangadharan et al., 2013). Although there is a general consensus that LPI acts as an agonist for GPR55, it has been also reported that LPI modulates large-conductance Ca^{2+} -activated potassium (K^+) channels (BK_{Ca}) (Bondarenko et al., 2011a,b), 2-pore domain potassium (K^+)-channels (TREK-1) (Maingret et al., 2000; Danthi et al., 2003) and the potassium (K^+) channel subfamily K member 4 (KCNK4 or TRAAK) (Maingret et al., 2000), transient receptor potential (TRPV2; Monet et al., 2009; Harada et al., 2017), and transient receptor potential (TRPM8; Vanden Abeele et al., 2006; Andersson et al., 2007) channels. All these channels are expressed in the primary nociceptive pathway and their activation either modulates or amplifies sensory information (Basbaum et al., 2009). Therefore, the pharmacological data with LPI should be taken with caution. Furthermore, LPI is not the sole GPR55 activator. The hydrophilic glycerophospholipid lyso-phosphatidyl- β -D-glucoside (LysoPtdGlc) was recently reported as a regulator of the nociceptive central axon projections by activating GPR55 with high affinity (Guy et al., 2015). This indicates that glycerophospholipids could play a role modulating nociceptive inputs *in vivo*.

Nerve damage increases GPR55 mRNA expression in the spinal cord and DRG of rats (Malek et al., 2016) suggesting the participation of these receptors in neuropathic pain. It has been shown that the synthetic GPR55 agonist O-1602 reduces movement-evoked firing of nociceptive C fibers in a rat model of acute joint inflammation, and this effect is blocked by the GPR55 receptor antagonist O-1918 (Schuelert and McDougall, 2011). O-1602 also has protective effects in a murine model of experimentally induced colitis, but this anti-inflammatory effect could not be mediated by GPR55 (Schicho et al., 2011).

On the other hand, other studies have reported that GPR55 knockout mice show a reduced tumor-induced mechanical hypersensitivity (Gangadharan et al., 2013). GPR55 agonist O-1602 produces pronociceptive effects in neuropathic rats

TABLE 2 | Possible role of GPR18 and GPR55 receptors in different animal models of pain.

Pain model/specie	Drug treatment	Dose	Route	Outcome	Proposed mechanisms of action	Reference
Formalin /rat	NAGly	275 nmol	i.pl.	Suppression of phase II response	Non-CB1 mediated mechanism	Huang et al., 2001
	CID16020046	10 μ M	Intra-ACC	Attenuation of phase II response Reduction of p-ERK in the ACC Attenuation of spinal <i>c-fos</i> expression in the spinal cord	Endogenous activation of GPR55 signaling. Modulatory effects of GPR55 signaling in the ACC on the descending pain pathway	Okine et al., 2016
Formalin/mouse	N/T	N/T	N/T	No differences between WT and GPR55 ^{-/-} mice in mechanical, cold and heat hypersensitivity	Non-GPR55 mediated mechanism	Carey et al., 2017
CFA /rat	NAGly	70–700 nmol	i.t.	Attenuation of mechanical and thermal hyperalgesia	Non-cannabinoid mediated mechanism	Succar et al., 2007
CFA/mouse	N/T	N/T	N/T	Absence of mechanical hyperalgesia in GPR55 ^{-/-} mice	GPR55 signaling	Staton et al., 2008
Capsaicin/mouse	N/T	N/T	N/T	GPR55 ^{-/-} and WT mice display comparative levels of capsaicin-evoked nociceptive behavior, mechanical and thermal hyperalgesia	Non-GPR55 mediated mechanism	Carey et al., 2017
PNL/rat PNL/Mouse PNL/Mouse	NAGly N/T N/T	70–700 nmol N/T N/T	i.t. N/T N/T	Reduction of mechanical allodynia Absence of mechanical hyperalgesia in GPR55 ^{-/-} mice GPR55 ^{-/-} and WT mice develop similar levels of hypersensitivity to mechanical, heat, and cold stimulation	CB1 and CB2 independent mechanism GPR55 signaling Non-GPR55 mediated mechanism	Vuong et al., 2008 Staton et al., 2008 Carey et al., 2017
CCI/rat	O-1602 AA-5-HT	1–10 mg/kg 100–1000 nM	i.p. i.t.	Pronociceptive properties in neuropathic pain induced by O-1602 (atypical cannabinoid) Upregulation of CB2, GPR18, and GPR55 mRNA in the spinal cord and/or DRG after CCI. Increased pain threshold to mechanical and thermal stimuli following AA-5HT	Pronociceptive role of GPR55. Possible role of GPR18 Involvement of CB2, GPR18 and GPR55 receptors	Breen et al., 2012 Malek et al., 2016
Paclitaxel/mouse	N/T	N/T	N/T	GPR55 ^{-/-} and WT mice develop similar levels of paclitaxel-induced mechanical and cold allodynia	Non-GPR55 mediated mechanism	Carey et al., 2017
LPI-induced pain/mouse	LPI	2 pmol–6 nmol	i.pl.	WT mice: Sensitization against non-painful and painful mechanical stimuli. GPR55 ^{-/-} mice: reduction of LPI-induced acute allodynia, attenuation of LPI-induced long-term mechanical hyperalgesia	GPR55, Gα _{q/11} , and Gα13 pathways, and their signaling via RhoA-ROCK as well as ERK1/2	Gangadharan et al., 2013
Hot plate test/rat	LPI	1 μ g	Intra-PAG	Reduction in nociceptive threshold that is abolished by a pretreatment with ML-193, a GPR55 antagonist.	Pro-nociception mediated by GPR55 activation at central levels. Blockade of GPR55 signaling in the PAG may promote analgesia	Deliu et al., 2015

CFA, Complete Freund's Adjuvant; PNL, partial ligation of the sciatic nerve; CCI, chronic constriction injury; NAGly, N-arachidonylglycine; LPI, lysophosphatidylinositol; AA-5-HT, N-arachidonoyl-serotonin; WT, wild type; ACC, anterior cingulated cortex; PAG, periaqueductal gray; N/T, not tested.

(Breen et al., 2012). At the central nervous system, local injection of the GPR55 putative inverse agonist CID16020046 into the anterior cingulated cortex (ACC) produces antinociception in the formalin test by decreasing the extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in the ACC and *c-fos* mRNA expression in the spinal cord (Okine et al., 2016). Moreover, LPI administration into the periaqueductal gray (PAG) attenuates nociceptive latencies in a hot-plate test and also produces a concentration-dependent increase in intracellular Ca^{2+} levels in dissociated rat PAG neurons expressing GPR55 mRNA (Deliu et al., 2015). Although the exact mechanisms underlying the GPR55-mediated antinociceptive effects remain to be elucidated, it has been suggested that some cytokines (e.g., IL-4 and IL-10) are responsible for the modulatory effects observed during inflammatory pain conditions (Staton et al., 2008).

Using cell lines, other studies have shown that GPR55 couples to $\text{G}_{\alpha 13}$ and activates GTPases RhoA, Cdc42 and Rac1 (Ryberg et al., 2007; Henstridge et al., 2009). Some efforts have tried to elucidate the G-protein signaling pathway activated by GPR55 agonists *in vivo*. Using pharmacological and conditional genetic tools in mice, the research group headed by Rohini Kuner showed that LPI-mediated hypersensitivity depends on the activation of $\text{G}_{\alpha 13}$ and $\text{G}_{\alpha q/11}$, which in turn activate ERK1/2 (Gangadharan et al., 2013). In support of these results, it has been shown that LPI produces β -arrestin trafficking, MAPK, ERK1/2 phosphorylation and activates the G-protein signaling by a PKC β II-independent mechanism (Oka et al., 2007; Kapur et al., 2009). Interestingly, the effects on β -arrestin GPR55 complex formation, ERK1/2 phosphorylation and internalization of GPR55 are blocked by the GPR55 antagonist/partial agonist CP55,940 (Kapur et al., 2009), suggesting that a complex mechanism triggered upon GPR55 activation modulates G-coupled signaling pathways. Moreover, it has been documented that activation of GPR55 leads to additional p38 MAPK (Oka et al., 2010) and AKT phosphorylation (Pineiro et al., 2011). These events are related to the subsequent activation of several major transcription factors such as the nuclear factor of activated T-cells (NFAT) (Waldeck-Weiermair et al., 2008; Henstridge et al., 2009, 2010), CREB (Henstridge et al., 2010), NF- κ B (Waldeck-Weiermair et al., 2008; Henstridge et al., 2010), and ATF2 (Oka et al., 2010).

Certainly, there is extensive literature indicating that signaling pathways involving MAPK and transcription factors such as NF- κ B play an important role in pain (Niederberger and Geisslinger, 2008; Ji et al., 2009). However, it is worth emphasizing that most of the signaling mechanisms reported for GPR55 receptors have been obtained *in vitro* using cell lines and may not be completely translated to *in vivo* models. This is particularly important due to the recent discrepancies in the pain field using GPR55 knock-out mice. It was originally reported that mice lacking GPR55 show no differences in baseline pain responses compared to wild-type mice, but mechanical hyperalgesia is absent following either intraplantar CFA injection or partial nerve ligation (Staton et al., 2008). However, a recent study using knock-out mice suggests that GPR55 is dispensable for the development of inflammatory

and neuropathic pain (Carey et al., 2017). According to these authors, GPR55 knock-out mice have no differences in mechanical, cold or heat hypersensitivity after intraplantar capsaicin, formalin or CFA injection. Likewise, development and maintenance of neuropathic pain after paclitaxel administration or partial nerve ligation is undistinguishable between GPR55 knock-out and wild-type mice. While the explanation for this discrepancy is not clear, Carey et al. have suggested that these differences could be due to multiple factors, including the way the GPR55 knock-out mice were made, the battery of tests used, freely moving animals versus restrained animals during the test, sex differences, body weight, and age of animals. Evidently, more behavioral studies using controlled experimental conditions will be necessary to define the importance of GPR55 receptors in modulating pain responses.

CONCLUSION

Cannabinoids, *via* CB₁ receptors, mainly induce inhibition of pain integration that seems to be useful particularly in the treatment of chronic pain, whereas CB₂ stimulation mainly causes antiinflammation *via* negative modulation of the immune system. GPR18 and GPR55 have a role in integrating, transmitting and/or alleviating pain. However, further studies using more selective pharmacological tools combined with genetic tools to generate cell-specific ablation or reactivation of GPR18/GPR55 receptors in specific cell populations will help to clarify the functional role of these receptors to take advantage of them in therapeutics.

AUTHOR CONTRIBUTIONS

RG-A, PB-I, and EV-M developed the manuscript and discussed central ideas of it. AG-H and MC-L adapted the manuscript, designed graphs, and discussed central ideas of it. VG-S corrected the style and reviewed and edited the manuscript. MR supervised the project, worked on the conceptualization and acquired funding. BM-C conceived of the presented idea, integrated and edited information, and developed some central themes.

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REFERENCES

- Agarwal, N., Pacher, P., Tegeder, I., Amaya, F., Constantin, C. E., Brenner, G. J., et al. (2007). Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat. Neurosci.* 10, 870–879. doi: 10.1038/nn1916
- Agrawal, R. P., Goswami, J., Jain, S., and Kocher, D. K. (2009). Management of diabetic neuropathy by sodium valproate and glyceryl trinitrate spray: a prospective double-blind randomized placebo-controlled study. *Diabetes Res. Clin. Pract.* 83, 371–378. doi: 10.1016/j.diabres.2008.12.018
- Ahluwalia, J., Urban, L., Capogna, M., Bevan, S., and Nagy, I. (2000). Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neuroscience* 100, 685–688. doi: 10.1016/S0306-4522(00)00389-4
- Akerman, S., Kaube, H., and Goadsby, P. J. (2004). Anandamide is able to inhibit trigeminal neurons using an in vivo model of trigeminovascular-mediated nociception. *J. Pharmacol. Exp. Ther.* 309, 56–63. doi: 10.1124/jpet.103.059808
- Albe-Fessard, D., Berkley, K. J., Kruger, L., Ralston, H. J. III, and Willis, W. D. Jr. (1985). Diencephalic mechanisms of pain sensation. *Brain Res.* 356, 217–296. doi: 10.1016/0165-0173(85)90013-X
- Alexander, S. P. (2012). 2012 cannabinoid themed section. *Br. J. Pharmacol.* 167, 1573–1574. doi: 10.1111/j.1476-5381.2012.02238.x
- Alexander, S. P., Christopoulos, A., Davenport, A. P., Kelly, E., Marrion, N. V., Peters, J. A., et al. (2017). The concise guide to pharmacology 2017/18: G protein-coupled receptors. *Br. J. Pharmacol.* 174(Suppl. 1), S17–S129. doi: 10.1111/bph.13878
- Anand, U., Otto, W. R., Sanchez-Herrera, D., Facer, P., Yiangou, Y., Korchev, Y., et al. (2008). Cannabinoid receptor CB2 localisation and agonist-mediated inhibition of capsaicin responses in human sensory neurons. *Pain* 138, 667–680. doi: 10.1016/j.pain.2008.06.007
- Andersson, D. A., Nash, M., and Bevan, S. (2007). Modulation of the cold-activated channel TRPM8 by lysophospholipids and polyunsaturated fatty acids. *J. Neurosci.* 27, 3347–3355. doi: 10.1523/JNEUROSCI.4846-06.2007
- Attal, N., Brasseur, L., Guirimand, D., Clermond-Gnamien, S., Atlami, S., and Bouhassira, D. (2004). Are oral cannabinoids safe and effective in refractory neuropathic pain? *Eur. J. Pain* 8, 173–177.
- Ballantyne, J. C., and Shin, N. S. (2008). Efficacy of opioids for chronic pain: a review of the evidence. *Clin. J. Pain* 24, 469–478. doi: 10.1097/AJP.0b013e31816b2f26
- Barann, M., Molderings, G., Bruss, M., Bonisch, H., Urban, B. W., and Gothert, M. (2002). Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulator site. *Br. J. Pharmacol.* 137, 589–596. doi: 10.1038/sj.bjp.0704829
- Basbaum, A. I., Bautista, D. M., Scherrer, G., and Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell* 139, 267–284. doi: 10.1016/j.cell.2009.09.028
- Basbaum, A. I., and Woolf, C. J. (1999). Pain. *Curr. Biol.* 9, R429–R431. doi: 10.1016/S0960-9822(99)80273-5
- Beaulieu, P. (2006). Effects of nabilone, a synthetic cannabinoid, on postoperative pain. *Can. J. Anaesth.* 53, 769–775. doi: 10.1007/BF03022793
- Beltramo, M., Bernardini, N., Bertorelli, R., Campanella, M., Nicolussi, E., Fredduzzi, S., et al. (2006). CB2 receptor-mediated antihyperalgesia: possible direct involvement of neural mechanisms. *Eur. J. Neurosci.* 23, 1530–1538. doi: 10.1111/j.1460-9568.2006.04684.x
- Belvisi, M. G., Patel, H. J., Freund-Michel, V., Hele, D. J., Crispino, N., and Birrell, M. A. (2008). Inhibitory activity of the novel CB2 receptor agonist, GW833972A, on guinea-pig and human sensory nerve function in the airways. *Br. J. Pharmacol.* 155, 547–557. doi: 10.1038/bjp.2008.298
- Berman, J. S., Symonds, C., and Birch, R. (2004). Efficacy of two cannabis based medicinal extracts for relief of central neuropathic pain from brachial plexus avulsion: results of a randomised controlled trial. *Pain* 112, 299–306. doi: 10.1016/j.pain.2004.09.013
- Bhosale, U. A., Quraishi, N., Yegnanarayanan, R., and Devasthale, D. (2015). A comparative study to evaluate the cardiovascular risk of selective and nonselective cyclooxygenase inhibitors (COX-Is) in arthritic patients. *J. Basic Clin. Physiol. Pharmacol.* 26, 73–79. doi: 10.1515/jbcpp-2014-0005
- Bondarenko, A. I., Malli, R., and Graier, W. F. (2011a). The GPR55 agonist lysophosphatidylinositol acts as an intracellular messenger and bidirectionally modulates Ca²⁺-activated large-conductance K⁺ channels in endothelial cells. *Pflugers Arch.* 461, 177–189. doi: 10.1007/s00424-010-0898-x
- Bondarenko, A. I., Malli, R., and Graier, W. F. (2011b). The GPR55 agonist lysophosphatidylinositol directly activates intermediate-conductance Ca²⁺-activated K⁺ channels. *Pflugers Arch.* 462, 245–255. doi: 10.1007/s00424-011-0977-7
- Booker, L., Kinsey, S. G., Abdullah, R. A., Blankman, J. L., Long, J. Z., Ezzili, C., et al. (2012). The fatty acid amide hydrolase (FAAH) inhibitor PF-3845 acts in the nervous system to reverse LPS-induced tactile allodynia in mice. *Br. J. Pharmacol.* 165, 2485–2496. doi: 10.1111/j.1476-5381.2011.01445.x
- Bouaboula, M., Hilairet, S., Marchand, J., Fajas, L., Le Fur, G., and Casellas, P. (2005). Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. *Eur. J. Pharmacol.* 517, 174–181. doi: 10.1016/j.ejphar.2005.05.032
- Breen, C., Brownjohn, P. W., and Ashton, J. C. (2012). The atypical cannabinoid O-1602 increases hind paw sensitisation in the chronic constriction injury model of neuropathic pain. *Neurosci. Lett.* 508, 119–122. doi: 10.1016/j.neulet.2011.12.039
- Breuer, B., Pappagallo, M., Knotkova, H., Guleyupoglu, N., Wallenstein, S., and Portenoy, R. K. (2007). A randomized, double-blind, placebo-controlled, two-period, crossover, pilot trial of lamotrigine in patients with central pain due to multiple sclerosis. *Clin. Ther.* 29, 2022–2030. doi: 10.1016/j.clinthera.2007.09.023
- Bridges, D., Rice, A. S., Egertova, M., Elphick, M. R., Winter, J., and Michael, G. J. (2003). Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using in situ hybridisation and immunohistochemistry. *Neuroscience* 119, 803–812. doi: 10.1016/S0306-4522(03)00200-8
- Buffum, M., and Buffum, J. C. (2000). Nonsteroidal anti-inflammatory drugs in the elderly. *Pain Manag. Nurs.* 1, 40–50. doi: 10.1053/jpmn.2000.7779
- Burstein, S. H., McQuain, C. A., Ross, A. H., Salmons, R. A., and Zurier, R. E. (2011). Resolution of inflammation by N-arachidonoylglycine. *J. Cell. Biochem.* 112, 3227–3233. doi: 10.1002/jcb.23245
- Burston, J. J., and Woodhams, S. G. (2014). Endocannabinoid system and pain: an introduction. *Proc. Nutr. Soc.* 73, 106–117. doi: 10.1017/S0029665113003650
- Calabro, R. S., Gervasi, G., Marino, S., Mondo, P. N., and Bramanti, P. (2010). Misdiagnosed chronic pelvic pain: pudendal neuralgia responding to a novel use of palmitoylethanolamide. *Pain Med.* 11, 781–784. doi: 10.1111/j.1526-4637.2010.00823.x
- Calignano, A., La Rana, G., and Piomelli, D. (2001). Antinociceptive activity of the endogenous fatty acid amide, palmitoylethanolamide. *Eur. J. Pharmacol.* 419, 191–198. doi: 10.1016/S0014-2999(01)00988-8
- Callen, L., Moreno, E., Barroso-Chinea, P., Moreno-Delgado, D., Cortes, A., Mallol, J., et al. (2012). Cannabinoid receptors CB1 and CB2 form functional heteromers in brain. *J. Biol. Chem.* 287, 20851–20865. doi: 10.1074/jbc.M111.335273
- Carey, L. M., Gutierrez, T., Deng, L., Lee, W. H., Mackie, K., and Hohmann, A. G. (2017). Inflammatory and neuropathic nociception is preserved in GPR55 knockout mice. *Sci. Rep.* 7:944. doi: 10.1038/s41598-017-01062-2
- Celorrio, M., Rojo-Bustamante, E., Fernandez-Suarez, D., Saez, E., Estella-Hermoso De Mendoza, A., Muller, C. E., et al. (2017). GPR55: a therapeutic target for Parkinson's disease? *Neuropharmacology* 125, 319–332. doi: 10.1016/j.neuropharm.2017.08.017
- Chen, J., Li, L., Chen, S. R., Chen, H., Xie, J. D., Sirieh, R. E., et al. (2018). The alpha2delta-1-NMDA receptor complex is critically involved in neuropathic pain development and gabapentin therapeutic actions. *Cell Rep.* 22, 2307–2321. doi: 10.1016/j.celrep.2018.02.021
- Chiang, N., Dalli, J., Colas, R. A., and Serhan, C. N. (2015). Identification of resolinin D2 receptor mediating resolution of infections and organ protection. *J. Exp. Med.* 212, 1203–1217. doi: 10.1084/jem.20150225
- Chu, L. F., Angst, M. S., and Clark, D. (2008). Opioid-induced hyperalgesia in humans: molecular mechanisms and clinical considerations. *Clin. J. Pain* 24, 479–496. doi: 10.1097/AJP.0b013e31816b2f43
- Clapper, J. R., Moreno-Sanz, G., Russo, R., Guijarro, A., Vacondio, F., Duranti, A., et al. (2010). Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nat. Neurosci.* 13, 1265–1270. doi: 10.1038/nrn2632
- Clayton, N. M., Wilson, A. W., Collins, S. D., Giblin, G. M., Mitchell, B. L., Goldsmith, P., et al. (2004). Anti-hypersensitive and anti-inflammatory activity

- of the potent and selective CB2 agonist GW842166X. *Proc. Br. Pharmacol. Soc.* 21:050P. Available at: <http://www.pa2online.org/Vol2Issue4abst050P.html>
- Clermont-Gnamien, S., Atlani, S., Attal, N., Le Mercier, F., Guirimand, F., and Brasseur, L. (2002). [The therapeutic use of D9-tetrahydrocannabinol (dronabinol) in refractory neuropathic pain]. *Presse Med.* 31, 1840–1845.
- Condés-Lara, M., Martínez-Lorenzana, G., Rubio-Beltrán, E., Rodríguez-Jiménez, J., Rojas-Piloni, G., and González-Hernández, A. (2015). Hypothalamic paraventricular nucleus stimulation enhances c-Fos expression in spinal and supraspinal structures related to pain modulation. *Neurosci. Res.* 98, 59–63. doi: 10.1016/j.neures.2015.04.004
- Conigliaro, R., Drago, V., Foster, P. S., Schievano, C., and Di Marzo, V. (2011). Use of palmitoylethanolamide in the entrapment neuropathy of the median in the wrist. *Minerva Med.* 102, 141–147.
- Console-Bram, L., Brailou, E., Brailou, G. C., Sharir, H., and Abood, M. E. (2014). Activation of GPR18 by cannabinoid compounds: a tale of biased agonism. *Br. J. Pharmacol.* 171, 3908–3917. doi: 10.1111/bph.12746
- Console-Bram, L., Ciuciu, S. M., Zhao, P., Zipkin, R. E., Brailou, E., and Abood, M. E. (2017). N-arachidonoyl glycine, another endogenous agonist of GPR55. *Biochem. Biophys. Res. Commun.* 490, 1389–1393. doi: 10.1016/j.bbrc.2017.07.038
- Corchero, J., Avila, M. A., Fuentes, J. A., and Manzanares, J. (1997a). delta-9-Tetrahydrocannabinol increases prodynorphin and proenkephalin gene expression in the spinal cord of the rat. *Life Sci.* 61, 39–43.
- Corchero, J., Fuentes, J. A., and Manzanares, J. (1997b). delta-9-Tetrahydrocannabinol increases proopiomelanocortin gene expression in the arcuate nucleus of the rat hypothalamus. *Eur. J. Pharmacol.* 323, 193–195. doi: 10.1016/S0014-2999(97)00144-1
- Corey-Bloom, J., Wolfson, T., Gamst, A., Jin, S., Marcotte, T. D., Bentley, H., et al. (2012). Smoked cannabis for spasticity in multiple sclerosis: a randomized, placebo-controlled trial. *CMAJ* 184, 1143–1150. doi: 10.1503/cmaj.110837
- Costa, B., Bettini, I., Petrosino, S., Comelli, F., Giagnoni, G., and Di Marzo, V. (2010). The dual fatty acid amide hydrolase/TRPV1 blocker, N-arachidonoyl-serotonin, relieves carrageenan-induced inflammation and hyperalgesia in mice. *Pharmacol. Res.* 61, 537–546. doi: 10.1016/j.phrs.2010.02.001
- Crawley, J. N., Corwin, R. L., Robinson, J. K., Felder, C. C., Devane, W. A., and Axelrod, J. (1993). Anandamide, an endogenous ligand of the cannabinoid receptor, induces hypomotility and hypothermia in vivo in rodents. *Pharmacol. Biochem. Behav.* 46, 967–972. doi: 10.1016/0091-3057(93)90230-Q
- Crean, R. D., Tapert, S. F., Minassian, A., Macdonald, K., Crane, N. A., and Mason, B. J. (2011). Effects of chronic, heavy cannabis use on executive functions. *J. Addict. Med.* 5, 9–15. doi: 10.1097/ADM.0b013e31820cd57
- D'Agostino, G., La Rana, G., Russo, R., Sasso, O., Iacono, A., Esposito, E., et al. (2007). Acute intracerebroventricular administration of palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor-alpha agonist, modulates carrageenan-induced paw edema in mice. *J. Pharmacol. Exp. Ther.* 322, 1137–1143. doi: 10.1124/jpet.107.123265
- Deliu, E., Sperow, M., Console-Bram, L., Carter, R. L., Tilley, D. G., Kalamarides, D. J., et al. (2015). The lysophosphatidylinositol receptor GPR55 modulates pain perception in the periaqueductal gray. *Mol. Pharmacol.* 88, 265–272. doi: 10.1124/mol.115.099333
- Danthi, S., Enyeart, J. A., and Enyeart, J. J. (2003). Modulation of native TREK-1 and Kv1.4 K⁺ channels by polyunsaturated fatty acids and lysophospholipids. *J. Membr. Biol.* 195, 147–164. doi: 10.1007/s00232-003-0616-0
- Davis, M. P. (2014). Cannabinoids in pain management: CB1, CB2 and non-classic receptor ligands. *Expert Opin. Investig. Drugs* 23, 1123–1140. doi: 10.1517/13543784.2014.918603
- Demuth, D. G., and Molleman, A. (2006). Cannabinoid signalling. *Life Sci.* 78, 549–563. doi: 10.1016/j.lfs.2005.05.055
- Deng, L., Guindon, J., Cornett, B. L., Makriyannis, A., Mackie, K., and Hohmann, A. G. (2015). Chronic cannabinoid receptor 2 activation reverses paclitaxel neuropathy without tolerance or cannabinoid receptor 1-dependent withdrawal. *Biol. Psychiatry* 77, 475–487. doi: 10.1016/j.biopsych.2014.04.009
- Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., et al. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946–1949. doi: 10.1126/science.1470919
- Di Marzo, V., Bifulco, M., and De Petrocellis, L. (2004). The endocannabinoid system and its therapeutic exploitation. *Nat. Rev. Drug Discov.* 3, 771–784. doi: 10.1038/nrd1495
- Dowell, D., Haegerich, T. M., and Chou, R. (2016). CDC guideline for prescribing opioids for chronic pain—united states, 2016. *JAMA* 315, 1624–1645. doi: 10.1001/jama.2016.1464
- Drewes, A. M., Andreassen, A., and Poulsen, L. H. (1994). Valproate for treatment of chronic central pain after spinal cord injury. A double-blind cross-over study. *Paraplegia* 32, 565–569.
- Dumas, E. O., and Pollack, G. M. (2008). Opioid tolerance development: a pharmacokinetic/pharmacodynamic perspective. *AAPS J.* 10, 537–551. doi: 10.1208/s12248-008-9056-1
- Dunn, S. L., Wilkinson, J. M., Crawford, A., Bunning, R. A. D., and Le Maitre, C. L. (2016). Expression of cannabinoid receptors in human osteoarthritic cartilage: implications for future therapies. *Cannabis Cannabinoid Res.* 1, 3–15. doi: 10.1089/can.2015.0001
- Eisenberg, E., Lurie, Y., Braker, C., Daoud, D., and Ishay, A. (2001). Lamotrigine reduces painful diabetic neuropathy: a randomized, controlled study. *Neurology* 57, 505–509. doi: 10.1212/WNL.57.3.505
- Elmes, S. J., Jhaveri, M. D., Smart, D., Kendall, D. A., and Chapman, V. (2004). Cannabinoid CB2 receptor activation inhibits mechanically evoked responses of wide dynamic range dorsal horn neurons in naive rats and in rat models of inflammatory and neuropathic pain. *Eur. J. Neurosci.* 20, 2311–2320. doi: 10.1111/j.1460-9568.2004.03690.x
- Evans, R. M., Scott, R. H., and Ross, R. A. (2004). Multiple actions of anandamide on neonatal rat cultured sensory neurones. *Br. J. Pharmacol.* 141, 1223–1233. doi: 10.1038/sj.bjp.0705723
- Fabbro, F., and Crescentini, C. (2014). Facing the experience of pain: a neuropsychological perspective. *Phys. Life Rev.* 11, 540–552. doi: 10.1016/j.plrev.2013.12.010
- Fan, P. (1995). Cannabinoid agonists inhibit the activation of 5-HT3 receptors in rat nodose ganglion neurons. *J. Neurophysiol.* 73, 907–910. doi: 10.1152/jn.1995.73.2.907
- Felder, C. C., Joyce, K. E., Briley, E. M., Mansouri, J., Mackie, K., Blond, O., et al. (1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol. Pharmacol.* 48, 443–450.
- Finnerup, N. B., Sindrup, S. H., and Jensen, T. S. (2010). The evidence for pharmacological treatment of neuropathic pain. *Pain* 150, 573–581. doi: 10.1016/j.pain.2010.06.019
- Fischbach, T., Greffrath, W., Nawrath, H., and Treede, R. D. (2007). Effects of anandamide and noxious heat on intracellular calcium concentration in nociceptive DRG neurons of rats. *J. Neurophysiol.* 98, 929–938. doi: 10.1152/jn.01096.2006
- Fox, A., Kesingland, A., Gentry, C., Mcnair, K., Patel, S., Urban, L., et al. (2001). The role of central and peripheral Cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. *Pain* 92, 91–100. doi: 10.1016/S0304-3950(00)00474-7
- Fu, J., Gaetani, S., Oveis, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F., et al. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* 425, 90–93. doi: 10.1038/nature01921
- Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., et al. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* 232, 54–61. doi: 10.1111/j.1432-1033.1995.tb20780.x
- Gangadharan, V., Selvaraj, D., Kurejova, M., Njoo, C., Gritsch, S., Skorikova, D., et al. (2013). A novel biological role for the phospholipid lysophosphatidylinositol in nociceptive sensitization via activation of diverse G-protein signalling pathways in sensory nerves in vivo. *Pain* 154, 2801–2812. doi: 10.1016/j.pain.2013.08.019
- Gantz, I., Muraoka, A., Yang, Y. K., Samuelson, L. C., Zimmerman, E. M., Cook, H., et al. (1997). Cloning and chromosomal localization of a gene (GPR18) encoding a novel seven transmembrane receptor highly expressed in spleen and testis. *Genomics* 42, 462–466. doi: 10.1006/geno.1997.4752
- Gaoni, Y., and Mechoulam, R. (1964). Isolation, structure, and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* 86, 1646–1647. doi: 10.1021/ja01062a046
- Gatti, A., Lazzari, M., Gianfelice, V., Di Paolo, A., Sabato, E., and Sabato, A. F. (2012). Palmitoylethanolamide in the treatment of chronic pain caused by different etiopathogenesis. *Pain Med.* 13, 1121–1130. doi: 10.1111/j.1526-4637.2012.01432.x

- Gauldie, S. D., McQueen, D. S., Pertwee, R., and Chessell, I. P. (2001). Anandamide activates peripheral nociceptors in normal and arthritic rat knee joints. *Br. J. Pharmacol.* 132, 617–621. doi: 10.1038/sj.bjp.0703890
- Gee, N. S., Brown, J. P., Dissanayake, V. U., Offord, J., Thurlow, R., and Woodruff, G. N. (1996). The novel anticonvulsant drug, gabapentin (Neurontin), binds to the alpha₂delta subunit of a calcium channel. *J. Biol. Chem.* 271, 5768–5776. doi: 10.1074/jbc.271.10.5768
- Giblin, G. M. P., O’shaughnessy, C. T., Naylor, A., Mitchell, W. L., Eatherton, A. J., Slingsby, B. P., et al. (2007). Discovery of 2-[(2,4-dichlorophenyl)amino]-N-[(tetrahydro-2H-pyran-4-yl)methyl]-4-(trifluoromethyl)-5-pyrimidinecarboxamide, a selective CB2 receptor agonist for the treatment of inflammatory pain. *J. Med. Chem.* 50, 2597–2600. doi: 10.1021/jm061195+
- Glass, M., Dragunow, M., and Faull, R. L. (1997). Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. *Neuroscience* 77, 299–318. doi: 10.1016/S0306-4522(96)00428-9
- Godlewski, G., Goertert, M., and Malinowska, B. (2003). Cannabinoid receptor-independent inhibition by cannabinoid agonists of the peripheral 5-HT3 receptor-mediated von Bezold-Jarisch reflex. *Br. J. Pharmacol.* 138, 767–774. doi: 10.1038/sj.bjp.0705114
- Gong, J. P., Onaivi, E. S., Ishiguro, H., Liu, Q. R., Tagliaferro, P. A., Brusco, A., et al. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res.* 1071, 10–23. doi: 10.1016/j.brainres.2005.11.035
- González-Hernández, A., Martínez-Lorenzana, G., Rodríguez-Jiménez, J., Rojas-Piloni, G., and Condés-Lara, M. (2015). Intracisternal injection of palmitoylethanamide inhibits the peripheral nociceptive evoked responses of dorsal horn wide dynamic range neurons. *J. Neural Transm.* 122, 369–374. doi: 10.1007/s00702-014-1255-6
- Griffin, M. R., Piper, J. M., Daugherty, J. R., Snowden, M., and Ray, W. A. (1991). Nonsteroidal anti-inflammatory drug use and increased risk for peptic ulcer disease in elderly persons. *Ann. Intern. Med.* 114, 257–263. doi: 10.7326/0003-4819-114-4-257
- Guy, A. T., Nagatsuka, Y., Ooashi, N., Inoue, M., Nakata, A., Greimel, P., et al. (2015). Glycerophospholipid regulation of modality-specific sensory axon guidance in the spinal cord. *Science* 349, 974–977. doi: 10.1126/science.aab3516
- Hanus, L., Abu-Lafi, S., Fride, E., Breuer, A., Vogel, Z., Shalev, D. E., et al. (2001). 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3662–3665. doi: 10.1073/pnas.061029898
- Hanus, L., Breuer, A., Tchilibon, S., Shiloah, S., Goldenberg, D., Horowitz, M., et al. (1999). HU-308: a specific agonist for CB2, a peripheral cannabinoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14228–14233. doi: 10.1073/pnas.96.25.14228
- Harada, K., Kitaguchi, T., Kamiya, T., Aung, K. H., Nakamura, K., Ohta, K., et al. (2017). Lysophosphatidylinositol-induced activation of the cation channel TRPV2 triggers glucagon-like peptide-1 secretion in enteroendocrine L cells. *J. Biol. Chem.* 292, 10855–10864. doi: 10.1074/jbc.M117.788653
- Heinricher, M. M. (2016). Pain modulation and the transition from acute to chronic pain. *Adv. Exp. Med. Biol.* 904, 105–115. doi: 10.1007/978-94-017-7537-3_8
- Henstridge, C. M., Balenga, N. A., Ford, L. A., Ross, R. A., Waldhoer, M., and Irving, A. J. (2009). The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca²⁺ signaling and NFAT activation. *FASEB J.* 23, 183–193. doi: 10.1096/fj.08-108670
- Henstridge, C. M., Balenga, N. A., Schroder, R., Kargl, J. K., Platzer, W., Martini, L., et al. (2010). GPR55 ligands promote receptor coupling to multiple signalling pathways. *Br. J. Pharmacol.* 160, 604–614. doi: 10.1111/j.1476-5381.2009.00625.x
- Herzberg, U., Eliav, E., Bennett, G. J., and Kopin, I. J. (1997). The analgesic effects of R(+)-WIN 55,212-2 mesylate, a high affinity cannabinoid agonist, in a rat model of neuropathic pain. *Neurosci. Lett.* 221, 157–160. doi: 10.1016/S0304-3940(96)13308-5
- Hillsley, K., McCaul, C., Aerssens, J., Peeters, P. J., Gijsen, H., Moehars, D., et al. (2007). Activation of the cannabinoid 2 (CB2) receptor inhibits murine mesenteric afferent nerve activity. *Neurogastroenterol. Motil.* 19, 769–777. doi: 10.1111/j.1365-2982.2007.00950.x
- Hohmann, A. G., Briley, E. M., and Herkenham, M. (1999). Pre- and postsynaptic distribution of cannabinoid and mu opioid receptors in rat spinal cord. *Brain Res.* 822, 17–25. doi: 10.1016/S0006-8993(98)01321-3
- Hohmann, A. G., and Herkenham, M. (1999). Localization of central cannabinoid CB1 receptor messenger RNA in neuronal subpopulations of rat dorsal root ganglia: a double-label in situ hybridization study. *Neuroscience* 90, 923–931. doi: 10.1016/S0306-4522(98)00524-7
- Horl, W. H. (2010). Nonsteroidal anti-inflammatory drugs and the kidney. *Pharmaceuticals* 3, 2291–2321. doi: 10.3390/ph3072291
- Howlett, A. C. (2002). The cannabinoid receptors. *Prostaglandins Other Lipid Mediat.* 68–69, 619–631. doi: 10.1016/S0090-6980(02)00060-6
- Hsieh, G. C., Pai, M., Chandran, P., Hooker, B. A., Zhu, C. Z., Salyers, A. K., et al. (2011). Central and peripheral sites of action for CB2 receptor mediated analgesic activity in chronic inflammatory and neuropathic pain models in rats. *Br. J. Pharmacol.* 162, 428–440. doi: 10.1111/j.1476-5381.2010.01046.x
- Huang, S. M., Bisogno, T., Petros, T. J., Chang, S. Y., Zavitsanos, P. A., Zipkin, R. E., et al. (2001). Identification of a new class of molecules, the arachidonyl amino acids, and characterization of one member that inhibits pain. *J. Biol. Chem.* 276, 42639–42644. doi: 10.1074/jbc.M107351200
- Ibrahim, M. M., Deng, H., Zvonok, A., Cockayne, D. A., Kwan, J., Mata, H. P., et al. (2003). Activation of CB2 cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10529–10533. doi: 10.1073/pnas.1834309100
- Ishac, E. J., Jiang, L., Lake, K. D., Varga, K., Abood, M. E., and Kunos, G. (1996). Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. *Br. J. Pharmacol.* 118, 2023–2028. doi: 10.1111/j.1476-5381.1996.tb15639.x
- Jagger, S. I., Hasnje, F. S., Sellaturay, S., and Rice, A. S. (1998). The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanamide in visceral and somatic inflammatory pain. *Pain* 76, 189–199. doi: 10.1016/S0304-3959(98)00041-4
- Jensen, B., Chen, J., Furnish, T., and Wallace, M. (2015). Medical marijuana and chronic pain: a review of basic science and clinical evidence. *Curr. Pain Headache Rep.* 19:50. doi: 10.1007/s11916-015-0524-x
- Jeong, H. J., Vandenberg, R. J., and Vaughan, C. W. (2010). N-arachidonyl-glycine modulates synaptic transmission in superficial dorsal horn. *Br. J. Pharmacol.* 161, 925–935. doi: 10.1111/j.1476-5381.2010.00935.x
- Ji, R. R., Gereau, R. W. T., Malcangio, M., and Strichartz, G. R. (2009). MAP kinase and pain. *Brain Res. Rev.* 60, 135–148. doi: 10.1016/j.brainresrev.2008.12.011
- Johanek, L. M., Heitmiller, D. R., Turner, M., Nader, N., Hodges, J., and Simone, D. A. (2001). Cannabinoids attenuate capsaicin-evoked hyperalgesia through spinal and peripheral mechanisms. *Pain* 93, 303–315. doi: 10.1016/S0304-3959(01)00336-0
- Johanek, L. M., and Simone, D. A. (2004). Activation of peripheral cannabinoid receptors attenuates cutaneous hyperalgesia produced by a heat injury. *Pain* 109, 432–442. doi: 10.1016/j.pain.2004.02.020
- Julius, D., and Basbaum, A. I. (2001). Molecular mechanisms of nociception. *Nature* 413, 203–210. doi: 10.1038/35093019
- Kalliomäki, J., Segerdahl, M., Webster, L., Reimfelt, A., Huizar, K., Annas, P., et al. (2013). Evaluation of the analgesic efficacy of AZD1940, a novel cannabinoid agonist, on post-operative pain after lower third molar surgical removal. *Scand. J. Pain* 4, 17–22. doi: 10.1016/j.jspain.2012.08.004
- Kaplan, J. S., Stella, N., Catterall, W. A., and Westenbroek, R. E. (2017). Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 114, 11229–11234. doi: 10.1073/pnas.1711351114
- Kapur, A., Zhao, P., Sharir, H., Bai, Y., Caron, M. G., Barak, L. S., et al. (2009). Atypical responsiveness of the orphan receptor GPR55 to cannabinoid ligands. *J. Biol. Chem.* 284, 29817–29827. doi: 10.1074/jbc.M109.050187
- Keppel Hesselink, J. M., Kopsky, D. J., and Sajben, N. L. (2014). Vulvodynia and proctodynia treated with topical baclofen 5 % and palmitoylethanamide. *Arch. Gynecol. Obstet.* 290, 389–393. doi: 10.1007/s00404-014-3218-4
- Kinsey, S. G., Naidu, P. S., Cravatt, B. F., Dudley, D. T., and Lichtman, A. H. (2011). Fatty acid amide hydrolase blockade attenuates the development of collagen-induced arthritis and related thermal hyperalgesia in mice. *Pharmacol. Biochem. Behav.* 99, 718–725. doi: 10.1016/j.pbb.2011.06.022
- Knerlich-Lukoschus, F., Noack, M., Von Der Ropp-Brenner, B., Lucius, R., Mehndorn, H. M., and Held-Feindt, J. (2011). Spinal cord injuries induce changes in CB1 cannabinoid receptor and C-C chemokine expression in brain

- areas underlying circuitry of chronic pain conditions. *J. Neurotrauma* 28, 619–634. doi: 10.1089/neu.2010.1652
- Ko, M. C., and Woods, J. H. (1999). Local administration of delta9-tetrahydrocannabinol attenuates capsaicin-induced thermal nociception in rhesus monkeys: a peripheral cannabinoid action. *Psychopharmacology* 143, 322–326. doi: 10.1007/s002130050955
- Kohno, M., Hasegawa, H., Inoue, A., Muraoka, M., Miyazaki, T., Oka, K., et al. (2006). Identification of N-arachidonoylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochem. Biophys. Res. Commun.* 347, 827–832. doi: 10.1016/j.bbrc.2006.06.175
- Kraft, B., Frickey, N. A., Kaufmann, R. M., Reif, M., Frey, R., Gustorff, B., et al. (2008). Lack of analgesia by oral standardized cannabis extract on acute inflammatory pain and hyperalgesia in volunteers. *Anesthesiology* 109, 101–110. doi: 10.1097/ALN.0b013e31817881e1
- LaBuda, C. J., Koblish, M., and Little, P. J. (2005). Cannabinoid CB2 receptor agonist activity in the hindpaw incision model of postoperative pain. *Eur. J. Pharmacol.* 527, 172–174. doi: 10.1016/j.ejphar.2005.10.020
- Langford, R. M., Mares, J., Novotna, A., Vachova, M., Novakova, I., Notcutt, W., et al. (2013). A double-blind, randomized, placebo-controlled, parallel-group study of THC/CBD oromucosal spray in combination with the existing treatment regimen, in the relief of central neuropathic pain in patients with multiple sclerosis. *J. Neurol.* 260, 984–997. doi: 10.1007/s00415-012-6739-4
- Laprairie, R. B., Kelly, M. E., and Denovan-Wright, E. M. (2012). The dynamic nature of type 1 cannabinoid receptor (CB1) gene transcription. *Br. J. Pharmacol.* 167, 1583–1595. doi: 10.1111/j.1476-5383.2012.02175.x
- Lauckner, J. E., Jensen, J. B., Chen, H. Y., Lu, H. C., Hille, B., and Mackie, K. (2008). GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2699–2704. doi: 10.1073/pnas.0711278105
- Li, H.-L. (1974). An archaeological and historical account of cannabis in China. *Econ. Bot.* 28, 437–448. doi: 10.1007/BF02862859
- Li, J., Daughters, R. S., Bullis, C., Bengiamin, R., Stucky, M. W., Brennan, J., et al. (1999). The cannabinoid receptor agonist WIN 55,212-2 mesylate blocks the development of hyperalgesia produced by capsaicin in rats. *Pain* 81, 25–33. doi: 10.1016/S0304-3959(98)00263-2
- Lim, G., Sung, B., Ji, R. R., and Mao, J. (2003). Upregulation of spinal cannabinoid-1-receptors following nerve injury enhances the effects of Win 55,212-2 on neuropathic pain behaviors in rats. *Pain* 105, 275–283. doi: 10.1016/S0304-3959(03)00242-2
- Llorca-Torralba, M., Borges, G., Neto, F., Mico, J. A., and Berrocoso, E. (2016). Noradrenergic Locus caeruleus pathways in pain modulation. *Neuroscience* 338, 93–113. doi: 10.1016/j.neuroscience.2016.05.057
- Lo Verme, J., Fu, J., Astarita, G., La Rana, G., Russo, R., Calignano, A., et al. (2005). The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol. Pharmacol.* 67, 15–19. doi: 10.1124/mol.104.006353
- Loyd, D. R., and Murphy, A. Z. (2009). The role of the periaqueductal gray in the modulation of pain in males and females: are the anatomy and physiology really that different? *Neural Plast.* 2009:462879. doi: 10.1155/2009/462879
- Lu, V. B., Puhl, H. L. III, and Ikeda, S. R. (2013). N-Arachidonyl glycine does not activate G protein-coupled receptor 18 signaling via canonical pathways. *Mol. Pharmacol.* 83, 267–282. doi: 10.1124/mol.112.081182
- Luchicchi, A., and Pistis, M. (2012). Anandamide and 2-arachidonoylglycerol: pharmacological properties, functional features, and emerging specificities of the two major endocannabinoids. *Mol. Neurobiol.* 46, 374–392. doi: 10.1007/s12035-012-8299-0
- Lynch, M. E., Cesar-Rittenberg, P., and Hohmann, A. G. (2014). A double-blind, placebo-controlled, crossover pilot trial with extension using an oral mucosal cannabinoid extract for treatment of chemotherapy-induced neuropathic pain. *J. Pain Symptom Manage.* 47, 166–173. doi: 10.1016/j.jpainsymman.2013.02.018
- Ma, H., Chen, S. R., Chen, H., Li, L., Li, D. P., Zhou, J. J., et al. (2018). Alpha2delta-1 is essential for sympathetic output and NMDA receptor activity potentiated by angiotensin II in the hypothalamus. *J. Neurosci.* 38, 6388–6398. doi: 10.1523/JNEUROSCI.0447-18.2018
- Mackie, K. (2005). Cannabinoid receptor homo- and heterodimerization. *Life Sci.* 77, 1667–1673. doi: 10.1016/j.lfs.2005.05.011
- Mackie, K. (2008a). Cannabinoid receptors: where they are and what they do. *J. Neuroendocrinol.* 20(Suppl. 1), 10–14. doi: 10.1111/j.1365-2826.2008.01671.x
- Mackie, K. (2008b). Signaling via CNS cannabinoid receptors. *Mol. Cell. Endocrinol.* 286, S60–S65. doi: 10.1016/j.mce.2008.01.022
- Maingret, F., Patel, A. J., Lesage, F., Lazdunski, M., and Honore, E. (2000). Lysophospholipids open the two-pore domain mechano-gated K⁺ channels TREK-1 and TRAAK. *J. Biol. Chem.* 275, 10128–10133. doi: 10.1074/jbc.275.14.10128
- Maione, S., Costa, B., Piscitelli, F., Morera, E., De Chiaro, M., Comelli, F., et al. (2013). Piperazinyl carbamate fatty acid amide hydrolase inhibitors and transient receptor potential channel modulators as “dual-target” analgesics. *Pharmacol. Res.* 76, 98–105. doi: 10.1016/j.phrs.2013.07.003
- Maione, S., De Petrocellis, L., De Novellis, V., Moriello, A. S., Petrosino, S., Palazzo, E., et al. (2007). Analgesic actions of N-arachidonoyl-serotonin, a fatty acid amide hydrolase inhibitor with antagonistic activity at vanilloid TRPV1 receptors. *Br. J. Pharmacol.* 150, 766–781. doi: 10.1038/sj.bjp.0707145
- Malan, T. P. Jr., Ibrahim, M. M., Vanderah, T. W., Makriyannis, A., and Porreca, F. (2002). Inhibition of pain responses by activation of CB2 cannabinoid receptors. *Chem. Phys. Lipids* 121, 191–200. doi: 10.1016/S0009-3084(02)00155-X
- Malan, T. P. Jr., Ibrahim, M. M., Deng, H., Liu, Q., Mata, H. P., Vanderah, T., et al. (2001). CB2 cannabinoid receptor-mediated peripheral antinociception. *Pain* 93, 239–245. doi: 10.1016/S0304-3959(01)00321-9
- Malek, N., Kostrzewska, M., Makuch, W., Pajak, A., Kucharczyk, M., Piscitelli, F., et al. (2016). The multiplicity of spinal AA-5-HT anti-nociceptive action in a rat model of neuropathic pain. *Pharmacol. Res.* 111, 251–263. doi: 10.1016/j.phrs.2016.06.012
- Malek, N., Mrugala, M., Makuch, W., Kolosowska, N., Przewlocka, B., Binkowski, M., et al. (2015). A multi-target approach for pain treatment: dual inhibition of fatty acid amide hydrolase and TRPV1 in a rat model of osteoarthritis. *Pain* 156, 890–903. doi: 10.1097/j.pain.0000000000000132
- Manzanares, J., Corchero, J., Romero, J., Fernandez-Ruiz, J. J., Ramos, J. A., and Fuentes, J. A. (1999). Pharmacological and biochemical interactions between opioids and cannabinoids. *Trends Pharmacol. Sci.* 20, 287–294. doi: 10.1016/S0165-6147(99)01339-5
- Marichal-Cancino, B. A., Fajardo-Valdez, A., Ruiz-Contreras, A. E., Méndez-Díaz, M., and Prospero-García, O. (2017). Advances in the physiology of GPR55 in the central nervous system. *Curr. Neuropharmacol.* 15, 771–778. doi: 10.2174/1570159X14666160729155441
- Marichal-Cancino, B. A., Fajardo-Valdez, A., Ruiz-Contreras, A. E., Méndez-Díaz, M., and Prospero-García, O. (2018). Possible role of hippocampal GPR55 in spatial learning and memory in rats. *Acta Neurobiol. Exp.* 78, 41–50. doi: 10.21307/ane-2018-001
- Marichal-Cancino, B. A., Manrique-Maldonado, G., Altamirano-Espinoza, A. H., Ruiz-Salinas, I., González-Hernández, A., Maassenvandenbrink, A., et al. (2013). Analysis of anandamide- and lysophosphatidylinositol-induced inhibition of the vasopressor responses produced by sympathetic stimulation or noradrenaline in pithed rats. *Eur. J. Pharmacol.* 721, 168–177. doi: 10.1016/j.ejphar.2013.09.039
- Marichal-Cancino, B. A., Sánchez-Fuentes, A., Méndez-Díaz, M., Ruiz-Contreras, A. E., and Prospero-García, O. (2016). Blockade of GPR55 in the dorsolateral striatum impairs performance of rats in a T-maze paradigm. *Behav. Pharmacol.* 27, 393–396. doi: 10.1097/FBP.0000000000000185
- Martell, B. A., O’Connor, P. G., Kerns, R. D., Becker, W. C., Morales, K. H., Kosten, T. R., et al. (2007). Systematic review: opioid treatment for chronic back pain: prevalence, efficacy, and association with addiction. *Ann. Intern. Med.* 146, 116–127. doi: 10.7326/0003-4819-146-2-200701160-00006
- Martin-Sánchez, E., Furukawa, T. A., Taylor, J., and Martin, J. L. (2009). Systematic review and meta-analysis of cannabis treatment for chronic pain. *Pain Med.* 10, 1353–1368. doi: 10.1111/j.1526-4637.2009.00703.x
- Mason, P. (2001). Contributions of the medullary raphe and ventromedial reticular region to pain modulation and other homeostatic functions. *Annu. Rev. Neurosci.* 24, 737–777. doi: 10.1146/annurev.neuro.24.1.737
- Massi, P., Vaccani, A., Rubino, T., and Parolaro, D. (2003). Cannabinoids and opioids share cAMP pathway in rat splenocytes. *J. Neuroimmunol.* 145, 46–54. doi: 10.1016/j.jneuroim.2003.09.006
- McGaraughty, S., Chu, K. L., Dart, M. J., Yao, B. B., and Meyer, M. D. (2009). A CB2 receptor agonist, A-836339, modulates wide dynamic range neuronal activity in neuropathic rats: contributions of spinal and peripheral CB2 receptors. *Neuroscience* 158, 1652–1661. doi: 10.1016/j.neuroscience.2008.11.015

- McHugh, D., Hu, S. S., Rimmerman, N., Juknat, A., Vogel, Z., Walker, J. M., et al. (2010). N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. *BMC Neurosci.* 11:44. doi: 10.1186/1471-2202-11-44
- Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., et al. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50, 83–90. doi: 10.1016/0006-2952(95)00109-D
- Meng, I. D., Manning, B. H., Martin, W. J., and Fields, H. L. (1998). An analgesia circuit activated by cannabinoids. *Nature* 395, 381–383. doi: 10.1038/26481
- Merriam, F. V., Wang, Z. Y., Guerios, S. D., and Bjorling, D. E. (2008). Cannabinoid receptor 2 is increased in acutely and chronically inflamed bladder of rats. *Neurosci. Lett.* 445, 130–134. doi: 10.1016/j.neulet.2008.08.076
- Mico, J. A., Ardid, D., Berrocoso, E., and Eschalier, A. (2006). Antidepressants and pain. *Trends Pharmacol. Sci.* 27, 348–354. doi: 10.1016/j.tips.2006.05.004
- Mika, J., Zychowska, M., Makuch, W., Rojewska, E., and Przewlocka, B. (2013). Neuronal and immunological basis of action of antidepressants in chronic pain - clinical and experimental studies. *Pharmacol. Rep.* 65, 1611–1621. doi: 10.1016/S1734-1140(13)71522-6
- Miller, S., Leishman, E., Oehler, O., Daily, L., Murataeva, N., Wager-Miller, J., et al. (2016). Evidence for a GPR18 role in diurnal Regulation of intraocular pressure. *Invest. Ophthalmol. Vis. Sci.* 57, 6419–6426. doi: 10.1167/iovs.16-19437
- Millns, P. J., Chapman, V., and Kendall, D. A. (2001). Cannabinoid inhibition of the capsaicin-induced calcium response in rat dorsal root ganglion neurones. *Br. J. Pharmacol.* 132, 969–971. doi: 10.1038/sj.bjp.0703919
- Moffat, R., and Rae, C. P. (2011). Anatomy, physiology and pharmacology of pain. *Anaesth. Intensive Care Med.* 12, 12–15. doi: 10.1016/j.mpac.2010.10.011
- Monet, M., Gkika, D., Lehen'kyi, V., Pourtier, A., Vanden Abeele, F., Bidaux, G., et al. (2009). Lysophospholipids stimulate prostate cancer cell migration via TRPV2 channel activation. *Biochim. Biophys. Acta* 1793, 528–539. doi: 10.1016/j.bbamcr.2009.01.003
- Morera, E., De Petrocellis, L., Morera, L., Moriello, A. S., Ligresti, A., Nalli, M., et al. (2009). Synthesis and biological evaluation of piperazinyl carbamates and ureas as fatty acid amide hydrolase (FAAH) and transient receptor potential (TRP) channel dual ligands. *Bioorg. Med. Chem. Lett.* 19, 6806–6809. doi: 10.1016/j.bbamcr.2009.09.033
- Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365, 61–65. doi: 10.1038/365061a0
- Narang, S., Gibson, D., Wasan, A. D., Ross, E. L., Michna, E., Nedeljkovic, S. S., et al. (2008). Efficacy of dronabinol as an adjuvant treatment for chronic pain patients on opioid therapy. *J. Pain* 9, 254–264. doi: 10.1016/j.jpain.2007.10.018
- Niederberger, E., and Geisslinger, G. (2008). The IKK-NF- κ B pathway: a source for novel molecular drug targets in pain therapy? *FASEB J.* 22, 3432–3442. doi: 10.1096/fj.08-109355
- Nurmikko, T. J., Serpell, M. G., Hoggart, B., Toomey, P. J., Morlion, B. J., and Haines, D. (2007). Sativex successfully treats neuropathic pain characterised by allodynia: a randomised, double-blind, placebo-controlled clinical trial. *Pain* 133, 210–220. doi: 10.1016/j.pain.2007.08.028
- Oka, S., Kimura, S., Toshida, T., Ota, R., Yamashita, A., and Sugiura, T. (2010). Lysophosphatidylinositol induces rapid phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor 2 in HEK293 cells expressing GPR55 and IM-9 lymphoblastoid cells. *J. Biochem.* 147, 671–678. doi: 10.1093/jb/mvp208
- Oka, S., Nakajima, K., Yamashita, A., Kishimoto, S., and Sugiura, T. (2007). Identification of GPR55 as a lysophosphatidylinositol receptor. *Biochem. Biophys. Res. Commun.* 362, 928–934. doi: 10.1016/j.bbrc.2007.08.078
- Okamoto, Y., Wang, J., Morishita, J., and Ueda, N. (2007). Biosynthetic pathways of the endocannabinoid anandamide. *Chem. Biodivers.* 4, 1842–1857. doi: 10.1002/cbdv.200790155
- Okine, B. N., Madasu, M. K., McGowan, F., Prendergast, C., Gaspar, J. C., Harhen, B., et al. (2016). N-palmitoylethanolamide in the anterior cingulate cortex attenuates inflammatory pain behaviour indirectly via a CB1 receptor-mediated mechanism. *Pain* 157, 2687–2696. doi: 10.1097/j.pain.0000000000000687
- Onaiyi, E. S., Ishiguro, H., Gong, J. P., Patel, S., Perchuk, A., Meozzi, P. A., et al. (2006). Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Ann. N. Y. Acad. Sci.* 1074, 514–536. doi: 10.1196/annals.1369.052
- Oshita, K., Inoue, A., Tang, H. B., Nakata, Y., Kawamoto, M., and Yuge, O. (2005). CB(1) cannabinoid receptor stimulation modulates transient receptor potential vanilloid receptor 1 activities in calcium influx and substance P Release in cultured rat dorsal root ganglion cells. *J. Pharmacol. Sci.* 97, 377–385. doi: 10.1254/jphs.FP0040872
- Otto, M., Bach, F. W., Jensen, T. S., and Sindrup, S. H. (2004). Valproic acid has no effect on pain in polyneuropathy: a randomized, controlled trial. *Neurology* 62, 285–288. doi: 10.1212/WNL.62.2.285
- Park, C. K., Xu, Z. Z., Liu, T., Lu, N., Serhan, C. N., and Ji, R. R. (2011). Resolvin D2 is a potent endogenous inhibitor for transient receptor potential subtype V1/A1, inflammatory pain, and spinal cord synaptic plasticity in mice: distinct roles of resolvin D1, D2, and E1. *J. Neurosci.* 31, 18433–18438. doi: 10.1523/JNEUROSCI.4192-11.2011
- Patel, H. J., Birrell, M. A., Crispino, N., Hele, D. J., Venkatesan, P., Barnes, P. J., et al. (2003). Inhibition of guinea-pig and human sensory nerve activity and the cough reflex in guinea-pigs by cannabinoid (CB2) receptor activation. *Br. J. Pharmacol.* 140, 261–268. doi: 10.1038/sj.bjp.0705435
- Pertwee, R. G. (2009). Emerging strategies for exploiting cannabinoid receptor agonists as medicines. *Br. J. Pharmacol.* 156, 397–411. doi: 10.1111/j.1476-5381.2008.00048.x
- Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P. H., Di Marzo, V., Elphick, M. R., et al. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB₁ and CB₂. *Pharmacol. Rev.* 62, 588–631. doi: 10.1124/pr.110.003004
- Pineiro, R., Maffucci, T., and Falasca, M. (2011). The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene* 30, 142–152. doi: 10.1038/onc.2010.417
- Pini, L. A., Guerzoni, S., Cainazzo, M. M., Ferrari, A., Sarchielli, P., Tiraferri, I., et al. (2012). Nabilone for the treatment of medication overuse headache: results of a preliminary double-blind, active-controlled, randomized trial. *J. Headache Pain* 13, 677–684. doi: 10.1007/s10194-012-0490-1
- Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.* 4, 873–884. doi: 10.1038/nrn1247
- Porter, A. C., Sauer, J. M., Knierman, M. D., Becker, G. W., Berna, M. J., Bao, J., et al. (2002). Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J. Pharmacol. Exp. Ther.* 301, 1020–1024. doi: 10.1124/jpet.301.3.1020
- Price, T. J., Helesic, G., Parghi, D., Hargreaves, K. M., and Flores, C. M. (2003). The neuronal distribution of cannabinoid receptor type 1 in the trigeminal ganglion of the rat. *Neuroscience* 120, 155–162. doi: 10.1016/S0306-4522(03)00333-6
- Pugh, G. Jr., Smith, P. B., Dombrowski, D. S., and Welch, S. P. (1996). The role of endogenous opioids in enhancing the antinociception produced by the combination of delta 9-tetrahydrocannabinol and morphine in the spinal cord. *J. Pharmacol. Exp. Ther.* 279, 608–616.
- Quartilho, A., Mata, H. P., Ibrahim, M. M., Vanderah, T. W., Porreca, F., Makriyannis, A., et al. (2003). Inhibition of inflammatory hyperalgesia by activation of peripheral CB2 cannabinoid receptors. *Anesthesiology* 99, 955–960. doi: 10.1097/000000542-200310000-00031
- Quintero, G. C. (2017). Review about gabapentin misuse, interactions, contraindications and side effects. *J. Exp. Pharmacol.* 9, 13–21. doi: 10.2147/JEP.S124391
- Rahn, E. J., and Hohmann, A. G. (2009). Cannabinoids as pharmacotherapies for neuropathic pain: from the bench to the bedside. *Neurotherapeutics* 6, 713–737. doi: 10.1016/j.nurt.2009.08.002
- Rahn, E. J., Thakur, G. A., Wood, J. A., Zvonok, A. M., Makriyannis, A., and Hohmann, A. G. (2011). Pharmacological characterization of AM1710, a putative cannabinoid CB2 agonist from the cannabiolactone class: antinociception without central nervous system side-effects. *Pharmacol. Biochem. Behav.* 98, 493–502. doi: 10.1016/j.pbb.2011.02.024
- Rajapakse, C. S., Lisai, M., Deregnaucourt, C., Sinou, V., Latour, C., Roy, D., et al. (2015). Synthesis of new 4-aminoquinolines and evaluation of their in vitro activity against chloroquine-sensitive and chloroquine-resistant plasmodium falciparum. *PLoS One* 10:e0140878. doi: 10.1371/journal.pone.0140878
- Ranganathan, M., and D'Souza, D. C. (2006). The acute effects of cannabinoids on memory in humans: a review. *Psychopharmacology* 188, 425–444. doi: 10.1007/s00213-006-0508-y

- Ray, P., Torck, A., Quigley, L., Wangzhou, A., Neiman, M., Rao, C., et al. (2018). Comparative transcriptome profiling of the human and mouse dorsal root ganglia: an RNA-seq-based resource for pain and sensory neuroscience research. *Pain* 159, 1325–1345. doi: 10.1097/j.pain.0000000000001217
- Ray, W. A., Meredith, S., Thapa, P. B., Hall, K., and Murray, K. T. (2004). Cyclic antidepressants and the risk of sudden cardiac death. *Clin. Pharmacol. Ther.* 75, 234–241. doi: 10.1016/j.cpt.2003.09.019
- Rice, W., Shannon, J. M., Burton, F., and Fiedeldey, D. (1997). Expression of a brain-type cannabinoid receptor (CB1) in alveolar Type II cells in the lung: regulation by hydrocortisone. *Eur. J. Pharmacol.* 327, 227–232. doi: 10.1016/S0014-2999(97)89665-3
- Roa-Coria, J. E., Navarrete-Vázquez, G., Fowler, C. J., Flores-Murrieta, F. J., Déciga-Campos, M., and Granados-Soto, V. (2012). N-(4-Methoxy-2-nitrophenyl)hexadecanamide, a palmitoylethanolamide analogue, reduces formalin-induced nociception. *Life Sci.* 91, 1288–1294. doi: 10.1016/j.lfs.2012.09.024
- Roberts, J. D., Gennings, C., and Shih, M. (2006). Synergistic affective analgesic interaction between delta-9-tetrahydrocannabinol and morphine. *Eur. J. Pharmacol.* 530, 54–58. doi: 10.1016/j.ejphar.2005.11.036
- Roberts, L. A., Christie, M. J., and Connor, M. (2002). Anandamide is a partial agonist at native vanilloid receptors in acutely isolated mouse trigeminal sensory neurons. *Br. J. Pharmacol.* 137, 421–428. doi: 10.1038/sj.bjp.0704904
- Rog, D. J., Nurmiikko, T. J., Friede, T., and Young, C. A. (2005). Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. *Neurology* 65, 812–819. doi: 10.1212/01.wnl.0000176753.45410.8b
- Rosenkilde, M. M., Benned-Jensen, T., Andersen, H., Holst, P. J., Kledal, T. N., Luttschau, H. R., et al. (2006). Molecular pharmacological phenotyping of EBI2. An orphan seven-transmembrane receptor with constitutive activity. *J. Biol. Chem.* 281, 13199–13208. doi: 10.1074/jbc.M602245200
- Ross, R. A. (2003). Anandamide and vanilloid TRPV1 receptors. *Br. J. Pharmacol.* 140, 790–801. doi: 10.1038/sj.bjp.0705467
- Ross, R. A., Coutts, A. A., McFarlane, S. M., Anavi-Goffer, S., Irving, A. J., Pertwee, R. G., et al. (2001). Actions of cannabinoid receptor ligands on rat cultured sensory neurones: implications for antinociception. *Neuropharmacology* 40, 221–232. doi: 10.1016/S0028-3908(00)00135-0
- Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermansson, N. O., Leonova, J., et al. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *Br. J. Pharmacol.* 152, 1092–1101. doi: 10.1038/sj.bjp.0707460
- Sagar, D. R., Jhaveri, M., and Chapman, V. (2009). Targeting the cannabinoid system to produce analgesia. *Curr. Top. Behav. Neurosci.* 1, 275–287. doi: 10.1007/978-3-540-88955-7_11
- Salio, C., Fischer, J., Franzoni, M. F., and Conrath, M. (2002). Pre- and postsynaptic localizations of the CB1 cannabinoid receptor in the dorsal horn of the rat spinal cord. *Neuroscience* 110, 755–764. doi: 10.1016/S0306-4522(01)00584-X
- Santha, P., Jenes, A., Somogyi, C., and Nagy, I. (2010). The endogenous cannabinoid anandamide inhibits transient receptor potential vanilloid type 1 receptor-mediated currents in rat cultured primary sensory neurons. *Acta Physiol. Hung.* 97, 149–158. doi: 10.1556/APhysiol.97.2010.2.1
- Sanudo-Pena, M. C., Strangman, N. M., Mackie, K., Walker, J. M., and Tsou, K. (1999). CB1 receptor localization in rat spinal cord and roots, dorsal root ganglion, and peripheral nerve. *Zhongguo Yao Li Xue Bao* 20, 1115–1120.
- Scavone, J. L., Sterling, R. C., and Van Bockstaele, E. J. (2013). Cannabinoid and opioid interactions: implications for opiate dependence and withdrawal. *Neuroscience* 248, 637–654. doi: 10.1016/j.neuroscience.2013.04.034
- Schicho, R., Bashashati, M., Bawa, M., McHugh, D., Saur, D., Hu, H. M., et al. (2011). The atypical cannabinoid O-1602 protects against experimental colitis and inhibits neutrophil recruitment. *Inflamm. Bowel Dis.* 17, 1651–1664. doi: 10.1002/ibd.21538
- Schuelert, N., and McDougall, J. J. (2011). The abnormal cannabidiol analogue O-1602 reduces nociception in a rat model of acute arthritis via the putative cannabinoid receptor GPR55. *Neurosci. Lett.* 500, 72–76. doi: 10.1016/j.neulet.2011.06.004
- Schuelert, N., Zhang, C., Mogg, A. J., Broad, L. M., Hepburn, D. L., Nisenbaum, E. S., et al. (2010). Paradoxical effects of the cannabinoid CB2 receptor agonist GW405833 on rat osteoarthritic knee joint pain. *Osteoarthritis Cartilage* 18, 1536–1543. doi: 10.1016/j.joca.2010.09.005
- Serpell, M., Ratcliffe, S., Hovorka, J., Schofield, M., Taylor, L., Lauder, H., et al. (2014). A double-blind, randomized, placebo-controlled, parallel group study of THC/CBD spray in peripheral neuropathic pain treatment. *Eur. J. Pain* 18, 999–1012. doi: 10.1002/ejp.1532-2149.2013.00445.x
- Shiue, S. J., Peng, H. Y., Lin, C. R., Wang, S. W., Rau, R. H., and Cheng, J. K. (2017). Continuous intrathecal infusion of cannabinoid receptor agonists attenuates nerve ligation-induced pain in rats. *Reg. Anesth. Pain Med.* 42, 499–506. doi: 10.1097/AAP.0000000000000601
- Silver, M., Blum, D., Grainger, J., Hammer, A. E., and Quesey, S. (2007). Double-blind, placebo-controlled trial of lamotrigine in combination with other medications for neuropathic pain. *J. Pain Symptom Manage.* 34, 446–454. doi: 10.1016/j.jpainsymman.2006.12.015
- Simon, G. M., and Cravatt, B. F. (2006). Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for alpha/beta-hydrolase 4 in this pathway. *J. Biol. Chem.* 281, 26465–26472. doi: 10.1074/jbc.M604660200
- Sinha, S., Schreiner, A. J., Biernaskie, J., Nickerson, D., and Gabriel, V. A. (2017). Treating pain on skin graft donor sites: review and clinical recommendations. *J. Trauma Acute Care Surg.* 83, 954–964. doi: 10.1097/TA.0000000000001615
- Skrabek, R. Q., Galimova, L., Ethans, K., and Perry, D. (2008). Nabilone for the treatment of pain in fibromyalgia. *J. Pain* 9, 164–173. doi: 10.1016/j.jpain.2007.09.002
- Smart, D., Gunthorpe, M. J., Jerman, J. C., Nasir, S., Gray, J., Muir, A. I., et al. (2000). The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.* 129, 227–230. doi: 10.1038/sj.bjp.0703050
- Smith, F. L., Fujimori, K., Lowe, J., and Welch, S. P. (1998). Characterization of delta9-tetrahydrocannabinol and anandamide antinociception in nonarthritic and arthritic rats. *Pharmacol. Biochem. Behav.* 60, 183–191. doi: 10.1016/S0093-3057(97)00583-2
- Snider, W. D., and McMahon, S. B. (1998). Tackling pain at the source: new ideas about nociceptors. *Neuron* 20, 629–632. doi: 10.1016/S0896-6273(00)81003-X
- Sousa-Valente, J., Varga, A., Ananthan, K., Khajuria, A., and Nagy, I. (2014). Anandamide in primary sensory neurons: too much of a good thing? *Eur. J. Neurosci.* 39, 409–418. doi: 10.1111/ejn.12467
- Stahl, S. M., Grady, M. M., Moret, C., and Briley, M. (2005). SNRIs: their pharmacology, clinical efficacy, and tolerability in comparison with other classes of antidepressants. *CNS Spectr.* 10, 732–747. doi: 10.1017/S1092852900019726
- Staton, P. C., Hatcher, J. P., Walker, D. J., Morrison, A. D., Shapland, E. M., Hughes, J. P., et al. (2008). The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. *Pain* 139, 225–236. doi: 10.1016/j.pain.2008.04.006
- Steeds, C. E. (2009). The anatomy and physiology of pain. *Surgery* 27, 507–511.
- Stein, A. T., Ufret-Vincenty, C. A., Hua, L., Santana, L. F., and Gordon, S. E. (2006). Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. *J. Gen. Physiol.* 128, 509–522. doi: 10.1085/jgp.200609576
- Stella, N., Schweitzer, P., and Piomelli, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388, 773–778. doi: 10.1038/42015
- Stevens, A. J., and Higgins, M. D. (2017). A systematic review of the analgesic efficacy of cannabinoid medications in the management of acute pain. *Acta Anaesthesiol. Scand.* 61, 268–280. doi: 10.1111/aas.12851
- Straiker, A., Wager-Miller, J., Hutchens, J., and Mackie, K. (2012). Differential signalling in human cannabinoid CB1 receptors and their splice variants in autaptic hippocampal neurones. *Br. J. Pharmacol.* 165, 2660–2671. doi: 10.1111/j.1476-5381.2011.01744.x
- Succar, R., Mitchell, V. A., and Vaughan, C. W. (2007). Actions of N-arachidonylglycine in a rat inflammatory pain model. *Mol. Pain* 3:24. doi: 10.1186/1744-8069-3-24
- Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., et al. (1995). 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* 215, 89–97. doi: 10.1006/bbrc.1995.2437
- Sugiura, T., and Waku, K. (2000). 2-Arachidonoylglycerol: a possible multifunctional lipid mediator in the nervous and immune systems. *Ann. N. Y. Acad. Sci.* 905, 344–346. doi: 10.1111/j.1749-6632.2000.tb06575.x

- Svendsen, K. B., Jensen, T. S., and Bach, F. W. (2004). Does the cannabinoid dronabinol reduce central pain in multiple sclerosis? Randomised double blind placebo controlled crossover trial. *BMJ* 329:253. doi: 10.1136/bmj.38149.566979.AE
- Takenouchi, R., Inoue, K., Kambe, Y., and Miyata, A. (2012). N-arachidonoyl glycine induces macrophage apoptosis via GPR18. *Biochem. Biophys. Res. Commun.* 418, 366–371. doi: 10.1016/j.bbrc.2012.01.027
- Tognetto, M., Amadesi, S., Harrison, S., Creminon, C., Trevisani, M., Carreras, M., et al. (2001). Anandamide excites central terminals of dorsal root ganglion neurons via vanilloid receptor-1 activation. *J. Neurosci.* 21, 1104–1109. doi: 10.1523/JNEUROSCI.21-04-01104.2001
- Toth, C., Mawani, S., Brady, S., Chan, C., Liu, C., Mehina, E., et al. (2012). An enriched-enrolment, randomized withdrawal, flexible-dose, double-blind, placebo-controlled, parallel assignment efficacy study of nabilone as adjuvant in the treatment of diabetic peripheral neuropathic pain. *Pain* 153, 2073–2082. doi: 10.1016/j.pain.2012.06.024
- Touw, M. (1981). The religious and medicinal uses of Cannabis in China, India and Tibet. *J. Psychoactive Drugs* 13, 23–34. doi: 10.1080/02791072.1981.10471447
- Turcotte, D., Le Dorze, J. A., Esfahani, F., Frost, E., Gomori, A., and Namaka, M. (2010). Examining the roles of cannabinoids in pain and other therapeutic indications: a review. *Expert Opin. Pharmacother.* 11, 17–31. doi: 10.1517/14656560903413534
- Ueda, N., Tsuboi, K., Uyama, T., and Ohnishi, T. (2011). Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. *Biofactors* 37, 1–7. doi: 10.1002/biof.131
- Valenzano, K. J., Tafesse, L., Lee, G., Harrison, J. E., Boulet, J. M., Gottshall, S. L., et al. (2005). Pharmacological and pharmacokinetic characterization of the cannabinoid receptor 2 agonist, GW405833, utilizing rodent models of acute and chronic pain, anxiety, ataxia and catalepsy. *Neuropharmacology* 48, 658–672. doi: 10.1016/j.neuropharm.2004.12.008
- Valverde, O., Noble, F., Beslot, F., Dauge, V., Fournie-Zaluski, M. C., and Roques, B. P. (2001). Delta9-tetrahydrocannabinol releases and facilitates the effects of endogenous enkephalins: reduction in morphine withdrawal syndrome without change in rewarding effect. *Eur. J. Neurosci.* 13, 1816–1824. doi: 10.1046/j.0953-816x.2001.01558.x
- van Amerongen, G., Kanhai, K., Baakman, A. C., Heuberger, J., Klaassen, E., Beumer, T. L., et al. (2017). Effects on spasticity and neuropathic pain of an oral formulation of delta9-tetrahydrocannabinol in patients with progressive multiple sclerosis. *Clin. Ther.* 40, 1467–1482. doi: 10.1016/j.clinthera.2017.01.016
- Vanden Abeele, F., Zholos, A., Bidaux, G., Shuba, Y., Thebault, S., Beck, B., et al. (2006). Ca²⁺-independent phospholipase A2-dependent gating of TRPM8 by lysophospholipids. *J. Biol. Chem.* 281, 40174–40182. doi: 10.1074/jbc.M605779200
- Vanegas, H., and Schaible, H. G. (2004). Descending control of persistent pain: inhibitory or facilitatory? *Brain Res. Brain Res. Rev.* 46, 295–309. doi: 10.1016/j.brainresrev.2004.07.004
- Vasileiou, I., Fotopoulos, G., Matzourani, M., Patsouris, E., and Theocharis, S. (2013). Evidence for the involvement of cannabinoid receptors' polymorphisms in the pathophysiology of human diseases. *Expert Opin. Ther. Targets* 17, 363–377. doi: 10.1517/14728222.2013.754426
- Vassilatis, D. K., Hohmann, J. G., Zeng, H., Li, F., Ranchalis, J. E., Mortrud, M. T., et al. (2003). The G protein-coupled receptor repertoires of human and mouse. *Proc. Natl. Acad. Sci. U.S.A.* 100, 4903–4908. doi: 10.1073/pnas.0230374100
- Veress, G., Meszar, Z., Muszil, D., Avelino, A., Matesz, K., Mackie, K., et al. (2013). Characterisation of cannabinoid 1 receptor expression in the perikarya, and peripheral and spinal processes of primary sensory neurons. *Brain Struct. Funct.* 218, 733–750. doi: 10.1007/s00429-012-0425-2
- Vestergaard, K., Andersen, G., Gottrup, H., Kristensen, B. T., and Jensen, T. S. (2001). Lamotrigine for central poststroke pain: a randomized controlled trial. *Neurology* 56, 184–190. doi: 10.1212/WNL.56.2.184
- Vizi, E. S., Katona, I., and Freund, T. F. (2001). Evidence for presynaptic cannabinoid CB1 receptor-mediated inhibition of noradrenaline release in the guinea pig lung. *Eur. J. Pharmacol.* 431, 237–244. doi: 10.1016/S0014-2999(01)01413-3
- Vuong, L. A., Mitchell, V. A., and Vaughan, C. W. (2008). Actions of N-arachidonoyl-glycine in a rat neuropathic pain model. *Neuropharmacology* 54, 189–193. doi: 10.1016/j.neuropharm.2007.05.004
- Waldeck-Weiermair, M., Zoratti, C., Osibow, K., Balenga, N., Goessnitzer, E., Waldhoer, M., et al. (2008). Integrin clustering enables anandamide-induced Ca²⁺ signaling in endothelial cells via GPR55 by protection against CB1-receptor-triggered repression. *J. Cell Sci.* 121, 1704–1717. doi: 10.1242/jcs.020958
- Walker, J. M., and Hohmann, A. G. (2005). Cannabinoid mechanisms of pain suppression. *Handb. Exp. Pharmacol.* 168, 509–554. doi: 10.1007/3-540-26573-2_17
- Walker, J. M., and Huang, S. M. (2002). Cannabinoid analgesia. *Pharmacol. Ther.* 95, 127–135. doi: 10.1016/S0163-7258(02)00252-8
- Wallace, M., Schulteis, G., Atkinson, J. H., Wolfson, T., Lazzaretto, D., Bentley, H., et al. (2007). Dose-dependent effects of smoked cannabis on capsaicin-induced pain and hyperalgesia in healthy volunteers. *Anesthesiology* 107, 785–796. doi: 10.1097/01.anes.0000286986.92475.b7
- Wallace, M. S., Marcotte, T. D., Umlauf, A., Gouaux, B., and Atkinson, J. H. (2015). Efficacy of inhaled cannabis on painful diabetic neuropathy. *J. Pain* 16, 616–627. doi: 10.1016/j.jpain.2015.03.008
- Wang, S., Lim, G., Mao, J., Sung, B., Yang, L., and Mao, J. (2007). Central glucocorticoid receptors regulate the upregulation of spinal cannabinoid-1 receptors after peripheral nerve injury in rats. *Pain* 131, 96–105. doi: 10.1016/j.pain.2006.12.019
- Wang, Z. Y., McDowell, T., Wang, P., Alvarez, R., Gomez, T., and Bjarling, D. E. (2014). Activation of CB1 inhibits NGF-induced sensitization of TRPV1 in adult mouse afferent neurons. *Neuroscience* 277, 679–689. doi: 10.1016/j.neuroscience.2014.07.041
- Whiting, P. F., Wolff, R. F., Deshpande, S., Di Nisio, M., Duffy, S., Hernandez, A. V., et al. (2015). Cannabinoids for medical use: a systematic review and meta-analysis. *JAMA* 313, 2456–2473. doi: 10.1001/jama.2015.6358
- Wiles, A. L., Pearlman, R. J., Rosvall, M., Aubrey, K. R., and Vandenberg, R. J. (2006). N-Arachidonoyl-glycine inhibits the glycine transporter, GLYT2a. *J. Neurochem.* 99, 781–786. doi: 10.1111/j.1471-4159.2006.04107.x
- Wilsey, B., Marcotte, T., Deutsch, R., Gouaux, B., Sakai, S., and Donaghe, H. (2013). Low-dose vaporized cannabis significantly improves neuropathic pain. *J. Pain* 14, 136–148. doi: 10.1016/j.jpain.2012.10.009
- Wotherspoon, G., Fox, A., McIntyre, P., Colley, S., Bevan, S., and Winter, J. (2005). Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. *Neuroscience* 135, 235–245. doi: 10.1016/j.neuroscience.2005.06.009
- Yamamoto, W., Mikami, T., and Iwamura, H. (2008). Involvement of central cannabinoid CB2 receptor in reducing mechanical allodynia in a mouse model of neuropathic pain. *Eur. J. Pharmacol.* 583, 56–61. doi: 10.1016/j.ejphar.2008.01.010
- Yao, B. B., Hsieh, G. C., Frost, J. M., Fan, Y., Garrison, T. R., Daza, A. V., et al. (2008). In vitro and in vivo characterization of A-796260: a selective cannabinoid CB2 receptor agonist exhibiting analgesic activity in rodent pain models. *Br. J. Pharmacol.* 153, 390–401. doi: 10.1038/sj.bjp.0707568
- Yin, H., Chu, A., Li, W., Wang, B., Shelton, F., Otero, F., et al. (2009). Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. *J. Biol. Chem.* 284, 12328–12338. doi: 10.1074/jbc.M806516200
- Yrjola, S., Kalliokoski, T., Laitinen, T., Poso, A., Parkkari, T., and Nevalainen, T. (2013). Discovery of novel cannabinoid receptor ligands by a virtual screening approach: further development of 2,4,6-trisubstituted 1,3,5-triazines as CB2 agonists. *Eur. J. Pharm. Sci.* 48, 9–20. doi: 10.1016/j.ejps.2012.10.020
- Yu, X. H., Cao, C. Q., Martino, G., Puma, C., Morinville, A., St-Onge, S., et al. (2010). A peripherally restricted cannabinoid receptor agonist produces robust anti-nociceptive effects in rodent models of inflammatory and neuropathic pain. *Pain* 151, 337–344. doi: 10.1016/j.pain.2010.07.019
- Zhang, M. J., Sansbury, B. E., Hellmann, J., Baker, J. F., Guo, L., Farmer, C. M., et al. (2016). Resolin D2 enhances postischemic revascularization while resolving inflammation. *Circulation* 134, 666–680. doi: 10.1161/CIRCULATIONAHA.116.021894
- Zhu, W., and Oxford, G. S. (2007). Phosphoinositide-3-kinase and mitogen activated protein kinase signaling pathways mediate acute NGF sensitization of TRPV1. *Mol. Cell. Neurosci.* 34, 689–700. doi: 10.1016/j.mcn.2007.01.005
- Zhuang, Z. Y., Xu, H., Clapham, D. E., and Ji, R. R. (2004). Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. *J. Neurosci.* 24, 8300–8309. doi: 10.1523/JNEUROSCI.2893-04.2004

Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., et al. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400, 452–457. doi: 10.1038/22761

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Vitamin B₁₂ Enhances Nerve Repair and Improves Functional Recovery After Traumatic Brain Injury by Inhibiting ER Stress-Induced Neuron Injury

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Traumatic brain injury (TBI) is one of the most common causes of neurological damage in young human populations. Vitamin B₁₂ has been reported to promote axon growth of neuronal cells after peripheral nerve injury, which is currently used for the treatment of peripheral nerve damage in the clinical trial. Thus, we hypothesized that TBI can be attenuated by vitaminB₁₂ treatment through its beneficial role on axon regeneration after nerve injury. To confirm it, the biological function of vitaminB₁₂ was characterized using hematoxylin and eosin (H&E) staining, Luxol fast blue (LFB) staining, western blot analysis, and immunohistochemistry staining. The results showed that the neurological functional recovery was improved in the VitaminB₁₂-treated group after TBI, which may be due to downregulation of the endoplasmic reticulum stress-related apoptosis signaling pathway. Moreover, the microtubule stabilization, remyelination and myelin reparation were rescued by vitamin B₁₂, which was consistent with the treatment of 4-phenylbutyric acid (4-PBA), an endoplasmic reticulum stress inhibitor. The study suggests that vitamin B₁₂ may be useful as a novel neuroprotective drug for TBI.

Keywords: vitamin B₁₂, traumatic brain injury, endoplasmic reticulum stress, microtubule, myelin

INTRODUCTIONS

Traumatic brain injury (TBI) is commonly found following traffic accidents in adults or perinatal asphyxia in newborns, which causes brain swelling with an increase in intracranial pressure and a subsequent decrease in cerebral perfusion leading to ischaemia (Fink et al., 2012). Previous therapeutic approaches have focused on protecting the blood-brain-barrier at the early stage of injury to mitigate damages such as ionic homeostasis disturbances, secondary cerebral oedema, inflammation and the generation of free radicals (Mayer et al., 2013; Katz et al., 2015; Ma et al., 2017). The repair processes following TBI are severely limited due to a failure to entirely replenish the neuronal population (Sun, 2014). Moreover, the degeneration and necrosis of axons are also the pivotal pathological event of acute TBI (Zhang et al., 2015). Therefore, inhibiting neuronal

apoptosis and promoting axon regeneration are of great significance in promoting the functional recovery from TBI (Sun, 2014; Zhang et al., 2016).

Microtubules stability is a major determinant of axonal growth and neuronal polarization during axon formation (Murillo and Sousa, 2018). A previous study had demonstrated that the administration of fibroblast growth factor 13 (FGF13) maintained microtubule stability, and promoted axon formation and neuronal polarization after spinal cord injury (Li et al., 2018). Microtubule-stabilizing proteins (MSPs), as cytoskeletal proteins, cross cells and transport nutrients to ensure the integrity of cell function (Nogales, 2000). Members of the MSP family, such as microtubule-associated proteins (MAPs), Tau and doublecortin (DCX) can promote microtubule assembly, stabilize the microtubule, and play key roles in directing neuronal migration into the cerebral cortex and axon regeneration at the early stage of neuronal migration (Takei et al., 2000; Kapitein and Hoogenraad, 2015; Brunden et al., 2017). Thus, a drug that reduces neuronal apoptosis and promotes microtubule stability may represent a promising approach for the clinical treatment of TBI (Brunden et al., 2017; Gao et al., 2018).

Vitamin B₁₂ (Mecobalamin, MeCbl) is an important micronutrient that is required in numerous biological processes (Rathod et al., 2016). It is considered a coenzyme in folate metabolism and nucleotide biosynthesis, which makes it crucial in the metabolism of fatty acids and some amino acids and normal nervous system function (Field et al., 2018). Furthermore, vitamin B₁₂ deficiency results in methionine deficiency, leading to the dyse-synthesis of both phospholipids and myelin (Gröber et al., 2013). Currently, combination therapy with vitamin B₁₂ is widely combined and used in clinical patients with nerve diseases. It has been reported that systemic administration of vitamin B₁₂ promoted the recovery process from peripheral nerve damage in experimental rats (Hobbenaghi et al., 2013). Additionally, vitamin B₁₂ was recently shown to be a superoxide scavenger contributing to neuronal cells axonal growth (Chan et al., 2018). Thus, we hypothesized that vitamin B₁₂ could enhance axon formation after TBI via stabilizing microtubule and reducing neuronal apoptosis.

The accumulation of misfolded protein in the endoplasmic reticulum (ER) leads to ER dysfunction, which is known as ER stress (Hughes and Mallucci, 2018). Recent studies have demonstrated that ER stress is involved in a range of neurological diseases, including cerebral ischaemia, neurodegenerative disorders and Alzheimer's disease (Roussel et al., 2013; Hood et al., 2018). Our previous study indicated that ER stress inhibition significantly protected against neuronal apoptosis after spinal cord injury (He et al., 2017), but the role of ER stress in TBI is still unclear. An increasing number of studies suggested that vitamin B₁₂ regulated ER homeostasis (Sukocheva et al., 2001; Ghemrawi et al., 2013). Furthermore, it was reported that vitamin B₁₂ deficiency activated ER stress pathways by increasing the phosphorylation of PERK and IRE1α and the expression of ATF6 (Ghemrawi et al., 2013). Given our previous work on ER stress and its participation in nerve disease, we hypothesized that vitamin B₁₂ could promote nerve regeneration after TBI by regulating ER stress.

In this study, we explored the effect of vitamin B₁₂ on nerve regeneration after TBI both *in vivo* and *in vitro*. Meanwhile, we investigated the role of ER stress during the vitamin B₁₂ treatment for TBI by inspecting changes in neuronal apoptosis, microtubule, myelin regeneration, and axonal growth.

MATERIALS AND METHODS

Animals

Adult C57BL/6 male mice aged 6–8 weeks and weighing 20–26 g were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). Animals were housed under a 12-h light/dark cycle at 21–23°C and provided access to food and water *ad libitum*. The care and use of all animals were approved by the Ethics Committee of Wenzhou Medical University and conformed to the guidelines set forth by the Chinese National Institutes of Health. Mice were allocated to the following four groups using a random number table: the sham animal group, which received only anesthesia and a craniotomy; the TBI group; and two vitamin B₁₂ treatment groups (doses of 0.5 mg/kg/day and 1.5 mg/kg/day) that received vitamin B₁₂ intraperitoneally following surgery.

Cell Culture and OGD/Re-oxygenation Model

PC12 cells were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The PC12 cells were highly differentiated and adhered. Cells were cultured with RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, and then incubated in a humidified atmosphere containing 5% CO₂ at 37°C. For oxygen-glucose deprivation (OGD), cells were incubated in an anaerobic chamber for 6 h at an oxygen level that remained below 0.5% with normal growth medium or FBS-free medium, and then cells were incubated for another 12 h under normal culture conditions. Vitamin B₁₂ (200 µM) pretreatment was administered for 2 h before OGD. To further estimate the effect of ER stress activation during OGD, cells were pretreated with 4-phenylbutyric acid (4-PBA) (1 mM) for 1 h. All experiments were performed at least three times.

Surgical Procedures

TBI procedures were performed in male mice by controlled cortical impact (CCI). Briefly, all male mice were anesthetized with isoflurane via brain stereotaxis, and an incision was made in the middle of the scalp to expose the skull. After removing the bone flap, CCI was performed with a controlled impactor device at a speed of 4 m/s, a depth of 1 mm and a 150-ms impact duration (Impact One™ Stereotaxic Impactor, Leica, Milan, Italy). Postoperatively, the incision was sutured and treated with a local antibiotic application (cefazolin sodium salt). The mice were placed on a heating pad at 37°C to recover from anesthesia. All experiments were conducted with all efforts to reduce the number of animals used and their suffering as much as possible.

Drug Administration

Vitamin B₁₂ (V2876, Sigma) and 4-PBA (P21005, Sigma) were used as drugs in this study. The vitamin B₁₂ was dissolved in normal saline as 150 mg/ml stock. The mice were treated with vitamin B₁₂ in 0.5 mg/kg or 1.5 mg/kg by intraperitoneal injection from 1 day after injury for 2 consecutive weeks. The 4-PBA (100 mg/kg) was administered before vitamin B₁₂ treatment via intraperitoneal injection. Every drug was filtered by 0.2 μm microfiltration membrane before injection.

Brain Water Content

Mice were decapitated under deep anesthesia and perfusion at 24 h after TBI induction. The olfactory bulbs and brain stems were removed from their brains. Then brains were divided into two segments: right hemispheres and left hemispheres. Each part of the brain was weighed immediately to determine the wet weight. The samples were dried at 72°C for 72 h to obtain dry weights. Brain water contents were calculated as follows: [(wet weight - dry weight)/wet weight] × 100%.

Garcia Neurobehavioral Score

According to Garcia neurobehavioral score, the mice in each group were scored on day 1, day 7, and day 14 after TBI. The recovery of nerve function after TBI was observed by double-blind method. Briefly, (1) spontaneous activity (0 ≤ 3 points). (2) symmetrical movement of limbs (0 ≤ 3 points). (3) forelimb extension (0 ≤ 3 points). (4) climbing (0–3 points). (5) body trunk reaction (0 ≤ 3 points). (6) tentacles reaction (0 ≤ 3 points). (7) lateral rotation reaction (0 ≤ 3 points).

Tissue Preparation

Animals were anesthetized with isoflurane at specific time points following TBI. For immunofluorescence staining, brain tissues were dissected out, post-fixed by 4% paraformaldehyde (PFA) for 12 h, embedded in paraffin, cut into 5 mm sections and mounted on slides for subsequent staining. For western blot, a brain segment was dissected and stored at –80°C immediately. Animal tissues were lysed with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM EDTA, pH 7.4) supplemented with 10 μl/ml protease inhibitor cocktail (GE Healthcare Biosciences, Pittsburgh, PA, United States). The samples were homogenized using mechanical disruption and ultrasound cell breaker (30 s/times in three times). The tissue lysates were incubating at 4°C for 15 min. The supernatants were collected after centrifuging at 12,000 rpm at 4°C for 15 min.

Animal Experiment

Luxol fast blue (LFB) staining: The sections were deparaffinized, rinsed in dimethylbenzene I, dimethylbenzene II, absolute ethyl alcohol I, absolute ethyl alcohol II, 95% ethanol, 90% ethanol, 80% ethanol, 70% ethanol, and distilled water and then incubated in an LFB solution (0.01% in 95% ethanol) overnight at 60°C. Gray and white matter tissues were differentiated by immersing the slides in 0.05% lithium carbonate solution for 5 – 10 s,

followed by 2 changes of 70% ethanol for 1 min each, and then rinsed in distilled water. The sections were observed under a microscope to confirm proper differentiation. Differentiation steps were repeated until a sharp contrast was achieved between blue-stained white matter and colorless gray matter.

Nissl staining: We dewaxed the paraffin sections and stained them with 5% toluidine blue at room temperature for 10 min. Sections were soaked in 95% ethanol for 2 min, dimethylbenzene for 3 min and sealed with neutral balsam. The Nissl-stained slides were observed using a Nikon microscope.

H&E staining: An H&E staining kit was purchased from Beyotime Company (Jiangsu, China), and the experimental procedure was performed according to the kit instructions.

Immunohistochemistry: Paraffin sections were deparaffinized by dimethylbenzene and then rehydrated in an alcohol gradient. After incubated in 3% oxydol for 15 min, antigen retrieval was performed in citric acid by a pressure cooker, and then the slides were cooled for 2 h. BSA at 5% was used for pre-incubation at 37°C for 30 min, and the sides were incubated with the primary antibodies at 4°C for 24 h. The following primary antibody was used: MBP (1:1000, Cell Signaling Technologies, America). In the next step, glass slides were incubated with goat anti-rabbit IgG (H+L) HRP secondary polyclonal antibody (1:1000, Yession) at 37°C for 1 h and then with DAB for approximately 5 min. Stained sections were photographed with a fluorescence microscope (Nikon, Tokyo, Japan).

Western Blot

Proteins from tissues and cells were quantified with BCA reagents. 40 μg proteins were separated on a 12% gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, United States). The membranes were blocked for 120 min with 5% (w/v) milk dissolved in 0.1% Tween-20 in TBS at room temperature, and then were incubated overnight at 4°C with the following primary antibodies: Caspase12 (1:1000, Abcam, United States), IRE1α (1:1000, Abcam, United States), GRP78 (1:1000, Abcam, United States), XBP-1 (1:1000, Abcam, United States), CHOP (1:1000, Cell Signaling Technologies, United States), Ace-tubulin (1:2000, Cell Signaling Technologies, United States), Tau (1:1000, Abcam, United States), MAP2 (1:2000, Cell Signaling Technologies, United States), myelin basic protein (MBP) (1:1000, Cell Signaling Technologies, United States), and GAPDH (1:10000, Bio-world, United States). Subsequently, the membranes were washed thrice with TBST and the membranes were incubated with horseradish-peroxidase conjugated secondary antibodies rabbit/mouse polyclonal antibody (1:1000, Yession) for 60 min. A ChemiDoc™ XRS imaging system (Bio-Rad, United States) was used to visualize the signals. Quantity One was used to analyze the relative band densities, and the band densities of target proteins were normalized to that of GAPDH. All experiments were repeated at least in triplicate.

Immunofluorescence Staining

Cells were fixed with 4% PFA for 30 min at 37°C. The prepared tissue sections and cells were blocked with 5% BSA for 30 min and then incubated at 4°C overnight with the following primary

antibodies: MAP-2 (1:500, Abcam, United States), Ace-tubulin (1:500, Cell Signaling Technologies, United States), Tyr-tubulin (1:500, Sigma Aldrich, United States), GRP78 (1:1000, Abcam, United States), and MBP (1:500, Cell Signaling Technologies, United States). Next, the sections and cells were incubated with an Alexa Fluor 594/647 donkey anti-mouse/rabbit secondary antibody (1:1000, Abcam, United States) for 1 h at 37°C. Nuclei were stained with DAPI. The samples were imaged under a Nikon ECLIPSE Ti microscope (Nikon, A1 PLUS, Tokyo, Japan). 10 images were captured from each sample in the cortex randomly. The quantitative of immunofluorescence was performed by Image J. All experiments were repeated at least in triplicate.

TUNEL Staining

The TUNEL analysis kit (40307ES20, Yeason) was used for detecting the level of DNA damage. The brain sections were deparaffinized and rehydrated by difference concentrated-ethanol. After washing with PBS, the sections were treated with 10.2 mM sodium citrate buffer. The sections were stained with Alexa Fluor 488-12-dUTP Labeling Mix. The sections were counter staining with DAPI after washing with PBS. The images were captured by the fluorescence microscope (Olympus Inc., Tokyo, Japan). The TUNEL positive cells in six random fields of each section were counted for analysis.

Statistical Analysis

Statistically analyzed data were presented as the mean ± standard error of the mean (SEM). Student's *t*-test was used to determine statistical significance between two groups. For comparison of three or more groups, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used to analyze the results. The difference was considered statistically significant when the *P*-value was <0.05.

RESULTS

Vitamin B12 Decreased En-Cephaloedema and Motor Neuron Loss After TBI

To evaluate the therapeutic role of vitamin B12 in the treatment of TBI, vitamin B12 was administered intraperitoneally immediately following TBI. Brain water content was detected at 24 h after TBI. The brain water content of the TBI group was obviously increased relative to the sham group (91.91 ± 1.488 vs. 83.53 ± 0.7815 , Figure 1A, Mean ± SEM, $n = 6$), and the brain water contents of the vitamin B12-treated TBI groups were reduced to differing degrees relative to the TBI group (86.11 ± 1.070 in 0.5 mg/kg vitamin B12 dose group and 85.31 ± 0.7846 in 1.5 mg/kg vitamin B12 dose group vs. 91.91 ± 1.488 in TBI group, Mean ± SEM, $n = 6$). These results indicated that vitamin B12 significantly alleviated ipsilateral brain oedema after TBI injury.

Motor function recovery was estimated for 14 days after injury using the 21-point Garcia test. As shown in Figure 1B, the sham-operated group had an average score of 21, representing a normal motor function. The TBI+vitamin B12 group showed better

functional recovery after 7 days. Additionally, H&E and Nissl staining were performed to assess the histological morphology in each group. As shown in Figure 1C, there was obvious severe cerebral cortex tissue loss in the TBI group relative to the sham group. Consistent with the Garcia scores, the vitamin B12-treated groups showed less tissue damage and neuronal apoptosis (Figure 1D), indicating that vitamin B12 reduced tissue damage, protected neurons in the cortex and ameliorated the pathological morphology of the lesion area after TBI in mice.

Vitamin B12 Alleviated Caspase12-Dependent Neuronal Apoptosis

To determine whether vitamin B12 treatment could decrease apoptosis in brain tissues, immunofluorescence staining was performed after TBI. As shown in Figure 2A, vitamin B12 treatment obviously reduced the amount of cleaved-caspase12 positive neurons relative to the TBI group. Meanwhile, western blot results indicated that the vitamin B12-treated groups showed reduced levels of cleaved-caspase12 expression relative to their untreated counterparts in Figures 2B,C. Moreover, TUNEL staining was performed on 7 days after injury to further verify the anti-apoptotic effect of vitamin B12. As shown in Figures 2D,E, significantly increased number of apoptotic cells was found in the TBI group relative to the sham group. In comparison, vitamin B12 treatment greatly reversed TBI-induced apoptosis. Therefore, these findings demonstrated that vitamin B12 attenuated TBI-induced neuronal cell apoptosis.

Vitamin B12 Inhibited ER Stress Signaling Pathway

Under chronic ER stress, the associated apoptosis may contribute to pathophysiological processes involved in a number of prevalent diseases, including neurodegenerative diseases, diabetes, atherosclerosis and renal disease (Tabas and Ron, 2011). Hu et al. (Wu et al., 2018) reported that the ER-localized E3 ligase RNF183 triggered apoptosis in response to prolonged ER stress. Meanwhile, our previous study indicated that ER stress signaling induced neuronal apoptosis in spinal cord injury (He et al., 2017). To clarify the relationship between vitamin B12 and the regulation of ER stress, we detected ER stress signaling pathway proteins and the downstream apoptosis-related proteins by western blot and immunofluorescence staining. As shown in Figures 3A–F, the levels of ER stress-related proteins (GRP78, IRE1α, XBP-1, and CHOP) were significantly increased at 3 days and decreased after vitamin B12 treatment. Meanwhile, immunofluorescence staining revealed that the number of GRP78 positive cells was significantly increased in the TBI group, and vitamin B12 reversed this trend to a differing degree (Figure 3B). These findings suggested that vitamin B12 alleviated the level of ER stress.

Vitamin B12 Inhibited Microtubule Damage Through ER Stress After TBI

Numerous studies have indicated that vitamin B12 has neuroprotective effects on TBI (Sun et al., 2012; Chen et al., 2015).

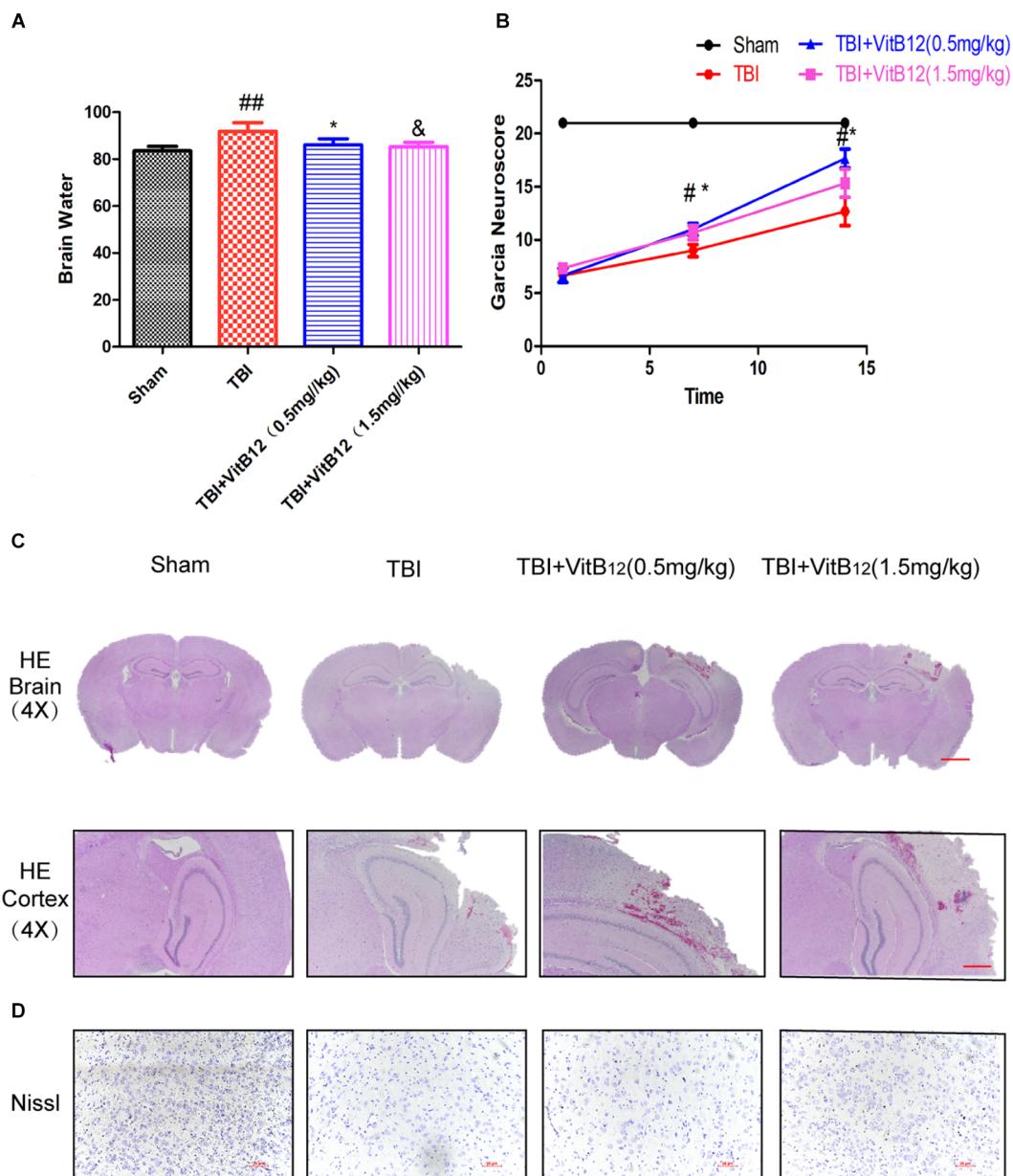


FIGURE 1 | Vitamin B12 treatment reserves tissue structure damage and protects functional recovery after TBI. **(A)** Quantification of brain water content in the ipsilateral brain cortex at 1 day after TBI. ** $P < 0.01$ vs. the sham group. * $P < 0.05$ and & $P < 0.05$ vs. the TBI group values represent the mean \pm SEM, $n = 6$. **(B)** Garcia test evaluation at 7 days, 14 days after TBI. # $P < 0.05$ vs. the sham group. * $P < 0.05$ vs. the TBI group values represent the mean \pm SEM, $n = 5$. **(C–D)** Representative images of H&E and Nissl staining in the cortex at 14 days post-TBI.

We hypothesized that vitamin B12 may play a neuroprotective role by maintaining microtubule stability. We detected the expressions of MAP-2 and Tau, promote axon microtubulin bundling and dynamics and stabilize microtubule. As shown in Figures 4A–C, the expressions of MAP-2 and Tau decreased after injury, and the Tau expression was significantly higher in the vitamin B12-treated group relative to the TBI group after 7 days. Interestingly, only the 1.5 mg/kg dose of vitamin B12 reversed the loss of MAP-2. Then we examined MAP-2

expression by immunofluorescence. The results revealed that the MAP-2-positive neurons in the TBI group were significantly disorganized 7 days after injury. However, the vitamin B12-treated groups showed good neurological morphology, which was similar to the morphology observed in the sham group relative to the TBI group (Figure 4D). In addition, this result was further confirmed by immunofluorescence in PC12 cells with OGD, as shown in Figure 4E. The Ace-tubulin and Tyr-tubulin proteins were detected to examine stable microtubules

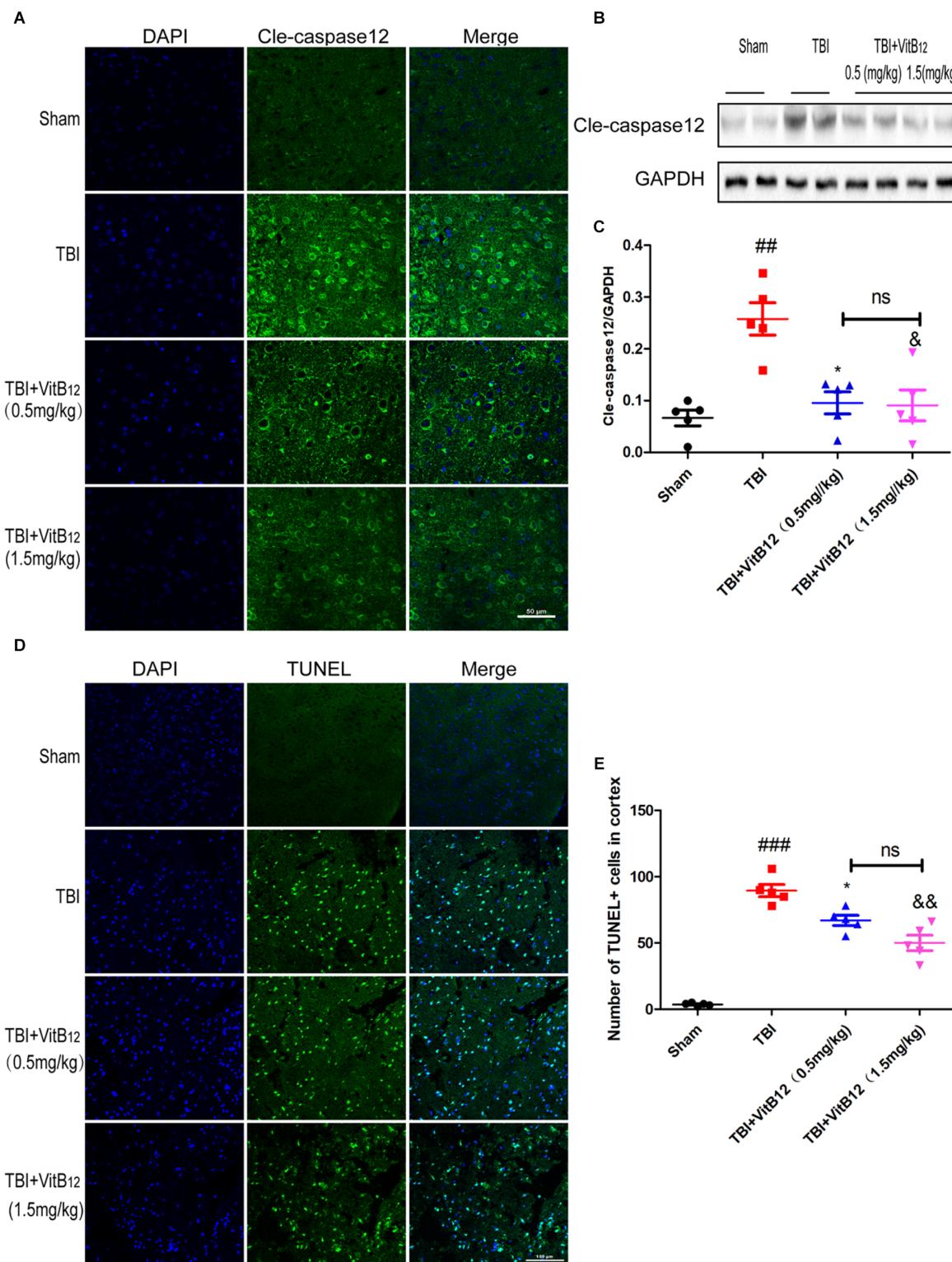


FIGURE 2 | Vitamin B₁₂ protects neurons from apoptosis. **(A)** Immunofluorescence staining of cleaved caspase-12 (green) in the cortex at 7 days post-injury. Scale bar = 50 μ m. **(B,C)** The expression and quantification of cleaved caspase-12 proteins in the cortex in the 7 days post-injury. $^{##}P < 0.01$ vs. the sham group. $^{*}P < 0.05$ and $^{&}P < 0.05$ vs. the TBI group values represent the mean \pm SEM, $n = 5$. **(D,E)** Representation and quantification of immunofluorescence staining of TUNEL (green) in the cortex at 7 days post-injury. $^{##}P < 0.01$ vs. the sham group. $^{*}P < 0.05$ and $^{&}P < 0.05$ vs. the TBI group values represent the mean \pm SEM, $n = 5$. Scale bar = 100 μ m.

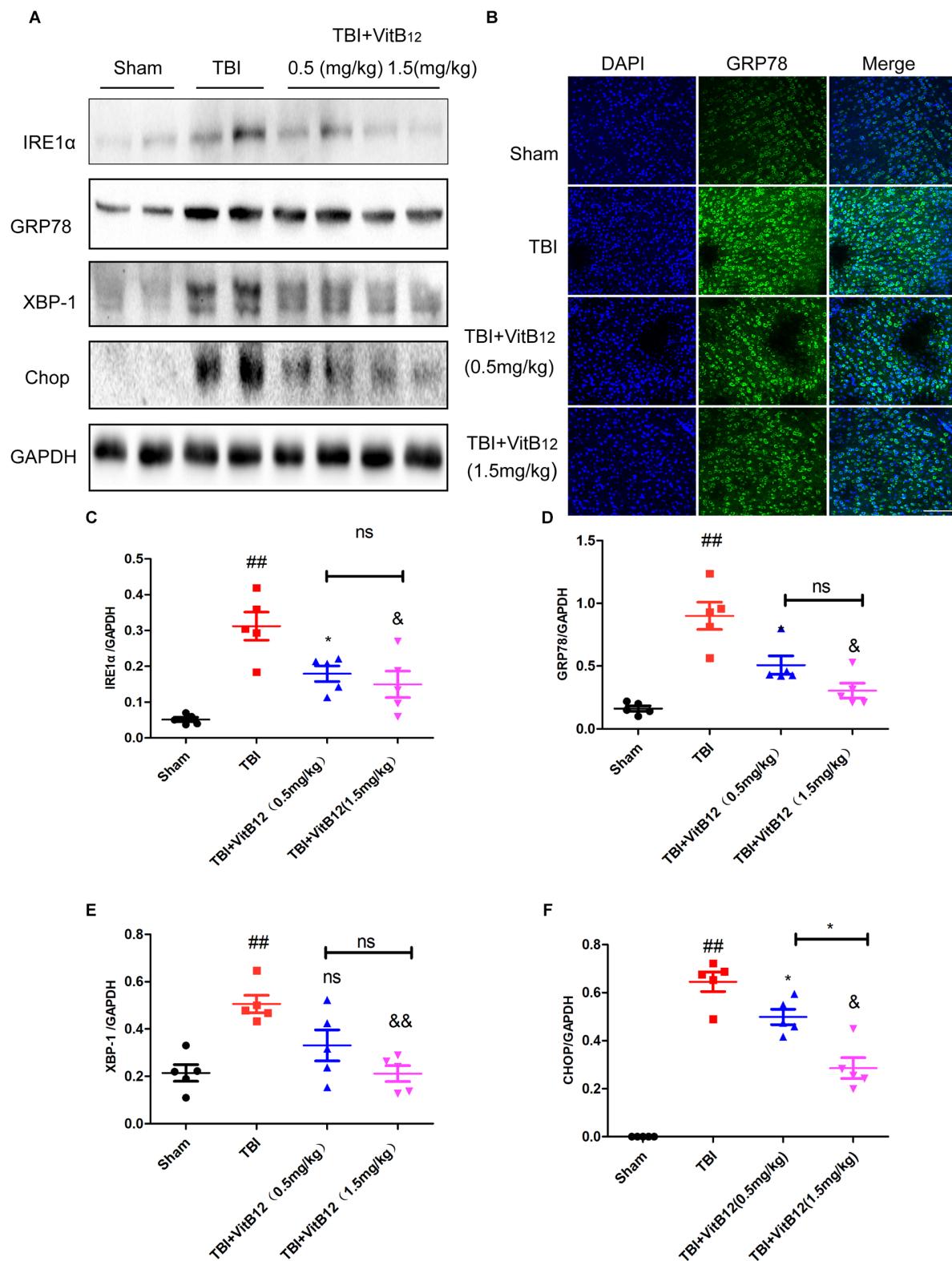
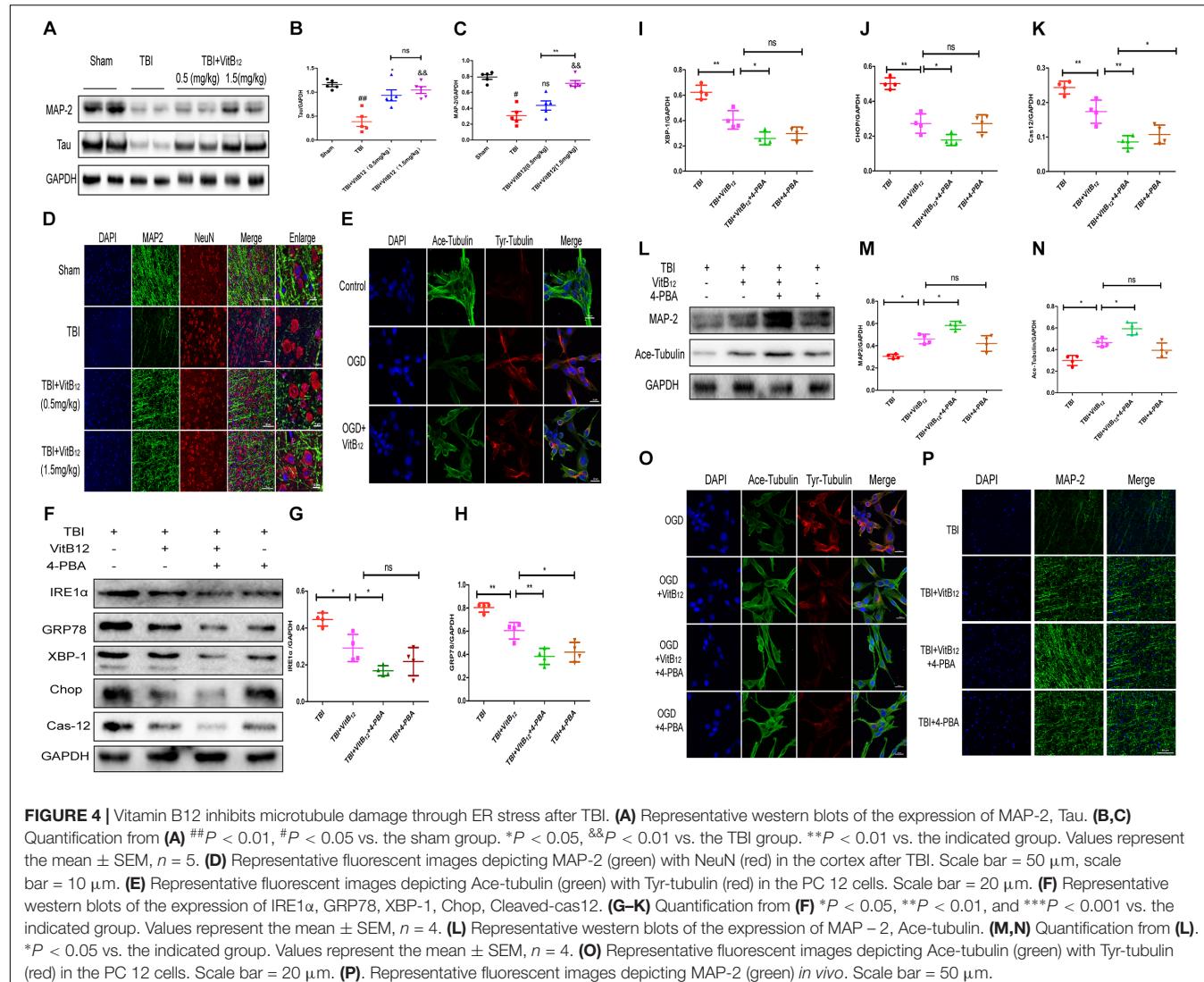


FIGURE 3 | Vitamin B₁₂ reduces the level of ER stress. **(A)** Representative western blots of the expression of IRE1 α , GRP78, XBP-1, Chop. **(B)** Immunofluorescence staining of GRP78 (green) in the cortex at 7 days post-injury. Scale bar = 100 μ m. **(C–F)** Quantification from **(A)**. $^{##}P < 0.01$ vs. the sham group. $^{*}P < 0.05$, $^{&}P < 0.05$, and $^{&&}P < 0.01$ vs. the TBI group. $^{*}P < 0.05$ vs. the indicated group. Values represent the mean \pm SEM, $n = 5$.



and dynamic microtubules, respectively. The results showed that the Ace-tubulin/Tyr-tubulin ratio after vitamin B12 treatment was increased relative to the OGD treatment group. Taken together, vitamin B12 exerted microtubule stabilizing effect.

We next explored the relationship between vitamin B12 protection and ER stress by using 4-PBA, which is the most commonly used ER stress inhibitor. As shown in Figures 4F–K, ER stress-induced apoptosis signaling pathway levels were substantially down-regulated in the vitamin B12 and 4-PBA co-treatment group relative to the vitamin B12 group. Meanwhile, microtubule stabilization protein expressions (MAP-2 and Ace-tubulin) were significantly increased in the co-treatment group relative to the vitamin B12 group, which indicated that vitamin B12-mediated protection was most likely achieved by inhibiting ER stress (Figures 4L–N). Next, we used immunofluorescence to detect Ace-tubulin and Tyr-tubulin in PC12 cells *in vitro* and MAP-2 *in vivo*. The results showed that the Ace-tubulin/Try-tubulin ratio in the

vitamin B12+4-PBA group was increased relative to the vitamin B12 group, which was consistent with MAP-2 in mice (Figures 4O,P). Taken together, these results suggested that vitamin B12 was able to stabilize microtubules by reducing ER stress level.

Vitamin B12 Promoted Myelin Regeneration by ER Stress After TBI

Myelin regeneration is a key factor in sensory and motor function recovery after brain injury (Cantuti-Castelvetro et al., 2018). We explored the effect of vitamin B12 on re-myelination by LFB staining. Histopathology with LFB staining revealed that the TBI group showed white matter lesions in the corpus callosum relative to the sham group (Figure 5A). Vacuolar changes and microtubule loss from the myelin sheath were also observed in the TBI group. The vitamin B12 treatments reduced the degree of myelin sheath destruction, and 1.5 mg/kg dose of vitamin B12 showed slightly better effects than the 0.5 mg/kg

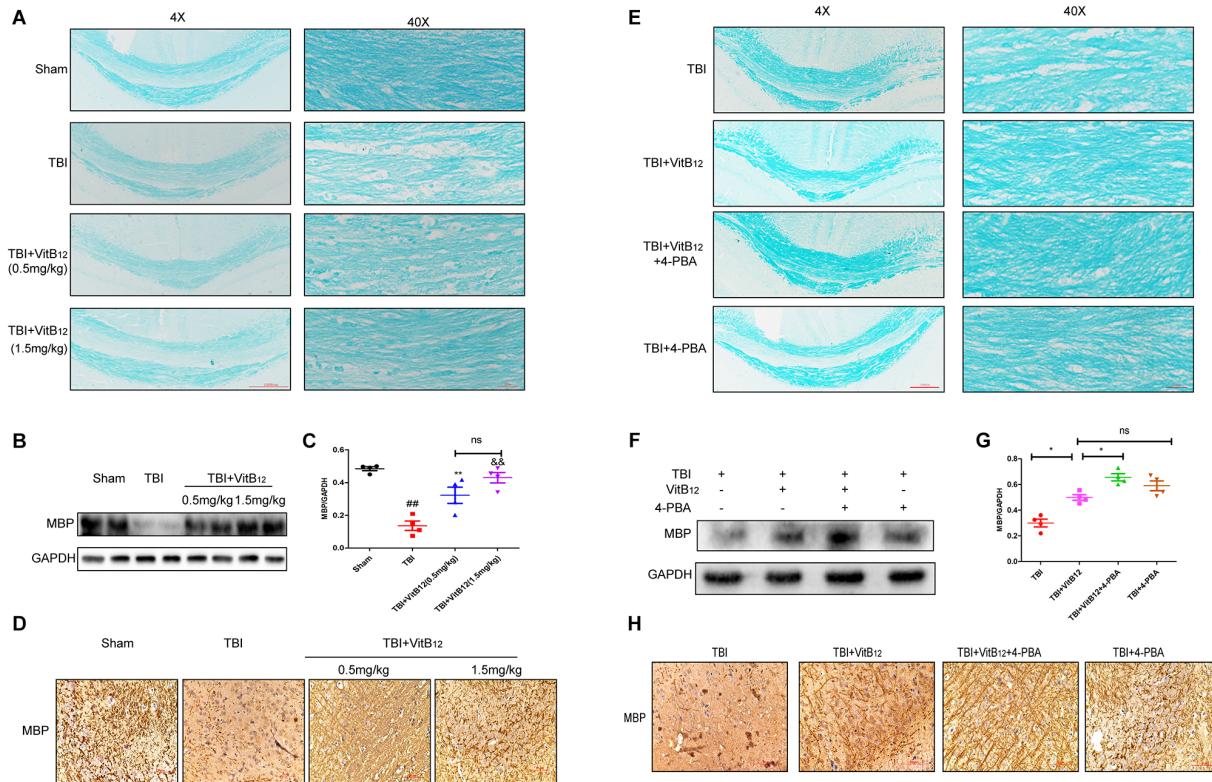


FIGURE 5 | Vitamin B12 protects against myelin damage after TBI through ER Stress. **(A)** Representative images of white matter with LFB staining images of the myelin sheath at 14 days. Scale bar = 1000 μ m, Scale bar = 250 μ m. **(B,C)** Protein expressions and quantification data of MBP in each group. ##P < 0.01 vs. the sham group, **P < 0.01 and &&P < 0.01 vs. the TBI group. Values represent the mean \pm SEM, n = 4. **(D)** Immuno-histochemistry staining of MBP in each group. Representative images of white matter with LFB staining images of the myelin sheath at 14 days. Scale bar = 1000 μ m, Scale bar = 250 μ m. **(E)** Protein expressions and quantification data of MBP in each group. *P < 0.05 vs. the indicated group values represent the mean \pm SEM, n = 4. **(F,G)** Protein expressions and quantification data of MBP in each group. *P < 0.05 vs. the indicated group values represent the mean \pm SEM, n = 4. **(H)** Immuno-histochemistry staining of MBP in each group.

dose. Next, MBP, which is constituents of the myelin sheath was detected by western blot and immunohistochemistry. As shown in Figures 5B–D, MBP protein expression was significantly decreased after TBI, but increased by vitamin B12; there was no statistical significance between the two groups receiving different vitamin B12 concentrations. Taken together, these results revealed that vitamin B12 promoted myelin regeneration after TBI. We next explored whether ER stress was involved in the protective effect of vitamin B12 on remyelination. LFB staining revealed that the myelin sheath in the corpus callosum was clear, and the microtubules in the axons were tightly arranged in the vitamin B12+4-PBA group relative to vitamin B12 treatment group (Figure 5E). Moreover, the expression of MBP was consistent with the LFB staining. As shown in Figures 5F,G, the expression of MBP was significantly increased in the co-treatment group relative to the vitamin B12 treatment groups. Meanwhile, immunohistochemistry results showed that the vitamin B12+4-PBA group presented tighter and more continuous MBP positive myelin relative to that in the vitamin B12 groups (Figure 5H). All these results indicated that the vitamin B12 enhanced re-myelination by inhibiting ER Stress.

DISCUSSION

Increased understanding of neuroprotection in TBI has helped to establish the concept of promoting nerve cell function recovery to potentially improve functional recovery (Wieloch and Nikolich, 2006; Loane and Faden, 2010). Numerous studies have reported that microtubule stabilization and remyelination play pivotal roles in several CNS diseases (Sengottivel and Fischer, 2011; Abu-Rub and Miller, 2018; Li et al., 2018). There is a consistent evidence that vitamin B12 promotes the synthesis of neurotrophic factors, which in turn support neurite outgrowth and survival (Okada et al., 2010). However, whether vitamin B12 can alleviate the damage caused by brain trauma is still unclear. To assess this possibility, vitamin B12 was administered intraperitoneally immediately following TBI. And the results showed that vitamin B12 was able to promote nerve repair after TBI.

The ER is the main organelle responsible for protein folding, lipid biosynthesis and Ca²⁺ storage (Tsai and Weissman, 2010). Increased ER stress may be a self-protective signal transcription pathway after mild injury (Xie et al., 2016). In contrast, excessive ER stress triggers extensive neuronal death

via CHOP activation. CHOP is the downstream of ER stress-induced apoptosis. The cytoplasmic calcium-activated calpain cleaves and activates caspase-12 in response to ER-released calcium (Hood et al., 2018; Tan et al., 2018). It has been confirmed that, ER stress is also involved in TBI (Hood et al., 2018). Vitamin B12 has been currently used to treat peripheral nerve damage in the clinic (Hobbenaghi et al., 2013). In this study, we found that the ER stress was involved in the process of vitamin B12 treating TBI (**Figure 3**). As showed in **Figure 3A**, IRE1α, GRP78, XBP-1 and CHOP were increased significantly after TBI injury, and reversed following the 3-day treatment of vitamin B12. Meanwhile, vitamin B12 alleviated ER stress-induced apoptosis. Taken together, ER stress plays an important role in neuronal apoptosis induction after TBI, and vitamin B12 inhibits neuronal death by down-regulating ER stress.

A growing number of studies has reported that microtubule stabilization might play a pivotal role in axon regeneration in several CNS diseases (O'Donovan, 2016; Abu-Rub and Miller, 2018; Chuckowree et al., 2018). In an injured brain, microtubules are vulnerable to misalignment and dissolution in neurons. And microtubules also implicate in the injury-induced glial responses and adaptive neuroplasticity in the aftermath of injury (Chuckowree et al., 2018). Tau protein is involved in regulating axonal microtubule assembly and disassembly. It has been reported that the plasma phospho-tau levels and phospho tau/total tau ratio during the acute phase and chronic TBI were superior to total/tau levels as discriminating indices for the severity and status of neurotrauma patients from healthy controls (Rubenstein et al., 2017). This study also demonstrated that the vitamin B12 could maintain the stability of the microtubule after TBI. As shown in **Figure 4**, vitamin B12 exerted microtubule-stabilizing effect *in vitro* and *in vivo*. Meanwhile, the high dose (1.5 mg/kg) of vitamin B12 treatment showed better effectiveness than the 0.5 mg/kg dose in TBI mice. In addition, the combination therapy with the ER stress inhibitor 4-PBA partially strengthened the neuro-protective effect of vitamin B12. However, whether vitamin B12 directly inhibits the ER stress signaling pathway requires further investigation.

CNS injury-induced growth cone collapse and retraction of the axonal cytoskeleton are closely related to growth inhibitory molecules associated with myelin (Stassart et al., 2018). The myelinating cells surrounding axons not only accelerate the propagation of electrical impulses, but also provide metabolic support for axons and refine neural circuits (Figlia et al., 2017). Myelin has a significant role in the progression of white matter pathology after TBI and in the potential for plasticity and subsequent recovery (Armstrong et al., 2016). Besides, myelin is an active form of vitamin

B12, and the MeCbl plays an essential role in the synthesis and maintenance of myelin (Gröber et al., 2013). Vitamin B12 accelerates Schwann cells differentiation by suppressing Erk1/2 activities (Nishimoto et al., 2015). In addition, vitamin B12 has been reported to promote the remyelination in focal demyelination rat (Nishimoto et al., 2015). Thus, we evaluated the effect of vitamin B12 on remyelination after TBI. These data suggested that vitamin B12 increased the level of MBP, which plays vital roles in the myelination process and the appropriate formation of myelin thickness and compactness. Meanwhile, LFB staining showed that vitamin B12 restored myelin by reducing the vacuolar changes in the myelin sheath after TBI. We also used the ER stress inhibitor 4-PBA to assess the role of ER stress in remyelination. The results showed that ER stress was involved in the treatment of TBI induced myelin damage by vitamin B12. Therefore, we can conclude that the vitamin B12 enhance the survival of the nerve cell in the TBI mouse by inhibiting ER stress. In our further study, we will investigate the effects of vitamin B12 on glial cells and the relationship between membrane-associated proteins and autophagy signals, ultimately meeting the goal of deeper understand of the treatment mechanism of vitamin B12 in TBI.

ETHICS STATEMENT

The care and use of all animals were approved by the Ethics Committee of Wenzhou Medical University and conformed to the guidelines set forth by the Chinese National Institutes of Health.

AUTHOR CONTRIBUTIONS

AH and DC conceived and designed the experiments. FW, KX, LL, MZ, and CT performed the experiments, analyzed data and wrote or revised the manuscript. KZ, LX, HT, YH, and YX provided assistance with experiments. HZ was responsible for experiment supplementation and manuscript modification. All the above authors discussed the results and approved the manuscript submission.

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REFERENCES

- Abu-Rub, M., and Miller, R. (2018). Emerging cellular and molecular strategies for enhancing central nervous system (CNS) remyelination. *Brain Sci.* 8:111. doi: 10.3390/brainsci8060111
- Armstrong, R. C., Mierzwa, A. J., Sullivan, G. M., and Sanchez, M. A. (2016). Myelin and oligodendrocyte lineage cells in white

- matter pathology and plasticity after traumatic brain injury. *Neuropharmacology* 110, 654–659. doi: 10.1016/j.neuropharm.2015.04.029
- Brunden, K. R., Lee, V. M.-Y., Smith, A. B., Trojanowski, J. Q., and Ballatore, C. (2017). Altered microtubule dynamics in neurodegenerative disease: therapeutic potential of microtubule-stabilizing drugs. *Neurobiol. Dis.* 105, 328–335. doi: 10.1016/j.nbd.2016.12.021

- Cantuti-Castelvetri, L., Fitzner, D., Bosch-Queralt, M., Weil, M.-T., Su, M., Sen, P., et al. (2018). Defective cholesterol clearance limits remyelination in the aged central nervous system. *Science* 359, 684–688. doi: 10.1126/science.aan4183
- Chan, W., Almasieh, M., Catrinescu, M.-M., and Levin, L. A. (2018). Cobalamin-associated superoxide scavenging in neuronal cells is a potential mechanism for vitamin b 12 -deprivation optic neuropathy. *Am. J. Pathol.* 188, 160–172. doi: 10.1016/j.japath.2017.08.032
- Chen, C., Huang, Y., and Jaw, F. (2015). Ultrasound-guided perineural vitamin b12 injection for peripheral neuropathy. *J. Med. Ultrasound* 23, 104–106. doi: 10.1016/j.jmdu.2015.02.001
- Chuckowree, J. A., Zhu, Z., Brizuela, M., Lee, K. M., Blizzard, C. A., and Dickson, T. C. (2018). The microtubule-modulating drug epothilone d alters dendritic spine morphology in a mouse model of mild traumatic brain injury. *Front. Cell Neurosci.* 12:223. doi: 10.3389/fncel.2018.00223
- Field, M. S., Kamynina, E., Chon, J., and Stover, P. J. (2018). Nuclear folate metabolism. *Annu. Rev. Nutr.* 38, 219–243. doi: 10.1146/annurev-nutr-071714-034441
- Figlia, G., Gerber, D., and Suter, U. (2017). Myelination and mTOR. *Glia* 66, 693–707. doi: 10.1002/glia.23273
- Fink, E. L., Kochanek, P. M., and Clark, R. S. B. (2012). “Cerebral resuscitation and traumatic brain injury,” in *Pediatric Critical Care Study Guide*, eds S. Lucking, F. Maffei, R. Tamburro, and N. Thomas (London: Springer), 643–667. doi: 10.1007/978-0-85729-923-9_31
- Gao, Y.-Y., Zhang, Z.-H., Zhuang, Z., Lu, Y., Wu, L.-Y., Ye, Z., et al. (2018). Recombinant milk fat globule-EGF factor-8 reduces apoptosis via integrin β3/FAK/PI3K/AKT signaling pathway in rats after traumatic brain injury. *Cell Death Dis.* 9:845. doi: 10.1038/s41419-018-0939-5
- Ghemrawi, R., Pooya, S., Lorentz, S., Gauchotte, G., Arnold, C., Gueant, J.-L., et al. (2013). Decreased vitamin B12 availability induces ER stress through impaired SIRT1-deacetylation of HSF1. *Cell Death Dis.* 4:e553. doi: 10.1038/cddis.2013.69
- Gröber, U., Kisters, K., and Schmidt, J. (2013). Neuroenhancement with vitamin B12—underestimated neurological significance. *Nutrients* 5, 5031–5045. doi: 10.3390/nu5125031
- He, Z., Zhou, Y., Huang, Y., Wang, Q., Zheng, B., Zhang, H., et al. (2017). DL-3-n-butylphthalide improves functional recovery in rats with spinal cord injury by inhibiting endoplasmic reticulum stress-induced apoptosis. *Am. J. Transl. Res.* 9, 1075–1087.
- Hobbenaghi, R., Javanbakht, J., Hosseini, E., Mohammadi, S., Rajabian, M., Moayeri, P., et al. (2013). Neuropathological and neuroprotective features of vitamin B12 on the dorsal spinal ganglion of rats after the experimental crush of sciatic nerve: an experimental study. *Diagn. Pathol.* 8:123. doi: 10.1186/1746-1596-8-123
- Hood, K. N., Zhao, J., Redell, J. B., Hylin, M. J., Harris, B., Perez, A., et al. (2018). Endoplasmic reticulum stress contributes to the loss of newborn hippocampal neurons after traumatic brain injury. *J. Neurosci.* 38, 2372–2384. doi: 10.1523/jneurosci.1756-17.2018
- Hughes, D., and Mallucci, G. R. (2018). The unfolded protein response in neurodegenerative disorders - therapeutic modulation of the PERK pathway. *FEBS J.* 286, 342–355. doi: 10.1111/febs.14422
- Kapitein, L. C., and Hoogenraad, C. C. (2015). Building the neuronal microtubule cytoskeleton. *Neuron* 87, 492–506. doi: 10.1016/j.neuron.2015.05.046
- Katz, P. S., Sulzer, J. K., Impastato, R. A., Teng, S. X., Rogers, E. K., Molina, P. E., et al. (2015). Endocannabinoid degradation inhibition improves neurobehavioral function, blood-brain barrier integrity, and neuroinflammation following mild traumatic brain injury. *J. Neurotrauma* 32, 297–306. doi: 10.1089/neu.2014.3508
- Li, J., Wang, Q., Wang, H., Wu, Y., Yin, J., Chen, J., et al. (2018). Lentivirus mediating fgf13 enhances axon regeneration after spinal cord injury by stabilizing microtubule and improving mitochondrial function. *J. Neurotrauma* 35, 548–559. doi: 10.1089/neu.2017.5205
- Loane, D. J., and Faden, A. L. (2010). Neuroprotection for traumatic brain injury: translational challenges and emerging therapeutic strategies. *Trends Pharmacol. Sci.* 31, 596–604. doi: 10.1016/j.tips.2010.09.005
- Ma, M. W., Wang, J., Zhang, Q., Wang, R., Dhandapani, K. M., Vadlamudi, R. K., et al. (2017). NADPH oxidase in brain injury and neurodegenerative disorders. *Mol. Neurodegener.* 12:7. doi: 10.1186/s13024-017-0150-7
- Mayer, C. L., Huber, B. R., and Peskind, E. (2013). Traumatic brain injury, neuroinflammation, and post-traumatic headaches. *Headache* 53, 1523–1530. doi: 10.1111/head.12173
- Murillo, B., and Sousa, M. M. (2018). Neuronal intrinsic regenerative capacity: the impact of microtubule organization and axonal transport. *Dev. Neurobiol.* 78, 952–959. doi: 10.1002/dneu.22602
- Nishimoto, S., Tanaka, H., Okamoto, M., Okada, K., Murase, T., and Yoshikawa, H. (2015). Methylcobalamin promotes the differentiation of schwann cells and remyelination in lysophosphatidylcholine-induced demyelination of the rat sciatic nerve. *Front. Cell Neurosci.* 9:298. doi: 10.3389/fncel.2015.00298
- Nogales, E. (2000). Structural insights into microtubule function. *Annu. Rev. Biochem.* 69, 277–302. doi: 10.1146/annurev.biochem.69.1.27
- O’Donovan, K. J. (2016). Intrinsic axonal growth and the drive for regeneration. *Front. Neurosci.* 10:486. doi: 10.3389/fnins.2016.00486
- Okada, K., Tanaka, H., Temporin, K., Okamoto, M., Kuroda, Y., Moritomo, H., et al. (2010). Methylcobalamin increases Erk1/2 and Akt activities through the methylation cycle and promotes nerve regeneration in a rat sciatic nerve injury model. *Exp. Neurol.* 222, 191–203. doi: 10.1016/j.expneurol.2009.12.017
- Rathod, R., Khaire, A., Kale, A., and Joshi, S. (2016). A combined supplementation of vitamin B12 and n-3 polyunsaturated fatty acids across two generations improves nerve growth factor and vascular endothelial growth factor levels in the rat hippocampus. *Neuroscience* 339, 376–384. doi: 10.1016/j.neuroscience.2016.10.0
- Roussel, B. D., Kruppa, A. J., Miranda, E., Crowther, D. C., Lomas, D. A., Marciniaik, S. J., et al. (2013). Endoplasmic reticulum dysfunction in neurological disease. *Lancet Neurol.* 12, 105–118. doi: 10.1016/s1474-4422(12)70238-7
- Rubenstein, R., Chang, B., Yue, J. K., Chiu, A., Winkler, E. A., and Puccio, A. M. (2017). Comparing plasma phospho tau, total tau, and phospho tau–total tau ratio as acute and chronic traumatic brain injury biomarkers. *JAMA Neurol.* 74:1063. doi: 10.1001/jamaneurol.2017.0655
- Sengottuvvel, V., and Fischer, D. (2011). Facilitating axon regeneration in the injured CNS by microtubules stabilization. *Commun. Integr. Biol.* 4, 391–393. doi: 10.4161/cib.4.4.15552
- Stassart, R. M., Möbius, W., Nave, K.-A., and Edgar, J. M. (2018). The axon-myelin unit in development and degenerative disease. *Front. Neurosci.* 12:467. doi: 10.3389/fnins.2018.00467
- Sukocheva, O. A., Abramov, A. Y., Levitskaya, J. O., Gagelgans, A. I., and Carpenter, D. O. (2001). Modulation of intracellular Ca²⁺ concentration by vitamin B12 in rat thymocytes. *Blood Cell Mol. Dis.* 27, 812–824. doi: 10.1006/bcmd.2001.0450
- Sun, D. (2014). The potential of endogenous neurogenesis for brain repair and regeneration following traumatic brain injury. *Neural. Regen. Res.* 9, 688–692. doi: 10.4103/1673-5374.131567
- Sun, H., Yang, T., Li, Q., Zhu, Z., Wang, L., Bai, G., et al. (2012). Experimental research dexamethasone and vitamin B12 synergistically promote peripheral nerve regeneration in rats by upregulating the expression of brain-derived neurotrophic factor. *Arch. Med. Sci.* 5, 924–930. doi: 10.5114/aoms.2012.31623
- Tabas, I., and Ron, D. (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat. Cell Biol.* 13, 184–190. doi: 10.1038/ncb0311-184
- Takei, Y., Teng, J., Harada, A., and Hirokawa, N. (2000). Defects in axonal elongation and neuronal migration in mice with disrupted tau and map1b genes. *J. Cell Biol.* 150, 989–1000. doi: 10.1083/jcb.150.5.989
- Tan, H. P., Guo, Q., Hua, G., Chen, J. X., and Liang, J. C. (2018). Inhibition of endoplasmic reticulum stress alleviates secondary injury after traumatic brain injury. *Neural. Regen. Res.* 13, 827–836. doi: 10.4103/1673-5374.232477
- Tsai, Y. C., and Weissman, A. M. (2010). The unfolded protein response: degradation from the endoplasmic reticulum, and cancer. *Genes Cancer* 1, 764–778. doi: 10.1177/1947601910383011
- Wieloch, T., and Nikolic, K. (2006). Mechanisms of neural plasticity following brain injury. *Curr. Opin. Neurobiol.* 16, 258–264. doi: 10.1016/j.conb.2006.05.011
- Wu, Y., Li, X., Jia, J., Zhang, Y., Li, J., Zhu, Z., et al. (2018). Transmembrane E3 ligase RNF183 mediates ER stress-induced apoptosis by degrading Bcl-xL. *Proc. Natl. Acad. Sci. U.S.A.* 115, E2762–E2771. doi: 10.1073/pnas.1716439115

- Xie, Y., Ye, S., Zhang, J., He, M., Dong, C., Tu, W., et al. (2016). Protective effect of mild endoplasmic reticulum stress on radiation-induced bystander effects in hepatocyte cells. *Sci. Rep.* 6:38832. doi: 10.1038/srep38832
- Zhang, J., Niu, F., Dong, H., Liu, L., Li, J., Li, S., et al. (2015). Characterization of protein alterations in damaged axons in the brainstem following traumatic brain injury using fourier transform infrared microspectroscopy: a preliminary study. *J. Forensic Sci.* 60, 759–763. doi: 10.1111/1556-4029.12743
- Zhang, Y., Chopp, M., Liu, X. S., Katakowski, M., Wang, X., Tian, X., et al. (2016). Exosomes derived from mesenchymal stromal cells promote axonal growth of cortical neurons. *Mol. Neurobiol.* 54, 2659–2673. doi: 10.1007/s12035-016-9851-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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New Trends in Migraine Pharmacology: Targeting Calcitonin Gene–Related Peptide (CGRP) With Monoclonal Antibodies

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Migraine is a common neurologic disorder characterized by attacks consisting of unilateral, throbbing headache accompanied by photophobia, phonophobia, and nausea which remarkably reduces the patients' quality of life. Not migraine-specific non-steroidal anti-inflammatory drugs (NSAIDs) are effective in patients affected by mild episodic migraine whilst in moderate or severe episodic migraine and in chronic migraineurs triptans and preventative therapies are needed. Since these treatments are endowed with serious side effects and have limited effectiveness new pharmacological approaches have been investigated. The demonstrated pivotal role of calcitonin gene-related peptide (CGRP) has fostered the development of CGRP antagonists, unfortunately endowed with liver toxicity, and monoclonal antibodies (mAbs) toward circulating CGRP released during migraine attack or targeting its receptor. Currently, four mAbs, eptinezumab, fremanezumab, galcanezumab for CGRP and erenumab for CGRP canonical receptor, have been studied in clinical trials for episodic and chronic migraine. Apart from the proven effectiveness, these antibodies have resulted well tolerated and could improve the compliance of the patients due to their long half-lives allowing less frequent administrations. This study aims at investigating the still poorly clear pathogenesis of migraine and the potential role of anti-CGRP mAbs in the scenario of prophylaxis of migraine.

Keywords: migraine, pharmacology of migraine, CGRP, treatment, anti-CGRP, monoclonal antibodies anti-CGRP

INTRODUCTION: MIGRAINE CLINIC

Migraine consists in unilateral headache accompanied by a cluster of other sensory, autonomic and cognitive symptoms and it has been identified by the global burden of disease (GBD) study 2016 as the sixth most prevalent disorder and one of the main causes of disability all over the world, often occurring in working age and in young adult and middle-aged women (Collaborators, 2018). Hence, migraine represents a very serious social issue in terms of years of life lived with

disability (YLDs) and the most important cause of YLDs within 15 to 49 years of age (see Steiner et al., 2018). The burden of such disease has not been identified until recently because: migraine is not a cause of permanent disability or death; headache occasionally occurs in general population (Collaborators, 2018). Although a definite pathogenesis for migraine is not known, the extracranial circulation is involved (Drummond and Lance, 1983). The clinical course of migraine is articulated in different following or concomitant stages: premonitory, aura, headache, and postdrome (similar to the premonitory phase) (Goadsby et al., 2017a). The premonitory phase includes irritability, food craving, stiff neck and can occur from 2 to 72 h prior to the attack and continues over the other phases. According to the definitions of the International Classification of Headache Disorders third edition (ICHD-3):

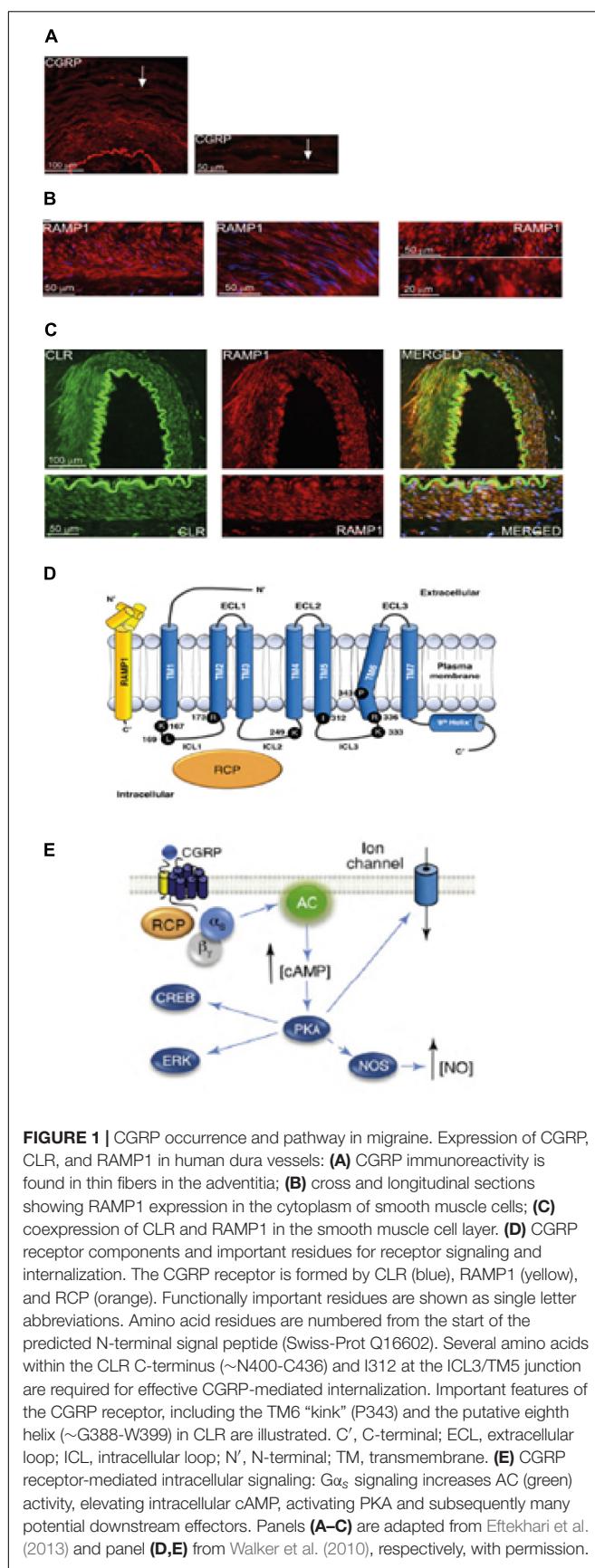
- aura is characterized by one or more transient, reversible neurological deficits, of which at least one has to have a unilateral localization, that develop over 5 min or more and of which each deficit lasts between 5 and 60 min;
- migraine consists in headache attacks lasting 4–72 h accompanied by nausea, photophobia and phonophobia, or both (see Goadsby et al., 2017a).

Cutaneous allodynia, defined as the perception of pain following non-painful stimuli, occurs in more than the 70% of patients (Lambru et al., 2018). Severe headache attenuates to stop during the postdrome phase, while other symptoms such as asthenia, somnolence and photophobia keep affecting the patient, thus displaying the complex neural basis underlying migraine (Lambru et al., 2018). Depending on the days per month affected, migraine is classified as episodic (fewer than 15 migraine or headache days) or chronic (at least 15 days, among which 8 or more are migraine days) (see Goadsby et al., 2017b). This aspect influences remarkably the impact of the disease and the therapeutic options (Giamberardino et al., 2017). With 1 to 3 attacks per month it is possible to use only abortive symptomatic drugs, while if 4 to 14 attacks per month occur it is mandatory to add preventative treatments; the latter treatments are needed to avoid chronicification of the disease (Giamberardino and Martelletti, 2015; Giamberardino et al., 2017) and to reduce the risk of medication overuse headache and refractory migraine (Martelletti, 2017).

PATHOPHYSIOLOGY OF MIGRAINE AND CALCITONIN GENE-RELATED PEPTIDE (CGRP)

The exact cause of migraine attacks is not well known yet, but the current research has been highlighting the importance of sensitization processes within the trigeminovascular system and the whole brainstem, as well as the observation of reduced gray matter in pain processing areas (see Goadsby et al., 2017a). From the trigeminal ganglion containing the cell body pseudo-unipolar primary afferents synapse on the blood vessels and on the trigeminocervical complex from which second-order fibers

synapse on third-order thalamocortical neurons and on *locus coeruleus*, periaqueductal gray and hypothalamus (Goadsby et al., 2017a). The nociceptive fibers from the trigeminal ganglion and the cervical dorsal root ganglia innervate the dura mater vessels and their terminals release vasoactive neuropeptides such as calcitonin gene-related peptide (CGRP) inducing vasodilation. Expression of CGRP, CLR, and RAMP1 in human dura vessels is shown in **Figures 1A–C**. Indeed, the autonomic nervous fibers innervating extracerebral vasculature contain several neurotransmitter vasoactive molecules: noradrenaline, serotonin, acetylcholine, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), substance P (SP), neurokinin A (NU), and CGRP (Goadsby et al., 1990). The CGRP receptor antagonists act blocking the nociceptive information from the dura mater: it is processed in the thalamic nuclei from which it reaches higher cortical regions. These inputs from the trigeminovascular system are subjected to modulation by the brainstem descending pathway: the 5-hydroxytryptamine (5HT)1B/1D receptor agonists, i.e., triptans, are thought to act via this system and to counteract the occurred vasodilation. While propranolol and topiramate as preventive treatment, the latter drugs are used in acute episodes of migraine. In particular, apart from their vasoconstrictor activity, triptans directly acting on 5HT1B/D presynaptic receptors inhibit the release of mediators like CGRP involved in nociception. Furthermore, sumatriptan has experimentally been demonstrated to inhibit the inward current mediated by transient receptor potential vanilloid 1 (TRPV1) in the trigeminal ganglia (Evans et al., 2012). CGRP is released during migraine attacks and it displays several roles as the most vasoactive neuropeptide whose craniovascular levels increase in the course of the disorder (Goadsby et al., 1990). CGRP has been shown to undergo alterations also in cerebrospinal fluid (van Dongen et al., 2017). The triggers to altered central excitability and the following breakthrough of migraine pain episodes have long been investigated. The stimulation of nerve fibers can foster both orthodromic and antidromic action potentials and, in particular, the activation of dural peptidergic primary sensory afferents that express the transient receptor potential (TRP) can induce the release of several molecules including CGRP which trigger inflammatory tissue reactions known as neurogenic inflammation (Xanthos and Sandkuhler, 2014). In particular, CGRP is involved in cranial nociception and in the epiphrenomenon of vasodilation binding its receptors on meningeal and cerebral blood vessels (Deen et al., 2017). These processes promote the sensitization of the trigeminal second order fibers taking amplified painful stimuli to higher regions like thalamus, hypothalamus and cortex, thus originating migraine (Dussor et al., 2014). TRPs are subjected to activation in response to several environmental irritant stimuli as temperature and pH variations that in predisposed individuals can trigger migraine pain. CGRP is produced by tissue-specific alternative splicing of the calcitonin gene CALC I on chromosome 11, also encoding for calcitonin. On the contrary, β CGRP is produced from CALC II gene located on a different site of chromosome 11. α CGRP neuropeptide is present in the central nervous system as α isoform of 37 amino acids and its signal transduction is mediated by two receptors.



The first one is known as canonical CGRP receptor and is a G α_s protein coupled receptor which requires the receptor activity-modifying protein (RAMP) 1 to be functional (Goadsby et al., 2017a). The second one is the human amylin subtype 1 receptor (AMY1): amylin belongs to the calcitonin gene family and it has hormonal activity. When CGRP binds to its receptor, it is subjected to dynamin/clathrin dependent internalization after having been complexed with β -arrestin (see Walker et al., 2010). Conformational changes occur inducing activation of adenylate cyclase (AC), increase in cAMP and activation of protein kinase A (PKA) that can promote vasodilation through direct activation of endothelial nitric oxide synthase and pain responses. This latter receptor can be coupled to G $\alpha_q/11$ with the activation of phospholipase C (PLC), to mitogen-activated protein kinase (MAPK) and to the release of NO (see Walker et al., 2010) (see Figures 1D,E for schematic representation of CGRP receptor-mediated intracellular signaling). The administration of CGRP to migraineurs triggers attacks and this evidence supports its pivotal role in the pathogenesis of migraine (Lassen et al., 2002). A signal termination system has not been identified yet: CGRP is metabolized by neprilysin, insulin-degrading enzyme and endothelin-converting enzyme-1 and a reuptake active transport system has been hypothesized (see Russell et al., 2014). Moreover, when the receptor is transiently activated by CGRP it is internalized into endosomes and fastly recycled back to the cell membrane, while chronic stimulation of the receptor induces desensitization and lysosomal degradation (see Russell et al., 2014).

PHARMACOLOGICAL INTERVENTIONS ON CGRP PATHWAY

Migraine is a multifaceted disabling neurovascular disorder and the current therapy with oral triptans is effective in acute attacks, though some 40% patients are resistant to treatment (Ferrari et al., 2001; Edvinsson, 2015). These selective 5-HT1B/1D agonists, of which the most commonly used is sumatriptan mostly active for subcutaneous route, exert their therapeutic action through vasoconstriction of cranial vessels, and inhibition of the trigeminal and trigeminocervical system (see Ferrari et al., 2001). However, vasoconstriction represents a limit for the cardiovascular side effects of these drugs. Furthermore, the treatment with triptans cannot last longer than 9 days per month because of the risk of drug-induced headache. In 2010 botulinum toxin type A (BoNT-A) was approved by the Food and Drug Administration (FDA) in the prevention of migraine in unresponsive chronic migraineurs and its complex administration (it requires injection in 31 sites) limits the compliance of patients. Due to the pivotal role played by CGRP in the pathophysiology of migraine, antagonists of its receptor have been developed. These drugs belong to the class of gepants. Telgaceptant, the first oral gepant, resulted well tolerated in migraineurs affected by coronary artery disease (ClinicalTrials.gov NCT00662818, Ho et al., 2012), but it presented hepatotoxicity, thus, in spite of continuous research

on these small molecules, a new approach consisting in the development of monoclonal antibodies (mAbs) toward CGRP (neuzumabs) or CGRP receptor (numabs) was proposed. These drugs are believed to inhibit the action of the circulating CGRP molecules to prevent migraine attacks and, since they do not cross blood brain barrier (BBB) because of their size, the sites of action should be in the trigeminal system (Edvinsson, 2015). In particular, dural vessels are not included in BBB. Moreover, CGRP likely does not cross BBB and acts as a paracrine modulator since it is expressed by half neurons of trigeminal ganglion which do not present the CGRP receptor, expressed by satellite glial cells and the 30% of neuronal soma of trigeminal ganglion (see Yuan et al., 2017). Currently, there are four mAbs, eptinezumab, fremanezumab, galcanezumab and erenumab studied in clinical trials for episodic and chronic migraine. The first three mAbs are humanized antibodies directed toward CGRP, while erenumab is a human antibody toward its canonical receptor. The half-life of these drugs is quite long, thus allowing no more than one administration per month and this is a matter of interest since mAbs need to be administered for intravenous or subcutaneous route. In particular, eptinezumab only is administered intravenously (Israel et al., 2018). The phase III PROMISE 1 (PRevention Of Migraine via Intravenous eptinezumab Safety and Efficacy 1) trial (ClinicalTrials.gov NCT02559895) assessed Eptinezumab effectiveness on prevention of frequent episodic migraine. The results showed a significant reduction in the primary endpoint consisting in decrease of monthly migraine days (MMDs) from mean baseline of 8.5 days over weeks 1–12 to 4.3 MMDs with the dose of 300 mg, 3.9 with 100 mg, and 4.0 with 30 mg respect to 3.2 days of placebo (Saper et al., 2018). For the evaluation of eptinezumab in the prevention of chronic migraine 1121 participants have been enrolled in the PROMISE 2 (ClinicalTrials.gov NCT02974153). Galcanezumab was assessed for efficacy in the prevention of episodic migraine in the EVOLVE-1 phase 3 double-blind, randomized, placebo-controlled study (ClinicalTrials.gov NCT02614183) dissected in 4 periods and in the EVOLVE-2 Phase 3 randomized controlled double-blind 6 month-clinical trial (ClinicalTrials.gov NCT02614196). In the EVOLVE-1 both doses of galcanezumab (120 and 240 mg) met the primary outcome of significant reduction of monthly migraine headache days of 4.7 days and 4.6 days, respectively, in comparison with the 2.8 days of the placebo (Stauffer et al., 2018). In the EVOLVE-2 patients received monthly injection of 120 or 240 mg of galcanezumab. The mean monthly migraine headache days were reduced of 4.3 and 4.2 days, respectively, compared to 2.3 days reduction obtained with placebo ($p < 0.001$) (Skljarevski et al., 2018). Also the secondary endpoints of reduction of functional impairment assessed through the Role Function-Restrictive (R-FR) domain score of the Migraine-Specific Quality of Life Questionnaire (MSQ) and improvement of the score of the Patient Global Impression of Severity (PGI-S) and of the Migraine Disability Assessment (MIDAS; time point = month 6) were met (Skljarevski et al., 2018). 147 (65.0%) and 163 (71.5%) of the patients treated with galcanezumab, 120 and 240 mg, respectively, and 287 (62.3%) with placebo presented

adverse events, among which acute myocardial infarction and transient ischemic attack within a group of seven patients under treatment with galcanezumab 240 mg (Skljarevski et al., 2018). There were not statistically significant differences in mean change from baseline for systolic/diastolic blood pressure. The 19 (8.6%), 11 (5.1%), and 2 (0.5%) patients in the galcanezumab 120 mg, galcanezumab 240 mg, and placebo groups, respectively, showed treatment-emergent anti-drug antibodies (ADA). In the REGAIN phase III trial evaluating galcanezumab against chronic migraine MMDs were reduced of 4.8 days with 120 mg and of 4.6 days with 240 mg dose compared to 2.7 of placebo (see Yuan et al., 2017). Moreover, the treatment with galcanezumab was demonstrated to be endowed with statistically significant persistence of the effect (Forderreuther et al., 2018). Fremanezumab was investigated in the phase III HALO trial for the preventive treatment of migraine (ClinicalTrials.gov NCT02638103). It reduced MMDs of episodic migraine at 12 weeks of 3.7 days at 225 mg monthly for 3 months and of 3.4 days at 675 mg once in quarterly dose regimen when compared to the 2.2 days decrease of placebo (see Yuan et al., 2017). The effectiveness of erenumab in episodic migraine prevention was studied in the phase 3, randomized, double-blind, placebo-controlled study ARISE (ClinicalTrials.gov NCT02483585). MMDs were reduced by 70 mg monthly subcutaneous erenumab of 2.9 days in comparison with 1.8 days of placebo ($p < 0.001$) and it was effective in the secondary endpoints of at least a 50% reduction in MMDs and change in monthly migraine-specific medication treatment days (MSMD) (Dodick et al., 2018). The proportion of patients presenting adverse events was similar between the group treated with the mAb and the placebo; 4.3% tested positive for anti-erenumab-binding antibodies through week 12, one of whom transiently positive for neutralizing antibodies but only at week 4 (Dodick et al., 2018). Also the phase III STRIVE clinical trial (ClinicalTrials.gov NCT02456740) assessed the effectiveness of erenumab for the prevention of episodic migraine. Erenumab was administered at 70 or 140 mg monthly for 6 months (Goadsby et al., 2017b). The baseline mean number of MMDs was 8.3 and the 70 mg and 140 mg doses reduced it of 3.2 and 3.7, respectively, compared to 1.8 days of placebo (with $p < 0.001$ for each dose vs. placebo) (Goadsby et al., 2017b). Also over the final 3 months of treatment each dose of erenumab fulfilled the secondary endpoints of at least a 50% reduction from baseline in the mean number of migraine days per month and reduction from baseline in both the Migraine Physical Function Impact Diary (MPFID) everyday-activities (MPFID-EA) and physical-impairment (MPFID-PI) (Goadsby et al., 2017b). In those patients with anti-erenumab antibodies only one in the group treated with 70 mg tested positive for neutralizing antibodies and the mAb was overall well tolerated in terms of creatinine levels, liver toxicity, total neutrophil counts and electrocardiographic function (Goadsby et al., 2017b). According to the authors one of the limits of this trial is that patients that had not shown therapeutic response to more than two classes of migraine-preventive drugs were excluded (Goadsby et al., 2017b). Furthermore, there are clinical trials investigation the effectiveness of fremanezumab (NCT02945046 and NCT02964338) and galcanezumab (NCT

02397473 and NCT02438826) in the prevention cluster headache (see Israel et al., 2018), that is primary headache characterized by severe unilateral pain in the periorbital area accompanied by tearing, conjunctival redness, and rhinorrhea (Vollesen et al., 2018). Indeed, it was demonstrated that continuous intravenous infusion through infusion pump of 1.5 µg/min of CGRP for 20 min (in 2 days separated by at least 7 days) to patients with episodic (active or remission phase) or chronic cluster headache elicited cluster-like attacks in patients in active phase or with chronic cluster headache, but not in remission phase (ClinicalTrials.gov NCT02466334) (Vollesen et al., 2018). The main PK advantages of mAbs are represented by long elimination half-life thus limiting the need for daily dosing and clearance by proteolysis. Due to the effects of gepants on liver and because of the vasodilatory properties of CGRP, the most feared risks of the inhibition of CGRP signaling consist in the hypothesized hepatotoxicity and cardiovascular theoretical risk (Yuan et al., 2017). The European Headache Federation (EHF) produced guidelines on the use of anti-CGRP mAbs applying to the Grading of Recommendation, Assessment, Development and Evaluation (GRADE) method and, when not possible, relying on the opinion of a panel of experts (Sacco et al., 2019).

PHARMACOKINETICS (PK)/ PHARMACODYNAMICS (PD) RELATIONSHIP OF ANTI-MIGRAINE mAbs

The development of mAbs has represented a completely new approach to inhibit the CGRP pathway. The main PD improvement achieved using mAbs instead of small molecules is that it is easier to target the broad CGRP receptor-ligand-binding site (Taylor, 2018). The main PK advantages of mAbs are represented by long elimination half-life without need for daily dosing and clearance by proteolysis and the most feared risks of the inhibition of CGRP signaling consist in the hypothesized hepato-toxicity, due to the effects of gepants on liver, and cardiovascular theoretical risk, because of the vasodilatory properties of CGRP. However, following the journey of these mAbs from their production to the target, the first hindrance that these molecules meet is their administration because of their scarce oral bioavailability. Therefore, a parenteral route of administration is required and, in order to favor adherence to the treatment, half-life needs to be long (Taylor, 2018). Immunoglobulins (Ig)G1, 2, or 4 are the possibilities (Wang et al., 2008; Taylor, 2018). Apart from the origin of the IgG, eventual cross-reactivity and individual modifications of catabolism can affect half-life (Bonilla, 2008; Taylor, 2018). The technology used to produce mAbs relies on the use of hybridomas composed of cells in continuous division which produce determined clones of a single type of antibody, with no or low variability (Taylor, 2018). Based on the origin of the amino acids composing the mAb, it is possible to distinguish among chimeric (rodent immunization; maintenance of rodent Fragment antigen-binding

region Fab, but introduction of human Fragment crystallizable region Fc), humanized (zumabs, with mouse Complementarity Determining Regions, CDRs, grafted to human Fab regions) and human (introduction of the whole sequence of the human antibody gene in the mouse that becomes humanized, as for instance the XenoMouse) mAbs (see Taylor, 2018). The CDRs influence the fitting to the target. Among the 4 mAbs anti-migraine only erenumab is human, while the grafted CDRs are from mouse for fremanezumab, rabbit for eptinezumab and likely mouse for galcanezumab (see Taylor, 2018). In particular, the only anti-CGRP receptor mAb is erenumab, which is a human IgG2λ, and the other three mAbs directed against sites of CGRP are: eptinezumab, a genetically engineered humanized IgG1k; fremanezumab, a humanized IgG2k; galcanezumab, a humanized IgG4 (Edvinsson et al., 2018). Erenumab (Tmax 3–14 days) requires monthly subcutaneous administration, as well as fremanezumab (Tmax 3–20 days) and galcanezumab (Tmax 7–14 days) (Taylor, 2018). On the contrary, eptinezumab (Tmax 4.8 h) is intravenously administered once every 3 months; it associates more rapidly and dissociates more slowly than fremanezumab and galcanezumab. Since mAbs are large sized proteins, they cannot easily cross BBB. Some CGRP receptors are outside the BBB, thus allowing the action of anti-CGRP mAbs (Edvinsson, 2018). Before binding, the antibody enters vascular endothelial cells via pinocytosis. Large apparent distribution volumes could depend on the tissue and the related capacity of binding of the mAb (Lobo et al., 2004; Taylor, 2018). Erenumab is the most novel approach, since it is the only one to target a fusion protein of the extracellular domains of human G protein-coupled receptor calcitonin receptor-like receptor CALCRL (required in the receptors for CGRP and adrenomedullin) and RAMP1 including the CGRP binding pocket (Edvinsson et al., 2018). It has been demonstrated to competitively inhibit the binding of [¹²⁵I]-CGRP to the human CGRP receptor in human neuroblastoma cells (SK-N-MC) with a Ki of 0.02 ± 0.01 nM (Shi et al., 2016). Erenumab exerted potent and full antagonism of CGRP-stimulated cAMP accumulation with an IC₅₀ of 2.3 ± 0.9 nM in functional assays performed in SK-N-MC (Shi et al., 2016). Moreover, it resulted 5000-fold more selective for CGRP receptor showing no agonist/antagonist effect on other human calcitonin family receptors including adrenomedullin, calcitonin, and amylin receptors up to the highest concentration tested of 10 µM (Shi et al., 2016). Fremanezumab might exert its effect on different vessels; in fact, it causes concentration dependent inhibition of CGRP induced vasodilation in pre-contracted human cerebral, meningeal and peripheral abdominal arteries (Ohlsson et al., 2018). In addition, CGRP may act also on neurons and glial cells and on the glymphatic (lymphatic-like), the latter being implicated in the expression of aura (see Messlinger, 2018). The likely absence of metabolism by liver enzymes could avoid drug-drug interactions. Elimination occurs through renal proteolysis of amino acids. Although clinical trials report that these antibodies are overall well tolerated, apart from the highlighted injection site pain, there is concern with their immunogenicity through production of ADA (Taylor, 2018). During a double-blind, randomized, placebo-controlled clinical trial (ClinicalTrials.gov NCT 01337596)

evaluating the treatment with different regimens of single and multiple doses of galcanezumab, 11 of treated patients (26%) presented low titers (1:10 – 1:80) of treatment emergent-ADA (in 3 patients the pre-existing antibodies increased in titer), without dose-response and detected effects on PK and PD (Monteith et al., 2017). The study of immunogenicity is essential because the presence of ADA can accelerate drug removal or, worse, foster end organ damage (Taylor, 2018). The main characteristics of anti-migraine mAbs are summarized in **Table 1**.

CONCLUSION

Migraine is a disabling and debilitating neurovascular painful condition representing more than 90% of cases of recurrent headache and toward which the tendency can be inherited (MacGregor, 2017). Divalproex sodium, sodium valproate, topiramate, metoprolol, propranolol, and timolol have proven strong, level A, evidence for migraine prevention (American Academy of Neurology and American Headache Society, 2015). However, all the classic oral preventative treatments including tricyclic antidepressants, beta blockers, 5-HT2 antagonists ergots and anti-epileptic drugs were not developed for migraine and provide 50% reduction in the number of monthly days of migraine pain only up to the 45% of migraineurs (D'Amico and Tepper, 2008), also because of low adherence due to scarce tolerability. Medication persistence and discontinuation was

examined in a retrospective US claims analysis (Hepp et al., 2017) and the results suggest low persistence to the initial drug used and high amount of discontinuation by 6 months independently on drug class. Due to the fundamental role of CGRP in sustaining neuroinflammation and central sensitization in the pathway from trigeminal ganglion and brainstem to higher regions involved in the physiopathology of migraine, novel mAbs toward CGRP and its receptor have been developed. The first small molecules CGRP antagonists showed to induce liver toxicity, but mAbs did not produce either toxic metabolites or cardiovascular side reactions because of the inhibition of vasodilation (see Deen et al., 2017). Central side effects were not highlighted, as well. The potential long-term effects of blocking CGRP still need to be studied but, a fundamental advantage of these antibodies stems from their long half-life allowing monthly or less frequent injections, which can remarkably improve adherence to the treatment and its following effectiveness (Deen et al., 2017). Thus, anti-CGRP mAbs could represent effective tools in the therapeutic arsenal against unresponsive migraine; however, deep monitoring of efficacy and safety (i.e., production of toxic metabolites, immunogenicity with ADA, neutralizing antibodies and tissue cross-reactivity and side effects) is mandatory (Taylor, 2018). In fact, evidence from older biotech products show that therapies with anti-tumor necrosis factor α (TNF α) mAbs can be subjected to secondary failure (in contrast with primary non-responders) of the initial therapeutic response because these drugs induce the production of antibodies that may degrade the mAb but may also neutralize its action before

TABLE 1 | Main characteristics of fremanezumab, eptinezumab, galcanezumab, and erenumab.

mAb	PK	PD	Outcome
Fremanezumab	Monthly subcutaneous administration (Tmax 3–20 days) (Taylor, 2018).	Humanized anti-CGRP IgG2 (Edvinsson et al., 2018).	HALO (NCT02638103). Decrease in MMDs of episodic migraine at 12 weeks of 3.7 days at 225 mg monthly for 3 months and of 3.4 days at 675 mg once in quarterly dose regimen, in comparison with the 2.2 days of placebo (see Yuan et al., 2017).
Eptinezumab	Intravenously administered once every 3 months (Tmax 4.8 h) (Taylor, 2018).	Humanized IgG1 toward CGRP (Edvinsson et al., 2018).	PROMISE 1 (NCT02559895): activity in prevention of frequent episodic migraine from mean baseline of 8.5 days over weeks 1–12 to 4.3 MMDs with the dose of 300 mg, 3.9 with 100 mg and 4.0 with 30 mg respect to 3.2 days of placebo (Saper et al., 2018).
Galcanezumab	Monthly subcutaneous administration (Tmax 7–14 days) (Taylor, 2018).	Humanized anti-CGRP IgG4 (Edvinsson et al., 2018).	EVOLVE-2 (NCT02614196): effectiveness in prevention of episodic migraine. 120 mg or 240 mg of galcanezumab reduced the mean monthly migraine headache days of 4.3 and 4.2 days, respectively, compared to 2.3 days with placebo (Skljarevski et al., 2018). REGAIN: chronic migraine. MMDs were reduced of 4.8 days with 120 mg and of 4.6 days with 240 mg dose compared to 2.7 of placebo (see Yuan et al., 2017).
Erenumab	Monthly subcutaneous administration (Tmax 3–14 days) (Taylor, 2018).	Anti-CGRP receptor human IgG2 (Edvinsson et al., 2018).	ARISE (NCT02483585): prevention of episodic migraine. Reduction in MMDs by 70 mg monthly subcutaneous erenumab of 2.9 days in comparison with 1.8 days of placebo (Dodick et al., 2018). STRIVE (NCT02456740): prevention of episodic migraine. 70 mg and 140 mg doses (monthly for 6 months) reduced baseline mean number of MMDs (8.3) of 3.2 and 3.7, respectively, compared to 1.8 days of placebo (Goadsby et al., 2017b).

The administration route and frequency, structure of the mAb and target and the main results of the clinical trials of the four mAbs acting on CGRP-pathway are outlined.

binding to the target (see Prado et al., 2017). Immunogenicity can be influenced by glycosylation, type of mAb, number of epitopes and impurities in formulation (see Prado et al., 2017). Moreover, several apparently unexplainable serious side effects are associated with immunogenicity of anti-TNF α . Local and systemic hypersensitivity reactions, immunodeficiency with increased susceptibility to infections, and immune complex formation which can lead even to death have been highlighted (see Prado et al., 2017). Therefore, the use of therapeutic diagnostics (theranostics) may improve the knowledge and

correct management of these pharmacological devices (Bendtzen, 2013; Prado et al., 2017).

AUTHOR CONTRIBUTIONS

MTC, LAM, PT, and GB conceived the study. DS collected the trial results, analyzed the literature, and wrote the manuscript. AA, LR, and MN participated in the literature survey. All authors read and approved the final manuscript.

REFERENCES

- American Academy of Neurology and American Headache Society (2015). Appendix a: summary of evidence-based guideline for clinicians, update: pharmacologic treatment for episodic migraine prevention in adults. *Continuum* 21, 1165–1166. doi: 10.1212/01.CON.0000470901.87438.80
- Bendtzen, K. (2013). Personalized medicine: theranostics (therapeutics diagnostics) essential for rational use of tumor necrosis factor-alpha antagonists. *Discov. Med.* 15, 201–211.
- Bonilla, F. A. (2008). Pharmacokinetics of immunoglobulin administered via intravenous or subcutaneous routes. *Immunol. Allergy Clin. North Am.* 28, 803–819. doi: 10.1016/j.iac.2008.06.006
- Collaborators, G. B. D. H. (2018). Global, regional, and national burden of migraine and tension-type headache, 1990–2016: a systematic analysis for the global burden of disease study 2016. *Lancet Neurol.* 17, 954–976. doi: 10.1016/S1474-4422(18)30322-3
- D'Amico, D., and Tepper, S. J. (2008). Prophylaxis of migraine: general principles and patient acceptance. *Neuropsychiatr. Dis. Treat.* 4, 1155–1167.
- Deen, M., Correnti, E., Kamm, K., Kelderman, T., Papetti, L., Rubio-Beltran, E., et al. (2017). Blocking CGRP in migraine patients - a review of pros and cons. *J. Headache Pain* 18:96. doi: 10.1186/s10194-017-0807-1
- Dodick, D. W., Ashina, M., Brandes, J. L., Kudrow, D., Lanteri-Minet, M., Osipova, V., et al. (2018). ARISE: a phase 3 randomized trial of erenumab for episodic migraine. *Cephalgia* 38, 1026–1037. doi: 10.1177/0333102418759786
- Drummond, P. D., and Lance, J. W. (1983). Extracranial vascular changes and the source of pain in migraine headache. *Ann. Neurol.* 13, 32–37. doi: 10.1002/ana.410130108
- Dussor, G., Yan, J., Xie, J. Y., Ossipov, M. H., Dodick, D. W., and Porreca, F. (2014). Targeting TRP channels for novel migraine therapeutics. *ACS Chem. Neurosci.* 5, 1085–1096. doi: 10.1021/cn500083e
- Edvinsson, L. (2015). CGRP receptor antagonists and antibodies against CGRP and its receptor in migraine treatment. *Br. J. Clin. Pharmacol.* 80, 193–199. doi: 10.1111/bcpt.12618
- Edvinsson, L. (2018). The CGRP pathway in migraine as a viable target for therapies. *Headache* 58(Suppl. 1), 33–47. doi: 10.1111/head.13305
- Edvinsson, L., Haanes, K. A., Warfvinge, K., and Krause, D. N. (2018). CGRP as the target of new migraine therapies - successful translation from bench to clinic. *Nat. Rev. Neurol.* 14, 338–350. doi: 10.1038/s41582-018-0003-1
- Eftekhari, S., Warfvinge, K., Blixt, F. W., and Edvinsson, L. (2013). Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. *J. Pain* 14, 1289–1303. doi: 10.1016/j.jpain.2013.03.010
- Evans, M. S., Cheng, X., Jeffry, J. A., Disney, K. E., and Premkumar, L. S. (2012). Sumatriptan inhibits TRPV1 channels in trigeminal neurons. *Headache* 52, 773–784. doi: 10.1111/j.1526-4610.2011.02053.x
- Ferrari, M. D., Roon, K. I., Lipton, R. B., and Goadsby, P. J. (2001). Oral triptans (serotonin 5-HT(1B/1D) agonists) in acute migraine treatment: a meta-analysis of 53 trials. *Lancet* 358, 1668–1675. doi: 10.1016/S0140-6736(01)06711-3
- Forderreuther, S., Zhang, Q., Stauffer, V. L., Aurora, S. K., and Lainez, M. J. A. (2018). Preventive effects of galcanezumab in adult patients with episodic or chronic migraine are persistent: data from the phase 3, randomized, double-blind, placebo-controlled EVOLVE-1, EVOLVE-2, and REGAIN studies. *J. Headache Pain* 19:121. doi: 10.1186/s10194-018-0951-2
- Giamberardino, M. A., Affaitati, G., Costantini, R., Cipollone, F., and Martelletti, P. (2017). Calcitonin gene-related peptide receptor as a novel target for the management of people with episodic migraine: current evidence and safety profile of erenumab. *J. Pain Res.* 10, 2751–2760. doi: 10.2147/JPR.S128143
- Giamberardino, M. A., and Martelletti, P. (2015). Emerging drugs for migraine treatment. *Expert Opin. Emerg. Drugs* 20, 137–147. doi: 10.1517/14728214.2015.999040
- Goadsby, P. J., Edvinsson, L., and Ekman, R. (1990). Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann. Neurol.* 28, 183–187. doi: 10.1002/ana.410280213
- Goadsby, P. J., Holland, P. R., Martins-Oliveira, M., Hoffmann, J., Schankin, C., and Akerman, S. (2017a). Pathophysiology of migraine: a disorder of sensory processing. *Physiol. Rev.* 97, 553–622. doi: 10.1152/physrev.00034.2015
- Goadsby, P. J., Reuter, U., Hallstrom, Y., Broessner, G., Bonner, J. H., Zhang, F., et al. (2017b). A controlled trial of erenumab for episodic migraine. *N. Engl. J. Med.* 377, 2123–2132. doi: 10.1056/NEJMoa1705848
- Hepp, Z., Dodick, D. W., Varon, S. F., Chia, J., Matthew, N., Gillard, P., et al. (2017). Persistence and switching patterns of oral migraine prophylactic medications among patients with chronic migraine: a retrospective claims analysis. *Cephalgia* 37, 470–485.
- Ho, T. W., Ho, A. P., Chaitman, B. R., Johnson, C., Mathew, N. T., Kost, J., et al. (2012). Randomized, controlled study of telcagepant in patients with migraine and coronary artery disease. *Headache* 52, 224–235. doi: 10.1111/j.1526-4610.2011.02052.x
- Israel, H., Neeb, L., and Reuter, U. (2018). CGRP monoclonal antibodies for the preventative treatment of migraine. *Curr. Pain Headache Rep.* 22:38. doi: 10.1007/s11916-018-0686-4
- Lambru, G., Andreou, A. P., Guglielmetti, M., and Martelletti, P. (2018). Emerging drugs for migraine treatment: an update. *Expert Opin. Emerg. Drugs* 23, 301–318.
- Lassen, L. H., Haderslev, P. A., Jacobsen, V. B., Iversen, H. K., Sperling, B., and Olesen, J. (2002). CGRP may play a causative role in migraine. *Cephalgia* 22, 54–61. doi: 10.1046/j.1468-2982.2002.00310.x
- Lobo, E. D., Hansen, R. J., and Balthasar, J. P. (2004). Antibody pharmacokinetics and pharmacodynamics. *J. Pharm. Sci.* 93, 2645–2668. doi: 10.1002/jps.20178
- MacGregor, E. A. (2017). Migraine. *Ann. Intern. Med.* 166, ITC49–ITC64. doi: 10.7326/ITC201704040
- Martelletti, P. (2017). The application of CGRP(r) monoclonal antibodies in migraine spectrum: needs and priorities. *BioDrugs* 31, 483–485. doi: 10.1007/s40259-017-0251-4
- Messlinger, K. (2018). The big CGRP flood - sources, sinks and signalling sites in the trigeminovascular system. *J. Headache Pain* 19:22. doi: 10.1186/s10194-018-0848-0
- Monteith, D., Collins, E. C., Vandermeulen, C., Van Hecken, A., Raddad, E., Scherer, J. C., et al. (2017). Safety, tolerability, pharmacokinetics, and pharmacodynamics of the CGRP binding monoclonal antibody LY2951742 (Galcanezumab) in healthy volunteers. *Front. Pharmacol.* 8:740. doi: 10.3389/fphar.2017.00740
- Ohlsson, L., Kronvall, E., Stratton, J., and Edvinsson, L. (2018). Fremanezumab blocks CGRP induced dilatation in human cerebral, middle meningeal and abdominal arteries. *J. Headache Pain* 19:66. doi: 10.1186/s10194-018-0905-8

- Prado, M. S., Bendtzen, K., and Andrade, L. E. C. (2017). Biological anti-TNF drugs: immunogenicity underlying treatment failure and adverse events. *Expert Opin. Drug Metab. Toxicol.* 13, 985–995. doi: 10.1080/17425255.2017.1360280
- Russell, F. A., King, R., Smillie, S. J., Kodji, X., and Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol. Rev.* 94, 1099–1142. doi: 10.1152/physrev.00034.2013
- Sacco, S., Bendtsen, L., Ashina, M., Reuter, U., Terwindt, G., Mitsikostas, D. D., et al. (2019). European headache federation guideline on the use of monoclonal antibodies acting on the calcitonin gene related peptide or its receptor for migraine prevention. *J. Headache Pain* 20:6. doi: 10.1186/s10194-018-0955-y
- Saper, J., Lipton, R., Kudrow, D., Hirman, J., Dodick, D., Silberstein, S., et al. (2018). Primary results of PROMISE-1 (prevention of migraine via intravenous eptinezumab safety and efficacy-1) trial: a phase 3, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of eptinezumab for prevention of frequent episodic migraines (S20.001). *Neurology* 90(15 Suppl.), S20.001.
- Shi, L., Lehto, S. G., Zhu, D. X., Sun, H., Zhang, J., Smith, B. P., et al. (2016). Pharmacologic characterization of AMG 334, a potent and selective human monoclonal antibody against the calcitonin gene-related peptide receptor. *J. Pharmacol. Exp. Ther.* 356, 223–231. doi: 10.1124/jpet.115.227793
- Skljarevski, V., Matharu, M., Millen, B. A., Ossipov, M. H., Kim, B. K., and Yang, J. Y. (2018). Efficacy and safety of galcanezumab for the prevention of episodic migraine: results of the EVOLVE-2 Phase 3 randomized controlled clinical trial. *Cephalalgia* 38, 1442–1454. doi: 10.1177/0333102418779543
- Stauffer, V. L., Dodick, D. W., Zhang, Q., Carter, J. N., Ailani, J., and Conley, R. R. (2018). Evaluation of galcanezumab for the prevention of episodic migraine: the EVOLVE-1 randomized clinical trial. *JAMA Neurol.* 75, 1080–1088. doi: 10.1001/jamaneurol.2018.1212
- Steiner, T. J., Stovner, L. J., Vos, T., Jensen, R., and Katsarava, Z. (2018). Migraine is first cause of disability in under 50s: will health politicians now take notice? *J. Headache Pain* 19:17. doi: 10.1186/s10194-018-0846-2
- Taylor, F. R. (2018). CGRP, amylin, immunology, and headache medicine. *Headache* 59, 131–150. doi: 10.1111/head.13432
- van Dongen, R. M., Zielman, R., Noga, M., Dekkers, O. M., Hankemeier, T., van den Maagdenberg, A. M., et al. (2017). Migraine biomarkers in cerebrospinal fluid: a systematic review and meta-analysis. *Cephalalgia* 37, 49–63. doi: 10.1177/0333102415625614
- Vollesen, A. L. H., Snoer, A., Beske, R. P., Guo, S., Hoffmann, J., Jensen, R. H., et al. (2018). Effect of infusion of calcitonin gene-related peptide on cluster headache attacks: a randomized clinical trial. *JAMA Neurol.* 75, 1187–1197. doi: 10.1001/jamaneurol.2018.1675
- Walker, C. S., Conner, A. C., Poyner, D. R., and Hay, D. L. (2010). Regulation of signal transduction by calcitonin gene-related peptide receptors. *Trends Pharmacol. Sci.* 31, 476–483. doi: 10.1016/j.tips.2010.06.006
- Wang, W., Wang, E. Q., and Balthasar, J. P. (2008). Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin. Pharmacol. Ther.* 84, 548–558. doi: 10.1038/clpt.2008.170
- Xanthos, D. N., and Sandkuhler, J. (2014). Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. *Nat. Rev. Neurosci.* 15, 43–53. doi: 10.1038/nrn3617
- Yuan, H., Lauritsen, C. G., Kaiser, E. A., and Silberstein, S. D. (2017). CGRP monoclonal antibodies for migraine: rationale and progress. *BioDrugs* 31, 487–501. doi: 10.1007/s40259-017-0250-5

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