

# **Neurobiology and Physiology of the Endocannabinoid System**

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# Dedication

**I dedicate this book to my late mother, Indumati B. Patel.**

**Vinood B. Patel**

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# Preface

Extracts from the plant *Cannabis sativa* contain scores of psychoactive compounds. The principal agent is tetrahydrocannabinol. Refined extracts include cannabidiol, cannabinol, cannabigerol, cannabichromene, and approximately 80 other active compounds, many of which have been reported to have neuroactive effects. Crude preparations or extracts from the cannabis plant are among the most widely used drugs on a global basis, and the World Health Organization has indicated that 2.5% of the world's population uses cannabis. Adverse effects of cannabis have been reported, which are dependent on the different forms and amounts in which it is used. For example, a small proportion of cannabis users will develop psychoses or exhibit extreme behavioral problems such as paranoia. Neurological changes may be transient or long term. Some synthetic cannabinoids have also been used recreationally with drastic consequences.

On the other hand, there is also a growing body of scientific publications to support or promote the usage of cannabis and cannabis-related products in alleviating or preventing symptoms in different diseases and conditions. These include the prevention of nausea in HIV and cancer. The World Health Organization has even mentioned that there is potential usage of cannabis and cannabis-related products in asthma and glaucoma. In other words, it seems that a hitherto described psychoactive drug has health benefits. This is like the scenario of alcohol where consumption of small amounts has been shown to reduce cardiovascular disease but in very high amounts, there's increased rate of mortality. The problem with cannabis is that in many cases, there is insufficient evidence to partition the differences between beneficial and adverse effects. However, it is important to point out that the usage of any component or regimen requires scientifically vigorous trials and investigations. Treatments and pathways seen in modeling systems or *in vitro* need to be verified *in vivo*. Adverse effects also need to be investigated. The gold standard is randomized controlled trials and with due consideration of toxic and adverse effects; these chapters provide a framework only.

Recent advances in neuroscience have seen an emergence of research into the endocannabinoid system. This system, in simple terms, encompasses endocannabinoids (lipid-based compounds), cannabinoid receptors, and postreceptor responses. The endocannabinoid system has been implicated in various neurological conditions or processes and included, for example, Alzheimer's, Parkinson's, and Huntington's diseases, sleep, fear, memory, appetite, and pain. Endogenous cannabinoid receptors can also be activated by exogenous cannabinoids that may be derived from plants or synthesized artificially. Potentially the modulation of the endocannabinoid system may confer neurobiological advantages and provide novel treatment regimes.

The editors have sought to overcome these limitations and advance the understanding of neurosciences and cannabis with a series of three books collectively called **The Neuroscience of Cannabis and Cannabinoids**.

It brings together information relating to cannabis and cannabinoids in relation to neurological processes and systems.

The three books are

Book 1: *Cannabis Use, Neurobiology, Psychology, and Treatment*

Book 2: *Medicinal Usage of Cannabis and Cannabinoids*

Book 3: *Neurobiology and Physiology of the Endocannabinoid System*

**The Neuroscience of Cannabis and Cannabinoids** transcends both the disciplinary and intellectual divides as each chapter has:

- **Abstract (published online)**
- **Applications to other areas**
- **Key facts**
- **Mini-dictionary of terms**
- **Summary points**

The section **Applications to other areas** is particularly important as it highlights the translational aspect of cannabis and cannabinoids-related research. The **Key facts** section gives important information about individual components in each

chapter. The **Mini-dictionary of terms** is suited for both the novice and those working in other scientific fields or areas. The section **Summary points** encapsulates the entire chapter in brief sets of simple sentences.

This book **Neurobiology and Physiology of the Endocannabinoid System** is divided into seven parts as follows:

- 1. Setting the scene and introductory chapters**
- 2. Neurobiology of the endocannabinoid system**
- 3. Receptor biology and responses**
- 4. Profiles and behavior of selective endocannabinoids**
- 5. Exogenous, synthetic other compounds linking in the endocannabinoid system**
- 6. Comparative studies on nonneurological systems**
- 7. Resources**

It has been difficult to ascribe particular chapters to different parts of the book as many chapters can be placed into two or more sections. Nevertheless, the navigation of the chapters, areas, and key aspects related to cannabis and cannabinoids is aided by the excellent index at the end of the book.

This book is designed for research and teaching purposes. It is suitable for neurologists, psychologists, psychiatrists, behavioral scientists, health scientists, healthcare workers, pharmacologists, and research scientists. The audience also includes federal and state program directors. It is valuable as a personal reference book and also for academic libraries that covers the domains of neurology, health sciences, or substance misuse. Contributions are from leading national and international experts including those from world-renowned institutions. The book is suitable for undergraduates, post-graduate, lecturers, and academic professors.

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## Chapter 1

# Distribution of the endocannabinoid system: Linking signaling and development

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>2-OG</b>	2-oleoylglycerol
<b>5HT1A</b>	serotonin 5-hydroxytryptamine 1A receptor
<b>AA</b>	arachidonic acid
<b>ABHD</b>	alpha-beta hydrolase domain protein
<b>AEA</b>	<i>N</i> -arachidonoyl ethanolamine
<b>ATP</b>	adenosine triphosphate
<b>BDNF</b>	brain derived growth factor
<b>BMP</b>	bone morphogenetic protein
<b>cAMP</b>	cyclic adenosine monophosphate
<b>CB1</b>	cannabinoid receptor subtype 1
<b>CB2</b>	cannabinoid receptor subtype 2
<b>CDON</b>	cell-adhesion molecule-related/downregulated by oncogenes
<b>CNS</b>	central nervous system
<b>CRIP1a</b>	cannabinoid receptor interacting protein 1a
<b>DAGL</b>	diacylglycerol lipase
<b>DCC</b>	deleted in colorectal cancer
<b>eCB</b>	endogenous (endo)cannabinoid
<b>ECS</b>	endocannabinoid system
<b>ERK</b>	extracellular signal-regulated kinase
<b>FAAH</b>	fatty acid amide hydrolase
<b>FGF</b>	fibroblast growth factor
<b>FGFR</b>	fibroblast growth factor receptor tyrosine kinase
<b>G<math>\alpha</math>i/o</b>	heterotrimeric G protein alpha subunits i/o
<b>GABA</b>	gamma-aminobutyric acid
<b>GABAA</b>	ionotropic GABA receptor A
<b>GPCR</b>	G-protein-coupled receptor
<b>LPI</b>	lysophosphatidylinositol
<b>LysoPtdGlc</b>	lysophosphatidylglucoside
<b>MAGL</b>	monoacylglycerol lipase
<b>MPS</b>	membrane-associated periodic skeleton
<b>NAPE-PLD</b>	<i>N</i> -acyl phosphatidylethanolamine-specific phospholipase D
<b>NGF</b>	nerve growth factor
<b>NIH3T3</b>	a fibroblast cell line that was isolated from a mouse NIH/Swiss embryo
<b>pCB</b>	plant-derived (phyto)cannabinoid
<b>Rho</b>	Ras (rat sarcoma virus) homologous
<b>ROCK</b>	Rho-associated protein kinase
<b>SGIP1</b>	SH3-containing GRB2-like protein 3-interacting protein 1

<b>Shh</b>	sonic hedgehog
<b>SMO</b>	smoothened
<b>THC</b>	Δ9-tetrahydrocannabinol
<b>Trk</b>	tropomyosin receptor kinase
<b>TRPV</b>	transient receptor potential vanilloid cation channels
<b>Wnt</b>	wingless/integrated

## Introduction

The endogenous cannabinoid signaling system (ECS) has been identified following the isolation of Δ9-tetrahydrocannabinol (THC)—the main psychoactive plant-derived cannabinoid (pCB) from the *Cannabis sativa*—and the endogenous receptors that it activates (reviewed in [Hillard, 2015](#)). The classical, and most extensively studied, roles of ECS in the central nervous system (CNS) are linked to the regulation of synaptic activity.

However, following the pioneering research ([Berghuis et al., 2007](#)) highlighting the role of ECS in axon guidance, cannabinoid signaling has been implicated in various neurodevelopmental phases, including proliferation and specification of neural and glial progenitors, differentiation of forebrain and striatal neurons, neuron migration, apoptosis, and synaptogenesis (reviewed in [Keimpema et al., 2011](#)). Setting up a conceptual framework for the field, Keimpema, Mackie, and Harkany proposed that ECS plays a key role in neurodevelopment by providing “focal instructive signals,” i.e., the precise impacts of ECS signaling on neuronal circuit assembly critically depend on the timing and the location of expression of the relevant signaling components; and that pCBs, such as THC, disrupt neural circuit development primarily through interfering with the spatial and temporal specificity of endogenous cannabinoid signaling (eCB) ([Keimpema et al., 2011](#)).

Classical neurodevelopmental signals are either secreted or cell-surface-associated proteins, which elicit specific responses only in the cells expressing appropriate receptors. Thus, their activity is defined by the time and place of their production and by their biochemical properties, allowing them to either act at a very short range (contact-mediated signaling) or diffuse, generating concentration gradients. eCBs are hydrophobic lipids, and their biochemical properties in developing tissues—such as the range of diffusion and effective concentrations for activating receptors *in vivo*—are still actively explored. In this chapter, we will review the research positioning ECS expression at the right place and time to directly influence specific phases of neurodevelopment, side by side with the research showing specific neurodevelopmental aspects disrupted by deregulation of ECS signaling. We will highlight developmental phases where the evidence supports direct instructive roles of cannabinoid signaling.

## Chemical signaling orchestrates neurodevelopment

The intricate anatomical patterning and functional compartmentalization of the nervous system are generated through stereotypical sequences of developmental phases: a script of gene expression patterns determining the ballet moves of nervous tissue morphogenesis and cellular differentiation, thus regulating the emergence of functional neuronal networks. This multitude of phases and developmental mechanisms is orchestrated and a range of chemical signals, which include expansive extracellular gradients (generated by organizing centers in early embryos), local paracrine contact-mediated signals from neighboring cells and environmental signaling guideposts, and autocrine signaling ([Dorskind & Kolodkin, 2021](#)). eCBs are among the most recent signaling families recognized to play a role in neural circuit formation. In this chapter, we will discuss the current literature on the expression and role of cannabinoid signaling during the different developmental phases. We will evaluate whether eCB signaling regulates the numbers, location, and connectivity patterns of neurons generated during development.

## Main text

### Endocannabinoid system

The ECS is composed of endogenous signaling lipids and their biosynthetic and hydrolyzing enzymes and receptors, as well as their interacting proteins.

#### Endocannabinoids

The two classical eCBs in the central nervous system (CNS) are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). 2-AG is typically expressed at much higher levels than AEA: it is found at ~100-fold higher concentrations in adult human

serum and in many regions of adult rodent CNS (Hillard, 2018). Many additional signaling lipids have been identified, which are classified as eCB-like, since they exhibit varying affinities for allosteric and orthosteric sites in eCB receptors. Some, like 2-oleoylglycerol (2-OG), are found in serum and tissue at several-fold higher concentrations than 2-AG (Hillard, 2018), but their roles in development are almost completely unexplored.

AEA and 2-AG are small lipophilic molecules, synthesized from phospholipid precursors within, and released through diffusion along and across, lipid membranes. Unlike classical signaling molecules, their bioavailability is primarily controlled through the balance of activity of biosynthetic and hydrolyzing enzymes, rather than a regulated release from intracellular vesicles. Transfer of several signaling lipids (AEA, 2-AG, lysophosphatidylinositol (LPI)) from lipid membranes to extracellular space and between cells was suggested to involve ATP-dependent transporters (Chicca et al., 2012). Other signaling lipids, such as lysophosphatidylglucoside (LysoPtdGlc), are hydrolyzed from membrane glycerophospholipids by secreted enzymes, forming extracellular matrix-associated signaling droplets, or guideposts (Guy & Kamiguchi, 2021).

As we discuss below, ongoing research is starting to identify local concentrations of eCBs and eCB-like signaling lipids in different anatomical regions at specific stages of neurodevelopment; mechanisms facilitating or limiting their diffusion; whether they act as gradients, contact-mediated paracrine signals, or in an autocrine manner; the repertoire of their cognate receptors; and the specific developmental mechanisms which they regulate.

### eCB synthesizing and hydrolyzing enzymes

A complicated ecosystem of multiple alternative biosynthetic, hydrolyzing, and oxidizing pathways for eCBs and eCB-like lipids is beyond the scope of this chapter but has been recently reviewed (Baggelaar et al., 2018; Guy & Kamiguchi, 2021; Maccarrone, 2017). Here we will focus on a few enzymes, recognized for their key roles in the synthesis and the hydrolysis of classical eCBs. *N*-arachidonoyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) plays a major role in AEA synthesis in the CNS, and fatty acid amide hydrolase (FAAH)—in the CNS AEA hydrolysis (Maccarrone, 2017).

Diacylglycerol lipase  $\alpha$  (DAGL $\alpha$ ) is a major synthesizing enzyme for 2-AG, responsible for the production of about 80% of 2-AG in the adult CNS (Bisogno et al., 2003). Its activity in mature neurons is regulated by calcium concentration, activity-dependent phosphorylation, and tight synaptic localization through interactions with postsynaptic scaffold protein HOMER (Jung et al., 2007). DAGL $\beta$  exhibits an almost identical enzymatic domain but lacks a cytoplasmic tail responsible for activity- and HOMER-dependent regulation exhibited by DAGL $\alpha$  (Blankman et al., 2008). A major enzyme hydrolyzing 2-AG in the adult CNS is monoacylglycerol lipase (MAGL) (Dinh et al., 2002). In addition,  $\alpha$ - $\beta$  hydrolase domain proteins ABHD-6 and ABHD-12 were shown to contribute to 2-AG hydrolysis (Blankman et al., 2008) and possibly also synthesis (van Esbroeck et al., 2019).

### Receptors

Both AEA and 2-AG are agonists of the canonical cannabinoid receptors—CB1 and CB2—from the G-protein-coupled receptors (GPCRs) family. eCB and pCB regulate activity of additional GPCRs, including GPCR55, GPCR119, and serotonin 5-hydroxytryptamine 1A receptor (5HT1A) (Hillard, 2015). It is important to note that G $\alpha$ i/o, a major G-protein regulated by these GPCRs, is abundantly expressed in the developing CNS and is enriched in developing neurons and axons (Bromberg et al., 2008), highlighting the importance of GPCR signaling in neurodevelopment. In addition, eCBs can act directly on some ionotropic receptors, including transient receptor potential vanilloid family (TRPV), glycine, and GABA $A$  (Hillard, 2015), which are also expressed in developing neurons and axons.

CB1 is by far the most abundant cannabinoid receptor in the adult CNS, highly expressed in restricted subtypes of inhibitory GABAergic and, at lower levels, in excitatory glutamatergic neurons (Mackie, 2005). In mature neurons, ECS components exhibit polarized localization: CB1 is localized primarily to axons and presynaptic terminals; DAGL $\alpha$ —to somatodendritic postsynaptic compartments, while MAGL is primarily presynaptic. Thus, ECS-dependent synaptic plasticity is mediated by retrograde 2-AG signaling through presynaptic CB1, and this directionality depends on the compartmentalization of signal-synthesizing and signal-receiving cellular compartments (Kreitzer & Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson & Nicoll, 2001). In a distinct example of compartmentalized paracrine ECS signaling, presynaptically synthesized AEA acts as an anterograde signal, mediating synaptic depression through postsynaptic TRPV1 receptors (Chávez et al., 2010; Grueter et al., 2010). Contrary to the well-established directionality of ECS signaling in mature neural circuits, the subcellular distribution of ECS components, directionality, and range of ECS signaling in developing neurons are very different. ECS components are expressed in a cell-type and developmental stage-specific manner during development: for example, in the developing cerebellum, DAGL $\alpha$  is highly expressed in Purkinje cells, while CB1 is expressed in axons of afferent fibers navigating toward the Purkinje cell layer, suggesting possible paracrine retrograde 2-AG

signaling in the regulation of cerebellar afferent axon development (Martinez et al., 2020). On the other hand, ECS receptors, synthesizing and hydrolyzing enzymes, were shown to co-localize in the developing axons, underlying the importance of autocrine ECS signaling during neurodevelopment (reviewed in Maccarrone et al., 2014).

ECS signaling differs in two important aspects from the classical paracrine neurotrophic or chemoattractant and chemorepulsive signaling mechanisms implicated in neurodevelopment. First, many GPCRs, including CB1, exhibit significant constitutive activity and thus can exert their neurodevelopmental effects in a ligand-independent fashion. Second, some developmental effects may be mediated by autocrine signaling mechanisms if the same cell expresses both eCBs and ECS receptors.

## Prelude to neurodevelopment: Embryo implantation and placental function

ECS system is expressed in both uterus and placenta and plays multiple functional roles in the earliest stages of reproduction, including oviductal retention of embryos, placentation, and embryo resorption rates (reviewed in Correa et al., 2016). Cannabis exposure during early pregnancy is associated with increased placental weight, reduced fetal growth rates, and altered glucose transport (Natale et al., 2020), suggesting that early disruptions in ECS signaling may have indirect widespread effects on development. Even though these effects are outside the scope of this chapter, which focuses specifically on neurodevelopment, they are important to keep in mind when interpreting the role of cannabinoid signaling in neurodevelopmental mechanisms.

## ECS and the phases of neurodevelopment

### Brain patterning and regionalization

#### *CB1 is not expressed in progenitors, but gets upregulated in differentiating neurons*

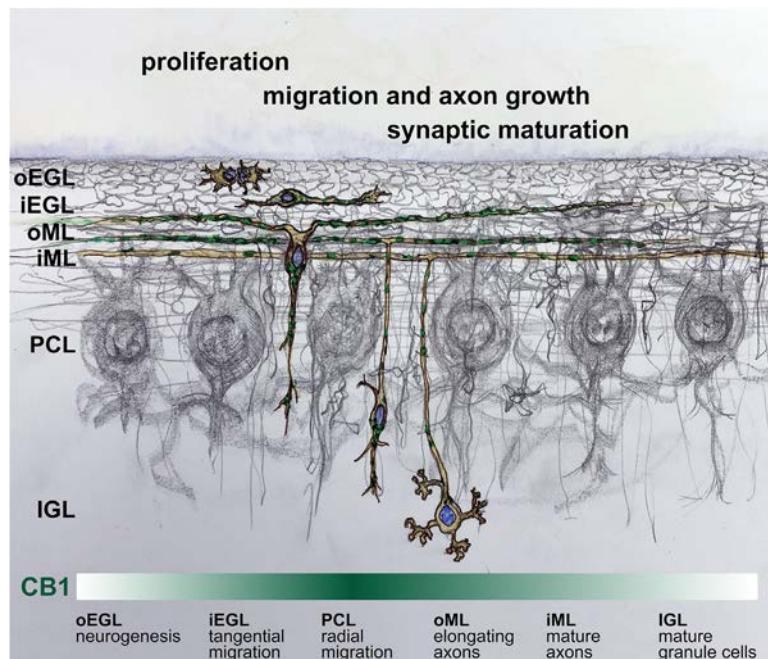
CNS morphogenesis—the emergence of the complex, regionalized, three-dimensional brain and spinal cord structures by uneven expansion, bending, and stretching of the embryonic neural tube—depends on location-specific proliferation, migration, and apoptosis of progenitors. In early embryonic CNS, two broadly defined cellular populations are involved in morphogenetic signaling: clusters of early-specified cells secreting morphogens (i.e., organizing centers), and progenitors in proliferative zones responding to these signals. Importantly, sonic hedgehog (Shh) is the main morphogen required to keep progenitor cells in the proliferating stage (Machold et al., 2003). Three additional signaling families are prominently involved in CNS morphogenesis: bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and wingless/integrated (Wnt). The four signaling systems converge on key intracellular effector cascades, acting either synergistically or antagonistically to generate a multiverse of outcomes. For example, at the midbrain/hindbrain boundary, BMP and FGF signaling synergizes to induce noradrenergic locus caeruleus, while FGF and Shh signaling synergizes to induce serotonergic raphe nucleus (Basson, 2012). Mutations or exposure to exogenous factors that interfere with morphogenetic signaling are teratogenic, i.e., result in obvious anatomical alterations.

No mutations in ECS components leading to obvious teratogenic phenotypes have been described, either in humans or in experimental animals. Similarly, embryonic exposure to physiological ranges of pCBs does not produce obvious teratogenic phenotypes.

Therefore, it is unlikely that ECS components play direct obligatory roles in morphogenetic signaling. On the other hand, more subtle yet consequential neurodevelopmental alterations are documented in ECS knockouts and following developmental pCB exposure, and some studies specifically implicate cannabinoid signaling in the regulation of progenitor proliferation and specification (Díaz-Alonso et al., 2015; Galve-Roperh et al., 2013; Xapelli et al., 2013). We will discuss here the possible mechanisms of ECS crosstalk with morphogenetic signaling programs shaping the early embryonic nervous system development.

Developmental expression of CB1 is well characterized. Throughout the brain, CB1 expression is very low/undetectable in proliferative zones but gets upregulated in some neuronal types after they exit from the cell cycle and begin differentiating (Morozov et al., 2020; Watson et al., 2008). In the embryonic forebrain, CB1 expression is high in the cortical plate in excitatory early-differentiating developmentally-transient Cajal-Retzius cells and in early pyramidal cells (Mulder et al., 2008), but absent from the proliferative domain in the ventricular zone (Lo et al., 2021). In the developing cerebellum, CB1 expression is not detected in the external granule cell layer (granule cell proliferative zone) but is high in granule cells after they exit from the proliferative zone and while their axons actively elongate (Martinez et al., 2020)—as shown in Fig. 1.

In sum, based on this expression pattern, eCB signaling through CB1 is unlikely to play a direct role in regulating progenitor proliferation. However, it could regulate progenitor proliferation indirectly, through regulation of the activity of the



**FIG. 1** CB1 expression during cerebellar granule cell development. Depiction of granule cell development is inspired by Ramón y Cajal (1995). Relative levels and distribution of CB1 expression in the layers of the developing cerebellum and compartments of differentiating granule cells are shown in green and summarized in a gradient bar at the bottom. Distinct cytology of cerebellar cell types and crystalline laminar organization of the cerebellum facilitate clear visualization of the developmental phases. Top to bottom: cerebellar granule cells proliferate in the outer external granule cell layer (oEGL)—CB1 expression is absent from oEGL; following exit from the cell cycle, granule cells initiate tangential migration in the inner external granule cell layer (iEGL)—CB1 is primarily expressed in intracellular vesicles in the soma, along the leading process, and in the core region of the growth cone; granule cells migrate radially through Purkinje cell layer (PCL—large diameter cells in the background) to settle in their target region, the inner granule cell layer (IGL)—CB1 continues to be expressed primarily in intracellular vesicles in the soma, along the leading process, and in the core region of the growth cone; granule cell axons continue to elongate parallel to the pia after the granule cell soma begins descending toward the IGL; axons of later-born granule cells are stuck on top of the earlier born axons, thus outer molecular layer (oML) contains immature actively elongating axons, while the inner molecular layer (iML) contains more mature axons—CB1 expression is several folds higher in elongating, compared with more mature axons.

early-differentiated signaling cells in the organizing centers. More research is needed to assess this possibility, as well as the expression and function in proliferating progenitor cells of other ECS receptors: CB2, TRPV1, etc.

### *CB1 acts directly as Shh signaling antagonist by competing with Shh for binding to SMO receptors*

Recent work highlights intriguing additional mechanisms of ECS crosstalk with classical morphogenetic signaling mediators. Exposure to both eCBs and pCBs can downregulate Shh signaling (Khaliullina et al., 2015). Shh signaling is transduced through a smoothened (SMO) receptor complex assembled in cell cilia. In knockout mice lacking SMO co-receptor CDON (which exhibit reduced Shh activity but no overt phenotype), embryonic THC exposure induces teratogenic defects—failed induction of ventral midline structures—typical of Shh inhibition (Lo et al., 2021). Intriguingly, THC-induced deficit in Shh signaling happens in the absence of CB1 expression in progenitor cells that express Shh receptors and does not depend on CB1 expression in the NIH3T3 cell line, suggesting that eCB and pCB regulation of Shh signaling is independent of the classical ECS signaling cascade, but acts directly as Shh signaling antagonist by competing with Shh for binding to SMO receptors (Lo et al., 2021).

In sum, CB1 expression in embryonic CNS is largely absent from proliferative zones but is upregulated in differentiating neurons. Nevertheless, CB1 knockouts exhibit moderate reductions in the size of some brain regions (Blázquez et al., 2015; Martinez et al., 2020), and in combination with genetic vulnerability impairing Shh signaling, THC exposure can be pro-teratogenic (Lo et al., 2021). These results suggest that cannabinoid signaling can interfere with morphogenetic signaling code, but cannabinoid signaling effects on proliferation and differentiation in the embryonic CNS proliferative zones are likely mediated through mechanisms that are either cannabinoid-receptors-independent (such as direct binding to Shh receptor SMO) or indirect (such as affecting the function of early-differentiated signaling cells within organizing centers).

Since Shh and other morphogenetic signaling systems continue to play important roles in later developmental phases, contributing to the regulation of neuron migration, axon growth, and synaptogenesis, the effects of crosstalk between Shh and ECS are likely to extend to later phases of neurodevelopment.

## Neuronal migration

Upon exit from the cell cycle, neurons get specified, i.e., set on a path to differentiate into a specific neuronal type. Their exact identity depends on the timing and place of their specification, migration routes that they take to get from their birthplaces to their appropriate anatomical positions, and the patterns of synaptic connectivity that they establish. Therefore, the routes and rates of migration are important not only to determine neuronal final positions but also to fine-tune their final identity.

Neuronal migration involves dynamic changes in cellular morphology: extension and elongation of the leading process, retraction of the trailing process, and nuclear translocation. These are controlled by signaling cascades converging on the regulation of cytoskeletal dynamics. In addition, interactions between migrating neurons and their environment are important for the regulation of their migration: adhesive and signaling contact-mediated interactions with substrates can either promote or inhibit migration; soluble signaling gradients can regulate direction and rate of movement.

The role of ECS in neuron migration has been primarily studied in cortical development. Global congenital CB1 knockout, as well as targeted CB1 downregulation in the developing cortex, is associated with reduced pyramidal cell migration, abnormal multipolar morphology of the migratory pyramidal neurons, mild lamination abnormalities, and increased susceptibility to seizures (Díaz-Alonso et al., 2017). However, these knockout strategies do not allow differentiating whether cannabinoid signaling regulates neuron migration directly by controlling signaling cascades in the migrating neurons, or indirectly, by regulating cellular properties of the signaling cells.

Glutamatergic pyramidal neurons migrate along radial glial fibers from their birthplace in the ventricular zone to the cortical plate, where they detach from the glial fibers and settle in the developing cortical layers. Detachment from radial glial fibers is regulated by reelin signaling from Cajal-Reitzus cells that reside in the cortical plate. CB1 expression is high in Cajal-Retzius cells (Morozov et al., 2009; Vitalis et al., 2008). However, whether CB1 activation regulates Cajal-Retzius signaling, affecting neuronal migration and cortical lamination, is unknown.

CB1 expression is upregulated in glutamatergic neurons as they exit the proliferative zone and start migrating (Díaz-Alonso et al., 2017; Morozov et al., 2020), and it is further upregulated during the subsequent axon elongation phase (Wu et al., 2010). Unlike guidance receptors, CB1 does not accumulate in growth cone filopodia on the leading edge of migrating pyramidal neurons. On the contrary, CB1 is primarily localized to intracellular vesicles abundant in the soma, especially around the centrosome (microtubule organizing center), in puncta throughout the leading process, and at the core region of the growth cone (Morozov et al., 2020). CB1 expression is also upregulated in neocortical GABAergic interneurons upon their specification and initiation of migration. Similar to glutamatergic neurons, CB1 is expressed primarily in perisomatic intracellular vesicles in migrating GABAergic neurons (Morozov et al., 2009). An effector cascade downstream from CB1 involving activation of small GTPase RhoA (which plays a key role in the regulation of cytoskeletal dynamics) was proposed to regulate the migration of pyramidal cortical neurons (Díaz-Alonso et al., 2017).

Sources of cannabinoid signaling at these early stages of telencephalon development are poorly understood. 2-AG synthesizing enzymes—DAGL $\alpha$  and DAGL $\beta$ —are not expressed in the developing cortex at this early stage (Morozov et al., 2020); and the expression of AEA biosynthetic enzymes, such as NAPE-PLD, has been difficult to verify. If there are no local cortical sources of eCBs to activate CB1 receptors on radially migrating neurons, it is possible that ligand-independent activation of CB1 plays a role or that 2-AG or AEA is produced by adjacent tissues: perhaps secreted from thalamocortical axons that begin to innervate cortical plate or produced by pial cells.

In sum, the onset of CB1 expression upon neuron specification and initiation of migration has been described in several glutamatergic and GABAergic cell types. CB1 in migrating neurons is expressed at moderate levels and is primarily localized to intracellular vesicles. Effector cascades regulating neuron migration downstream from CB1 activation may involve small GTPases or additional mechanisms that are currently unknown. Numerous questions remain open: specific cellular mechanisms regulated by CB1 signaling during neuronal migration, sources and identities of eCB signals activating CB1 receptors on migrating cortical neurons, autocrine versus paracrine mode of cannabinoid signaling, etc.

## Axon extension and guidance

Neuron polarization and guided axon growth generate the cellular substrate for selective synaptic connectivity, enabling information processing in neural circuits. Neurons polarize by extending neurites, among which, the fastest growing is

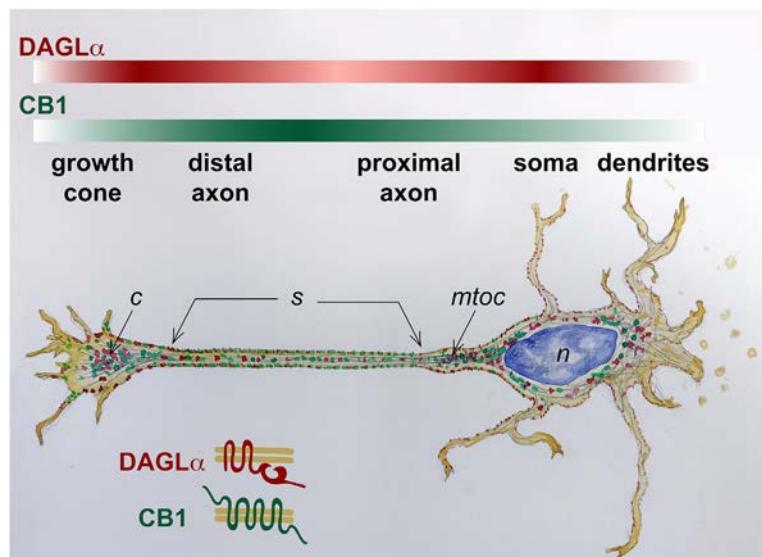
specified as an axon, molecularly and functionally distinct from the somatodendritic compartment. The axon emerges from the cell body led by the growth cone, a specialized motile compartment packed with receptors and effectors generating directional movement in response to sensing guidance cues. Thus, classical axon guidance receptors, directing the trajectory of axon growth, typically localize to the plasma membrane in filopodia and lamellipodia of growth cones (Schelski & Bradke, 2017).

Elongating axons of glutamatergic and GABAergic neurons express prominently high levels of CB1 (Berghuis et al., 2007; Martinez et al., 2020; Mulder et al., 2008; Vitalis et al., 2008; Wu et al., 2010), but in the contrast to the classical axon guidance receptors, a large pool of CB1 is localized in intracellular vesicles in core regions of growth cones, and in neuronal somata, with the most prominent surface expression of CB1 being along the axon shafts (as illustrated in Fig. 2) (Leterrier et al., 2006; Li et al., 2020), suggesting that signaling through CB1 is unlikely to directly influence growth cone chemoattraction and chemorepulsion. Yet, pharmacological and genetic disruptions of CB1 lead to axon pathfinding and fasciculation abnormalities (Díaz-Alonso et al., 2012; Martinez et al., 2020; Tortoriello et al., 2014; Wu et al., 2010), indicating an important role of CB1 in axon growth.

If not through direct control of growth cone steering, how does cannabinoid signaling influence axon elongation and guidance? Could it contribute to asymmetrical regulation of general cellular mechanisms—transport and incorporation of lipid membrane vesicles and axon-targeted proteins, trafficking of signaling receptors and adhesion molecules between surface and intracellular membranes, cytoskeletal polymerization and reorganization, local protein translation, and energy metabolism—and thus play a role in axon elongation. In the following paragraphs, we discuss recent research highlighting the role of ECS signaling in these cellular processes.

### *Bidirectional regulation of ECS and actin polymerization*

CB1 activation by eCBs or pCBs was shown to regulate cytoskeletal dynamics controlling cellular motility (Njoo et al., 2015; Roland et al., 2014; Saez et al., 2020). The key molecular targets downstream from ECS and upstream from actin rearrangements are mitogen-activated protein kinases, such as extracellular signal-regulated kinase (ERK), which can regulate the localization and activity of actin-binding proteins (Njoo et al., 2015; Roland et al., 2014; Saez et al., 2020; Zhou et al., 2019). Additionally, CB1 was shown to transactivate neurotrophin and signaling-factor receptor tyrosine kinases, such as tropomyosin receptor kinase B (TrkB) and fibroblast growth factor receptor tyrosine



**FIG. 2** Distribution of CB1 and DAGL $\alpha$  in a generalized diagram depicting axon growth in a developing neuron. Neuronal compartments are marked above the diagram. The cell nucleus (n) is blue, microtubules are black, and actin-filament-rich compartments are darker ochre. Distribution gradients of CB1 (green) and DAGL $\alpha$  (red) along the different cellular compartments are shown on top. The majority of CB1 expression is intracellular, primarily concentrated along the axon shaft (s), in the core region of the growth cone (c), and in cell soma, especially at the front pole of the nucleus around the microtubule-organizing center (mtoc). CB1 localization to the plasma membrane is most prominent in the axon shaft. DAGL $\alpha$  co-localizes with CB1 in the plasma membrane in the axon shaft. DAGL $\alpha$  expression is enriched in the distal axon and the core region of the growth cone, suggesting that localization of DAGL $\alpha$  to distal axons may generate a gradient of 2-AG axonal distribution (high distally). An additional region of higher DAGL $\alpha$  expression is in the soma.

kinase (FGFR)—synergistically and even in the absence of their endogenous ligands brain-derived growth factor (BDNF) and FGF (Asimaki et al., 2011; Berghuis et al., 2005; Zhou et al., 2019); thus, ECS signaling through CB1 can elicit neurotrophin-like effects on axon development.

Recent super-resolution microscopy studies have shown that cell-surface CB1 receptors in axon shafts localize to the membrane-associated periodic skeleton (MPS—a lattice of intervening actin and spectrin rings), which serves as a platform for organizing and clustering cell-surface signaling receptors and their intracellular effectors, including CB1 and tyrosine kinases (Li et al., 2020; Zhou et al., 2019). The introduction of CB1 receptor agonists has a biphasic effect on surface CB1 receptor localization: at lower doses and with shorter exposure—promoting localization to MPS and plasma membrane, but at higher doses and following prolonged application—promoting internalization and ERK-dependent degradation of MPS (Daigle et al., 2008; Li et al., 2020; Zhou et al., 2019). This may explain conflicting reports, where in some cases CB1 agonist application promotes (Saez et al., 2020) and in others, inhibits (Berghuis et al., 2007) axon growth. In sum, subcellular localization of CB1 regulates its availability in signaling cascades and interactions with actin cytoskeleton regulating axon growth and depends upon ligand-binding-regulated subcellular localization, rather than total levels of CB1 expression in axons.

### *Availability of eCBs, intracellular transport, and crosstalk with other signaling systems*

Since CB1 receptors and ECS synthesizing and degrading enzymes are co-expressed in axons and somata of the developing neurons, the autocrine mode of eCB signaling, with eCBs diffusing *in cis* along the plasma membrane from biosynthetic enzymes to receptors, is likely to play a prominent role in ECS regulation of axon growth (Berghuis et al., 2007; Keimpema et al., 2010; Maccarrone et al., 2014). 2-AG synthesizing enzymes DAGL $\alpha$  and DAGL $\beta$  localize to distal axonal domains through regulated intracellular transport (Davies et al., 2022; Keimpema et al., 2010; Williams et al., 2003). This mechanism was suggested to play a key role in promoting axon elongation by creating a 2-AG gradient that is highest in growth cones. The establishment of a high-distal 2-AG gradient is additionally controlled by the localization and activity of MAGL (Keimpema et al., 2010). The importance of this mechanism is highlighted by a clinical neurodevelopmental syndrome resulting from abnormal intracellular transport leading to depletion of DAGL $\beta$  in distal axons (Davies et al., 2022).

DAGL inhibition prevents FGF-mediated axon growth (Williams et al., 2003), suggesting that neurotrophin/cytokine-dependent axon elongation requires 2-AG-mediated ECS signaling. Another level of crosstalk between neurotrophin and ECS signaling systems in axon growth involves neurotrophin control of eCB availability through regulation of expression and activity of eCB synthesizing and degrading enzymes (Keimpema et al., 2013; Maison et al., 2009). In addition, signaling cascades downstream from neurotrophin and cytokine receptor activation can regulate the activity of axonal motor proteins, such as kinesin-1, which, in turn, can regulate axonal transport of ECS components, such as CB1 (Saez et al., 2020), and possibly DAGL $\alpha$  and DAGL $\beta$ . Conversely, activation of ECS signaling can affect axonal transport of classical axon guidance receptors, as was shown for deleted in colorectal cancer (DCC) (Argaw et al., 2011).

### *Noncanonical ECS signaling and extracellular lipid guideposts*

In addition to classical eCBs, lysophosphatidylglucoside (LysoPtdGlc) was shown to regulate axon guidance. In this case, environmental signaling guideposts play a key role, since LysoPtdGlc is derived from glycerophospholipid phosphatidyl- $\beta$ -D-glucoside through hydrolysis by secreted extracellular enzymes (Guy & Kamiguchi, 2021). LysoPtdGlc guideposts are deposited in the dorsomedial spinal cord by radial glia and repel nociceptive sensory afferents from entering the territory where proprioceptive afferents predominate. The receptor on the nociceptive afferent axons mediating repulsion to LysoPtdGlc is GPCR55, and nociceptive afferent axons are misrouted to the dorsomedial spinal cord in GPCR55 knockouts (Guy et al., 2015). Effector cascade involving activation of G $\alpha$ i/o, Rho, and Rho-associated kinase (ROCK) was proposed to mediate this axon repulsion (Guy & Kamiguchi, 2021).

Lysophosphatidylinositol (LPI) was also identified as an endogenous GPR55 ligand and was proposed to play a role in regulating axon growth of GPCR55-expressing retinal ganglion neurons (Cherif et al., 2015). Highlighting the functional importance of these developmental mechanisms, GPCR55 knockout mice exhibit deficits in sensorimotor coordination (Wu et al., 2013). Another noncanonical eCB receptor that is expressed in axons and growth cones and implicated in the regulation of axon growth is TRPV1. AEA is one of the endogenous ligands that can activate TRPV1 (Zygmunt et al., 1999), leading to either promotion (Chandan & Tim, 2007) or stalling (Goswami et al., 2007) of axon growth. Effector cascades regulating axon growth through TRPV1 are not well understood: TRPV1 is a cation channel, and its activation leads to local depolarization and increased cytoplasmic calcium concentration, yet some TRPV1 effects on axon growth may be independent of its activation (Goswami et al., 2007).

### *Effector cascades and future directions*

Rapid changes in cytoplasmic concentrations of calcium downstream from activation of TRPV1 and CB1 are thought to play a key role in synaptic plasticity mediated by cannabinoid signaling in mature neurons. Whether molecular effector cascades downstream from cannabinoid signaling in developing axons can also act through regulation of cytoplasmic calcium concentrations is an intriguing question, considering that changes in cytoplasmic calcium concentrations affect multiple cellular mechanisms, from activation of small GTPases upstream from cytoskeletal dynamics to regulation of lipid vesicle recycling, and profoundly affect growth cones guidance, switching growth cone responses from attraction to repulsion (Henley & Poo, 2004).

Another interesting direction of future research relates to the role of cannabinoid signaling in mediating lateral (adhesive and signaling) interactions between shafts of long-range axons running parallel to each other within axon bundles. Even though the majority of axon guidance and development research is currently focused on the regulation of growth cone dynamics, recent years brought an appreciation that lateral interactions between adjacent axon shafts are important in mediating fasciculation and distribution of axons within axon tracts, adjusting axon diameters, and, ultimately, can impact the propagation of action potentials and topographical ordering of axon terminal within their target territories (Sitko & Mason, 2016).

In addition, it will be very interesting for future research to explore the role of kinase-dependent CB1 C-terminal phosphorylation and interactions with accessory proteins ( $\beta$ -arrestin (Leo & Abood, 2021), CRIP-1a (Blume et al., 2016), SGIP1 (Hájková et al., 2016)) in the regulation of CB1 intracellular localization in the context of axon development.

In sum, in multiple neuronal populations, cannabinoid receptors and eCB synthesizing and hydrolyzing enzymes are co-expressed in developing axons, highlighting the importance of cell-intrinsic cannabinoid regulation of axon growth. CB1 is expressed at high levels in developing axons, with the most prominent localization to plasma membrane along the axon shafts. Regulation of intracellular transport of cannabinoid receptors and synthesizing enzymes by motor proteins play a key role in their polarized targeting to axonal domains, and interactions with actin cytoskeleton are regulated by ligand binding and important for plasma membrane targeting of CB1 receptors. Crosstalk between neurotrophin, cytokine, and ECB signaling systems is likely to play a prominent role in the regulation of axon growth.

## **Applications to other areas**

In this chapter, we discussed the roles of cannabinoid signaling in the prenatally developing brain, from neurogenesis up to synaptogenesis. However, the neurodevelopment does not end with the conclusion of prenatal development—the majority of synaptogenesis and synaptic refinement occur after birth, and in some brain areas, such as the prefrontal cortex and the cerebellum, a critical synaptic elaboration and pruning period coincides with adolescence (Petanjek et al., 2011). Adolescence is also the time when the expression of CB1 receptors in the forebrain surges before the levels stabilize (Heng et al., 2011).

Several studies linked deregulation of ECS signaling to abnormal synaptic pruning in the prefrontal cortex (Filbey et al., 2015; Miller et al., 2019; Rubino et al., 2015). Hence, adolescence is a particularly vulnerable developmental period when exposure to pCBs can disrupt eCB-regulated refinement of synaptic connectivity leading to emotional and cognitive dysfunction, and increased risk of severe neuropsychiatric disease later in life, including schizophrenia (Caspi et al., 2005). Thus, understanding the function of ECS in neuronal development is important for the appreciation of the risks associated with developmental exposure to pCBs and for the potential of ECS-based pharmacological tools for therapeutic approaches to combat neurodevelopmental and neurodegenerative disorders.

## **Mini-dictionary of terms**

- **Autocrine signaling:** All of the signaling machinery is contained within the cell and is sufficient to mediate signaling without inputs from the environment.
- **Cajal-Retzius cells:** Earliest differentiated but developmentally transient glutamatergic neuronal type in the forebrain, “guidepost cells” essential for correct migration and synaptogenesis in the early embryonic neocortex and hippocampus.
- **Compartmentalization** Generation of structurally, molecularly, and functionally distinct subregions.
- **Differentiation:** A process of specialization by which a cell obtains its function and phenotype.
- **Endogenous:** from within
- **Exogenous:** from outside

- **Intrinsic:** contained within
- **Ligand:** Signaling molecule with affinity for a specific receptor.
- **Morphogenesis:** Developmental increase in shape complexity of cells, organs, or whole organisms.
- **Paracrine signaling:** Short-range or contact-mediated signaling.
- **Plasma membrane:** Lipid membrane enclosing a cell.
- **Proliferation:** Cell division generating increased cell numbers and tissue volume; only progenitor cells can proliferate.
- **Polarization:** Generation of structurally, molecularly, and functionally distinct sides (poles)
- **Signaling gradient:** A diffusible signal present at the highest concentration next to the source: the concentration is gradually decreasing away from the source.
- **Synaptogenesis:** Formation and maturation of synaptic junctions between neurons.

## Key facts of ECS impact on neurodevelopment

- ECS signaling does not directly regulate neuron progenitors' proliferation or specification. Classical eCB synthesizing enzymes are not expressed in proliferative zones, and neuron progenitors do not express classical ECS receptors. Unlike deregulation of morphogenetic signaling, mutations or pharmacological disruptions of ECS during early embryonic stages do not lead to dramatic changes in brain size or regionalization. However, ECS signaling can regulate neurogenesis and neuron specification through crosstalk with classical morphogenetic signaling systems. eCBs (and structurally similar pCBs) directly bind to and antagonize Shh receptors, affecting neuronal stem cell proliferation independently of ECS receptors.  
Additional, yet unexplored mechanisms through which ECS signaling can influence neurogenesis indirectly are through regulation of activity of early-differentiated cells that secrete morphogenetic signals (developmentally transient clusters of cells within organizing centers).
- Cajal-Retzius neurons are developmentally transient guidepost cells that express high levels of CB1. Cajal-Retzius cells are the earliest glutamatergic neurons to be specified in the developing forebrain. They are essential for regulating neuron migration and axon guidance in the cerebral cortex and hippocampus, signaling for the developing neurons where to stop migrating and settle, and designating synaptic target regions, where afferent axons stop advancing, stall, and begin elaborating synapses, even before their appropriate target neurons are differentiated and available for synaptogenesis (Del Río et al., 1997). Thus, Cajal-Retzius cells direct the formation of cortical layers and guide afferent and target neurons to form synapses in their appropriate locations, even when the timing of their differentiation is mismatched. Whether and how ECS signaling affects developmental functions of Cajal-Retzius cells has not been explored.
- Endocannabinoids are signaling lipids, and, as such, exhibit properties distinct from the classical hydrophilic signaling molecules that diffuse through the aqueous extracellular environment to form chemoattractive or chemorepulsive signaling gradients, directing the turning of the navigating axonal growth cones. In contrast, for signaling lipids, two major modes influence the direction and rate of axon growth: gradient of signaling lipid diffusing along the plasma membrane (regulated by the local activity of synthesizing and degrading enzymes) and contact-mediated repulsion from signaling guideposts (lipid droplets anchored to extracellular matrix). Thus, localization of ECS receptors and synthesizing enzymes within developing axons is the key mechanism of intrinsic and cell-autonomous promotion of axon growth.

## Summary points

- CB1 expression is not detected in proliferative zones in the embryonic forebrain and cerebellum but is upregulated in differentiating neurons, especially during axon elongation and guidance.
- In migrating neurons, CB1 is mainly localized in intracellular vesicles in neuronal somata, not in the plasma membrane, and not in growth cone filopodia.
- In elongating axons, the majority of CB1 surface expression is along the axon shafts, CB1 in growth cones is primarily localized in intracellular vesicles in the core regions.
- CB1 knockouts and mice embryonically exposed to pCBs exhibit subtle reductions in the size and differences in foliation and lamination of cerebral and cerebellar cortices.
- Disruptions in ECS signaling cause abnormalities in axon growth and fasciculation.
- Co-localization of ECS receptors and synthesizing enzymes within developing axons regulate axon growth.
- Intracellular transport and association with actin scaffolds are important in the regulation of ECS signaling in axon growth.

- Noncanonical ECS ligands (LysoPtdGlc, LPI) and receptors (GPR55, TRPV1) are involved in the regulation of axon growth.
- Bidirectional crosstalk between effector cascades downstream of neurotrophin, cytokine and ECS signaling plays important roles in multiple neurodevelopmental phases.

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## Chapter 2

# The endocannabinoid system, immunomodulation, and LPS-induced inflammation

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## Abbreviations

<b>2-AG</b>	2-arachidonoyl glycerol
<b>AEA</b>	<i>N</i> -arachidonoyl ethanolamine, anandamide
<b>BALF</b>	bronchoalveolar lavage fluid
<b>CB1</b>	cannabinoid receptor 1
<b>CB2</b>	cannabinoid receptor 2
<b>CBC</b>	cannabichromene
<b>CBD</b>	cannabidiol
<b>CBDA</b>	cannabidiolic acid
<b>CBDV</b>	cannabidivarin
<b>CBG</b>	cannabigerol
<b>CCL</b>	C-C Motif chemokine ligand
<b>CXCL</b>	C-X-C Motif chemokine ligand
<b>ERK1/2</b>	extracellular signal-regulated kinase 1/2
<b>FAAH</b>	fatty acid amide hydrolase
<b>GPCR</b>	G-protein coupled receptor
<b>IL</b>	interleukin
<b>LPS</b>	lipopolysaccharide, endotoxin
<b>MAGL</b>	monoacylglycerol lipase
<b>MAPK</b>	mitogen-activated protein kinase
<b>MOG</b>	myelin oligodendrocyte glycoprotein
<b>MPO</b>	myeloperoxidase
<b>NF-κB</b>	nuclear factor kappa B
<b>NO</b>	nitric oxide
<b>PAI-1</b>	plasminogen activator inhibitor-1
<b>PBMC</b>	peripheral blood mononuclear cells
<b>PPARs</b>	peroxisome-proliferator-activation receptors
<b>PMA</b>	phorbol myristate acetate
<b>ROS</b>	reactive oxygen species
<b>Δ9-THC</b>	Δ9-tetrahydrocannabinol
<b>THCA</b>	tetrahydrocannabinolic acid
<b>THCV</b>	tetrahydrocannabivarin
<b>TLR</b>	toll-like receptor
<b>TNFα</b>	tumor necrosis factor α
<b>TRP</b>	transient receptor potential
<b>TRPV1</b>	transient receptor potential vanilloid 1

## Introduction

*Cannabis* has been cultivated since ancient times and used for medicinal purposes including for the treatment of symptoms associated with inflammation, such as rheumatism, swelling, and pain. During the *Yersinia pestis*, 1830s epidemic in Egypt, treatment of infection survivors with hashish was observed to be associated with decreased fever, bronchitis, and pain (Crocq, 2020). Studies over the last half a century have demonstrated substantial immune effects of cannabinoids. In 1974, a human study demonstrated impaired cell-mediated immunity in chronic marijuana smokers (Nahas et al., 1974). This chapter describes the immune modulating effects of cannabinoids and the endocannabinoid system in acute inflammation and endotoxemia. There is a brief overview of the endocannabinoid system followed by descriptions of cannabinoid receptor expression by immune and endothelial cells, effects of cannabinoids in vitro on cellular inflammation and in vivo on inflammation, the effects of acute inflammation and endotoxemia on endogenous production of cannabinoids and will touch on some of the antiinflammatory mechanisms of cannabinoids.

## Overview of the endocannabinoid system

The endocannabinoid system is distributed throughout the body and has roles in the nervous system as well as in the vascular and immune systems. It is composed of endocannabinoids and endovanilloids, the enzymes involved in their synthesis and metabolism, and multiple classes of cannabinoid receptors, including G-protein-coupled receptors (GPCRs), members of the transient receptor potential (TRP) family, and peroxisome-proliferator-activation receptors (PPARs). Arachidonic acid acts as a precursor for numerous endocannabinoids, including the acyl-dopamines *N*-oleoyl dopamine (OLDA) and *N*-arachidonoyl dopamine (NADA) (Hu et al., 2009). Other endocannabinoids, including *N*-arachidonoyl ethanolamine (AEA, anandamide) and 2-arachidonoyl glycerol (2-AG), are derived from *N*-acylphosphatidyl ethanolamine and arachidonate-containing diacylglycerols. There are multiple enzymes involved in the metabolism of cannabinoids, including fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), cyclooxygenases, lipoxygenases, and catechol-*O*-methyltransferase (Hu et al., 2009; Urquhart et al., 2015).

## Expression of CB1, CB2, and other putative GPCR cannabinoid receptors by leukocytes and endothelial cells

Endocannabinoids and *Cannabis*-derived cannabinoids (phytocannabinoids) bind with varying specificity and biological activity to multiple GPCR cannabinoid receptors (CBs), including CB1 and CB2, as well as other GPCRs, including GPR18, GPR55, and GPR119,  $\alpha_2$ -adrenergic receptors, and serotonin receptors (de Luca et al., 2018; Pertwee et al., 2010). CB1, CB2, and other GPCRs are expressed in many organs and by diverse cells, including neurons, astrocytes, microglia, circulating and tissue leukocytes, endothelial cells, and smooth muscle cells (Pertwee et al., 2010; Simard et al., 2022; Stanley et al., 2016; Wilhelmsen et al., 2014). CB1 is highly expressed in the CNS on presynaptic nerve terminals and axons of GABAergic and Glutaminergic interneurons and in sensory neurons of the DRG. Low levels of CB1 expression have been reported outside of the nervous system, in the bone marrow, thymus, and tonsils. In contrast, CB2 expression is low in the brain, but is robustly expressed in some peripheral immune tissues, including the spleen and tonsils. Furthermore, although CB2 is more highly expressed by leukocytes than CB1, multiple leukocyte populations have been reported to express both CB1 and CB2, including basophils, T cells, B cells, dendritic cells, eosinophils, monocytes, macrophages, NK cells, and microglia (Simard et al., 2022). The expression of microglial CB2 is dynamically modulated under different inflammatory conditions (Lopez et al., 2018).

Alternative GPCRs for endocannabinoids are also expressed in immune tissues as well as by leukocyte and endothelial cell populations. For example, GPR18 expression has been detected at high levels in multiple immune cell subsets, including macrophages, lymphocytes, and neutrophils (Takenouchi et al., 2012; Zhang et al., 2019). GPR55 is expressed in the spleen and by neutrophils, lymphocytes, mast cells, monocytes, and NK cells (Chiurchiu et al., 2015). Vascular endothelial cells express CB1, CB2, GPR18, and GPR55 (Wilhelmsen et al., 2014).

## Expression of TRPs and PPARs by leukocytes and endothelial cells

In addition to acting on GPCRs, cannabinoids act on multiple TRPs, including TRPV1-4, TRPA1, and TRPM8 (Pertwee et al., 2010). TRPs are nonselective cation channels that are expressed throughout the body in multiple cell types and play roles in sensing numerous stimuli, including temperature, pain, mechanical stimuli, light, smell, taste, and lipids. TRPV1 is the most extensively studied of the TRPs. TRPV1 is activated by capsaicin, heat, low pH, and endogenous lipids, including

the endocannabinoids AEA and *N*-arachidonoyl dopamine (NADA) (Caterina et al., 1997; Fenwick et al., 2017; Huang et al., 2002). TRPV1 is highly expressed in afferent sensory neurons of the trigeminal nerve, dorsal root ganglia (DRG), and vagus nerve (Helliwell et al., 1998). TRPV1 expression has also been reported in the spinal cord (Roberts et al., 2004) and in areas of adult mouse brains (Cavanaugh et al., 2011). Nonneuronal cells also express TRPV1, including glial cells (Talbot et al., 2012), peripheral leukocytes (Bertin et al., 2014; Omari et al., 2017), endothelial cells (Wilhelmsen et al., 2014), and smooth muscle cells (Cavanaugh et al., 2011).

The peroxisome-proliferator-activated receptors (PPARs), a family of nuclear receptors that are activated by a variety of lipids, including some cannabinoids, regulate the expression of multiple genes, including those involved in metabolism, cellular differentiation, adipogenesis, and immunity (Iannotti & Vitale, 2021). PPARs regulate the expression of several immune-related proteins, including AMPK, SIRT, and MAP kinases (Iannotti & Vitale, 2021). They are widely distributed throughout the body in peripheral organs, adipose tissue, skeletal muscle, nervous system, the vascular and immune systems, and in multiple cells, including neurons, microglia, astrocytes, adipocytes, endothelial cells, and leukocytes (Iannotti & Vitale, 2021).

## Modulation of endocannabinoids by inflammation and endotoxin

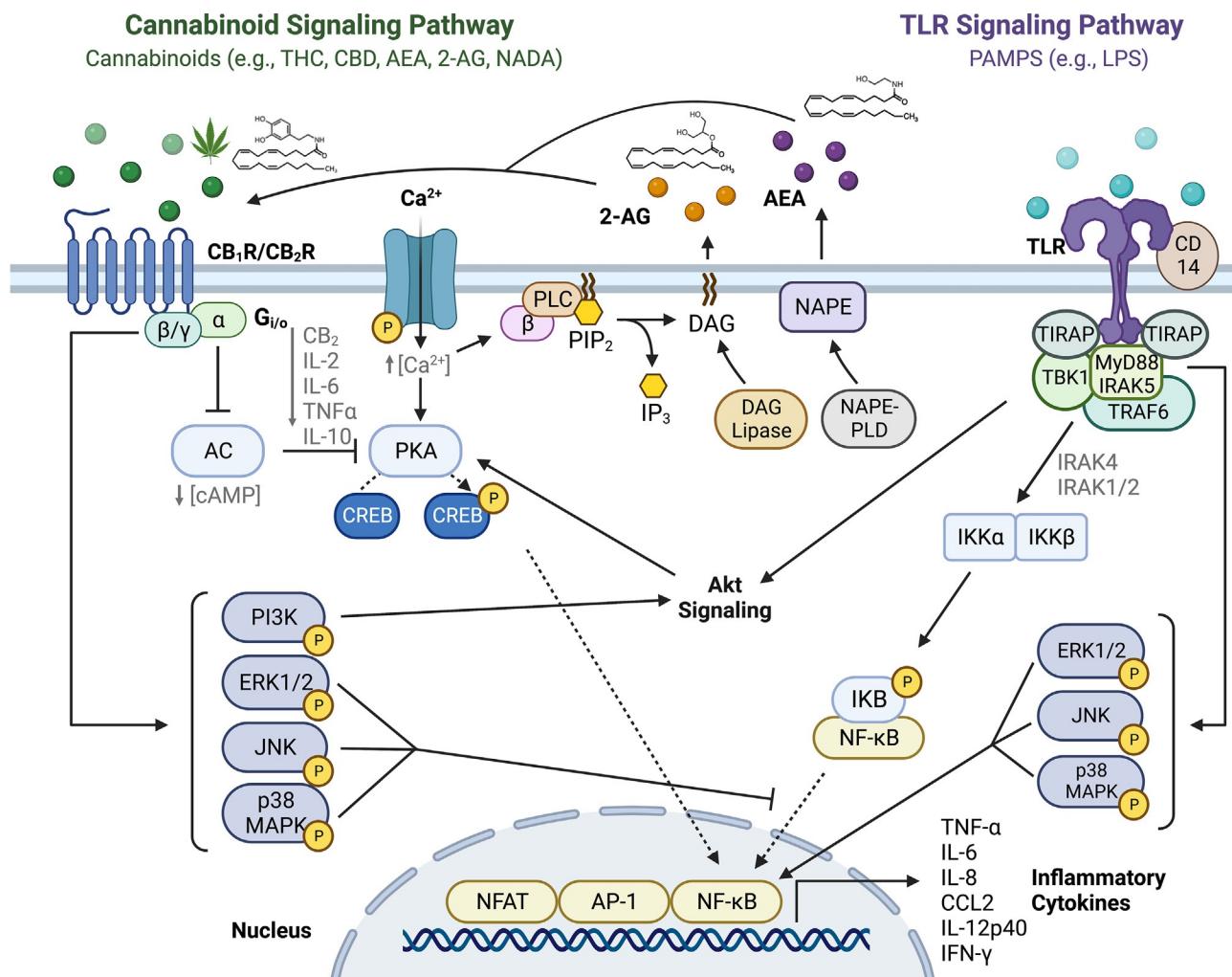
Endocannabinoids are produced at baseline and in response to inflammatory mediators and physiologic changes. Examples of conditions known to modulate endocannabinoid production include endotoxemia, rheumatoid arthritis, inflammatory pain, atherosclerosis, multiple sclerosis, and cerebral ischemia (Hillard, 2018; Wang et al., 2001). Although the specifics of the cellular and tissue sources of endocannabinoids and of the modulation of the various receptors have yet to be fully elucidated, the available data suggest that numerous immune and nonimmune tissues and cells produce endocannabinoids. For example, in vitro work demonstrated that treatment with LPS inhibits FAAH and upregulates AEA secretion by rodent macrophages (Liu et al., 2003). Challenge with LPS was reported to upregulate the secretion of 2-AG but not AEA by rat platelets, without changing FAAH expression (Varga et al., 1998). Activation of cultured T cell, B cells, and mast cells led to the upregulation of their secretion of 2-AG (Espinosa-Riquer et al., 2019; Sido et al., 2016). Nonimmune cells, such as hepatocytes, endothelial cells, and adipocytes, also produce endocannabinoids in response to inflammatory stimuli. For example, treatment of human vascular smooth muscle cells with inflammatory agonists such as IL-1 $\beta$  and TNF $\alpha$  was reported to induce the release of several endocannabinoids (Pfluger-Muller et al., 2020). This raises the possibility that nonimmune tissues may produce endocannabinoids in the local inflammatory environment to regulate inflammation and promote immune homeostasis.

## Modulation of cannabinoid receptor expression by inflammation and endotoxin

Cannabinoid receptors are also dynamically regulated during inflammation in a lineage-, time-, and agonist-dependent manner. For example, intravenous LPS challenge led to the upregulated expression of CB2 in the forebrain of endotoxemic rats beginning at  $T=6$  h and peaking at 24 h (Mukhopadhyay et al., 2006). LPS treatment was also reported to decrease microglial CB2 expression (Carlisle et al., 2002). CB2 mRNA abundance was decreased below baseline in LPS-treated splenic cultures but was increased above that of resident macrophages in Thioglycolate elicited peritoneal macrophages (Lee et al., 2001). Treatment with Freund's adjuvant, which is composed of inactivated mycobacteria, led to increased CB1 expression by DRG glial cells (Laprairie et al., 2012). Exposure to various pro-inflammatory cytokines, including IL-6, TNF- $\alpha$ , IFN- $\gamma$ , IL-4, and IL-1 $\beta$ , upregulated the expression of CB1 and CB2 by human cells, including peripheral blood mononuclear cells (PBMCs), T cells, and microglia (Jean-Gilles et al., 2015). We speculate that the dynamic regulation of cannabinoid receptor expression enables homeostatic antiinflammatory functions of the endocannabinoid system by increasing the number of available receptors to respond to endocannabinoids released during inflammation. Cannabinoid receptor expression is also modulated by exposure to cannabinoids. For example, administration of  $\Delta$ 9-THC reportedly upregulates production of TGF- $\beta$ , which in turn downregulates CB2 immunoreactivity in peripheral tissues (Gardner et al., 2002).

## Links between signaling pathways engaged by cannabinoids and inflammatory agonists (Fig. 1)

As outlined in Fig. 1, the intracellular signaling pathways engaged by cannabinoids and by LPS and other inflammatory agonists have several points of convergence. Both systems signal through several mitogen-activated protein kinases (MAPKs) and NF- $\kappa$ B. The innate immune receptors, including the Toll-like receptors (TLRs), activate NF- $\kappa$ B and several



**FIG. 1** Links between signaling pathways engaged by cannabinoids and inflammatory agonists. The intracellular signaling pathways engaged by signaling through innate immune receptors and cannabinoid receptors both converge upon several MAPks and NF- $\kappa$ B. The activation of innate immune receptors, including the TLRs, induces the activation of NF- $\kappa$ B, ERK1/2, p38 MAPK, and JNK, leading to the downstream expression and secretion of pro-inflammatory mediators, including cytokines and chemokines. CBR activation decreases PKA, which reduces CREB phosphorylation and activation, thereby reducing NF- $\kappa$ B activation and downstream pro-inflammatory cytokine secretion. CBRs also activate PI-3K as well as ERK1/2, p38 MAPK, and JNK (Howlett, 2005). (Created with BioRender.com.)

MAPks, including the extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, and Jun-N terminal kinase (JNK). In this context, MAPks promote the inflammatory response. Their activation leads to upregulated production of pro-inflammatory cytokines and chemokines (Arthur & Ley, 2013). The activation of CB1 or CB2 leads to decreased intracellular cAMP, diminished phosphokinase (PKA) activation, and lack of phosphorylation and inactivation of cAMP-response element binding protein (CREB), which results in reduced activation of NF- $\kappa$ B, which subsequently limits pro-inflammatory cytokine secretion (Howlett, 1985; Wen et al., 2010). Cannabinoid receptors also activate phosphatidylinositol-3 kinase (PI-3K) as well as ERK1/2, p38 MAPK, and JNK (Howlett, 2005). Cannabinoid activation of these signaling pathways leads to an antiinflammatory profile and downregulates the activation innate immune signaling pathways through incompletely defined mechanisms.

## In vitro effects of endocannabinoids on activation of immune and endothelial cells (Table 1; Fig. 2)

**2-Arachidonoyl glycerol:** 2-AG, an agonist of CB1 and CB2, has been shown to downregulate inflammatory activation of mouse peritoneal and bone-marrow-derived macrophages, mast cells, microglia, human PBMC, T-cells, and glial cells

**TABLE 1** In vitro effects of endocannabinoids on activation of immune and endothelial cells.

Endocannabinoid	Cellular model	Origin of cells	Inflammatory context	Effects on inflammation	Reference(s)
2-AG	Thioglycollate-elicited peritoneal macrophages	Mouse	LPS	↓ TNFalpha	Gallily et al. (2000)
	Peritoneal and bone marrow derived macrophages	Mouse	LPS	↓ PGE2, TXB2, IL-1beta, IL-1alpha, CCL2, IL-6	Habib et al. (2019)
	J774 Macrophages	Mouse	LPS	↓ IL-6; ↑ NO	Chang et al. (2001)
	Bone marrow-derived mast cells	Mouse	LPS	↓ IKK, p65NF-kappaB Phosphorylation; ↓ TNFalpha	Espinosa-Riquer et al. (2019)
AEA	Peripheral blood mononuclear cells	Human	LPS	↓ TNFalpha, IL-8, IL-6	Berdyshev et al. (1997)
	J774 Macrophages	Mouse	LPS	↓ IL-6, NO	Chang et al. (2001)
	Primary T cells	Human	PMA/Ionomycin	↓ TNFalpha, IFN-gamma, IL-17	Cencioni et al. (2010)
	Primary T cells	Human	Anti-CD3, anti-CD28 antibody	↓ IL-2	Cencioni et al. (2010)
	Primary microglia	Mouse	LPS/IFN-gamma	↑ IL-10	Correa et al. (2010)
	Primary microglia	Mouse	LPS	↑ PGE2, PGD2, 8-iso-PGF2a	Navarrete et al. (2009)
	Primary astrocytes	Mouse	LPS	↑ PGE	Navarrete et al. (2009)
	Aortic endothelial cells	Human	N/A	Vasorelaxation	Stanley et al. (2016)
NADA	Primary bone marrow cells; thioglycollate-elicited peritoneal	Mouse	LPS, bacterial lipopeptide <sup>a</sup>	↓ IL-6	Lawton et al. (2017)
	Jurkat T-cells	Human	Staphylococcal enterotoxin B	↓ T cell proliferation, transcriptional activity of TNFalpha, IL-2	Sancho et al. (2004)
	Primary microglia	Mouse	LPS, bacterial lipopeptide <sup>a</sup>	↓ IL-7	Lawton et al. (2017)
	Primary microglia	Mouse	LPS	↓ PGE2, 8-iso-PGF2a	Navarrete et al. (2009)
	BV-2 microglia	Rat	LPS	↑ IL-10	Arnold et al. (2021)

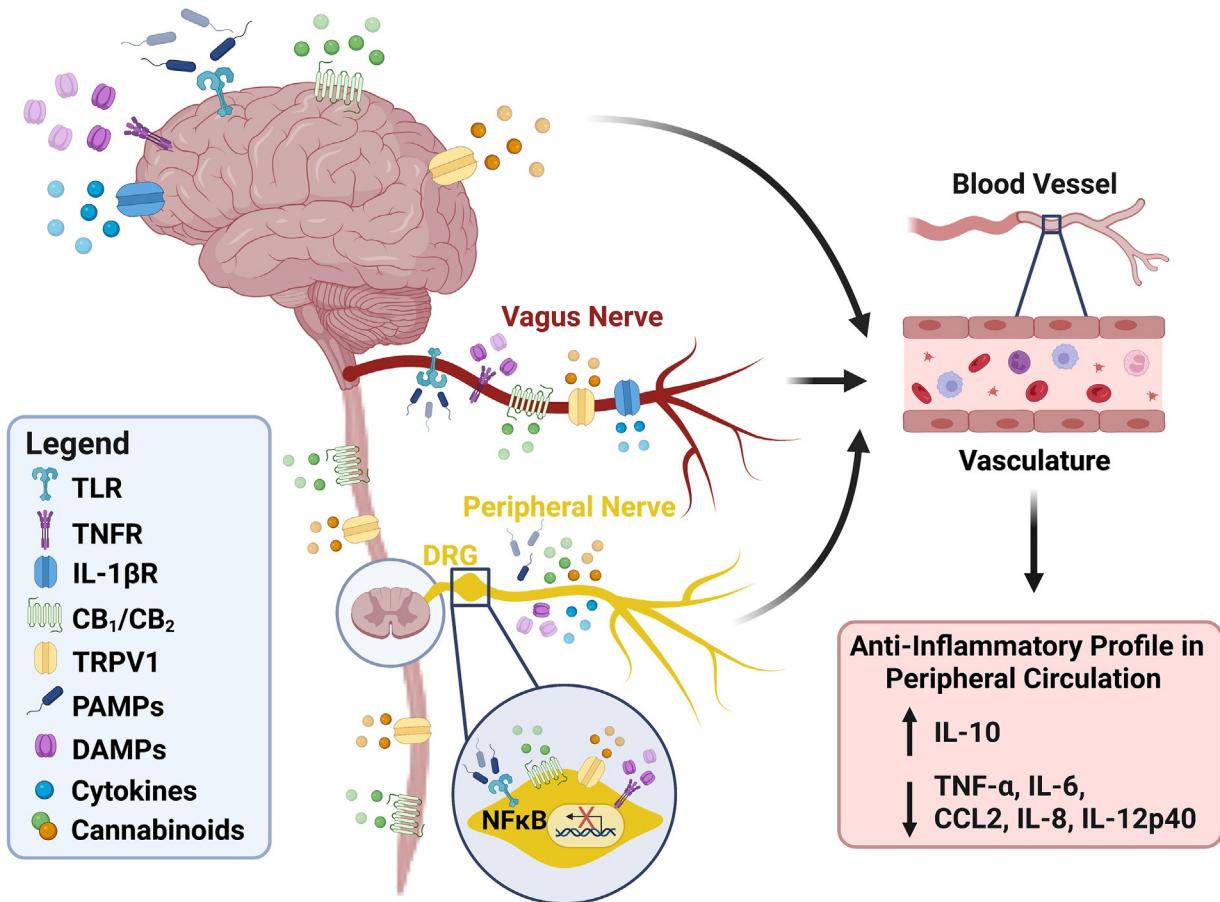
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**TABLE 1** In vitro effects of endocannabinoids on activation of immune and endothelial cells—cont'd

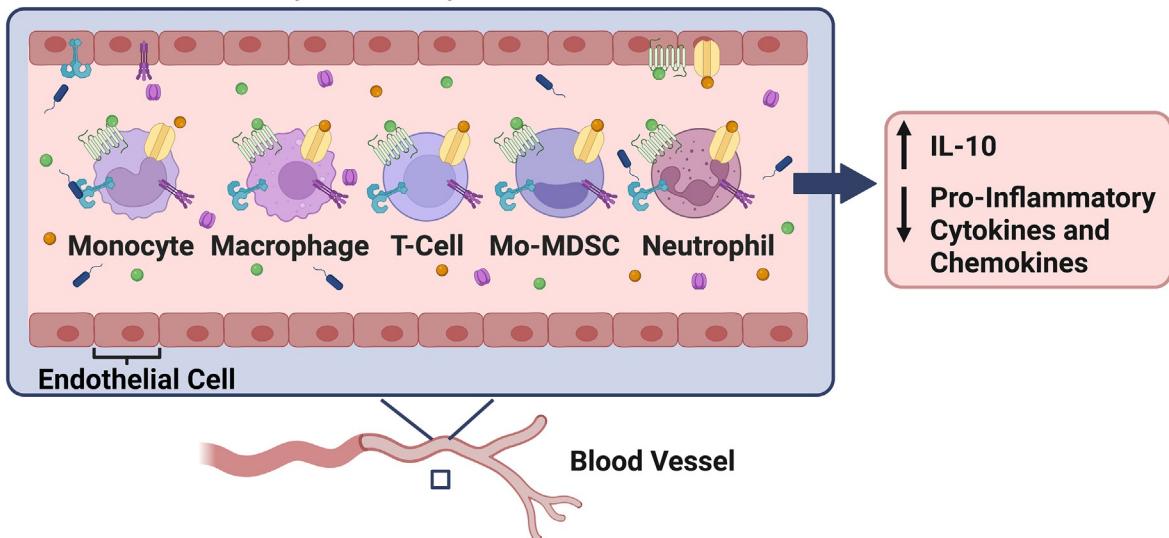
Endocannabinoid	Cellular model	Origin of cells	Inflammatory context	Effects on inflammation	Reference(s)
Anandamide	Astrocytes	Mouse	LPS	↓ PGE2	Navarrete et al. (2009)
	Lung microvascular endothelial cells	Human	LPS, FSL-1, TNFalpha	↓ IL-6, IL-8; ↑ [Ca2+]i	Wilhelmsen et al. (2014)
	Lung microvascular endothelial cells	Mouse	LPS, FSL-1, TNFalpha	↓ IL-6, IL-8; ↑ [Ca2+]i	Wilhelmsen et al. (2014)
	Lung microvascular endothelial cells	Mouse	LPS, FSL-1, TNFalpha	↓ IL-6, IL-8; ↑ [Ca2+]i	Lawton et al. (2017)
	b.end5 blood brain barrier endothelial cells	Mouse	LPS	↑ PGD2, COX-2; ↓ PGE2	Navarrete et al. (2010)
OLDA	Jurkat T-cells	Human	Staphylococcal enterotoxin B	↓ T cell proliferation	Sancho et al. (2004)

<sup>a</sup>Bacterial lipopeptide—TLR2 agonist.

**(a) Cannabinoids Modulate Systemic Inflammation by Acting on Nervous System Cells**



**(b) Cannabinoids Act Directly on Leukocytes and Endothelial Cells to Reduce Inflammation**



**FIG. 2** Potential sites of action responsible for the systemic antiinflammatory effects of cannabinoids. (A) Potential nervous system sites of action responsible for the systemic antiinflammatory effects of cannabinoids: The receptors for the cannabinoids, including CBRs and TRPs, are expressed by neurons and microglia of the brain, spinal cord, and peripheral nervous system, including the DRG and vagus nerve. Endocannabinoids are also produced in these CNS and PNS locations. We speculate that during systemic inflammation induced by infection or injury, cannabinoid-dependent activation of receptors in the nervous system leads to an antiinflammatory cytokine profile in the systemic circulation characterized by upregulated IL-10 and downregulated pro-inflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$ , and CCL2. The descending neuro-immune pathway connecting nervous system activation of cannabinoid receptors to the alterations in cytokines and chemokines in the peripheral circulation has yet to be defined. (B) Potential peripheral non-neuronal sites of action responsible for the antiinflammatory effects of cannabinoids: Outside of the nervous system, cannabinoids can act on various leukocyte populations as well as on vascular endothelial cells to reduce their production of pro-inflammatory cytokines and for some leukocytes to upregulate IL-10 production. (Created with BioRender.com.)

(Espinosa-Riquer et al., 2019; Gallily et al., 2000; Habib et al., 2019). For example, 2-AG inhibits LPS-induced TNF $\alpha$  secretion by peritoneal macrophages (Gallily et al., 2000) and reduces LPS-induced NF- $\kappa$ B activation and TNF $\alpha$  secretion by mast cells (Espinosa-Riquer et al., 2019). Peritoneal macrophages from mice with myeloid-specific knockdown of MAGL, which leads to elevated 2-AG levels, had reduced LPS-induced secretion of multiple pro-inflammatory mediators including IL-1 $\beta$ , IL-1 $\alpha$ , CCL2, IL-6, and PGE-2 (Habib et al., 2019).

**N-arachidonoyl ethanolamine:** AEA is a partial CB1/CB2 agonist with fourfold greater selectivity for CB1 (Pertwee et al., 2010) and low-affinity for TRPV1 (Fenwick et al., 2017). AEA reduces inflammatory activation of mouse microglia and macrophage cell lines, as well as human primary PBMC, T cells, and endothelial cells. In most of these cells, AEA reduces LPS-induced production of pro-inflammatory mediators, including TNF $\alpha$ , IL-6, IL-8, IFN- $\gamma$ , and nitric oxide. Interestingly, AEA has been found to enhance IL-10 production by LPS/IFN- $\gamma$ -activated microglia (Correa et al., 2010). AEA also reduces LPS-induced secretion of TNF $\alpha$ , IL-8, and IL-6 by human PBMC (Berdyshov et al., 1997) and IL-17 by activated human primary T cells (Cencioni et al., 2010). Finally, AEA has been found to increase phosphorylation of ERK1/2, JNK, NF- $\kappa$ B, AKT, STAT3, and STAT5 in human aortic endothelial cells and induce nitric oxide (NO)-dependent vasorelaxation of human mesenteric arteries (Stanley et al., 2016).

**N-acyl dopamines:** NADA is a potent TRPV1 agonist and a putative CB1 agonist and thus is considered an endocannabinoid and endovanilloid (Pertwee et al., 2010). NADA reduces LPS-induced pro-inflammatory cytokine secretion and reactive oxygen species (ROS) production by primary mouse macrophages, PBMC, microglia and upregulates IL-10 secretion by BV2 microglia (Arnold et al., 2021; Lawton et al., 2017; Navarrete et al., 2009). In Jurkat T cells, NADA and OLDA reduced IL-2 secretion and TNF $\alpha$  mRNA expression. NADA also blocked DNA binding and transcription activity of NF-AT and AP-1 downstream of JNK activation (Sancho et al., 2004). Additionally, NADA has been found to reduce activation of endothelial cells by LPS and other inflammatory agonists. NADA reduces the expression of pro-inflammatory mediators by and binding of nonactivated neutrophils to human lung microvascular endothelial cells activated with LPS (TLR4 agonist), FSL-1 (TLR2 agonist), or TNF $\alpha$  (Wilhelmsen et al., 2014). These effects of NADA on endothelial activation are hypothesized to be mediated through CB1 and CB2, and this is supported as the TRPV1 antagonist AMG9810 enhanced the antiinflammatory effects of NADA on LPS-induced endothelial activation (Wilhelmsen et al., 2014).

## In vitro effects of phytocannabinoids on activation of immune and endothelial cells (Table 2; Fig. 2)

**$\Delta 9$ -Tetrahydrocannabinol:**  $\Delta 9$ -THC, a CB1 and CB2 agonist, has antiinflammatory effects on leukocyte populations, including macrophages, T cells, and microglia (Chang et al., 2001; Kozela et al., 2010; Puffenbarger et al., 2000; Yuan et al., 2002). THC has been reported to inhibit LPS-induced production of IL-6 and nitric oxide (NO) levels, as well as COX-2 activity by J774 macrophages (Chang et al., 2001).  $\Delta 9$ -THC exposure reduces LPS-induced upregulation of microglial IL-6, IL-1 $\beta$ , IL-1 $\alpha$ , and TNF $\alpha$  mRNA expression (Kozela et al., 2010).  $\Delta 9$ -THC pretreatment was reported to decrease production of IFN- $\gamma$  and modify expression of Th1 and Th2 cytokines, reduce proliferation of ant9-CD3/CD28-activated primary human peripheral blood T-cells (Yuan et al., 2002), and reduce NF- $\kappa$ B activation in Jurkat T cells (Borner et al., 2009).

**Cannabidiol:** CBD has minimal binding affinity for CB1 and CB2, but may act as an antagonist at those receptors in the presence of  $\Delta 9$ -THC or as a negative allosteric modulator of CB1, thus reducing the potency of other cannabinoids (Vuckovic et al., 2018). CBD also affects the activity of TRPV1, TRPM8, TRPA1, 5-HT1A, adenosineA<sub>2A</sub>, and PPARs (Morales et al., 2017). Studies have demonstrated varied effects of CBD on inflammatory activation of different cell populations and even differences in effects on different inflammatory outcomes, sometimes showing the upregulation of some, and the downregulation of other inflammatory mediators. For example, in one study, CBD treatment reduced CCL2 and CCL5, but increased multiple pro-inflammatory mediators (e.g., IL-8, IL-16, IL-32, and MIF) by LPS-activated U937 monocytes (Muthumalage & Rahman, 2019). Similar mixed effects profiles were observed with CBD treatment of LPS-activated RAW264.7 macrophages, and CBD was also found to increase LPS-induced IL-8 production by BEAS-2B lung epithelial cells (Muthumalage & Rahman, 2019). In contrast, CBD decreased IL-1 $\beta$  and IL-6 production by LPS-activated BV-2 microglia and decreased IL-10 secretion by macrophages (Kozela et al., 2010; Yeisley et al., 2021). Furthermore, CBD upregulated activation of STAT3 transcription factor, which has a role in the induction of anti-inflammatory events, but decreased LPS-induced activation of STAT1, which is crucial to IFN- $\beta$ -dependent pro-inflammatory signaling (Kozela et al., 2010). CBD has been reported to inhibit proliferation T cells stimulated with phorbol myristate acetate (PMA)/Ionomycin (Dhital et al., 2017).

**TABLE 2** In vitro effects of phytocannabinoids on activation of immune and endothelial cells.

Phytocannabinoid	Cellular model	Origin of cells	Inflammatory context	Effects on inflammation	Reference(s)
THC	Raw 264.7 macrophages	Mouse	LPS	↓ TNFalpha	Fischer-Stenger et al. (1993)
	J774 macrophages	Mouse	LPS	↓ IL-6, NO, COX-2 activity	Chang et al. (2001)
	Peripheral blood T cells	Human	Allogenic dendritic cells, anti-CD3/CD28	↓ IFN-gamma, proliferation, Th1 cytokines<Th2 cytokine mRNA	Yuan et al. (2002)
	Jurkat T cells	Human	IL-4 ≥ anti-CD3/CD28	↑ cAMP; ↓ NF-kappaB, NF-AT phosphorylation	Borner et al. (2009)
	BV-2 microglia	Rat	LPS	↓ IL-1beta, IL-6	Kozela et al. (2010)
CBD	U937 monocytes	Human	LPS	↑ IL-1ra, IL-8, IL-16, IL-32, MIF; ↓ CCL2, CCL5, cell viability	Muthumalage and Rahman (2019)
	THP-1 derived macrophages	Human	LPS	↓ TNFalpha, IL-1beta, IL-6, IL-10 mRNA	Yeisley et al. (2021)
	Raw 264.7 macrophages	Mouse	LPS	↑ G-CSF, GM-CSF, IFN-gamma, IL-1alpha, IL-6, IL-27, I-TAC, M-CSF, CCL1, RANTES, TNFalpha; ↓	Muthumalage and Rahman (2019)
	T-regulatory cells	Mouse	Low level T-cell stimulation	↑ CD25, FoxP3 expression, IL-10; ↓ T cell proliferation	Dhital et al. (2017)
	BV-2 microglia	Rat	LPS	↓ IL-1beta, IL-6	Kozela et al. (2010)
	BEAS-2B lung epithelial cells	Human	LPS	↑ IL-8, Serpin E1	Muthumalage and Rahman (2019)
CBG	Primary astrocytes	Human	Oxygen-glucose deprivation ischemia model	↓ IL-6, VEGF, <sup>a</sup> LDH activity, DNA damage	Stone et al. (2021)
	Primary brain microvascular endothelial cells	Human	Oxygen-glucose deprivation ischemia model	↓ IL-6	Stone et al. (2021)
CBC	Thioglycollate-elicited peritoneal macrophages	Mouse	LPS	↓ Nitric oxide	Romano et al. (2013)
CBDV	Primary astrocytes	Human	Oxygen-glucose deprivation ischemia model	↓ IL-6, LDH, VEGF; ↑ DNA damage markers	Stone et al. (2021)
CBN	Primary monocytes	Human	LPS; <i>Porphyromonas gingivalis</i>	↓ TNFalpha, IL-6, IL-12p40 and IL-8; ↑ IL-10	Gu et al. (2019)

<sup>a</sup>LDH, lactate dehydrogenase—used as an indicator for acute/chronic tissue damage and inflammation.

**Other minor phytocannabinoids:** Cannabigerol (CBG), a nonpsychoactive phytocannabinoid, is a precursor of CBD and Δ9-THC reported to activate CB1, CB2, TRPV1, and the α2-adrenoceptor, and to act as a TRPM8 antagonist (Borrelli et al., 2014; Cascio et al., 2010; Pertwee et al., 2010). Limited in vitro studies have shown antiinflammatory actions of CBG on astrocytes and brain endothelial cells in an oxygen-glucose deprivation model of cellular ischemia (Stone et al., 2021). Cannabidivarin (CBDV) has been shown to reduce IL-6 secretion by primary astrocytes (Stone et al., 2021). Cannabichromene (CBC), a TRPA1 agonist, has been found to reduce LPS-induced IL-10, IFN-γ, and NO production by peritoneal macrophages (Romano et al., 2013). Cannabinol (CBN) has been reported to reduce TNFα, IL-6, IL-12p40, and IL-8 secretion and to increase IL-10 secretion by human monocytes activated with LPS- or *Porphyromonas gingivalis* (Gu et al., 2019).

### In vivo effects of endocannabinoids on inflammation and endotoxemia (Table 3; Fig. 2)

**2-AG:** The enzyme MAGL hydrolyzes 2-AG. MAGL inhibition or knockdown reduces 2-AG metabolism resulting in prolonged cannabinoid receptor signaling. MAGL inhibition with JZL184 reduced LPS-induced acute lung injury as evidenced by decreased lung vascular permeability and leukocyte migration, decreased bronchoalveolar lavage fluid (BALF) levels of CCL2, TNFα, and IL-6, and increased BALF levels of IL-10 at  $t=6$ , 24, and 48 h (Costola-de-Souza et al., 2013). These changes were accompanied by decreased β2-integrin and increased L-selectin expression in BALF

**TABLE 3** In vivo effects of endocannabinoids on inflammation in animal models of endotoxemia and injury.

Endocannabinoid	Animal model	Species	Intervention, dose, route	Suggested	Effects on inflammation and other outcomes	Reference(s)
2-AG	Formalin-induced inflammatory pain	Rat	2-AG, 0.1–100 mcg, subcutaneous into hind paw	CB2	↓ Pain responses	Guindon et al. (2007)
	Formalin-Induced Inflammatory Pain	Rat	URB602 <sup>a</sup> , 0.1–500 mcg, subcutaneous into hind paw	CB2	↓ Pain responses	Guindon et al. (2007)
	LPS-induced lung injury	Mouse	JZL 184 <sup>a</sup> , 16 mg/kg, intraperitoneal	CB1/CB2	↓ CCL2, TNFalpha, IL-6 in BALF; ↓ vascular permeability, leukocyte migration, ↓ neutrophil beta2-	Costola-de-Souza et al. (2013)
	TNBS-induced colitis	Mouse	JZL 184 <sup>a</sup> , 16 mg/kg, intraperitoneal	CB1/CB2	↓ CCL2, TNFalpha, IL-1beta, IL-12, IL-6	Alhouayek et al. (2011)
AEA	LPS-induced acute lung injury	Mouse	AEA, 0.075 nmol/kg, intranasal	N/A	↓ BALF neutrophil recruitment, TNFalpha	Berdyshev et al. (1998)
	LPS induced systemic inflammation	Mouse	AEA, 40 mg/kg, intravenous	TRPV1	↓ IL-6, CCL2, PAI-1; ↑ IL-10	Lawton et al. (2017)
NADA	Pam3Cys- or LPS-induced inflammation; polymicrobial	Mouse	NADA 5–10 mg/kg, intravenous	TRPV1	↓ IL-6, CCL-2, PAI-1, IL-1beta, TNFalpha, CCL2, CCL3, CCL4, CCL5, IL-12p40; ↑ IL-10	Lawton et al. (2017) and Joffre, Yeh, et al. (2020)
	Carageenan-induced thermal hyperalgesia	Rat	NADA, 1.5–50 mcg, intrathecal	CB1 and TRPV1	↓ Thermal hyperalgesia	Farkas et al. (2011)

<sup>a</sup>URB602, JZL184, KML29—MAGL inhibitors—result in a sustained increase in 2-AG levels.

and blood neutrophils. Inhibition of MAGL with JZL184 also reduced CCL2, TNF $\alpha$ , IL-1 $\beta$ , IL-12, and IL-6 in mice with TNBS-induced colitis (Alhouayek et al., 2011). Another selective MAGL inhibitor, KML29, decreased mechanical allodynia and paw edema in mice with carrageenan-induced inflammatory pain. In a model of formalin-induced inflammatory pain, intraplantar injection of 2-AG or the selective MAGL inhibitor, URB602, reduced pain responses (Guindon et al., 2007).

**AEA:** The intranasal administration of AEA was reported to downregulate BALF neutrophil recruitment and TNF $\alpha$  in mice with lung injury induced by intranasally administered LPS. However, this effect was not sustained at higher doses (Berdyshev et al., 1998). Treatment with high doses of AEA (40 mg/kg) led to the reduced levels of IL-6, CCL2, and plasminogen activator inhibitor (PAI)-1, and increased IL-10 in mice with LPS-induced endotoxemia (Lawton et al., 2017).

**N-acyl dopamines:** NADA has roles in pain modulation and has been shown to reduce thermal hyperalgesia in rats with inflammatory pain (Farkas et al., 2011). Administration of NADA has been found to have strong antiinflammatory effects in mice with systemic inflammation induced by the IV administration of bacterial lipopeptide (TLR2 agonist) or LPS (TLR4) and in mice with sepsis induced by perforation of the bowel (Lawton et al., 2017). The intravenous administration of NADA was found to reduce plasma levels of pro-inflammatory cytokines and augment plasma IL-10 at  $t=6$  h after inducing CLP-sepsis. NADA also reduced plasma levels of multiple pro-inflammatory mediators (e.g., IL-6, CCL2, IL-1 $\beta$ , TNF $\alpha$ , PAI-1, CCL3, CCL4, CCL5, and IL-12p40), while increasing plasma IL-10 within 2 h of LPS or bacterial lipopeptide challenge (Lawton et al., 2017). The antiinflammatory actions of NADA in mice treated with LPS or bacterial lipopeptide were absent in *Trpv1*<sup>-/-</sup> mice. In studies using bone marrow chimeras, NADA retained its antiinflammatory effect in endotoxemic WT mice transplanted with *Trpv1*<sup>-/-</sup> bone marrow, but its effects were lost in endotoxemic *Trpv1*<sup>-/-</sup> mice transplanted with WT bone marrow (Lawton et al., 2017). This suggests that nonhematopoietic TRPV1-expressing cells mediate NADA's antiinflammatory effects.

## In vivo effects of phytocannabinoids on inflammation and endotoxemia (Table 4; Fig. 2)

**$\Delta 9$ -THC:** The administration of  $\Delta 9$ -THC to mice with LPS-induced systemic inflammation was found to significantly reduce plasma levels of IL-6 and CCL2, strongly increase plasma IL-10, and activate NF- $\kappa$ B at early time points ( $T=2$ –6 h) (Joffre, Yeh, et al., 2020). In that study, mice treated with  $\Delta 9$ -THC had reduced mouse sepsis scores through the final timepoint of 48 h, reduced mRNA for lung inflammatory markers, fewer histological signs of organ injury, and a reduction in MHC II and CD40 expression on splenic leukocytes at 12 and 26 h. Similar effects were observed in splenic B cells and CD4+ T cells, but there were no alterations to T-regulatory cells. Neither global genetic ablation of TRPV1, CB2, and GPR18 nor pharmacological antagonization of GPR55 and GPR119 altered  $\Delta 9$ -THC-mediated IL-10 induction. The CB1 antagonist SR141716A reversed the antiinflammatory effects of  $\Delta 9$ -THC and subsequently normalized NF- $\kappa$ B activity. The cells responsible for the initial induction of IL-10 were determined by flow cytometric analysis in IL-10-GFP reporter mice to be CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>Hi</sup> monocytic-myeloid-derived suppressive cells (Mo-MDSCs). These results corroborate well with the abrogation of IL-10 augmentation when monocytes/macrophages are depleted with chlodronate (Joffre, Yeh, et al., 2020). In a mouse model of sublethal *Legionella pneumophila* bloodstream infection, pretreatment with  $\Delta 9$ -THC reduced serum levels IL-12 and IFN- $\gamma$  while concomitantly increasing spleen levels of IL-4. In this model,  $\Delta 9$ -THC also increased mortality (Klein et al., 2000). The administration of CB1 and CB2 antagonists reversed the suppressive effects of  $\Delta 9$ -THC on IFN- $\gamma$  and IL-12.

$\Delta 9$ -THC also exerts protective effects in rodent models of colitis. In a rat model of TNBS-induced colitis, pretreatment with  $\Delta 9$ -THC decreased macroscopic damage and reduced neutrophil infiltration and activity in the colon. These effects were increased when rats received  $\Delta 9$ -THC in combination with CBD (Hayakawa et al., 2008). In rats with CFA-induced inflammatory pain, daily administration of  $\Delta 9$ -THC was found to reduce pain-related behaviors including mechanical allodynia and heat hyperalgesia, but did not affect paw edema or alter inflammatory cytokine levels (Britch et al., 2020).

**CBD:** The studies on the inflammatory effects of CBD have yielded widely different results depending on dosage, route of administration, and the vehicle through which the drug is administered. In one study, CBD intraperitoneally administered to mice with LPS-induced lung injury reduced leukocyte migration into the lungs, BALF levels of TNF $\alpha$ , IL-6, CCL2, and CCL3, and lung myeloperoxidase activity suggesting a protective effect (Ribeiro et al., 2015). In contrast, a similar study on LPS-induced lung inflammation in mice showed enhanced lung infiltration of neutrophils and monocytes increased expression of cytokine mRNAs with orally administered CBD (Karwau et al., 2013). Intraperitoneally administered CBD (5 mg/kg) was reported to drastically reduce colon injury and wet weight/length ratio, and to induce iNOS expression, increase IL-10, and decrease IL-1 $\beta$  in colonic tissue (Borrelli et al., 2009). CBD has also been reported to reduce IL-1 $\beta$  levels in mice orally infected with *Porphyromonas gingivalis* via CB2 (Gu et al., 2019).

**TABLE 4** In vivo effects of phytocannabinoids on inflammation in animal models of endotoxemia and injury.

Endocannabinoid	Animal model	Species	Dose, route	Suggested receptor	Effects on inflammation and other outcomes	Reference(s)
THC	Systemic endotoxemia	Mouse	5mg/kg, intravenous	CB1	↓ IL-6, CCL2; ↑ IL-10; ↓ mouse sepsis scores, acute lung injury	Joffre, Yeh, et al. (2020)
	Sublethal <i>Legionella pneumophila</i> infection	Mouse	8mg/kg, intravenous	CB1/CB2	↓ IFN-gamma, IL-12; ↑ IL-4; ↑ mortality	Klein et al. (2000).
	Inflammatory pain—intraplantar CFA	Rat	4mg/kg, intraperitoneal	N/A	↓ Mechanical allodynia, thermal hyperalgesia	Britch et al. (2020)
CBD	LPS-induced lung inflammation	Mouse	20–80mg/kg, intraperitoneal	Alternative receptor;	↓ Lung MPO <sup>a</sup> ; ↓ TNFalpha, IL-6, CCL2, CCL3, total protein in BALF <sup>b</sup>	Ribeiro et al. (2015)
	LPS-induced lung inflammation	Mouse	75mg/kg, Oral	N/A	↑ Neutrophil and monocyte lung infiltration; ↑ TNFalpha, IL-6, IL-17A, IL-23, G-CSF	Karmaus et al. (2013)
	Oral infection with <i>Porphyromonas gingivalis</i>	Mouse	10mg/kg every other day, oral		↓ IL-1beta	Gu et al. (2019)
	DNBS-induced colitis	Mouse	5mg/kg, intraperitoneal	N/A	↑ iNOS, IL-10 in colon tissue; ↓ colon injury, IL-1beta	Borrelli et al. (2009)
	Poly(I:C)-induced acute respiratory distress syndrome	Mouse	5mg/kg, intraperitoneal	N/A	↓ IL-6, TNFalpha, and IFN-gamma; ↓ neutrophil and monocyte infiltration	Khodadadi et al. (2020)
	Combined model of systemic inflammation induced by abdominal	Rat	60mg/kg, oral	N/A	↓ Sickness behavior; ↓ IL-1beta, MMP-9, IL-6, TNFalpha	Trivedi et al. (2022)
CBG	Carrageenan-induced hyperalgesia	Rat	10mg/kg, oral	TRPV1	↑ Paw withdrawal latency; ↓ hyperalgesia	Rock et al. (2018)
	DNBS-induced colitis	Mouse	30mg/kg, intraperitoneal	Alternative receptor	↓ MPO, iNOS; ↓ intestinal permeability; normalization of colonic expression of IL 1beta, IFN	Borrelli et al. (2013)
CBDV	DNBS-induced colitis	Mouse	0.3–10mg/kg, intraperitoneal; 3–30mg/kg, Oral	TRPA1	↓ IL-1beta, IL-6, CCL2	Pagano et al. (2019)
CBDA	Carrageenan-induced inflammatory pain	Rat	0.01–0.1mg/kg, intraperitoneal	TRPV1	↓ Hyperalgesia and edema	Rock et al. (2018)
THCV	Inflammatory pain	Mouse	0.3–1mg/kg, intraperitoneal	CB2	↓ Hyperalgesia and edema	Bolognini et al. (2010)
THCA	3-Nitropropionic acid-induced striatal neurodegeneration	Mouse	20 mg/kg, intraperitoneal	PPAR-gamma	↓ TNFalpha, IL-6, iNOS, COX2 mRNA; ↓ cytotoxicity; ↑ mitochondrial mass	Nadal et al. (2017).

<sup>a</sup>MPO, myeloperoxidase, reflects neutrophil activity.<sup>b</sup>BALF, bronchoalveolar lavage fluid.

In a rat model of systemic inflammation induced by administration of a combination of cecal slurry, LPS, and *Escherichia coli*, treatment with CBD reduced sickness behaviors culminating in increased locomotion, increased grip strength, and reductions of IL-1 $\beta$ , MMP-9, IL-6, and TNF $\alpha$  compared with vehicle-treated controls (Trivedi et al., 2022). Similarly, in a model of TLR3 agonist (PolyI:C)-induced acute respiratory distress syndrome, CBD reduced clinical severity and systemic pro-inflammatory cytokine levels (Khoddadadi et al., 2020). CBD has been reported to reduce hyperalgesia in carrageenan-induced inflammatory pain, decrease pain associated with osteoarthritis and sciatic nerve injury paired with decreases in pro-inflammatory cytokines and eicosanoids, as well as reduce the severity of collagen-induced arthritis (Rock et al., 2018).

**Other minor phytocannabinoids:** There is limited information on the in vivo effects of other minor phytocannabinoids on inflammation and endotoxemia. In mouse models of carrageenan-induced inflammatory pain, Tetrahydrocannabivarin (THCV) has been demonstrated to decrease pain and reduce edema (Bolognini et al., 2010). Tetrahydrocannabinolic acid (THCA) has been reported to reduce multiple pro-inflammatory cytokines in mice with 3-nitropropionic acid-induced striatal neurodegeneration (Nadal et al., 2017). In mice with DNBS-induced colitis, CBDV has been demonstrated to decrease neutrophil infiltration and intestinal permeability and reduce IL-1 $\beta$ , IL-6, and CCL2 production (Pagano et al., 2019). This attenuation was sensitive to TRPA1 antagonist, thus implicating the ion channel in the protective effect. Pretreatment with Cannabidiolic acid (CBDA) was reported to induce antihyperalgesia and antiinflammatory effects in mice with inflammatory pain induced by carrageenan challenge (Rock et al., 2018). In a model of murine DNBS-induced colitis, CBG reduced colonic myeloperoxidase activity and iNOS (Borrelli et al., 2013).

## Applications to other areas

The results of the basic and translational studies reviewed above suggest that during acute inflammation and endotoxemia, cannabinoids have substantial effects on multiple immunologic functions, and that the endocannabinoid system is involved in modulating systemic and organ inflammation. So far most of the data on immunomodulation by the endocannabinoid system are from animal models and in vitro studies using cultured cells. Much remains to be understood about the endocannabinoid system in human health and disease, but there are some limited data suggesting that the endocannabinoid system is also active in humans with acute inflammation. For example, studies have demonstrated increased circulating AEA and 2-AG in humans with endotoxemia or acute myocardial infarction (Maeda et al., 2009; Wang et al., 2001).

These data may have broad implications for a wide range of acute and chronic disorders and diseases, particularly those are driven by or complicated by inflammation. Examples of acute processes that could be substantially impacted by the immunomodulatory effects of the endocannabinoid system include sepsis, infection with SARS-CoV-2, traumatic injuries, and localized or global ischemia-reperfusion from vascular blockage, low blood pressure, or cardiac arrest. Chronic processes that may be impacted by activation or suppression of the endocannabinoid system include inflammatory bowel disease, chronic inflammatory or neuropathic pain, rheumatoid arthritis, and neuroinflammatory and neurodegenerative diseases, such as Alzheimer's disease. Additionally, because inflammation plays a role in the pathogenesis of some cancers, the effects of the endocannabinoid on inflammation may also have applicability to cancer pathogenesis and therapy.

## Key facts

### Key facts of IL-10 modulation by cannabinoids under conditions of acute inflammation or endotoxemia

The antiinflammatory cytokine IL-10 is upregulated in response to inflammatory stimuli, including exposure to LPS. Under inflammatory conditions, exposure to multiple cannabinoids further upregulates IL-10.

In vivo treatment with AEA, NADA, and  $\Delta$ 9-THC has been found to increase circulating IL-10 levels at early timepoints mice with endotoxemia or sepsis (Joffre, Yeh, et al., 2020; Lawton et al., 2017).

The  $\Delta$ 9-THC-induced upregulation of circulating IL-10 in endotoxemic mice requires peripheral monocytes/macrophages. Monocytic-Myeloid-Derived Suppressive Cells may be the source of the upregulated IL-10 in endotoxemic mice treated with  $\Delta$ 9-THC.

In vitro exposure of LPS-treated cells with cannabinoids upregulates IL-10 secretion by monocytes, microglia, astrocytes, T-cells, and Muller glia (Arnold et al., 2021; Correa et al., 2010; Dhital et al., 2017; Gu et al., 2019), but has also been reported to decrease IL-10 secretion by macrophages, (Yeisley et al., 2021).

## Key facts of the antiinflammatory effects of NADA in acute inflammation

Treatment with the endocannabinoid/endovanilloid NADA induces an antiinflammatory profile in mice treated with innate immune receptor agonists, including bacterial lipopeptide (TLR2) or LPS (TLR4) (Lawton et al., 2017).

NADA treatment decreases blood levels of multiple proinflammatory mediators, including IL-6, IL-1 $\beta$ , TNF- $\alpha$ , CCL2, CCL3, CCL4, CCL5, and IL-12p40, and increases blood levels of the antiinflammatory cytokine IL-10 in LPS-treated mice.

In mice with abdominal sepsis induced by perforation of the bowel, NADA treatment leads to decreased IL-6, CCL2, and PAI-1 and increased IL-10 in the systemic circulation.

NADA's anti-inflammatory effects are dependent on TRPV1 expressed outside of the myeloid compartment, which suggest involvement of TRPV1 by nonleukocyte cells, such as neurons, which highly express TRPV1.

## Mini-dictionary of terms

**Endothelial cells:** Vascular endothelial cells play active roles in responses to infection and injury (Joffre, Hellman, et al., 2020). Endothelial cells express innate immune receptors and inflammatory signaling intermediaries (e.g., NF- $\kappa$ B, MAPKs) and produce cytokines. Furthermore, they regulate leukocyte recruitment to organs and the movement of fluids and leukocytes between the intravascular and extravascular spaces. Sepsis and other forms of acute inflammation cause endothelial dysfunction leading to vascular leak, coagulopathy, and increased levels of activated neutrophils in organs, which ultimately culminates in shock, tissue edema, and organ injury and failure.

**Innate immunity:** The innate immune system plays an early role in sensing and initiating the immune response to infection or tissue injury. The engagement of host or microbial factors with innate immune receptors activates and intracellular signaling cascade that ultimately leads to the upregulated expression and secretion of cytokines, chemokines, and other inflammatory mediators that play roles in antimicrobial defenses and in initiating reparative responses to tissue injury. Under dysfunctional conditions, such as can occur with sepsis or trauma, dysregulated inflammatory responses can lead to shock, organ injury and failure, and suppressed host defenses.

**Interleukin 10:** IL-10, an inducible cytokine, is secreted by multiple leukocyte lineages in response to host and microbial inflammatory triggers (Saraiva et al., 2020). Its binding to the IL-10 receptor (IL-10R) leads to the activation of JAK/STAT signaling and several MAPKs, ultimately resulting in reduction of the expression and secretion of pro-inflammatory mediators such as cytokines and ROS.

**Sepsis:** Sepsis is defined as a “Life-threatening organ dysfunction caused by a dysregulated host response to infection” (Singer et al., 2016). It is a global health problem that is estimated to cause up to 11 million deaths worldwide each year (Rudd et al., 2020).

**Toll-like receptors (TLRs):** TLRs are a family of innate immune receptors that are activated by different microbial and host factors. TLR4 is the receptor for LPS, a pro-inflammatory lipid in the outer membrane of Gram-negative bacteria. TLR2 is the receptor for bacterial lipoproteins, which are expressed by Gram-positive and Gram-negative bacteria.

## Summary points

- (1) Endocannabinoids are synthesized by numerous immune and nonimmune cells, and their production is modulated by exposure of cells to inflammatory agonists such as LPS.
- (2) Endocannabinoids and phytocannabinoids have strong antiinflammatory actions in vitro and in vivo in models of acute inflammation and endotoxemia.
- (3) Cannabinoids modulate inflammation at a cellular and systemic level via CB1, CB2, and other GPCRs, as well as TRPs, in particular TRPV1, and PPARs.
- (4) CB1 is dominantly expressed in the CNS, with low levels of expression peripherally on astrocytes, microglia, circulating and tissue-resident leukocytes, and endothelial cells.
- (5) CB2 is more highly expressed by leukocytes, and it is often co-expressed alongside CB1 and alternative GPCRs.
- (6) TRPV1 is a nonselective cation channel that binds the endocannabinoids AEA and NADA and is expressed in sensory neurons of the trigeminal nerve, DRG, and vagus nerve. TRPV1 is also expressed by nonneuronal cells such as astrocytes, microglia, multiple subpopulations of peripheral leukocytes, endothelial cells, and smooth muscle cells.
- (7) Cannabinoid receptor expression is dynamically regulated during inflammation.
- (8) Signaling cascades engaged by cannabinoids result in the inhibition of NF- $\kappa$ B activity, as well as the activation of PI-3K, ERK1/2, p38MAPK, and JNK, which ultimately induces an antiinflammatory profile.

- (9) In vivo treatment of mice with endotoxemia and sepsis with some cannabinoids (e.g., AEA, NADA, and Δ9-THC) decreases levels of pro-inflammatory cytokine, and strongly upregulates IL-10 blood levels.
- (10) The antiinflammatory actions of NADA in endotoxemic mice were found to be mediated through nonhematopoietic TRPV1.

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## Chapter 3

# Physiology of the endocannabinoid system: Imaging and the use of positron emission tomography (PET)

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## Abbreviations

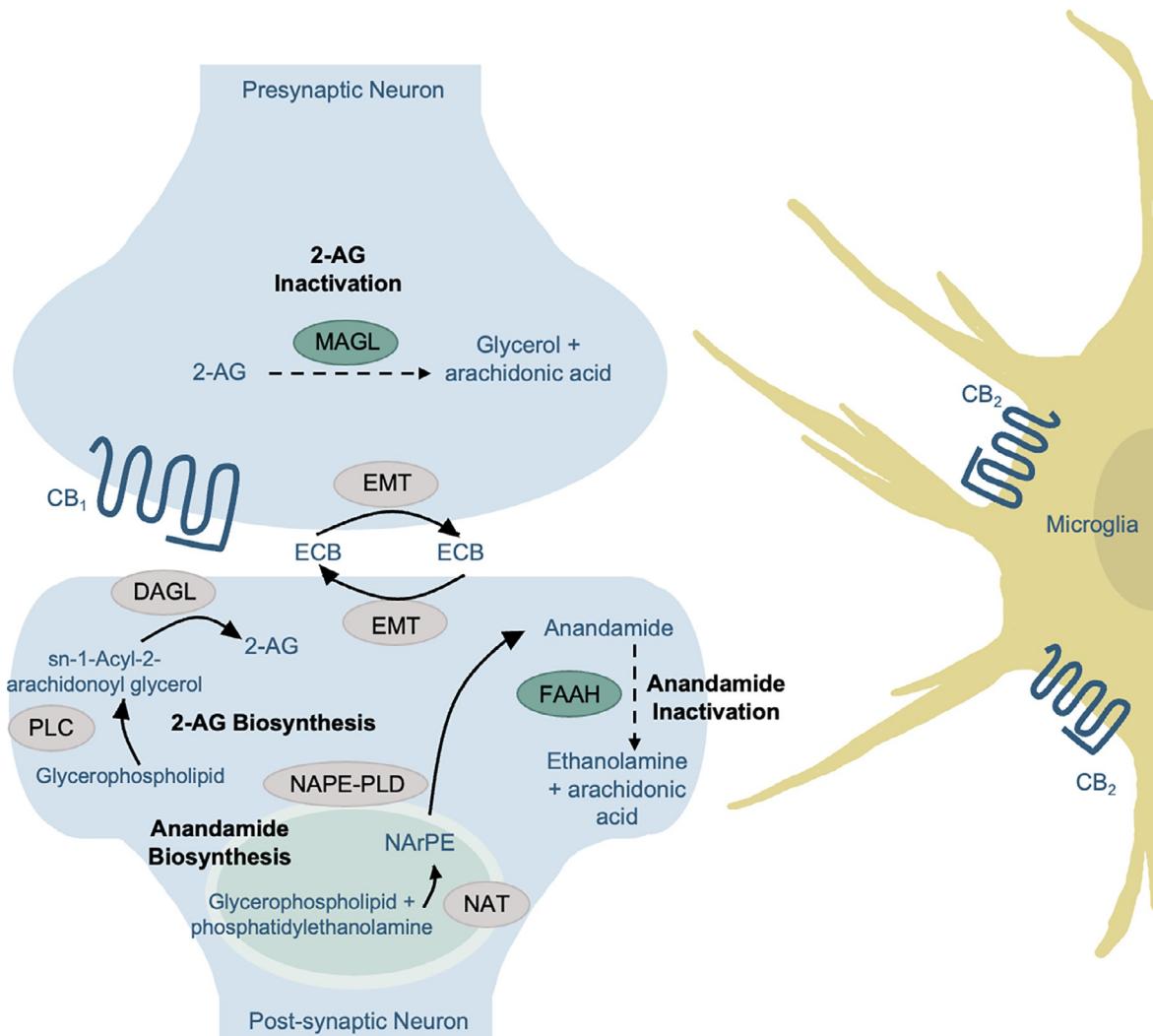
<b>2-AG</b>	2-arachidonoyl glycerol
<b>AEA</b>	anandamide
<b>AUD</b>	alcohol use disorder
<b>BMI</b>	body mass index
<b>CB1/CB1R</b>	cannabinoid 1 receptor
<b>CB2/CBR2</b>	cannabinoid 2 receptor
<b>CUD</b>	cannabis use disorder
<b>ECS</b>	endocannabinoid system
<b>FAAH</b>	fatty acid amide hydrolase
<b>fMRI</b>	functional magnetic resonance imaging
<b>MAGL</b>	monoacylglycerol lipase
<b>MRI</b>	magnetic resonance imaging
<b>PET</b>	positron emission tomography
<b>ROI</b>	region of interest
<b>THC</b>	tetrahydrocannabinol

## Introduction

The endocannabinoid system (ECS) has become a focus of mainstream neuroscience research as it's unique on-demand, lipid-based, retrograde signaling sets it apart from "classical" neurotransmitter systems (Katona & Freund, 2012). This form of communication is known to fine-tune information flow within all major neurotransmitter pathways and contributes to short and long-term synaptic plasticity in several brain regions thought to be involved in neuropsychiatric disorders including addictions, psychotic, externalizing, anxiety, and stress disorders (Vinod & Hungund, 2005). Recent preclinical research has suggested ECS components (depicted in Fig. 1) as potential therapeutic targets. This has sparked increased efforts to both translate preclinical findings and better understand the status of the ECS *in vivo* in human—necessary first steps for developing ECS-targeting therapeutics. In this regard, positron emission tomography (PET) imaging is a valuable clinical research tool (Heilig et al., 2016).

## Brief overview of PET imaging

PET imaging is a molecular imaging technique with beneficial applications for the identification of disease biomarkers and drug development, as it allows for the measurement of human biological functions *in vivo*. PET is a noninvasive, functional imaging method that uses a radioligand or radiotracer, a positron-emitting radioactive isotope, which is often bound to a



**FIG. 1** Endocannabinoid system components. The ECS comprises: (1) the G-protein-coupled “brain” and “peripheral” cannabinoid receptors, CB1R, localized presynaptically on both GABAergic and glutamatergic neurons cite, and the most abundant G-protein-coupled receptor in the nervous system and CB2, mainly found in the immune system; (2) endocannabinoid ligands, including the widely studied anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), which have complementary and perhaps mutually inhibitory function as a partial and full agonist at CB1R, respectively; (3) the biosynthetic enzymes, *N*-acylphosphatidylethanolamine phospholipase D and diacylglycerol lipase, for AEA and 2-AG respectively; (4) the hypothetic reuptake AEA transporter and (5) the metabolizing enzymes, monoacylglycerol lipase (MAGL) for 2-AG and fatty acid amide hydrolase (FAAH) for AEA.

pharmaceutical compound with affinity for the desired target. The most commonly used radioisotopes for PET imaging are <sup>11</sup>C, <sup>13</sup>N, <sup>15</sup>O, and <sup>18</sup>F (Lameka et al., 2016), which are short-lived radioisotopes that undergo positron decay. Positron decay is a process characterized by the conversion of a proton into a neutron causing the emission of a positron. When a positron is emitted, it can unite with a neighboring electron producing a pair of photons traveling in opposite directions (Lameka et al., 2016). These photons are simultaneously detected by pairs of sensors colinearly aligned in the PET scanner and are used to construct three-dimensional images of radiotracer concentrations within the body using iterative reconstruction algorithms (Lameka et al., 2016). By leveraging the radioactive emissions from the radiotracer, the resultant three-dimensional image provides visualization and quantification of the binding site for the radioligand over time. Praised as a tool for “precision pharmacology,” PET imaging assists with biomarker identification and allows for more precise detection of interindividual differences in physiology within a population (Heilig et al., 2016; Matthews et al., 2012). Caveats of this technology have been cited including the high cost to obtain data and strict on-site laboratory requirements to synthesize the radiotracers (Heilig et al., 2016).

## PET radiotracers to map *in vivo* physiology

Several characteristics contribute to radiotracer success *in vivo*. As such, multiple radiotracers often exist to image a single target of interest. Selectivity for one target is critical, as off-target binding can confound analysis and inaccurately represent target expression if not identified (Patel & Gibson, 2008). For central nervous system PET imaging, brain penetration is also a challenging hurdle to overcome and is influenced by several factors such as lipophilicity and binding characteristics. The kinetics of a radiotracer and reversibility allow for kinetic modeling to predict a time course of regional radioactivity concentration based on local physiological variables and the available radiotracer concentration in the plasma (Carson, 2005). In addition to these physicochemical and biochemical properties, radiochemistry is also a determinate of the utility of a radiotracer. The synthesis time, radiochemical yield, resulting molar activity, and ease for automation contribute to the feasibility of radiosynthesis. The radioisotope used for labeling can also provide its own benefits with respect to half-lives that allow for multiple scans in a single day or transportation of the radiotracer to facilitate multicenter clinical trials (Varlow et al., 2021).

There is an increasing focus on understanding the ECS through molecular imaging. To date, multiple PET radiotracers that target components of the ECS have been developed and validated for use in humans (Table 1). While past efforts have primarily focused on receptor-targeting PET ligands (e.g., CB1R), radiotracer development has broadened to include ligand development for additional ECS targets, including CB2R, FAAH, and most recently, MAGL. The present narrative review will discuss the existing body of literature that has used PET imaging of ECS components in the human brain to investigate the status of the ECS in neuropsychiatric and other neurological conditions.

## Using PET imaging to investigate neurophysiology and neuropsychiatric conditions

Over the last decade and a half, researchers have leveraged PET imaging to better understand the status of the ECS in the brain of living humans. Due to radiotracer availability, most studies have investigated CB1R (reviewed in Table 2) and FAAH binding (reviewed in Table 3), while some have more recently explored other components related to the ECS (reviewed in Table 4). The main conclusions from these studies that contribute to our understanding of the neurophysiology of the ECS in healthy human brain, as well as in various neuropsychiatric conditions, are reviewed below. All studies were conducted in the human brain, with the exception of Ahmad et al., 2013.

### Healthy controls

PET imaging has been particularly useful to provide insight into how demographic factors including sex, body mass index (BMI), age, and genotype might impact components of the ECS.

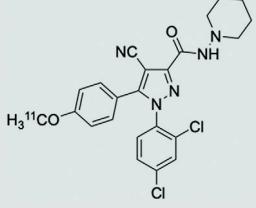
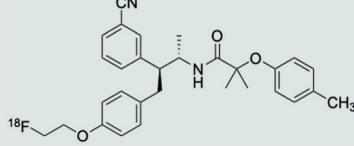
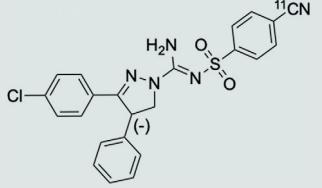
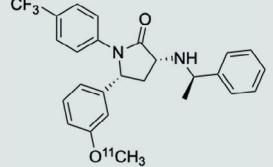
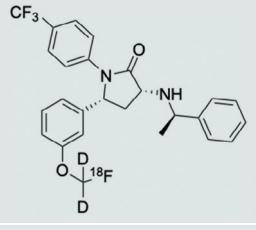
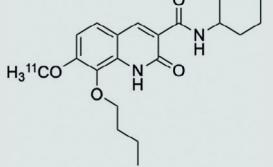
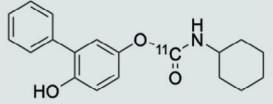
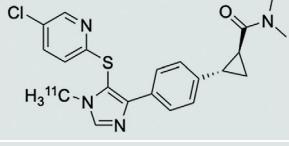
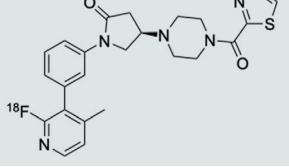
#### *Biological sex differences*

Three PET studies have assessed biological sex differences in CB1R availability. One used PET imaging to assess sex differences in CB1R availability in a small sample and found 23% higher CB1R in females compared with males (Normandin et al., 2015). In contrast, two larger studies found increased CB1R availability in males (Laurikainen et al., 2019; Van Laere et al., 2008). Findings regarding FAAH levels between males and females are also inconsistent, with one study reporting elevated FAAH in females (Watts et al., 2020) and another reporting no difference (Best et al., 2020). The former study used a combined sample of healthy volunteers and individuals with schizophrenia, while the latter was conducted in a sample of healthy volunteers. Preclinical studies have reported potential sex-dependent effects on CB1R and FAAH and FAAH levels are, in part, regulated by progesterone and estrogen (Maccarrone et al., 2003; Waleh et al., 2002), the levels of which fluctuate differently in males and females. It is possible that levels and function of ECS components are sex-dependent, and future PET studies should not only take this into account, but endeavor to better understand these differences.

#### *Body mass index*

Global CB1R availability was inversely related to BMI in male HC, though the underlying mechanism is unclear (Hirvonen et al., 2012). Preclinical studies have reported a possible inverse relationship between adipose tissue and peripheral FAAH levels (e.g., Cable et al., 2011). To investigate this relationship *in vivo* in humans, a cross-sectional PET study assessed FAAH levels in the brain and peripheral blood samples to measure endocannabinoid levels in healthy controls. Outcomes were compared between participants with  $BMI < 25$  and  $BMI \geq 25$  and revealed no significant difference in FAAH in brain.

**TABLE 1** PET radiotracers validated for use in human to measure endocannabinoid system components in brain.

Name	Target	Structure	Reversible/irreversible	Mechanism	Reference
[ <sup>11</sup> C] OMAR	CB1		Reversible	Antagonist/ Inverse agonist	Wong et al. (2010)
[ <sup>18</sup> F]MK-9470			Reversible	Inverse agonist	Burns et al. (2007)
[ <sup>11</sup> C] SD5024			Reversible	Antagonist	Tsujikawa et al. (2014)
[ <sup>11</sup> C] MePPEP			Reversible	Mixed inverse agonist/antagonist	Terry et al. (2009)
[ <sup>18</sup> F] FMPEP-d2			Not reported/reversible	Inverse agonist	Terry et al. (2010)
[ <sup>11</sup> C] NE40	CB2		Reversible	Inverse agonist	Ahmad et al. (2013)
[ <sup>11</sup> C] CURB	FAAH		Irreversible	Inhibitor	Rusjan et al. (2013)
[ <sup>11</sup> C]MK-3168			Reversible	Inhibitor	Postnov et al. (2018)
[ <sup>18</sup> F]T-401	MAGL		Reversible	Inhibitor	Takahata et al. (2022)

CB1R, cannabinoid 1 receptor; CB2R, cannabinoid 2 receptor; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase.

**TABLE 2** PET studies of CB1R availability *in vivo* in human brain.

Population	Control sample		Experimental sample		Radiotracer used	Main study objective “To assess CB1R availability...”	Change in CB1R in experimental group compared to control	Region of interest	Reference
	n	M/F	n	M/F					
HC	7	4/3			[ <sup>11</sup> C]MePPEP	Radiotracer binding specificity			Terry et al. (2010)
HC	10	5/5			[ <sup>11</sup> C]OMAR	Sex differences	↑ CB1R in females (+23%)	Whole brain	Normandin et al. (2015)
HC	22	11/11			[ <sup>18</sup> F]FMPEP-d2	Sex differences	↑ CB1R in males (+41%)	PCC, retrosplenial cortices	Laurikainen et al. (2019)
HC	50	25/25			[ <sup>18</sup> F]MK-9470	Relation with aging and sex differences	↑ CB1R with ↑ age, only in females  ↑ CB1R in males	Basal ganglia, lateral temporal cortex, limbic system, hippocampus  Limbic system and cortico-striato-thalamic-cortica	Van Laere et al. (2008)
HC	10	10/0			[ <sup>11</sup> C]OMAR	Relation with aging	CB1R positively correlated with aging No relationship	Globus pallidus Other ROI	Wong et al. (2010)
HC, acute THC	14	14/0			[ <sup>11</sup> C]MePPEP	Relation with THC effects Relation with fear-processing task	CB1R (+)-correlated with THC induced anxiety & amygdala activation during fear processing	RAmygdala	Bhattacharyya et al. (2017)
CC	28	28/0	30 14	30/0 14/0	[ <sup>18</sup> F]FMPEP-d2	HC vs. CC CC (early abstinence) vs. CC (later abstinence)	↓ CB1R (~20%) ↑ CB1R	Neocortex, Limbic cortex	Hirvonen et al. (2012)
CUD	10	8/2	10	7/3	[ <sup>18</sup> F]MK-9470	HC vs. CUD (early abstinence)	↓ CB1R (~11.2–13.5%)	Temporal, ACC, PCC, Nucleus Accumbens	Ceccarini et al. (2015)
CUD	19	19/0	11	11/0	[ <sup>11</sup> C]OMAR	HC vs. CUD (nonabstinent) HC vs. CUD (early abstinence) HC vs. CUD (later abstinence)	↓ CB1R (~15%) ↔ CB1R ↔ CB1R	Whole brain	D’Souza et al. (2019)
AUD	8		8		[ <sup>11</sup> C]OMAR	HC vs. AUD (later abstinence)	↑ CB1R (+20%)	Amygdala, hippocampus, putamen, insula, ACC, PCC and orbitofrontal cortex	Neumeister et al. (2012)

*Continued*

**TABLE 2** PET studies of CB1R availability *in vivo* in human brain—cont'd

Population	Control sample		Experimental sample		Radiotracer used	Main study objective “To assess CB1R availability...”	Change in CB1R in experimental group compared to control	Region of interest	Reference
	n	M/F	n	M/F					
AUD	18	18/0	19	19/0	[ <sup>18</sup> F]FMPEP-d2	HC vs. AUD (early abstinence) HC vs. AUD (later abstinence)	↓ CB1R (-20–30%) ↓ CB1R (-20–30%)	Whole brain	Hirvonen et al. (2013)
ALC-ACU AUD	20	20/0	26	26/0	[ <sup>18</sup> F]MK-9470	HC vs. Acute Alcohol use in HED HC HC vs. AUD (early abstinence) HC vs. AUD (later abstinence)	↑ CB1R (+15.8%) ↓ CB1R (-16.1%) ↓ CB1R (-17.0%)	Whole brain	Ceccarini et al. (2014)
SCZ	10	10/0	9	9/0	[ <sup>11</sup> C]OMAR	HC vs. SCZ	↑ CB1R in SCZ (+15–23%)	Whole brain	Wong et al. (2010)
SCZ	18	18/0	50	50/0	[ <sup>11</sup> C]OMAR	HC vs. SCZ (SCZ-MED +SCZ-UNMED)	↓ CB1R in SCZ (-12%); ↔ SCZ-MED vs. SCZ-UNMED	Whole brain	Ranganathan et al. (2016)
feSCZ	11	11/0	7	7/0	[ <sup>18</sup> F]FMPEP-d2	HC vs. feSCZ	↓ CB1R	ACC, hippocampus, thalamus, striatum	Borgan et al. (2019)
feSCZ	20	20/0	20	20/0	[ <sup>11</sup> C]MePPEP	HC vs. feSCZ	↓ CB1R	ACC, hippocampus, thalamus, striatum	Borgan et al. (2019)
feSCZ	20	20/0	20	20/0	[ <sup>11</sup> C]MePPEP	Relation with ACC-GluMRS	CB1R (-)-correlation, in HC only	Striatum, hippocampus	Borgan et al. (2021)
feSCZ*	10 11		8 15		[ <sup>18</sup> F]FMPEP-d2 [ <sup>11</sup> C]MePPEP	Relation with peripheral endocannabinoid in HC vs. feSCZ	CB1R (-)-correlation with eCBs in HCs CB1R no correlation with eCBs in SCZ	PCC	Dickens et al. (2020)
FD	12	n/s	12	n/s	[ <sup>18</sup> F]MK-9470	HC vs. FD	↑ CB1R in FD (+19.2–27.7%)	Whole brain	Ly et al. (2015)
Migraine	18	0/18	20	0/20	[ <sup>18</sup> F]MK-9470	HC vs. Migraine	↑ CB1R (+16%)	Cortical gray matter	Van Der Schueren et al. (2012)

**TABLE 2** PET studies of CB1R availability in vivo in human brain—cont'd

Population	Control sample		Experimental sample		Radiotracer used	Main study objective “To assess CB1R availability...”	Change in CB1R in experimental group compared to control	Region of interest	Reference
	n	M/F	n	M/F					
AN BN	19	0/19	14 16	0/14 0/16	[ <sup>18</sup> F]MK-9470	HC vs. AN vs BN	↑ CB1R in AN vs. HC (+24.5%) ↔ BN vs. HC ↑ CB1R in AN vs. BN (+14.7%)	Inferior frontal, temporal cortex, insula	Gérard et al. (2011)
PTSD	35	18/17	25	11/14	[ <sup>11</sup> C]OMAR	HC+TR-HC vs. PTSD	↑ CB1R in PTSD-UNMED, mainly in females CB1R (+)-correlation with age	Whole brain	Neumeister et al. (2013)

\*Study conducted at two sites using two tracers.

ACC, anterior cingulate cortex; AN, anorexia nervosa; AUD, alcohol use disorder; BN, bulimia nervosa; CB1R, cannabinoid 1 receptor; CUD, cannabis use disorder; early abstinence: ~1–7 days after last substance use; FD, functional dyspepsia; feSCZ, first episode psychosis schizophrenia/psychotic disorder; HC, healthy control; later abstinence: ~2–4 weeks after last substance use; M/F, male/female; PCC, posterior cingulate cortex; PTSD, posttraumatic stress disorder; ROI, region of interest; RAmygdala, right amygdala; SCZ, schizophrenia/psychotic disorder; THC, Δ9-tetrahydrocannabinol; TR-HC, trauma-exposed healthy control. Not applicable; n/s, not specified. Note: only main study objective and corresponding results reported. Results at the significance level of  $P < .05$  were reported as changes in CB1R.

**TABLE 3** PET Studies of FAAH Availability in vivo in human brain.

Population	Control sample		Experimental sample		Radiotracer used	Main study objective “To assess FAAH binding...”	Change in CB1R in experimental group compared to control	Region of interest	Reference
	n	M/F	n	M/F					
HC	6	3/3			[ <sup>11</sup> C]CURB	First-in-human study; kinetic modeling			Rusjan et al. (2013)
HC	6	3/3			[ <sup>11</sup> C]CURB	Biodistribution and dosimetry			Boileau et al. (2014)
HC: CC FAAH Genotype vs A-Allele Carriers (AC + AA Genotype)	14	8/6	10	4/6	[ <sup>11</sup> C]CURB	Between FAAH genotypes	↓ FAAH in A allele carriers (-23%)	Whole brain	Boileau, Tyndale, et al. (2015)
HC	5	2/3			[ <sup>11</sup> C]CURB	Test-retest reliability	Excellent reproducibility and good reliability (test-retest variability=9%)	Whole brain	Boileau, Rusjan, et al. (2015)
HC	6	4/2			[C-11]CURB	In vivo specificity with highly specific FAAH inhibitor, PF-04457845	Oral administration of PF-04457845 reduced [(11)C]CURB binding to a homogeneous level at all three doses, with λk3 values decreased by ≥91%	Whole brain	Boileau, Rusjan, et al. (2015)
HC	20	n/s	10	n/s	[ <sup>11</sup> C]CURB	BMI<25 vs. BMI≥25	↔ FAAH	Whole brain	Best et al. (2017)
HC	51	23/28			[ <sup>11</sup> C]CURB	Relation with Impulsivity	FAAH (-)-correlation with Barratt Impulsiveness Total Score	PFC	Mansouri et al. (2018)
HC	61	29/32			[ <sup>11</sup> C]CURB	Sex Differences Relation with age & BMI	↔ FAAH FAAH no correlation	Whole brain	Best et al. (2020)
HC	31	13/18			[ <sup>11</sup> C]CURB	Relation with amygdala-FC	FAAH (-)-correlation	Amygdala	Green et al. (2021)
HC	15	15/0			[ <sup>11</sup> C]MK-3168	Binding profile of a FAAH inhibitor drug, JNJ-42165279		Whole brain	Postnov et al. (2018)

**TABLE 3** PET Studies of FAAH Availability in vivo in human brain—cont'd

Population	Control sample		Experimental sample		Radiotracer used	Main study objective “To assess FAAH binding...”	Change in CB1R in experimental group compared to control	Region of interest	Reference
	n	M/F	n	M/F					
CUD	22	11/11	10	7/3	[ <sup>11</sup> C]CURB	CON vs. CUD (early abstinence)	↓ FAAH (-14–20%)	Whole brain	Boileau et al. (2016)
HCY-CU	16	8/8	13	9/4	[ <sup>11</sup> C]CURB	HCY vs. HCY-CU (early abstinence)	↓ FAAH (-12%)	Whole brain	Jacobson et al. (2021)
HCY-HED	17	n/s	14	n/s	[ <sup>11</sup> C]CURB	AUD-FH- vs. AUD-FH+	↔ FAAH	Whole brain	Best et al. (2018)+
HCY-HED	31	14/17			[ <sup>11</sup> C]CURB	Relation with alcohol sensitivity	FAAH (+)-correlation with negative effects of alcohol	PFC	Best et al. (2018)
AUD	25	13/12	14	13/1	[ <sup>11</sup> C]CURB	CON vs. AUD (early abstinence) CON vs. AUD (later abstinence)	↓ FAAH (-9%) ↔ FAAH, later abstinence	Whole brain	Best et al. (2020)
PTSD	29	n/s	15	n/s	[ <sup>11</sup> C]CURB	CON vs. PTSD	↔ FAAH	Whole brain	Gaudette et al. (2020)
SAD	34	14/20	10	3/7	[ <sup>11</sup> C]CURB	CON vs. SAD	↑ FAAH	Whole brain	Ahmed et al. (2020)
SCZ	36	19/17	27	22/5	[ <sup>11</sup> C]CURB	CON vs. SCZ Sex differences (CON+SCZ)	↔ FAAH ↑ FAAH, females	Whole brain	Watts et al. (2020)
BPD	20	10/10	20	2/18	[ <sup>11</sup> C]CURB	CON vs. BPD	↑ FAAH	Amygdala; Prefrontal Cortex	Kolla et al. (2020)
BPD+ASPD			31	13/18	[ <sup>11</sup> C]CURB	Relation with trait neuroticism Relation with trait agreeableness	FAAH (+)-correlation FAAH no correlation	PFC; dorsal putamen	Kolla et al. (2022)
ASPD	16	16/0	16	16/0	[ <sup>11</sup> C]CURB	CON vs. ASPD	↓ FAAH (-12.5%) ↔ FAAH	Amygdala; Orbitofrontal Cortex	Kolla et al. (2021)

ASPD, antisocial personality disorder; AUD, alcohol use disorder; AUD-FH+, positive family history of alcohol use disorder; AUD-FH-, negative family history of alcohol use disorder; BMI, body mass index; BPD, borderline personality disorder; CON, control group; CUD, cannabis use disorder; early abstinence: ~1–7 days after last substance use; FAAH, fatty acid amide hydrolase; FC, functional connectivity; HC, healthy control; HCY, healthy control youth; HCY-CU, healthy control youth cannabis user; HCY-HED, healthy control youth heavy episodic (binge) alcohol drinker; later abstinence: ~2–4 weeks after last substance use; M/F, male/female; PFC, prefrontal cortex; PTSD, posttraumatic stress disorder; SAD, social anxiety disorder; SCZ, schizophrenia/psychotic disorder; Striatum, ventral striatum.  
Not applicable.

Note: only main study objective and corresponding results reported. Results at the significance level of  $P < .05$  were reported as changes in FAAH.

**TABLE 4** PET studies of other ECS components *in vivo* in human.

Population	Healthy control sample		Experimental sample		Radiotracer used	ECS target	Main study objective	Experimental change in ECS target	Region of interest	Reference
	n	M/F	n	M/F						
HC	6	6/0			[ <sup>11</sup> C]NE40	CB2	Assess safety and tolerability radiotracer		Whole body	Ahmad et al. (2013))
HC	4	2/2			[ <sup>11</sup> C]NE40	CB2	Test-retest reliability	Test-retest variability 28%–30%	Whole brain	Ahmad et al. (2016)
HC, AD	8	4/4	9	7/2	[ <sup>11</sup> C]NE40	CB2	HC vs. AD	↓ CB2R	Whole brain	Ahmad et al. (2016))
HC	7	7/0			[ <sup>18</sup> F]T-401	MAGL	To visualize MAGL in human brain	CB2R distribution in brain: cerebral cortex > thalamus, putamen > brainstem, white matter	Whole brain	Takahata et al. (2022)
HC	6	6/0			[ <sup>18</sup> F]T-401	MAGL	Test-retest reliability	Test-retest variability 8%–9%	Whole brain	Takahata et al. (2022)

AD: Alzheimer's disease; CB2: cannabinoid 2 receptor; M/F: male/female; MAGL: monoacylglycerol lipase.

Not applicable.

Further, BMI did not correlate with FAAH levels, as inferred by [<sup>11</sup>C]CURB binding (Best et al., 2017), nor in a larger sample of healthy volunteers (Best et al., 2020).

### Aging

Region-dependent increases in CB1R availability were associated with increasing age; however, this was only reported in the females (Van Laere et al., 2008). FAAH levels in the brain were not correlated with age in a sample of male and female healthy volunteers aged 19–58 years (Best et al., 2020).

### FAAH genotype

Using the FAAH radioprobe [<sup>11</sup>C]CURB, Boileau, Rusjan, et al. (2015) used PET imaging to assess the functionality of a common genetic polymorphism in the gene encoding FAAH, variance in which has been associated with heightened substance use (e.g., Sipe et al., 2002), fear response (Hariri et al., 2009), and anxiety (Spagnolo et al., 2016). Each participant completed one PET scan and was genotyped for the *FAAH* C385A polymorphism (rs324420). Boileau, Rusjan, et al. (2015) reported that relative to the C/C genotype, those with the minor A allele (A/C or A/A genotype) showed lower tracer binding in vivo (~23%) (Boileau, Rusjan, et al., 2015), suggesting that genotypic variability relative to the *FAAH* C385A polymorphism is measurably associated with variance in FAAH binding in the brain and may have functional consequences for endocannabinoid tone (Boileau, Rusjan, et al., 2015). This is consistent with literature suggesting constitutively lower levels of FAAH activity and higher FAAH substrates (e.g., AEA) (Dincheva et al., 2015; Mayo et al., 2020) in both central and peripheral tissues in those with the minor A allele (Chiang et al., 2004).

### Cannabis use and cannabis use disorder

Six studies have used PET imaging to investigate ECS components in individuals who use cannabis; four measuring CB1R and two measuring FAAH. Using a variety of radiotracers, CB1R availability was reported to be decreased in nonabstinent chronic cannabis users compared with controls (Ceccarini et al., 2015; D’Souza et al., 2016; Hirvonen et al., 2012). This decrease in CB1R did not persist into early or later abstinence (D’Souza et al., 2016; Hirvonen et al., 2012). Correlations suggest that length of time using cannabis (Hirvonen et al., 2012) as well as withdrawal symptom severity (D’Souza et al., 2016) during early and later abstinence inversely relates to CB1R availability, but not recent cannabis use (Ceccarini et al., 2015; Hirvonen 2012; D’Souza et al., 2016). One pilot study has investigated the status of FAAH during early abstinence from cannabis in chronic cannabis users ( $n = 10$ ) using the FAAH radioligand [<sup>11</sup>C]CURB (Boileau et al., 2016). In comparison to matched healthy controls, a significant decrease in FAAH was reported across all ROIs in early abstinence in chronic cannabis users and inversely related to THC metabolites in blood and trait impulsivity, but not withdrawal or cannabis-related outcomes (Boileau et al., 2016).

The effects of acute cannabis use were assessed in a cohort of cannabis using healthy controls completed PET scans using the radioligand [<sup>11</sup>C]MePPEP, as well as two fMRI scans with fear-processing tasks, after administration of either an oral dose of delta- or placebo and reported that acute THC administration induced anxiety, as well as modulated right amygdala activation during fMRI fear-processing tasks, and that this correlated positively to CB1R availability in this region (Bhattacharyya et al., 2017). A similar PET study was conducted in a sample of 14 regular cannabis using youth aged (23 years  $\pm$  5) who completed a PET scan using the radioligand [<sup>11</sup>C]CURB (Jacobson et al., 2021). They reported a significant global decrease in FAAH in young cannabis users compared with matched healthy controls. Overall, these studies suggest that CB1R and FAAH may be reduced in cannabis use disorder (CUD) and cannabis-dependent groups and that continued investigation of whether agonism of CB1R might help alleviate cannabis-related withdrawal is warranted.

### Alcohol use and alcohol use disorder

Five studies have used PET imaging to investigate the status of the ECS in relation to alcohol use and alcohol use disorder (AUD). Of the three that measured CB1R after chronic alcohol consumption in treatment-seeking individuals with AUD, two studies (Ceccarini et al., 2014; Hirvonen et al., 2013) found significantly reduced CB1R binding in all ROI in early abstinence (~1 week) from alcohol, which persisted into later abstinence (~2–4 weeks); the other reported an increase in CB1R after 1 month of abstinence from alcohol (Neumeister et al., 2012). With regard to FAAH, Best et al. (2020) reported a significant brain-wide reduction of FAAH during early abstinence from alcohol in treatment-seeking individuals with AUD compared with HC, which did not persist with sustained abstinence. However, this pilot study was under-powered

to reliably assess differences in FAAH at the second timepoint. Here, FAAH binding in early abstinence from alcohol was negatively related to recent alcohol consumption and may be related to increased risk for resuming alcohol consumption (Best et al., 2020). Together, these studies suggest that both CB1R and FAAH are reduced in AUD at early abstinence, with these changes in CB1R persisting with continued abstinence.

To better understand whether alcohol exposure alters components of the ECS, two studies used PET imaging to measure CB1R (Ceccarini et al., 2014) and FAAH (Best et al., 2018; Best et al., unpublished) in social heavy-episodic drinking (HED) individuals. In line with preclinical study findings (Ceccarini et al., 2014), social heavy drinkers had increased CB1R binding in all regions following the alcohol challenge, and this was negatively related to recent alcohol consumption per week (i.e., those who regularly consumed more alcohol had reduced changes in CB1R). More studies are warranted to better understand how the ECS relates to behaviors associated with alcohol use and symptomatology related to withdrawal from alcohol.

## Schizophrenia/psychotic disorder

Five studies have used PET imaging to investigate CB1R availability in the brain in individuals with psychotic disorders. One study found an increase in CB1R in those with schizophrenia compared with controls (Wong et al., 2010). Others have reported decreased CB1R in individuals with schizophrenia (both medicated and unmedicated) (Ranganathan et al., 2016) and those with a first episode of psychosis (Borgan et al., 2019). Dickens et al. (2020) used PET imaging of CB1R in males with first-episode psychosis and healthy controls to correlate with peripheral endocannabinoid serum levels. Peripheral endocannabinoid levels in serum were found to be inversely related to CB1R in HCs, but not in those with first episode psychosis. Borgan et al. (2021) combined PET and magnetic resonance spectroscopy imaging and reported that ACC glutamate levels were negatively associated with CB1R availability in the striatum and hippocampus in healthy controls but not those with schizophrenia.

Only one study has used PET imaging with [<sup>11</sup>C]CURB to investigate the status of FAAH in individuals with schizophrenia. Watts et al. (2020) reported no significant change between FAAH levels in SCZ compared with HCs. However, lower FAAH levels appeared to be indicative of increased psychotic symptomatology severity (Watts et al., 2020).

## Posttraumatic stress disorder

Due to a body of preclinical research suggesting the involvement of the ECS in stress response and fear extinction (Green et al., 2021; Mayo et al., 2021), elucidating the status of CB1R and FAAH has been a focus of previous and ongoing research. A PET study conducted by Neumeister et al. (2013) probed sex differences in CB1R availability in untreated PTSD, trauma-exposed controls, and healthy controls. Each participant completed one PET scan, which revealed elevated global CB1R in the untreated PTSD cohort, particularly in females, compared with the trauma-exposed control, and healthy control cohorts (Neumeister et al., 2013). A preliminary study investigating the status of FAAH in individuals with PTSD found no significant change as compared with healthy controls (Gaudette et al., 2020). However, these data may be limited by small sample sizes, and ongoing and future studies may well alter these conclusions.

## Externalizing disorders

A series of studies have investigated the involvement of FAAH in externalizing disorders such as borderline personality disorder (BPD) and antisocial personality disorder (ASPD) using PET imaging with [<sup>11</sup>C]CURB. These studies report that FAAH in the amygdala may be elevated in BPD (Kolla et al., 2020) and decreased in ASPD (Kolla et al., 2021), and that FAAH was positively related to trait neuroticism, but not agreeableness, in a combined sample of those with BPD or APSD (Kolla et al., 2022). Of note, the BPD sample consisted of mainly female participants, and the ASPD had only male participants due to the nature of the condition.

## Future directions

Many investigations have been the only study to use PET imaging of the ECS in various conditions including anorexia and bulimia nervosa, functional dyspepsia, migraine, and social anxiety disorder (Tables 2 and 3). These conditions, in addition to those described above and others in which the endocannabinoid system has been suggested to play a role such as depression (Gallego-Landin et al., 2021) or autism spectrum disorder (Zou et al., 2019), highlight possible areas of future research. Ongoing PET studies in CUD, AUD, PTSD, and SAD will provide further insight into the potential for endocannabinoid-based therapeutics for these conditions. In addition, radiotracers developed to image CB2R have been

underutilized. Only two PET studies have probed CB2R availability in the brain, using the CB2R-specific radioligand [<sup>11</sup>C] NE40. Of these, only one study has used PET imaging to investigate CB2R availability in a clinical population (Ahmad et al., 2016). Overall, the CB2R receptor has been the focus of minimal research as compared with CB1R, and with more research highlighting the presence of CB2R in the brain (on microglia, for example), future studies should endeavor to better understand the possible role of CB2R in neuropsychiatric conditions that involve inflammatory responses.

The continued development of new radiotracers to image enzymes involved in the ECS will contribute to the development and characterization of new drug candidates for conditions in which the ECS is implicated. These include 2-AG hydrolysis enzymes MAGL, ABHD6 and ABHD12, and the endocannabinoid biosynthetic enzymes, DAGL and NAPE-PLD. Historically, rodent studies have provided the greatest insight into MAGL function through genetic and pharmacological blockade resulting in analgesic, antinociceptive, and anti-anxiety phenotypes, driving the development of pharmacological probes (Grimsey et al., 2020). Recently, Takahata et al. (2022) has advanced the first MAGL PET radiotracer, [<sup>18</sup>F]T-401, for human translation, reporting high  $V_T$  in the cerebral cortex, moderate  $V_T$  in the thalamus and putamen, and lower  $V_T$  in the white matter and brainstem, as well as good test-retest reliability. The translation of this novel MAGL radiotracer provides opportunities for clinical PET research in psychiatric diseases for the first time and will facilitate investigation of MAGL inhibition as a potential therapeutic strategy. ABHD6 and ABHD12 are serine hydrolases that compliment MAGL activity, accounting for the remaining 2-AG hydrolysis activity and may also contribute in a specialized manner in cells with low MAGL expression (Hou et al., 2021). PET imaging of these targets may help reveal their role in 2-AG hydrolysis as well as providing an understanding for potential applications in investigating neurotransmitter modulation.

## Applications to other areas

PET imaging is a useful tool for clinical research, in general, as it provides an understanding of physiology or pathology *in vivo* at the molecular level. PET imaging is an invaluable tool for the identification of biomarkers that may predict or inform on vulnerability for diseases or behaviors that predispose the development of conditions. With respect to drug development, PET imaging can confirm target engagement by a compound at different doses. For example, future studies that leverage PET imaging of the ECS, and CB1R in particular, to investigate differences in CB1R engagement at varying doses of cannabinoid-based compounds would inform on an important gap in our current knowledge of cannabinoid and endocannabinoid-based therapies. There is currently no research informing on the optimal dose of cannabinoid-based therapies to maximize receptor occupancy and reduce the risk of off-target binding and/or adverse side effects. With the expansion of cannabinoid-based therapeutics being developed or recommended to treat a wide variety of conditions including pain, anxiety, and PTSD, it will be important to recommend evidence-based doses that both maximize therapeutic and minimize adverse effects.

In addition, PET imaging can assist in successful drug development by facilitating the identification of underlying differences in target availability between subjects that may account for variability in response to the compound within a population. For example, a subsample within a clinical population might share some underlying biological differences that could affect binding or efficacy of a pharmaceutical compound (e.g., functional genetic differences). Without identification of these differences to inform the design of a clinical trial, a compound that might successfully treat one subgroup of individuals within a clinical population might be disregarded prematurely.

## Conclusions

In summary, PET imaging is a valuable tool to translate preclinical findings into the human that is facilitating a better understanding of the status of the ECS in various neuropsychiatric conditions. PET studies have revealed that CB1R and FAAH may be reduced in cannabis and alcohol use disorders, and that continued research is necessary to understand how the ECS is involved in clinical symptomatology of these and other conditions such as schizophrenia, PTSD, and externalizing disorders. Continued development of new radiotracers for other ECS components will be useful to expand our understanding of this complex system.

## Minidictionary of terms

**Neuroimaging:** collection of quantitative, noninvasive imaging methods used to image both structure and function of the central nervous system.

**Positron emission tomography:** noninvasive, functional imaging method that uses a positron-emitting radioactive isotope, often bound to a radioligand or radiotracer.

**Radiotracer/radioligand:** a radioactive pharmaceutical compound developed to image a specific enzyme or receptor target in vivo.

## Key facts of physiology of the endocannabinoid system: Imaging and the use of positron emission tomography (PET)

- Positron emission tomography (PET) imaging is a valuable tool to understand physiology and pathophysiology in vivo.
- PET is a noninvasive, functional imaging method that uses a radioligand or radiotracer, a positron-emitting radioactive isotope, which is often bound to a pharmaceutical compound with affinity for the desired target.
- PET radiotracers exist for endocannabinoid components including CB1, FAAH, CB2R, and MAGL.
- PET imaging of CB1R and FAAH in various neuropsychiatric and other conditions have revealed alterations of the endocannabinoid system that may contribute to disease progression or symptomatology.
- The use of PET imaging in clinical research can aid in the development of new therapeutics by facilitating investigation of dose-dependent target engagement or underlying physiology at the molecular level that may contribute to variability in drug response.

## Summary points

- Preclinical research has suggested a role for the endocannabinoid system in the progression and symptomatology of various neuropsychiatric and other conditions.
- Positron emission tomography (PET) imaging of endocannabinoid system components can facilitate translation of these findings into humans in vivo.
- Studies leveraging PET imaging of endocannabinoid components, mainly FAAH and CB1R, reveal that reduced CB1R and FAAH may play a role in cannabis and alcohol use disorders.
- PET studies report less consistent results in other neuropsychiatric conditions including psychotic and externalizing disorders.
- Future research should focus on development of radiotracers to image other components of the endocannabinoid system and leverage existing tracers to inform development of endocannabinoid-based therapeutics.

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## Chapter 4

# The endocannabinoid system and aging

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>ABCA1</b>	ATP-binding cassette transporter 1
<b>AD</b>	Alzheimer's disease
<b>AEA</b>	<i>N</i> -arachidonylethanolamine
<b>AGES</b>	advanced glycation end-products
<b>ALS</b>	amyotrophic lateral sclerosis
<b>ApoE</b>	apolipoprotein E
<b>BCP</b>	$\beta$ -caryophyllene
<b>CB</b>	cannabinoid receptors
<b>CNS</b>	central nervous system
<b>Dagla</b>	diacylglycerol lipase a
<b>eCBs</b>	endocannabinoids
<b>ECS</b>	endocannabinoid system
<b>FAAH</b>	fatty acid amide hydrolase
<b>GM-CSF</b>	granulocyte/macrophage colony-stimulating factor
<b>IL-1RN</b>	IL-1 receptor antagonist
<b>IL-6</b>	Interleukin-6
<b>LTP</b>	long-term potentiation
<b>MCPs</b>	monocyte chemoattractant proteins
<b>MIPs</b>	macrophage inflammatory proteins
<b>NAE</b>	<i>N</i> -acylethanolamine
<b>NLRP3</b>	NLR family pyrin domain-containing 3
<b>PD</b>	Parkinson's disease
<b>PGC-1<math>\alpha</math></b>	peroxisome proliferator-activated receptor-gamma coactivator-1 alpha
<b>SASP</b>	senescence-associated secretory phenotype
<b>T2DM</b>	Type 2 diabetes mellitus
<b>TG</b>	triglyceride
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor-alpha
<b>TOMM20</b>	translocase of outer mitochondrial membrane 20

## Introduction

### Endocannabinoid system

The endocannabinoid system (ECS) consists of the two G-protein-coupled cannabinoid receptors, CB1 (type 1) and CB2 (type 2), and the endogenous ligands agonists known as endocannabinoids (eCBs) (Askari & Shafiee-Nick, 2019). The CB1 receptor is highly expressed in the central nervous system (CNS), including the cortex, hippocampus, cerebellum, and basal ganglia. However, it is also expressed in the peripheral tissues such as the liver, skeletal muscle, and adipose tissue. In contrast, the CB2 receptor is mainly expressed in the peripheral nervous and immune system, but also expressed in the CNS (Watkins, 2018).

Endocannabinoids are derived from arachidonic acid, and the most studied eCBs are *N*-acylethanolamine (NAE), *N*-arachidonylethanolamine (anandamide, AEA), and 2-arachidonoylglycerol (2-AG) (Hillard, 2018). It has been emphasized that eCBs modulate neuroplasticity, cognitive processing, food intake, emotions and mood, pain, and sleep (Rácz et al., 2015). Furthermore, they can be beneficial in treating multiple sclerosis, Parkinson's disease, and Alzheimer's disease due to their antiinflammatory effects (Askari et al., 2019).

## Aging

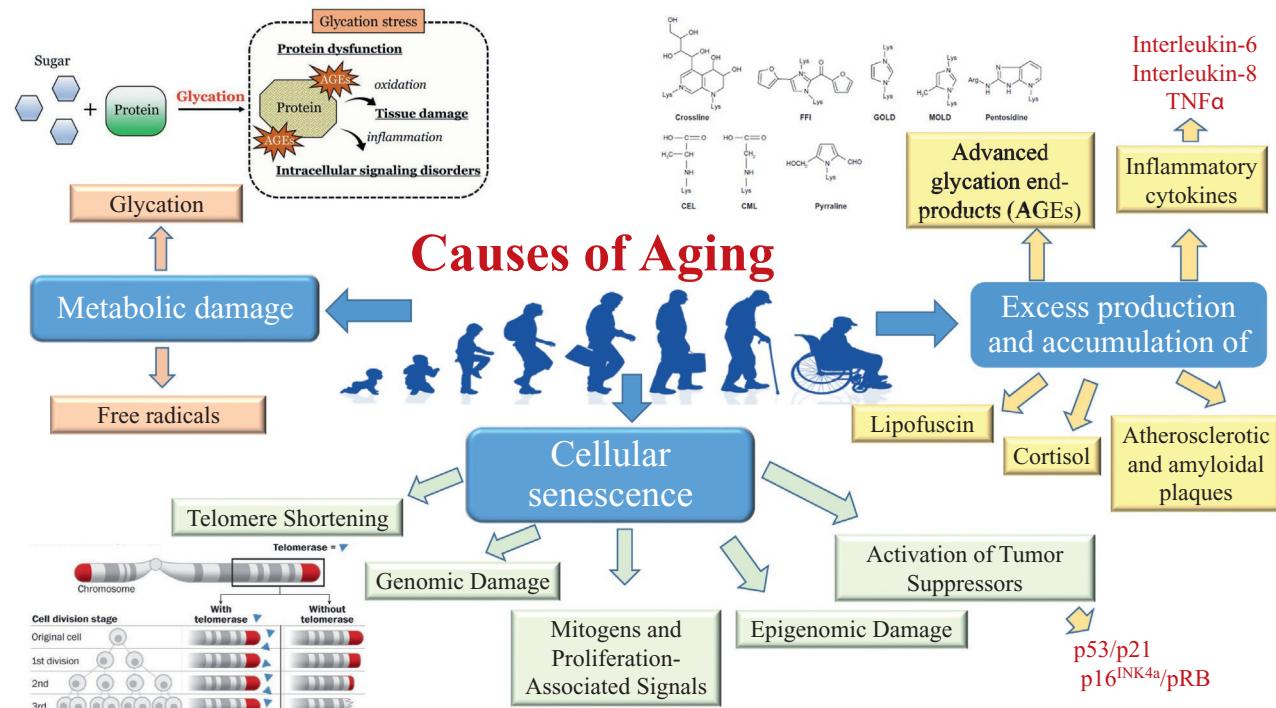
Nowadays, the population is becoming globally aged, and almost every country in the world faces an increase in the proportion of older persons. According to the United Nations World Population Aging, the 2019 revision, the population aged 65 years and over in the world is predicted to double from 703 million in 2019 (9%) to 1.5 billion in 2050 (16%). Eventually, the population of people aged  $\geq 65$  is globally increasing faster than all other younger age groups that one in six people will be aged 65 years or over in 2050 (United Nations Department of Economic and Social Affairs, 2020).

Aging is conceptualized as a progressive, time-related, accumulative, and natural phenomenon that affects almost all living organisms (Rahimi et al., 2018). It leads to an irreversible deterioration in the physiological functions of all molecules, cells, tissues, and organs of an organism (Campisi, 2013). In fact, aging presents as a mosaic and dynamic process that propagates the probability of death and limits the life of an organism. According to the reserve function of the various physiological system, aging manifests to a greater or lesser degree from successful aging to pathological aging (Fulop et al., 2010).

Accumulation of evidence emphasizes that aging gives rise to well-recognized pathologies, called age-related disorders, including osteoporosis, atherosclerosis, heart failure and cardiovascular diseases, pulmonary insufficiency, liver and renal failure, cancers, type 2 diabetes, immune system dysfunction, and neurodegenerative diseases, especially Alzheimer's disease and Parkinson's diseases (Rahimi et al., 2019). Recently, antiaging studies have attracted great attention due to the increased rate of aged persons and age-related disorders and better insight into aging complications.

## Aging mechanisms

Inflammation and oxidative stress are considered hallmarks of aging development. The leading causes of aging are illustrated in Fig. 1, including metabolic damage, excess production and accumulation of factors such as advanced glycation end-products (AGEs) and inflammatory cytokines, and cellular senescence (Mattson & Arumugam, 2018). Additionally, cellular senescence is defined as the irreversible arrest of cell proliferation and growth, and the final feature of the senescent



**FIG. 1** The leading causes of aging.

cells is known as the senescence-associated secretory phenotype (SASP). The SASP includes the secretion of chemokines, growth factors, proteases, and various inflammatory cytokines (Ogrodnik, 2021). Particularly, many SASP components, including Interleukin-6 (IL-6), IL-8, and tumor necrosis factor-alpha (TNF- $\alpha$ ), monocyte chemoattractant proteins (MCPs), macrophage inflammatory proteins (MIPs), and granulocyte/macrophage colony-stimulating factor (GM-CSF), lead to chronic inflammation. Eventually, the chronic inflammation caused by these markers is a major cause or main contributor to almost every age-related disorder (Birch & Gil, 2020).

## ECS and accelerated aging models

The alteration of the ECS in D-galactose-induced aging and memory impairment has been established (Kataoka et al., 2020; Li et al., 2020). D-Galactose is frequently used to induce accelerated aging in animals that strongly mimics the human aging symptoms (Rahimi et al., 2018). D-Galactose leads to elevated oxidative stress markers and reactive oxygen species generation, while reducing antioxidative markers in brain tissue and an impaired hippocampal long-term potentiation (LTP) and spatial memory loss in rats. CB1 protein level was notably stimulated at the early stage of D-galactose treatment (week 1) while remarkably attenuating in the hippocampus tissues in the following weeks of treatment (Li et al., 2020). These changes in the CB1 levels emphasized their role in protecting neurons from oxidative stress and inflammation (Paloczi et al., 2018). Additionally, the CB2 receptor meaningfully propagated following the D-galactose-induced aging indicating its antiinflammatory role in the brain (Braun et al., 2018). Moreover, D-galactose-induced aging markedly enhanced the fatty acid amide hydrolase (FAAH) level while alleviating the hippocampus's AEA levels (Li et al., 2020). Significantly decreased in the expression of diacylglycerol lipase a (Dagla), the synthesizing enzyme of 2-AG, and 2-AG levels have been reported in the hippocampus of aged mice (Piyanova et al., 2015) and human postmortem brains tissue (Long et al., 2012). Interestingly, selective FAAH inhibitor (URB597) mitigated the levels of inflammatory cytokines IL-6, IL-1 $\beta$ , and TNF- $\alpha$  and improved hippocampal long-term potentiation in naturally aged rats (Murphy et al., 2012). In fact, facilitating the ECS balance may be a promising way to prevent and improve age-related cognitive impairment (Table 1).

**TABLE 1** ECS changes in D-galactose and naturally aged animal models.

Study model	Animal	Results	Ref.
D-Galactose induced brain aging and memory impairment	Rat	↑ Oxidative stress markers and reactive oxygen species generation ↓ Antioxidative markers ↓ Hippocampal long-term potentiation ↓ Spatial memory ↓ CB1 expression in the hippocampus ↑ Fatty acid amide hydrolase (FAAH) level in the hippocampus ↓ AEA level in the hippocampus	Li et al. (2020)
D-Galactose induced kidney aging	Mice	↑ CB2 expression ↓ Mitochondrial mass ↑ $\beta$ -Catenin activation ↑ Angiotensin type-1 (AT1) expression ↑ Matrix metallopeptidase 7 (MMP7) expression ↓ ATP production ↓ PGC-1 $\alpha$ and TOMM20 expression	Zhou et al. (2021)
Naturally aged	C57BL/6J mice	↓ Diacylglycerol lipase a (DAGLA) expression ↓ 2-AG levels in the hippocampus	Piyanova et al. (2015)
Naturally aged	C57BL/6J CB1 knockout mice	↓ Serine 65-phosphorylated ubiquitin ↓ Mitochondrial autophagy in hippocampal neurons	Kataoka et al. (2020)
Naturally aged+FAAH inhibitor	Rat	↓ Levels of inflammatory cytokines IL-6, IL-1 $\beta$ and TNF- $\alpha$ in the hippocampus ↑ Hippocampal long-term potentiation	Murphy et al. (2012)

2-AG, 2-arachidonoylglycerol; AEA, anandamide; CB1R, cannabinoid receptor type 1; CB2R, cannabinoid receptor type 2; DAGLA, diacylglycerol lipase a; FAAH, fatty acid amide hydrolase; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; MMP7, matrix metallopeptidase 7; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor-gamma coactivator-1alpha; TOMM20, translocase outer mitochondrial membrane 20.

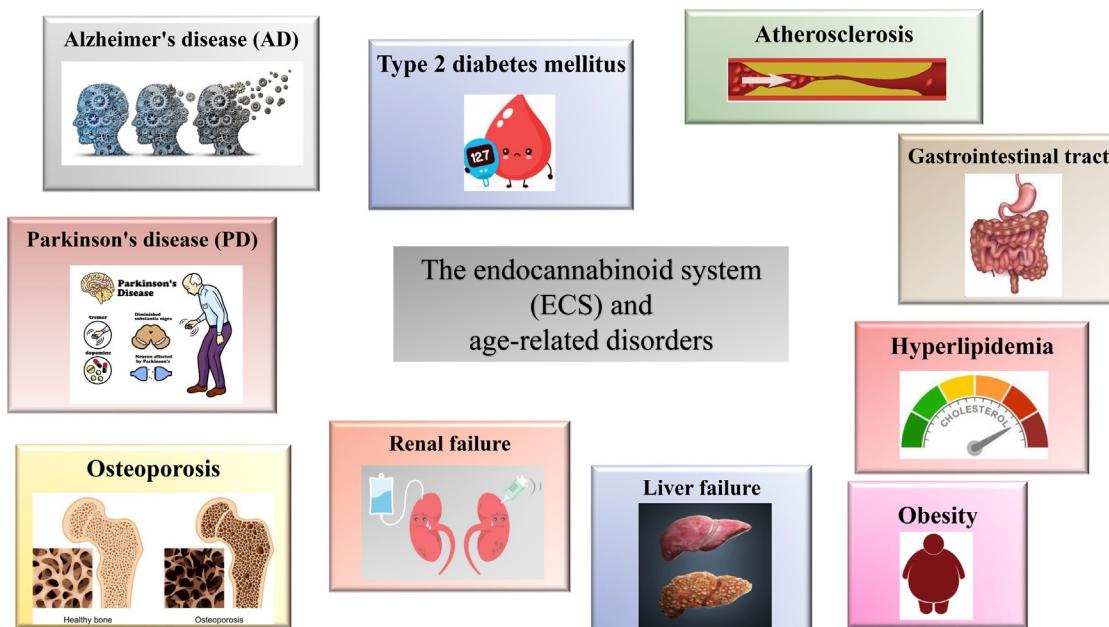
It has been emphasized that the CB2 receptor is critically associated with renal tubular mitochondrial dysfunction and kidney aging. In both naturally aged and D-galactose-induced aging mice, CB2 expression was significantly upregulated along with the reduced mitochondrial mass in the kidney tissue. Furthermore, D-galactose leads to upregulation of  $\beta$ -catenin, diminishes ATP production, and downregulated the peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1 $\alpha$ ), as a key factor in regulating mitochondrial biogenesis, and translocase of outer mitochondrial membrane 20 (TOMM20) as a mitochondrial marker. Additionally, gene deletion of CB2 markedly alleviated the  $\beta$ -catenin activation while stimulating the ATP production and PGC-1 $\alpha$  and TOMM20 expression. Taken together, the CB2 receptor is responsible for the D-galactose-induced kidney aging through activating  $\beta$ -catenin signaling (Zhou et al., 2021).

## ECS and age-related disorders

Numerous animal and human studies supported the age-related changes in ECS, indicating the important involvement of ECS in aging and age-related disorders. In this regard, there is plenty of evidence noticing the decrease in CB1 mRNA expression levels in old age rodents (Rodríguez De Fonseca et al., 1993), dogs (Freundt-Revilla et al., 2017), and also humans (Glass et al., 1997). Furthermore, CB1 receptor knockout mice presented symptoms of accelerated aging, such as neuronal loss, neuroinflammation (Albayram et al., 2011), memory deficits, and early loss of subdermal fat (Albayram et al., 2012). CB2 receptor knockout mice also showed severe osteoporosis reminiscent of accelerated aging (Bab & Zimmer, 2008). Taken together, disruption of ECS accelerates age-related damages in several tissues and organs that emphasize the implication of ECS in aging (Fig. 2).

### Brain aging and neurodegenerative disorders

The prevalence of multiple neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease, increases by age (Baker & Petersen, 2018). Previous studies emphasized that abnormal protein accumulation, excitotoxicity, lysosomal system impairment, oxidative stress, and neuroinflammation are responsible for the pathogenesis of brain aging (Si et al., 2021). Interestingly, the prominent role of neuroinflammation in brain aging and neurodegenerative diseases has been well established in the last two decades (Ruiz-Valdepeñas et al., 2010). Considering the high expression of ECS constituents in the CNS and their alteration in various age-related neurodegenerative diseases, determining that ECS may ameliorate these conditions leading to diminishing disease symptoms or progression (Di Marzo et al., 2015).



**FIG. 2** The endocannabinoid system (ECS) and age-related disorders.

The healthy brain represents high expressions of CB1 receptors in the dendritic tree and axon terminals and low levels of expression of CB2 receptors in the resting glial cells. Additionally, the production of eCBs is high in neurons and low in microglia in the healthy brain. However, during the age-related neurodegenerative diseases, the expression profile of ECS changes to lower expression of CB1 receptors in the dendritic tree and axon terminal of neurons and higher expression levels of CB2 receptors in the activated microglia. This provided a decreased production of eCBs by neurons and enhanced production of eCBs by microglia. (Stella, 2010). In this regard, an upregulated ECS with a strong upregulation of CB2 receptors in glial cells has been confirmed in dogs with Steroid-Responsive Meningitis-Arteritis and Intraspinal Spirocerosis as two models of inflammatory CNS disorders (Freundt-Revilla et al., 2018). CB1 receptor knockout mice represent age-dependent accelerated cognitive dysfunction, a faster loss of hippocampal CA1 and CA3 neurons, an elevated number of activated glial cells, and higher levels of inflammatory cytokines (Bilkei-Gorzo et al., 2012). These findings highlighted the ECS as a beneficial target in neuroinflammation-associated diseases (Tables 2 and 3).

### ECS and Alzheimer's disease (AD)

AD is one of the most common age-related disorders defined as a progressive decline of memory, cognition, and other mental functions. Plenty of studies evaluated the effects of ECS in AD in humans and animals. Different studies have reported a significant decrease (Solas et al., 2013) or no significant differences (Lee et al., 2010) in CB1 receptors in human postmortem brain samples from AD donors. Noteworthy, elevated levels of the CB1 receptor were observed in the early stages of AD, followed by a meaningful decrement as the disease progresses. These changes in CB1 expression levels may support its therapeutic potential in AD progression (Manuel et al., 2014). In contrast, the propagated expression levels of CB2 receptor in microglial cells have been reported in human AD patients (Ramírez et al., 2005). Interestingly, the expression levels of the CB2 receptor were correlated with the levels of A $\beta$ (42) and senile plaque score, as two molecular targets of AD (Solas et al., 2013). Additionally, it has been emphasized that the levels of AEA and its precursor N-arachidonoyl phosphatidylethanolamine (NArPE) notably diminished in the cortical areas (Jung et al., 2012) while FAAH levels markedly increased in the human AD samples (Benito et al., 2003).

Interestingly, in animal models of AD, WIN-55,212-2 (CB1R and CB2R agonist) and JWH-133 (selective CB2R agonist) remarkably alleviated neuroinflammation and A $\beta$  levels and improved cognitive performance (Fakhfouri et al., 2012; Martín-Moreno et al., 2012). Furthermore, administration of exogenous endocannabinoids such as AEA (Milton, 2002) and 2-AG (Janefjord et al., 2014) protects against A $\beta$ -induced neurotoxicity and neurodegeneration in cultured hippocampal neurons. Additionally, the indirect increment of endocannabinoid levels through the inhibition of MAGL and FAAH showed promising neuroprotective effects. Inactivation of MAGL using JZL184 decreased the A $\beta$  accumulation, neuroinflammation, and neurodegeneration, while improving spatial learning and memory in an animal model of AD (Chen et al., 2012) (Tables 2 and 3).

### ECS and Parkinson's disease (PD)

Parkinson's disease (PD) is considered the second common neurodegenerative and age-related disease that is frequently known by rigidity, tremor, and bradykinesia symptoms. It is usually characterized by the dopamine deficit caused by the progressive loss of dopaminergic neurons in the substantia nigra (Udupa et al., 2022). Plenty of evidence showed the involvement of ECS and the pathophysiology of PD. In this regard, the CB1 receptor mRNA level in postmortem brain tissues from PD patients was diminished in the caudate nucleus, anterior dorsal putamen, and an outer segment of the globus; however, no significant changes were observed in the other brain areas (Hurley et al., 2003). Moreover, the AEA level was elevated in the cerebrospinal fluid of PD patients (Pisani et al., 2005). The increment in CB2 receptor expression in microglial cells was also reported in the postmortem human substantia nigra of PD patients (Gómez-Gálvez et al., 2016; Navarrete et al., 2018).

In addition to clinical studies, the contribution of ECS and PD was also supported in numerous animal and cellular models of PD. In LPS-lesioned mice, CB2 receptor expression was stimulated in the striatum and substantia nigra (Gómez-Gálvez et al., 2016). Furthermore, it has been emphasized that the levels of 2-AG (Mounsey et al., 2015) and AEA notably propagated in the striatum while FAAH activity strikingly reduced in the animal model of PD (Maccarrone et al., 2003). Interestingly, in the genetically mutant mice PD model, the CB1 receptor mRNA level and CB1 receptor binding significantly attenuated in the caudate-putamen, substantia nigra, and globus pallidus at early stages. In contrast, both mRNA levels and binding for the CB1 receptor markedly increased in these areas at older ages (García-Arencibia et al., 2009).

In addition, treatment with nonselective cannabinoid receptor agonists (WIN55,212-2 and HU210) meaningfully elevated dopamine neurons survivals in substantia nigra, dopamine levels in the striatum, and motor function, while

remarkably mitigating the production of reactive oxygen species and expression of proinflammatory cytokines from activated microglia (Chung et al., 2011). Treatment with HU308 (selective CB2 receptor agonist) provided a significant decrement in LPS-induced neurodegeneration, CD68 immunostaining, and iNOS expression in mice (Gómez-Gálvez et al., 2016). In rotenone-induced PD in rats, treatment with  $\beta$ -caryophyllene (BCP), a naturally occurring CB2 receptor agonist, notably alleviated the levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , NF- $\kappa$ B, COX-2, iNOS, and oxidative stress while markedly elevating the antioxidant enzyme activity and dopaminergic neurons survival in the substantia nigra (Javed et al., 2016). Moreover, AEA injection significantly diminished the IL-1 $\beta$  protein levels in the hypothalamus, while stimulating the IL-1 $\beta$  and IL-1 receptor antagonist (IL-1RN) in the hypothalamic structures following LPS-induced inflammation ewes (Tomczyk et al., 2021) (Tables 2 and 3).

**TABLE 2** ECS changes in Alzheimer's disease and Parkinson's disease.

Study model	Changes in the ECS	Ref.
Postmortem brain tissues of AD patients	↓ CB1R expression in frontal cortex ↑ CB2R expression in frontal cortex CB2 expression was correlated with A $\beta$ (42) levels and senile plaque score	Solas et al. (2013)
Postmortem brain tissues of AD patients	Unchanged CB1R expression in the frontal cortex, anterior cingulate gyrus, hippocampus, and caudate nucleus	Lee et al. (2010)
Postmortem brain tissues of AD patients	↑ CB2 receptor expression in microglial cells	Ramírez et al. (2005)
Postmortem brain tissues of AD patients	↓ AEA and NArPE levels in the cortical areas	Jung et al. (2012)
Postmortem brain tissues of AD patients	↑ FAAH levels ↑ CB2R in neuritic plaque-associated astrocytes and microglia Unchanged CB1R in the whole brain	Benito et al. (2003)
Postmortem brain tissues of AD patients	↓ FAAH activity in the frontal cortex	Pascual et al. (2014)
Postmortem brain tissues of PD patients	↓ CB1 receptor mRNA level in the caudate nucleus, anterior dorsal putamen and an outer segment of the globus pallidus Unchanged CB1 receptor mRNA level in other brain areas	Hurley et al. (2003)
Cerebrospinal fluid of PD patients	↑ AEA level	Pisani et al. (2005)
Postmortem human substantia nigra of PD patients	↑ CB2 receptor expression in microglial cells	Gómez-Gálvez et al. (2016)
Postmortem brain tissues of PD patients	Unchanged CB1 receptor gene expression in the substantia nigra ↑ CB1 receptor gene expression in the putamen ↑ CB2 receptor gene expression (fourfold) in the substantia nigra ↓ CB2 receptor gene expression in the putamen ↓ MAGL gene expression in the substantia nigra and ↑ in the putamen	Navarrete et al. (2018)
LPS-lesioned mice	↑ CB2 receptor expression in striatum and substantia nigra ↑ CD68 immunostaining in striatum	Gómez-Gálvez et al. (2016)
MPTP-induced PD in mice	↑ 2-AG in substantia nigra	Mounsey et al. (2015)
6-Hydroxydopamine-induced PD in rat	↑ AEA in striatum ↓ FAAH activity	Maccarrone et al. (2003)
MPTP-induced PD in monkey	↑ 2-AG in striatum and substantia nigra ↑ AEA in striatum and globus pallidus	Van Der Stelt et al. (2005)
PARK1, PARK2 and PARK6 mutant mice model of PD	↓ CB1R-mRNA and receptor binding in caudate-putamen, substantia nigra and globus pallidus in early stages ↑ CB1R-mRNA and receptor binding in older age	García-Arencibia et al. (2009)

2-AG, 2-arachidonoylglycerol; AD, Alzheimer's disease; AEA: anandamide; A $\beta$ , beta-amyloid; CB1R, cannabinoid receptor type 1; CB2R, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NArPE, N-arachidonoyl phosphatidylethanolamine; PD, Parkinson's disease.

**TABLE 3** ECS targeted pharmacological compounds in Alzheimer's disease and Parkinson's disease.

Study model	Drugs	Changes in the ECS	Ref.
Transgenic amyloid precursor protein 2576 mice	WIN-55,212-2 JWH-133	↓ Neuroinflammation, COX-2 protein levels and TNF- $\alpha$ mRNA expression ↓ A $\beta$ levels ↑ Cognitive performance	Martín-Moreno et al. (2012)
A $\beta$ -induced neurodegeneration in rat hippocampus	WIN-55,212-2	↑ PARP signaling ↓ A $\beta$ -induced neuroinflammation and neurodegeneration ↑ Memory function ↓ TNF- $\alpha$ , active caspase 3, and nuclear NF- $\kappa$ B levels	Fakhfouri et al. (2012)
Human teratocarcinoma cell line, Ntera 2/cl-D1 neurons	AEA	↓ A $\beta$ -induced neurotoxicity	Milton (2002)
Cultured hippocampal neurons	2-AG	↓ A $\beta$ -induced neurodegeneration ↓ ERK1/2 and NF- $\kappa$ B phosphorylation ↓ COX-2 expression	Chen et al. (2011)
SHSY5Y neuroblastoma cells	2-AG	↓ A $\beta$ -induced neurotoxicity	Janefjord et al. (2014)
5XFAD APP transgenic mice	JZL184	↓ A $\beta$ accumulation, neuroinflammation, and neurodegeneration ↓ Spatial learning and memory	Chen et al (2012)
NO <sub>2</sub> -induced AD in C57BL/6J and APP/PS1 mice	JZL184	↓ PGE2 production and neuroinflammation ↓ A $\beta$ 42 accumulation	Yan et al. (2016)
LPS-lesioned mice	HU-308	↓ LPS-induced neurodegeneration ↓ CD68 immunostaining ↓ iNOS expression	Gómez-Gálvez et al. (2016)
MPTP-induced PD in mice	WIN55,212-2 and HU210	↑ Dopamine neurons survivals in substantia nigra ↑ Dopamine levels in the striatum ↑ Motor function ↓ Production of reactive oxygen species ↓ Expression of proinflammatory cytokines from activated microglia	Chung et al. (2011)
Rotenone-induced PD in rat	$\beta$ -Caryophyllene	↓ Levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , NF- $\kappa$ B, COX-2, and iNOS ↓ Oxidative stress ↑ Antioxidant enzyme activity ↑ Dopaminergic neurons survival	Javed et al. (2016)
LPS-induced inflammation in ewes	AEA	↓ IL-1 $\beta$ protein levels in the hypothalamus ↑ IL-1 $\beta$ and IL-1 receptor antagonist (IL-1RN) in the hypothalamic structures	Tomczyk et al. (2021)

2-AG, 2-arachidonoylglycerol; AD, Alzheimer's disease; AEA, anandamide; A $\beta$ , beta-amyloid; COX-2, cyclooxygenase-2; HU-210, CB1R and CB2R agonist; HU308, selective CB2R agonist; IL, interleukin; IL-1RN, IL-1 receptor antagonist; iNOS, inducible nitric oxide synthase; JWH-133, selective CB2R agonist; JZL184, irreversible MAGL inhibitor; LPS, lipopolysaccharide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF- $\kappa$ B, nuclear factor kappa B; NO, nitric oxide; PD, Parkinson's disease; PGE2, prostaglandin E2; TNF- $\alpha$ , tumor necrosis factor-alpha; WIN-55, 212 2, CB1R and CB2R agonist.

### ECS and atherosclerosis

Aging-related atherosclerosis and cardiovascular diseases are the most common leading cause of death and are associated with chronic arterial wall inflammation with subendothelial plaque formation (Gholoobi et al., 2021). Several studies emphasized the implication of ECS in the pathogenesis of atherosclerosis. In normal-weight coronary artery disease patients, the levels of AEA and 2-AG in serum were significantly greater than that in healthy controls. Moreover, the CB1 receptor mRNA expression was markedly enhanced in lipid-rich atheromatous plaques, especially in CD68 macrophages, than fibrous plaques. The CB1 receptor expression was increased while the CB2 receptor expression significantly

decreased in cultured human macrophages during monocyte-macrophage differentiation. In addition, CB1 receptor antagonist (rimonabant) provided a significant decrement in c-Jun N-terminal kinase phosphorylation, resulting in a meaningfully attenuated production of proinflammatory mediators including IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , and matrix metalloproteinase 9 in lipopolysaccharide-activated human macrophages (Sugamura et al., 2009). Furthermore, the atheroprotective role of CB2 was highlighted in various experiments; thus, JWH-133 (selective CB2 agonist) alleviated atherosclerotic lesion formation and atherogenesis and ROS generation while enhancing endothelial function following the high-cholesterol diet in Apolipoprotein E $-/-$  (ApoE $-/-$ )CB2 $-/-$  double knockout mice (Hoyer et al., 2011). Interestingly, pharmacological FAAH inhibitor URB597 propagated the development of unstable plaques and atherosclerotic plaque vulnerability in ApoE $-/-$  mice fed with a high-fat diet (Hoyer et al., 2014).

It has been noticed that ECS is also involved in regulating lipid metabolism. CB1 activation stimulates the bile/fatty acid synthesis and triglyceride (TG) accumulation while mitigating the apolipoprotein A1 secretion in the liver, leading to increased TG levels and decreased HDL levels in serum. Besides, CB1 activation elevated the CD36 uptake and diminished ATP-binding cassette transporter 1 (ABCA1) efflux in macrophages, resulting in intracellular cholesterol accumulation and NLR family pyrin domain-containing 3 (NLRP3) inflammasome activation. CB1 activation also stimulates fatty acid synthesis in the adipose tissue (Guillamat-Prats et al., 2019). Taken together, blocking CB1 signaling in the vasculature and peripheral organs may be a beneficial therapeutic target for improving atherosclerosis and related metabolic complications.

### *ECS and Type 2 diabetes mellitus (T2DM)*

Type 2 diabetes mellitus (T2DM) is closely related to the aging process and is considered one of the most widespread and alarming public health problems in the elderly ( $\geq 60$ –65 years old) in developed and even in developing countries (Longo et al., 2019). Increased ECS, primarily through activation of CB1 signaling, notably propagated food intake, energy storage, body fat accumulation resulting in obesity-associated metabolic disorders. In plasma of obese human subjects, the levels of EAE and 2-AG significantly elevated compared with nonobese controls (Little et al., 2018). Additionally, patients with T2DM also represented higher plasma levels of eCBs such as EAE and 2-AG than nondiabetic subjects with same adiposity. Chronic circulating eCBs lead to hampered beta-cell function, increased inflammation, and beta-cell apoptosis. Interestingly, the eCBs levels significantly reduced by interventions that ameliorate insulin resistance and dyslipidemia, including weight loss, dietary interventions, negative energy balance, and bariatric surgery (Veilleux et al., 2019).

Upregulation of CB1 receptor expression has also been reported in both visceral and subcutaneous white adipose tissue of patients with T2DM and insulin resistance independent of BMI (Sidibeh et al., 2017). Adipocyte-specific inducible deletion of the CB1 gene in mice ameliorated the diet-induced obesity, body weight, total adiposity while reducing insulin resistance and energy expenditure (Ruiz De Azua et al., 2017). CB1 blockade using rimonabant meaningfully normalized insulin secretion, glucose homeostasis, and protecting against  $\beta$ -cell loss in diabetic obese rats (Duvivier et al., 2009). Taken together, peripheral CB1 receptors may be an interesting target for the treatment of type 2 diabetes and dyslipidemia.

Although the CB2 receptor has been much less investigated in diabetes, CB2 receptor agonists such as JWH133 or SER601 notably improved glucose homeostasis, insulin sensitivity, and  $\beta$ -cell function in animal models of T2DM (Zhang et al., 2016). These studies may suggest the beneficial role of the CB2 receptor in T2DM.

## Applications to other areas

In this chapter, we have reviewed the implication of the endocannabinoid system on aging and age-related disorders. The population is becoming globally aged, and almost every country faces an increase in the proportion of older persons. Inflammation and oxidative stress are considered hallmarks of aging development. Numerous animal and human studies supported the age-related changes in ECS, indicating the important involvement of ECS in aging and age-related disorders. During the age-related neurodegenerative diseases, the expression profile of ECS changes to lower expression of CB1 receptors in the dendritic tree and axon terminal of neurons and higher expression levels of CB2 receptors in the activated microglia. This provided a decreased production of eCBs by neurons and enhanced production of eCBs by microglia. Considering the high expression of ECS constituents in the CNS and their alteration in various age-related neurodegenerative diseases such as AD and PD, ECS may alleviate these conditions, leading to diminishing disease symptoms or progression. Elevated levels of the CB1 receptor were observed in the early stages of AD, followed by a meaningful decrement as the disease progresses that may support its therapeutic potential in AD progression. In contrast, the propagated expression levels of CB2 receptor in microglial cells have been reported in human AD patients, which was correlated with the levels of A $\beta$ (42) and senile plaque score, as two molecular targets of AD. Additionally, the CB1 receptor mRNA level in post-mortem brain tissues from PD patients was diminished in the caudate nucleus, anterior dorsal putamen, and an outer

segment of the globus. In contrast, the increment in CB2 receptor expression in microglial cells was reported in the post-mortem human substantia nigra of PD patients.

Several studies also emphasized the implication of ECS in the pathogenesis of atherosclerosis. In normal-weight coronary artery disease patients, the levels of AEA and 2-AG in serum were significantly greater than those in healthy controls. Moreover, the CB1 receptor expression was increased while the CB2 receptor expression significantly decreased in cultured human macrophages during monocyte-macrophage differentiation. In addition, the CB1 receptor antagonist provided significant antiinflammatory effects in lipopolysaccharide-activated human macrophages, and the atheroprotective role of CB2 was highlighted in various experiments. Taken together, blocking CB1 signaling in the vasculature and peripheral organs may be a beneficial therapeutic target for improving atherosclerosis and related metabolic complications.

The changes in ECS in patients with T2DM have also been reported. Patients with T2DM also represented higher plasma levels of eCBs such as EAE and 2-AG than nondiabetic subjects with the same adiposity. The eCBs levels were significantly reduced by interventions that alleviate insulin resistance and dyslipidemia, including weight loss, dietary interventions, negative energy balance, and bariatric surgery. Upregulation of CB1 receptor expression has also been reported in both visceral and subcutaneous white adipose tissue of patients with T2DM and insulin resistance independent of BMI. CB1 blockade meaningfully normalized insulin secretion glucose homeostasis and protected against  $\beta$ -cell loss in diabetic obese rats. CB2 receptor agonists notably improved glucose homeostasis, insulin sensitivity, and  $\beta$ -cell function in animal models of T2DM. Taken together, peripheral CB1 receptors may be an exciting target for the treatment of type 2 diabetes and dyslipidemia.

## Minidictionary of terms

- **Aging:** A progressive, time-related, accumulative, and natural phenomenon that affects almost all living organisms and leads to an irreversible deterioration in the physiological functions of all molecules, cells, tissues, and organs of an organism.
- **Aging-related atherosclerosis:** are associated with chronic arterial wall inflammation with subendothelial plaque formation.
- **Age-related disorders:** Well-recognized pathologies that aging give rise to, including osteoporosis, atherosclerosis, heart failure and cardiovascular diseases, pulmonary insufficiency, liver and renal failure, cancers, type 2 diabetes, immune system dysfunction, and neurodegenerative diseases, especially Alzheimer's disease and Parkinson's diseases.
- **Alzheimer's disease (AD):** One of the most common age-related disorders defined as a progressive decline of memory, cognition, and other mental functions.
- **Endocannabinoid system (ECS):** Consists of the two G-protein-coupled cannabinoid receptors, CB1 (type 1) and CB2 (type 2), and the endogenous ligands agonists known as endocannabinoids (eCBs).
- **Parkinson's disease (PD):** The second common neurodegenerative and age-related disease that is frequently known by rigidity, tremor, and bradykinesia symptoms and is usually characterized by the dopamine deficit caused by the progressive loss of dopaminergic neurons in the substantia nigra.
- **Senescence-associated secretory phenotype (SASP):** the final feature of the senescent cells that includes the secretion of chemokines, growth factors, proteases, and various inflammatory cytokines.
- **Type 2 diabetes mellitus (T2DM):** Accounts for about 90–95% of diabetic patients, and also referred to as noninsulin-dependent diabetes, encompasses individuals who have hyperglycemia, insulin resistance, and relative insulin deficiency.

## Key facts of the endocannabinoid system and aging

- The population is becoming globally aged, and almost every country faces an increase in the proportion of older persons.
- The population of people aged  $\geq 65$  is globally increasing faster than all other younger age groups.
- Aging presents as a mosaic and dynamic process that propagates the probability of death and limits the life of an organism.
- The chronic inflammation caused by these markers is a significant cause or main contributor to almost every age-related disorder.
- D-Galactose is frequently used to induce accelerated aging in animals that strongly mimics the human aging symptoms.
- There is plenty of evidence noticing the decrease in CB1 mRNA expression levels in old-age rodents, dogs, and humans.

- The healthy brain represents expressions of CB1 receptors highly in the dendritic tree and axon terminals and low levels of expression of CB2 receptors in the resting glial cells along with the high production of eCBs in neurons and low in microglia.
- Aging-related atherosclerosis and cardiovascular diseases are the most common leading cause of death globally.
- Type 2 diabetes mellitus (T2DM) is closely related to the aging process and is considered one of the most widespread and alarming public health problems in the elderly ( $\geq 60$ – $65$  years old) in developed and even in developing countries.

## Summary points

- Numerous animal and human studies supported the age-related changes in ECS, indicating the important involvement of ECS in aging and age-related disorders.
- CB1 receptor knockout mice presented symptoms of accelerated aging, such as neuronal loss, neuroinflammation, memory deficits, and early loss of subdermal fat.
- CB2 receptor knockout mice also showed severe osteoporosis reminiscent of accelerated aging.
- Elevated levels of the CB1 receptor were observed in the early stages of AD, followed by a meaningful decrement as the disease progresses that may support its therapeutic potential in AD progression.
- The propagated expression levels of CB2 receptor in microglial cells have been reported in human AD patients, which was correlated with the levels of A $\beta$  (42) and senile plaque score, as two molecular targets of AD.
- The CB1 receptor mRNA level in postmortem brain tissues from PD patients was diminished in the caudate nucleus, anterior dorsal putamen, and an outer segment of the globus.
- The increment in CB2 receptor expression in microglial cells was reported in the postmortem human substantia nigra of PD patients.
- Blocking CB1 signaling in the vasculature and peripheral organs may be a beneficial therapeutic target for improving atherosclerosis and related metabolic complications.
- Patients with T2DM represented higher plasma levels of eCBs such as EAE and 2-AG than nondiabetic subjects with the same adiposity.
- Chronic circulating eCBs lead to hampered beta-cell function, increased inflammation, and beta-cell apoptosis.
- Upregulation of CB1 receptor expression has also been reported in both visceral and subcutaneous white adipose tissue of patients with T2DM and insulin resistance independent of BMI.

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## Chapter 5

# The endocannabinoid system and posttraumatic stress disorder (PTSD): A new narrative

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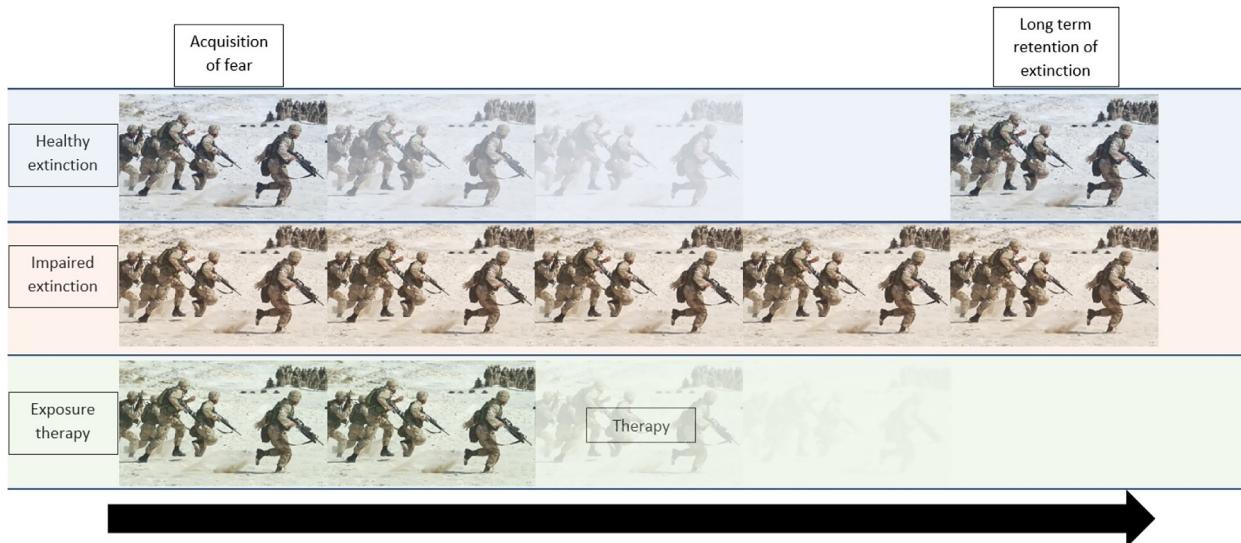
## Abbreviations

2-AG	2-arachidonoyl glycerol
AEA	anandamide
CB1	cannabinoid receptor 1
FAAH	fatty acid amide hydrolase
OEA	oleoylethanolamide
PEA	palmitoylethanolamide
PTSD	posttraumatic stress disorder
SEA	stearoylethanolamide
SNP	single nucleotide polymorphism
THC	delta9-tetrahydrocannabinol

## Introduction

Posttraumatic stress disorder (PTSD) is a highly debilitating psychiatric disorder that develops in some individuals following exposure to one or more traumatic events (e.g., physical and sexual assault, motor vehicle accidents, combat). PTSD is characterized by intrusive recollection of trauma-related memories, avoidance of people and places that trigger recall of trauma memories, negative alterations in cognition and mood, and prolonged physiological hyperarousal and vigilance toward perceived threats associated with the trauma (American Psychiatric Association, 2013). Global lifetime prevalence of PTSD is approximately 3.9% and tends to occur in up to 10% of people who experience trauma (Koenen et al., 2017). The negative personal and societal effects of PTSD are acknowledged to be among the most severe of psychiatric disorders (Ivanova et al., 2011). Attempts to treat PTSD often include psychotherapy alone or in combination with pharmacological approaches (e.g., selective serotonin reuptake inhibitors, SSRIs: sertraline and paroxetine). Exposure-based cognitive behavioral therapies (e.g., prolonged exposure) are the most commonly administered and most effective psychotherapeutic options for adults with PTSD—although there is room for improvement, as many fail to experience a significant reduction in symptoms and remission rates are around 50%–60% (Bisson et al., 2013).

Mechanistically, PTSD is believed to be a result of extreme stress reactivity to trauma, which results in maladaptive memory consolidation and impaired fear extinction of the event (Ney, Schenker, & Lipp, 2022; Pitman et al., 2012). In fact, exposure-based therapies are based on the mechanisms of fear conditioning and extinction learning (Bouton et al., 2020). For instance, trauma-related memories can be conceptualized as conditioned stimuli (CS+) that can trigger anxiety responses (conditioned responses), since details embedded within the trauma memory are associated with the occurrence of the actual trauma event(s) (unconditioned stimulus) (Crombie et al., 2022; Ney, Crombie, et al., 2022). As such, a major component of treatment involves gradual, repeated exposure (in a safe context) to trauma-related memories and cues (CS+) in an attempt to weaken the predictive value of the conditioned stimuli. This process is thought to weaken the ability of trauma-related stimuli and cues to elicit distress and anxiety (i.e., promotes safety learning). In other words, the association



**FIG. 1** Fear conditioning involves acquisition of fear memories, as well as learning and retention of an extinction memory. Fear is learned during the acquisition stage (e.g., during trauma) and then extinguished during extinction. Extinction can occur naturally or may be impaired (as in PTSD), in which case exposure therapies are used to encourage extinction through repeated exposure to the feared stimuli or situation in a safe context. The extinction memory competes with the fear memory and can be retained long term, but relapse is common both following treatment and following successful natural extinction of fear. Pharmacotherapies are being explored as potential ways to enhance extinction learning and retention, particularly in combination with exposure therapies. Note: In any condition a patient may relapse.

between the CS+ and unconditioned stimulus (i.e., the original fear memory) is not erased during exposure therapy; rather, therapy tries to promote new inhibitory learning that is ideally retrieved over time and in different contexts in the future (Bouton et al., 2020). Typically, someone who does not develop PTSD following trauma exposure will experience greater retrieval of the learned safety memory over time (Fig. 1). In contrast, individuals who develop PTSD often exhibit impaired extinction learning, which thereby contributes to poor retrieval of learned safety memories in the future. However, in all cases it is possible for an individual to experience the return of fear (i.e., relapse; see Fig. 1) regardless of whether extinction occurred naturally or as result of therapy (Bouton et al., 2020). Fear extinction learning can be fragile and the return of fear following successful fear extinction learning is a relatively common phenomenon. For instance, fear can return following the passage of time (spontaneous recovery), when in a context that is the same or similar to the context in which the original fear memory was formed (renewal) or following exposure to the unconditioned stimulus that instantiated the original fear learning (reinstatement).

The role of the endocannabinoid system in this process—particularly with respect to fear extinction—has been extensively explored in animal models of the disorder. Early studies identified that cannabinoid receptor 1 (CB1) is essential for fear extinction (Marsicano et al., 2002), and later that key components of endocannabinoid signaling such as arachidonoyl ethanolamide (AEA), 2-arachidonoyl glycerol (2-AG) and the degrading enzyme fatty acid amide hydrolase (FAAH) also regulate the efficiency of extinction learning in animals (Dincheva et al., 2015; Gunduz-Cinar et al., 2013; Hill et al., 2018; Ney et al., 2019). The emerging hypothesis from the preclinical literature is dysregulation of the endocannabinoid system—either as a direct result from trauma or due to genetic predisposition—may explain why fear extinction is impaired in individuals who develop PTSD. Consequently, activation of CB1 (e.g., via THC) or indirect activation through elevation of AEA through inhibition of the FAAH enzyme might improve PTSD symptoms in humans (Hill et al., 2018; Mayo et al., 2021).

## Association between PTSD and endocannabinoid biomarkers

The capacity to test whether these findings translate to human participants has improved considerably over the past decade (Ney, Crombie, et al., 2022). Cannabinoid receptors and enzymes can now be imaged using positron emission tomography (Chen et al., 2021; Hamilton et al., 2021; Wilson et al., 2011), and in addition to plasma or serum quantification, endocannabinoids are now quantifiable in saliva (Matias et al., 2012; Ney et al., 2020) and hair (Gao et al., 2020; Krumbholz et al., 2013; Ney, Felmingham, et al., 2021). Further, there are an increasing number of endocannabinoid single-nucleotide polymorphisms (SNPs; see Table 2) recognized to be important to human psychopathology and behavior

(Hillard et al., 2012), and measurements of endocannabinoids in blood samples are becoming more precise (Fanelli et al., 2012). Despite this, there are still relatively few clinical studies linking PTSD diagnosis and/or symptomology to endocannabinoid phenotypes.

Previous research suggested that circulating concentrations of endocannabinoids may be altered in PTSD, although findings have been mixed, possibly in part due to heterogeneous samples (e.g., combat, survivors of terrorist attacks, and motor vehicle accidents) and relatively small sample sizes (Table 1) (Ney et al., 2018). For instance, sampling from a cohort in close proximity to the World Trade Center 9/11 attacks, Hill et al. (2013) reported that plasma 2-AG but not AEA levels were significantly lower among participants who met the criteria for PTSD compared with those who did not. Similarly, Wilker et al. (2016) reported that Ugandan war survivors with PTSD had significantly lower oleoylethanolamide (OEA), but not palmitoylethanolamide (PEA) or stearoylethanolamide (SEA), compared with healthy war survivors, in hair samples as measured by mass spectrometry. Neumeister et al. (2013) found lower AEA plasma levels in a

**TABLE 1** Studies testing the association between endocannabinoid biomarkers and PTSD diagnosis and symptomology.

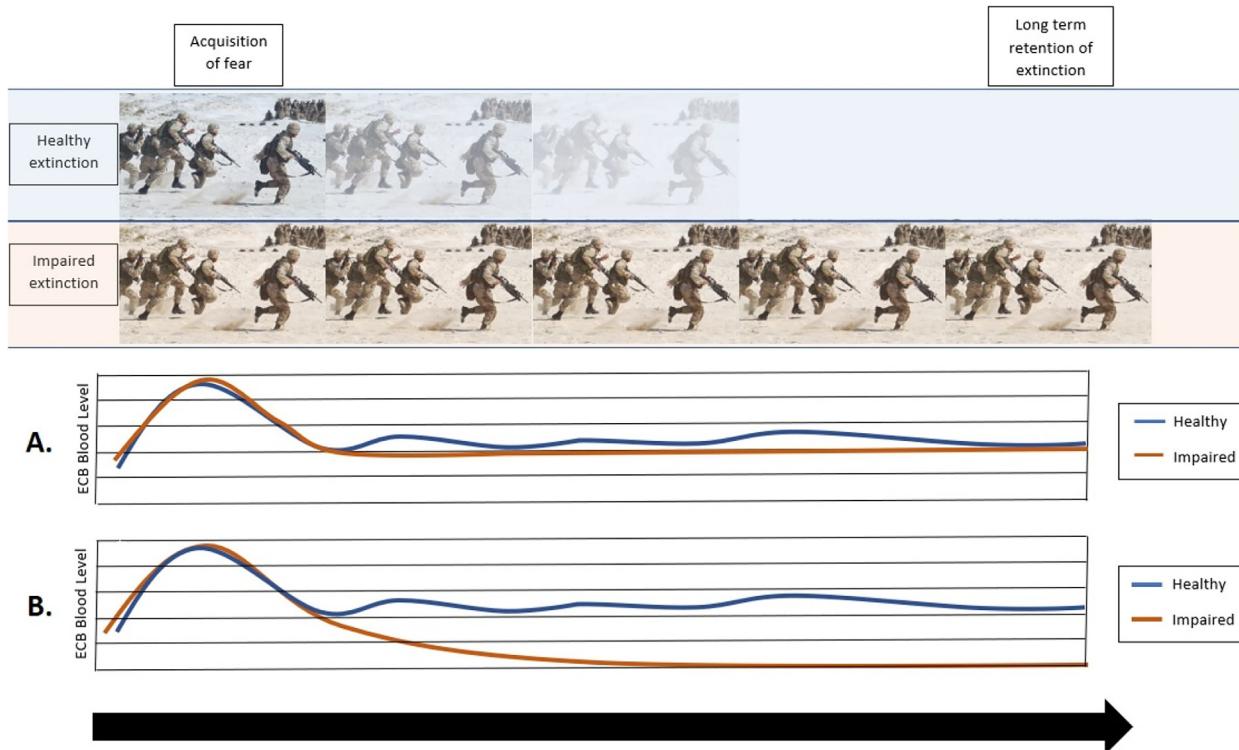
Study	Population	Sample size	Experimental design	Outcome
Hauer et al. (2013)	Trauma-exposed (war/trauma) and HCs, mixed race	48 (9 PTSD, 10 trauma controls)	Cohort study	Plasma AEA, 2-AG, OEA, and SEA were significantly higher compared to HCs and 2-AG and PEA were significantly higher compared to trauma controls
Hill et al. (2013)	Close proximity to World Trade Center attacks	46 (24 PTSD)	Cohort study	Plasma levels of 2-AG but not AEA were significantly lower in PTSD
Neumeister et al. (2013)	Noncombat related PTSD in men and women	60 (25 PTSD, 12 trauma controls)	Cohort study	PTSD group exhibited significantly lower AEA compared to trauma-exposed adults without PTSD and healthy controls
Schaefer et al. (2014)	German PTSD patients (childhood sexual abuse) and HCs	51 (21 PTSD)	Prospective cohort study	Significantly higher plasma OEA, trending lower 2-AG, no difference in AEA or PEA, in PTSD
Wilker et al. (2016)	Ugandan war veterans	76 (38 PTSD)	Cohort study	Hair levels of OEA, but not PEA or SEA, were significantly lower in PTSD
Crombie et al. (2018)	American adults (75% women) with PTSD and HCs	24 (12 PTSD)	Stress induction	No group differences in plasma lipids at baseline. AEA, OEA, and 2-AG significantly increased following exercise. HCs exhibited a greater magnitude of change for AEA, OEA, and 2-AG following exercise compared to PTSD
Crombie et al. (2019)	American women with IPV PTSD and HCs	20 (10 PTSD)	Stress induction	Higher plasma PEA but not AEA, 2-AG, or OEA in PTSD at baseline. All lipids showed stress reactivity except for 2-AG in PTSD group
Crombie, Cisler, et al. (2021) and Crombie, Sartin-Tarm, et al. (2021)	American women with IPV PTSD, trauma-exposed women without PTSD, trauma-free HC women	42 (14 PTSD, 14 trauma controls)	Stress induction	Significantly greater increase in plasma AEA, OEA, PEA, and 2-AG following an acute bout of moderate-intensity aerobic exercise compared to quiet rest control condition. Magnitude of change following aerobic exercise was greater for those without PTSD
deRoon-Cassini et al. (2022)	American patients in acute physical trauma hospital	170 (50 PTSD)	Prospective, longitudinal study	Plasma AEA and 2-AG levels elevated at hospitalization but not at 6-month follow-up

AEA, arachidonoyl ethanolamide; 2-AG, 2-arachidonoyl glycerol; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; SEA, stearoylethanolamide; HC, healthy controls; IPV, interpersonal violence.

noncombat-related PTSD group compared with healthy control and trauma-exposed groups. This study also reported higher CB1 availability relative to controls, which was measured using positron emission tomography (Neumeister et al., 2013).

In contrast to this, Hauer et al. (2013) found that participants with PTSD (mostly refugees with exposure to war and/or torture) had significantly higher AEA, 2-AG, OEA, and SEA plasma concentrations compared with healthy controls and higher 2-AG and PEA plasma concentrations compared with trauma-exposed controls. Schaefer et al. (2014) reported no differences between PTSD participants stemming from childhood sexual abuse and healthy controls in plasma AEA or PEA, but reported marginally lower 2-AG levels and significantly higher OEA levels. deRoon-Cassini et al. (2022) also reported that serum AEA and 2-AG levels were higher at hospital admission for participants who later went on to develop PTSD, but there were no group differences at a 6-month follow-up (Table 1). However, in participants belonging to racial/ethnic minorities, 2-AG was significantly positively correlated with PTSD severity at the follow-up and AEA levels were significantly, negatively correlated with PTSD severity at hospitalization (deRoon-Cassini et al., 2022). In the overall sample, the findings from this study are similar to a recent animal study that found significantly higher 2-AG (but not AEA) concentrations in PTSD-phenotype rats 1 day but not 8-day poststress exposure, where endocannabinoid concentrations were lower (Danan et al., 2021), suggesting that extreme endocannabinoid responses to stress in the short term may be associated with later PTSD onset.

The endocannabinoid-stress literature in healthy humans mostly supports the narrative that endocannabinoids increase immediately following stress as most, but not all, studies have observed 2-AG, and in some cases AEA, increases to experimental stress (Chouker et al., 2010; Crombie et al., 2019; Dlugos et al., 2012; Hill et al., 2009; Mayo, Asratian, Linde, et al., 2020; Ney, Stone, et al., 2021; Spohrs et al., 2022). Some studies also suggest that, during recovery following acute stress (~20+min), peripheral AEA levels are reduced relative to baseline, with lower AEA levels positively associated to increased subjective stress responses (Mayo, Asratian, Linde, et al., 2020; Spohrs et al., 2022). This suggests that endocannabinoids may follow the same long-term trajectory as cortisol following trauma (Fig. 2), where extreme cortisol responses are facilitated during acute trauma exposure (Pitman et al., 2012), whereas long-term PTSD is more likely characterized by reduced cortisol tone (Yehuda, 2009). Interestingly, there is evidence that PTSD is associated with blunted



**FIG. 2** Alteration of peripheral endocannabinoid reactivity and potentially long-term tone by extreme stress or trauma in impaired compared with healthy extinction learning and retention. Based on existing evidence, peripheral endocannabinoid concentrations appear to be elevated following extreme stress and trauma in humans. At the very least, available evidence is compelling that endocannabinoid reactivity to stress in PTSD is impaired long term (A), which may interfere with their capacity to learn and retain fear extinction. Long-term, endocannabinoid tone might also be reduced in PTSD participants (B).

2-AG increases to aerobic exercise (Crombie et al., 2018; Crombie, Cisler, et al., 2021; Crombie et al., 2019; Crombie, Sartin-Tarm, et al., 2021) and psychosocial stress (Crombie et al., 2019) compared with the responses observed in healthy controls without a diagnosis of PTSD; however, long-term self-reported stress is not correlated with hair endocannabinoid levels (Gao et al., 2021). In conclusion, more research is needed to confirm whether endocannabinoid levels are altered long term in PTSD, though existing evidence strongly suggests that endocannabinoid stress reactivity is impaired in PTSD.

## Relationship between human and animal models

It is important to note that the peripheral changes in phasic endocannabinoid concentrations following stress in humans do not entirely mimic the models proposed by animal models of stress (Balsevich et al., 2017; Hill & Tasker, 2012; Morena et al., 2016). Endocannabinoid animal models of stress reactivity postulate that central nervous system reductions in AEA tone—rather than increases—initiate the stress response, while phasic 2-AG elevations result in its termination (Hill & Tasker, 2012; Patel et al., 2004). In humans, reduction in peripheral AEA levels has been shown to occur during recovery from stress, rather than immediately following stress induction (Mayo, Asratian, Linde, et al., 2020; Spohrs et al., 2022). Since the endocannabinoid system is profuse and shows properties of an “on-demand” signalling system (Hillard, 2017), the findings from the human literature cannot be demonstrated to be consistent with the proposed animal models, which use invasive techniques to quantify central nervous system endocannabinoid concentrations. However, one study showed that peripheral AEA responses to acute stress in mice were decreased 15 min after stress induction compared with a nonstress control condition in FAAH rs324420 C/C homozygotes and A/C heterozygotes (but not A/A homozygotes), which replicates some of the findings in the human literature (Mayo, Asratian, Linde, et al., 2020). It is therefore likely that the differences between findings in humans and animals are due to the noninvasiveness of methods for biological sampling in humans, though further refinement of what the peripheral endocannabinoid concentrations reflect is warranted. Existing evidence suggests that circulating endocannabinoids originate from many sources, of which one is the brain (Hillard, 2017). It is possible that the acute increases in endocannabinoid blood levels following stress in humans may reflect the sympathetic nervous system response, whereas the later decrease in blood levels may reflect the state of the central nervous system.

## Relationship between fear conditioning and endocannabinoids in PTSD

Recent studies have also examined the relationship between the endocannabinoid system and fear conditioning in PTSD participants (for a comprehensive review, see Mayo et al., 2021). Substantial preclinical research has demonstrated the vital role of the CB1 in the extinction of a conditioned fear response (Hill et al., 2018; Marsicano et al., 2002; Ney, Akhurst, et al., 2021). These findings have been largely replicated in human models of fear conditioning involving healthy participants, with recent work showing that the presence of a minor allele (A) on the FAAH rs324420 SNP is associated with significantly elevated levels of AEA and improved fear extinction (Dincheva et al., 2015; Mayo, Asratian, Linde, et al., 2020; Zabik et al., 2021). It has also been reported that circulating AEA concentrations are associated with fear extinction efficacy and retention (Crombie, Sartin-Tarm, et al., 2021; Ney, Matthews, et al., 2021; Spohrs et al., 2021), though the role of CB1 receptor SNPs rs1049353 and rs2180618 in fear conditioning is inconsistent (Heitland et al., 2012; Ney, Matthews, et al., 2021). Further, pharmacological intervention with FAAH inhibitors resulted in improved fear extinction recall 24 h after extinction learning in one recent clinical trial in healthy participants (Mayo, Asratian, Lindé, et al., 2020), though there was no significant effect on within-session fear extinction. FAAH inhibition in this study also reduced subjective and autonomic stress responses, as well as negative affect following an acute stressor (Mayo, Asratian, Lindé, et al., 2020). Another recent clinical trial did not find any effect of FAAH inhibition on within-session fear extinction in healthy participants, which is consistent with Mayo, Asratian, Lindé et al. (Paulus et al., 2020), though this study did not assess extinction recall in a subsequent testing session.

Only three studies have tested the relationship between the endocannabinoid system and fear conditioning in PTSD participants (Table 2). In a 3-day behavioral study, Crombie, Cisler, et al. (2021) and Crombie, Sartin-Tarm, et al. (2021) found that administering moderate-intensity aerobic exercise after extinction training resulted in enhanced cognitive indices of extinction recall (reduced threat expectancy ratings following reinstatement) when tested in women with interpersonal violence related PTSD—an effect that was mediated by exercise-induced increases in AEA (Crombie, Cisler, et al., 2021; Crombie, Sartin-Tarm, et al., 2021).

Using fMRI, Crombie et al. (2022) found evidence for higher activation in frontoparietal network areas throughout fear acquisition and extinction in PTSD participants with a minor (A) allele on the FAAH rs324420 SNP compared with C/C homozygotes. Additionally, functional network analyses revealed divergent responding between CC homozygotes and

**TABLE 2** Studies testing the association between endocannabinoid biomarkers and genetics in PTSD participants.

Study	Population	Sample size	Polymorphisms	Experimental design	Outcome
Lu et al. (2008)	Finnish and Los Angeles populations	630 (42 PTSD)	rs1049353, rs806377, rs806368, rs6454674	Epidemiologic study (candidate gene)	Higher likelihood of PTSD in minor allele carriers (rs1049353)
Mota et al. (2015)	Predominantly African-American adults	487	rs1049353	Epidemiologic study (candidate gene)	Minor allele carriers had higher PTSD symptoms if they had previous childhood abuse
Spagnolo et al. (2016)	Adult AUD patients with PTSD	49	rs324420	Stress induction	Minor allele carriers had significantly less arousal to a stress task
Korem et al. (2021)	American war veterans	1372	rs1049353	Epidemiologic study (candidate gene)	Minor allele carriers had higher PTSD symptoms if they had previous childhood abuse
Ney, Matthews, et al. (2021)	Australian PTSD participants versus controls	220 (43 PTSD)	rs324420, rs1049353, rs2180619	Fear conditioning	Minor allele carriers (rs324420) had higher SCR to safe stimuli in PTSD group
Crombie, Cisler, et al. (2021) and Crombie, Sartin-Tarm, et al. (2021)	American women with IPV-related PTSD	35	N/A	Fear conditioning	Exercise-induced increases in AEA mediated relationship between aerobic exercise and enhanced extinction recall (reduced threat expectancy) following reinstatement
Crombie et al. (2022)	American women with IPV-related PTSD	85	rs324420	Fear conditioning	Higher activation in parietal regions in minor allele carriers during fear conditioning
deRoon-Cassini et al. (2022)	Patients in acute trauma hospital	170 (50 PTSD)	rs324420, rs806371, rs1049353, rs2180619	Prospective, longitudinal study	Minor allele (rs324420) associated with higher PTSD symptoms at 6-month follow-up

PTSD, posttraumatic stress disorder; SCRs, skin conductance responses. rs1049353, rs806377, rs806386, rs6454674, and rs2180619 belong to the cannabinoid receptor 1 gene and rs324420 belongs to the fatty acid amide hydrolase gene. PTSD prevalence was not reported in these studies as the focus was on dimensional PTSD symptomology.

A-allele carriers within the limbic and striatum networks throughout the task, with A-allele carriers' processing tuned to the CS+ and CC homozygotes processing tuned to the CS- (Crombie et al., 2022). Ney, Matthews, et al. (2021) found that A-allele carriers (compared with C/C homozygotes) with PTSD had significantly higher physiological responding to a safety cue during fear extinction, when compared with trauma-exposed and healthy controls. Given that higher reactivity to the safety cue during extinction is usually interpreted as an indicator of poor fear extinction learning (Duits et al., 2015), this result contrasted with the narrative that the A allele of the rs324420 SNP should be beneficial for extinction learning and a protective factor against PTSD. This finding is also a timely reminder of the importance of studying the endocannabinoid system in PTSD populations due to the substantial difference between the regulation of neurobiology of clinical and healthy cohorts, which was also found in Crombie et al. (2019).

It is critical that genetic and biological effects of the endocannabinoid system are explored in both PTSD and healthy populations before conclusions concerning their role in the disorder can be confirmed. This should involve exploration of PTSD symptomology outside of the fear-conditioning paradigm, of which the direct relevance to PTSD symptoms is debated (Ney, Schenker, & Lipp, 2022; Schevaneels et al., 2021). Future studies should explore whether treatment

responsiveness is moderated by endocannabinoid genotypes and metabolomic phenotypes. Further, available studies of endocannabinoid signaling in PTSD have not measured endocannabinoid concentrations at multiple time points. Longitudinal studies that measure endocannabinoids at different stages of disease course should be a topic for future research.

## Relationship between endocannabinoid genetics and PTSD

There are sparse studies of the relationship between endocannabinoid genotypes and PTSD diagnosis or symptomology (Table 2). In a sample of 170 participants, the G allele of rs8066371 (CB1 SNP) was associated with higher PTSD symptoms (deRoon-Cassini et al., 2022). This study reported trending, but not significant, correlations between minor alleles of the CB1 rs1049353 and rs2180618 SNPs and PTSD symptom severity (deRoon-Cassini et al., 2022). The minor allele of rs1049353 also interacted with childhood trauma in a veteran sample to predict higher PTSD symptomology, with the minor A allele associated with higher likelihood of developing PTSD (Korem et al., 2021; Mota et al., 2015). Similarly, the rs1049353 minor allele was associated with increased rate of PTSD diagnosis in a moderate adult sample of 320 participants from Los Angeles, but not in a similar sample of 310 participants from Finland (Lu et al., 2008).

In a small sample of 49 participants with comorbid PTSD and AUD, Spagnolo et al. (2016) found that participants with a minor allele (A,  $n = 25$ ) on the FAAH rs324420 SNP had significantly greater reductions in hyperarousal symptoms compared with C/C homozygotes overtime (30 days later) but no baseline differences in any symptoms. The A allele carriers also self-reported less anxiety in response to an acute alcohol-related stressor compared with C/C homozygotes (Spagnolo et al., 2016). This is what may have been predicted from the preclinical literature described above. It was reported elsewhere in a larger sample ( $N = 170$ ) that homozygote minor allele carriers (A/A,  $n = 20$ ) of the FAAH rs324420 SNP had significantly higher overall PTSD symptomology on follow-up compared with heterozygous ( $n = 67$ ) and C/C homozygous ( $n = 81$ ) participants (deRoon-Cassini et al., 2022), but no information regarding specific symptomology was reported in this study. Both in this study, as well as Ney, Matthews, et al. (2021) where poor fear extinction in the PTSD A allele group was observed, there is divergence between patterns observed in healthy compared with PTSD cohorts, which points to the FAAH rs324420 A allele being a risk factor for higher PTSD symptomology rather than a protective factor as suggested by studies in healthy participants (Mayo, Asratian, Linde, et al., 2020). Since these effects are associated with genetic factors, it is unlikely that stress reactivity to trauma is the sole cause of the divergence of neurobiological responses observed in studies such as Crombie et al. (2019). It is also possible that higher AEA, caused by the presence of an A allele on the rs324420 FAAH SNP, may result in both enhanced fear learning as well as enhanced fear extinction learning (Morena et al., 2019), with AEA facilitating nonspecific memory consolidation for both threat and safety learning in animals. Presence of an A allele may also result in a more complex phenotype that moderates PTSD symptom severity and treatment efficacy in ways that have not yet been explored in this budding literature. Significantly more genetics data are needed to replicate and extend these findings, and these divergences from healthy cohort research do not imply that cannabinoid- or even FAAH-based treatments for PTSD will be ineffective, particularly given that large changes in AEA availability following FAAH inhibition make clinical trial work with these drugs incomparable with natural research examining the effects of the FAAH rs324420 A allele.

## Effect of cannabinoids on PTSD symptomology

Ultimately, the best tests of the preclinical hypotheses are clinical trials of cannabinoid pharmaceuticals in PTSD patients. There are very few studies that have examined the effects of cannabinoid administration in patients (Table 3), though there are ongoing registered clinical trials (EudraCT 2020-001965-36, NCT04080427, NCT04597450). There is substantial anecdotal evidence for high potential of treating PTSD symptoms effectively with cannabinoids (Bonn-Miller et al., 2020; Greer et al., 2014; Lake et al., 2019), though these reports only provide low-level evidence for such effects that are not replicated across all cross-sectional and anecdotal data (Gradus et al., 2010; Loflin et al., 2017). There have been a number of case studies reporting efficacy of cannabinoids (cannabidiol, delta-9 tetrahydrocannabinol (THC)) on PTSD and anxiety symptomology (Elms et al., 2019; Passie et al., 2012; Shannon et al., 2019; Shannon & Opila-Lehman, 2016); however, these also only constitute low-quality evidence.

Nabilone and dronabinol have been shown to improve sleep quality, nightmares, and some waking symptoms in participants with PTSD in several retrospective and open-label studies (Cameron et al., 2014; Fraser, 2009; Jetly et al., 2015). Bonn-Miller et al. (2021) recently reported that PTSD patients who received 3 weeks of cannabis of varying potency had no significant improvements in symptomology compared with patients taking placebo medications. Bolsoni et al. (2022) also recently found that 300mg of orally administered cannabidiol (CBD) did not alter the anxiety, alertness, or discomfort of PTSD participants who recalled a traumatic experience compared with placebo; however, cognitive impairment was

**TABLE 3** Studies testing the effect of cannabinoids on PTSD symptomology.

Study	Population	Experimental design	Drug and dose	Outcome
Fraser (2009)	PTSD patients	Open label trial	Nabilone 0.2–4 mg oral daily	Reduced nightmares
Passie et al. (2012)	PTSD patient	Case study	Cannabis resin	Decrease in waking PTSD symptoms
Cameron et al. (2014)	PTSD inmates	Retrospective	Nabilone 0.5–6.0 mg oral daily	Improved sleep and reduced waking PTSD symptoms
Jetly et al. (2015)	PTSD patients	Clinical trial	Nabilone 0.5–3 mg oral daily	Reduced nightmares
Shannon and Opila-Lehman (2016)	PTSD patient	Case study	CBD 25 mg+oral daily	Improved sleep and reduced anxiety
Elms et al. (2019)	PTSD patients	Retrospective case series	CBD 2–100 mg oral daily	Decrease in waking PTSD symptoms
Rabinak et al. (2020)	PTSD participants	Experimental clinical trial	Dronabinol 7.5 mg oral single dose	Lowered amygdala threat activity, increased mPFC activity
Bonn-Miller et al. (2021)	PTSD patients	Randomized clinical trial	1.8 g/day smoked cannabis: high THC/low CBD, high CBD/low THC, CBD:THC	No effect of cannabinoids relative to placebo on symptoms
Bolsoni et al. (2022)	PTSD patients	Randomized clinical trial	CBD 300 mg oral single dose	No effect of cannabinoids relative to placebo on symptoms other than cognitive impairment

PTSD, posttraumatic stress disorder; THC, delta9-tetrahydrocannabinol; CBD, cannabidiol; mPFC, medial prefrontal cortex.

reported to be lower in the CBD group. However, 7.5 mg of orally administered dronabinol reduced threat-related neurobiological fMRI activity in PTSD participants during a fear conditioning task relative to placebo (Rabinak et al., 2020), suggesting that either the lack of precise dosing—both in terms of dose timing and formulation—in the two clinical trials above may have underpinned the lack of improvement in treatment outcome measures (Bolsoni et al., 2022; Bonn-Miller et al., 2021). Moreover, the Bonn-Miller et al. clinical trial used an ad libitum dosing schedule with participants spending the duration of the trial in their own homes, suggesting that the differences in findings may also have been due to differences in therapeutic context between the studies. Regardless, significantly more clinical research is required before the effectiveness of cannabinoid products in PTSD can be adequately determined (Table 3).

## Conclusions

While strongly implicated in preclinical and healthy human models, there is still insufficient human research demonstrating a direct relationship between the endocannabinoid system and PTSD. The available biomarker evidence is quite inconsistent, though emerging research suggests that endocannabinoids may show an extreme response during trauma and maintain a blunted profile posttrauma. Existing research is unable to determine whether low endocannabinoid tone post-trauma is a result of trauma or a cause of PTSD symptomology. Genetic data are sparse and inconsistent, and only two studies have tested the association between endocannabinoid markers and fear conditioning in humans. Significant anecdotal and uncontrolled research evidence suggests that cannabinoid administration can improve PTSD symptoms, but recent clinical trials have not been conducted to a standard that can replicate these translational findings. Research using more stringent research designs including better controlled dosing timing (relative to psychological therapy) and dosing formulations is critical to understanding how cannabinoids might improve outcomes for PTSD patients.

## Application to other areas

This chapter reviews the relationship between PTSD and the endocannabinoid system, as well as the potential evidence for treatment efficacy of PTSD with cannabinoid drugs. Endocannabinoid research in PTSD has thus far mostly followed the stringent preclinical-clinical translational research trajectory, with preclinical fear-conditioning models underpinning human conditioning research, and finally, most recently culminating in the conduct of randomized clinical trials in PTSD patients (Mayo et al., 2021; Ney, Crombie, et al., 2022).

In many ways, the tools used in this translational pathway are applicable to the study of other diseases. For instance, human research has encouraged the increasing development of sophisticated measurement tools capable to quantifying cannabinoid receptors in the human brain and endocannabinoid concentrations in various peripheral matrices. Other psychiatric disorders can learn from many of the lessons learned from the PTSD translational literature, where decades of high-quality research have been conducted and the fruits of which are only beginning to emerge. The PTSD literature is also a cautionary tale to pharmacological translational research, with the variety of methods trialled during the translational pathway often leading to conflicting results. For example, while Bonn-Miller et al. (2021) used an ad libitum, home-based model in their clinical trial, preclinical and translational experiments have typically focused on approaches using cannabinoid drugs as adjuncts immediately prior to fear extinction learning, which would translate to a clinical trial of cannabinoid immediately prior to exposure therapy. Further to this, basic findings from preclinical and healthy human work that are based on genetics information may not be transferrable to patient cohorts without significantly improved understanding of the exact relationship between endocannabinoid genetics, signaling, and disease.

Regardless, PTSD is a disorder characterized by a multitude of symptoms and comorbidities that interact with various other disorders and biological systems (Ney, Akhurst, et al., 2021). For example, PTSD can be considered a memory-related disorder, and some of the memory systems purported to be involved in PTSD (e.g., endocannabinoids, brain-derived neurotrophic factor) are shared by other memory disorders, such as dementia (Pitman et al., 2012). Similarly, PTSD is strongly characterized by alterations to mood and is usually associated with significant hyperarousal and behavioral avoidance. Depressive and anxiety disorders therefore share many of the symptoms of PTSD and are often treated as similar disorders with shared biological and neurobiological features. Many of the lessons learned from the PTSD-endocannabinoid field could easily be applied to anxiety and depression (as well as other similar disorders) and may serve as starting blocks for investigations into these disorders and related comorbidities.

## Mini-dictionary of terms

- **Extinction retention.** A test of how well the extinction memory is retained long term after extinction training.
- **Fear acquisition.** Pairing a neutral stimulus (e.g., a tone) with an aversive outcome (e.g., an electric shock) results in a conditioned fear response to the neutral stimulus alone.
- **Fear conditioning.** The learning processes and experimental paradigms describing fear acquisition, extinction, and extinction retention.
- **Fear extinction.** Unpairing of a conditioned stimulus from its aversive outcome (often by altering context) creates a parallel safety memory trace that competes with the original fear memory. A key concept in common exposure-based psychological therapies for PTSD.

## Key facts of PTSD

- PTSD occurs in up to 10% of people who experience trauma and has a global lifetime prevalence of approximately 3.9%.
- Trauma is defined as a “Criterion A trauma,” which results in threatened or actual injury and/or death, occurring to either the person directly or vicariously by witnessing the event occur to another person or by hearing about the event occurring to a significant other person.
- The key symptoms of PTSD are intrusive memories, hyperarousal, negative/blunted mood, and avoidance behaviors.
- Exposure-based psychotherapies for PTSD aim to reduce anxiety toward trauma reminders by enabling therapeutic safety learning. This type of safety learning is hypothesized to work via the mechanisms of fear extinction learning.
- Multiple pharmacotherapies have been trialled in PTSD, with the endocannabinoid system one recent promising candidate for treatment.

## Summary points

- This chapter focuses on existing evidence linking endocannabinoid phenotypes, genetics, and cannabinoid drugs with PTSD symptomology and treatment outcomes.
- It is unclear whether PTSD is associated with higher or lower endocannabinoid tone. Equivocal findings may be due to differences in sample size, heterogeneity of trauma types, and other differences between existing studies.
- There is some evidence that high endocannabinoid responses occur immediately following trauma, but these subside to blunted responding of circulating endocannabinoids long-term following acute stressors
- Although research in animals and healthy participants suggests that the minor A allele of FAAH rs324420 may be a protective factor, clinical evidence suggests that the A allele may be a risk factor for higher PTSD symptomology.
- Preliminary evidence suggests that cannabinoids can effectively reduce PTSD symptoms, although support from clinical trials is limited to date.
- Clinical trials have been effective in improving experimental fear extinction performance (e.g., enhanced extinction recall, reduced return of fear).
- Significantly higher quality research is needed to establish the efficacy of cannabinoid treatments in PTSD and the relationship between the endocannabinoid system and PTSD.

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## Chapter 6

# The protective effect of the endocannabinoid system in neurotoxin-induced damage to hippocampal neurons: a focus on light and electron microscopy

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## Abbreviations

AD	Alzheimer's disease
A $\beta$	$\beta$ -amyloid peptide
CB1R	type 1 cannabinoid receptors
CB2R	type 2 cannabinoid receptors
CBS	endocannabinoid system
eCBs	endocannabinoids
KA	kainic acid
TLE	temporal lobe epilepsy

## Introduction

Endogenous cannabinoids (eCBs) are regulators of synaptic transmission in the brain, mediate numerous forms of plasticity, and control neuron energy metabolism. eCBs exert influences using a series of mechanisms and interactions with neuromediators, neurotrophic factors, and neuropeptides (Di Marzo, 2018; Lutz, 2020). Scientific interest in eCBs and their role in brain function has been growing rapidly in recent years. However, despite the fact that the existence of the endocannabinoid system (CBS) in mammals was established in the early 1990s, the deciphering of the mechanisms of its functioning both in a healthy brain and in various neuropathologies is still far from the final stage.

In recent years, intensive investigation has been carried out on the importance of eCBs in brain damage; it was found that in these cases, they can have both protective and degenerative influences; there is evidence that eCBs can determine the "fate" of cells in pathological processes in the brain (Garcia-Arencibia et al., 2019). The CBS has been shown to play a significant role in the development of neurodegenerative diseases such as Alzheimer's disease (AD) (Aso & Ferrer, 2014; Ramírez et al., 2005), Huntington's disease (Pazos et al., 2008), and Parkinson's disease (Di Marzo et al., 2000).

In experimental models, in animals that were injected into the brain with neurotoxins kainic acid (KA, an agonist of glutamate receptors, provoking the development of seizure activity) and  $\beta$ -amyloid peptide (A $\beta$ ), one of the key factors in the pathogenesis of AD), a violation of synaptic function was revealed, which ultimately leads to memory loss characteristic for temporal lobe epilepsy (TLE) and AD (Gordon et al., 2017; Llorens-Martin et al., 2014; Raudino, 2017; Zhang et al., 2018).

The present review is analyzing of investigation on the protective role of CBS activation during neurotoxic effects on the brain in models of TLE and AD, in which damage and death of hippocampal neurons are observed. Possible mechanisms of such protective effects are discussed.

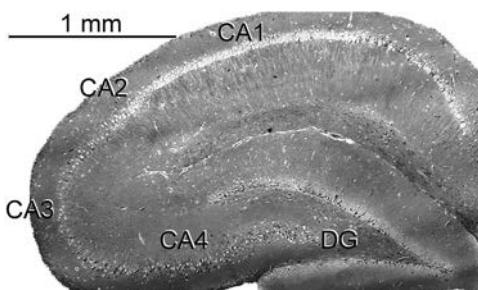
## Hyperexcitability and oxidative stress as the main factors leading to the development of temporal lobe epilepsy and Alzheimer's disease

Glutamatergic neurotransmission is believed to be the basis of the functioning of neural networks in the brain (Niciu et al., 2012; Reiner & Levitz, 2018). At the same time, the control of neuronal excitability is the main factor in maintaining the normal functioning and vitality of neural networks. Dysregulation of glutamatergic transmission provokes hyperactivation and oxidative stress, which underlies neurodegenerative diseases such as TLE and AD (Aso & Ferrer, 2016; Butterfield & Boyd-Kimball, 2018; Farooqui, 2010; Sultana & Butterfield, 2010).

In experimental models in animals that were injected with the neurotoxin kainic acid (KA, an agonist of glutamate receptors that provokes seizure activity) and  $\beta$ -amyloid peptide (one of the key factors in the pathogenesis of AD), a dysregulation of synaptic function was revealed, which ultimately leads to memory loss and other cognitive impairments characteristic of both TLE and AD (Gordon et al., 2017; Llorens-Martin et al., 2014; Raudino, 2017; Zhang et al., 2018). One of the main targets of these neurotoxins is the hippocampus, a key structure in memory processes. The hippocampus is a complex structure that includes the CA1–CA4 fields and the dentate gyrus (DG) (Fig. 1). The areas included in the hippocampus differ from each other physiologically, biochemically, and morphologically; this is the reason that their neurons have various thresholds of sensitivity to damaging factors (Ji & Maren, 2008; Vinogradova, 2001). The hippocampus includes the main glutamatergic neurons (most of hippocampal cell population) and inhibitory GABAergic interneurons. The hippocampal fields contain one type of glutamatergic neurons—pyramidal cells, while the DG includes two types of glutamatergic neurons—granular cells and mossy cells. Mossy neurons consist a significant portion of the DG neuron population.

The hippocampus receives information from most structures in the brain. The entorhinal cortex (EC) is the source of the cortical afferents reaching the hippocampus fields and DG via the perforant path (Llorens-Martin et al., 2014). The perforant path consists of two components: trisynaptic (EC – DG – CA3 – CA1) and monosynaptic, from the EC directly to the CA1 field. The trisynaptic path represents the projection of the neurons of the II layer of the EC on the granular cells of the DG, which, through their axons (mossy fibers), in turn are projected onto the mossy neurons and into the pyramidal neurons of the CA3 field, forming giant synapses on them. Then Schaffer's collaterals (axons of the CA3 pyramidal cells) are projected into the CA1 pyramidal cells. The monosynaptic path connects the neurons of the III layer of the EC directly with the neurons of the CA1 field.

It was shown that the internal molecular layer of DG, where glutamatergic signals from mossy cells (Johnston & Amaral, 2004) and from pyramidal CA3 neurons are coming, contains CB1 receptors (Monory et al., 2006). Both of these neuronal types, mossy cells and CA3 pyramidal neurons, express CB1 mRNA and synthesize the CB1 receptor protein (Campbell & Downer, 2008). Since receptors are key elements for the transmission of information in the hippocampus, as in other structures of the brain, it should be noted that neurons differ in the density of binding sites for such receptors as glutamatergic (NMDA, AMPA, kainate) and GABAergic (Lothmann et al., 2021). For example, neurons in the CA3 field of the dorsal hippocampus have more kainate receptors than in the CA1 field, while there are more NMDA and AMPA receptors in CA1 (Vincent & Mulle, 2009; Yeung, Calvo-Flores Guzmán, et al., 2020; Yeung, Palpagama, et al., 2020). Some authors believe that the vulnerability of hippocampal neurons is related to the distribution of AMPA and



**FIG. 1** Micrograph of a slice of the dorsal hippocampus in a control rat. The CA1, CA2, and CA3 fields and the dentate gyrus (DG) are outlined. (*Unpublished data.*)

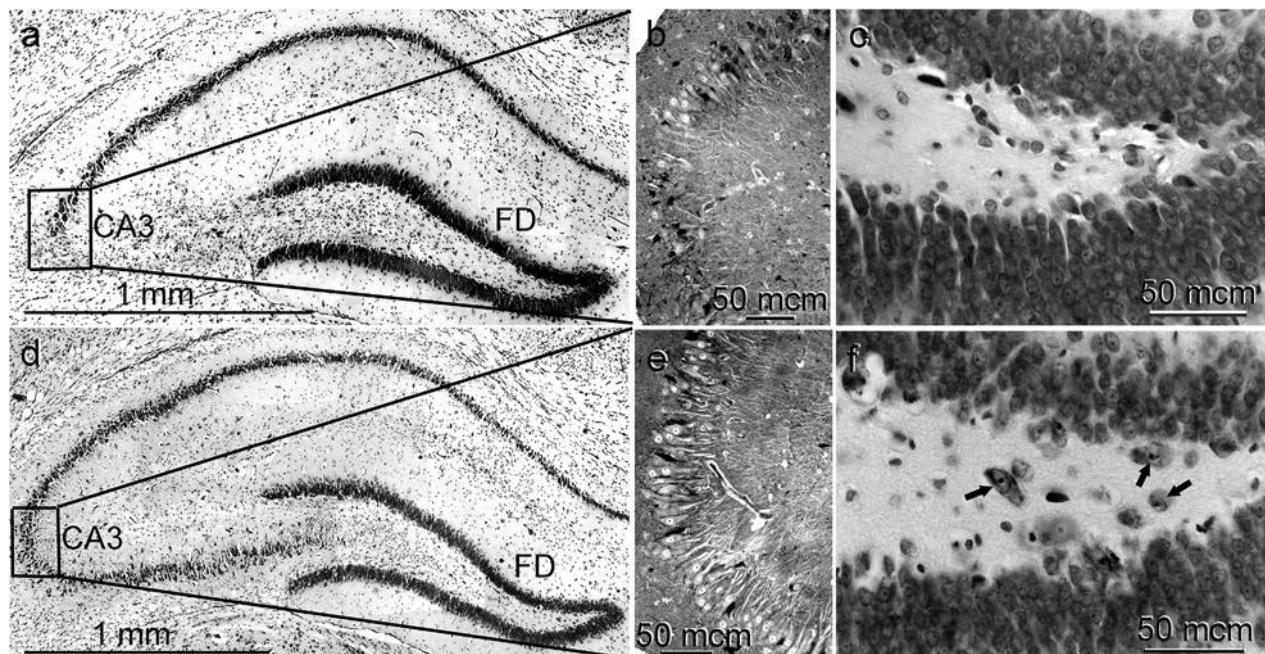
kainate receptors. Thus, it is the abundance of kainate receptors in CA3 pyramidal neurons of the hippocampus and their activation by, for example, kainic acid that lead to the development of oxidative stress (Méndez-Armenta et al., 2014), which is associated with the production of reactive oxygen species and impairment mitochondrial function (Zheng et al., 2011). Besides, the action of A $\beta$  leads to the reduction of the ratio of AMPA and NMDA receptors in hippocampal neurons, which also contributes to the development of hyperexcitation and oxidative stress (Hettinger et al., 2018; Sulzer et al., 2008).

## Analysis of degenerative processes caused by the action of KA and A $\beta$

### Cytological analysis of cellular components of neurons

Cytological analysis of cellular components plays a fundamental role in the study of both neuronal degenerations and their survival and recovery under the influence of neuroprotective agents. It should be noted that the generalized response of neurons to various types of damage has much in common. However, even in the same brain structure, neurons can react differently to a damaging factor. Thus, the action of KA and A $\beta$  revealed a different level of degeneration of hippocampal neurons (Gordon et al., 2015, 2017; Mikheeva et al., 2017). In animal models, 14 days after the administration of A $\beta$ , significant damage to the glutamatergic neurons of the CA1 field was noted and, after 45 days, to the pyramidal neurons of the CA3 field. On the contrary, with the introduction of KA after 14 days, the death of pyramidal neurons of the CA3 field is observed (Fig. 2A and B), and after a month, the death of the neurons of the CA1 field occurred. According to the literature, under the action of damaging factors, glutamatergic granular neurons of the DG underwent significant degeneration as well (Thodeson et al., 2018). Mossy neurons of DG hilus are especially sensitive to neurotoxins (Fig. 2C). Their damage can cause significant changes in the neural network of the hippocampus, since they innervate both granular cells and GABAergic interneurons of the DG (Scharfman, 2016).

According to the literature, neurotoxic effects initiate both apoptosis and necrosis, depending on the intensity of oxidative stress caused by them (Farooqui, 2010). Previously, oxidative stress was shown to be the main cause of neurodegenerative processes caused by such neurotoxins as KK and A $\beta$  (Méndez-Armenta et al., 2014; Singh et al., 2019). Oxidative stress triggers metabolic cascade, i.e., a set of interrelated pathological reactions, often irreversibly damaging the cell (Aso & Ferrer, 2016; Sultana & Butterfield, 2010).



**FIG. 2** Micrographs of slices of the rat dorsal hippocampus, the CA3 field and DG: (A–C) 2 weeks after intraventricular injection of KA; and (D–F) after combined administration of KA and URB. In the DG hilus region, mossy cells with a clearly defined nucleus, nucleolus and a cell membrane are visible, in contrast to the pattern observed after the only KA injection. A, C, D, F – staining with cresyl violet according Nissl; B, E – slices treated with osmium. (*Unpublished data.*)

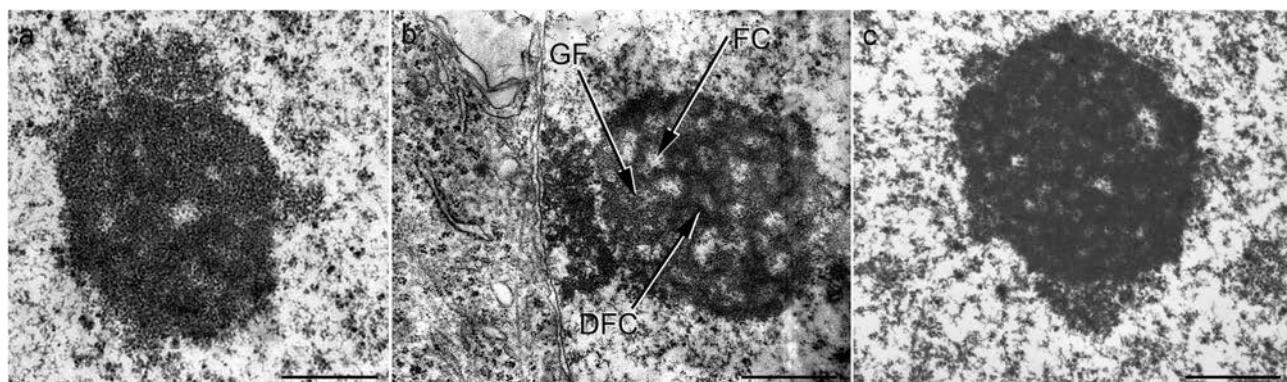
The changes in the state of mitochondria should be noted particularly, which are a key factor in the pathogenesis of both AD (Aso & Ferrer, 2016) and TLE (Mikheeva et al., 2017; Zheng et al., 2011). Mitochondrial dysfunction includes decreased energy metabolism, accumulation of reactive oxygen species, and membrane increased permeability. In all studied neurons, the lack of energy production leads to a disorder of protein metabolism, that is, to a change in the state of the protein synthesizing and catabolic systems, which are the main indicators of the state of the cell and damage to the structural integrity of the outer membranes.

### Ultrastructural analysis of cellular components of the protein synthesizing system

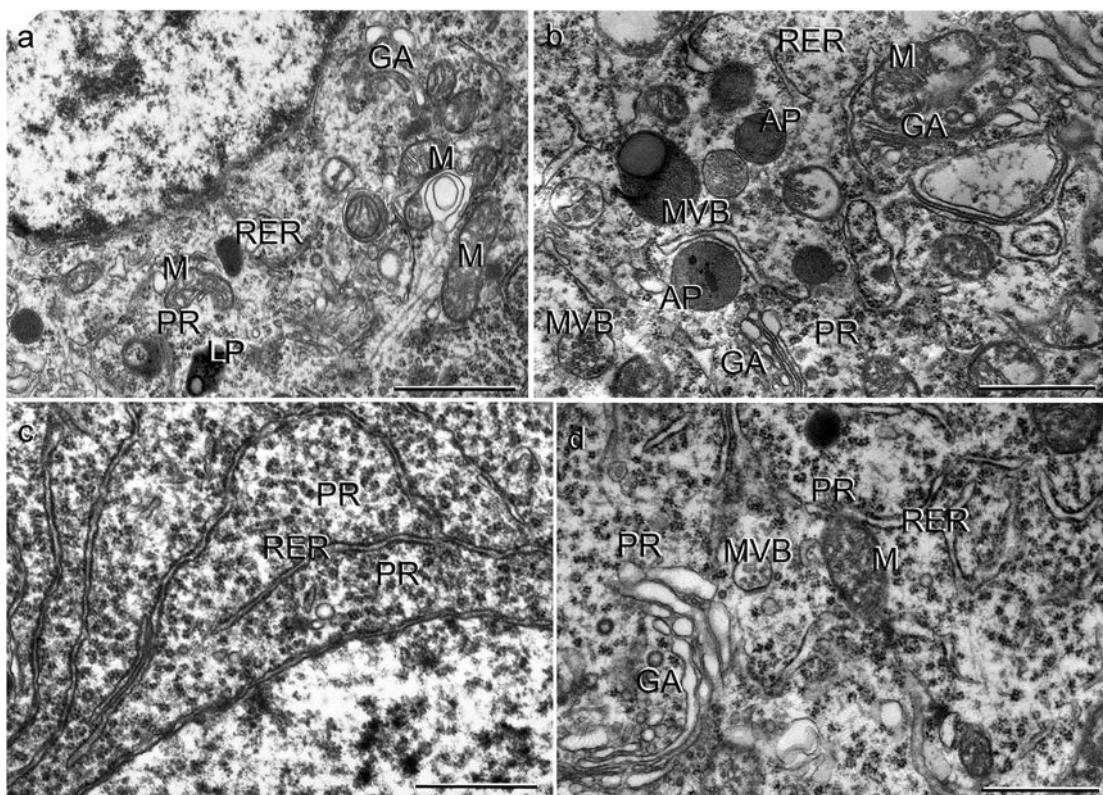
In pathology, in particular under the action of the neurotoxins, KA and A $\beta$  changes are primarily observed in the state of the nucleolus, which reflects the levels of the process of ribosome biogenesis: synthesis of preribosomal RNA, processing of ribosomal RNA, (rRNA), formation of ribosomal subunits, and their migration into the karyoplasm (Hetman et al., 2010; Kim et al., 1994). The nucleolus consists of a fibrillar center, on the surface of which transcription occurs, a dense fibrillar component, which represents transcripts of rRNA genes, and a granular component of preribosomal and ribosomal subunits at various stages of maturation. In the control, active nucleoli with intertwined dense fibrillar and granular components and faintly visible fibrillar centers predominate (Fig. 3A). For the state of the nucleoli of rat neurons exposed to the action of neurotoxins, segregation of the granular and dense fibrillar components is characteristic, the fibrillar center is distinguished (Fig. 3B). This change in the state of the components of the nucleolus reflects inhibition of transcription and processing. In the nuclei, there is a partial margination of chromatin located under the nuclear envelope (hyperchromatolysis of the nucleus), a weak condensation of chromatin is observed in the karyoplasm, which reflects the inactivation of transcription sites and is considered a pathology that sometimes leads to cell death (Hetman et al., 2010; Kim et al., 1994).

The transformation of the nucleus is accompanied by destructive changes in the cytoplasm. Protein synthesis is one of the early cellular processes disrupted by oxidative stress in. Oxidation of nucleic acids is one of the main mechanisms in which oxidative stress provokes cellular pathology. In the culture of nerve cells within 24 h after the onset of oxidative stress caused by the action of A $\beta$ , mRNA oxidation is observed, which leads to the loss of sliding of ribosomal subunits along the transcript, the appearance of heavy polyribosomes, and, accordingly, to a decrease in protein expression (Ding et al., 2007).

As a result of rough endoplasmic reticulum (RER) stress, accompanied by partial fragmentation of RER, swelling of cisternae, and detachment of ribosomes from RER membranes (Fig. 4A and B), a decrease in the synthesis of necessary proteins is accompanied by the synthesis and accumulation of misfolded proteins as a result of a decrease in the function of chaperones—proteins (Kudo et al., 2008). Golgi apparatus (GA), whose function is to modify proteins, “packing” secreted products into granules, formation of the cell membrane, formation of lysosomes, etc., is in dynamic equilibrium with EPR then changes in the structure of AG (swelling of cisternae, blurring of their membranes, partial dissociation of structures from the outer side), which indicates atrophy of the AG plates. Membrane curls, lamellar structures appear, which may be a consequence of impaired lipid synthesis. As a result, the cytoplasm accumulates aggregation of protein complexes in the



**FIG. 3** Ultrastructure of the nucleoli in neurons of the CA3 field on a slice of the dorsal hippocampus: (A) in control rat, an active nucleolus with intertwined dense fibrillar and granular components of rRNA and a hardly noticeable fibrillar center; (B) 2 weeks after intraventricular KA injection. In the nucleus segregation of these components are observed, the granular component decreases in size, which indicates inhibition of transcription and processing of rRNA; (C) 2 weeks after the combined administration of KA and URB, the amounts of dense fibrillar and granular components are significantly greater than in b, which indicates the activation of rRNA transcription and processing. *DFC*, dense fibrillar component; *FC*, fibrillar center; *GC*, granular component. The scale bar corresponds to 1 mcm. (*Unpublished data*.)



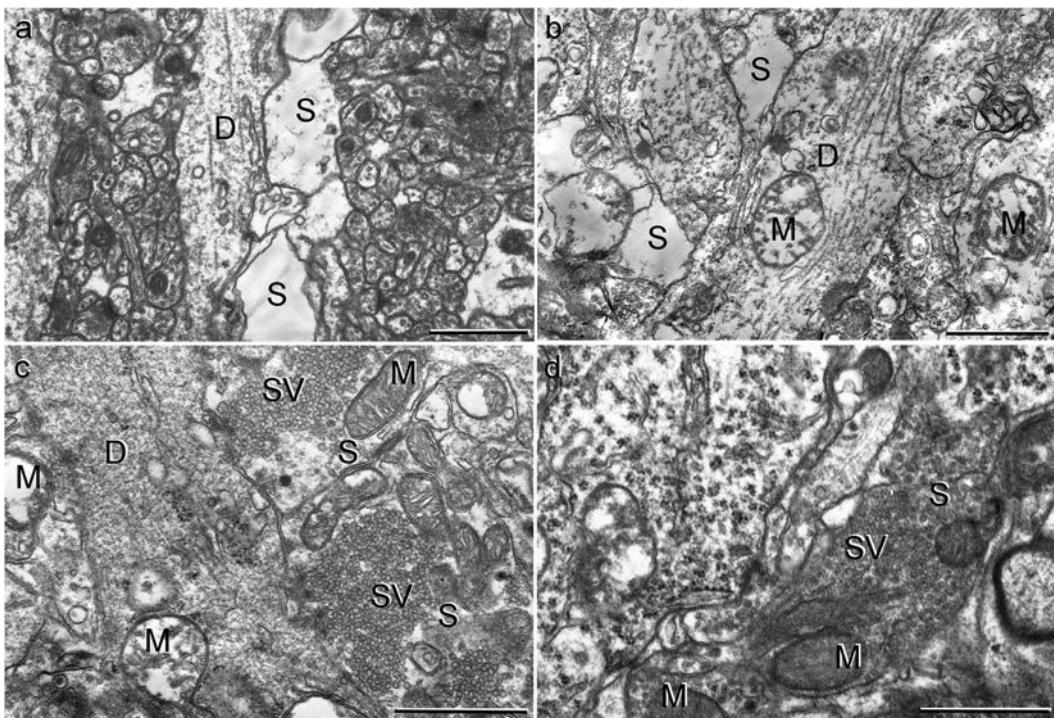
**FIG. 4** Ultrastructure of the cytoplasm of pyramidal neurons in the hippocampus 2 weeks after intraventricular injection of KA: (A, B) after combined administration of KA and URB; (C, D) AP, autophagosome; GA, Golgi apparatus; L, lysosomes; LP, lipofuscin granules; M, mitochondria; MVB, multi-vesicular bodies; PR, polyribosomes; RER, granular endoplasmic reticulum. Arrows indicate electron-dense material. The scale bars correspond to 1  $\mu$ m. (Unpublished data.)

form of a dark material not surrounded by a membrane, with consisting, possibly, of protein aggregates (Chen et al., 2020) and lipofuscin granules or secondary lysosomes. This occurs as a result of decreased proteolysis (Ding et al., 2007).

### Ultrastructural analysis of the cellular components of the catabolic system

The presence of such proteolysis systems in neurons as the ubiquitinproteasome system (nonlysosomal) and lysosomal autophagy in a normally functioning cell plays an important role in maintaining a controlled balance between anabolism and catabolism. Oxidative stress suppresses the process of binding of oxidized damaged protein to proteasomes, inhibiting the functioning of the ubiquitin proteasome system (UPS) (Chen et al., 2020). Inhibition of the ubiquitin-proteasome system increases the activity of autophagy, which plays a fundamental role in the degradation of dysfunctional cell organelles and toxic protein aggregates that are delivered to lysosomes, where they are degraded into biologically active molecules under critical conditions.

Oxidative stress during glutamate excitotoxicity stimulates aberrant autophagy in neurons, which is an accumulation of autophagosomes, the composition of which is unable to digest lysosomes (Menzies et al., 2017; Sulzer et al., 2008). The functional activity of lysosomes depends on the state of the membrane and the digestive power of enzymes. Partial or selective disruption of the permeability of lysosomal membranes, followed by the release of lysosomal enzymes into the cytosol, caused by the action of neurotoxins, in particular A $\beta$  provokes aberrant autophagy (O'Keefe & Denton, 2018). This autophagy has serious adverse consequences for normal cellular functions and is associated with numerous pathologies, including neurodegenerative diseases, in particular AD. As a result of damage to the functioning of lysosomes and autophagosomes, a significant amount of lipofuscin formations is observed in the cytoplasm (Fig. 4B), which indicates irreversible destructive and dystrophic processes in cells (Sulzer et al., 2008). Violation of the structural integrity of the outer membranes in neurons leads to active endocytosis. Increased permilization of lysosomes contributes to an increase in the number of secondary endosomes or multivesicular structures in the cytoplasm of neurons, for example, KA, stimulating



**FIG. 5** Ultrastructure of the synapses of the pyramidal neurons of the hippocampus: (A, B) 2 weeks after intraventricular KA injection. The synapses contain agglutinated synaptic vesicles and destroyed mitochondria. (C, D) after combined administration of KA and URB. (B) shows a watered dendrite with emptied synapses. *D*, dendrite; *S*, synapse; *SV*, synaptic vesicles. Other abbreviations are the same as in Fig. 4. The scale bars correspond to 1 mcm. (*Unpublished data.*)

kainate receptors, changes the fluidity and permeability of membranes (Farooqui, 2010) and increases the internalization of these receptors (Martin & Henley, 2004), and beta-amyloid promotes internalization of AMPA receptors.

Degeneration of neurons leads to damage to their dendrites, axons, and synaptic apparatus (Fig. 5A and B). Structural abnormalities are noted in a number of large and medium-sized dendrites. For some, focal swelling is characteristic—the earliest symptom of damage. In other dendrites, the number of microtubules decreased, or they were completely devoid of them (Zhvania et al., 2015). Dystrophic dendrites also accumulate immature autophagosomes and secondary endosomes (Shankar & Walsh, 2009). Synaptic endings in the CA3 neuropil underwent a light (edematous) type of destruction. The terminal swelled, brightened, the content of synaptic vesicles (SV) decreased, and the remaining SVs were subjected to agglutination, grouped either in the center or near the active zones. The contour of synaptic membranes was “blurred.” There were vacuoles or membrane structures in the space free from vesicles. Depleted buds alternated with ones with medium vesicle filling. Dysfunction of synapses inevitably leads to the progression of neurodegenerative processes and a decrease in interneuronal connections. In axons, signs of degeneration include loosening and thinning of myelin, unweaving of myelin membrane layers, and axoplasm emptying.

### Different levels of damage in hippocampal neurons

The above damage in the state of cellular components is characteristic to varying degrees for all glutamatergic cells of the hippocampus. However, it should be noted that, while the lifetime of pyramidal neurons of the hippocampal fields and mossy cells corresponds to the lifetime of an individual, granular cells are replaced continuously throughout life as a result of neurogenesis and differ in the degree of maturity. (Kron et al., 2010). Under the action of a damaging factor, a long-lived neuron either recovers or dies, most likely in a necrotic pattern. Moreover, the level of their vulnerability is various (Gordon, Mikheeva, et al., 2021; Yuan et al., 2015). While the pyramidal neurons of the hippocampus are characterized by different levels of damage under the action of KA, the mossy neurons of the hilus have the greatest damaging effect (Gordon, Mikheeva, et al., 2021). This may be due to the absence of autophagosomes in mossy neurons against the background of the ubiquitin proteosome system inhibition in all cells (Toda & Gage, 2018; Yuan et al., 2015).

As for granular cells, as a rule, they die by the apoptotic type. However, the action of neurotoxins, such as KA, contributes to the appearance of signs of necrosis. This is probably due to the fact that there is a critical stage in the development of granular cells, during which they are vulnerable to neurotoxin (Kron et al., 2010). The number of granular cells under the damaging effect of KA depends on the proportion of dead cells and on the degree of proliferation of neuronal precursors. Various factors can influence the balance between them. So, for example, in the case of prolonged action of the neurotoxin, the number of “young” neuronal cells is significantly reduced, and some of the remaining cells form abnormal connections with resident cells in the granular layer (Thodeson et al., 2018).

Degenerative processes occurring in the hippocampal fields and in the DG are likely to damage the so-called glutamatergic loop between granular and mossy cells, which, in turn, innervate GABAergic interneurons and project onto a large number of granular cells (Johnston & Amaral, 2004; Ratzliff et al., 2004). In addition, the neurons of the entorhinal cortex, which is the source of most of the cortical afferents in the hippocampus and DG, due to separate pathways (trisynaptic in the DG and fields CA3 and CA1, and monosynaptic in field CA1) are among the first to be damaged by the action of such damaging factors as A $\beta$  and KA (Drexel et al., 2012; Llorens-Martin et al., 2014). As a result of the degeneration of a part of the pyramidal neurons, the recurrent glutamatergic connections with the neurons of the CA3 field are also disrupted. An increase in the excitability of CA3 neurons under the action of KA and A $\beta$  stimulates Schaffer collaterals heading to the CA1 neurons, which induces degradation of these neurons and DG neurons (Melyan et al., 2004; Yeung, Palpagama, et al., 2020).

Under the action of KA in the DG, partial damage to both excitatory glutamate and inhibitory GABAergic inputs occurred. Moreover, normally, the primary effect of the influence of mossy cells is not the excitation of granular neurons, but their inhibition as a result of the activation of inhibitory interneurons, which, in turn, inactivate granular neurons (Scharfman, 2018). The imbalance between excitation and inhibition, leading to an excitotoxic effect, arises due to the fact that mossy cells, which primarily perform an inhibitory function, die under the action of KA. Since mossy cells are more vulnerable to hyperexcitation than granular cells (Scharfman, 2016), the degeneration of these cells is probably the most critical event in the system of morphological rearrangements caused by the effects of neurotoxins.

## **Activation of the endocannabinoid system as a possible protector in the development of temporal lobe epilepsy and Alzheimer’s disease**

### **CB receptors, endocannabinoids, and metabolic enzymes**

One of the mechanisms controlling glutamatergic pathways in the hippocampus under the action of strong excitatory factors is the activation of the endocannabinoid system (CBS) (Marsicano et al., 2003; Monory et al., 2006; Wallace et al., 2001, 2003). The main functional components of CBS in the brain are cannabinoid receptors, type 1 (CB1R) and type 2 (CB2R), their endogenous ligands—endocannabinoids (*N*-arachidonylethanolamide, or anandamide, and 2-arachidonylglycerol, 2-AG), as well as intracellular enzymes that synthesize, transport, and degrade them (Devane et al., 1992). Endocannabinoids (eCBs) are synthesized in neurons from membrane precursors “on demand,” depending on the current activity of the cells. The starting points for their synthesis can be, firstly, a strong depolarization of the cell, causing an intense entrance of Ca $^{2+}$  and a significant increase in its intracellular concentration, and secondly, the activation of phospholipase C through metabotropic Gq-protein-coupled receptors on neurons.

eCBs are produced and released retrogradely from postsynaptic cells, and by acting on CB1R located on the axonal terminals of the same or neighboring cells (present at a distance of no more than 20  $\mu$ m, Piomelli, 2003), they attenuate the release of various neurotransmitters. Using such a mechanism, neurons control the efficiency of their own synaptic inputs, as well as those of neighboring neurons (Freund et al., 2003).

CB1Rs are present in neurons of the main neurotransmitter systems (glutamatergic, GABAergic, serotoninergic, noradrenergic, cholinergic, and possibly dopaminergic). CB1R was found in almost all areas of the brain and in all major cell types, for example, neurons, glial cells (astrocytes, oligodendrocytes), and resident immune cells (microglia) (Fuerte-Hortigón et al., 2021). CB1 receptors are involved in various processes of synaptic and cellular plasticity, as well as in brain bioenergetics in a context-sensitive manner. Several studies have shown that CB1R activation reduces excitotoxic death of hippocampal neurons through several mechanisms (Shen & Thayer, 1998). The action of eCBs through CB1R located near the NMDA channels can weaken their activation and reduce Ca $^{2+}$  entry. Since the main anandamide-synthesizing enzyme (NAPE-PLD) is Ca $^{2+}$ -dependent, an increase in Ca $^{2+}$  will enhance eCB formation, leading to a decrease in neurotransmitter release (Alexander & Kendall, 2007). It was also found that neurons in mice with mutations at CB1R are more susceptible to neurodegeneration caused by excitotoxic cell death than in wild-type mice (Marsicano et al., 2003).

CB2 receptors are expressed in brain cells such as stem neurons, glutamatergic hippocampal cells, and dopaminergic neurons in the ventral tegmental region. CB2 receptors are involved in many neural functions and are highly activated in response to various stimuli, including neuroinflammation and stroke (Di Marzo, 2018; Lutz, 2020). The CBS is directly or indirectly involved in many physiological functions, including the processes of learning and memory. Extensive studies have shown that this system modulates cognitive processes, as shown using various animal models (Kruk-Slomka & Biala, 2016). Unlike conventional neurotransmitters, endocannabinoids are not stored in synaptic vesicles, but are synthesized in response to increased neuronal activity (Kano et al., 2009; Lupica et al., 2017). However, the lifetime of eCBs in the brain is short as a result of both their reuptake by neurons and degradation by enzymes. An effective increase in the level of eCBs and the duration of their functioning can be achieved by suppressing the processes that limit the actions of eCBs in the brain (Karanian et al., 2007; Marsicano et al., 2003). For this, in particular, an eCB reuptake inhibitor, AM404, and inhibitor of anandamide degradation enzyme, URB597, can be used.

### Morphological state of hippocampal neurons after the administration of neurotoxins and cannabinoid-related drugs

Light microscopic investigation of hippocampal neurons after the combined injection of KA+URB597 (anandamide-degrading enzyme inhibitor and combined administration of A $\beta$ 25-35+ AM404 (endocannabinoid reuptake inhibitor) showed more intact neurons in the CA1 and CA3 fields in comparison with the isolated administration of neurotoxins (Gordon, Makarova, et al., 2021; Gordon, Mikheeva, et al., 2021; Mikheeva et al., 2017). Ultrastructural analysis of these neurons demonstrated the preservation in this case of the structure of mitochondria and components of the protein-synthesizing system, in particular, the RER, GA, and polyribosomes (Fig. 4C and D). In the nucleus, there were no significant accumulations of near-membrane chromatin, the nucleolus remained at the control level (Fig. 3C). In the cytoplasm, dark protein aggregates were practically absent, which were seen under the isolated administration of neurotoxins (Gordon, Mikheeva, et al., 2021). These results indicate the ability of eCBs to maintain the functional activity of enzymes of the UPS. Due to its antioxidant activity, eCBs stabilize the state of lysosomes, reducing the permeabilization of their membranes (Noonan et al., 2010). This can explain the ability of lysosomes to maintain their enzymatic activity in the degradation of the composition of autophagosomes, as indicated fewer lipofuscin granules and multivesicular formations in the cytoplasm and processes.

The morphological state of hilar mossy cells after the combined administration of KA and URB597 also differed from their state after the isolated injection of KA: while the isolated KA infusion causes degeneration of almost all observed mossy cells, its combined administration with URB597 leads to the preservation of the cytoplasmic components in these cells (Fig. 2F). The giant synaptic buds were completely filled and corresponded to the control level after combined infusion of the drugs; the state of the dendrites was also nearly intact (Fig. 5C and D).

Thus, prolongation of eCBs action (by blocking their reuptake by neurons and enzymatic decay) promotes more intensive protection of neurons from damaging effects. It can be assumed that as a result of an increase in the level of anandamide and the time of its action, additional activation of CB1R reduces the release of excitatory neurotransmitters into the synaptic cleft and restores the excitation–inhibition balance in the neural network. As a result of tune regulation of the excitability of hippocampal networks, a stable morpho-functional state of synapses and neurons in the hippocampus is established (Kano et al., 2009; Lupica et al., 2017; Monory et al., 2006). Activation of the CB2 receptor reduces the neurotoxicity of A $\beta$  and decreases damage from oxidative stress (Aso & Ferrer, 2016).

### Final conclusion

In animal models, the prolonged action of endocannabinoids was shown the suppressed development of hyperactivity and oxidative stress, preventing the neurodegeneration of the components of the protein-synthesizing and catabolic systems necessary for the vital activity of the cell under the influence of such neurotoxins as KA and A $\beta$ . The role of eCBs in AD and epilepsy is supported by cellular and animal models (Campbell & Downer, 2008; Giuseppina et al., 2019). The value of studies on models lies in the fact that at the cellular level, it is possible to demonstrate conclusively, first, the level of neurodegeneration caused by the action of neurotoxins, which are key factors in neurodegenerative diseases (AD) or provoke signs of these diseases (KA); secondly, the degree of preservation of cells under the action of neuroprotectors, in particular, endocannabinoids. Thus, it can be assumed that in patients with neurodegenerative diseases, in particular with AD or epilepsy, activation of endocannabinoid system suppresses degenerative processes in neurons, maintaining the relationship between neurons of different brain structures, which contributes to memory retention and learning processes.

Thus, one of the potential therapeutic approaches of growing interest is the modulation of the endogenous cannabinoid system.

## Applications to other areas

The data of this review are able to translate to clinic. Now reliable medicines for treatment of neurodegenerative diseases such as temporal lobe epilepsy and Alzheimer's disease are absent. In recent years, the fundamental role of endocannabinoid system in regulation of neuroexcitability, energy metabolism, inflammatory, and many other processes playing an important role in neurodegenerative pathogenesis has been opened. It points to possibility of development of therapeutic approaches that use the preparations for activation of endocannabinoid system. Analysis of the available clinical data shows that preparations containing cannabinoids often give positive results, mainly with oral administration of cannabidiol, which has an independent CB receptor effect. Thus, cannabidiol-enriched cannabis can be used in pediatric treatment-resistant epilepsy.

As a possible therapy for Alzheimer's disease, many efforts have been made to reduce A<sub>β</sub> levels. Many different therapeutic agents have been tried in this direction, including the use of β- and γ-secretase inhibitors and immunization against A<sub>β</sub>, which, unfortunately, did not lead to improvements, and sometimes even an increase in cognitive dysfunctions occurred; this does not allow the use of these approaches for the treatment of AD. Given the gradual development of AD, it can be argued that this disease is a relatively tolerant degenerative process that has a destructive effect only after reaching a certain threshold of impairment. Based on this, the progression of the disease can be halted or mitigated by exposure to specific targets. Over the past several years, work has emerged showing that effects on the endocannabinoid system may be a potential therapeutic target for early treatment of Alzheimer's disease.

## Minidictionary of terms

- **Hilus:** Part of the dentate gyrus (DG), located between two layers of granular cells in the hippocampal formation.
- **Granular neurons:** The main glutamatergic neurons of the DG, located in the granular layer; they are numerous and densely packed. These neurons receive cortical and subcortical afferent pathways, which serve as the basis for the involvement of the hippocampus in cognitive functions.
- **Mossy cells:** The second type of glutamatergic neurons in DG. They are much less numerous than granular cells and are scattered throughout the hilus.
- **Interneurons:** The third type of cells in the DG, they are GABAergic, interact with other DG neurons and CA3 pyramidal cells, and inhibit them.
- **Oxidative stress:** Oxidative stress is a condition produced by the imbalance between oxidants and antioxidants, plays an important role in neurodegenerative diseases.

## Key facts

1. Temporal lobe epilepsy (TLE) is a neurological disorder that is one of the most common forms of focal epilepsy, characterized by recurrent spontaneous seizures, hippocampal sclerosis, and memory deficits. TLE is often caused by genetic damage or cerebral strokes (meningitis, trauma, ischemia, etc.).
2. The prognosis for TLE largely depends on the degree of brain damage; in general, in one-third of patients, the use of pharmacological drugs is not able to stop the progression of the disease.
3. The epileptic focus in TLE is localized in the medial temporal structures, most often in the hippocampus or amygdala (or in both structures). Key structures for the spread and generalization of seizure activity are also the perirhinal, parahippocampal, frontoparietal, and entorhinal cortex.
4. Alzheimer's disease (AD) is the most common form of neurodegenerative diseases, characterized by a progressive and irreversible decline in the cognitive functions of the brain. It is usually found in people over the age of 65, although early Alzheimer's, a rare form of the disease, also exists. About 50%–80% of patients with dementia suffer from AD.
5. The changes in the brains of AD patients differ from those observed during normal aging. Currently, AD is incurable, and therapy is limited to only partial relief of symptoms. It should be noted that the development of the disease from early stages to sympathetic can take decades; only after a distinct manifestation of cognitive impairment and dementia does the disease begin to progress much faster, and the loss of brain function leads to death.

6. The exact etiology of AD is not entirely clear. For a fairly long period and until now, there is a tendency to consider A $\beta$  as triggers of plaque formation, tau phosphorylation, and disease progression. Genetic research identified mutations in the APP and presenilins 1 and 2 genes responsible for the occurrence of inherited AD. Subsequently, many studies have shown an increase in the production of amyloidogenic A $\beta$  peptides associated with mutations in early familial AD, which provided strong support for the amyloid hypothesis.
7. The majority of AD patients suffer not from a genetic but from a sporadic form of this disease resulting from a combination of the vulnerability of several genes with the action of external factors. It has been shown that in sporadic AD, tau protein hyperphosphorylation precedes the formation of A $\beta$  deposits in many brain regions and that misfolded tau protein plays an important role in AD. It has been also shown that soluble oligomeric A $\beta$ 42 (A $\beta$ 42o) species, capable of diffusion derivatives of the A $\beta$  ligand, are even more associated with neurotoxicity and cognitive deficits than insoluble polymerized A $\beta$ 42 monomers (A $\beta$ 42m, or A $\beta$  plaques or fibrils), with which the development of AD is mainly associated. Based on this, it was suggested that the etiology of AD is more complex than the amyloid hypothesis and suggests that factors other than A $\beta$  play a key role in the pathogenesis of AD.

## Summary points

1. A literature review showed that under the influence of neurotoxic factors, such as  $\beta$ -amyloid peptide and kainic acid, the damage and death of hippocampal neurons were observed.
2. Oxidative stress was shown to underly neurodegenerative processes caused by neurotoxins. Oxidative stress triggers the set of interrelated pathological reactions, often irreversibly damaging the cell.
3. Hippocampal neurons are characterized by varying degrees of vulnerability to the damaging effects of neurotoxins. At the same time, the ultrastructural components of both the protein synthesizing and catabolic systems undergo significant changes.
4. Prolongation of the action of endocannabinoids in the brain (by blocking their reuptake by neurons and enzymatic decay) promotes intensive protection of neurons from the damaging action of neurotoxins, reducing the level of damage to cellular components.
5. Endocannabinoids may act as neuroprotective factors in a brain damage. The protective effect is mediated primarily through the activation of neuronal CB1 receptors. CB2 receptor activation may also contribute to the neuroprotective effect of activation of endocannabinoid system.

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## Chapter 7

# Endocannabinoid signaling at excitatory and inhibitory synapses

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>ABHD6</b>	alpha/beta-hydrolase domain containing protein 6
<b>AC</b>	adenylyl cyclase
<b>AEA</b>	anandamide
<b>cAMP</b>	cyclic adenosine monophosphate
<b>CCK</b>	cholecystokinin
<b>DAGL</b>	diacylglycerol lipase
<b>eCB</b>	endocannabinoid
<b>ECS</b>	endocannabinoid signaling system
<b>FAAH</b>	fatty acid amide hydrolase
<b>GIRK</b>	G-protein-coupled inward rectifier potassium
<b>GPCR</b>	G-protein-coupled receptor
<b>LPP</b>	lateral perforant path
<b>LTD</b>	long-term depression
<b>LTP</b>	long-term potentiation
<b>MAGL</b>	monoacylglycerol lipase
<b>NAPE</b>	<i>N</i> -arachidonoyl phosphatidyl ethanol
<b>PLD</b>	phospholipase D
<b>RIM<math>\alpha</math></b>	Rab-interacting protein $\alpha$
<b>SST</b>	somatostatin
<b>TRPV1</b>	transient receptor potential vanilloid type-1
<b>VGCC</b>	voltage-dependent calcium channels

## Introduction

Communication between neurons mostly occurs via synapses, and disturbed synaptic connections are thought to underlie a wide range of neurological diseases (Lepeta et al., 2016; Zoghbi & Bear, 2012). Synapses consist of three components: the presynaptic terminal, the synaptic cleft, and the postsynaptic terminal. When the presynaptic neuron fires an action potential, chemical neurotransmitters are released from the presynaptic axonal terminal. The neurotransmitter molecules diffuse across the synaptic cleft and activate receptors in the postsynaptic membrane. Postsynaptic receptors are ligand-gated ion channels, which transform the chemical neurotransmission signal into an electric signal, inducing a small fluctuation of the membrane potential of the postsynaptic neuron. In case of excitatory transmission, the membrane potential of the postsynaptic neuron depolarizes, which means that the postsynaptic neuron is more likely to fire an action potential. In contrast, inhibitory synaptic currents hyperpolarize the membrane and reduce the probability in the postsynaptic neuron to fire an action potential. In the brain, the vast majority of synapses are excitatory, and only 10%–15% are inhibitory.

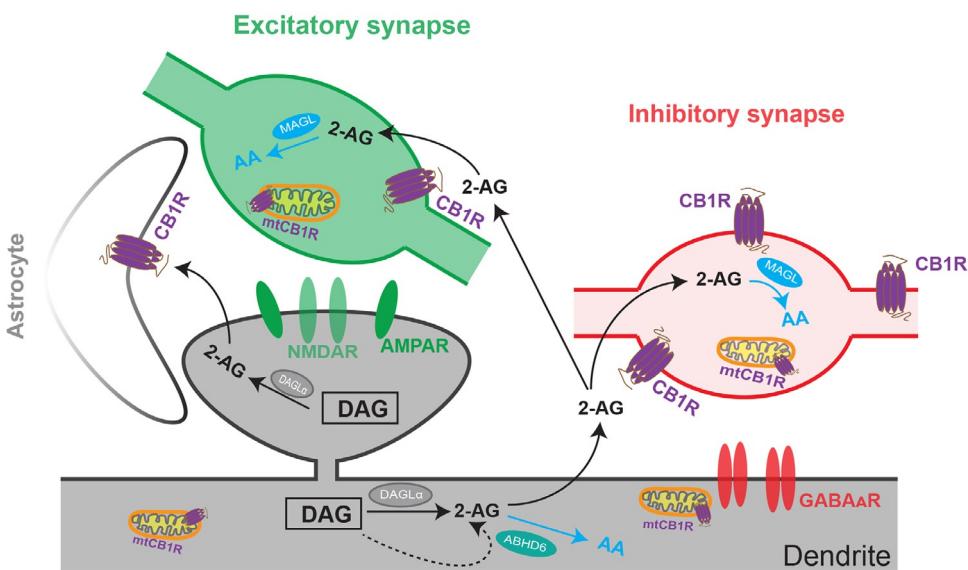
synapses. However, inhibitory synapses are of crucial importance in shaping brain activity and controlling information processing (Herstel & Wierenga, 2021).

The strength of synaptic connections is continuously adapting in response to environmental stimuli and neuronal activity. This synaptic plasticity is crucial for one of the most remarkable features of the brain: its capacity to adjust to changing circumstances and to incorporate previous experiences. It is important to note that adaptation of brain function involves changes at both excitatory and inhibitory synapses (Field et al., 2020; Herstel & Wierenga, 2021; Vogels et al., 2013). The way information is processed in the brain is strongly influenced by the behavioral context, such as stress or attention. At the cellular level, context signals are often provided by neuromodulatory signals, which alter synaptic connections and neuronal information transfer in neuronal networks (Abraham, 2008; Hattori et al., 2017). The most well-known neuromodulators include dopamine and serotonin, but endocannabinoids are the most abundant neuromodulator (Nadim & Bucher, 2014). Understanding how neuromodulators, including endocannabinoids, regulate synaptic transmission and plasticity will be crucial to advance our understanding of neuropsychiatric disorders and for future design of new therapies. In this chapter, we will provide a concise overview of the function of endocannabinoids at excitatory and inhibitory synapses in the brain.

The dominant endogenous cannabinoid in the central nervous system is 2-AG, which is present at higher levels than AEA and has a wider abundance over several brain regions (Araque et al., 2017; Piomelli, 2014). Therefore, we will focus on 2-AG in this chapter. Neurons synthesize endocannabinoids on demand, in an activity-dependent fashion (Hashimotodani et al., 2013). 2-AG synthesis is performed by the diacylglycerol lipase (DAGL) enzyme, with DAGL $\alpha$ , rather than DAGL $\beta$ , as the dominant isoform in neurons (Gao et al., 2010; Tanimura et al., 2010). CB1 receptors are G-protein-coupled receptors, which mostly signal via the inhibitory G $i/o$  proteins, although they can also interact with other G proteins, including G $q$  (Lauckner et al., 2005), G $12/13$  (Roland et al., 2014), and G $s$  (Fan & Yazulla, 2004; Finlay et al., 2017; Glass & Felder, 1997). In addition, downstream signaling pathways that are G-protein-independent have also been described in neurons (Delgado-Peraza et al., 2016; Nguyen et al., 2012).

## Many components of the endocannabinoid signaling system are present at synapses

The 2-AG production enzyme DAGL $\alpha$  is located in dendrites and at postsynaptic spines (Katona et al., 2006; Uchigashima et al., 2011; Yoshida et al., 2006), where excitatory synapses are located. In contrast, the predominant enzyme for 2-AG degradation, monoacylglycerol lipase (MAGL), is mainly located at axonal terminals (Fig. 1) (Gulyas et al., 2004; Uchigashima et al., 2011). Postsynaptic dendrites also contain alpha/beta-hydrolase domain containing protein 6 (ABHD6),



**FIG. 1** Localization of components of the endocannabinoid signaling system at synapses. The endogenous cannabinoid 2-AG is synthesized from DAG in postsynaptic dendrites and spines by the production enzyme DAGL $\alpha$ . The main endocannabinoid receptor CB1R is localized at presynaptic terminals of excitatory and inhibitory synapses. Expression levels of CB1R are particularly high at specific inhibitory axons. CB1 receptors are also present at astrocytes and intracellularly in mitochondrial membranes. The predominant enzyme for 2-AG degradation, MAGL, is located presynaptic, while ABHD6 is the main 2-AG hydrolyzing enzyme in dendrites (but it may also synthesize 2-AG).

which is involved in breakdown of 2-AG (Ludányi et al., 2011; Marrs et al., 2010), but it is also capable of 2-AG synthesis (van Esbroeck et al., 2019). Interestingly, the expression of these synthesis and degradation enzymes is highly specific and differs per brain region, cell, and synapse type (Yoshida et al., 2011). For instance, activity of MAGL strongly regulates tonic activation of presynaptic CB1 receptors at perisomatic, but not dendritic, inhibitory synapses in the hippocampal CA1 area (Lee et al., 2015; Lenkey et al., 2015).

The expression of CB1 receptors in the brain is also not homogenous. CB1 receptors are expressed at particularly high levels in a specific subtype of inhibitory neurons, which also express cholecystokinin (CCK). In these neurons, CB1 receptor localization is strongly enriched at the axonal shaft and presynaptic terminals (Fig. 1) (Dudok et al., 2015; Katona et al., 1999). In addition, CB1 receptors are expressed by excitatory neurons and their synapses, although the expression level is much lower compared with inhibitory neurons (Katona et al., 2006). Intriguingly, CB1 receptors are not only localized in the cell membrane, but they are also present in intracellular membranes of the mitochondria and endosomes (Bénard et al., 2012; Busquets-Garcia et al., 2018; Hebert-Chatelain et al., 2016; Zou & Kumar, 2018). Furthermore, CB1 receptors are also present at astrocytes, where they can regulate synaptic plasticity and memory formation (Han et al., 2012; Navarrete & Araque, 2008). The specific expression and subcellular location of the different components of the endocannabinoid system indicate that endocannabinoid signaling is tightly regulated by local synthesis and degradation and strongly dependent on cell type and brain region. In addition, the endocannabinoid system is highly dynamic and adaptable (Ogasawara et al., 2015). This is important to keep in mind when studying the endocannabinoid system in reduced models or under (often unphysiological) experimental conditions.

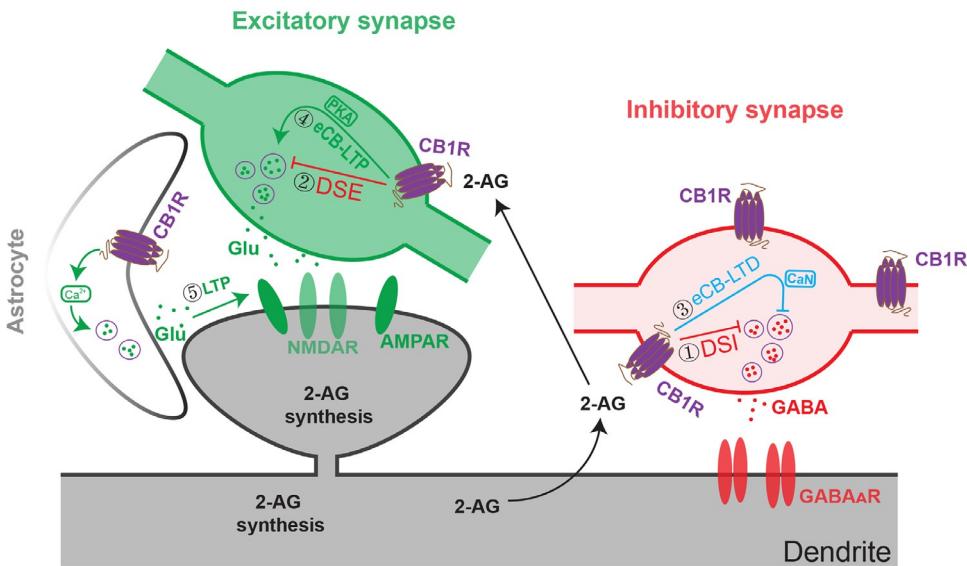
## Suppression of synaptic neurotransmitter release

In the central nervous system, endocannabinoids are well-known modulators of synaptic function in many brain regions, including the hippocampus and cortex. Their best described function is to serve as retrograde messengers, when they are produced in the postsynaptic dendrite and act on presynaptic CB1 receptors (Castillo et al., 2012; Lu & MacKie, 2016). CB1 receptors are G-protein-coupled receptors (GPCRs). In the presynaptic terminals, CB1 receptor activation activates G-protein signaling, which inhibits presynaptic voltage-dependent calcium channels (VGCCs) and activates inward-rectifying K<sup>+</sup> channels (Wilson et al., 2001) via the G-protein  $\beta\gamma$  subunits. This results in a hyperpolarization of the terminal and a reduced presynaptic calcium influx in response to an action potential and therefore a reduction in evoked neurotransmitter release (Castillo et al., 2012; Chevaleyre et al., 2007; Kano et al., 2009).

In most cases, suppression of neurotransmitter release is only temporary and lasts as long as endocannabinoids are available. For instance, when postsynaptic neurons are depolarized for several minutes, they produce endocannabinoids, which transiently suppress neurotransmitter release from incoming synapses (Kreitzer & Regehr, 2001; Ohno-Shosaku et al., 2001). This phenomenon is often referred to as depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE), depending on the type of incoming synapses that are involved (Fig. 2). Excitatory synapses are generally less sensitive to endocannabinoids compared to inhibitory synapses (Kreitzer & Regehr, 2001). This is not simply reflecting the more prominent expression of CB1 receptors in inhibitory neurons, as the amount of DSI is not directly correlated to the number of CB1 receptors that are present on the presynaptic terminal, but also depends on the abundance of specific signaling partners (Dudok et al., 2015; Lee et al., 2010; Lenkey et al., 2015). DSI and DSE are present in many brain regions and therefore likely represent a general form of short-term modulation of synaptic strength (Castillo et al., 2012). An important function of DSI is to temporarily reduce inhibitory inputs to facilitate the induction of plasticity at nearby excitatory synapses (Carlson et al., 2002; Castillo et al., 2012; Maglio et al., 2018).

## Long-term depression

Besides short-term suppression of neurotransmitter release, prolonged or repeated CB1 receptor activation can also lead to sustained or long-term depression (eCB-LTD) (Fig. 2) (Araque et al., 2017; Castillo et al., 2012). The induction of eCB-LTD requires prolonged activation of CB1 receptors and is often induced in experiments by 20 min application of the CB1 receptor agonist WIN55,212-2 or by theta burst stimulation. This type of presynaptic plasticity occurs at both excitatory and inhibitory synapse and extends to multiple brain regions, including the hippocampus and amygdala. Presynaptic CB1 receptors are typically coupled to G<sub>i/o</sub> protein signaling. Activated G<sub>i/o</sub> proteins reduce cyclic adenosine monophosphate (cAMP) levels by inhibiting the adenylyl cyclase (AC) via their  $\alpha_{i/o}$  subunits. In the presynaptic terminals, a prolonged decrease in cAMP levels results in LTD via a reduction of PKA-dependent phosphorylation of the Rab-interacting protein  $\alpha$  (RIM $\alpha$ ) (Chevaleyre et al., 2007; Tsetsernis et al., 2011). Furthermore, the calcium-sensitive



**FIG. 2** Short and long-term synaptic plasticity mediated by endocannabinoids. Endocannabinoids are synthesized in the postsynaptic dendrite in response to neuronal activity. Brief activation of presynaptic CB1 receptors leads to a transient suppression of neurotransmitter release (few seconds), which is termed ① depolarization-induced suppression of inhibition (DSI) at inhibitory synapses, or ② depolarization-induced suppression of excitation (DSE) at excitatory presynaptic terminals. Prolonged activation of CB1 receptors (minutes to hours) triggers long-term depression (③ eCB-LTD). This process involves multiple downstream effectors including calcineurin (CaN). In addition, in some synapses strong CB1 receptor activation can trigger potentiation of synaptic transmission (④ eCB-LTP) via presynaptic PKA activity. Finally, activation of CB1 receptors on astrocytes can facilitate ⑤ LTP at excitatory synapses via astrocytic glutamate release. LTP induction at excitatory synapses can also be facilitated by endocannabinoid-mediated suppression of GABA release from nearby inhibitory synapses (not shown).

phosphatase calcineurin is required for eCB-LTD induction, which assures that plasticity is restricted to active synapses (Heifets et al., 2008). Interestingly, local mTOR-based protein synthesis and ubiquitination (but not protein degradation) are also involved (Monday et al., 2020; Younts et al., 2016). In addition, G-protein-independent pathways have been reported in eCB-LTD. For instance, activation of CB1 receptors can mediate synaptic depression via ERK-mediated phosphorylation, and ultimately degradation, of Munc18-1 (Schmitz et al., 2016).

One of the main functions of eCB-LTD is to mediate heterosynaptic LTP. For instance, CB1-receptor-mediated short-term or long-term depression of GABA release facilitates excitatory synaptic transmission and mediates EPSP-to-spike (E-S) potentiation, increasing the excitability of the postsynaptic neuron (Chevaleyre & Castillo, 2003; Kim et al., 2019). In addition, endocannabinoid-mediated LTD at inhibitory synapses onto L2/3 neurons is important for development of the visual cortex (Jiang, Huang, et al., 2010; Jiang, Sohya, et al., 2010), and eCB-LTD of inhibitory synapses may be important for flexible learning (Tsetseris et al., 2011).

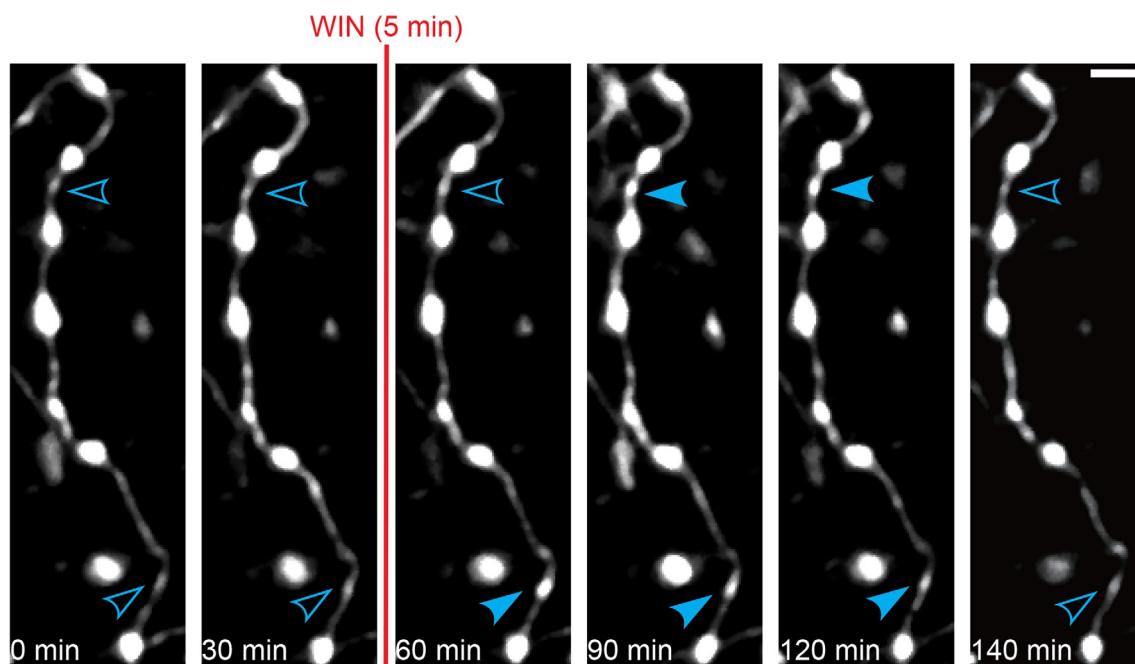
## Long-term potentiation

Until recently, the sole function of endocannabinoids at synapses was thought to modulate context-dependent suppression of transmission. However, a number of exciting recent studies have revealed that endocannabinoids are well capable of bidirectional modulation of synaptic transmission (Monday & Castillo, 2017). For instance, in corticostriatal synapses, endocannabinoids were shown to mediate both synaptic depression and potentiation, depending on the precise activation pattern of pre- and postsynaptic neurons (Cui et al., 2015, 2016). These studies suggest that short activation of CB1 receptors with relatively high levels of endocannabinoids induces synaptic potentiation, while prolonged CB1 activation with moderate levels of endocannabinoids induces synaptic depression. Activation of TrkB by BDNF modulates endocannabinoid synthesis and can thereby influence the direction of the eCB-induced plasticity (Gangarossa et al., 2019). While eCB-LTD requires presynaptic calcineurin, eCB-LTP appears to be mediated by presynaptic PKA (Fig. 2) (Cui et al., 2016). Endocannabinoid-dependent potentiation was also reported at synapses from the lateral perforant path (LPP) in the dentate gyrus, which requires interactions between presynaptic CB1 and integrin  $\beta 1$  receptors and involves actin remodeling (Wang et al., 2016, 2018).

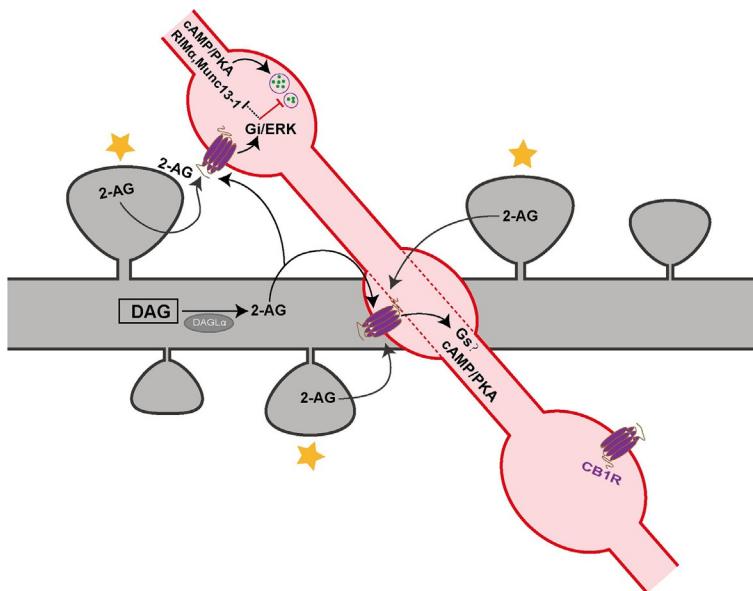
Another form of eCB-LTP has been described via astrocytes (Fig. 2). Activation of CB1 receptors on astrocytes leads to an increase in astrocytic calcium levels. This promotes astrocytic glutamate release, which facilitates long-term synaptic potentiation in nearby synapses (Gómez-Gonzalo et al., 2015; Navarrete et al., 2014; Navarrete & Araque, 2008, 2010). Astrocytic CB1 receptors can also be involved in cortical LTD (Han et al., 2012; Min & Nevian, 2012).

## Local coordination between excitatory and inhibitory synapses in dendrites

We recently observed another, rather unexpected, role for endocannabinoids in the brain. We discovered that endocannabinoid signaling mediates local coordination between excitatory and inhibitory synaptic inputs in dendrites of hippocampal CA1 neurons. Strong local excitatory activity can trigger the formation of a new inhibitory bouton at the same dendrite (Hu et al., 2019). This process was mediated by the endogenous cannabinoid 2-AG, which is synthesized in the dendrite and activates CB1 receptors on the inhibitory axon to promote synapse formation. In this way, dendrites may use retrograde endocannabinoid signaling to restore the balance between excitation and inhibition after excessive dendritic activity. Consistent with this first observation, brief activation of CB1 receptors at inhibitory axons is sufficient to initiate new bouton formation (Fig. 3) (Liang et al., 2021). Remarkably, downstream signaling mechanisms are independent of  $G_{i/o}$  signaling. Instead, CB1-receptor-mediated bouton formation required cAMP/PKA activity (Liang et al., 2021), suggesting that axonal CB1 receptors, in stark contrast to most presynaptic CB1 receptors, may be coupled to  $G_s$  proteins (Fig. 4). Intriguingly, cAMP signaling in Drosophila, zebrafish, and Aplysia axons has previously been implicated in the formation of presynaptic varicosities (Bailey & Kandel, 1993; Koon et al., 2011; Maiellaro et al., 2016; Upreti et al., 2019; Yoshida & Mishina, 2005). This suggests that endocannabinoid signaling via axonal CB1 receptors converges onto an intracellular cAMP/PKA pathway that is common with other neuromodulators, including dopamine, acetylcholine, and serotonin, to trigger axonal bouton formation. Downstream effectors of PKA are currently unknown, but may involve the actin cytoskeleton (Njoo et al., 2015; Roland et al., 2014; Zhou et al., 2019) or adhesion molecules (Frias et al., 2019; Klatt et al., 2021; Li et al., 2021).



**FIG. 3** Activation of CB1 receptors can trigger bouton formation at inhibitory axons. Time-lapse two-photon microscopy of GFP-expressing inhibitory axons in an organotypic hippocampal slice from a GAD65-GFP mouse. After 40 min, CB1 receptors were transiently activated by bath application of the synthetic agonist WIN (20  $\mu$ M) for 5 min (red line). This triggered formation of new boutons in the inhibitory axon (Liang et al., 2021). Solid arrowheads indicate the new boutons, empty arrowheads indicate the same axonal location at time points when no bouton was present. Scale bar is 2  $\mu$ m.



**FIG. 4** Local coordination between excitatory and inhibitory synapses. Strong local stimulation of excitatory synapses (on dendritic spines) can trigger the formation of a new inhibitory synapse nearby. Stimulated spines (indicated with the yellow stars) induce local synthesis of 2-AG. While presynaptic CB1 receptors mostly mediate synaptic suppression via Gi-protein signaling, activation of axonal CB1 receptors triggers bouton formation via an increase in cAMP and PKA activity, suggesting that they are coupled to G proteins.

## Applications to other areas

In this chapter, we have discussed different actions of endocannabinoids at synapses. However, our concise summary does certainly not cover all neuromodulatory actions of endocannabinoids in the brain. For instance, besides regulating synaptic transmission and plasticity, endocannabinoids can also interfere with cellular properties and synaptic plasticity via ion channel modulation and the actin cytoskeleton (Njoo et al., 2015; Roland et al., 2014). In particular, some inhibitory neurons, including CCK- and SST-expressing interneurons, can synthesize endocannabinoids themselves, and they use it to induce slow self-inhibition after repeated firing by hyperpolarization via G-protein-coupled inward rectifier potassium (GIRK) channels (Bacci et al., 2004; Glickfeld & Scanziani, 2005; Marinelli et al., 2008). Furthermore, in addition to CB1 receptors, endocannabinoids can also activate transient receptor potential vanilloid type-1 (TRPV1) receptors, and these two endocannabinoid receptors often have opposing effects on synaptic transmission (Egaña-Huguet et al., 2021; Jamieson et al., 2021). Notably, alteration of the metabolic processing of 2-AG may result in changes in pathways that indirectly influence synaptic transmission and/or plasticity. For example, 2-AG degradation by MAGL is the source of arachidonic acid, which is upstream of prostaglandin synthesis. This pathway is involved in neuroinflammation (Nomura et al., 2011).

The overall picture that emerges is that endocannabinoid-mediated modulation of synapses is highly synapse- and context-specific. This indicates that endocannabinoid actions in the brain are widespread, but at the same time region-specific. This corresponds well with the important role for endocannabinoid modulation during brain development (Harkany et al., 2008), in learning (DePoy et al., 2013; Ioannidou et al., 2021), and its link to many diseases (Lu & MacKie, 2016; Zou et al., 2019). A better understanding of the endocannabinoid action at the different type of synapses in the brain may hold the key for developing targeted treatments for brain diseases in the future.

## Minidictionary of terms

- **Synaptic plasticity.** Activity/experience-dependent modulation of synaptic strength and connectivity. In Hebbian forms of plasticity, synapses between active cells grow stronger, while synapses made by inactive cells will become weaker. Synaptic plasticity is often considered the biological process underlying learning.
- **Long term potentiation (LTP).** Long-lasting (hours to days) increase in synaptic strength. In many synapses, LTP is expressed by an increase in the number of postsynaptic receptors. LTP at inhibitory synapses is often referred to as iLTP.
- **Long term depression (LTD).** Long-lasting (hours to days) reduction in synaptic strength (often via a decrease in post-synaptic receptors). LTD at inhibitory synapses is often referred to as iLTD.
- **DSI.** Depolarization-induced suppression of inhibition.

- **DSE.** Depolarization-induced suppression of excitation.
- **Bouton.** Axonal varicosity (swelling). Mature boutons contain presynaptic terminals, but immature boutons may not.
- **Excitation/inhibition balance.** Coordination between excitatory and inhibitory synaptic signals in a neuronal circuit or postsynaptic neuron.
- **cAMP.** Intracellular second messenger, downstream of G-protein-coupled receptors. High levels of cAMP activate the protein kinase PKA.

## Key facts of endocannabinoids mediated synaptic plasticity

- Endocannabinoids can bidirectionally modulate synaptic transmission.
- Endocannabinoid-mediated plasticity is highly specific per cell type and brain region.

## Summary points

- Endocannabinoid signaling is tightly regulated by local synthesis, degradation, and specific signaling
- In most synapses, activation of presynaptic CB1 receptors leads to a transient suppression of neurotransmitter release.
- Prolonged CB1 receptor activation induces LTD, while strong, but brief, CB1 receptor activation can induce LTP.
- Activation of axonal CB1 receptors can trigger synapse formation via cAMP increase.

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## Chapter 8

# The endocannabinoids and potassium channels—An updated narrative

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>2-AGE</b>	2-arachidonoyl glyceryl ether; noladin ether
<b>AA</b>	arachidonic acid
<b>AEA</b>	<i>N</i> -arachidonylethanamide; anandamide
<b>APD</b>	action potential duration
<b>ARA-S</b>	<i>N</i> -arachidonoyl-L-serine, an endogenous bioactive endocannabinoid-related molecule
<b>BK<sub>Ca</sub></b>	large-conductance calcium-activated potassium (channel)
<b>BSA</b>	bovine serum albumin, a lipid scavenger
<b>Cav</b>	voltage-gated calcium (channel)
<b>CB1/CB2</b>	cannabinoid type 1 or type 2 receptor
<b>CHO</b>	Chinese hamster ovary (cell line)
<b>EC<sub>50</sub></b>	half-maximal effective concentration (the concentration required to produce the half-maximal response)
<b>GIRK</b>	G-protein-gated inwardly rectifying potassium (channel)
<b>HEK293</b>	human embryonic kidney 293 (cell line)
<b>hKv1.5</b>	human Kv1.5 (channel)
<b>Hv1</b>	voltage-gated (depolarization-activated) proton (channel)
<b>I<sub>A</sub></b>	A-current; fast inactivating A-type K <sup>+</sup> current
<b>IC<sub>50</sub></b>	half-maximal inhibitory concentration
<b>I<sub>K1</sub></b>	cardiac classical inward rectifier K <sup>+</sup> current
<b>I<sub>KDR</sub></b>	delayed outward rectifier K <sup>+</sup> current
<b>I<sub>Kso</sub></b>	standing-outward K <sup>+</sup> current, proposed to be principally carried by TASK-1 in cerebellar granule neurons
<b>I<sub>Kur</sub></b>	ultra-rapidly activating delayed rectifier K <sup>+</sup> current, the ultrarapid component of delayed rectifier K <sup>+</sup> current
<b>I<sub>M</sub></b>	M-current, a noninactivating, voltage-dependent K <sup>+</sup> current carried by Kv7 (KCNQ) channels
<b>I<sub>ss</sub></b>	steady-state outward K <sup>+</sup> current, proposed to reflect the activity of delayed rectifier potassium channels
<b>I<sub>sus</sub></b>	atrial end pulse sustained outward K <sup>+</sup> current
<b>I<sub>to</sub></b>	transient outward K <sup>+</sup> current, rapid activating and inactivating in response to depolarization, contributing to the early repolarization—phase 1—of the action potential
<b>I<sub>to, fast</sub></b>	fast component of transient outward K <sup>+</sup> current in cardiac cells, a major repolarization current in human atrium
<b>I<sub>to, slow</sub></b>	slow component of transient outward K <sup>+</sup> current in cardiac cells
<b>K<sub>2P</sub></b>	two-pore domain potassium (channel)
<b>K<sub>ATP</sub></b>	ATP-sensitive potassium (channel)
<b>Kir</b>	Inwardly rectifying potassium (channel)
<b>Kv</b>	voltage-gated potassium (channel)
<b>LTD</b>	long-term synaptic depression
<b>LTD-IE</b>	LTD of intrinsic excitability
<b>methAEA</b>	methanandamide, a synthetic, nonhydrolyzable analog of AEA
<b>NAGly</b>	<i>N</i> -arachidonoyl glycine, an endogenous lipoamino acid structurally and metabolically related to AEA
<b>O-LM</b>	oriens-lacunosum moleculare (cells), a major class of GABAergic interneurons in hippocampus
<b>PTX</b>	pertussis toxin
<b>TASK-1</b>	TWIK-related acid-sensitive potassium channel-1; KCNK3

TEA	tetraethylammonium
THC	$\Delta^9$ -tetrahydrocannabinol
TRP	transient receptor potential cation (channel)
TWIK	tandem of pore domains in a weak inward rectifying K <sup>+</sup> (channel)

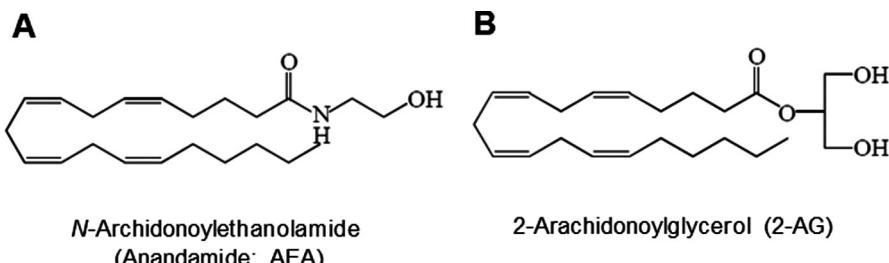
## Introduction

Endocannabinoids are a class of small signaling lipids consisting of amides, esters, and ethers of long-chain polyunsaturated fatty acids derived naturally from lipid precursors in plasma membranes of animal organisms (Burstein, 2014) and can be found throughout the human body. Endocannabinoids are part of the endocannabinoid system consisting of endocannabinoids, their metabolic enzymes for biosynthesis and biodegradation, the cannabinoid type 1 (CB1) and type 2 (CB2) receptors, and the endocannabinoid membrane transporter (De Petrocellis & Di Marzo, 2009). The endocannabinoid system operates as a homeostatic regulator in essentially all organ systems of physiological processes such as neurodevelopment and cell fate, synaptic transmission, learning and memory, nociception, stress and emotions, immunomodulation, hormone secretion, food intake and energy balance, digestive tract motility and secretion, bone mass, and reproduction, among others (Almeida et al., 2021; Maccarrone et al., 2015; Mechoulam & Parker, 2013). Aside from the physiological roles, elements of the endocannabinoid system may serve as potential therapeutic targets in various pathological conditions (Aizpurua-Olaizola et al., 2017). Indeed, pharmacological manipulation of the endocannabinoid system has yielded anti-nociceptive, anticonvulsive, anxiolytic, antiinflammatory, antiemetic, and orexigenic outcomes, alleviating the symptoms or slowing the progression of different diseases (Fraguas-Sánchez & Torres-Suárez, 2018; Reddy et al., 2020).

N-Arachidonylethanolamine (anandamide; AEA) (Fig. 1A), 2-arachidonoylglycerol (2-AG) (Fig. 1B), and 2-arachidonylglyceryl ether (2-AGE; noladin ether) represent the most notable endocannabinoids. AEA has widespread actions in the brain (Fride, 2002); it is also produced in the peripheral tissues where it exerts local effects such as regulation of vascular tone and of embryo transport/implantation (Maccarrone et al., 2015). While many of AEA's effects involve activation of CB1 or CB2 receptors (Lovering, 2008), pharmacological evidence nevertheless reveals the existence of additional targets of cannabinoids, such as orphan G-protein-coupled receptors, peroxisome proliferator-activated receptors, equilibrative nucleoside transporter-1, Cys-loop ligand-gated ion channels, transient receptor potential (TRP) channels, and a variety of voltage-gated ion channels (De Petrocellis & Di Marzo, 2010; Pistics & O'Sullivan, 2017). The presence of additional targets of cannabinoids besides the canonical CB receptors illustrates the complexity of the endocannabinoid system and its extensive signaling network.

Ion channels are integral membrane proteins with intrinsic ion-conducting pores and play a principal role in regulating membrane electrical properties and cellular excitability. Ion channels are crucially involved in a multitude of biological processes, ranging from neurotransmission to cell survival. Ion channel proteins are subject to posttranslational modulation mediated by cellular enzymes and messengers; notably, cannabinoids, a structurally heterogeneous group of lipid-soluble compounds that include endocannabinoids, phytocannabinoids, and synthetic cannabinoids, have been shown to modulate a variety of ion channel types independently of CB1/CB2 receptors (De Petrocellis et al., 2017; Oz, 2006).

In this chapter, I summarize evidence for the canonical CB receptor-independent actions of endocannabinoids on potassium channels, with an emphasis on the interactions between potassium channels and AEA. The aim is to put into perspective the biological relevance of ion channels serving as molecular targets of endocannabinoids as well as open questions that remain to be addressed. Mechanistic understanding of how ion channels are modulated directly by endocannabinoids and endocannabinoid-like compounds may reveal new possibilities for developing cannabinoid-based therapeutics to improve human health.



**FIG. 1** Chemical structures of *N*-arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG), two major endocannabinoids. (A) AEA. (B) 2-AG.

## Discussion

### Interactions between endocannabinoids and potassium channels

The potassium channels are encoded by over 80 genes in human genome (González et al., 2012). They are present in virtually all types of cells in all organisms and are crucial for setting the resting membrane potential, determining the shape, duration, and frequency of action potential, and damping excitatory signals (Hille, 2001). By controlling potassium flow across cell membrane, potassium channels regulate a wide spectrum of biological functions, including neurotransmitter/hormone release, vascular tone, heart rate, muscle contraction, cell volume, proliferation and migration, and cell survival, among others (González et al., 2012). Owing to their diverse molecular compositions, expression patterns, and physiological roles, potassium channels constitute attractive drug targets for potential treatment of metabolic, neurological, and cardiovascular disorders, autoimmune diseases, and cancer (Huang & Jan, 2014; Maljevic & Lerche, 2013; Wickenden, 2002; Wulff et al., 2009).

AEA has been implicated in a plethora of physiological and pathophysiological processes, such as embryonic development, vascular tone, motor functions, pain reduction, cognition functions, stress response, sleep, immunomodulation, neuroprotection, feeding and appetite, obesity, reward, neurodegenerative diseases, mood disorders, and cancer (Fride, 2002; Maccarrone et al., 2011; Pertwee, 2015). Many biological actions of AEA result from binding and activating the inhibitory G<sub>i/o</sub>-protein-coupled CB1 and CB2 receptors. For example, presynaptic suppression of neurotransmission induced by endocannabinoids (Lovinger, 2008) is likely mediated by CB receptor-dependent inhibition of N-type voltage-gated calcium (Cav) channels (Guo & Ikeda, 2004) as well as activation of G-protein-gated inwardly rectifying potassium (GIRK) channels (Guo & Ikeda, 2004) and A-type (fast inactivating) voltage-gated potassium (Kv) channels (Deadwyler et al., 1995). On the other hand, AEA may target ion channels and other proteins independently of CB1 or CB2 receptor activation to elicit its effects (De Petrocellis & Marzo, 2010). Indeed, several members in the potassium channel superfamily may interact with cannabinoids to mediate non-CB1, non-CB2 receptor-dependent actions (Tables 1–3).

### Modulation of large-conductance calcium-activated potassium channels by endocannabinoids

Large-conductance calcium-activated potassium (BK<sub>Ca</sub>) channels are activated by membrane depolarization and an elevated intracellular calcium concentration. They are expressed in most mammalian brain regions and hormone-secreting

**TABLE 1** CB1/CB2 receptor-independent effects of endocannabinoids or analogs on BK<sub>Ca</sub> channels.

Cannabinoids	Channel targets	Changes in channel activity	Cell models	IC <sub>50</sub> /EC <sub>50</sub> (concentration range) <sup>a</sup>	Reference
AEA/methAEA (indirect)	BK <sub>Ca</sub> ( $\alpha$ or $\alpha\beta$ )	↑	HEK293 and mouse aortic myocytes	(0.3–3.0 $\mu$ M)	Sade et al. (2006)
AEA 2-AG ARA-S <sup>b</sup> (extracellular)	BK <sub>Ca</sub> ( $\alpha$ )	↑ No change ↑	HEK293	5.27 $\mu$ M (to 10 $\mu$ M) 5.63 $\mu$ M	Godlewski et al. (2009)
AEA <sup>b</sup>	BK <sub>Ca</sub>	↑	EA.hy926 human endothelial-derived cell line	1.10 $\mu$ M	Bondarenko et al. (2017)
NAGly <sup>b</sup>	BK <sub>Ca</sub>	↑	Endothelial cells and isolated mouse aorta	(0.1–30 $\mu$ M)	Bondarenko et al. (2018)
AEA (extracellular) WIN 55,212-2 (extracellular)	TEA-sensitive BK <sub>Ca</sub> -like current	↓	Rat retinal ganglion cells	(10 $\mu$ M) 4.7 $\mu$ M	Zhang et al. (2013)

<sup>a</sup>Numbers in parentheses denote the concentration or the range of concentrations being tested.

<sup>b</sup>This effect is sensitive to membrane cholesterol depletion.

**TABLE 2** CB1/CB2 receptor-independent effects of endocannabinoids or analogs on Kv channels.

Cannabinoids	Channel targets	Changes in channel activity	Cell models	IC <sub>50</sub> /EC <sub>50</sub> (concentration range) <sup>a</sup>	Reference
AEA (extracellular <sup>b</sup> ) 2-AG methAEA	(h)Cardiac Kv4.3/KChIP2 ( $\cong I_{to}$ , fast)	↓ ↓ ↓	CHO and human right atrial appendage myocytes	0.4 μM 0.3 μM 0.6 μM	Amorós et al. (2010)
AEA	$I_{to}$	↓	Rat ventricular myocytes	(1–100 nM)	Li et al. (2012)
2-AG <sup>c</sup>	$I_A$	↓	Mouse midbrain dopaminergic neurons	(0.03–30 μM)	Gantz and Bean (2017)
AEA (extracellular) (also THC)	Kv1.2	↓	Murine fibroblasts (B82 cell line)	2.7 μM	Poling et al. (1996)
AEA (extracellular) methAEA WIN 55,212-2	Delayed rectifier Kv	↓ ↓ ↓	Rat aortic vascular smooth myocytes	0.6 μM (10 μM) (20 μM)	Van den Bosche and Vanheel (2000)
AEA (either side of membrane)	Kv3.1 (noninactivating)	↓ (into $I_A$ -type)	Xenopus oocytes and rat hippocampal slices	(3 μM)	Oliver et al. (2004)
AEA (extracellular) 2-AG methAEA <sup>b</sup>	Delayed rectifier Kv	↓	Rat primary cortical astrocytes and neocortical slices	~0.3 μM (1 μM) (1 μM)	Vignali et al. (2009)
2-AG	Delayed rectifier Kv	↓	Mouse insulinoma R7T1 β-cells	20 μM	Spivak et al. (2012)
AEA (extracellular) and 2-AG <sup>d</sup>	hKv1.5 $I_{sus}$ APD	↓ ↓ ↑	Mouse fibroblasts Human atrial cells Mouse left atria	0.9–2.5 μM	Barana et al. (2010)
AEA (intracellular) (open-channel block)	hKv1.5	↓	HEK293	~0.2 μM	Moreno-Galindo et al. (2010)
AEA (intracellular) and AA (intracellular) (open-channel block)	Kv1.1	↓	Xenopus oocytes and HEK293	1.3 μM 1.5 μM	Decher et al. (2010)
AEA	Delayed rectifier ( $I_{ss}$ )	No change	Rat ventricular myocytes	(1–100 nM)	Li et al. (2012)
AEA/2-AG ARA-S (extracellular)	hKv7.2/3 hKv7.2/3	No change ↑	Xenopus oocytes	3–7 μM	Larsson et al. (2020)
2-AG and ARA-S (extracellular)	M-current (Kv7.2/3)	↑	Rat O-LM interneurons	(30 μM)	Incontro et al. (2021)

<sup>a</sup>Numbers in parentheses denote the concentration or the range of concentrations being tested.<sup>b</sup>This effect does not involve lipid rafts/caveolae.<sup>c</sup>This effect was measured at 37°C (in contrast to room temperature at which other studies referenced in this chapter were performed).<sup>d</sup>This effect of 2-AG was only tested on hKv1.5, not on  $I_{sus}$  or APD.

**TABLE 3** CB1/CB2 receptor-independent effects of endocannabinoids or analogs on Kir and K<sub>2P</sub> channels.

Cannabinoids	Channel targets	Changes in channel activity	Cell models	IC <sub>50</sub> /EC <sub>50</sub> (concentration range) <sup>a</sup>	Reference
AEA	I <sub>K1</sub>	No change	Rat ventricular myocytes	(1–100 nM)	Li et al. (2012)
2-AG (intracellular)	K <sub>ATP</sub>	↓	Mouse insulinoma R7T1 β-cells	1.0 μM	Spivak et al. (2012)
AEA	K <sub>ATP</sub> (cromakalin-induced)	↓	Follicle-enclosed Xenopus oocytes	8.1 μM	Oz et al. (2007)
AEA <sup>b</sup> (CB2-dependent)	K <sub>ATP</sub>	↑	Rat ventricular myocytes	(1–100 nM)	Li et al. (2012)
AEA/methAEA 2-AG/THC methAEA	TASK-1 TASK-1 IKso	↓ No change ↓	COS/CHO/HEK293 COS-7 Cerebellar neurons	0.7 μM (10 μM) (10 μM)	Maingret et al. (2001)

<sup>a</sup>Numbers in parentheses denote the concentration or the range of concentrations being tested.

<sup>b</sup>This effect is CB2 receptor-dependent and CB1 receptor-independent.

cells where they modulate neurotransmitter and hormone release (Berkefeld et al., 2006). BK<sub>Ca</sub> channels are also present in vascular smooth muscle cells where they contribute to regulation of vascular contractile tone (Jackson, 2017). BK<sub>Ca</sub> channels may participate in the anticonvulsant and vasorelaxant effects of cannabinoids and mediate cannabinoid-induced peripheral analgesia and firing-suppressing effects in primary sensory afferents after nerve injury (Li et al., 2019).

### Effects of endocannabinoids on vascular BK<sub>Ca</sub> channels

It has been reported that the whole-cell current of BK<sub>Ca</sub> channels acquired in both transfected human embryonic kidney 293 (HEK293) and native aortic myocytes is potentiated by AEA and methanandamide (methAEA), a synthetic, nonhydrolyzable analog of AEA (Sade et al., 2006). The onset of BK<sub>Ca</sub> current potentiation induced by AEA or methAEA is gradual, taking around 6–8 min to develop a peak response. Notably, the BK<sub>Ca</sub>-potentiating effect of methAEA is insensitive to pretreatment with a potent CB1 receptor antagonist AM251 or with pertussis toxin (PTX) that prevents activation of G<sub>i/o</sub>-protein-coupled receptors, thus excluding an involvement of CB1 receptors (Sade et al., 2006). The CB2 receptor is unlikely to be involved, either, as JWH133, a selective and potent agonist for the CB2 receptor, does not reproduce the BK<sub>Ca</sub>-potentiating effect of AEA/methAEA. MethAEA also enhances BK<sub>Ca</sub> current acquired in aortic myocytes. It is thus suggest that, by activating BK<sub>Ca</sub> channels in vascular smooth muscle independently of CB1 and CB2 receptors, endocannabinoids may hyperpolarize membrane potential, reduce cell excitability, and consequently elicit vasodilation, providing neuroprotection after an ischemic stroke and/or suppressing excess activity of vascular smooth muscle tissues. However, BK<sub>Ca</sub> is probably not a direct target of AEA/methAEA, as methAEA only enhances BK<sub>Ca</sub> activity in whole-cell and cell-attached patch configurations but not in excised inside-out membrane patches (Sade et al., 2006). Cytosolic factors that potentially mediate the endocannabinoid-elicited potentiation of vascular BK<sub>Ca</sub> remain to be determined.

### Effects of ARA-S on recombinant human BK<sub>Ca</sub> channels

N-Arachidonoyl L-serine (ARA-S) is an endogenous lipoamino acid structurally similar to the endocannabinoid AEA. Both ARA-S and AEA, but not 2-AG (another major endocannabinoid), enhance the whole-cell outward K<sup>+</sup> current in HEK293 cells stably transfected with the α subunit of human BK<sub>Ca</sub> (hSlo) channels (Godlewski et al., 2009). AEA appears to be equipotent as ARA-S in enhancing BK<sub>Ca</sub> current (EC<sub>50</sub> ~ 5 μM). Interestingly, only externally applied ARA-S potentiates BK<sub>Ca</sub> current, whereas the effect is lost after patch excision. Moreover, the BK<sub>Ca</sub>-potentiating effect of ARA-S is independent of activation of CB receptors, G proteins, protein kinases, or calcium-dependent processes. Furthermore, ARA-S induces relaxation of rat isolated, intact, and denuded, small mesenteric arteries in a BK<sub>Ca</sub> channel blocker-sensitive manner. The findings by Godlewski et al. (2009) thus suggest that ARA-S directly interacts with BK<sub>Ca</sub> to potentiate channel function, through which ARA-S may contribute to the endothelium-independent mesenteric vasorelaxation.

### *Effects of endocannabinoids on endothelial BK<sub>Ca</sub> channels*

Bondarenko et al. (2017) have demonstrated that AEA concentration-dependently facilitates BK<sub>Ca</sub> single-channel activity ( $EC_{50}=1.1\text{ }\mu\text{M}$ ) in cell-free, inside-out patches obtained from human endothelial-derived EA.hy926 cells within a physiological Ca<sup>2+</sup> range, which suggests that AEA directly modifies BK<sub>Ca</sub> channel activity to induce endothelium-dependent vasorelaxation. N-Arachidonoyl glycine (NAGly), an endogenous lipoamino acid structurally and metabolically related to AEA, also exhibits vasorelaxant, analgesic, anti-inflammatory, and proinflammatory-resolving properties (Burstein, 2014). It has been reported by Bondarenko et al. (2018) that NAGly activates BK<sub>Ca</sub> channels in excised inside-out as well as outside-out patches obtained from human endothelial-derived cells and causes BK<sub>Ca</sub> channel blocker-sensitive membrane hyperpolarization in *in situ* mouse aortic endothelium, a critical event to initiate endothelium-dependent vasorelaxation. BK<sub>Ca</sub> channels are thus identified as cellular sensors for cannabinoids in *in vitro* and *in situ* endothelial cells. The NAGly effect does not require activation of CB1/CB2 receptors or GPR18 (a postulated endothelial cannabinoid receptor), suggesting that NAGly induces a CB1/CB2 receptor- and GPR18-independent activation of endothelial BK<sub>Ca</sub> channels, which might contribute to vasodilation by cannabinoids (Bondarenko et al., 2018). Interestingly, the action of cannabinoids and cannabinoid-like compounds on endothelial cells is at least partially underpinned by modulation of cholesterol level in caveolae, as the stimulatory response of endothelial BK<sub>Ca</sub> channels to NAGly (Bondarenko et al., 2018) or to AEA (Bondarenko et al., 2017) is prevented following cholesterol depletion.

### *Effects of endocannabinoids on neuronal BK<sub>Ca</sub>-like current*

Contrary to the BK<sub>Ca</sub>-potentiating effects described above, extracellularly applied AEA or WIN 55,212-2 (a high-affinity CB1/CB2 receptor agonist) has been shown to concentration-dependently reduce tetraethylammonium (TEA)-sensitive outward K<sup>+</sup> current in rat retinal ganglion cells, in a CB1/CB2-independent fashion (Zhang et al., 2013). These findings suggest that endocannabinoids (and certain synthetic cannabinoids) may regulate the excitability of retinal ganglion cells and thereby influence their output signals, by modulating K<sup>+</sup> conductance in a CB1/CB2-independent manner. The TEA-sensitive (but 4-AP- and glibenclamide-insensitive) outward current might be conducted by BK<sub>Ca</sub> channels (Zhang et al., 2013); however, its identity remains to be verified.

## Modulation of voltage-gated potassium channels by endocannabinoids

Kv channels shape the action potential by controlling its repolarization phase and determine the membrane potential and duration of the interspike interval. The rapidly inactivating A-type Kv channels space repetitive responses and help a cell fire at low frequencies, whereas the slowly or noninactivating delayed rectifier-type Kv channels function to keep single action potentials short and to permit high-frequency trains of action potentials (Hille, 2001).

### *Effects of endocannabinoids on A-type potassium channels*

The A-type potassium current ( $I_A$ ) is calcium-independent Kv current that undergoes rapid activation and inactivation.  $I_A$  has been identified and characterized in neuronal, cardiac, vascular, genitourinary, and gastrointestinal smooth muscle cells.

#### (1) Cardiac A-type K<sup>+</sup> current ( $I_{to}$ )

Cardiac potassium channels are the basis for the change in action potential configuration in response to variation in heart rate, and they are highly regulated.  $I_A$  in atrial and ventricular myocytes is referred to as “transient” outward current ( $I_{to}$ ). A complex formed by Kv4.2, Kv4.3, and KChIP2 may underlie the fast component of transient outward current ( $I_{to, \text{ fast}}$ ) in cardiac muscle, while Kv1.4 may underlie a slower transient outward current ( $I_{to, \text{ slow}}$ ) (González et al., 2012).

Endocannabinoids are involved in the regulation of cardiovascular function (Hiley, 2009). It has been demonstrated by Amorós et al. (2010) that the whole-cell current of human cardiac Kv4.3/KChIP2 channels ( $I_{Kv4.3}$ ; reproducing  $I_{to, \text{ fast}}$ ) stably expressed in Chinese hamster ovary (CHO) cells is inhibited by AEA, 2-AG, and methAEA in a concentration-dependent fashion ( $IC_{50}\sim 0.3\text{--}0.6\text{ }\mu\text{M}$ ). The inhibition of  $I_{Kv4.3}$  by endocannabinoids is accompanied by accelerated inactivation and a hyperpolarization shift in the voltage dependence of inactivation; moreover, these  $I_{Kv4.3}$ -suppressing effects of endocannabinoids are not mediated by activation of CB1/CB2 receptors or by modifications of the lipid order and microviscosity of the cell membrane. The putative AEA-interacting site may reside in Kv4.3 at its extracellular surface, as AEA and methAEA only block  $I_{Kv4.3}$  when administered extracellularly. In line with the findings obtained from recombinant Kv4.3/KChIP2 channels (reproducing  $I_{to, \text{ fast}}$ ), AEA also inhibits native  $I_{to, \text{ fast}}$  acquired from isolated human atrial myocytes in the presence of rimonabant and AM630, antagonists selective for CB1 and CB2 receptors, respectively, lending

support for a CB1/CB2 receptor-independent mechanism (Amorós et al., 2010). Human cardiac Kv4.3 channels thus represent a novel molecular target of AEA, 2-AG and cannabinoid analogs; the potent, direct inhibition of human cardiac  $I_{Kv4.3}$  and  $I_{to, \text{fast}}$  by endocannabinoids would increase the height and prolong the plateau duration of human cardiac action potential (Amorós et al., 2010), altering human cardiac electrical activity.

Li et al. (2012) examined  $I_{to}$  in isolated rat ventricular myocytes and reported that AEA concentration-dependently decreases  $I_{to}$ , by shifting the steady-state inactivation curve to the left and the recovery curve to the right, which suggests that AEA accelerates the voltage-dependent, steady-state inactivation of  $I_{to}$  and suppresses  $I_{to}$  recovery from inactivation. By contrast, AEA does not significantly alter the activation kinetics. The maximal effect of AEA on  $I_{to}$  develops slowly but can be measured within 8 min of initial exposure. Neither the CB1 receptor antagonist AM251 nor CB2 receptor antagonist AM630 abolishes the  $I_{to}$ -inhibiting effect of AEA, suggesting that AEA reduces  $I_{to}$  through a non-CB1 and non-CB2 receptor-mediated mechanism. On the other hand, AEA exerts no effect on steady-state outward K<sup>+</sup> current ( $I_{ss}$ ; proposed to reflect the activity of delayed rectifier Kv channels) (Li et al., 2012). Cardiac  $I_{to}$  channels represent an important target of class III antiarrhythmic drugs, owing to their critical role in defining resting membrane potential, heart rate, and action potential shape and duration in cardiac sinus node cells and cardiac myocytes (Niwa & Nerbonne, 2010). Direct inhibition of  $I_{to}$  may therefore account for, at least in part, the antiarrhythmic action of AEA.

## (2) Neuronal A-type K<sup>+</sup> current

Endocannabinoids are involved in the regulation of neuronal excitability (Augustin & Lovinger, 2018). In substantia nigra pars compacta dopaminergic neurons, the fast inactivating  $I_A$  is mediated by Kv4.3 channels co-assembled with the auxiliary subunits KChIP3.1 (Liss et al., 2001). Gantz and Bean (2017) reported that 2-AG, the dominant endocannabinoid in the brain, evokes a robust acceleration of action potential firing of isolated mouse midbrain dopaminergic neurons at sub-micromolar concentrations. 2-AG also acutely reduces  $I_A$  in a concentration-dependent manner, and the effect does not require CB receptor activation or G protein signaling. Moreover, 2-AG produces gating changes, including a shift of the voltage-dependent activation of  $I_A$  in the depolarizing direction, acceleration of the inactivation kinetics, and reduction of the maximal current for large depolarizations. The  $I_A$ -suppressing effect elicited by 2-AG likely results from a direct action on the channel through a membrane lipid interaction; specifically, binding of 2-AG may modify the interaction of the voltage-sensing S4-S5 region of the channel with the S6 region that controls channel opening and closing (Gantz & Bean, 2017). It is thus suggested that 2-AG and related lipid signaling molecules may tune neuronal excitability in a cell-autonomous manner, by direct modulation of  $I_A$ .

## *Effects of endocannabinoids on delayed rectifiers*

Classical delayed rectifiers such as Kv1.2 (in the absence of β subunits), Kv2.1, and Kv3.1 do not exhibit inactivation in the millisecond time scale, and they are involved in terminating action potentials, restoring the dominant potassium permeability of the resting membrane potential, and shaping the action potential (González et al., 2012).

### (1) Recombinant Kv1.2 delayed rectifier channels

AEA has been demonstrated to inhibit recombinant *Shaker*-related Kv1.2 channels (Poling et al., 1996); specifically, via accelerating inactivation, externally applied AEA concentration-dependently reduces Kv1.2 current recorded at whole-cell, cell-attached, and outside-out patch modes in stably transfected B82 murine fibroblasts, whereas intracellularly dialyzed AEA is without effect. At the single-channel level, the block of Kv1.2 by AEA is associated with a shortened open time and a prolonged closed time, reflecting stabilization of the long closed state and destabilization of the long open state. The inhibitory effect of AEA on Kv1.2 current ( $IC_{50} = 2.7 \mu\text{M}$ ) does not require activation of CB1 receptors or G protein signaling since neither CB1 receptor antagonists nor PTX prevents the AEA block, suggestive of direct channel modulation. Furthermore, externally applied Δ<sup>9</sup>-THC, the major psychotropic constituent of cannabis, is capable of mimicking the inhibitory action of AEA on the Kv1.2 channel with a comparable potency. Poling et al. (1996) thus propose that an acceptor site on the extracellular side of the Kv1.2 channel recognizes AEA and other cannabinoid-like molecules to generate functional suppression of the channel.

### (2) Native vascular delayed rectifier current

In vascular smooth muscle cells, modulation of potassium channel activity plays an essential role in regulating membrane potential, which in turn influences the open probability of vascular Ca<sub>v</sub> channels, the contractile tone of vascular smooth muscle, and blood flow (Nelson & Quayle, 1995). Externally applied AEA has been shown to concentration-dependently ( $IC_{50} = 0.6 \mu\text{M}$ ) reduce the native delayed rectifier K<sup>+</sup> current ( $IK_{DR}$ ) acquired in the whole-cell mode from dissociated rat

aortic smooth muscle cells in a CB1 receptor-independent manner ([Van den Bossche & Vanheel, 2000](#)). The reduction in  $IK_{DR}$  by AEA is associated with accelerated current decay. In addition, both methAEA and WIN 55212-2 elicit similar inhibition of  $IK_{DR}$  as does AEA, and their effects are also CB receptor-independent. By contrast, internally applied AEA or methAEA is ineffective. [Van den Bossche and Vanheel \(2000\)](#) thus postulated that cannabinoids likely bind to an external site on or near the delayed rectifier Kv channel of aortic vascular smooth muscle cells to modulate the channel activity. The molecular details for this effect remain to be elucidated.

### (3) Native astroglial delayed rectifier current

In both primary cultured rat cortical astrocytes and astroglial cells in cortical slices, low micromolar concentrations of AEA (and 2-AG) potently reduce  $IK_{DR}$  ( $IC_{50} \approx 0.3 \mu M$ ) ([Vignali et al., 2009](#)). The inhibition is voltage-independent (i.e., does not require channel being open to occur), and AEA does not alter the current kinetics. Pharmacological blockade experiments further uncovered that the AEA-induced inhibition is independent of CB1 receptor activation, AEA metabolism, and  $Ca^{2+}$  signaling. AEA's inhibitory effect in astrocytes is likely mediated by its interaction with the extracellular leaflet of the plasma membrane, because only extracellularly, not intracellularly, applied AEA effectively reduces  $IK_{DR}$  and only extracellularly applied bovine serum albumin (a lipid scavenger) recovers  $IK_{DR}$  suppressed by AEA. The inhibitory effect of AEA on astrocyte  $IK_{DR}$  does not involve an interaction of AEA with lipid rafts and/or caveolae as cholesterol-extracting agents fail to abrogate the AEA effect. Moreover, the  $IK_{DR}$ -suppressing effect of AEA is unaffected by removal of extracellular calcium, ruling out an involvement of TRP channels. AEA also inhibits  $IK_{DR}$  in passive and complex astrocytes and NG2 glia cells examined in neocortical slices. Collectively, the findings made by [Vignali et al. \(2009\)](#) suggest that AEA stabilizes the closed state of the astroglial delayed rectifier potassium channel by binding to hydrophobic determinants of the protein complex from the extracellular side. Their findings support that endocannabinoids may modulate CNS function through regulating potassium channel-mediated homeostatic function of the astroglial syncytium, which might account for some nonneuronal effects of the endocannabinoid system.

### (4) Native pancreatic $\beta$ -cell delayed rectifiers

Kv channels in the pancreas contribute to the regulation of insulin secretion by controlling the repolarization of  $\beta$ -cell action potential ([MacDonald & Wheeler, 2003](#)). The delayed rectifier is considered the dominant Kv current in  $\beta$ -cells ([Smith et al., 1990](#)) and has received much attention as potential therapeutic targets for type 2 diabetes. [Spivak et al. \(2012\)](#) reported that 2-AG concentration-dependently inhibits whole-cell  $IK_{DR}$  in the mouse insulinoma cell line R7T1 ( $IC_{50} = 20 \mu M$ ), causing a fast current decay; the 2-AG effect is CB receptor-independent. The predominant delayed rectifier potassium channel in murine  $\beta$ -cells is the Kv2.1 type; delayed rectifiers from Kv1 (such as Kv1.5), Kv2, and Kv3 subfamilies are also present ([MacDonald & Wheeler, 2003](#)). It is possible that multiple distinct Kv channels comprise the  $IK_{DR}$  of the human  $\beta$ -cell. How different types of delayed rectifier potassium channels present in  $\beta$ -cells are modulated by endocannabinoids remains to be deciphered.

### (5) Conversion of delayed rectifiers into fast-inactivating A-type Kv channels

Examining cloned Kv channels expressed in *Xenopus* oocytes has revealed that membrane lipids such as phosphatidylglycerol 4,5-bisphosphate, by removing fast inactivation, can convert A-type Kv channels into delayed rectifiers, whereas AEA and arachidonic acid, by conferring fast inactivation, convert noninactivating delayed rectifiers into rapidly inactivating A-type channels ([Oliver et al., 2004](#)). The proposed mechanism of action was that arachidonic acid and AEA, following being inserted into the membrane from either side of the membrane, cause conformational alterations in the selectivity filter, which then allosterically induces rapid closure of the channel pore. The findings by [Oliver et al. \(2004\)](#) thus imply that AEA may control the coding properties of neurons and synapses beyond the characteristics set by the expression profile of Kv channel subunits. It is suggested that the mechanism of action is a lipid-induced gating process rather than an open-channel block; but the molecular details remain to be determined.

### (6) Recombinant and native Kv1.5 channels

Cardiac Kv1.5 channels underlie the ultra-rapidly activating delayed rectifier  $K^+$  current ( $I_{Kur}$ ) prominent in atria but negligible in ventricles; these channels are critical for determining the height and duration of the human atrial action potential and represent a potential target for treating atrial arrhythmias ([Ravens & Wettwer, 2011](#)). Two different mechanisms of action with distinct interaction sites for endocannabinoid-induced modulation of cardiac Kv1.5 have been proposed (see below).

[Barana et al. \(2010\)](#) reported that both AEA and 2-AG exert a high-potency inhibition ( $IC_{50} \approx 0.9-2.5 \mu M$ ) on human cardiac Kv1.5 (hKv1.5) whole-cell current in stably transfected mouse fibroblasts. The hKv1.5-inhibiting effect of

endocannabinoids is independent of CB1/CB2 receptor activation and of changes in the order and microviscosity of the membrane; in other words, the potencies of blockade are unrelated to the liposolubility of the compounds. AEA, 2-AG, and methAEA all induce a fast inactivation component and slow the time course of deactivation of hKv1.5 current. Moreover, the blockade by AEA is evident exclusively when AEA is applied at the external surface of the cell membrane. Notably, the inhibitory effect of AEA or 2-AG is diminished by mutation of R487 located at the external vestibule entryway of the pore, a residue that determines Kv1.5 sensitivity to external TEA. The AEA block of hKv1.5 can be reproduced in native cells, as manifested by an inhibition by AEA of atrial end pulse sustained K<sup>+</sup> current (*I*<sub>lus</sub>, mainly carried by Kv1.5) in human atrial myocytes in the presence of CB1/CB2 receptor antagonists. AEA also prolongs the action potential duration (APD) in mouse left atria (Barana et al., 2010). These findings thus support that the endocannabinoids inhibit human cardiac Kv1.5 channels via specific interaction at the extracellular TEA binding site of the channel, a mechanism by which the endocannabinoids regulate the shape of atrial action potentials (Barana et al., 2010).

Moreno-Galindo et al. (2010) examined hKv1.5 overexpressed in HEK293 cells and demonstrated that AEA potently blocks hKv1.5 current ( $IC_{50} \approx 0.2 \mu M$ ) in a CB receptor-independent manner when applied from the cytoplasmic membrane surface. The Kv1.5-inhibiting effect of AEA is concentration- and voltage-dependent. Moreover, AEA accelerates the inactivation rate of Kv1.5 current while slowing its deactivation, and the onset of AEA block is slowed by internal TEA (a pore blocker), all consistent with an open-channel block mechanism. Following alanine-scanning mutagenesis analysis and molecular modeling, V505 and I508, two pore-facing residues located in the lower portion of the S6 transmembrane domain that lines the channel vestibule, were proposed as the AEA binding sites, which, however, are distinct from the extracellularly located interaction site (i.e., R487) suggested by Barana et al. (2010). V505 and I508 of Kv1.5, both highly conserved among Kv family members, face toward the central cavity of the pore and constitute a motif that forms a hydrophobic ring around the ion conduction pathway. Moreno-Galindo et al. (2010) thus suggest that the conserved hydrophobic ring motif may be a critical determinant of CB receptor-independent modulation by AEA in other K<sup>+</sup> channel families.

### (7) Recombinant Kv1.1 channels

Kv1.1 channels are delayed rectifier potassium channels expressed in the brain, retina, heart, and skeletal muscle, and malfunction of Kv1.1 is associated to episodic ataxia type 1 with myokymia (González et al., 2012). Studying cloned Kv1.1 expressed in oocytes and HEK293, Decher et al. (2010) reported that highly unsaturated lipids such as arachidonic acid and AEA reduce the steady-state outward Kv1.1 current and induce apparent fast inactivation. Specifically, I400, a highly conserved, pore-facing residue located at the S6 domain, is identified as the interaction site for highly unsaturated fatty acids, based on the evidence that an isoleucine-to-valine mutation of this residue abolishes lipid-induced fast inactivation. Of note, I400 in Kv1.1 is homologous to I508 in Kv1.5, a residue identified by Moreno-Galindo et al. (2010) as one of two pore-facing residues directly interacting with AEA to confer open-channel block. I400 in Kv1.1 is also equivalent to I428 in Kv1.3 (another delayed rectifier), and in a similar vein, mutating I428 in Kv1.3 (to alanine or valine) profoundly reduces fast inactivation induced by arachidonic acid (Decher et al., 2010). Through analyzing the effects of highly unsaturated lipids on Kv1.1 deactivation kinetics and their competition with internal TEA and Kvβ, Decher et al. (2010) suggest that arachidonic acid and other highly unsaturated lipids induce fast inactivation of Kv1.1 by causing open-channel block from the intracellular side. The changes in fast inactivation of Kv channels would affect cell excitability and activity-dependent signaling.

### *Effects of endocannabinoids on neuronal M-current (Kv7.2/Kv7.3)*

The Kv7 subfamily of Kv channels comprises five subunits (Kv7.1–Kv7.5), each exhibiting distinct tissue distribution and physiological properties (Soldovieri et al., 2011). Neuronal M-current ( $I_M$ ) is primarily carried by the heteromeric Kv7.2/Kv7.3 channel.  $I_M$  is important for dampening neuronal excitability by contributing to the negative resting membrane potential; it inhibits neuronal hyperexcitability and causes spike frequency adaptation during sustained depolarizations. Genetic mutations of Kv7.2 and Kv7.3 subtypes are associated with inherited epilepsy in humans; and Kv7.2/Kv7.3 channels have emerged as an appealing target for pharmacological interventions of human hyperexcitability disorders (Soldovieri et al., 2011).

### (1) Recombinant Kv7.2/Kv7.3 channels

ARA-S is an endogenous lipoamino acid structurally similar to AEA and a weak activator of the CB1 receptor. It has been recently shown by Larsson et al. (2020) that extracellular application of ARA-S potently activates human Kv7.2/Kv7.3 channels (i.e., neuronal M-channels) expressed in *Xenopus* oocytes ( $EC_{50} \approx 3\text{--}7 \mu M$ ), by shifting the voltage dependence

of activation to more hyperpolarized membrane potential and by increasing the maximal conductance. ARA-S also facilitates the activation kinetics while slowing the deactivation; by contrast, endocannabinoids such as AEA, 2-AG and *N*-arachidonoyl dopamine are without effect. Through charge-neutralization mutagenesis analysis, several arginine residues in the S4 and S6 domains of hKv7.2 and hKv7.3 are identified as important sites for ARA-S-elicited activation. Larsson et al. (2020) speculated that the negative charge of the head group of endocannabinoids or endocannabinoid analogs is required to enable electrostatic interaction with the arginine residues in the S4 and S6 domains of Kv7.2/Kv7.3 channels. The selectivity of ARA-S for the Kv7.2/Kv7.3 channel over other Kv7 subtypes suggests that combining low concentrations of ARA-S and antiepileptic drugs may effectively enhance M-currents with an improved Kv7 subtype selectivity while limiting the off-target effect (Larsson et al., 2020).

## (2) Native neuronal M-channels

Kv7.2/Kv7.3 channels are highly expressed in the dendrites of oriens-lacunosum moleculare (O-LM) interneurons, where they primarily control the interspike interval (Lawrence et al., 2006). Incontro et al. (2021) have demonstrated that Kv7 channel activity in rat hippocampal O-LM interneurons is upregulated following induction of presynaptic long-term synaptic depression (LTD) of excitatory input, which yields a synergistic LTD of intrinsic excitability (LTD-IE). Both LTD and LTD-IE in O-LM interneurons are mediated by biosynthesis of endocannabinoids. Extracellular application of 2-AG or ARA-S reduces intrinsic excitability of O-LM interneurons and enhances  $I_M$ . Moreover, molecular modeling uncovered direct interaction of 2-AG with the Kv7.2 channel, with a high-affinity binding site for 2-AG located at the junction of two adjacent Kv7.2 subunits in the outer leaflet of the plasma membrane, suggesting a persistent binding and possibly a persistent activation by 2-AG (Incontro et al., 2021). ARA-S appears to share the same binding pocket on the channel. Thus, direct interaction of endocannabinoids with postsynaptic Kv7.2/Kv7.3 channels induces LTD-IE in O-LM interneurons, which may represent a novel involvement of endocannabinoids in long-lasting plasticity of intrinsic excitability in interneurons.

## Modulation of inwardly rectifying potassium channels by endocannabinoids: $K_{ATP}$ channels

The ATP-sensitive potassium ( $K_{ATP}$ ) channel functions as a high-fidelity metabolic sensor that couples intracellular metabolic state to membrane electrical activity (Nichols, 2006), serving a homeostatic role ranging from blood glucose regulation to cardioprotection (Tinker et al., 2018). The molecular/subunit compositions of the widely expressed  $K_{ATP}$  channels exhibit tissue specificity (Babenko et al., 1998).

### *Effects of endocannabinoids on pancreatic $\beta$ -cell $K_{ATP}$ channels*

$K_{ATP}$  channels in pancreatic  $\beta$ -cells regulate insulin secretion in response to plasma glucose levels. Spivak et al. (2012) reported that single-channel  $K_{ATP}$  currents acquired at 2 mM glucose in the inside-out patch configuration in a mouse insulinoma  $\beta$ -cell line R7T1 are concentration-dependently inhibited by 2-AG applied from the cytosolic side ( $IC_{50}=1\ \mu M$ ). Although CB1 receptors are expressed in murine  $\beta$ -cells, 2-AG inhibition of  $K_{ATP}$  current is unaffected by the CB1 receptor antagonist AM251, indicating that CB1 receptors do not mediate the effect. The blockade of  $\beta$ -cell  $K_{ATP}$  channels by 2-AG at low glucose concentrations would depolarize the  $\beta$ -cell and stimulate insulin secretion; it is therefore suggested that 2-AG may increase insulin secretion by directly interacting with the  $K_{ATP}$  channel in a manner similar to sulphonylureas ( $K_{ATP}$  channel blockers) (Spivak et al., 2012).

### *Effects of endocannabinoids on $K_{ATP}$ channels in follicular oocytes*

Endogenous  $K_{ATP}$  channels in follicle-enclosed oocytes from *Xenopus laevis* are subject to modulation by gonadotropins (Honoré & Lazdunski, 1991) and may play important roles in oocyte maturation and hormonal regulation of oocyte development. The effect of endocannabinoids on cromakalim (a  $K_{ATP}$  channel opener)-activated  $K_{ATP}$  currents has been investigated in follicular oocytes (Oz et al., 2007). AEA reversibly suppresses cromakalim-activated  $K_{ATP}$  currents in a noncompetitive manner ( $IC_{50}=8.1\ \mu M$ ), and the effect is independent of CB1 and CB2 receptors and of  $G_{i/o}$ -protein-coupled receptors, as manifested by the ineffectiveness of CB receptor antagonists and PTX, respectively, to prevent AEA-induced block. Furthermore, inhibitors of the biodegradative enzymes for AEA also fail to alter the  $K_{ATP}$ -suppressing effect of AEA, indicating that the effect of AEA is not mediated by its metabolic products (Oz et al., 2007). These findings thus suggest that AEA may regulate the hormonal maturation process in *Xenopus* oocytes by directly modulating  $K_{ATP}$  channels.

### *Effects of endocannabinoids on ventricular myocardial K<sub>ATP</sub> channels*

AEA concentration-dependently increases whole-cell K<sub>ATP</sub> currents induced by dinitrophenol, a mitochondrial uncoupler, in isolated rat ventricular myocytes (Li et al., 2012). The stimulatory effect of AEA is reduced by the CB2 receptor antagonist AM630 but not by the CB1 receptor antagonist AM251. These findings suggest that AEA augments ventricular myocardial K<sub>ATP</sub> currents through a CB2 receptor-dependent pathway (Li et al., 2012), which may underlie the antiarrhythmic and cardioprotective action of AEA. By contrast, AEA exerts no effect on the classical inward rectifier current ( $I_{K1}$ ) in ventricular myocytes (Li et al., 2012), indicating that the AEA effect on K<sub>ATP</sub> is specific. Whether AEA is capable of modulating myocardial K<sub>ATP</sub> channels in cell-free, excised membrane patches remain to be determined. Endocannabinoid production is induced when the cardiovascular system is functioning under deleterious conditions (Hiley, 2009). Enhancement of K<sub>ATP</sub> current by endogenously released AEA under pathophysiological conditions may contribute to the cardioprotection afforded by K<sub>ATP</sub> channels.

### **Modulation of two-pore domain potassium channels by endocannabinoids: TASK-1**

TWIK-related acid-sensitive potassium channel 1 (TASK-1), a member in the K<sub>2P</sub> channel subfamily, encodes an acid- and anesthetic-sensitive background K<sup>+</sup> current (González et al., 2012). TASK-1 sets the resting membrane potential of both cerebellar granule neurons and somatic motoneurons and may contribute to anesthetic-induced immobilization (Sirois et al., 2000). It has been shown that TASK-1 expressed in transfected COS-7, CHO or HEK293 cells is blocked by sub-micromolar concentrations of AEA, an effect independent of canonical CB receptors and of G proteins (Maingret et al., 2001). The inhibition of TASK-1 by AEA is specific, not mimicked by 2-AG or by Δ<sup>9</sup>-THC; additionally, AEA hydrolysis is not involved, as methAEA, the nonhydrolyzable analog of AEA, is similarly effective. AEA also blocks the standing-outward K<sup>+</sup> current (IKso) principally carried by TASK-1 and induces depolarization in cerebellar granule neurons (Maingret et al., 2001). These findings suggest that TASK-1 constitutes a novel, sensitive molecular target of AEA. Cannabinoids including AEA profoundly affect locomotion, exerting a dose-related biphasic effect (Chaperon & Thiébot, 1999). Direct modulation of TASK-1 by low doses of AEA might thus account for some of the biphasic, CB1 receptor-independent effects observed with AEA on locomotion.

### **Potential mechanisms**

In the majority of studies reviewed here, the CB1/CB2 receptor-independent modulatory effects exerted by endocannabinoids or endocannabinoid analogs on potassium channels are effective only when endocannabinoids are introduced to the extracellular side of the membrane. However, in several studies, endocannabinoids are effective only when administrated at the cytoplasmic side of the membrane. These observations imply the presence of distinct interaction sites or mechanisms of action, which may be attributable to differences in the types of ion channels or endocannabinoids investigated, cell models/cellular environments channels being exposed to, or experimental protocols adopted.

On the other hand, although membrane environment seems to be critical for the regulation of signal transduction pathways triggered by G-protein-coupled receptors such as CB1 (Maccarrone et al., 2011), current evidence does not support an involvement of changing membrane fluidity or altering lipid bilayer properties in mediating the CB receptor-independent actions of AEA on potassium channels (Amorós et al., 2010; Maingret et al., 2001; Vignali et al., 2009). It is worth noting that most of AEA (~70%) is found in nonlipid raft fractions of the membrane, unlike 2-AG, which is entirely localized in lipid rafts in dorsal root ganglion cells (Rimmerman et al., 2008). It is therefore less likely that changes in membrane fluidity serve as a primary mechanism of action responsible for AEA's CB receptor-independent modulation of potassium channels. The presence of membrane cholesterol, however, appears to be relevant for direct modulation of endothelial BK<sub>Ca</sub> by AEA and NAGly (Bondarenko et al., 2017, 2018) and for ARA-S modulation of recombinant BK<sub>Ca</sub> (Godlewski et al., 2009).

Lipid signals such as endocannabinoids and structurally related fatty acids may modify gating of voltage-gated ion channels through a direct action on or near the channel via a membrane-lipid interaction (Amorós et al., 2010; Gantz & Bean, 2017). A model for direct interactions between potassium channel proteins and endocannabinoids is further supported by identification of specific residues in several channel proteins crucial for the CB receptor-independent modulatory actions exerted by endocannabinoids (Barana et al., 2010; Decher et al., 2010; Moreno-Galindo et al., 2010) and by endocannabinoid-like lipids (Larsson et al., 2020).

## Conclusions and perspectives

The endocannabinoid system not only provides important homeostatic regulation in essentially all organs and tissues, it is also involved in pathophysiological conditions where its activation (or inhibition) renders symptom relief or protection against the progression of certain disorders. Existing evidence suggests that various types of potassium channels, voltage-gated  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and Hv1 proton channels, TRP channels, and neurotransmitter-gated ion channels are directly modulated by AEA (or 2-AG), extending the known role of AEA as a retrograde messenger that indirectly modulates ion channel function via activation of presynaptic CB receptors. Considering the importance of the endocannabinoid system and ion channels in human health and disease, and their promising therapeutic prospects in a vast number of medical conditions, further research endeavors to delineate the molecular mechanisms underlying the interactions between different types of cannabinoids and their ion channel targets are warranted.

## Applications to other areas

In this chapter, we have reviewed evidence for the CB1/CB2 receptor-independent actions of endocannabinoids on  $\text{K}^+$  channels, which reinforces the notion that ion channels may serve as direct targets of endocannabinoids and thereby contribute to the physiological and pathophysiological roles of endocannabinoids in biological processes governed by the activity level of respective channel proteins. Molecular details concerning endocannabinoid's  $\text{K}^+$  channel-modulating effects are limited at present. Several mechanisms of action are proposed based on Kv channel studies. One model describes modification of the channel gating process, with endocannabinoids binding to the selectivity filter or the voltage sensor, and the other model postulates an open-channel block mechanism, with endocannabinoids physically occluding the ion permeation pathway of the channel; alternatively, endocannabinoids may also bind to sites at the junction of two adjacent channel subunits to alter channel function. It is tempting to speculate that similar mechanisms may account for interactions between endocannabinoids and other potassium channel subtypes, because some of the interaction sites/residues are conserved in evolutionarily related channels. The information may also be useful in studying potential direct interactions of ion channels with phytocannabinoids or endocannabinoid-like lipids, particularly those with low/no affinities toward canonical CB receptors, to achieve a mechanistic understanding of the medicinal effects associated with these cannabinoids.

## Minidictionary of terms

- **A-type channels.** A class of Kv channels that displays fast activation and fast inactivation during depolarization pulses, generating a transient outward current  $I_A$ .  $I_A$  spaces repetitive responses and helps a cell fire at low frequencies.
- **Delayed rectifiers.** A class of Kv channels that exhibit slow or no inactivation of the outward  $\text{K}^+$  current when activated during depolarization pulses; they keep single action potentials short and permit high-frequency trains of action potentials.
- **Gating.** The opening and closing process of ion channels.
- **Ion channels.** Pore-forming membrane proteins that when open allow the flow of ions across membranes down the electrochemical gradient, generating current.
- **$I_{\text{to}}$ .**  $I_A$  in atrial and ventricular myocytes, including a fast component ( $I_{\text{to, fast}}$ ) and a slower component ( $I_{\text{to, slow}}$ ).

## Key facts of potassium channels

- There are over 80 mammalian genes encoding potassium channel subunits.
- Potassium channels are present in virtually all types of cells in all organisms.
- The potassium channel superfamily consists of four families: voltage-gated potassium (Kv), calcium-activated potassium ( $\text{K}_{\text{Ca}}$ ), inwardly rectifying potassium (Kir), and two-pore domain potassium ( $\text{K}_{2\text{P}}$ ) channels.
- Potassium channels are important for setting the resting membrane potential, determining the shape, duration, and frequency of action potential, and damping excitatory inputs on a cell.
- By controlling potassium flow across cell membranes with high selectivity, potassium channels regulate a wide plethora of biological processes, from neurotransmission to cell survival.
- Potassium channels constitute attractive drug targets for potential treatment of metabolic, neurological, and cardiovascular disorders, autoimmune diseases, and cancer.

## Summary points

- Endocannabinoids are the endogenous ligands for the cannabinoid CB1 and CB2 receptors.
- While many effects of endocannabinoids involve activation of CB1 or CB2 receptors, pharmacological evidence reveals the existence of additional targets of cannabinoids.
- Noticeably, BK<sub>Ca</sub>, A-type Kv, select delayed rectifier-type Kv, Kv7 (KCNQ), K<sub>ATP</sub>, and TASK-1 channels have been shown to be modulated by the endocannabinoid AEA in a CB1 and CB2 receptor-independent manner.
- 2-AG also modulates select A-type, delayed rectifier Kv, KCNQ, and K<sub>ATP</sub> channels.
- Endocannabinoids may modify the gating process or act as an open-channel blocker, by directly interacting with their channel targets.
- However, molecular details at present are limited, and further mechanistic studies are warranted.

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## Chapter 9

# Insights into the endocannabinoid system from investigations of the development of social behavior in rodents of both sexes

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## Abbreviations

2-AG	2-arachidonyl glycerol
AEA	<i>N</i> -arachidonyl ethanolamine
BNST	bed nucleus of the stria terminalis
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid type-1 receptor
CB2	cannabinoid type-2 receptor
CG	cingulate gyrus
CPP	conditioned place preference
ECS	endocannabinoid system
ERK1/2	extracellular signal-regulated protein kinase-1 and -2
FAAH	fatty acid amide hydrolase
GIRK	G-protein-coupled inwardly rectifying potassium channel
GPCR	G-protein-coupled receptor
JNK	c-Jun N-terminal kinase
MAGL	monoacylglycerol lipase
mPFC	medial prefrontal cortex
mRNA	messenger RNA
NAc	nucleus accumbens
THC	Δ <sup>9</sup> -tetrahydrocannabinol
URB	URB597
VGCC	voltage-gated calcium channels
WIN	WIN55,212-2

## Introduction

The last 30 years have seen a tremendous advancement in the understanding of the endocannabinoid system (ECS) and its contributions to many facets of mammalian physiology and behavior. Among these advancements is an increased understanding of endocannabinoid contributions to social behavior and the development of underlying social brain networks. Here, we discuss the interactions between the ECS and social behaviors in rodents, with a focus on both the developmental and acute contributions of the ECS on sociality. We first provide an overview of social behaviors and their underlying neural networks, and a discussion of how these differ between the sexes and across development in rodents. Next, we briefly describe the ECS in terms of its composition, organization, and ability to drive changes in brain development and behavior. In particular, we focus on the influence of the ECS on social brain and behavior development, with an emphasis on development across the adolescent period and into adulthood. We describe evidence for a role of the ECS in the acute regulation of social behaviors, highlighting age-, sex-, and behavioral-specificity of this system's contributions. Further, there are well-defined sex differences in the social brain and associated behaviors, as well as in the expression and function of

the ECS and its various components, which often depend on developmental stage. Thus, we discuss potential mechanisms driving sex differences in ECS regulation of social behavior and the development of its underlying neurocircuitry, highlighting key areas that warrant further research.

## The endocannabinoid system

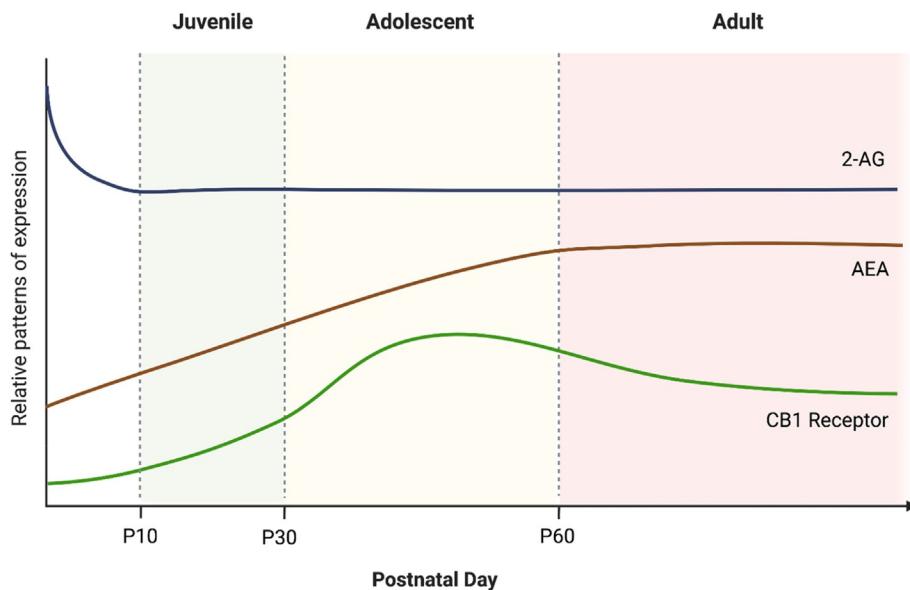
The ECS represents a diverse network of endogenously produced lipid-based signaling molecules comprising two main inhibitory G-protein-coupled receptors (GPCRs), the cannabinoid type-1 (CB1) and type-2 (CB2) receptors, the endogenous ligands that bind to and activate these receptors (endocannabinoids), as well as the enzymatic machinery that regulates their synthesis, degradation, and transport. Despite similarities in the endocannabinoids that target CB1 and CB2 receptors, most notably *N*-arachidonylethanolamine (AEA) and 2-arachidonyl glycerol (2-AG), as well as in the downstream effects of their activation, there is diversity in their spatial and cell-type expression patterns across organisms that are indicative of their functionality. For instance, CB1 receptors are highly localized to the brain and central nervous system, with abundant expression in corticolimbic regions involved in the regulation of cognitive and affective behaviors, and of relevance to this chapter, of social behaviors. Regions of importance to social behavior regulation that are rich in CB1 receptor expression include the medial prefrontal cortex (mPFC), the hippocampus, the amygdala, and the nucleus accumbens (NAc), among others (Herkenham et al., 1991; Tsou et al., 1998). Within the brain, CB1 receptors activation leads to the reduction of neurotransmitter release through inhibition of voltage-gated calcium channels (VGCCs) and activation of G-protein-coupled inwardly rectifying potassium (GIRK) channels (Guo & Ikeda, 2004). In addition to regulating cell-to-cell communication, CB1 receptor activation is coupled to the activation of a variety of intracellular signaling pathways that are associated with neurodevelopment and synaptic plasticity, most notably the cAMP-dependent Protein Kinase A, phosphoinositide 3-kinase/Protein Kinase B, c-Jun N-terminal Kinase (JNK), and extracellular-regulated protein kinase-1, and -2 (ERK1/2) pathways (Pertwee, 1997). As such, endocannabinoids, via CB1 receptor activation, can drive both acute effects on neural activity and long-term developmental and synapto-plastic changes that influence future behavioral and physiological responses.

In contrast to CB1 receptor expression and function, CB2 receptors are localized primarily to immune cells of the periphery, most notably within the spleen and lymphatic system (Svíženská et al., 2008). Although there is debate around the extent to which CB2 receptors are expressed centrally, recent evidence has identified both neural- and glial-expressed CB2 receptors, though greatest central expression appears to be restricted to microglia (Cabral et al., 2008). In terms of functional output, central CB2 receptor activation is best described in terms of regulating cytokine release and glial cell migration, with the current understanding of the functional role of neural-expressed CB2 receptors incomplete. Thus, most of this chapter focuses on CB1 receptors in social behavior regulation, though CB2 receptor contributions are discussed where evidence permits.

## The development of the endocannabinoid system in rats

The functional contributions of the ECS to early neurodevelopment were best characterized in terms of its involvement in neurogenesis and synaptogenesis (reviewed in Galve-Roperh et al., 2006). CB1 receptor mRNA was reported at embryonic day (E) 11 in rodent fetal brain tissue (Buckley et al., 1997), with expression increasing with age (Berrendero et al., 1999). However, the specific pattern of CB1 receptor development during gestation depends on the cell and tissue type assessed, with CB1 receptor expression on cortical excitatory pyramidal cells first appearing at E14–15, with little expression on inhibitory interneurons prior to the perinatal period (Mulder et al., 2008).

ECS development in the post-natal brain is somewhat protracted, with greatest developmental shifts in adolescence and into adulthood (Simone et al., 2022). Corticolimbic CB1 expression increased from the juvenile stage (postnatal day (P) 10) into early adolescence (P30–40) in rats, with expression tapering to more stable levels in adulthood (P70) (reviewed in Simone et al., 2020) (see Fig. 1). Although decreasing receptor expression from adolescence into adulthood is thought to occur throughout the brain, the timing of this shift in expression seems to be region-specific, with progressive reductions in limbic CB1 receptors evident across adolescence, and reductions in cortical CB1 receptors evident in mid-late adolescence (Heng et al., 2011). Further, the pattern of ECS development is sex-specific. In juvenile rats (P10), expression of mid-brain CB1 receptors was greater in females than in males (de Fonseca et al., 1994). However, the direction of the sex difference was transient, with a greater abundance of receptors detected in males than females after P15 (de Fonseca et al., 1994). Despite the greater abundance of CB1 receptors in the brain of adolescent males, CB1-mediated G-protein activation is greater in females (Burston et al., 2010), and expression of the receptor peaks earlier in female (P30) compared with male (P40) brains (Romero et al., 1997).



**FIG. 1** Relative developmental pattern of primary components 2-AG (blue line), AEA (red line), and CB1 receptors (green line), across post-natal development (reviewed in [Simone et al., 2022](#)). (Created with [Biorender.com](#).)

Sex differences in the development of the ECS throughout adolescence are mirrored by sex differences in the development of underlying behaviors. That developmental shifts in the ECS are most evident in corticolimbic brain regions associated with social behavior is in accordance with the hypothesis that the ECS is a critical regulator of social behavior development.

## The development of social behavior in rats

Positive social interactions with peers throughout development promote health by enhancing resilience to stressors (social buffering) in many species including humans (reviewed in [Hostinar et al., 2014](#)). Rodents such as rats continue to be the most relied on animal models in neuroscience, including investigations of the role of the endocannabinoid system in social development. Rats are altricial species, hairless, eyes-closed until about P14, auditory function begins at about P13, and adult-like locomotion at P15. The main behaviors evident in the first 2 weeks of life are orienting behavior toward the dam, suckling, and huddling with littermates as a means of temperature regulation. Although able to eat foods in the third week of life, rats are not fully weaned until the third week of life in the laboratory and until P40–P45 in wild Norway rats ([Calhoun, 1962](#)). Social behavior with peers in rodents begins with social grooming of littermates at about P13 ([Bolles & Woods, 1964](#)). Social play, or play fighting, begins at about P18–20, peaks in frequency around puberty, and is observed less often in adulthood (>P70) ([Klein et al., 2010](#); [Perkins et al., 2016](#); [Primus & Kellogg, 1989](#)). When sex differences are observed, it is typically males that show more social play than females, although the extent of a sex difference—if any—depends on the test conditions and the strain of rat ([Northcutt & Nwankwo, 2018](#)), and may be confounded by the difference in onset of puberty of females (~P33) and males (~P40). The sex difference in social play is best characterized as a qualitative difference rather than a quantitative difference, particularly when male rats change their patterns of defense at puberty ([Pellis et al., 1997](#)). Although most rats are separated from dams to live in small same sex and age groups in the laboratory at about P21, permanent nest dispersal in wild rats did not occur until P60 ([Calhoun, 1962](#)).

Investigations in laboratory rats highlight the importance of appropriate social experience in adolescence for the development of adult social behavior (e.g., [Bell et al., 2010](#); [Einon et al., 1978](#); [Fone & Porkess, 2008](#); [Pellis & Pellis, 2007](#)). Although social behavior takes many forms, for this review the focus is on same-sex social interactions that are pro-social, with the main measures considered being social play, social interaction, social approach, and social recognition. Isolation housing or housing with an adult female (which does not engage in social play to the same extent) of juveniles impaired adult social behavior ([Hol et al., 1999](#); [Toth et al., 2011](#)). For example, as adults, both male and female rats display more aggression toward a peer after isolation housing in adolescence ([Dawud et al., 2020](#); [Day et al., 1982](#); [de Moura Oliveira et al., 2019](#)) and may elicit more aggression from peers ([Luciano & Lore, 1975](#)). Some studies report increased social interaction after adolescent isolation housing in males only ([Ferdman et al., 2007](#)), whereas others have reported an increase in

both sexes (Dawud et al., 2020; Wall et al., 2012). Nevertheless, the morphology of neurons of the medial prefrontal cortex and orbitofrontal cortex, regions that when lesioned impair adult social behavior, is differentially shaped by social experience in adolescence; the medial prefrontal cortex is sensitive to the presence versus absence of peer interactions and the orbitofrontal cortex is responsive to the number of peer partners (Bell et al., 2010). Thus, social play is critical for developing brain regions that support appropriate adult social behavior.

Although the social interaction test was developed as a measure of anxiety (Johnston & File, 1991), it is used extensively in the literature to measure social behavior beyond social play. The behaviors that typically are measured in sessions of 10–15 min in pairs of rats include sniffing, following, grooming, kicking, mounting, jumping on, wrestling, and boxing with crawling under or over the partner, although usually these behaviors are tallied into a single measure (File, 1980). Adolescent male rats show more social interaction than do adolescent female rats. The sex differences diminish in adulthood although social interactions generally remain higher in male than in female rats (e.g., Asgari et al., 2021; Carrier & Kabbaj, 2012; Johnston & File, 1991; Lian et al., 2018; Thor et al., 1988). Whether male rats find social interaction more rewarding than do female rats is debatable. Operant conditioning tests whereby nose pokes can be made for access to a social peer suggest a greater reward value of social interaction in male than in female rats (P35–P41, Schatz et al., 2019; P30–P70, Murray et al., 2022). On the other hand, there is evidence that test conditions may influence the extent to which male rats exhibit more social reward than do females. Tests of conditioned place preference (CPP) for a chamber paired with a social stimulus also are used to measure social reward. Male and female rats form social CPP (e.g., Calcagnetti & Schechter, 1992), although the social CPP was greater in male than in female rats when housed in isolation and not when housed in pairs (Douglas et al., 2004). In contrast, at P55 there was no sex difference and only those in isolation-housing had a social CPP (Douglas et al., 2004). That test conditions moderate the sex difference makes it difficult to conclude that male and female rats differ in the reward value of social interaction. Adolescent rats, however, typically develop stronger conditioned CPPs to social stimuli than do adult rats (Douglas et al., 2004; Yates et al., 2013), and there is some evidence for a peak in social reward at P50 in male rats in operant conditioning tasks (Murray et al., 2022). Thus, social interaction seems to have a greater reward value in adolescents than in adults.

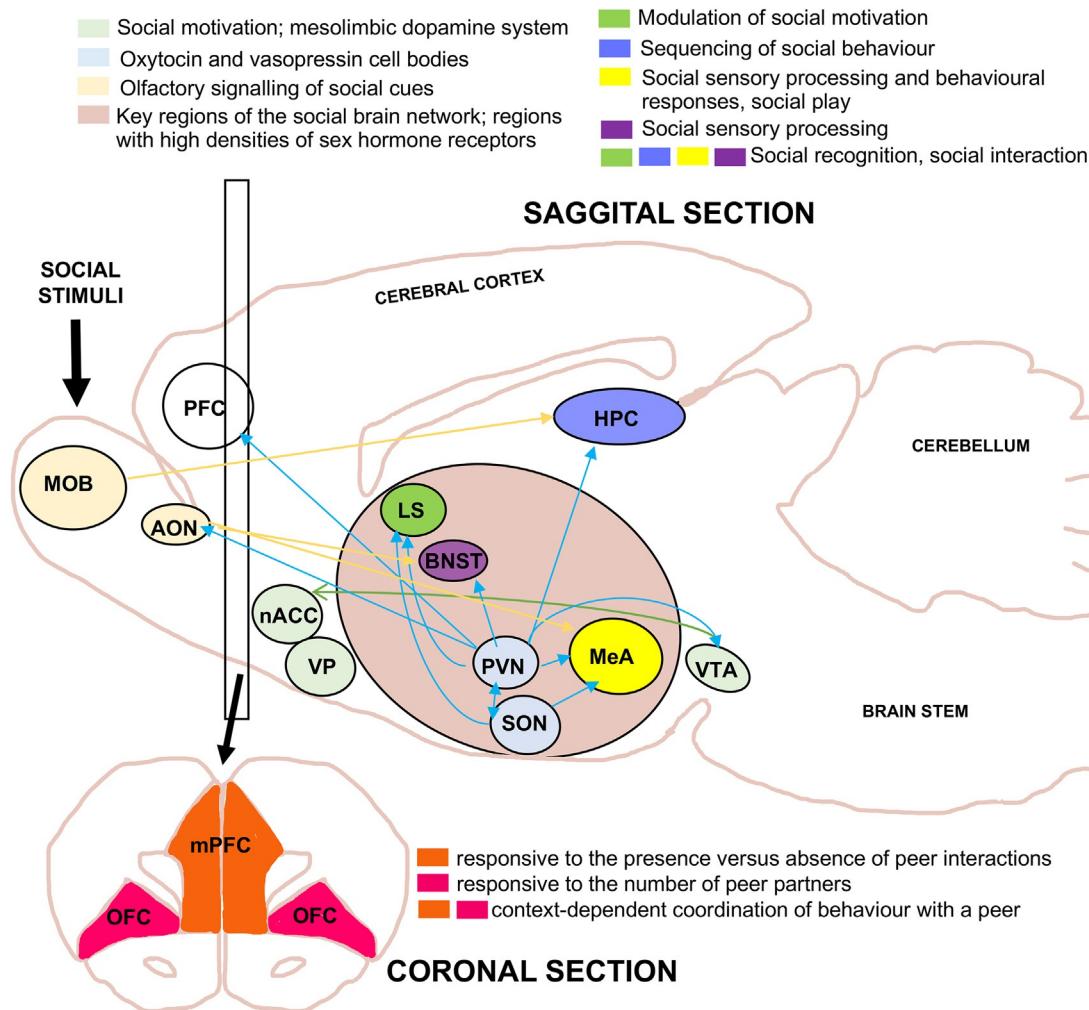
Social approach is also a commonly used measure of social behavior in rats and involves measuring the time spent in proximity to a peer under conditions that limit physical interaction (e.g., separated by mesh). Social approach is a behavior separate from social interaction, with evidence that some manipulations can both enhance social approach and lessen social interaction (Green et al., 2013; Hedges et al., 2017). Some have found that adolescent rats are higher in social approach than are adult rats (Willey & Spear, 2013) and that the sexes do not differ either in adolescence or in adulthood in social approach (Reppucci et al., 2020). We found no age difference, although female rats had lower social approach relative to male rats (Herlehy et al., 2022). These differences across studies may reflect the different strains involved (Sprague Dawley, Wistar, and Long Evans, respectively) (McCormick, 2022). However, not only have few studies investigated age and sex differences in social approach, there are few direct comparisons of strains in the literature for most measures.

Social recognition is used to evaluate changes in social cognition because it captures the perceiving, recognizing, and evaluating social stimulus aspects of such cognition (reviewed in McCall & Singer, 2012; van der Kooij & Sandi, 2012). Social recognition is adaptive by promoting stability in social hierarchies, distinguishing intruders from non-intruders, and modulating affiliative and aggressive behaviors (Aspesi & Choleris, 2022). The two basic forms of this test are a habituation/dishabituation paradigm or a social discrimination paradigm (reviewed in Hedges & McCormick, 2019). Both forms of the test capitalize on rats' preference for novelty. When sex differences are found in adults, female rats have better social recognition than do male rats (Bluthe & Dantzer, 1990; Markham & Juraska, 2007). Irrespective of a behavioral difference, the mechanisms underlying social recognition are sex-specific (Veenema et al., 2012). The available evidence for the development of these sex differences is mixed. Both no sex differences (Dumais et al., 2016) or sex differences favoring males (Veenema et al., 2012) were reported in P26–P30 Wistar rats.

In sum, there are notable changes in the expression and form of social behaviors throughout adolescence into adulthood. Although the evidence for sex differences in these behaviors across development is mixed, the underlying neural mechanisms are sex-specific and differ across development.

## Neural mechanisms of social behavior

The social brain involves circuits of brain regions that reliably underlie a diversity of social behaviors and includes several nuclei of the hypothalamus, the medial amygdala, basolateral amygdala, bed nucleus of the stria terminalis (BNST), hippocampus, habenula, lateral septum, ventral tegmental area, nucleus accumbens, and prefrontal cortex (Newman, 1999; O'Connell & Hofmann, 2011; Prounis & Ophir, 2020) (see Fig. 2). A full description of this circuitry is beyond the scope of this review, and only some main functions are described in specific regions. The most important players in signaling



**AOB** = accessory olfactory nucleus; **OFC** = Orbitofrontal cortex **PFC** = prefrontal cortex; **HPC** = hippocampus; **LS** = lateral septum; **nACC** = nucleus accumbens; **BNST** = bed nucleus of the stria terminalis; **MOB** = main olfactory bulb; **MeA** = Medial amygdala; **mPFC** = medial prefrontal cortex; **PVN** = paraventricular nucleus; **VTA** = Ventral tegmental area; **VP** = ventral pallidum.

**FIG. 2** Brain regions underlying social behavior. Blue arrows show oxytocinergic projections. Yellow arrows show main pathways for the processing of olfactory social cues. Green arrow shows dopaminergic projections. (Created with [BioRender.com](#).)

among these brain regions are the neuropeptides oxytocin and vasopressin and the steroids testosterone and estradiol. The motivation to perform social behaviors relies on the ventral tegmental area, nucleus accumbens, and ventral pallidum, with a central role of dopaminergic transmission in these functions. Oxytocin signaling in the nucleus accumbens was found necessary for social reward (Dölen et al., 2013). The orbital frontal cortex and medial prefrontal cortex are involved in context-dependent coordination of behavior with a peer (Bell et al., 2009; Himmler et al., 2014). The hippocampus also contributes to the appropriate sequencing of social behavior with another (Maaswinkel et al., 1997). Cell bodies for oxytocin are located primarily in the paraventricular nucleus and supraoptic nucleus. Oxytocin projections from the paraventricular nucleus of the hypothalamus innervate the medial prefrontal cortex, hippocampus, and nucleus accumbens (Froemke & Young, 2021). Further, the medial prefrontal cortex is a key node for the social buffering of anxiety (Lungwitz et al., 2014), and oxytocin signaling in the medial prefrontal cortex reduces anxiety-like behavior (Sabih et al., 2017).

The medial amygdala is an important node for modulating a broad range of social behavior, relating social information from the accessory olfactory bulb and vomeronasal organ throughout the brain. In turn, the medial amygdala regulates how social behavior is enacted (Raam & Hong, 2021). Oxytonergic projections from the paraventricular nucleus to the BNST

are involved in the processing of chemosignals during social behavior (Petrulis, 2013). The lateral septum modulates the motivation for affiliative behavior and contains a high density of oxytocin receptors (Dumais & Veenema, 2016). Vasopressin is synthesized more broadly than is oxytocin, with cell bodies for vasopressin in the paraventricular nucleus, supraoptic nucleus, suprachiasmatic nucleus, BNST, and medial amygdala (Dumais & Veenema, 2016). Vasopressin signaling and oxytocin signaling in the medial amygdala and lateral septum enhance social recognition (reviewed in Maroun & Wagner, 2016; Caldwell, 2017). Vasopressin signaling in the BNST and hippocampus is also implicated in social recognition.

The sex steroids testosterone and estradiol also are crucial for the appropriate expression of social behavior, in part through actions on oxytocin and vasopressin. For example, male rats have a higher density of vasopressinergic fibers than do female rats, a sex difference that is dependent upon testosterone. In turn, estradiol promotes the synthesis of oxytocin and oxytocin receptor densities (Dellovade et al., 1999; Gabor et al., 2012). The medial amygdala contains a high density of androgen and estrogen receptors, as do other regions of the social brain network (Newman, 1999). Thus, it comes as no surprise that many of the mechanisms that underlie social behavior are sex-specific.

Work of the McCarthy lab on the development of the medial amygdala highlights the role of endocannabinoids in shaping sex differences in brain and behavior (VanRyzin et al., 2019). Male rats have fewer cells in the amygdala than do female rats because of the higher endocannabinoid tone in males than in females, which in turn is linked to the higher concentrations of testosterone in neonatal males. The higher testosterone in males leads to higher 2-AG content, which increases the number of phagocytic microglia leading to a reduction in astrocytes in the medial amygdala (and thus higher numbers of astrocytes in females), and ultimately underlies the sex difference in social play evident in juvenile rats (VanRyzin et al., 2019). The next section reviews the role of endocannabinoids in social behavior in adolescence into adulthood.

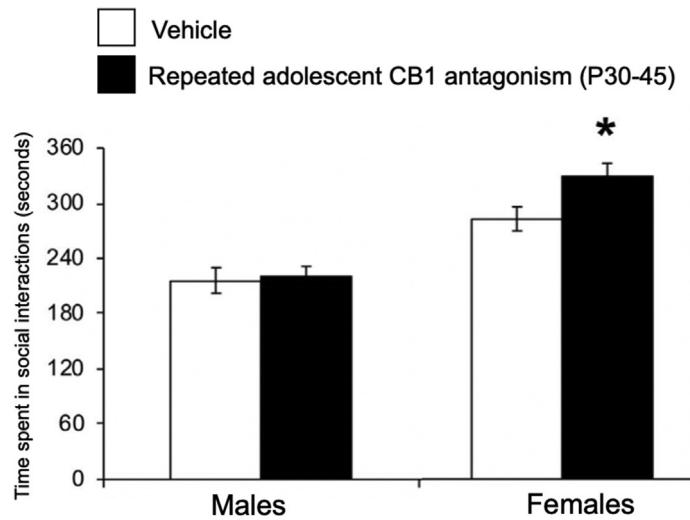
## Endocannabinoid system influences on social behavior

Developmental shifts in the ECS during adolescence are mirrored by shifts in social behaviors and their underlying brain regions, suggestive of a regulatory influence of this system in social development. Indeed, a genetic gain of function of CB1 receptors in adult rats resulted in heightened social interactions typical of adolescent rats (Schneider et al., 2015) and is in accordance with the increased expression of CB1 receptors in the adolescent versus adult brain. Conversely, CB1 receptor knockout mice displayed impaired social behaviors relative to their wild-type counterparts (Haller et al., 2004), though the extent to which these effects are driven by a lack of acute CB1 signaling during behavioral assessment, by developmental consequences of perturbed ECS signaling, or a combination thereof is not clear. Thus, while these findings support the hypothesis that the ECS is a key regulator of sociality in rodents, investigations with cannabinoid receptor agonists and antagonists allow for greater dissociation of the role of the ECS in both social behavior development and acute regulation.

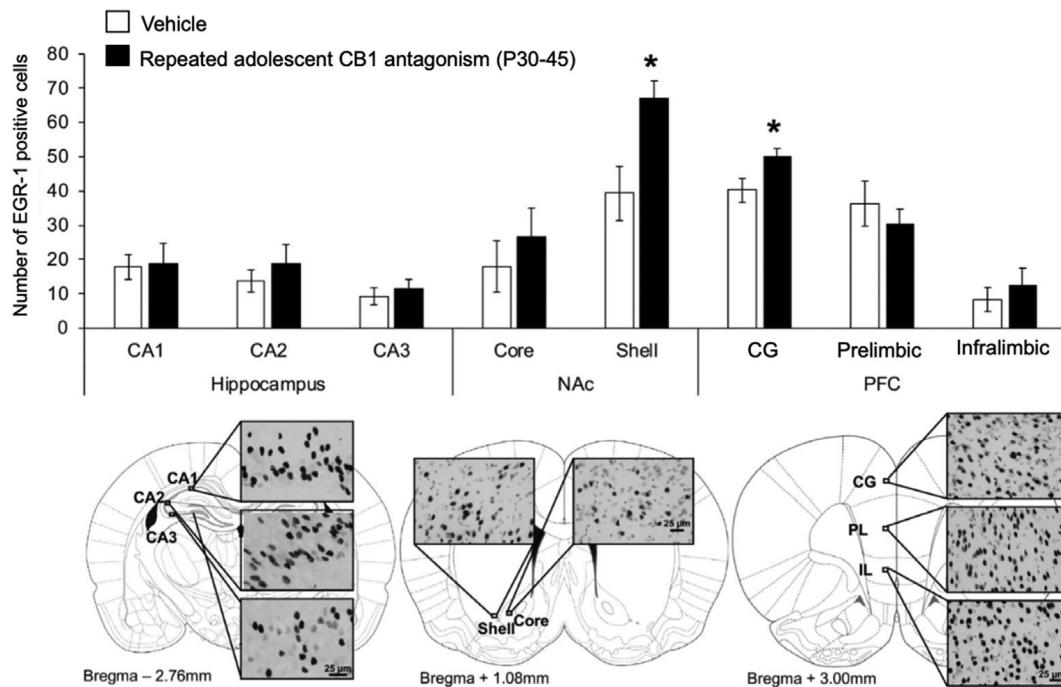
For instance, repeated exposure to the CB1 receptor antagonist/inverse agonist AM251 in mid-late adolescence (exposures once daily from P30 to P45) led to increased social interactions 24 h (Simone et al., 2018) and 5 days (Simone et al., 2020) after cessation of treatment in female, but not male, rats (see Fig. 3). Notably, the increase in social interactions in female rats after disruption of normative adolescent endocannabinoid signaling was independent of any effects on anxiety-like behaviors, novelty-seeking, or effects on locomotor behavior, suggesting that effects may be driven by increased social reward. In support of this hypothesis, the increased social interactions of female rats exposed to AM251 in adolescence were associated with greater neural activity in the NAc shell and in the cingulate gyrus of the mPFC (Simone et al., 2020; see Fig. 4), areas implicated in the regulation of social reward. Nevertheless, further research is warranted to discern the extent to which ECS signaling in adolescence drives development of social reward in female rats.

In contrast to the sex-specific increase in social interactions observed after downregulation of adolescent CB1 receptor signaling, increased endocannabinoid signaling during the juvenile period (P10–16) via inhibition of the AEA hydrolyzing enzyme Fatty Acid Amide Hydrolase (FAAH) led to increased social interactions in male rats tested at P36 (Carr et al., 2020). Alternatively, repeated adolescent CB1 receptor agonism (THC, WIN, CP55, 940) led to a lasting reduction in social interaction (O’Shea et al., 2004, 2006), social recognition (Renard et al., 2017; Schneider et al., 2008), and social motivation (Renard et al., 2017). Although female rats were not included in these studies, the differential results from those reported in adolescent females suggest that the contributions of endocannabinoid signaling to social development are likely dependent on sex, developmental stage, and the specific social behaviors investigated.

Consistent with the reported effects of ECS signaling on social behavior development, acute regulation of sociality is dependent on age, sex, and behavior. In adolescent male rats, single (Malone et al., 2009) or repeated (Keeley et al., 2021) exposure to the CB receptor partial agonist  $\Delta^9$ -tetrahydrocannabinol (THC) reduced social interactions and social play compared with vehicle-treated rats. Similarly, exposure to the CB receptor agonist WIN55,212-2 (WIN) reduced social play in adolescent male rats independent of effects on social exploration (Trezza et al., 2008). Notably, the indirect CB



**FIG. 3** Repeated exposure to the CB1 receptor antagonist/inverse agonist AM251 in adolescence (P30–45) increased social interactions in females, but not males, when tested 5 days after the final drug exposure. (Adapted from Simone, J.J., Baumbach, J.L., McCormick, C.M. (2018). Effects of CB1 receptor antagonism and stress exposures in adolescence on socioemotional behaviours, neuroendocrine stress responses, and expression of relevant proteins in the hippocampus and prefrontal cortex in rats. *Neuropharmacology*, 128, 433–447, <https://doi.org/10.1016/j.neuropharm.2017.10.029>.)



**FIG. 4** Female rats exposed daily (P30–45) to the CB1 receptor antagonist/inverse agonist AM251 had a greater amount of neural activity (as measured by EGR-1 positive cell counts) in the nucleus accumbens shell and in the CG subdivision of the mPFC after the completion of social interaction testing (P50) than did vehicle rats. (Adapted from Simone, J.J., Baumbach, J.L., McPherson, J., McCormick, C.M. (2020). Adolescent CB1 receptor antagonism influences subsequent social interactions and neural activity in female rats. *International Journal of Developmental Neuroscience*, 80(4), 319–333, <https://doi.org/10.1002/jdn.10028>.)

receptor agonist URB597 (URB) increased social play in adolescent rats while reducing social exploration (Trezza et al., 2008). The discrepancy in the direction of social effects resulting from WIN compared with URB likely reflects the differential mode of action of the two drugs. Whereas WIN acts as a direct agonist at CB receptors, URB indirectly agonizes CB receptors via the inhibition of AEA hydrolysis. Thus, the effects of WIN reflect global activation of CB receptors throughout the organism irrespective of their endogenous activity state, whereas URB effects occur in a spatially restricted

manner, elevating AEA levels only where it is actively being produced. Thus, the influence of the ECS on acute social behavior regulation is likely dependent on the specific brain regions and cell types being activated.

Consistent with hypothesized region-specific effects of ECS signaling on social behavior regulation, administration of the CB receptor agonist WIN into the ventral subiculum impaired acquisition, consolidation, and retrieval processes in a social discrimination task, whereas administration directly to the medial amygdala impaired only acquisition (Segev & Akirav, 2011). In support of these region-specific effects of ECS signaling on social behavior, levels of the endocannabinoid AEA were elevated in the amygdala and NAc, but not the hippocampus or PFC, after social play in adolescent male rats (Trezza et al., 2012). Further, enhancement of AEA signaling in the amygdala and NAc via direct application of the AEA hydrolysis inhibitor URB increased social play, though antagonism of CB1 signaling prevented this effect only when administered to the amygdala, with no effect of CB1 blockade on the URB-mediated increases in social play in the NAc (Trezza et al., 2012). Thus, whereas endocannabinoid signaling appears to regulate social behavior via CB1 receptor activation in the amygdala, effects in the NAc are likely because of interactions of AEA with CB2 or non-cannabinoid receptor targets such as GPR55. Indeed, direct administration of the endocannabinoid palmitoylethanolamine (PEA) into the ventral hippocampus disrupted social behavior and was attributed to actions at GPR55 (Kramar et al., 2017).

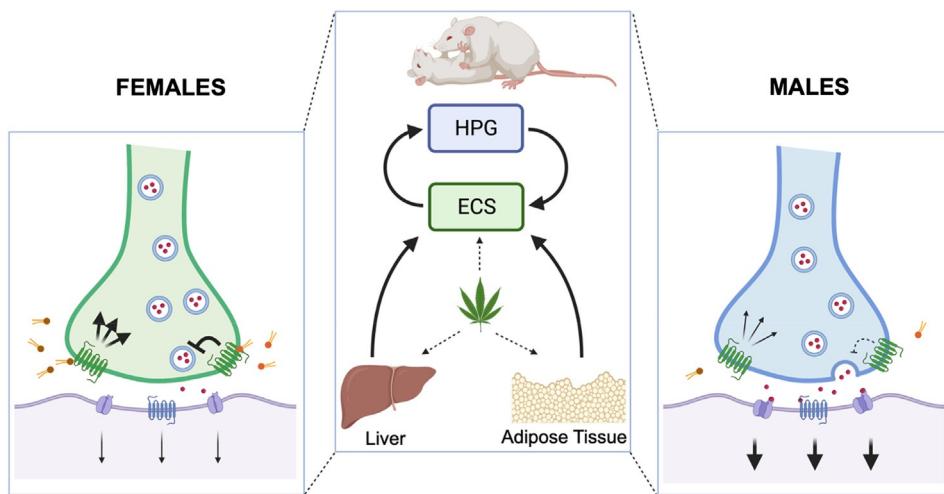
Although considerably less is known regarding the involvement of CB2 receptors to social behavior regulation compared with CB1, available evidence does suggest a role for these receptors. Mice lacking CB2 receptors displayed increased aggression during a social interaction test compared with wild-type counterparts (Rodríguez-Arias et al., 2015), providing evidence for endogenous CB2 receptor contributions to social behavior, though the extent to which these effects are driven by CB2 receptors on glial cells, neurons, or a combination of both remains unknown. Thus, investigations of ECS influences on sociality have remained focused on CB1 receptor contributions to both the development of social networks in the brain and the acute regulation of social behavior.

## Potential mechanisms underlying sex differences in ECS regulation of social behavior

Sex differences in ECS biology and behavioral pharmacology are well documented and are in part explained by sex differences in pharmacokinetics (reviewed in Craft et al., 2013). Female rats tend to display a greater sensitivity to the behavioral effects of cannabinoid drugs, and this has been attributed to a lower body fat composition compared with males, and thus, a greater concentration of bioavailable drug; cannabinoid drugs are lipophilic and are more readily absorbed into adipose tissue. Further, sex differences in the ECS are thought to involve bidirectional interactions between the ECS and gonadal hormones. In support of this hypothesis, dynamic expression of both CB1 receptors and of neural endocannabinoids was observed across brain regions and estrous cycle phases in female rats (Castelli et al., 2014; de Fonseca et al., 1994; González et al., 2000). Consistent with the hypothesis that CB1 receptor expression is positively correlated with circulating estrogens, ovariectomized female rats had lower CB1 receptor densities compared with ovariectomized females replaced with estradiol (González et al., 2000). Further, estrogens may exert inhibitory influences on the endocannabinoid hydrolyzing enzyme FAAH, thereby elevating AEA concentrations and driving activation of cannabinoid receptors (Hill et al., 2007). In male rats, orchectomy resulted in a reduction in CB1 receptor mRNA in the anterior pituitary, though this effect was not reversed after testosterone replacement (González et al., 2000). Conversely, ECS signaling influences gonadal hormone signaling. Specifically, CB1 receptor activation reduced circulating testosterone and estradiol through the inhibition of luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH) (reviewed in Gorzalka & Dang, 2012). Thus, the sexual dimorphism of the endocannabinoid system, and thus of its contributions to social behavior, likely involves both differences in the metabolism and distribution of cannabinoid drugs and bidirectional interactions between endocannabinoid signaling and gonadal hormone systems (see Fig. 5).

## Applications to other areas

This chapter discussed contributions of the ECS to social behavior and its development during adolescence. The role of the ECS in social regulation occurs through influences on the organization and development of social centers in the brain and their associated behaviors and through the acute regulation of social behaviors via modulation of neurotransmission. Whereas the developmental contributions of ECS signaling are best described in terms of adolescent development, acute ECS regulation of sociality occurs across the lifespan, though the specific outcomes are dependent on sex and developmental stage. Further, sex differences in the ECS and its influence over sociality involve bidirectional interactions between cannabinoid signaling and gonadal hormones, though further research in this area is warranted. Overall, the results presented in this chapter provide a lens to view abnormal social behavior and highlight the ECS as a potential target for novel therapies against social-specific disorders such as social anxiety and social withdrawal.



**FIG. 5** Sex differences in endocannabinoid regulation of social behavior are thought to involve differences in cannabinoid drug metabolism and distribution, as well as bidirectional interactions with gonadal hormone systems. (Adapted from Simone, J. J., Green, M. R., & McCormick, C. M. (2022). Endocannabinoid system contributions to sex-specific adolescent neurodevelopment. *Progress in Neuropsychopharmacology and Biological Psychiatry*, 113(8).)

## Mini-dictionary of terms

**Social behavior:** Behaviors that are directed at peers, including social play, social approach, social interaction, and social recognition.

**Gestation:** The period of development prior to birth when the animal is in the womb.

**Adolescence:** A transitional period of development between the juvenile and adult stages that involves maturation and refinement of brain and behavior.

**Neurodevelopment:** Diverse processes including cell proliferation/neurogenesis, axonal pathfinding and target selection, synapse formation and maintenance, and synaptic pruning across the lifespan.

**Gonadal hormone:** Steroid hormone produced by the gonads and circulated throughout the organism that influences brain and behavior development.

## Key facts

### Key facts of social behavior

Adolescence is a critical period for development of social behaviors and underlying brain regions.

Key brain regions involved in social behavior include the olfactory bulb, medial prefrontal cortex, medial amygdala, hippocampus, and nucleus accumbens, among others.

Sex differences in social behavior are driven largely by effects of gonadal hormones and neuropeptides.

The endocannabinoid system influences the development and the acute regulation of social behavior.

Sex differences in endocannabinoid regulation of social behavior involve differences in PK/PD and bidirectional interactions with gonadal hormones.

## Summary points

- The endocannabinoid system acts to regulate brain and behavior development through actions at cannabinoid receptors and activation of intracellular signaling pathways.
- The endocannabinoid system undergoes protracted development in adolescence in social brain regions.
- Adolescence is a key period for social brain and behavior development.
- Changes in the endocannabinoid system in adolescence are thought to underlie developmental of social behavior.
- Sex differences in endocannabinoid regulation of social behavior likely involve bidirectional interactions with gonadal hormone systems.

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## Chapter 10

# Exercise and Parkinson's disease: Linking in the cannabinoid type 1 (CB1) and type 2 (CB2) and mu-opioid receptors

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>6-OHDA</b>	6-hydroxydopamine
<b>ACC</b>	anterior cingulate cortex
<b>AE</b>	aerobic exercise
<b>BBB</b>	blood-brain barrier
<b>CB1</b>	cannabinoid receptor type 1
<b>CB2</b>	cannabinoid receptor type 2
<b>CNS</b>	central nervous system
<b>CPU</b>	caudate putamen
<b>DA</b>	dopamine
<b>DOR</b>	delta-opioid receptor
<b>eCB</b>	endocannabinoid
<b>GPCR</b>	G-protein-coupled receptors
<b>KOR</b>	kappa-opioid receptor
<b>L-DOPA</b>	L-3,4-dihydroxyphenylalanine
<b>MOR</b>	mu-opioid receptor
<b>MRI</b>	magnetic resonance imaging
<b>PAG</b>	periaqueductal gray matter
<b>PD</b>	Parkinson's disease
<b>PET</b>	positron emission tomography
<b>PPARY</b>	peroxisome proliferator-activated receptor $\gamma$
<b>SN</b>	substantia nigra
<b>THC</b>	tetrahydrocannabinol
<b>TRPV1</b>	transient receptor potential vanilloid 1

## Introduction

The first descriptions of Parkinson's disease (PD) were made in 1817 by James Parkinson, focusing on classic motor symptoms. This is the second most common neurodegenerative disorder after Alzheimer's Disease. The protein  $\alpha$ -synuclein plays an important driving role in PD occurrence, development and is essential for neuropathological diagnosis. A prerequisite for the *postmortem* diagnosis of both the presymptomatic and symptomatic phases of the pathological process underlying PD is evidence of specific inclusion bodies, protein aggregation in those bodies develops spindle- or thread-like Lewy neurites in cellular processes, and in the form of globular Lewy bodies in neuronal perikarya. The pathological process targets specific induction sites: lesions initially occur in the dorsal motor nucleus of the glossopharyngeal and vagal nerves, as well as in anterior olfactory nucleus, being the long prodromal period. Later, there is the

spread to the locus coeruleus and substantia nigra (SN) and progressively to other areas of the brain. Clinical symptoms are directly correlated with the spreading of  $\alpha$ -synuclein protein aggregation (Braak et al., 2003). Although it is not confirmed, it is worth mentioning that there are current hypotheses suggesting that  $\alpha$ -synuclein is produced in the intestine, which later spreads into the central nervous system through the vagus nerve (Li et al., 2021). However, the main damage observed in the nervous system of PD patients is a slow and progressive degeneration of SN dopaminergic neurons projection to the striatum, inducing a decrease in the production of dopamine (DA) (Deumens et al., 2002; Wirdefeldt et al., 2011). DA loss causes bradykinesia and/or akinesia, resting tremors and muscle rigidity, basis for PD diagnosis (Blandini et al., 2000; Zigmond et al., 1990), and significantly affects the life quality of patients.

In addition, nonmotor symptoms, which have been underestimated for years, are very prevalent in the disease and contribute to the severity of a patient's disability. Those impairment can be, for example, cognitive alterations, sleep disorders, constipation, and sensory abnormalities, such as pain (Engelender & Isacson, 2017). The influence of the neurodegenerative processes of PD on pain is not fully understood; however, there is some evidence that these processes could lead to impaired pain processing, potentially influencing the experience of pain in people with PD (Allen et al., 2015). Pain is a distressing nonmotor symptom experienced by up to 85% of people with PD (Broen et al., 2012). Many knowledge gaps remain in the treatment of pain in PD. There is difficulty in separating PD-related pain from other types of pain; this causes many pain symptoms to be poorly treated in patients (Qureshi et al., 2021).

Today, therapies for PD focus on symptom relief, including motor and nonmotor symptoms, such as pain, depression, constipation. Despite dopamine replacement therapy with levodopa/L-DOPA being the most effective symptomatic treatment for PD, it is also a big challenge for medicine since the patients present severe side effects over time. Many patients experience periods of return of PD symptoms, such as dyskinesias and compulsive behaviors, intermittently through the day, which can be related to a progression of dopaminergic neurons loss. Neurochemical changes are long-lasting and difficult to contrast by pharmacological interventions, since all drugs that are effective on dyskinesia impair motor performance. Therefore, new strategies to contrast dyskinesia in PD tackle both pharmacological and nonpharmacological aspects. These strategies mainly aim at mitigating the abnormal functional changes caused by dopamine replacement in the striatum and other basal ganglia areas as well as in cortico-striatal loops, thereby restoring motor function.

Thus, other nonpharmacological strategies are considered, including cell transplant therapy and deep brain stimulation. Regarding noninvasive procedures, physical training and transcranial magnetic field stimulation are also taken into consideration. Pain management in PD patients is often a complicated task. In the last 10 years, different pain relief techniques have appeared such as: exercise, massage, hydrotherapy, acupuncture, and neuromodulation are some of the most common methods used to reduce pain. Exercise protocols have been widely used in PD since are well tolerated by patients and reveal great effects on PD symptoms, especially on pain, focus of our discussion in this chapter.

## Physical activity as nonpharmacological alternatives for overcoming symptoms in PD

Physical exercise is a very common activity performed by humans and in a way, depending on the modality, quite simple. Exercise is capable of providing numerous benefits to the central nervous system; however, there is a dose-response relationship between exercise duration/intensity and quality of life, in which the best results are associated with light/moderate exercise (Larson et al., 2006). The various plastic processes triggered by physical exercise are described in healthy animals, such as angiogenesis, increase in antiinflammatory responses and decrease in inflammatory responses, improved mitochondrial functions, decreased oxidative damage and modulation of brain-derived neurotrophic factor, among others, important for healthy aging (Vecchio et al., 2018).

In addition to descriptions related to motor symptoms in patients and animal models of PD, studies with clinical populations have already shown that exercise promotes analgesia in conditions such as low back pain, osteoarthritis, fibromyalgia, myofascial pain, and chronic fatigue syndrome (Lima et al., 2017). It is noteworthy that the same effect was observed in studies with patients diagnosed with PD who assessed pain, in which a significant reduction in the number and intensity of patients reporting pain in the neck, hip, and ilio-sacral joint after 6 weeks was seen (Hagell & McKenna, 2003). Another 12-week study on flexibility, strength, and aerobic exercise described an 8% decrease in patient-reported pain (Rodrigues de Paula et al., 2006). Despite descriptions of pain improvement in patients, it is not known whether pain is secondary to the motor symptom or occurs independently of the motor condition, as motor improvement does not seem to be directly related to pain improvement. Thus, it appears that motor and nonmotor symptoms use distinct yet unknown mechanisms (Cury et al., 2016). Thus, it is possible to highlight that studies using physical exercise as a nonpharmacological intervention for pain in PD are very scarce, as is knowledge of the mechanisms by which there is an improvement in pain in both patients and animal models.

Many works emphasize that physical exercise is able to modulate the release of endogenous opioids and the expression of opioid receptors depending on the period of execution of the exercise protocol, and these may be the mechanisms responsible for improving the nociceptive threshold in animal models of neuropathic pain (Stagg et al., 2011). Furthermore, it also promotes activation of the endocannabinoid system that appears to mediate central and peripheral aerobic exercise-induced antinociception (Galdino et al., 2014). Thus, we can suggest that the endocannabinoid and opioid systems play a major role in mediating the pain threshold provided by physical exercise, indicating potential targets for studies on induced nociception in the PD model in rats (Binda et al., 2020; Ferreira et al., 2021).

## Studies in PD patients

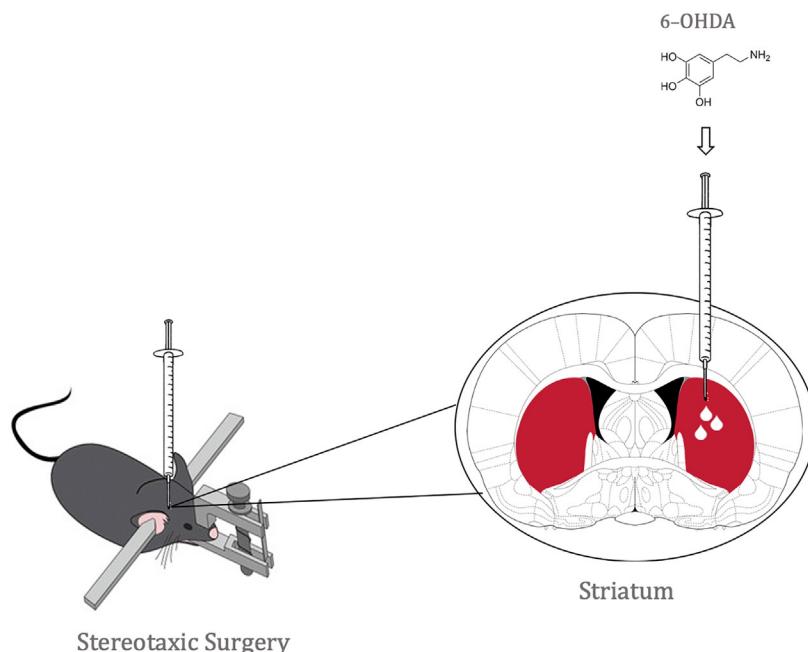
Improvements in bradykinesia, gait, balance, muscular force, and even quality of life have been observed in patients who performed some form of physical activity, compared with sedentary patients. Improving pain treatment is crucial as PD is often associated with multiple conditions. One such condition is its correlation for depression in PD patients who report pain. Two groups comprising 120 patients with PD and 120 controls were tested using the Pain Disability Index and Brief Pain Inventory. The authors found a statistical correlation between pain severity, pain disability, and depression among patients (Rana et al., 2017).

There is additional support for this claim as Tinazzi et al. (2006) found that the severity of pain in PD had a significant correlation with the severity of motor complications. Furthermore, researchers have found that pain sensitivity seems to be higher in PD patients than in controls, especially as the disease progresses (Mylius et al., 2011).

Recent reviews established a rationale for the use of resistance training and highlight findings related to positive effects of progressive resistance exercise in people with PD. Furthermore, even simple exercises such as walking have shown a reduction in pain in PD patients reported that after 5 weeks of physical/exercise therapy, an 85-year-old PD patient showed a significant reduction in back pain (Rosarion, 2018). It's possible that it's not just the strength of muscle contraction that determines people's ability with PD to perform physical activities; the muscle strength can be another important contributor.

## Studies in experimental animal

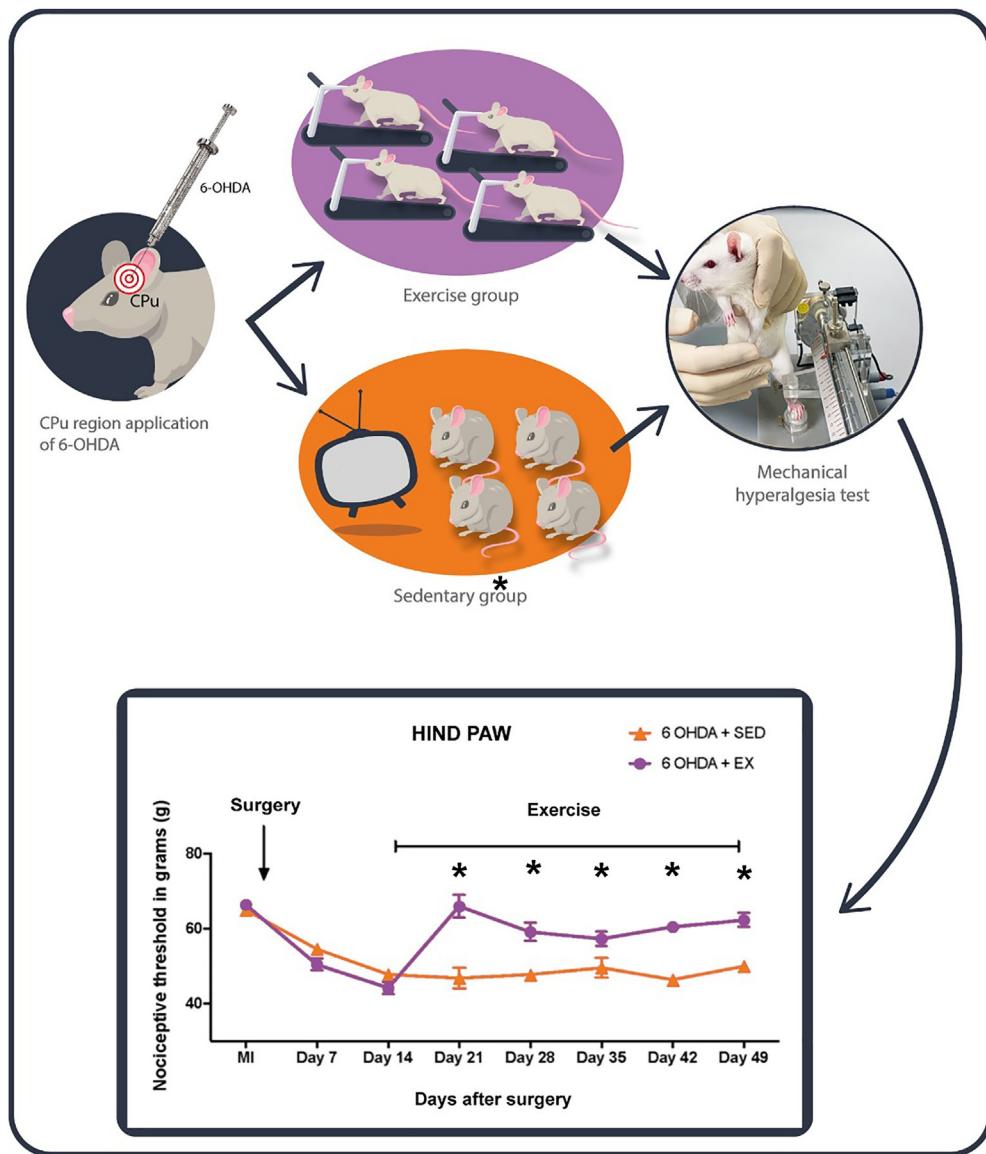
Evidence from rodent models of PD suggests that physical activity can counteract parkinsonian-type motor deficits and pain that is often overlooked. In this context, a few interesting data were obtained in rats unilaterally injured by 6-OHDA characterized by hemiparkinsonism (Ferreira et al., 2021), suggesting that physical exercise can be an important nonpharmacological intervention to reduce painful symptoms and improve motor ability by inhibiting apoptosis. The 6-OHDA is the most widely used model for PD animal model (Fig. 1).



**FIG. 1** Stereotaxic rodent model of Parkinson's disease. 6-Hydroxydopamine (6-OHDA), hydroxylated analog of dopamine, injection at striatum brain area.

In addition, studies with 6-OHDA-injured rats, which resulted in forced use of the impaired forelimb, exhibited reduced forelimb use asymmetry when evaluated within 60 days (Schallert et al., 2000). In agreement with this, studies have shown that the same animal model and obtaining movement performance by forcing rats to place their impaired forelimbs in response to vibration stimulation, a sensorimotor task or making them exercise in a treadmill (Dutra et al., 2012). Another study in parkinsonian rodents showed that behavioral parameters indicative of L-DOPA-induced motor complications are sensitive to exercise performance (Aguiar Jr. et al., 2013).

Furthermore, L-DOPA was observed to elicit lower severity AIMs in mice that performed voluntary exercise on running wheels, compared with sedentary hemiparkinsonian mice (Aguiar et al., 2013). These data suggest that movement stimulated by voluntary exercise can promote distinct neuroplastic adaptations in the basal ganglia, which can lead to divergent results regarding its role in motor complications. Although there are numerous sources of support for these procedures to reduce pain, their effects in PD patients are still limited. Additional research through methodologically robust studies must be conducted in order to bring relief to patients suffering from this disease, leading to an improvement in the quality of life. Considering previous studies, there are a high evidence of opioid and endocannabinoid systems roles on pain modulation pf PD after exercise intervention, which will be better described below (Fig. 2).



**FIG. 2** Experimental design and measurement of the mechanical nociceptive threshold. 6-OHDA + SED—sedentary animals, which received 6-OHDA injection; 6-OHDA + EX—exercised animals, which received 6-OHDA injection. \* $p < 0.05$  compared with groups.

## Endocannabinoid system

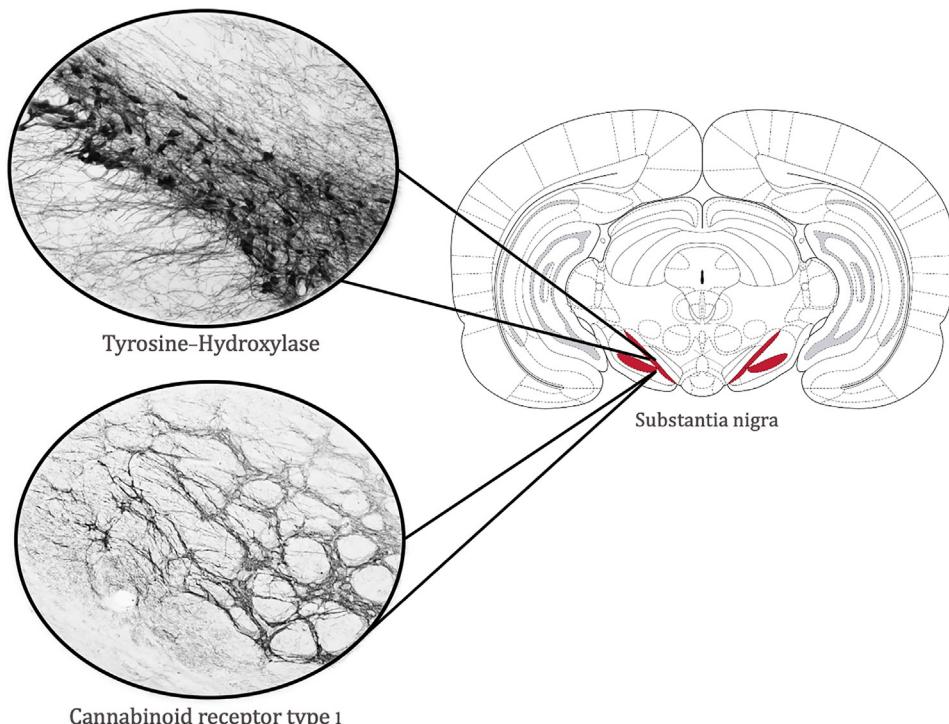
The endocannabinoid (eCB) system is an important brain modulatory network. The control of the system is lost in neurodegenerative diseases, including PD, may be increased or decreased. The eCB system is involved in regulation of cell, tissue, brain development, neurotransmitter release, synaptic plasticity, organ, and organism homeostasis. Additionally, eCB modulates cytokine release from microglia, which can be related to pain symptoms, both/either in the brain and/or in the periphery (de Melo Reis et al., 2021). The endocannabinoid system is complicated by promiscuity of mediators, overlap with other pathways and alternative metabolic processes. Then, modulation of its components affects a wider endocannabinoid-related network and makes it difficult to assess the pure role of eCB system in the benefits observed in neurodegenerative diseases (Fig. 3).

Recent review highlighted the role of eCB in the neurodegenerative diseases, such as the lipids anandamide (ethanolamine of arachidonic acid) and 2-arachidonoylglycerol (2-AG). Anandamide and 2-AG are endogenous endocannabinoids with high affinity and efficacy for CB1 and CB2, which are expressed in brain and intestinal samples. CB1 is mostly located presynaptically in excitatory and inhibitory neurons, suggesting that specific binders, particularly 2-AG, can act as inhibitory retrograde neuromodulator. On the other hand, the major role of CB2 is immune modulation and can be important for blood-brain barrier (BBB) permeability regulation. Haemopressins and related peptides are allosteric modulators of CB1 and CB2.

Endocannabinoids act also in other targets, named as endocannabinoidome receptors, for example, 2-AG activates TRPV1 channels and GABA<sub>A</sub> receptors. Moreover, anandamide can activate TRPV1 and PPAR $\gamma$ , whereas inhibits Ca<sub>v3.2</sub> Ca<sup>2+</sup> channels and transient receptor potential cation channel subfamily M member 8 channels. PPAR $\alpha$  and PPAR $\gamma$  are expressed in neurons, astrocytes, and microglia in the brain, where they have antiinflammatory and neuroprotective effects during acute and chronic neuroinflammatory insults. Role of GPR18 in brain physiology is poorly described; however, expression in microglia suggests that this receptor has a function in neuroinflammation (Chung et al., 2016).

## Endocannabinoid system and Parkinson's disease

As therapeutically concerned, the eCB system is essential for maintaining homeostasis. Thus, enhancers or inhibitors of endocannabinoid signaling can be a target for therapeutic strategies. In neurological disorders, the timing of CB1



**FIG. 3** Digital images of rat substantia nigra staining. Cannabinoid receptors type 1 (CB1) and tyrosine-hydroxylase, the rate-limiting enzyme for dopamine synthesis.

activation and the distribution of the receptor between inhibitory and excitatory terminals might be altered, thereby leading to profound alterations of CB1 function (Cristino et al., 2020). In PD, there is evidence in animal models and in patients that there is modulation of cannabinoid receptors, and in general, in most studies, endocannabinoid levels are increased. Biphasic dysregulation of CB1 (hypoactivity in presymptomatic and early PD and hyperactivity at later stages) occurs in different models, including  $\alpha$ -synuclein and parkin knockout animals, 6-hydroxydopamine (6-OHDA)-treated rats, and a monkey model of treatment-induced dyskinesia (Cristino et al., 2020).

PET and MRI have shown that CB1 levels are increased in patients with PD (Navarrete et al., 2018; Van Laere et al., 2012), and imaging in rats and patients has revealed CB2 upregulation (Gómez-Gálvez et al., 2016; Navarrete et al., 2018). The classical treatment for PD, levodopa, has been associated with regulation of eCB, suggesting that this system is related to disease symptoms. The eCB system has been studied systematically since the elucidation of the structure of tetrahydrocannabinol (THC) from Cannabis (Mechoulam & Gaoni, 1965) and later recognized as a physiological circuit breaker with the discovery of membrane receptors, enzymes, and endocannabinoid-like mediators (De Petrocellis et al., 2004; Katona & Freund, 2008). Alternatively, more people became interested in cannabinoids for alleviation of motor symptoms, which has been extensively explored in preclinical studies.

Cannabis products that contain the tetrahydrocannabinol (THC) cannabinoid are emerging as promising therapeutic agents for the treatment of medical conditions such as chronic pain. There are descriptions that taking cannabis for chronic back pain in PD patients can promote worsening tremors and vivid hallucinations. This patient case suggests that cannabis use in combination with dopamine-promoting drugs, especially in a patient with genetic variants, can increase the risk for vivid hallucinations (Pizzolato et al., 2021). In addition, the eCB system has a major role in regulating myenteric neuron activity, vagal and sympathetic nerve function, and the release of gastrointestinal. Then, it can in turn modulate endocannabinoid levels, acting on gut-brain axis (Sharkey & Wiley, 2016). PD patients show both dysmotility and alterations in the microbiota composition, but which one comes first is not clear yet. There is increasing evidence linking gut dysbiosis to the severity of PD's motor symptoms as well as nonmotor symptoms, such as gastrointestinal dysregulation and pain associated to somatosensory hypersensitivities (Klann et al., 2021). CB1 has been implicated in dysbiosis-induced increases in intestinal permeability, the ensuing systemic inflammation, and modulation of the microbiota composition in a way that favors dysmetabolism. Conversely, evidence suggests that CB2 activation partly mediates the analgesic effects of probiotics against visceral pain (Sharkey & Wiley, 2016). Considering that the exercise can modulate the eCB system, we can suggest that it can be also acting in the pain relief due to regulation of gut-brain axis. However, it is only evidence, there are no studies that prove it, deserving further investigation.

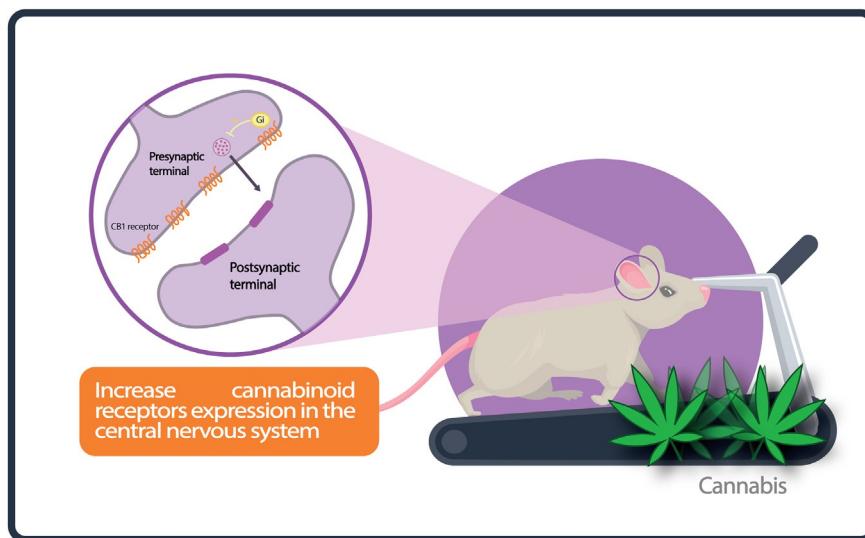
### **Endocannabinoid system and exercise on Parkinson's disease**

In addition to pharmacological treatment, eCB can be regulated by regular exercise and together with neurotrophic factors play important beneficial effects on pain mechanisms, such as inflammation. And most importantly, no known relevant adverse effects. The reason for exercise modulating eCB production is the fact that arachidonic acid, a precursor of eCB, is present in every membrane cell of the body and on demand eCB synthesis is regulated by electrical activity and calcium shifts. Despite the endocannabinoid system having emerged as a focal point to ascertain the mechanisms for how exercise benefits the body and how it reduces or controls pain (Watkins, 2018), many gaps remain unclear.

Plasma anandamide and 2-AG were increased in rats after AE exercise. Activation of CB1 receptors in the rat brain and immunofluorescence analysis demonstrated an increase of activation and expression of CB1. The increase in CB1 receptors was found in neurons of the periaqueductal gray matter (PAG) after exercise. Pretreatment of rats with CB1 receptor antagonist (AM251) and CB2 inverse agonist (AM630) blocked the antinociception induced by AE protocol in both mechanical and thermal nociceptive tests and aerobic exercise. The investigators conclude from their results that the eCB mediates AE-induced antinociception at peripheral and central levels in rats (Galdino et al., 2014). A study with a unilateral 6-OHDA rat model revealed that light/moderate treadmill exercise 3 times per week for 4 weeks is able to improve nociceptive threshold. The exercise is able to modulate cannabinoid receptors in brain regions involved with pain pathways, corroborating previous study, for example, increasing expression of CB2 in anterior cingulate cortex (ACC) and PAG, and CB1 in PAG (Binda et al., 2020) (Fig. 4).

### **Opioidergic system**

Opioid receptors play an important role in pain treatment (Stein, 2018), and they are widely found in different areas, for example, in the brain, peripheral nerves, spinal cord, digestive tract, and cells of immune system (Brezchova et al., 2020). More specifically in the brain, they can be expressed in different regions involved with both pain processing and motor



**FIG. 4** Treadmill exercise modulates endocannabinoid system. Exercise can induce cannabinoid receptors increase and/or decrease in specific areas of the brain in the PD animal models, which can be associated with improvement of PD symptoms. In addition, cannabis is an exogenous cannabinoid that has been used to treat symptoms in neurodegenerative diseases, including PD.

regulation, for example, thalamus (Binda et al., 2021) and striatum (Shokri-Kojori et al., 2022), respectively. There are three major subtypes of opioid receptors: MOR ( $\mu$ —Morphine, is the typical recognized exogenous ligand), DOR ( $\delta$ —Vas deferens, the tissue within which it was first isolated) and KOR ( $\kappa$ —ketocyclazocine, the first ligand to act at this receptor) (Lešnik et al., 2021). All those subtypes are G-protein-coupled receptors (GPCRs) and constituted by seven transmembrane domains.

Their mechanism of action is associated with the presence of inhibitory G protein, which when activated inhibits the production of cyclic AMP or acts directly on ion channels of the cell membrane ( $K^+$  or  $Ca^{2+}$ ) (Stein & Zällner, 2009). This can decrease neural excitability, as well as the release of proinflammatory neuropeptides, for example, as substance P and calcitonin (Stein & Zällner, 2009; Yaksh, 1988), which can modulate and, in general, reduce the painful information. Regarding opioid ligands, they can be of two types: endogenous, which can have an overlapping effect between ligand and the receptors, and exogenous. Endogenous ligand are, for example: Proenkephalin that is cleaved and forms met-enkephalin and leu-enkephalin, dynorphin that it is derived from prodynorphin (Fricker et al., 2020) and  $\beta$ -endorphin that has an important role in food intake and sexual behavior (Veening & Barendregt, 2015). Also, the most common exogenous ligand is morphine, extract of poppy plant (*Papaver somniferum*), which binds to MOR. However, despite morphine being the most powerful drug for severe pain, its use is strongly associated with side effects such as breathing problems, nausea, and addiction (Zällner & Stein, 2007).

### Opioidergic system in Parkinson's disease

Evidence shows complex interactions between opioidergic and dopaminergic systems (Blanchet & Brefel-Courbon, 2018), for instance, the opioid-induced analgesia effect is blocked after intranigral injection of 6-OHDA, neurotoxin that compromises the dopaminergic system (Morgan & Franklin, 1990). It suggests that dopaminergic degeneration that occurs in PD seems to arrest analgesia induced by opioid. Moreover, dopamine release can be stimulated within 10–30 min after exogenous opioids (Di Chiara & Imperato, 1988) and opioid agonists (Spanagel et al., 1992) injection in rats. Therefore, this timing indicates that opioid and dopamine system apparently interact and work closely, playing an important role in the descending inhibitory pain pathway.

Reduction in MOR-opioid receptor expression in 6-OHDA rat model was observed in the thalamus, area in which most of painful pathway ascends (Binda et al., 2020, 2021). Furthermore, previous PET studies in PD patients with levodopa-induced dyskinésias also demonstrated reduction in opioid receptors in the thalamus, as well as in the striatum and cingulate, while an increase in prefrontal cortex was shown (Piccini et al., 1997). Indeed, more specifically to MOR receptor, [<sup>3</sup>H]-Tyr-o-Ala-Gly-fN(Me)Phe-Gly-ol low binding was observed in the caudate nucleus and putamen (anterior and posterior) in *postmortem* tissue of PD patients. Additionally, MOR receptor levels were lower in the substantia nigra (*pars*

*compacta*), and highest in the striatum, cerebral cortex, and amygdala in *postmortem* tissue of PD patients (Delay-Goyet et al., 1987). Then, it is important to note that low level of MOR receptors could be associated with abnormal pain processing and consequently, generates central sensitization and painful symptoms in PD patients (Cury et al., 2016).

Increased levels of opioid peptides, more specifically, dynorphin, were observed in the substantia nigra (Ljungdahl et al., 2011) and striatum (Hanrieder et al., 2011) in a rat model of PD. That increase might be involved in a compensatory mechanism to alleviate striatal overstimulation of D1 receptor by unregulated L-dopa activity of L-dopa drugs (Hanrieder et al., 2011) as well as to compensate low levels of opioid receptor in PD (Cury et al., 2016).

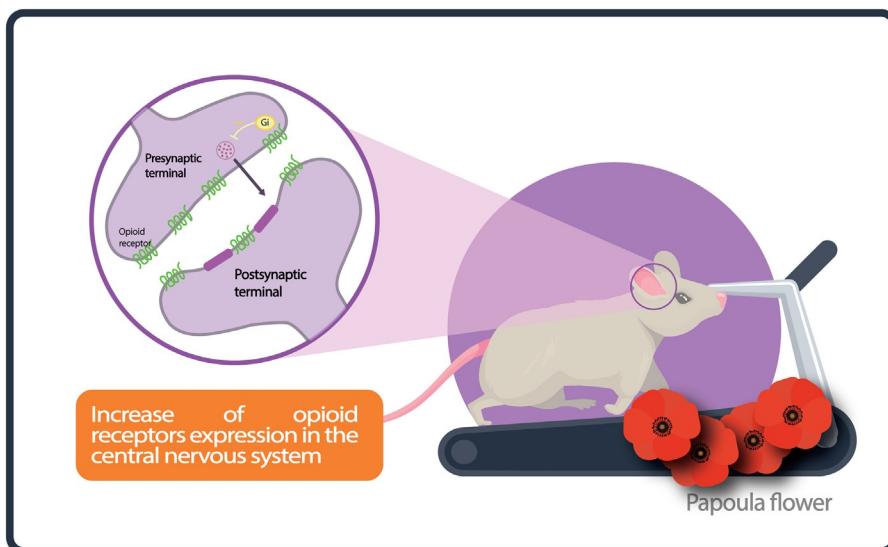
### Exercise elicits the opioidergic system modulation in Parkinson's disease

Physical exercise is well known to increase endorphins in the organism, which is strongly associated with well-being effect in the body (Dinas et al., 2011). Additionally, treadmill exercise also increases endorphin and enkephalin in the periaqueductal gray matter and in the cerebrospinal fluid in rat model of hypertension (Hoffmann et al., 1990) and neuropathic pain (Stagg et al., 2011).

Regarding opioid receptors, acute treadmill exercise increases hippocampal MOR opioid receptors in health rats, while chronic exercise is able to decrease those levels (de Oliveira et al., 2010). Moreover, another nonpharmacological therapeutic intervention (neural mobilization) also modulates opioid receptor (DOR and KOR) in the periaqueductal gray matter in a neuropathic pain model. This modulation can be directly associated with improvement in painful symptoms in those animals (Santos et al., 2014). It is also important to mention that antagonist of opioid receptor hinders beneficial effect of treadmill exercise in nociception (Stagg et al., 2011). Therefore, all those evidences demonstrate that physical exercise modulates opioid receptors, which could directly influence hyperalgesia in PD model (Fig. 5).

### Applications to other areas

In this chapter, we reviewed studies showing the use of exercise as nontherapeutic procedure for improving nonmotor symptoms of patients with PD. Studies have been showing the involvement of opioids and endocannabinoids receptors to improve pain related to PD either in humans or animals. Those receptors were increased in different areas from the brain after different exercise protocol. Thus, it is relevant to develop nonpharmacological interventions, such as physical exercise, which contribute to reducing excessive consumption of analgesics and improvement of quality of life of PD patients. In other words, may be patient who cannot practice exercise can benefit by the use of cannabidiol, for example.



**FIG. 5** Treadmill exercise modulates opioidergic system. Treadmill exercise induces opioid receptors increase and/or decrease in specific areas of the brain in the PD animal models, which can be associated with improvement of PD symptoms. In addition, Papoula flower is an exogenous opioid substrate, considering that it contains opium, a principal precursor of analgesic opiates such as morphine, which have been used to handle the pain in neurological disorders.

## Mini-dictionary of terms

**Parkinson's disease** is a degenerative brain disorder that leads to motor symptoms, such as shaking, stiffness, and difficulty with walking, balance, and coordination, and nonmotor symptoms, including depression, urinary problems or constipation, pain, sleep disruptions.

**Physical activity** is any movement produced by our bodies or skeletal muscles that results in increase of energy, caloric expenditure, and improved physical fitness.

**Exercise** is a subcategory of physical activity that attributes and focuses on improvement or maintenance of physical fitness.

**Receptor** is molecule that binds a ligand; this ligand could be a molecule, protein, or other neurotransmitters. After ligand binds the specific site, the receptor changes its conformational and alters the protein function exerting a number of different functions.

**Nociception** is a signal arriving at the central nervous system as a result of the stimulation of specialized sensory receptors in the peripheral nervous system called nociceptors. This signals process noxious stimuli.

**Pain** is a multidimensional phenomenon that is subjective and unique to each individual. In general, pain refers to unpleasant and uncomfortable feeling.

## Key facts of exercise and Parkinson's disease

- Changes in dopamine levels induce change in neural activity, leading to symptoms of Parkinson's disease. Physical activity promotes an improvement in the nociceptive and other symptoms of PD patients.
- Studies have been shown that environmental causes may help trigger Parkinson's disease.
- Prevention, control, or even reversal of risk factors such as: cigarette smoking, hypertension, hyperglycemia, obesity, physical inactivity, and poor nutrition are realized through leading a healthy lifestyle deaccelerating process of aging.
- Work-related musculoskeletal injuries are the most common cause of absence of work among health professionals. Adding exercise in PD patients helps to achieve a better quality of life.
- Lifestyle change promotes an improvement in quality of life of Parkinson's disease patients.
- Special cases for cannabis therapy are addressed for different neurologic diseases, and patients should be monitored.

## Summary points

- Abnormal central pain processing occurs in Parkinson's disease.
- Exercise modulates opioid and cannabinoid receptors in Parkinson's disease.
- Treadmill exercise improves nociceptive threshold in Parkinson's disease.
- Painful symptoms are observed in the majority of Parkinson's disease patients and have positive correlation with severity of motor symptoms.
- Nonpharmacological intervention as a positive response for PD patients

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## Chapter 11

# Endocannabinoids and inhalant misuse: Neuropsychological aspects

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## Abbreviations

2-AG	2-arachidonyl-glycerol
AUD	alcohol use disorder
BK	big potassium channel
BLA	basolateral amygdala
CB1	cannabinoid type 1 receptor
CPP	conditioned place preference
DREADD	designer receptor exclusively activated by designer drug
EC	endocannabinoid
EPSC	excitatory postsynaptic current
GABA	$\gamma$ -amino-butyric acid
GIRK	G-protein-coupled inwardly rectifying potassium channel
IL	infralimbic
mPFC	medial prefrontal cortex
MSN	medium spiny neuron
NAc	nucleus accumbens
NMDA	<i>N</i> -methyl-D-aspartate
OFC	orbitofrontal cortex
PL	prelimbic
SUD	substance use disorder

## Introduction

The use of substances for their intoxicating or mood-altering effects has a long history with records of use dating back to antiquity. These substances span a wide variety of compounds with varying chemical structures including alcohol, nicotine, opioids, psychostimulants (e.g., cocaine, amphetamine, methamphetamine), cannabis, hallucinogens (e.g., LSD, PCP, ketamine), sedative-hypnotics (e.g., benzodiazepines, barbiturates) steroids, inhalants, and others. In the United States, surveys of drug use (SAMHSA, 2021) reveal a high lifetime prevalence of alcohol (~80%) and nicotine (~61%) use with cannabis having the highest rate among illicit drugs (~46%). While the lifetime prevalence of inhalant use is lower (~10%), it is most often reported by younger members of the population that also show high rates of cannabis use (Johnston et al., 2021).

While studies of co-use of cannabis and inhalants are relative few, they reveal a striking correlation between combined cannabis/inhalant use and the prevalence of alcohol and substance abuse disorders (AUD, SUD). Among 12–17 year olds who reported any use of both cannabis and inhalants, ~35% had an AUD while ~39% had an SUD (Wu et al., 2005). This is compared with rates of SUD of 6% for inhalant only users and 16% for cannabis only users. In a follow-up study (Wu et al., 2008), 96% of adults (18 and older) who had a lifetime use of inhalants had at least one SUD including that for alcohol (87%), cannabis (68%), or inhalants (20%). These findings illustrate that inhalant users, who often begin as children and adolescents, have a higher risk of developing drug and alcohol problems later in life. Whether this reflects inhalant-induced alterations in the development of brain circuitry involved in reward and decision-making behavior is not completely known although abnormalities in brain volume, myelination, and gene expression in these and other areas have been reported following chronic exposure to inhalants (reviewed in Cruz & Bowen, 2021).

At the cellular level, an interaction between cannabis and inhalants at first seems unlikely due to differences in their targets. Most of the psychoactive and rewarding properties of cannabis are thought to be due to activation of endogenous G-protein-coupled, type 1 cannabinoid receptors (CB1R) by  $\Delta 9$ -tetrahydrocannabinol ( $\Delta 9$ -THC). CB1Rs are also activated by endogenous cannabinoids (ECs) such as anandamide and 2-arachidonoyl-glycerol (2-AG) that are synthesized in the postsynaptic neuron following periods of heightened synaptic activity. ECs act as retrograde messengers to dampen the action potential-dependent release of GABA and glutamate, thus providing mechanisms for inducing local forms of plasticity (Busquets-Garcia et al., 2018; Zlebnik & Cheer, 2016). Unlike  $\Delta 9$ -THC and ECs, the neuronal targets of inhalants are diverse and include members of the large class of voltage and ligand-gated ion channel family involved in the moment-to-moment control of neural activity. Recent findings, however, have revealed a novel mechanism linking inhalants to the activation of the endocannabinoid signaling pathway, thus providing an additional potential mode of action underlying the effects of inhalants on behavior. The following sections more fully describe this interaction.

## Brief review of inhalants and their uses

Inhalants are compounds used to produce intoxication following voluntary inhalation through the nose and mouth. They have been classified into multiple categories based on their chemical and structural makeup (Balster, 2009). As shown in Table 1,

**TABLE 1** Classes of commonly abused inhalants.

Category	Chemicals	Most Common Sources
<b>Volatile Organic Solvents</b>		
<i>Aromatic</i>	Toluene	
<i>Hydrocarbons</i>	Ethylbenzene Xylene	adhesives, spray paint, thinner, lacquer, leather tanner, disinfectant, cleaner, petroleum, octane booster
<i>Halocarbons</i>	Trichloroethylene (TCY) 1,1,1-trichloroethane (TCE) Tetrachloroethylene (PERC) n-Propyl Bromide (nPBr)	degreasing agent, coffee decaffeination film cleaner, correction fluid dry cleaning agent, degreasing agent metal cleaner, adhesive
<i>Aliphatic</i>	Propane	domestic and industrial fuel
<i>Hydrocarbons</i>	Butane n-Hexane Iso-Octane	lighter fluid adhesive automotive fuel
<b>Inhaled Anesthetics</b>		
<i>Halogenated</i>	Isoflurane	
<i>Ethers</i>	Halothane Desflurane Sevoflurane	general anesthetics
Nitrous Oxide	Nitrous Oxide	adjunct anesthetic, aerosol propellant
Alkyl Nitrites	Amyl Nitrite Isobutyl Nitrite Isopropyl Nitrite	vasodilator, heart disease treatment air freshener, electronics cleaner cyanide poisoning antidote

Modified from Beckley, J. T., & Woodward, J. J. (2013). Volatile solvents as drugs of abuse: Focus on the cortico-mesolimbic circuitry. *Neuropsychopharmacology*, 38(13), 2555–2567, used with permission of the publisher.

these include: (i) volatile organic solvents (e.g., toluene, trichloroethane, butane, among others); (ii) gases used for anesthesia (nitrous oxide, isoflurane, and desflurane); and (iii) volatile alkyl nitrites (amyl nitrite). Inhalant use is widespread throughout the world and is most often associated with children and adolescents, particularly those who are economically disadvantaged. This has been especially well studied in Mexico City where inhalation of glues, toluene (called activo), and paint thinner (turpentine) is observed among young children forced to live on the street. In the United States, inhalants rank only behind cannabis among 12–14 year olds although the prevalence of use is fairly low (~3%).

## Brain targets of inhalants

Studies on the brain targets of inhalants have historically lagged behind that of other abused substances. This likely reflects the assumption that structurally simple compounds commonly used as inhalants (e.g., benzene and toluene) are unlikely to produce selective effects on brain signaling pathways but rather cause nonspecific disruption of phospholipid membranes. This assumption has been challenged by results from a growing number of studies that have found a surprising degree of selectivity of inhalant effects on key ion channels that regulate neuronal excitability. The following is a brief summary of these findings that are discussed in more detail in a number of excellent previous reviews on this subject ([Cruz & Bowen, 2021](#); [Woodward & Beckley, 2014](#)).

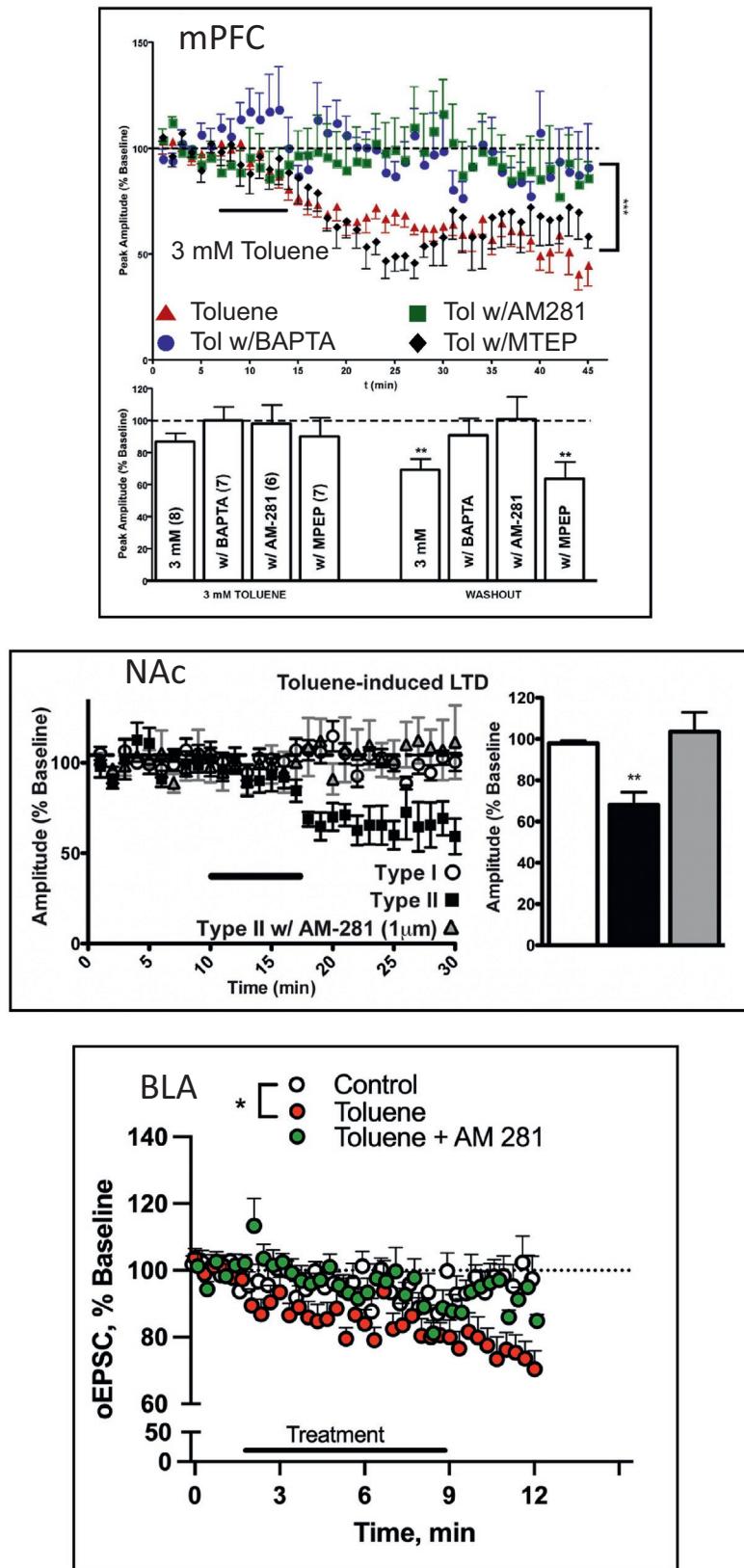
For example, the abused inhalant toluene has been shown to inhibit various subtypes of voltage-gated sodium channels (Nav1.4 and 1.5), potassium channels (BK and GIRK2), and calcium channels (Cav1 and Cav2) while having no effect on other subtypes (e.g., GIRK1/2 and GIRK1/4; native sodium channels in PFC and hippocampal neurons). A similar degree of selectivity was observed among members of the ionotropic glutamate receptor family. Toluene inhibited glutamate-activated currents generated by GluN1/GluN2B NMDA receptors with an  $IC_{50}$  value of 170  $\mu\text{M}$  while other NMDA subtypes (GluN1/GluN2A and GluN1/GluN2C) required millimolar concentrations to achieve the same degree of inhibition ([Cruz et al., 1998](#)). However, ion channel activity of various members of the related AMPA family of ionotropic glutamate receptors (e.g., GluA1 and GluA1/GluA2) was unaffected by high concentrations of toluene or in some cases (e.g., GluA6) was potentiated ([Cruz et al., 1998](#)). Subunit-dependent differences in toluene sensitivity were also reported for other ion channels including nicotinic acetylcholine receptors ([Bale et al., 2002](#)), ATP-gated P2X ion channels ([Davies et al., 2005](#)), and glycine and GABA<sub>A</sub> channels ([Beckstead et al., 2000, 2001](#)).

## Role of endocannabinoids in the cellular actions of toluene

As reviewed above, inhalants such as toluene have been shown to affect a number of ion channels that are critically involved in regulating neuronal excitability. Most of these studies utilized recombinant receptors expressed in heterologous cells (e.g., oocytes and HEK293 cells) allowing one to elucidate the sensitivity of specific subtypes to various inhalants. Armed with this knowledge, follow-up studies have begun to characterize the effects of inhalants on principal neurons within key addiction areas of brain including the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and recently, the basolateral amygdala (BLA). The following section summarizes results from these studies and highlights novel findings suggesting that the endocannabinoid system may be an important regulator of toluene action.

## Toluene and the mPFC

The mPFC is a key component of the frontal cortex that also includes the orbitofrontal cortex (OFC), anterior cingulate (ACC), and anterior insular cortex (AIC). Glutamatergic pyramidal neurons within the mPFC are critically involved in higher-order cognitive behaviors including assessment of risk and reward that will be discussed in a later section. Electrophysiological studies have revealed selective effects of toluene on the intrinsic excitability and synaptic transmission of deep layer mPFC neurons. In the first study of its kind, bath application of toluene had little effect on current-evoked action potential firing of mPFC pyramidal neurons in brain slices from adolescent (postnatal day 21–28) rats ([Beckley & Woodward, 2011](#)). However, the same concentration of toluene produced a delayed and long-lasting inhibition of synaptically evoked AMPA-mediated excitatory postsynaptic currents (Fig. 1, top panel). This was unexpected given previous reports that recombinant AMPA receptors are not inhibited by toluene ([Cruz et al., 1998](#)). Subsequent experiments showed that the toluene-induced reduction in AMPA EPSCs was endocannabinoid-dependent as it was blocked by a CB1 receptor antagonist (AM281) or by buffering intracellular calcium in the recorded neuron with the calcium chelator BAPTA ([Beckley & Woodward, 2011](#)). In this same study, toluene also enhanced GABA-mediated synaptic transmission in mPFC neurons although this effect occurred rapidly and fully reversed upon washout of the toluene solution suggesting that endocannabinoids were not involved. Subsequent studies by this same group used similar brain slice recording techniques to



**FIG. 1** Toluene inhibits AMPA-mediated excitatory synaptic currents via endocannabinoids. **Top:** Time course of toluene (3 mM) inhibition of AMPA EPSCs in deep-layer pyramidal neurons in the medial prefrontal cortex. By itself, toluene (3 mM) induced a significant decrease in AMPA EPSC amplitude that was reduced by the CB1R antagonist AM281 or by intracellular perfusion with the calcium chelator BAPTA. **Middle:** Time course of toluene inhibition of AMPA EPSCs in medium spiny neurons in the nucleus accumbens. Toluene (3 mM) inhibited AMPA currents only in Type II neurons subsequently identified as D2-expressing medium spiny neurons. This inhibition was blocked by the CB1R antagonist AM281. **Bottom:** Time course of toluene inhibition of AMPA EPSCs in pyramidal neurons in the basolateral amygdala evoked by optical stimulation of channelrhodopsin-2 expressing mPFC terminals. By itself, toluene (3 mM) induced a significant decrease in AMPA EPSC amplitude that was blocked by the CB1R antagonist AM281. For all panels, data are mean  $\pm$  SEM expressed as a percent of pretoluene baseline. Symbols: \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ . (**Top:** Figure from Beckley, J. T., & Woodward, J. J. (2011). *The abused inhalant toluene differentially modulates excitatory and inhibitory synaptic transmission in deep-layer neurons of the medial prefrontal cortex*. *Neuropharmacology*, 53(7), 1531–1542 used with permission of the publisher. **Middle:** Figure from Beckley, J. T., Randall, P. K., Smith, R. J., Hughes, B. A., Kalivas, P. W., & Woodward, J. J. (2016). *Phenotype-dependent inhibition of glutamatergic transmission on nucleus accumbens medium spiny neurons by the abused inhalant toluene*. *Addiction Biology*, 21(3), 530–546, used with permission from the publisher. **Bottom:** Unpublished data from the author's laboratory.)

examine how in vivo exposure to intoxicating concentrations of toluene vapor affects the excitability of mPFC neurons that project to different areas of the nucleus accumbens (Wayman & Woodward, 2018b). One day following a brief exposure to 10,500 ppm toluene vapor, current-evoked spiking of mPFC neurons was altered in layer, subdivision, and target-specific manner. Layer 5/6 NAc-core projecting neurons in the prelimbic (PL) mPFC showed reduced excitability while no change was observed in layer 2/3 NAc-core projecting PL neurons. In contrast, layer 5 and layer 2/3 NAc-core projecting neurons in the infralimbic (IL) mPFC showed enhanced firing following toluene exposure while PL and IL neurons projecting to the NAc-shell showed no change in excitability. The effects of toluene vapor on mPFC excitability were short-lived as no changes were observed 7 days after toluene vapor and were restricted to young animals as no effect was seen following toluene exposure in adult animals.

The studies discussed above all used a single exposure to toluene either applied to the bath solution or during a brief vapor exposure. To more closely mimic conditions associated with human inhalant use, the same group exposed adolescent rats to repeated pairings of toluene vapor previously shown to induce a conditioned place preference (CPP) that reflects the rewarding aspects of toluene. In toluene-CPP rats, the current-evoked spiking of IL 5/6 NAc-core projecting mPFC neurons was enhanced while firing was reduced in those projecting to the NAc-shell (Wayman & Woodward, 2018a). No changes were found in PL 5/6 mPFC neurons projecting to either the NAc-core or NAc-shell. In addition, alterations in firing of the NAc-projecting IL 5/6 mPFC neurons persisted for at least 7 days as did the preference for the toluene-paired chamber while no differences in firing or CPP were observed 30 days after the last vapor exposure. Finally, to test which subpopulation of mPFC neurons was responsible for the expression of toluene CPP, activity of these pathways was manipulated using chemogenetic DREADDs (designer receptors exclusively activated by designer drugs). Toluene CPP was blocked by activating the excitatory DREADD hM3Dq expressed in IL 5/6 NAc-shell projecting mPFC neurons while reducing activity in IL 5/6 NAc-core projecting mPFC neurons with the inhibitory hM4Di DREADD had no effect.

## Toluene and the NAc

The NAc is the most ventral part of the striatum and is involved in reward and goal-directed behaviors. The vast majority (~95%) of NAc neurons are GABAergic medium-spiny neurons (MSNs) that project to downstream targets including the ventral pallidum and ventral tegmental areas. As outlined above, NAc neurons that receive input from the mPFC are involved in toluene-based reward as measured by the conditioned place preference assay. As these inputs are glutamatergic, they might be expected to show toluene-induced effects similar to those previously reported in mPFC neurons. In a study exploring that idea, bath application of toluene had no effect on the current-evoked spiking of NAc MSNs although there were subtle effects on the resting membrane potential and the fast after-hyperpolarization driven by BK potassium channels (Beckley et al., 2016). However, like mPFC neurons, toluene induced a long-lasting depression in AMPA-mediated EPSCs although this was only observed in approximately half of the recorded neurons (Fig. 1, middle panel). In these neurons, the toluene-induced inhibition of AMPA EPSCs was blocked by the CB1 antagonist AM281. NAc MSNs are classified into two major categories based on their expression of dopamine receptors and neuropeptides. D1 MSNs express D1-dopamine receptors and the peptides substance P and dynorphin while D2 MSNs express D2-dopamine receptors (and adenosine 2A receptors) and the neuropeptide enkephalin. Previous studies indicated that D2, but not D1, MSNs can undergo an endocannabinoid-dependent depression in AMPA EPSCs suggesting that the toluene-sensitive MSNs were of the D2 subtype (Grueter et al., 2010). Using a partial least squares–discriminative analysis of the electrophysiological parameters of the recorded neurons, Beckley et al. (2016) reported that the putative identity of the toluene-sensitive neurons was the D2 subtype, and this was subsequently verified by immunohistochemical detection of the D2 marker enkephalin. As D2 MSNs are thought to oppose D1 MSN-mediated reward pathways, toluene inhibition of these neurons may enhance the development of compulsive drug seeking although that remains to be tested.

As reviewed above, in vivo exposure of adolescent rats to toluene vapor revealed layer, subregion, and target-specific changes in the intrinsic excitability of NAc-projecting mPFC neurons. Whether in vivo exposure to toluene vapor also induces selective effects on NAc MSN excitability is currently unknown although results from preliminary studies from the author's laboratory suggest this possibility. In this study, adolescent rats underwent a brief exposure to an intoxicating concentration of toluene vapor (10,500 ppm) identical to that used in the mPFC study mentioned previously (Wayman & Woodward, 2018b). Control animals underwent the same treatment but were only exposed to air. Twenty-four hours later, slices containing the NAc core and shell were prepared and current-evoked spiking of MSNs was measured. Using electrophysiological criteria previously shown to predict MSN subtype (Beckley et al., 2016), neurons were putatively identified as D1 or D2 MSNs. In the NAc-core, there was no difference in intrinsic excitability in "D1" MSNs from toluene-exposed rats while spiking in "D2" MSNs was enhanced as compared with air controls. MSNs in the NAc-shell showed a reduction in current-evoked spiking, and this was observed in both "D1" and "D2" subtypes. Although

preliminary, these data suggest that exposure to binge-like levels of toluene vapor produces cell and sub-region-dependent effects on NAc MSN function that may contribute to behavioral impairments discussed below.

## Toluene and the BLA

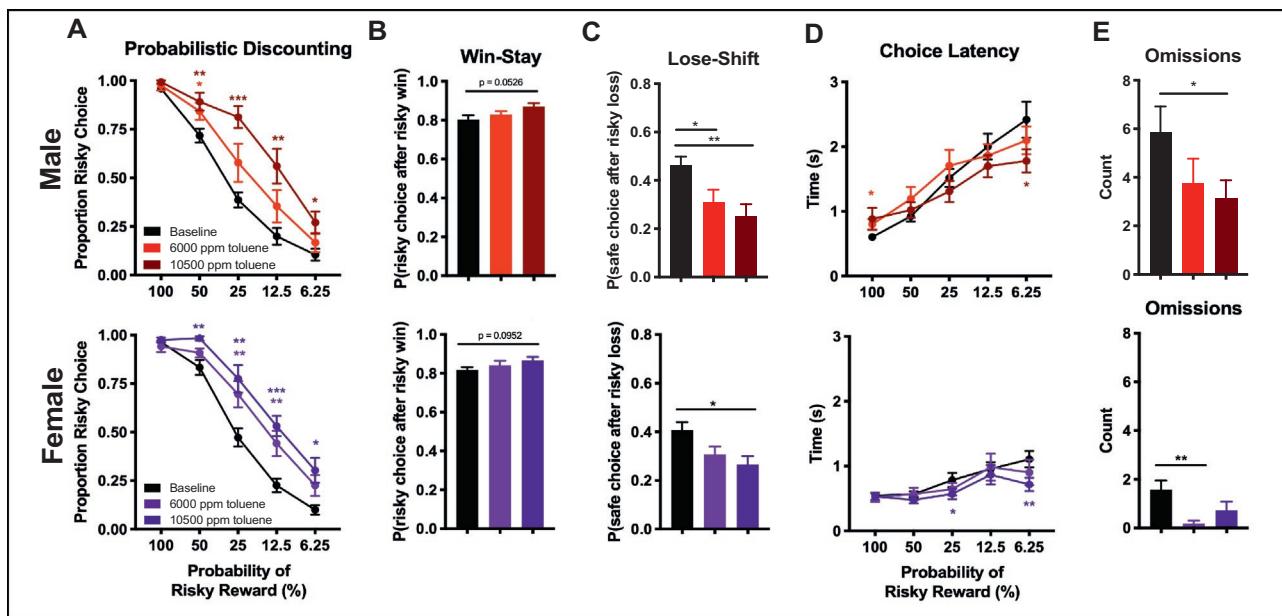
The amygdala is part of the limbic system that processes emotional information and is particularly involved in regulating reactions to fear and anxiety. There are multiple subdivisions of the amygdala, and the basolateral amygdala (BLA) is predominantly composed of excitatory glutamatergic neurons that project to the central amygdala as well as other limbic and cortical structures including the NAc and PFC. Disconnecting the mPFC-BLA circuit results in impaired behavioral flexibility ([Jenni et al., 2017](#); [St Onge et al., 2012](#)). Given toluene's action on glutamatergic transmission in the mPFC and NAc described above and its effect on behavioral flexibility described in a later section, it is important to assess whether BLA neurons are affected by toluene. While there are no published studies to date on this topic, preliminary studies examining toluene action on BLA neurons have been conducted in the author's lab ([Braunscheidel, 2020](#)). Findings from these brain slice electrophysiology studies indicate that bath application of toluene increases current-evoked firing of glutamatergic BLA neurons accompanied by changes in the rise time and decay time of action potentials and a reduction in the after-hyperpolarization. These effects reversed following washout of the toluene solution. To examine the effects of toluene on glutamatergic inputs from the mPFC to the BLA, an AAV virus encoding the light-activated ion channel channelrhodopsin-2 (ChR2) was infused in the mPFC of adolescent rats. Following recovery and time for ChR2 expression, brain slices containing the BLA were used for whole-cell recordings of light-activated AMPA-mediated EPSCs. Pairs of light pulses were used to detect overall changes in EPSC amplitude and paired-pulse plasticity caused by bath application of toluene. Treatment with 0.3 mM toluene, 3.0 mM toluene, or 3.0 mM toluene with 0.75 μM AM281 did not alter the paired pulse ratio (EPSC2/EPSC1) over the course of 15 min of testing. However, 3.0 mM toluene decreased the peak amplitude of EPSC1 in a time-dependent manner, and this did not reverse following washout of the toluene solution ([Fig. 1](#), bottom panel). Co-application of the CB1R antagonist AM281 blocked the toluene-induced inhibition of AMPA EPSCs. These findings suggest that endocannabinoids play a role in mediating toluene inhibition of EPSCs in BLA neurons similar to that previously reported for mPFC and NAc neurons.

## Effects of toluene and endocannabinoids on decision-making—Preclinical studies

### Effects of toluene on prefrontal-cortex-dependent risk taking

Preclinical studies of the behavioral effects of inhalants show that these agents act as general central nervous depressants with hyperactivity observed after the initial exposure followed by sedation and loss of consciousness. In addition, as mentioned earlier, inhalants produce signs of reward or reinforcement as demonstrated by greater time spent in the toluene-conditioned side of an experimental chamber. Studies of human inhalant users report deficits in executive control over behavior thought to involve areas of the frontal cortex and their connection to subcortical structures. Preclinical studies in rats have demonstrated effects of inhalants including toluene on various measures of learning and cognition including those thought to be mediated by the mPFC ([Baydas et al., 2005](#); [Braunscheidel et al., 2017](#); [Dick et al., 2014](#); [Furlong et al., 2016](#)).

Recently, studies carried out in the author's lab evaluated the effects of toluene vapor using a rat model of decision making in the face of uncertainty or risk ([Braunscheidel et al., 2019](#)). In this task, rats are trained to choose a lever that always delivers a small reward (safe choice) or a lever that gives a larger reward on a probabilistic basis (0%–100% or reverse). Previous work using lesions or inactivation approaches show that this form of decision-making involves areas of the frontal cortex including the medial orbitofrontal cortex and mPFC and downstream targets such as the NAc and BLA ([St Onge & Floresco, 2010](#); [St Onge et al., 2012](#)). Rats exposed to toluene vapor during adolescence and tested as adults performed the task equally well as those exposed only to air suggesting no long-lasting effects following the adolescent exposure. However, rats given an acute exposure to toluene vapor just prior to the task showed impaired behavioral flexibility evidenced by shifts in their proportion of risky choices and choice strategy following successful (e.g., win-stay) or unsuccessful (e.g., lose-shift) delivery of the large reward ([Fig. 2](#), top panel) suggesting impaired function of the mPFC ([Braunscheidel et al., 2019](#)). This was verified with *in vivo* calcium (GCaMP6f) fiber photometry that measured mPFC activity during various epochs of the task. In air control animals, GCaMP6f recordings showed peaks in activity just prior to lever choice (deliberation phase) and a decline during reward consumption that varied as a function of safe/risky choice ([Fig. 2](#), bottom panel). In contrast, neuronal calcium signals from toluene-treated rats showed no discrimination between



**FIG. 2** Toluene impairs flexible decision-making and mPFC neural activity during probabilistic discounting. **Top:** Male and female rats were trained at least 20 days on the probabilistic discounting task with descending odds until responding stabilized. (A) Proportion of risky choice within each probability block following acute exposure to air or toluene (6000; 10,500 ppm). (B and C) Choice strategy across all trials. Win-stay (B) indicates choice of risky lever after risky win, whereas lose-shift (C) indicates choice of safe lever after risky loss. (D) Time to choice selection within each probability block. Omissions across all trials (E) indicate no lever press within 10 s time period. Data shown are mean  $\pm$  SEM; all  $N = 12$ ; two-way ANOVA and Dunnett's test comparing each dose to baseline, \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ; colored to match dose. **Bottom:** Aggregate calcium responses in male rats expressing the genetically encoded calcium indicator GCaMP6f in the mPFC during entire probabilistic discounting task (1081 trials) from air ( $N = 8$ ) and toluene-treated animals ( $N = 8$ ). Lines represent mean  $\pm$  SEM  $\Delta F/F$  for safe choice/win (green), risky choice/win (blue), risky loss (pink). An increase in calcium activity was detected during deliberation, ~1.5 s before choice (shaded column throughout figure). Arrow indicates moment of lever press. Note loss of discrimination between safe and risky win in toluene-treated animals. (All figures from Braunscheidel, K. M., Okas, M. P., Hoffman, M., Mulholland, P. J., Floresco, S. B., & Woodward, J. J. (2019). The abused inhalant toluene impairs medial prefrontal cortex activity and risk/reward decision-making during a probabilistic discounting task. The Journal of Neuroscience, 39(46), 9207–9220 used with permission of the publisher.)

safe and risky choices during the deliberation or consumption phases. Overall, the results from this study suggest that a brief exposure to toluene vapor impairs the ability to recognize changes in the relative value of previously learned choices.

### **Endocannabinoids and toluene-induced impairment of decision-making**

As reviewed above, acute exposure to toluene vapor impairs the ability of rats to update their choices during probabilistic discounting (Braunscheidel et al., 2019) and AMPA-mediated EPSCs in mPFC, NAc, and BLA neurons are inhibited by toluene in a cannabinoid-dependent manner (Beckley et al., 2016; Beckley & Woodward, 2011; Braunscheidel, 2020). These findings suggest that the effect of toluene on risky decision-making behavior may be modulated by enhanced production of endocannabinoids and activation of CB1Rs on glutamatergic terminals. A recent study by the author's lab tested this hypothesis using peripheral or intra-mPFC injections of CB1R ligands (Braunscheidel et al., 2022). Results from these studies confirmed earlier findings and showed that toluene exposure impaired shifts in choice biases reflected as an increase in risky choice specifically during times when the probability of obtaining the large reward was uncertain (50%, 25%, and 12.5% blocks) but not when knowing whether obtaining the larger reward was easily predicted (100% and 6.25% blocks). The effect of toluene on choice behavior was not blocked in animals pretreated with an i.p. injection of the CB1R antagonist AM281 (Fig. 3, top panel). However, AM281 itself did affect choice strategy by reducing the probability of choosing the risky lever following a risky win (win-stay). Interestingly, this was only observed in air-exposed animals as AM281 had no effect on win-stay behavior in toluene-treated rats. In contrast, the effect of recent negative feedback on choice strategy indicated by the probability choosing the safe lever following a risky loss (lose-shift) did not differ across treatment groups.

Additional aspects of the risk task used in that study that may reflect decision-making include the delay in making a choice (latency) and whether responses were omitted. In this regard, although toluene did not affect choice latency, AM281 treatment increased this value. Again, this occurred only during epochs of the task where the probability of obtaining the large reward was uncertain. In addition, AM281-treated animals also omitted more responses than their vehicle-treated counterparts. Taken together, the data suggest that the alterations in decision-making induced by toluene in a probabilistic discounting task likely do not involve activation of CB1Rs systemically although blocking CB1Rs may cause animals to delay or disengage from the task when it becomes too difficult.

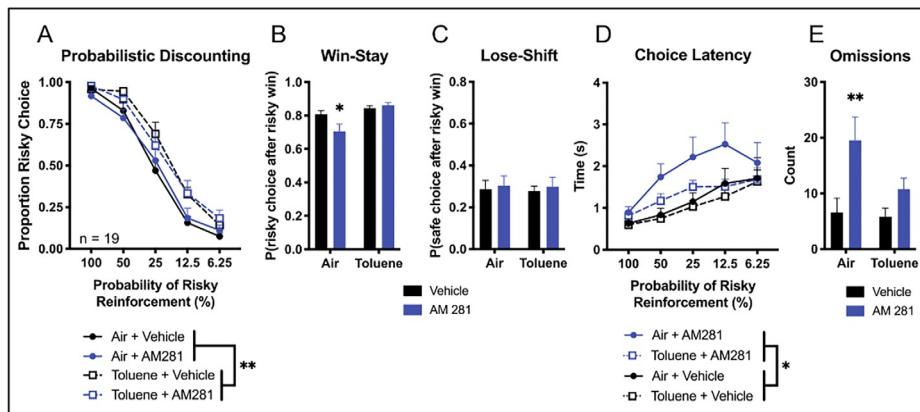
Peripheral injection of a CB1R antagonist would be expected to blunt endocannabinoid signaling throughout the brain and body and could occlude effects on decision-making by impairing circuitry with opposing effects on this behavior. To address this question, Braunscheidel et al. (2022) microinfused the CB1R antagonist AM251 bilaterally into the prelimbic mPFC followed by toluene exposure and testing of risk preference. The toluene-induced shift in risk preference and choice behavior (win-stay; lose-shift) was not altered by an intra-PFC infusion of AM251 nor did it affect choice latency or omissions (Fig. 3, middle panel). However, by itself, AM251 reduced win-stay behavior similar to results observed with i.p. administration of AM281. In addition, although toluene exposure alone reliably shifted risk preference and produced differential effects on choice behavior, these actions were not reproduced following intra-mPFC microinfusion of the CB1R agonist WIN-55-212 (Fig. 3, bottom panel).

Overall, these findings show that while toluene inhibition of AMPA-mediated EPSCs measured in brain slices is clearly EC-CB1R-dependent, the effects of toluene on risky decision-making as measured by the probabilistic discounting task appear to involve mechanisms other than altered endocannabinoid signaling within the mPFC. This may reflect differences in the concentration of toluene experienced by neurons in the slice as compared with that following passive vapor inhalation or that EC-CB1R-dependent actions of toluene on behavioral flexibility are mediated by regions outside the mPFC such as the NAc or BLA that also display toluene-sensitive AMPA-mediated responses. Future work is needed to address these possibilities.

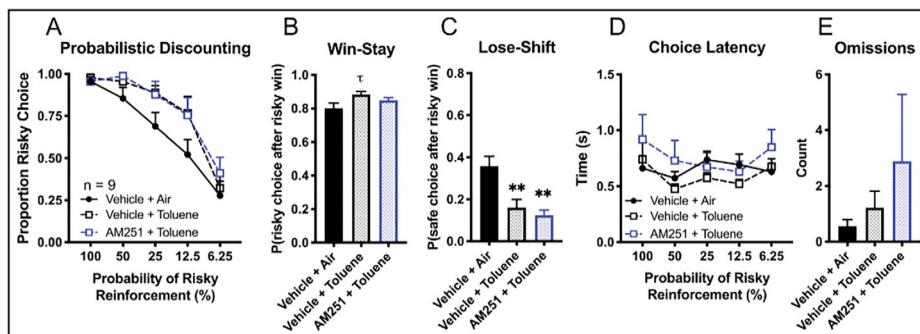
### **Applications to other areas**

The findings discussed in this review illustrate the ability of toluene to induce activation of the EC-CB1R system and dampen excitatory glutamatergic synaptic transmission in areas of the addiction neurocircuitry. This action is in addition to the direct effects of toluene on a wide variety of ion channels that regulate the excitability of neurons in the addiction neurocircuitry and as such extends the repertoire of toluene-sensitive processes. An open question is whether toluene's ability to induce EC-CB1R-dependent effects is shared among other abused inhalants or other drugs of abuse. It seems likely that compounds in the family of alkylbenzenes (e.g., benzene, ethylbenzene, and xylene) of which toluene is a member would also produce similar effects on EC-CB1R signaling although this has not yet been tested. To date, the actual mechanisms linking toluene exposure to production of ECs are not clear although it was reported that this effect is prevented by chelating intra-neuronal calcium or by blocking the ryanodine ion channel receptor that mediates calcium

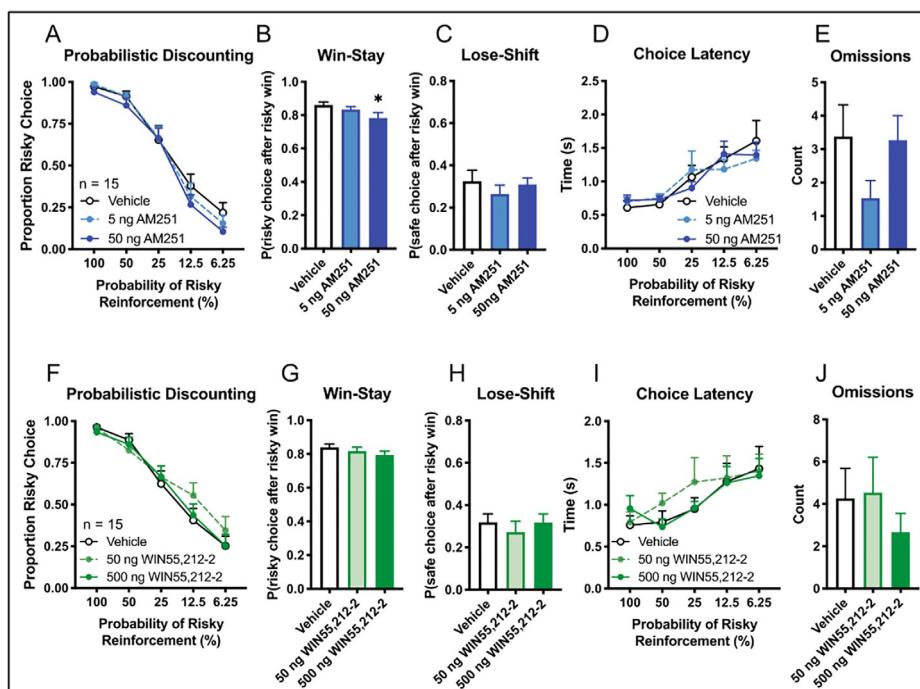
### i) Toluene + Systemic CB1R Antagonist



### ii) Toluene + Intra-mPFC CB1R Antagonist



### iii) Intra-mPFC CB1R Antagonist or Agonist



**FIG. 3** See figure legend on next page

**FIG.3, CONT'D** Effects of cannabinoid ligands on toluene-induced alterations in risky decision making. **Top (i)**: Well-trained rats were treated with a combination of peripheral injections (2 mg/kg AM281 or vehicle, i.p.) and vapor exposure (toluene or air) prior to task performance. (A) Proportion of risky choice within each probability block across treatments. (B and C) Choice strategies employed across all trials. Win-stay (B) indicates choice of risky lever after risky win while lose-shift (C) indicates choice of safe lever after risky loss. (D) Time to choice selection within each probability block. (E) Omissions across all trials indicate no lever press within the 10s trial period. Data shown are mean + SEM; all  $n = 19$ ; three-way ANOVA main effects, \* $P < .05$ , \*\* $P < 0.01$ ; Tukey's post hoc, \* $P < .05$ , \*\* $P < .01$ . **Middle (ii)**: Using a within-subject design, well-trained rats were given the following treatments prior to task performance across three test days: vehicle mPFC microinjection + air exposure, vehicle mPFC microinjection + toluene, or 50ng AM251 mPFC microinjection + toluene. (A) Proportion of risky choice within each probability block across treatments. (B and C) Choice strategies employed across all trials. Win-stay (B) indicates choice of risky lever after risky win while lose-shift (C) indicates choice of safe lever after risky loss. (D) Time to choice selection within each probability block. (E) Omissions across all trials indicate no lever press within the 10s trial period. Data shown are mean + SEM; all  $n = 9$ ; \* $P < .05$ ; Tukey's post hoc, \* $P = .063$ , \* $P < .05$ , \*\* $P < .01$ . **Bottom (iii)**: Using a within-subjects design and two separate cohorts of animals, a CB1R inverse agonist AM251 (5ng, 50ng; A–E), CB1R agonist WIN55,212-2 (50ng, 500ng; F–J) or vehicle was bilaterally microinfused into the mPFC of well-trained rats prior to task performance. (A and F) Proportion of risky choice within each probability block across treatments. (B, C, G, and H) Choice strategies employed across all trials. Win-stay (B and G) indicates choice of risky lever after risky win while lose-shift (C and H) indicates choice of safe lever after risky loss. (D and I) Time to choice selection within each probability block. (E and J) Omissions across all trials indicate no lever press within the 10s trial period. Data shown are mean + SEM; all  $n = 15$ ; Dunnett's post hoc, \* $P < .05$ . (All figures from Braunscheidel, K. M., Okas, M. P., Floresco, S. B., & Woodward, J. J. (2022). Cannabinoid receptor type 1 antagonists alter aspects of risk/reward decision making independent of toluene-mediated effects. Psychopharmacology used with permission from the publisher.)

efflux from the endoplasmic reticulum (Beckley & Woodward, 2011). Thus, any compound that evokes sufficient release of calcium from intracellular stores to activate EC synthesis could trigger a reduction in AMPA-mediated EPSCs. Interestingly, work from the author's lab showed that unlike toluene, ethanol, even at concentrations of 88 mM (equivalent to a blood ethanol concentration of 0.4%), had no effect on AMPA EPSCs evoked in mPFC neurons suggesting no ethanol-induced EC-CB1R signaling (Weitlauf & Woodward, 2008). While evidence supports a role for ECs in toluene action, an important and as yet overlooked area of research is how the combined use of abused inhalants and cannabis might affect behavioral flexibility, decision-making, and the development of a substance use disorder. There could be synergistic effects of having both toluene and psychoactive constituents of cannabis (e.g., d9-THC) present in the brain at the same time or novel adaptations in brain circuitry following long-term use of both compounds.

## Mini-dictionary of terms

**Abused inhalants:** This term covers a broad range of volatile gases and chemicals that are voluntarily inhaled to produce feelings of euphoria and intoxication. Inhalants are found in common commercial and household products, thus providing easy access to children and adolescents who may try inhalants as their first drug of abuse.

**Excitatory postsynaptic current (EPSC):** This reflects glutamate-mediated activation of cation-selective ion channels such as AMPA and NMDA receptors on the postsynaptic membrane. The amplitude of these currents is a measure of the strength of the synapse.

**Behavioral flexibility:** A term used to indicate the ability of an individual to adjust their behavior based on current and past outcomes associated with choosing between multiple alternatives.

**Probabilistic decision-making:** This refers to a task that monitors an individual's ability to determine the cost/benefit ratio of a decision. In animal models, this is usually tested using a small food reward that is always delivered upon a lever press (safe win) versus a large food reward that is provided on a probabilistic schedule (risky win). Changes in the proportion of safe versus risky choices determine the animals risk-taking threshold and indicate a change in the brain circuitry involved in decision-making.

**Addiction neurocircuitry:** A collection of brain areas that are thought to be involved in the acquisition and maintenance of drug seeking and taking behavior. They include but are not limited to areas of the prefrontal cortex, dorsal and ventral striatum, ventral tegmental area, and basolateral and central nuclei of the amygdala. These areas are also involved in decision-making and behavioral flexibility that are often impaired in individuals with drug and alcohol use disorders.

## Key facts of endocannabinoids and inhalants

- Abused inhalants are a large class of volatile agents that are voluntarily inhaled for their intoxicating effects.
- Toluene is a prototypical abused inhalant and is found in commonly used household and commercial products that are often used as inhalants by children and adolescents.
- The use of inhalants by adolescents has risen over the last several years and is second only to marijuana among illicit substances used by eighth graders.

- Individuals with a lifetime use of inhalants have a higher prevalence of substance use disorder than those who have never used inhalants.
- Despite their simple structure, toluene and other abused inhalants show a surprising degree of selectivity in their ability to affect the function of voltage-gated and ligand-gated ion channels critically involved in synaptic transmission.
- Endocannabinoids are neuromodulators produced on-demand in the postsynaptic neuron following high-intensity synaptic transmission. They act in a retrograde fashion by activating presynaptic CB1R receptors that reduce the release of neurotransmitters such as glutamate and GABA, thus impairing normal synaptic activity.

## Summary points

- Exposure of brain slices to toluene induces a slow but persistent depression in excitatory synaptic transmission that is mediated by an EC-CB1R decrease in glutamate release.
- This effect has been demonstrated in brain areas involved in cognitive control over behavior such as the mPFC, NAc, and BLA that are often impaired in individuals with a substance abuse disorder.
- In vivo, toluene impairs performance in a probabilistic discounting task that tests an animal's tolerance for risk by monitoring the ability to shift between risky and safe choices when the chances of winning diminish.
- The effect of toluene on probabilistic discounting is accompanied by impairments in the ability of mPFC neurons to differentiate between safe and risky rewards.
- Toluene's effect on risky decision-making was not prevented in animals treated systemically with a CB1R antagonist or in those receiving the drug directly into the mPFC.
- By themselves, CB1 antagonists altered behavior by reducing the animal's probability of choosing the risky lever following a risky win.
- The lack of effect of mPFC administered CB1R antagonists on decision-making may indicate that the toluene-induced production of ECs and their subsequent effect on risk performance are mediated in areas outside the mPFC such as the NAc and BLA.

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## Chapter 12

# Fatty acid amide hydrolase (FAAH) in substance use disorders: FAAH regulation and inhibition in cannabis, alcohol, nicotine, stimulant, and opioid use disorders

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## Abbreviations

AEA	anandamide
AUD	alcohol use disorder
CB <sub>1</sub>	cannabinoid subtype 1
CUD	cannabis use disorder
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
KO	knockout
PET	positron emission tomography
PFC	prefrontal cortex
PF-04457845	a FAAH inhibitor
PF-3845	a FAAH inhibitor
SNP	single-nucleotide polymorphism
SUD	substance use disorder
THC	Δ <sup>9</sup> -tetrahydrocannabinol
URB597	a FAAH inhibitor
WT	wild type

## Introduction

Substance use disorders (SUDs) have significant public health burdens. However, there are few effective pharmacotherapies available for treating many SUDs. Evidence is accumulating that the endocannabinoid system (ECS), which includes cannabinoid CB<sub>1</sub> receptors that are distributed widely throughout the brain and with particularly high density in the basal ganglia, hippocampus, cortex, and cerebellum, is a major player in the reward pathway (Spanagel, 2020). Additionally, CB<sub>1</sub>-receptor-dependent modulation of other neurotransmitters (e.g., GABA, glutamate, and dopamine) within the reward pathway may be one of the most important neural signaling pathways in development of substance abuse and dependence (Scharma et al., 2019). Evidence suggests that CB<sub>1</sub> receptor activation enhances reward to addictive substances, such as nicotine, while decreasing or blocking CB<sub>1</sub> receptor function has the opposite effect. Unfortunately, despite promising preclinical studies demonstrating direct CB<sub>1</sub> receptor engagement as a potential therapeutic target for SUD, these have not translated to comparable results in humans due to either intolerable adverse effects or lack of efficacy.

Fatty acid amide hydrolase (FAAH) is a serine hydrolase enzyme in the ECS that degrades multiple compounds, notably the endocannabinoid and CB<sub>1</sub> receptor partial agonist anandamide (AEA). Correspondingly, an increase of FAAH concentration or activity leads to a decrease in AEA, and a decrease of FAAH concentration or inhibition of activity leads to an increase in AEA. While both FAAH and CB<sub>1</sub> receptors are found widely throughout the brain, they are often, but not always, co-localized to the same neurons, suggesting that FAAH modulation may confer some neuroanatomical spatial specificity to CB<sub>1</sub> receptor stimulation compared with systemic CB<sub>1</sub> receptor targeted drugs. As well, by dynamically regulating AEA concentration following its synthesis and release, FAAH provides temporal specificity to CB<sub>1</sub> receptor signal transduction and, therefore, its downstream-mediated effects (including modulation of other neurotransmitters). Exemplifying the neuroanatomical and temporal importance of AEA regulation, endocannabinoid neurotransmission has been well described as an important component in long-term potentiation and long-term depression, and the neurophysiological process of reversal learning, all of which are time-sensitive processes primarily mediated through the hippocampus and limbic system.

FAAH has become an attractive research target for treatment of SUDs given its regulation of AEA concentration in the brain and thereby CB<sub>1</sub> receptor activity. Recent research has explored FAAH's role in the development, maintenance, withdrawal, and abstinence from various SUD. This chapter will discuss FAAH and the resulting effects of its altered genetic expression or pharmacological inhibition as it relates to the abovementioned salient characteristics of various SUDs including cannabis, alcohol, nicotine, stimulants, and opioids. Though FAAH inhibitors have been advanced to clinical studies, at this time of this writing, reports of utilizing FAAH inhibitors for addiction are available only for the treatment of cannabis use disorder (CUD) and not of other SUD. Therefore, this chapter will focus on FAAH expression in human SUD, preclinical studies on the use of FAAH inhibitors or genetic manipulation of FAAH, and clinical studies data available for FAAH inhibitors in CUD.

## **FAAH can be genetically altered or pharmacologically inhibited**

FAAH has variant expression through a naturally occurring genetic mutation in human populations. *FAAH*, the gene encoding FAAH, has one common single-nucleotide polymorphism (SNP) leading to a missense mutation (C385A), which converts a proline residue to threonine (P129T). Carriers of this SNP (A/A or A/C) have a FAAH variant that functions normally but is more susceptible to degradation (Sipe et al., 2002). As a result, the more rapid degradation of FAAH due to the SNP leads to an increase in AEA concentration in the central nervous system (Dincheva et al., 2015).

FAAH expression can also be directly altered by genetic manipulation in preclinical animal studies. For example, the C385A SNP has been expressed in rodents to interrogate its effect on behaviors of addiction and domains of SUD. As well, genetic ablation of *FAAH* in rodents, generating an FAAH knockout (KO), allows for a more thorough assessment of the role of FAAH. However, as FAAH KO occurs throughout development and the lifetime of the animal, corresponding outcomes may have limited applicability to humans due to neurophysiological or behavioral developmental changes resulting from compensated AEA neurotransmission and other consequences of *FAAH* deletion.

FAAH activity can be inhibited by highly selective drugs, either in specific brain regions via targeted intracranial injections or globally by peripheral administration (e.g., intraperitoneal injection). Several reversible and irreversible FAAH inhibitors have been developed, which result in a decrease of FAAH activity, including URB597, PF-3845, and PF-04457845; for review, see (Otrubova et al., 2011). Reversible inhibitors have a pharmacological action lasting approximately 3–24 h resulting from biological clearance of the compound, while irreversible inhibitors have a pharmacological action corresponding to the translation of FAAH protein to replace the irreparably damaged copies. For example, pharmacologic inhibition of FAAH has been shown to increase the concentration of anandamide in the brain up to fivefold for 12 h in the case of URB597 and up to 15-fold for 36 h in the case of PF-3845 (Muldoon et al., 2013).

## **Genetic variability of FAAH may impact risk for SUD**

While addiction has historically been viewed as a disease of choice and moral behavior, the last several decades have yielded evidence supporting the contribution of genetic risk as a premorbid factor for developing an SUD. For example, twin studies have indicated that genetic vulnerability can confer risk to development of SUD, with more than one genetic factor contributing to risk, and risk involving each stage from initiation to addiction (for review, see (Ducci & Goldman, 2012)). The following section describes how variation in *FAAH* expression and/or function may relate to susceptibility or severity of SUD.

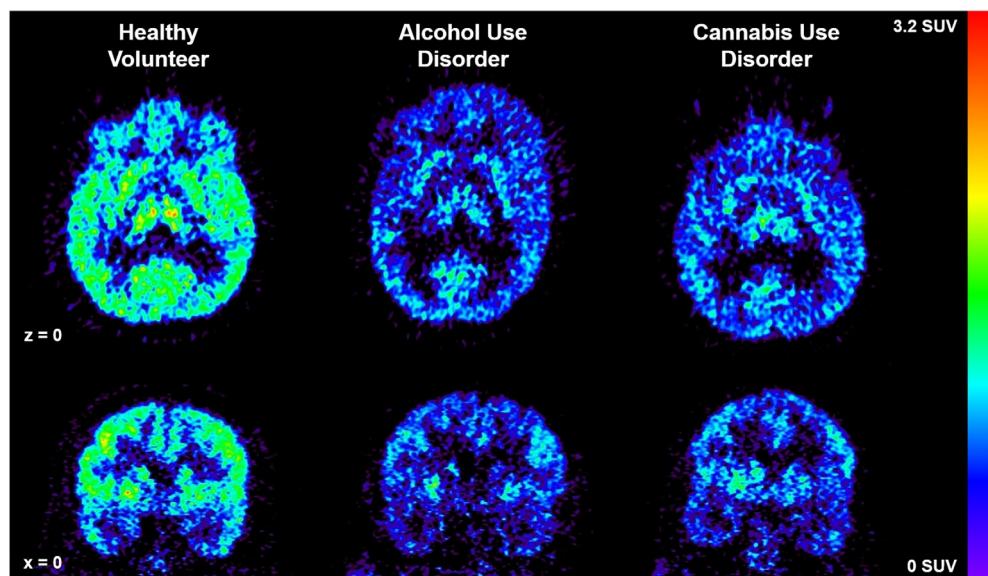
Variations in FAAH, such as from the C385A SNP, have been associated with characteristics that contribute to substance abuse or dependence. However, the evidence is mixed and may be dependent on substance and ancestral

background. For instance, a positron emission tomography (PET) study evaluating FAAH in the brain found that lower FAAH binding is associated with higher impulsiveness, a known behavioral phenotype in CUD (Boileau et al., 2016). Several other studies have found that the A allele of C385A (A/A or A/C carriers) is strongly associated with problem alcohol, street drug, and nicotine use (Sipe et al., 2002; Sloan et al., 2018; Spagnolo et al., 2016). Additionally, this SNP was found to be overrepresented in individuals addicted to cannabis, alcohol, cocaine, methamphetamine, and individuals who are addicted to multiple drugs when compared with nondrug-using controls (Arias Horcajadas et al., 2021; Flanagan et al., 2006; Patel et al., 2018; Sim et al., 2013; Sloan et al., 2018; Zhang et al., 2020).

Yet, other studies have found that the C/C genotype of the SNP may increase the risk of SUD. For instance, individuals with the C/C genotype displayed an increase in happiness and more severe withdrawal symptoms after smoking cannabis than A/A or A/C individuals, potentially making them more vulnerable to the positive and negative reinforcement of the drug (Schacht et al., 2009). Another study found that those carrying the C allele were significantly more likely to become dependent on  $\Delta^9$ -tetrahydrocannabinol (THC) among individuals who had ever tried it, whereas there was an insignificant increase in risk of A/A individuals to use THC (Tyndale et al., 2007). They theorized that the increase in AEA associated with the A allele may decrease anxiety, which may result in increased risk taking. However, it may also contribute to reduced withdrawal and craving in that group, leading to a reduced risk of dependence on the substance. Further, a study using MRI found that heavy cannabis users with the C/C genotype had greater activation encompassing several areas of the reward system when exposed to cannabis associated cues than the A/A or A/C genotypes (Filbey et al., 2010). In terms of other SUDs, investigations have not found an association between the C385A SNP and alcohol, methamphetamine, nicotine, or opioid dependence in certain populations (Iwasaki et al., 2007; Morita et al., 2005; Proudnikov et al., 2010; Sloan et al., 2018; Tyndale et al., 2007).

One explanation for the differing results is that the contribution of the C385A SNP may be dependent on ancestral background. For example, while Sloan et al., 2018 found that the SNP was overrepresented in Caucasians who were dependent on alcohol, they did not find this same association in African American participants. Morita et al., 2005 noted that there is a rarity of the mutant homozygote in Japanese populations when compared with Caucasians, which may result in a lack of genetic risk.

Variations in FAAH may also impact the severity of addiction. The C385A SNP is associated with a self-reported increased number of drinking and binge drinking days, increased tobacco use, and increased cocaine use in the past month (Patel et al., 2018; Sloan et al., 2018). Additionally, individuals with lower whole brain FAAH levels inversely correlated with the number of self-reported alcoholic drinks per week (Fig. 1) (Best et al., 2020).



**FIG. 1** Relative brain uptake and distribution of  $[^{11}\text{C}]$ CURB in healthy volunteer and patients with alcohol and cannabis use disorder.  $[^{11}\text{C}]$ CURB, radioligand that is selective and specific for FAAH, was imaged using PET in a healthy volunteer (left), a patient with alcohol use disorder (AUD, middle), and a patient with cannabis use disorder (CUD, right), all with the C/C genotype for the FAAH C385A SNP. The standardized uptake value (SUV) images were acquired over 60 min, and transverse (top) and coronal (bottom) brain images are illustrative of relative FAAH activity, which is grossly less in AUD and CUD compared with the healthy control throughout cortical and subcortical regions. Figure courtesy of Dr. Isabelle Boileau, Centre for Addiction and Mental Health, Toronto, Canada.

## How is FAAH activity affected in SUD?

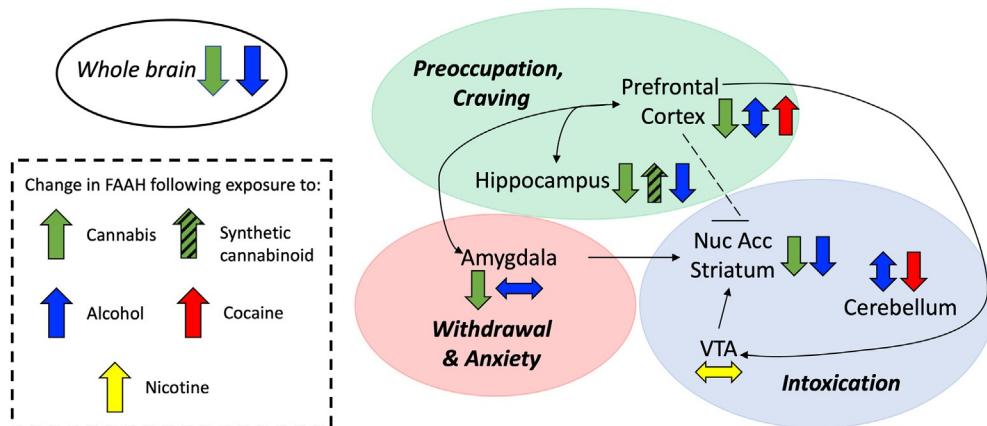
That the C385A SNP was found to be associated with an increased risk of susceptibility to various SUD suggests that FAAH activity may be involved in the brain reward pathway. This pathway, which critically includes the limbic system and the prefrontal cortex (PFC), is largely responsible for reward and reinforcement. Essentially all substances of abuse interfere with the neurotransmission of dopamine, GABA, and/or glutamate in these regions. Therefore, FAAH, which is highly expressed in these regions and indirectly influences these neurotransmitters via AEA modulation, has a critical role in core reward and reinforcement pathways (Fig. 2). This section will describe the differences in FAAH activity in regions of the brain associated with the reward pathway.

FAAH activity in the brains of humans and animals differ based on brain region, species, and length of drug exposure. For example, a PET study of chronic cannabis users found that FAAH binding in several brain regions was negatively correlated with blood and urine cannabinoid metabolite concentrations (Fig. 1) (Boileau et al., 2016). FAAH activity was found to be significantly lower in the ventral striatum of patients with alcohol dependence (Vinod et al., 2010), and in the ventral striatum, hippocampus, and PFC of rats and mice following either very light or chronic exposure to alcohol (Ferrer et al., 2007; Rubio et al., 2009; Vinod et al., 2006, 2012). FAAH expression was also significantly lower in the cerebellum of mice after cocaine sensitization (Palomino et al., 2014), while FAAH levels significantly increased in the PFC of cocaine-sensitized mice after a cocaine prime (Blanco et al., 2015). FAAH levels were significantly higher in the hippocampus of adult mice that had been injected with synthetic cannabinoids in adolescence (Gleason et al., 2012). However, FAAH activity was not found to be altered in the PFC of humans who abuse alcohol, in the amygdala, caudate, putamen, nucleus accumbens, or cerebellum of rats with acute exposure to alcohol (Erdozain et al., 2015; Ferrer et al., 2007; Rubio et al., 2009; Serrano et al., 2018), or in the ventral tegmental area of rats following nicotine exposure (Buczynski et al., 2013). The changes in FAAH activity may be time-dependent as the decreased FAAH activity in the hippocampus of rats after exposure to alcohol was only present for 45 min (Ferrer et al., 2007).

The question arises whether the abovementioned FAAH dysregulation confers pre-morbid susceptibility or is the result of drug exposure. In an effort to answer this question, studies in alcohol preferring rats found that FAAH expression and activity are decreased in the PFC and hippocampus before any alcohol exposure (Hansson et al., 2007; Vinod et al., 2012). The same reduction was not found in the striatum or cerebellum.

## How does FAAH affect the SUD behavior of preference?

As noted in the introduction, substances of abuse might undergo a CB<sub>1</sub>-receptor-dependent mechanism to mediate reward. Thus, inhibition or downregulation of FAAH indirectly increases the concentration of AEA, thereby facilitating alterations



**FIG. 2** Schematic overview of FAAH expression following substance exposure and its role in the addiction pathway. Following exposure to substances of abuse, FAAH concentration, or activity changes in brains regions salient to positive and negative reinforcement pathways, contributing to addiction. Brain regions and circuitry (black arrows; dashed line identifies inhibitory control) are highlighted for their role in an established model of reward pathways. Shaded circles overlap brain regions associated with salient features of SUD: green = regions associated with preoccupation and craving; blue = regions associated with intoxication and reward salience; red = region associated with withdrawal and associated anxiety. The resulting change in FAAH following exposure to cannabis or THC (green arrow), synthetic cannabinoids (green striped arrow; administered during adolescence), alcohol (blue arrow), cocaine (red arrow), or nicotine (yellow arrow) consumption is indicated by the direction of the arrow (increase or decrease). Double-headed horizontal arrows indicate no change in results; double-headed vertical arrows indicate mixed results reported in the literature. Whole brain FAAH is reduced following cannabis or alcohol exposure. Nuc Acc = nucleus accumbens; VTA = ventral tegmental area.

to the reward pathway. This section describes available evidence about the effect of FAAH inhibition on preference for a substance at initial exposure and preference after chronic exposure ([Table 1](#)).

The effect of FAAH inhibition on preference before the animal becomes dependent on a reinforcing substance may depend on the substance. Adolescent female mice with the C385A SNP that are exposed to THC demonstrate increased preference for THC that persists into adulthood. However, this same effect was not seen in adult mice ([Burgdorf et al., 2020](#)). Further, mice with global FAAH inhibition through genetic modification or pharmacologic inhibition had increased preference and greater consumption of alcohol ([Basavarajappa et al., 2006; Blednov et al., 2007; Vinod et al., 2008; Zhou et al., 2016](#)). In contrast, rats that received an FAAH inhibitor before exposure to nicotine had decreased preference for nicotine ([Scherma et al., 2008](#)).

After an animal has been trained to self-administer a substance, the effect of FAAH inhibition on preference may be dependent on a substance, its dose, and dose of the FAAH inhibitor. FAAH inhibition directly in the central and basolateral amygdala or ventral tegmental area decreased preference for alcohol in alcohol-preferring rats, with no effect in the control Wistar rats ([Stopponi et al., 2018](#)), whereas FAAH inhibition directly in the PFC increased preference for alcohol in Wistar rats ([Hansson et al., 2007](#)). Global FAAH inhibition decreased preference for a mid-level dose of nicotine, a dose of nicotine that had led to the highest level of self-administration in squirrel monkeys ([Justinova et al., 2015](#)). However, that same study found that FAAH inhibition led to an increase in nicotine preference at higher levels of nicotine, indicating that the effects of FAAH inhibition can be overcome by taking more nicotine. Further, low-dose FAAH inhibition increased

**TABLE 1** Effects of decreased FAAH on specific outcomes by substance and method of FAAH modification.

Substance	Method of FAAH modification	Species	Preference	Sensitivity	Withdrawal	Reinstatement
Alcohol	Genetic modification	Mice	Increased	Decreased	Decreased	
	Global pharmacologic inhibition	Mice	Increased		Decreased	Decreased
		Rats	No change		Decreased	Decreased
	Central/basolateral amygdala/ventral tegmental area	AA Rats	Decreased			
		Wistar rats	No effect			
	Prefrontal cortex	Wistar rats	Increased			
Nicotine	Genetic modification				No change	
	Global pharmacologic inhibition	Squirrel monkeys	Decreased <sup>a</sup>			
		Mice	Increased <sup>a</sup>	No effect		
		Rats	No change		Decreased	Decreased
Stimulants	Genetic modification	Humans		Increased		
	Global pharmacologic inhibition	Squirrel monkeys	No change			
		Rats	No change			Decreased
Opioids	Genetic modification	Mice			Decreased <sup>b</sup>	
	Global pharmacologic inhibition	Rats	No change	Increased	Decreased <sup>b</sup>	No change
		Mice		Increased	Decreased <sup>b</sup>	

<sup>a</sup>Dependent on dose of nicotine.

<sup>b</sup>Dependent on specific withdrawal symptom.

the preference for nicotine in mice at a subthreshold dose of nicotine; the same effect was not seen when there were higher doses of FAAH inhibition (Merritt et al., 2008; Muldoon et al., 2013). However, FAAH inhibition did not significantly alter preference for alcohol, nicotine, cocaine, heroin, or morphine in rats (Adamczyk et al., 2009; Cippitelli et al., 2008; Forget et al., 2009; Manwell et al., 2009; Solinas et al., 2005), nor did it change preference for THC or cocaine in squirrel monkeys (Justinova et al., 2008).

In some cases where FAAH inhibition had no effect on preference, the doses of FAAH inhibitor were much lower than the doses that were found to have a significant effect, indicating that there may be a dose-dependent response. Additionally, Adamczyk et al. (2009) suggested that the ECS may not mediate cocaine-induced enhancement of the reward system, increasing the likelihood that the effect of FAAH inhibition on preference is dependent on substance.

## FAAH might influence sensitivity to substances

Sensitivity to a substance may contribute susceptibility to abuse and therefore risk to developing an SUD. Increased sensitivity is characterized by either increased susceptibility of rewarding quality of a substance or reduced susceptibility to aversive experience related to use of the substance. For example, a low response to alcohol may increase risk of later heavy drinking and alcohol problems (Schuckit et al., 2011). The next section discusses information on the effect of FAAH on sensitivity to various substances (Table 1).

FAAH function may alter sensitivity to various substances of abuse. For example, FAAH inhibition prevented the development of, and reversed tolerance to, the antinociceptive effect of morphine in rats and mice (Fotio et al., 2020; Hasanein & Ghafari-Vahed, 2016). As well, the C385A SNP is associated with an increase in postoperative nausea and vomiting in children after opioid usage during surgery (Sadhasivam et al., 2015). The SNP is also associated with a heightened response to cocaine in humans (Patel et al., 2018), whereas C/C carriers had subjectively higher arousal levels and less fatigue with low-dose amphetamine ingestion (Dlugos et al., 2010). However, Dlugos et al. (2010) noted that the subjects did not report a difference in arousal with higher doses of amphetamine, and there were no associations between the genotype and objective measurements of arousal such as heart rate and blood pressure.

Yet, there is also evidence that a reduction in FAAH is associated with a decreased or lack of sensitivity to addictive substances. For example, multiple investigations have demonstrated that FAAH KO mice demonstrate decreased sensitivity to alcohol though there was no difference in blood alcohol content or clearance (Basavarajappa et al., 2006; Blednov et al., 2007; Pavón et al., 2019). Additionally, FAAH inhibition did not affect nicotine-induced nociception or hypothermia in mice, indicating no change in sensitivity (Merritt et al., 2008). Further, FAAH KO mice did not demonstrate any change in sensitivity to THC when compared with WT mice (Cravatt et al., 2001).

## FAAH expression and effect of inhibition during substance withdrawal

As the negative effects of withdrawal are one of the most important drivers for continued substance use, it is an attractive target to prevent relapse (Koob, 2015). This section discusses FAAH in relation to withdrawal, including changes in FAAH during withdrawal and the effect of FAAH manipulation on the severity of withdrawal symptoms (Table 1).

FAAH has been shown to be dysregulated during withdrawal. For instance, PET studies found that compared with healthy controls, FAAH binding was lower in the brains of chronic cannabis users in early abstinence from cannabis (Jacobson et al., 2021) and lower in the brain of treatment seeking patients with AUD in early abstinence from alcohol (Fig. 1) (Best et al., 2020). Though both studies note that this did not correlate with self-reported drug use, craving, withdrawal severity, or days of abstinence, others found that FAAH activity is correlated to the concentration of cannabinoids and metabolites in blood and urine (Boileau et al., 2016). Similarly, multiple studies found that FAAH mRNA is significantly decreased in the amygdala, but not the PFC, of rats in early abstinence from alcohol (Serrano et al., 2012; Zhou et al., 2017). However, all studies mentioned above found that this change in FAAH activity and expression was not present after maintained abstinence.

Among individuals who use cannabis daily, A allele carriers of the C385A SNP report less severe withdrawal symptoms than C/C carriers (Schacht et al., 2009). Indeed, in many cases, FAAH inhibition seems to ameliorate some of the negative signs of withdrawal. For instance, a 4-week placebo-controlled randomized trial of individuals with CUD found that daily treatment with the FAAH inhibitor PF-04457845 reduced cannabis withdrawal symptoms such as depression, anxiety, irritability, and sleep deficits in men (D'Souza et al., 2019). In rats, FAAH inhibition prevented nicotine withdrawal symptoms including anxiety (Cippitelli et al., 2011). FAAH inhibition also decreased the severity of opioid withdrawal in rats and mice by decreasing naloxone precipitated and spontaneous physical withdrawal symptoms (Ramesh et al., 2011, 2013; Shahidi & Hasanein, 2011), which was also observed in FAAH KO mice in comparison with WT mice (Ramesh et al.,

2011). FAAH inhibition also decreased the anxiogenic effects of alcohol withdrawal in both early and late withdrawal in rats and mice (Cippitelli et al., 2008; Serrano et al., 2018). Finally, FAAH KO mice had a significant reduction in alcohol withdrawal severity after chronic alcohol consumption, though this same effect was not seen after acute alcohol exposure (Blednov et al., 2007; Vinod et al., 2008).

In other cases, the evidence is mixed. For example, while FAAH KO mice had decreased opioid withdrawal-induced weight loss compared with their WT littermates (Ramesh et al., 2011), pharmacologic FAAH inhibition decreased withdrawal-induced weight loss in rats but not in mice (Ramesh et al., 2011, 2013; Shahidi & Hasanein, 2011). (However, the differing effects in rats and mice may have been due to the use of different FAAH inhibitors.) As well, whereas FAAH inhibition reduced the naloxone precipitated and spontaneous number of withdrawal induced jumps in rats and mice (Ramesh et al., 2011, 2013; Shahidi & Hasanein, 2011), it had no significant effect on the relative change of opioid withdrawal-induced jumping in mice (Gamage et al., 2015). Acute administration of an FAAH inhibitor to THC-dependent mice decreased some rimonabant-induced withdrawal responses in WT mice, such as paw tremors, yet had no effect in FAAH KO mice (Schlosburg et al., 2009). This same study found that FAAH inhibition decreased other signs of rimonabant-induced THC withdrawal symptoms, such as head twitches, in both genotypes. The authors theorized that as the variability was dependent upon genotype, both of which experienced withdrawal symptoms, it suggests that absence of FAAH does not affect the development of physical dependence on THC. However, the variability based on withdrawal symptoms may indicate that some behaviors in withdrawal, such as paw tremors, are specific to FAAH activity, while others are FAAH-independent.

In some cases, FAAH inhibition seemed to have no effect on signs of withdrawal, even occasionally worsening them. FAAH inhibitors failed to significantly alter opioid withdrawal-induced diarrhea in mice (Ramesh et al., 2011, 2013). Both FAAH inhibition in rats and mice and genetic reduction of FAAH in mice failed to decrease nicotine withdrawal severity and were even found to worsen global withdrawal scores (Cippitelli et al., 2011; Merritt et al., 2008).

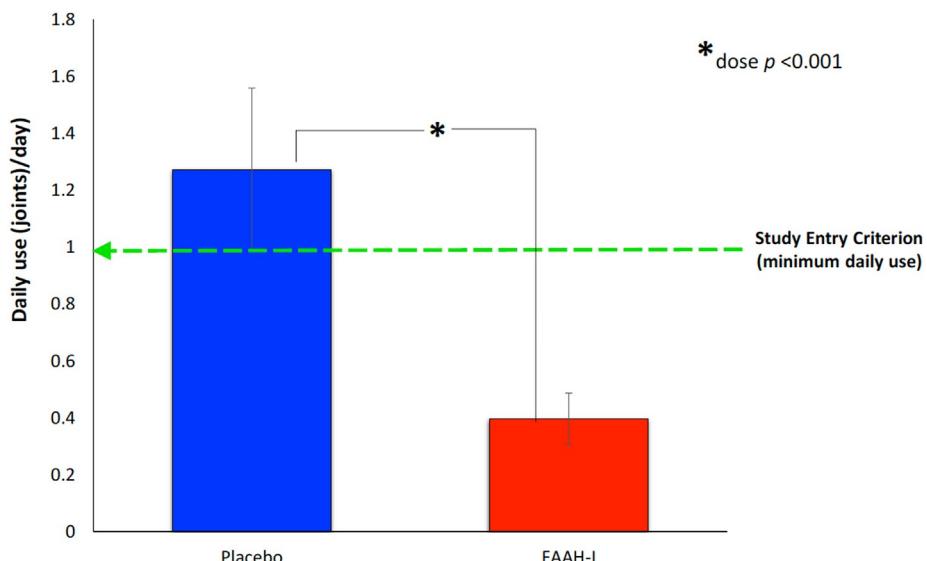
The behavioral effects of FAAH inhibition on substance withdrawal have also been mixed. Several investigations have found that FAAH inhibition did not affect naloxone precipitated conditioned floor aversion in rats (Gamage et al., 2015; McCallum et al., 2010; Wills et al., 2014). However, others found that FAAH inhibition facilitated this same induced withdrawal behavior (Manwell et al., 2009), while having no effect on floor aversion reinstatement upon rechallenge with naloxone precipitated morphine withdrawal (McCallum et al., 2010). Together, these results suggest that FAAH inhibition facilitates reversal learning to aversive morphine withdrawal, but not by acute rescue or memory ablation, as the effects were only observed after repeated trials and were rapidly reinstated. Wills et al. (2014) also theorized that the difference in effects could be due to species of animal used, length of dependence on opioids, and the difference in brain regions involved with physical versus motivational withdrawal.

These results suggest that the utility of FAAH inhibitors on withdrawal may depend on substance, timing, withdrawal symptom, and species. In the case of CUD, it is likely that THC directly contributes to CB<sub>1</sub> receptor downregulation as observed by PET in cannabis users (Hirvonen et al., 2012), and a reduction of demand for AEA and therefore FAAH (Boileau et al., 2016); while during cannabis abstinence, the absence of CB<sub>1</sub> receptor stimulation and associated upregulation results in a net reduction of AEA tone. Therefore, an FAAH inhibitor rationally provides an indirect support for the reduced AEA tone and is likely the mechanism for reducing cannabis withdrawal symptoms clinically. However, for other substances that do not directly target the ECS, the regulation of FAAH and AEA is likely compensatory and in concert with other neurotransmitter systems. For instance, chronic exposure to other toxic substances such as alcohol can lead to alterations in the levels of CB<sub>1</sub> receptors in several brain regions (Hirvonen et al., 2013; Vinod et al., 2006), and it is possible that the decreased FAAH levels in early abstinence are part of a homeostatic renormalizing AEA, and the benefit of FAAH inhibition found during withdrawal enhances this effect.

## FAAH regulation and inhibition during substance abstinence or reinstatement

As SUD is characterized by its relapsing and remitting nature, the regulation and inhibition of FAAH during abstinence are an important consideration. A decrease of endocannabinoids in the brain has been shown to be a crucial factor for reinstating drug-seeking behaviors, though the pharmacologic mechanism remains unclear (Nawata et al., 2019). In this section, we discuss the effect of FAAH inhibition on maintaining abstinence and reducing relapse.

In humans, a 4-week randomized trial in men with CUD found that daily treatment with a FAAH inhibitor decreased self-reported cannabis use and urinary concentrations of THC metabolites compared with placebo (Fig. 3) (D'Souza et al., 2019). Additionally, in daily cannabis users, the C385A SNP was associated with a significant decrease in craving of cannabis during abstinence compared with C/C carriers (Haughey et al., 2008). However, another study with a similar



**FIG. 3** FAAH inhibitor PF-04457845 versus placebo in cannabis use disorder. Compared with placebo ( $n = 24$ ), FAAH inhibitor PF-04457845 ( $n = 46$ ) given for 4 weeks, with the first 5 days in controlled abstinence, was associated with significantly less cannabis use as assessed by the timeline follow-back. Values represent means  $\pm$  S.E.M. (Figure reprinted with permission from D'Souza, D. C., Cortes-Briones, J., Creatura, G., Bluez, G., Thurnauer, H., Deaso, E., Bielen, K., Surti, T., Radhakrishnan, R., Gupta, A., Gupta, S., Cahill, J., Sherif, M. A., Makriyannis, A., Morgan, P. T., Ranganathan, M., & Skosnik, P. D. (2019). Efficacy and safety of a fatty acid amide hydrolase inhibitor (PF-04457845) in the treatment of cannabis withdrawal and dependence in men: a double-blind, placebo-controlled, parallel group, phase 2a single-site randomised controlled trial. *The Lancet Psychiatry*, 6(1), 35–45.)

population found no difference in craving between genotypes (Schacht et al., 2009) and theorized that their sample size was too small to detect a change in this phenotype.

The effect of FAAH inhibition on reinstatement of substance use after extinction may be dependent on the reinstatement paradigm and substance. In the absence of cues, rats that received an FAAH inhibitor daily for 28 days during abstinence had a reduction in the amount of cocaine seeking during extinction sessions (Chauvet et al., 2014). Additionally, FAAH inhibition in single and repeated doses reduced alcohol consumption in mice and rats in early withdrawal and in a relapse-like paradigm when given choice between alcohol and water (Serrano et al., 2018; Zhou et al., 2017). As well, FAAH inhibition significantly attenuated methamphetamine-induced drug-seeking behavior of methamphetamine (Nawata et al., 2019), cocaine, stress, and cue-induced drug-seeking behavior of cocaine (Adamczyk et al., 2009; Chauvet et al., 2014), and nicotine and cue-induced drug seeking of nicotine (Forget et al., 2009, 2016; Scherma et al., 2008) in rats who had previously self-administered these substances. However, it did not attenuate reinstatement after foot shock induced reinstatement of methamphetamine (Nawata et al., 2019), or cue-induced or stress-induced reinstatement of alcohol use, in rats (Cippitelli et al., 2008). Additionally, pharmacologic FAAH inhibition did not modify prime-induced reinstatement of conditioned floor preference for morphine in rats (Manwell et al., 2009; McCallum et al., 2010).

Nawata et al. (2019) proposed that the difference in results in cue-induced reinstatement of substance versus stress-induced reinstatement may be due to cognitive dysfunction in the setting of increased AEA interrupting memory consolidation.

## Conclusion

This chapter illustrates that FAAH has a role in the susceptibility, maintenance, withdrawal, and reinstatement of various SUDs, though the available evidence remains limited. Just as different substances of abuse modulate neurotransmitters along partially overlapping pathways, variations in the density, localization, and actions of FAAH and CB<sub>1</sub> receptors may contribute to some of the heterogeneous results reported here. For example, AEA has effects at other sites of action (e.g., TRPV1 and PPAR receptors), and so FAAH inhibition likely has effects beyond AEA's action at CB<sub>1</sub> receptors. This makes the generalizability of FAAH's roles and actions across all SUDs difficult to ascertain. For instance, mixed results linking reduction in FAAH (particularly due to the C385A SNP) to an increase in self-reported use of alcohol, nicotine, cocaine, methamphetamine, or opioids may be dependent on ancestry or substance. Thus, more research is needed to understand the contribution of FAAH in genetic susceptibility to SUD.

As for therapeutic development, FAAH inhibition during withdrawal and abstinence has the strongest evidence for benefit. In particular, use of FAAH inhibitors in CUD is a rational approach, as it provides indirect partial agonist therapy directly within the ECS, which is deficient in tone during acute THC withdrawal. Across other substances, the ability of FAAH inhibition to decrease anxiety and facilitate reversal learning or extinction of aversive experiences in withdrawal is especially promising. FAAH inhibition may also ameliorate somatic signs of withdrawal from nicotine, opioids, and alcohol. Moreover, FAAH inhibition may help prevent relapse as it was found to decrease seeking or reinstatement of cannabis, cocaine, nicotine, alcohol, and amphetamine.

Yet, it remains unclear if FAAH inhibition would be therapeutic for other signs, symptoms, and behaviors of SUD. For example, there is evidence that FAAH inhibition is unable to interrupt or extinguish memories of rewarding stimuli. Additionally, regarding sensitivity to and preference for various substances, the effect of FAAH inhibition may be dependent on numerous variables. For instance, though FAAH inhibition may decrease preference for certain nicotine concentrations in rodents, it may increase preference for alcohol across several different concentrations of alcohol. This indicates that during active substance use, FAAH inhibition may have beneficial or deleterious effects depending on the substance and amount of use.

The conclusions in this chapter are limited by the fact that most of the studies reviewed measured the effects of FAAH primarily in male subjects or animals. FAAH is influenced by gonadal hormones ([Gorzalka & Dang, 2012](#)) and by largely excluding female subjects or laboratory animals, the effect of FAAH on the above characteristics of addiction do not take this fully into account. As previously suggested, further research should include both sexes and control or adjust for the estrous cycle ([Niemela & Terry, 2021](#)).

As previously noted, most available data on the effect of FAAH inhibition on SUD have been gleaned from preclinical studies. While a concerted focus on the study of FAAH inhibitors for use in SUD therapeutic development remains recommended, the potential negative consequences on applying FAAH inhibitors in those with SUD should also be given ample attention. The ongoing development of FAAH inhibitors for neuropsychiatric disorders provides promise that medications with novel mechanisms of action could one day be available, supplying a desperate need. However, as SUD is highly comorbid in persons with neuropsychiatric disorders, the potential benefit or risk FAAH inhibitors present to those with regular substance use or SUD should be addressed.

## Applications to other areas

In this chapter, we have reviewed evidence that pharmacologic FAAH inhibition may be associated with extinction of aversive experiences associated with SUDs. It has been well established that inhibition of FAAH and the corresponding increase in AEA attenuate anxiety-related behavior in rodents. There is more evidence that the anxiolytic-like effects of FAAH inhibition are more reliable under conditions of environmental stress ([S. Patel et al., 2017](#)). Further, acute stress can trigger an increase in FAAH activity, leading to a reduction of AEA, especially within the amygdala ([Spagnolo et al., 2016](#)). Therefore, we postulate that inhibition of FAAH may be useful in the prevention of posttraumatic stress disorder (PTSD) if used immediately following a traumatic event. For instance, FAAH inhibition immediately following a traumatic event may be able to counteract the increase in FAAH activity seen in acute stress. Moreover, when used prophylactically in the setting of a traumatic event, the ability of FAAH inhibition to decrease anxiety during periods of stress and facilitate extinction of aversive experiences could reduce the risk of development of PTSD.

There is intriguing, preliminary support for the efficacy of FAAH reduction in preventing PTSD. Early intervention with cognitive behavioral therapy in trauma-exposed youth decreased PTSD symptoms and associated anxiety. However, as not all individuals who experience a traumatic event will go on to develop PTSD, identification of genetic or other risk factors for PTSD is critical. In support of this, a pilot study sought to assess if early intervention with psychotherapy after a traumatic event in patients mitigated the genetic risk associated with development of PTSD ([Rothbaum et al., 2014](#)). Interestingly, individuals with comorbid PTSD and AUD with the C385A SNP were shown to have decreased PTSD-related hyperarousal symptoms when compared with WT carriers ([Spagnolo et al., 2016](#)), further supporting the idea that decreased FAAH activity may be helpful in attenuating PTSD severity.

## Key facts of substance use disorders (SUDs)

- In 2019, the United Nations estimated that 35 million persons worldwide have a SUD.
- According to the National Institute on Drug Abuse, abuse of tobacco, alcohol, and illicit drugs incur an estimated \$740 billion annual cost in the United States due to crime and legal costs, health care, and lost work productivity.

- The lifetime risk of developing an SUD for persons who ever use the substance is approximately 32% for tobacco, 23% for heroin, 17% for cocaine, 15% for alcohol, and 9% for cannabis.
- SUDs have been hypothesized to develop from associating substances with positive reinforcement (causing a rewarding or pleasurable experience) and negative reinforcement (escaping or removing an unpleasant experience, such as withdrawal).
- Of all individuals in the United States who have a SUD, only about 10–12% received specialty treatment.
- Successful pharmacotherapy options for SUDs have included agonist therapy, in which a medication replaces or simulates the addictive substance but has a safer, better tolerated, and/or longer duration of action (e.g., nicotine replacement for tobacco, methadone for opioids).
- There are no pharmacotherapies currently approved by the U.S. Food and Drug Administration for cannabis or stimulant use disorders.

## Mini-dictionary of terms

- **Amygdala:** a region of the brain responsible for emotional activation and threat processing, closely connected to the hippocampus and partially controlled by the prefrontal cortex
- **Hippocampus:** a region of the brain primarily responsible for memory formation
- **Reinstatement model:** A model used to measure drug relapse behavior after extinction of a previously acquired self-administration, often using some sort of stimulus.
- **Reversal learning:** the ability to adjust behavior when something that had an expected rewarding response has changed.
- **Substance use disorder:** A complex disorder that leads to a persons' inability to control their use of a substance, even in the event of harmful consequences.
- **Sensitivity:** An individual's response to either rewarding or aversive effects of a substance and tolerance of that response.
- **Withdrawal:** Physical and mental symptoms that occur after stopping or reducing intake of drug after a person has become physically or mentally accustomed to its use.

## Summary points

- This chapter focuses on fatty acid amide hydrolase (FAAH), which is an enzyme that degrades the endocannabinoid anandamide and its role and potential therapeutic target in substance use disorders (SUD).
- A common mutation of FAAH (the C385A SNP) may increase the risk of SUD and substance misuse in certain populations.
- Exposure to substances may induce changes in FAAH expression and activity in brain regions in the reward pathway.
- Inhibition of FAAH might affect the sensitivity to and preference for various substances.
- Inhibition of FAAH might decrease some negative aspects of withdrawal, in particular anxiety and extinction of aversive experiences.
- Inhibition of FAAH, in single or more regular dosing, may decrease drug seeking behavior during abstinence.
- However, most of these effects are dependent on substance, dose, time course, and population studied.
- Therefore, FAAH inhibitors show promise in the treatment of SUD, but the potential application requires additional research.

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## Chapter 13

# Treatment of pain with dual fatty acid amide hydrolase (FAAH) enzyme and human soluble epoxide hydrolase (sEH) enzyme inhibitors: Interlinking the endocannabinoid system

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## Abbreviations

AEA	anandamide
AUDA	12-[(tricyclo[3.3.1.13,7]dec-1-ylamino)carbonyl]amino]-dodecanoic acid
CB <sub>1</sub>	cannabinoid type-1 receptor
CB <sub>2</sub>	cannabinoid type-2 receptor
CL <sub>int</sub>	apparent intrinsic clearance
COX	cyclooxygenase
CYP	cytochrome P450
DHETs	dihydroxyeicosatrienoic acids
DML	designed multiple ligand
EpFAs	endogenous epoxy-fatty acids
FAAH	fatty acid amide hydrolase
HTS	high throughput screening
LMt <sub>1/2</sub>	liver microsomes half-lives
LOX	lipoxygenase
NSAID	nonsteroidal antiinflammatory drug
PGG2	prostaglandin G2
PGH2	prostaglandin H2
PF-3845	N-3-Pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide
PF-750	N-phenyl-4-(quinolin-2-ylmethyl)piperidine-1-carboxamide
PD	pharmacodynamic
PK	pharmacokinetic
SAR	structure-activity relationship
sEH	soluble epoxide hydrolase
THC	Δ <sup>9</sup> -tetrahydrocannabinol
TPPU	N-[1-(1-Oxopropyl)-4-piperidinyl]-N'-[4-(trifluoromethoxy)phenyl]urea
t-TUCB	Trans-4-[4-(3-trifluoromethoxyphenyl-1-ureido)-cyclohexyloxy]-benzoic acid
URB597	cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester
URB937	cyclohexylcarbamic acid 3'-carbamoyl-6-hydroxybiphenyl-3-yl ester

## Introduction

Pain is in general categorized as acute (pain that lasts up to a few weeks) or chronic (pain that persists beyond 3 months), whereas the terms mild, moderate, or severe are often used in a clinical setting to describe pain severity (Millan, 1999; Riedel & Neeck, 2001). The current medical management guidelines for treatment of moderate pain are to start with non-opioid analgesics such as nonsteroidal antiinflammatory drugs (NSAIDs), and if this is inadequate, then the opioid analgesic may be introduced (Chou et al., 2016; Schmidt-Hansen et al., 2018). The continued use of NSAIDs and opioid analgesics to treat pain results in severe acute and chronic adverse effects (e.g., gastric hemorrhages, ulcers, and constipation) and later includes the development of addiction and death due to overdose (Bjorkman & Kimmy, 1995; Cicero & Ellis, 2017; Gasior et al., 2016).

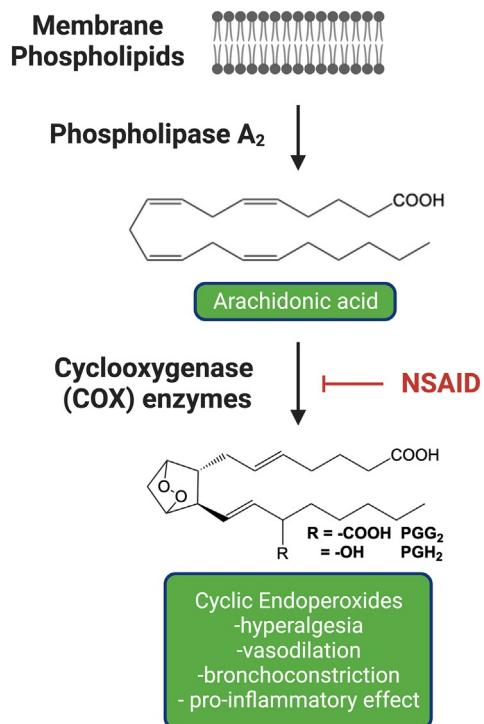
Chronic pain is the primary cause of disability worldwide. According to the National Institutes of Health, nearly 25.3 million Americans suffer from daily pain, and another 23.4 million Americans report significant pain. Worldwide, approximately one in five adults suffers from pain, and another one in 10 adults is diagnosed with chronic pain each year (Chronic Pain and High-Impact Chronic Pain among U.S. Adults, 2019, 2020). Despite its prevalence, chronic pain is poorly managed. Decades of research on pain and analgesia has yielded no novel therapies. Managing pain represents a unique challenge to health professionals that usually demands the use of multidisciplinary strategies.

## Current treatment options

- (a) *NSAIDs*: The most common nonopiod analgesics for mild-to-moderate pain are NSAIDs. Antiinflammatory action of NSAIDs is a result of their inhibitory effect on the cyclooxygenase (COX) enzymes, which are involved in metabolism of arachidonic acid into cyclic endoperoxides, including prostaglandins (Blobaum & Marnett, 2007; Kurumbail et al., 2001). The production of prostaglandins begins in membrane phospholipids, which are precursors to arachidonic acid (Malkowski et al., 2000). Under normal conditions, arachidonic acid is not available for metabolism and is conjugated to the cellular membrane. During inflammation, an enzyme phospholipase A2 releases a large amount of arachidonic acid into the cell; next, COX enzymes convert arachidonic acid to prostaglandin G2 (PGG2) and prostaglandin H2 (PGH2) that in turn sensitize pain pathways (Fig. 1) (Inceoglu et al., 2008). NSAIDs can treat only mild and moderate pain, and in addition, there are a number of adverse effects associated with their use, but most common are dyspepsia, an increased risk of gastric ulcer, and increased risk of myocardial infarction (Naesdal & Brown, 2006).
- (b) *Conventional opioids*: Prescription opioids (e.g., oxycodone) have high affinity for the  $\mu$ -opioid receptor and are commonly used to treat moderate-to-severe pain (Schmidt-Hansen et al., 2018). Opioid pain relievers are generally safe when taken for a short time and as prescribed by a doctor. However, in the past 20 years, there has been a dramatic increase in the use of prescription opioids for the treatment of chronic, noncancer pain, such as back pain or osteoarthritis, despite serious risks and the lack of evidence about their long-term effectiveness (Chou et al., 2016; Morlion et al., 2018). Prescription opioids are also euphorogenic agents, which is the main factor responsible for the growth of opioid abuse (Cicero & Ellis, 2017). The growth in opioid analgesics prescriptions led to increased availability, which in turn increased the adverse effects related to opioid-based drugs, addiction, and overdose deaths (Gasior et al., 2016). Currently, our nation is experiencing a crisis in opioid addiction not seen in decades. According to the Center for Disease Control and Prevention, on average, 115 Americans die every day from an opioid overdose (CDC, 2017). In 2016, the number of overdose deaths involving opioids was five times higher than in 1999, including both prescription and illicit opioids (Chronic Pain and High-Impact Chronic Pain among U.S. Adults, 2019, 2020).
- (c) Other nonopiod drug classes that are used in pain management (e.g., antidepressants) (Linton & Bergbom, 2011) are not commonly used due to relatively poor efficacy when compared with opioid or NSAIDs analgesics and due to the potential for serious side effects.

## Soluble epoxide hydrolase (sEH) inhibitors

Endogenous epoxy-fatty acids (EpFAs) are one of the metabolic derivatives of arachidonic acids (Spector et al., 2004) (Fig. 2) and exhibit vasodilatory effects in various arteries and have also been shown to possess analgesic and antiinflammatory properties (Nithipatikom & Gross, 2010). However, EpFAs are rapidly metabolized by soluble epoxide hydrolase (sEH) to generate less bioactive and pro-inflammatory dihydroxyeicosatrienoic acids (DHETs). sEH is a ubiquitous enzyme that has been detected in many organs and tissues, including the liver, kidneys, lungs, and vascular tissues



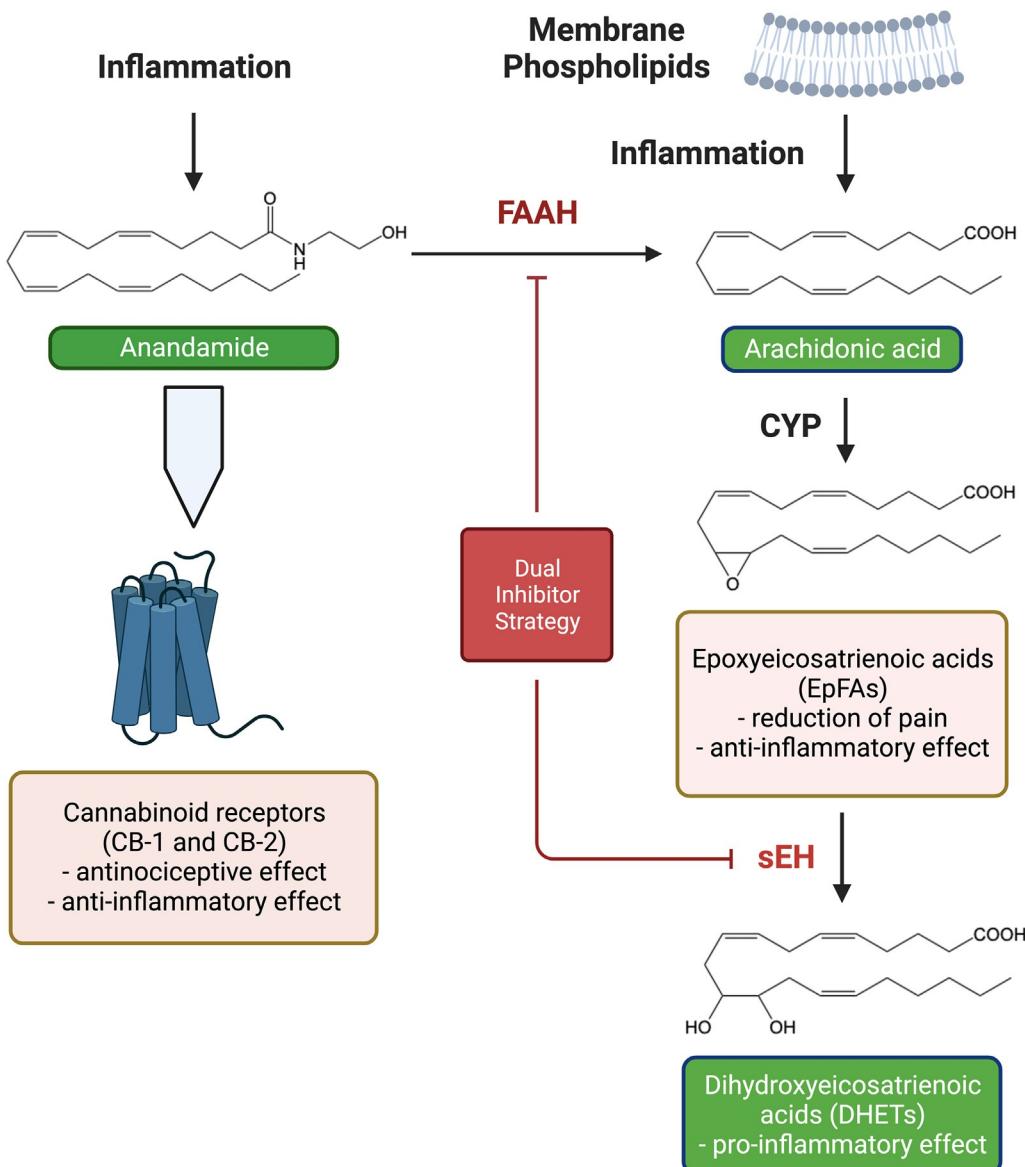
**FIG. 1** Synthesis of prostaglandins from fatty acids. During inflammation, arachidonic acid is released from plasma membrane by enzyme phospholipase A<sub>2</sub> and converted to different inflammatory mediators. Cyclic endoperoxides (e.g. prostaglandins PGG<sub>2</sub> and PGH<sub>2</sub>) are formed by oxidation and cyclization reactions initiated by an enzyme from the endoplasmic reticulum, cyclooxygenase (COX). Prostaglandins are potent mediators of inflammation. Nonsteroidal inflammatory drugs inhibit COX enzymes.

(Inceoglu et al., 2013). Arachidonic acid is metabolized through three major enzymatic pathways: COX, lipoxygenase (LOX), and cytochrome P450 (CYP) pathways (Imig & Hammock, 2009; Yang et al., 2013). It had been shown that inhibition of the enzyme sEH causes an increase in EpFAs concentration, which has beneficial therapeutic effects on pain, inflammation, and could be utilized in various cardiovascular diseases.

Initially, a large body of work has focused on a class of urea-based piperidine inhibitors for sEH (e.g., AUDA and TPPU) shown in Fig. 3 (Imig & Hammock, 2009). Numerous structure-activity relationship (SAR) studies led to the discovery of several active sEH inhibitors with IC<sub>50</sub>s in the low nanomolar range (Huang et al., 2010; Xie et al., 2009). Systemic administration of sEH inhibitors alleviated inflammatory pain and was more efficacious compared with the NSAID celecoxib (Hwang et al., 2011). Further, systemic administration of the sEH inhibitor *t*-TUCB (Fig. 3) produced antinociception that is equivalent to that produced by gabapentin without behaviorally disruptive side effects (Lee et al., 2014). These data indicate that sEH inhibitors produce antinociception against inflammatory and neuropathic pain in the absence of disruptive side effects, which highlight the utility of targeting sEH for pain reduction.

## Fatty acid amide hydrolase (FAAH) inhibitors

There are three known classes of cannabinoids: phytocannabinoids, synthetic cannabinoids, and endocannabinoids. Direct cannabinoid receptor agonists such as phytocannabinoid Δ<sup>9</sup>-tetrahydrocannabinol (THC) alleviate pain, but also produce sedation and prevent the restoration of function in humans (Pertwee, 1988). The endogenous cannabinoid system mainly functions through the cannabinoid type-1 (CB<sub>1</sub>) and type-2 (CB<sub>2</sub>) receptors, which are activated by the two endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol. One approach to increase concentrations of endogenous cannabinoids is to pharmacologically inhibit the enzymes that facilitate their hydrolysis. Fatty acid amide hydrolase (FAAH) is the enzyme responsible for regulating AEA (Fig. 2). The pharmacological inactivation of FAAH produces analgesic, antiinflammatory, anxiolytic, and mild antidepressant effects without the undesirable side effects of direct cannabinoid receptor agonists (Ahn, Johnson, & Cravatt, 2009). Inhibition of FAAH has produced antinociception without disruptive side effects in several rodent models of inflammatory pain (Ahn, Johnson, Mileni, et al., 2009; Cravatt et al., 2004).

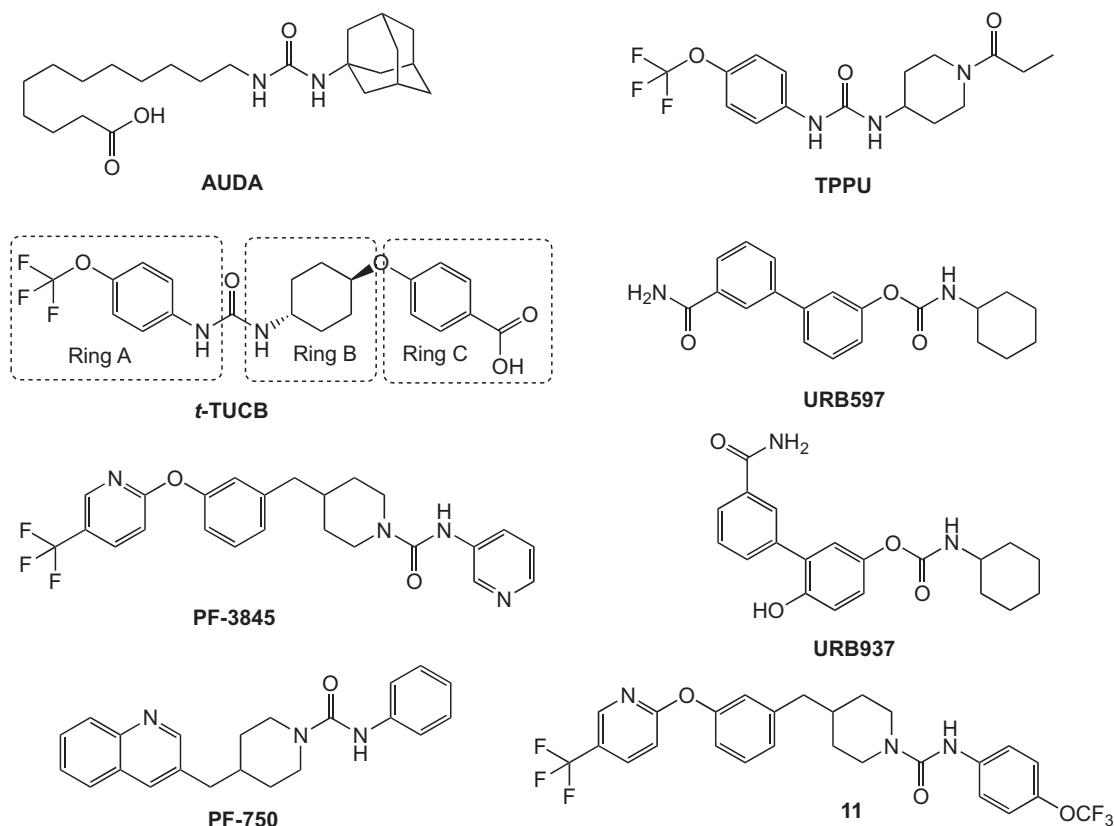


**FIG. 2** Metabolic pathways of fatty acid amide hydrolase and soluble epoxide hydrolase enzymes. Endocannabinoid anandamide (AEA) is released during inflammation and binds to cannabinoid receptors. AEA possesses antinociceptive and antiinflammatory effects. It is rapidly degraded by enzyme fatty acid amide hydrolase (FAAH) into arachidonic acid. Epoxyeicosatrienoic acids (EpFAs) are derivatives of arachidonic acid and exhibit analgesic and antiinflammatory properties. The enzyme soluble epoxide hydrolase mediates the addition of water to EpFAs, converting them to the corresponding diols, dihydroxyeicosatrienoic acids (DHETs), which show diminished biological activity. Dual, simultaneous inhibition of FAAH and sEH represents novel approach in the treatment of pain and inflammation.

Several classes of FAAH inhibitors have been previously reported with high inhibitory potency. Detailed SAR study led to discovery of the carbamate-based inhibitor URB597 and urea-based piperidine inhibitor PF-3845 (Fig. 3) (Seierstad & Breitenbacher, 2008; Wang et al., 2009).

## Simultaneous inhibition of sEH and FAAH has synergistic effects on pain

Recent evidence suggests that the antinociceptive effects of sEH inhibitors can be further enhanced when combined with other enzyme inhibitors including FAAH. Combinations of the sEH inhibitor TPPU and the FAAH inhibitor URB937 (Fig. 3) produced robust antinociception against carrageenan-induced acute inflammatory pain (Sasso et al., 2015). This suggests that there may be functional cross talk between endogenous cannabinoids and EpFAs in the alleviation of pain.



**FIG. 3** Known sEH, FAAH, and dual sEH/FAAH inhibitors. Structures of urea-based sEH inhibitors AUDA, TPPU, and *t*-TUCB. Structures of known FAAH inhibitors URB 597 and PF-3845 and PF-750. Structure-activity relationship studies performed by Kodani et al. (Kodani, Bhakta, et al., 2018; Kodani, Wan, et al., 2018) on *t*-TUCB yielded the first dual sEH/FAAH inhibitor 11 (modifications are done on sites labeled rings A, B, and C).

(Wang et al., 2021). Although the mechanism of synergy between sEH and FAAH is poorly understood, antinociceptive efficacy may be mediated through epoxy-fatty ethanolamides (EpFEAs). EpFEAs are CB<sub>2</sub> receptor agonists that are metabolized by both FAAH and sEH (McDougle et al., 2017; Snider et al., 2010), and the antinociceptive effects of sEH inhibitors alone are blocked by CB<sub>2</sub> antagonists (Wagner et al., 2011).

## Designed multiple ligands

Multitargeting compounds designed to enhance efficacy are known as Designed Multiple Ligands (DMLs) and are of particular interest in multifactorial diseases, such as cancer, chronic pain, inflammation, and neurodegenerative diseases (Bajda et al., 2011; Morphy & Rankovic, 2005; O'Boyle & Meegan, 2011). There are many advantages to develop DMLs (also known as polypharmacology), including a potential for higher efficacy and fewer side effects compared with cocktail drugs (Proschak et al., 2019). First, potential drug-drug interactions will be avoided; namely two drugs, which are safe when given independently of each other, cannot be assumed to be safe in combination. In addition, two drugs used together could produce highly unpredictable pharmacokinetic/pharmacodynamic (PK/PD) relationships, which could in turn increase the cost of both preclinical and clinical studies. Next, polypharmacology approach will decrease the time to determine the dosing for in vivo studies. Finally, the DMLs could provide potential synergism and more robust antinociceptive effect since both inhibited enzymes are involved in pain and inflammation pathways (Anighoro et al., 2014; Lillich et al., 2021).

Proschak et al. (2019) classified three types of DMLs according to their pharmacophoric structures: linked, fused, and merged pharmacophores. Linked DMLs are designed by simple linking (anchoring) two individual pharmacophores via linking group. Fused DMLs are similar, just the pharmacophores are connected directly, without a linking group. Both linked and fused types of multitarget compounds usually have molecular weights above 500 and increased lipophilicity, which are important in drug design and could lead to poor solubility and poor permeability according to Lipinski Rule of Five (Lipinski, 2000). However, these two types of conjugated pharmacophores are a valuable tool in the early SAR studies and the discovery of the multitarget activity. Merged pharmacophores are of the greatest interest in the multitarget drug

discovery. Here, the key pharmacophoric elements required to interact with each target of interest are combined (merged) into one single pharmacophore.

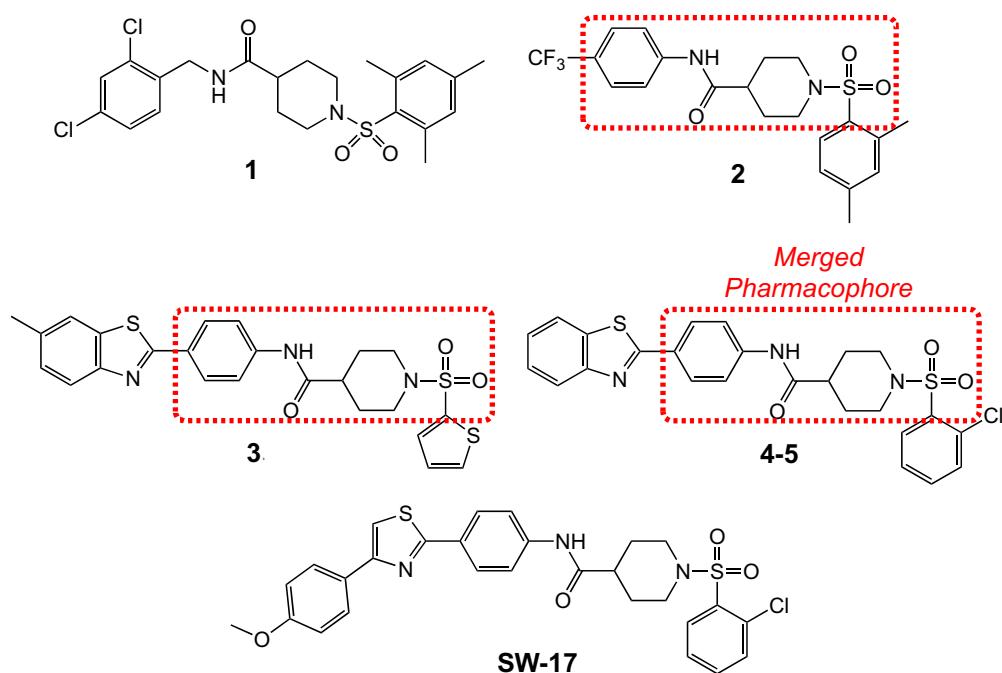
## Dual sEH/FAAH inhibitors

Given that inhibition at each enzyme has analgesic effect individually, and co-administration of sEH and FAAH inhibitors resulted in a significant synergistic reduction in pain behavior in animal models of pain, one logical medicinal chemistry approach is to design a DML, a single small molecule that could simultaneously target the two aforementioned enzymes.

The first study of dual inhibition of sEH and FAAH as targets for the polypharmacological approach for pain treatment was investigated in 2018 by Kodani et al. (Kodani, Bhakta, et al., 2018; Kodani, Wan, et al., 2018). Their design of dual sEH/FAAH inhibitors started with a potent sEH inhibitor *trans*-4-[4-(3-trifluoromethoxyphenyl-1-ureido)-cyclohexyloxy]-benzoic acid, *t*-TUCB ( $IC_{50} = 0.4\text{ nM}$ ), after they discovered that this compound possesses a moderate inhibitory potency for FAAH ( $IC_{50} = 260\text{ nM}$ ). Previously was demonstrated an excellent efficacy of *t*-TUCB in different models (Guedes et al., 2013; Wang et al., 2012), but was never investigated if this was due to its polypharmacology. In order to improve potency of *t*-TUCB for FAAH, Kodani et al. designed three libraries of analogs and explored the SARs of rings A, B, and C (Fig. 3). They reported several important observations: trifluoromethoxy group on the aromatic moiety on the ring A is important for the FAAH potency; substituting the cyclohexane ring (ring B) with aromatic moiety has no effect on the potency, while changing it to the piperidine moiety led to significant decrease in potency for FAAH. Further structural modifications of the ring C led to the discovery of the dual sEH/FAAH inhibitor with the activity against both enzymes in the nanomolar range. In addition, the most potent dual inhibitors discovered in this study seem to be reversible inhibitors of FAAH, since the potency did not change with time. The most potent FAAH inhibitors reported (e.g., URB597) are irreversible FAAH inhibitors (Ahn, Johnson, & Cravatt, 2009). It has to be noted that the FAAH inhibition of the newly discovered dual inhibitors was limited to human enzyme. The potency against FAAH enzymes from other species (mouse, rat, cat, and dog) was significantly lower. However, the dual inhibitors showed high selectivity toward FAAH, since they didn't inhibit any related enzymes, such as serine hydrolases and carboxylesterases.

In another study, in order to develop a dual sEH/FAAH inhibitor suitable for use in rodent model, Kodani et al. started with an urea group, a common pharmacophoric moiety present individually in both sEH and FAAH inhibitors (Kodani, Wan, et al., 2018). It has been reported previously that urea moiety binds within the catalytic pocket of sEH in the proximity of the key amino acid residues that are involved in the degradation of EpFAs and forms hydrogen bonds (Imig & Hammock, 2009). Urea-based FAAH inhibitors are also previously described in literature, and urea moiety forms a covalent bond with the serine residue located in the catalytic site of FAAH (Palermo et al., 2011). They selected a potent urea-based FAAH inhibitor, PF-750 ( $IC_{50} = 6.4\text{ nM}$ ), which was previously successfully used in several animal models of pain (Johnson et al., 2011). In addition, this commercially available FAAH inhibitor showed a moderate potency for sEH with an  $IC_{50}$  of  $360\text{ nM}$ . The detailed SAR showed that the introduction of the 4-trifluoromethoxy aniline moiety, typically present in the potent sEH inhibitors, led to a discovery of the potent dual sEH/FAAH inhibitor, **11** (Fig. 3) with activities for both sEH and FAAH enzymes in low nanomolar range, with  $IC_{50}$ s of  $5\text{ nM}$  and  $8\text{ nM}$ , respectively. Further evaluation revealed that all newly discovered dual inhibitors were less potent on rat and mouse FAAH enzyme. Similarly, the reduced potency for sEH in both rat and mouse enzymes was observed. However, **11** showed better potency on rodent sEH enzyme than the standard TPPU. The authors also performed several pharmacokinetic analyses in mice and rats in order to determine the potential use of the newly described dual inhibitors in *in vivo* rodent models. Dual inhibitor **11** had the good half-life of  $7.8\text{ h}$  and reached moderate blood concentration, but had poor water solubility. However, it had high selectivity for FAAH compared with other serin hydrolases and represents a promising candidate for studying dual sEH/FAAH inhibition in rodent pain models.

Wilt et al. explored amide-piperidine analogs as dual sEH/FAAH inhibitors (Wilt, Kodani, et al., 2020). They employed a DML strategy wherein pharmacophoric fragments from potent sEH and FAAH inhibitors were chemically joined to obtain dual inhibitors. Using high-throughput screening (HTS), Xie et al. identified a potent sEH inhibitor **1** (Fig. 4)—the derivative of isonipeptic acid (Xie et al., 2009). Next, Pecic et al. performed follow-up SAR studies and were able to successfully co-crystallize one of the nonurea inhibitors with human sEH (Pecic et al., 2012, 2013). Careful examination of the human sEH binding pocket revealed that the amide moiety of the crystallized sEH inhibitor is positioned in the same orientation as the urea group in urea sEH inhibitors, where the amide moiety, instead of urea group, is involved in hydrogen bonding with tyrosine and aspartic acid residues in the catalytic pocket of sEH. Using information obtained from SAR studies in combination with molecular modeling and crystallography data, they were able to determine a particular pharmacophore for this series of sEH inhibitors, e.g. inhibitor **2**, with pharmacophore shown in red (Fig. 4) required to inhibit the sEH enzyme. These data guided the follow-up SAR studies, and their synthetic efforts yielded



**FIG. 4** Research strategy that yielded merged sEH/FAAH dual pharmacophore. Structures of known nonurea piperidine-based sEH inhibitors 1 and 2, and known piperidine-based FAAH inhibitor 3. Dual sEH/FAAH inhibitors 4–5 discovered by [Wilt et al. \(2021\)](#). Dual (merged) pharmacophore is shown in red dotted box.

several inhibitors with efficacy in the picomolar range. However, screening of these inhibitors in liver microsomal stability assays revealed that sEH inhibitors with cycloalkyl substituents on the left side of the piperidine scaffold have poor metabolic profile, i.e., the most potent sEH inhibitor has a half-life of only 2.4 min. In order to improve the metabolic stability, they used a well-known medicinal chemistry approach—the introduction of isosteric substituents. Ten different analogs were synthesized by replacing hydrogens in various positions with fluorine or deuterium atoms, and it was observed a significant improvement in liver microsomes half-lives ( $LM_{1/2}$ ) and apparent intrinsic clearance ( $CL_{int}$ ), while the inhibition profile was kept in the same range as the lead compound ([Pecic et al., 2018](#)).

Wang et al. performed HTS and identified benzothiazole analog 3 (Fig. 4) as a potent FAAH inhibitor ([Wang et al., 2009](#)). Follow-up SAR studies indicated that the sulfonamide group, the piperidine ring, and benzothiazole were key components to their activity. Time-dependent preincubation study of compound 3 was consistent with it being a reversible inhibitor. Further biological evaluation of 3 in rat tissues revealed that it had exceptional selectivity and no off-target activity with respect to other serine hydrolases. In addition, the modeling study also indicated that hydrophobic interactions of the benzothiazole ring with the active site contributed to its extraordinary potency.

Since, the potent FAAH inhibitor 3 contains two key regions: a hydrophobic benzothiazole moiety on the left side connected to a central amide-piperidine core, Wilt et al. noticed that both sEH and FAAH inhibitors share structural similarities and selected benzothiazole moiety-amide-piperidine as a central core to design dual inhibitors. The rationale for this study was based on the fact that benzothiazole ring will contribute to the extraordinary inhibitory potency for FAAH enzyme, whereas previous SAR studies on sEH enzyme demonstrated that bulky, hydrophobic groups are well tolerated on the left side and will give potent sEH inhibition. This approach led to design of the dual inhibitors, and synthetic efforts yielded potent dual inhibitors of human sEH and FAAH enzymes in the low nanomolar range, with compound 4–5 (Fig. 4) being the most potent. SAR study revealed that halogens (fluoro-, chloro-, and bromo-) and methyl-groups, placed at the *ortho* and at both *ortho/para* positions, are all well tolerated in the human FAAH and human sEH enzymes leading to low nanomolar inhibition potencies on both enzymes. However, modifications of only *para* position with fluoro-, chloro-, bromo-, and methyl groups led to a loss of potency for FAAH, suggesting that the benefit from *ortho*- substitutions is greater than the loss from the *para*- substitutions. They proposed that pharmacophore for dual inhibition must possess three key regions: (a) a bulky hydrophobic/aryl group on the left side (benzothiazole ring), connected to (b) a central amide-piperidine moiety, which connects with the sulfonamide bond to (c) an aromatic ring. In the next, follow-up work, Wilt et al. conducted a systematic SAR study in order to determine the importance of the benzothiazole moiety for the dual sEH/FAAH inhibition

and identify suitable groups for attachment to the central pharmacophore core of **4–5** (Fig. 4—shown in red). Previously, in a separate study, Wilt et al. were able to utilize the 4-phenylthiazole moiety and were able to incorporate it in several potent FAAH inhibitors (Wilt, Rodriguez, et al., 2020). To explore the importance of the benzothiazole functionality, they kept the 2-chlorophenyl group connected to the sulfonamide bond of the pharmacophore and synthesized 16 analogs with various groups on the left side of the molecule (Fig. 4). The simplification strategy where the benzothiazole part was replaced with smaller groups led to complete loss of inhibition potency at the human sEH enzyme but led to moderate inhibition potency on the human FAAH enzyme. However, the introduction of the 4-phenylthiazole moiety on the left side led to excellent nanomolar potency with both enzymes, e.g., SW-17 showed inhibitory potency  $IC_{50}$  of 11.1 nM with FAAH and  $IC_{50}$  of 2.3 nM with sEH (Wilt et al., 2021). Next, the most potent dual sEH/FAAH inhibitor identified in their previous study, **4–5**, was used to demonstrate antinociception in a rat model of acute inflammatory pain (Wilt et al., 2021).

Demonstrating antinociception of the dual inhibitor **4–5** provided the first evidence that a dual sEH/FAAH inhibitor alleviates acute inflammatory pain induced by an intraplantar injection of dilute formalin (Wilt et al., 2021). The impact of these findings is that: (1) dual inhibitors are potent analgesics against acute pain; (2) dual inhibitors produce antinociception at lower doses than traditional NSAIDs; and (3) dual inhibitors are metabolically stable following systemic administration. Further, dual inhibitors only inhibit the inflammatory phase of the Formalin Test (Wilt et al., 2021), suggesting that the inhibitors interfere with the development and maintenance of inflammation. If this is true, then the utility of dual sEH/FAAH inhibitors may extend to many different types of pain increasing their therapeutic value.

Of course, candidate analgesics need to be tested extensively in rodent models of pain prior to drawing significant conclusions about the magnitude and duration of their analgesic effects. The last section will describe various preclinical pain tests that can be conducted to better understand the therapeutic potential of dual sEH/FAAH inhibitors.

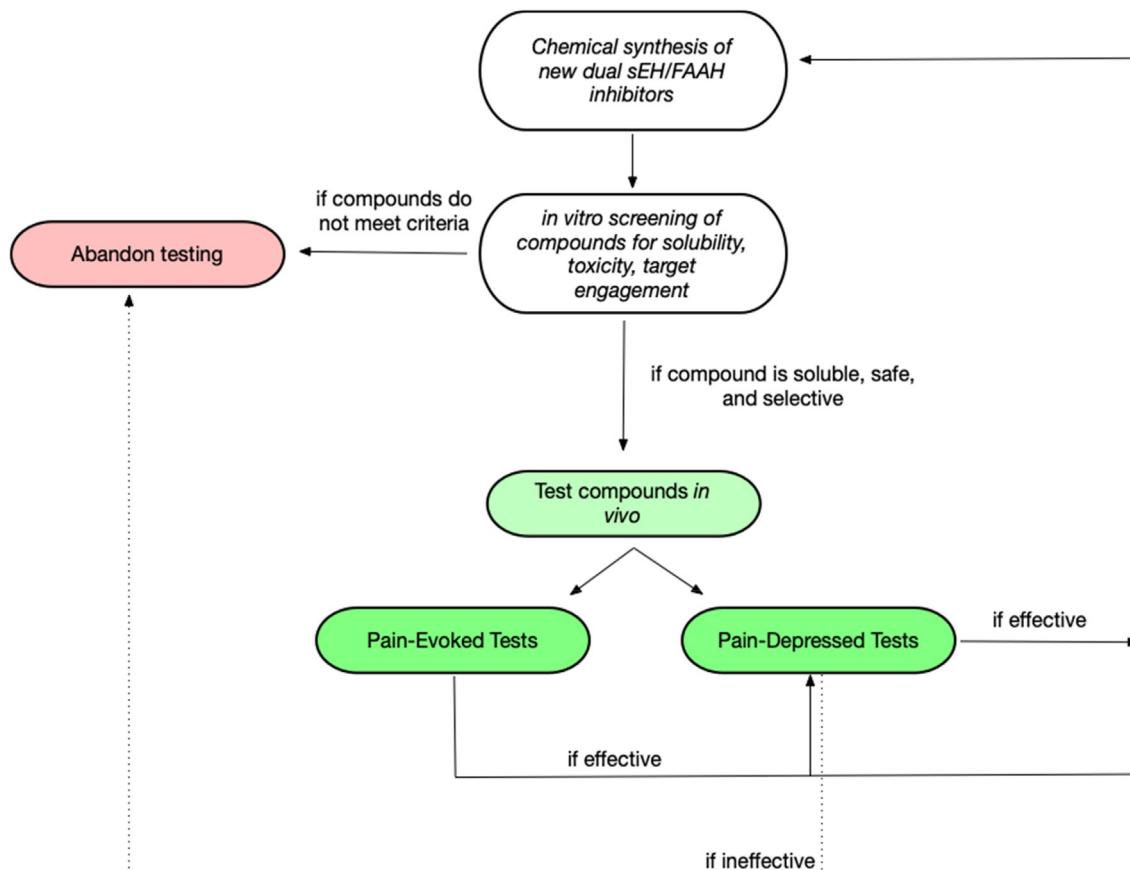
## Antinociceptive assessment of dual inhibitors

The discovery of new and effective dual sEH/FAAH inhibitors requires iterative synthesis and simultaneous behavioral testing (Fig. 5). Pain tests in rodents falls under two broad categories: (1) pain-evoked tests and (2) pain-depressed tests. Although there are differences in the stimuli and behavioral outcomes of these two tests, both categories of tests provide useful information that can be used to further refine development of dual sEH/FAAH inhibitors.

### Pain-evoked tests

The most commonly used measures to evaluate the magnitude and duration of pain are described as “pain-evoked behaviors.” Pain-evoked behaviors are defined as behaviors that increase in frequency, rate, duration, or intensity in response to a pain state (Negus, 2013; Negus et al., 2010). Examples of pain-evoked behaviors include withdrawal responses from innocuous mechanical or noxious thermal stimuli (e.g., paw withdrawal) or stretching/flinching responses to noxious stimuli (e.g., abdominal stretching elicited by intraperitoneal injection of acetic acid or flinching, licking, and guarding responses elicited by intraplantar injection of formalin) (Negus et al., 2010). There are several practical and ethical benefits to pain-evoked tests. Pain-evoked tests are beneficial in that they can quickly detect the antinociceptive potential of a candidate analgesic without long-lasting damage to the animal. Further, certain tests can be used several times in the same rat to generate cumulative dose-response curves eliminating the need to test doses in multiple different groups of rats (Darlington et al., 2012). As such, pain-evoked behaviors are an excellent starting point for testing dual sEH/FAAH inhibitors; however, exclusive reliance on these measures will not help advance development of the inhibitor.

The sole use of pain-evoked behaviors is dangerous because these measures do not account for the entire pain experience. The first problem with this is that discerning pain relief from motor impairment (e.g., sedation) is not possible (Kwilasz et al., 2014; Kwilasz & Negus, 2012). For example, sedating drugs may produce false positives and overestimate the antinociceptive properties of certain doses or inhibitors. That is, high doses of anesthetic will produce the same result as an effective analgesic on a pain-evoked test. The second problem is that pain-evoked behaviors do not typically correspond to the human pain experience (Negus et al., 2010). In other words, human patients typically do not complain of hypersensitivity to a mechanical or thermal stimulus, although this is the primary dependent measure used in animal studies. The third problem is that certain pain-evoked tests (e.g., tail flick and paw pressure) require the animal to be gently restrained for periods of time. This, in turn, can cause changes in nociception due to stress (Pilcher & Browne, 1983; Porro & Carli, 1988). This concept of stress-induced analgesia may confound the findings of certain preclinical pain studies. Lastly, most pain-evoked behaviors cannot be measured using automated equipment (Naesdal & Brown, 2006). Several pain tests require subjective evaluations (i.e., determining whether the rat flinched its paw). These subjective evaluations increase variability



**FIG. 5** Proposed preclinical testing paradigm to evaluate the analgesic potential of dual sEH/FAAH inhibitors. Following in vitro screening of new dual inhibitors, in vivo pain testing must occur. Pain evoked tests are advantageous in that they are quick and provide valuable data in terms of dose and timing. If a dual inhibitor produces a positive result on a pain-evoked test, this must be verified using a pain-depressed test (e.g., pain-depressed wheel running). A dual inhibitor that produces a positive result obtained on a pain-depressed test can be developed further. An iterative cycle of in vitro and in vivo testing will reveal the most potent dual inhibitors.

and decrease confidence in the findings. Maintaining the objectiveness of preclinical pain tests is important yet is a challenge with some pain-evoked tests.

In conclusion, many different doses and dose combinations can be tested quickly in rodents using pain-evoked tests. Importantly, initial dose exploration studies can be performed quickly to identify target dose ranges for further testing. However, exclusively relying on pain-evoked tests to reveal the therapeutic potential of dual sEH/FAAH inhibitors is not recommended due to the many problems of pain-evoked tests. These problems can largely be solved by using assays of pain-depressed, as opposed to pain-evoked, behavioral tests.

### Pain-depressed behaviors

Pain-depressed behaviors are defined as any behavior that decreases in frequency, rate, duration, or intensity in response to a noxious stimulus or pain state (Negus et al., 2010). Assessing pain-depressed behaviors provides several advantages. First, administration of analgesic compounds *increases* pain-depressed behaviors. For example, a high dose of morphine produces pain relief but will not restore a behavior depressed by pain due to its sedative effects. However, a moderate dose of morphine will provide pain relief and restore behaviors depressed by pain because there is likely a balance between antinociceptive efficacy and limited side effects. Thus, pain-depressed behaviors can help dissociate doses of drugs that produce pain relief and restore normal activity. Second, persistent pain states in humans present as a depression of behavior (Negus et al., 2010). A primary diagnostic criterion of pain is the inability to resume normal life activities due to pain as opposed to increased sensitivity to a particular stimulus such as radiant heat. Thus, the clinical relevance between pain-depressed tests and the human phenomenon is increased. Third, many pain-depressed behaviors can be assessed using automated equipment, which decreases experimenter bias (Negus et al., 2006). Data from certain types of pain-depressed

behaviors such as pain-depressed wheel running can be automatically captured using a computerized running wheel ([Kandasamy & Morgan, 2021](#)). Thus, the subjectivity of pain tests is eliminated. Lastly, some pain-depressed behavioral tests can be conducted during the animal's active phase and in the animal's home cage, which can be considered a stress-free environment ([Kandasamy et al., 2016](#)). Thus, the stress associated with behavioral testing can be eliminated when using certain pain-depressed behavioral tests.

Evaluating the analgesic potential of dual sEH/FAAH inhibitors using pain-depressed behavioral tests will allow for the objective measurement of pain relief. These approaches can be used after systemic, intrathecal, intraplantar, or topical administration. Importantly, assessing pain-depressed behaviors will also reveal the doses that produce pain relief and restore depressed behaviors versus doses that may not restore depressed behaviors. Thus, a positive result on a pain-evoked test may not automatically suggest that a dual inhibitor produces safe and effective analgesia. However, combining tests of pain-evoked and pain-depressed behaviors increases the likelihood of identifying a drug or dose of drug that produces safe and effective analgesia in preclinical studies ([Fig. 5](#)). Lastly, this approach must be iterative. Although this approach can be applied to virtually any pain condition and any analgesic, including dual sEH/FAAH inhibitors, it is important to revisit the screening and *in vitro* testing processes after results from pain-evoked and pain-depressed behavioral tests have been obtained. Rigorous *in vitro* and *in vivo* testing is required to identify the best dual sEH/FAAH inhibitors for human use.

## Conclusion

The development of effective nonopioid-based pharmacotherapies to treat pain and inflammation without severe side effects would be a significant scientific accomplishment. Polypharmacology has emerged as a new medicinal chemistry tool, and over the last few years, several drugs have been successfully identified using this approach providing a strong rationale for using the multitarget drug design concept in multifactorial diseases, such as cancer, chronic pain, and neurodegenerative diseases. Targeting sEH and FAAH enzymes with a single small molecule could be a beneficial and efficient strategy for the nonopioid pain management. Lastly, rigorous and iterative behavioral testing using both pain-evoked and pain-depressed behavioral tests will be required to unveil the therapeutic potential of dual sEH/FAAH inhibitors.

## Applications to other areas

In this chapter, we have reviewed the chemical and pharmacological effects of dual sEH/FAAH inhibitors. We have also described several preclinical pain testing approaches to ensure that the development of dual inhibitors is an iterative cycle that builds on both *in vitro* and *in vivo* findings. To our knowledge, we are at the forefront of dual sEH/FAAH inhibitors for the treatment of pain. Inhibitors of sEH and FAAH have been individually investigated as potential therapeutics in many diseases including cardiovascular, pulmonary, Alzheimer's, and Parkinson's disease. Dual inhibitors of both sEH and FAAH will be very useful to further develop as novel therapeutics for the diseases. The synthesis, screening, and development of dual sEH/FAAH inhibitors will also reveal compounds that may potently inhibit one enzyme over the other. If this is the case, then these drugs can also be investigated as candidate therapies for a variety of diseases. This exploration is especially promising in that a portfolio of new ligands will be identified that may be innovated as new therapies. Although the initial focus is to identify nonopioid treatments for chronic pain, this exploration has applications to several other areas opening the doors for further exploration of dual inhibitors for other disorders beyond chronic pain.

## Mini-dictionary of terms

- **Designed multiple ligands:** Drugs that act simultaneously at multiple different targets.
- **Pain-depressed behavior:** A behavior that decreases in frequency, rate, duration, or intensity following a pain stimulus.
- **Pain-evoked behavior:** A behavior that increases in frequency, rate, duration, or intensity following a pain stimulus.
- **Polypharmacology:** The design of drugs that act simultaneously at multiple different targets.
- **Structure-activity relationship:** The relationship between a drug's chemical structure and its pharmacological activity.

## Key facts of chronic pain

- Pain is defined an unpleasant sensory and emotional experience.
- Chronic pain is the number one cause of disability worldwide.
- Women suffered disproportionately more from chronic pain compared with men.
- Opioid analgesics are the gold standard to treat moderate-to-severe chronic pain.
- Pharmacological therapies for chronic pain must alleviate pain and restore normal activity.

## Summary points

- Few novel treatments for pain have been recently developed.
- Inhibition of soluble epoxide hydrolase (sEH) produces pain relief.
- Inhibition of fatty acid amide hydrolase (FAAH) produces pain relief without the adverse effects of direct cannabinoid agonists.
- Polypharmacology can be used to design, synthesize, and evaluate a completely novel class of drugs that modulate both sEH and FAAH simultaneously.
- The therapeutic potential of dual sEH/FAAH inhibitors can be further rigorously evaluated in vitro and in vivo using clinically relevant preclinical pain tests.

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## Chapter 14

# WWL70 targets the link between 2-arachidonoylglycerol and prostanoid pathways

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### Abbreviations

AA	arachidonic acid
2-AG	2-arachidonoylglycerol
ABHD6	$\alpha/\beta$ -hydrolase domain containing 6
ABPP	activity-based protein profiling
AEA	anandamide
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arg-1	arginase-1
BAL	bronchoalveolar lavage
BBB	blood-brain barrier
BMP	bis(monoacylglycero)phosphate
eCB	endocannabinoid
CB1/CB2 receptor	cannabinoid type 1/2 receptor
CCL2	chemokine (C-C motif) ligand2
COX	cyclooxygenase
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DSE/DSI	depolarization-induced suppression of excitation/inhibition
EAE	experimental autoimmune encephalopathy
ERK	extracellular-signal-regulated kinase
FAAH	fatty acid amide hydrolase
GABA <sub>A</sub> receptor	$\gamma$ -aminobutyric acid type A receptor
GFAP	glial fibrillary acidic protein
GSIS	glucose-induced insulin secretion
IBA1	ionized calcium binding adapter molecule1
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
LTD	long-term depression
MAG	monoacylglycerol
MAGL	monoacylglycerol lipase
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein-1
NF- $\kappa$ B	nuclear factor kappa B
NGF	nerve growth factor
NSAID	nonsteroidal antiinflammatory drug
PDI	postday of injury
PG	prostaglandin
PG-EA	prostaglandin ethanolamide
PG-G	prostaglandin glycerol ester

<b>PGES</b>	PGE <sub>2</sub> synthase
<b>PTZ</b>	pentylenetetrazole
<b>STZ</b>	streptozotocin
<b>TBI</b>	traumatic brain injury
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor- $\alpha$
<b>UCP-1</b>	uncoupling protein-1

## Introduction

A large number of literature has demonstrated that endocannabinoids (eCBs) can modulate neuronal activities to control homeostatic balance such as emotion (Mechoulam & Parker, 2013), pain (Starowicz & Finn, 2017), and energy expenditure (Silvestri & Di Marzo, 2013). The eCB system is also involved in the modulation of pathophysiological states in various diseases. This system comprises the lipid ligands, mainly 2-arachidonoyl glycerol (2-AG) and anandamide (AEA), the cannabinoid type 1 and type 2 (CB1 and CB2) receptors, and the biosynthetic and degradative enzymes of these endogenous lipids (Lu & Mackie, 2016). The eCB ligands can be hydrolyzed to produce arachidonic acid (AA) or oxygenated to form prostaglandin glycerol esters (PG-Gs) or prostaglandin ethanolamides (PG-EAs) (Duggan et al., 2011). Thus, the eCB metabolism and eicosanoid signaling are tightly intertwined. WWL70 was originally developed as a specific inhibitor of the 2-AG hydrolyzing enzyme,  $\alpha/\beta$ -hydrolase domain containing 6 (ABHD6) (Li et al., 2007). WWL70 has been demonstrated to possess potent therapeutic efficacies in several pathological conditions and animal models of diseases. In this chapter, we summarize experimental evidence unraveling that WWL70 not only acts as an inhibitor of 2-AG hydrolysis catalyzed by ABHD6 but also as a blocker of the PG biosynthesis.

## Enzymatic properties of ABHD6

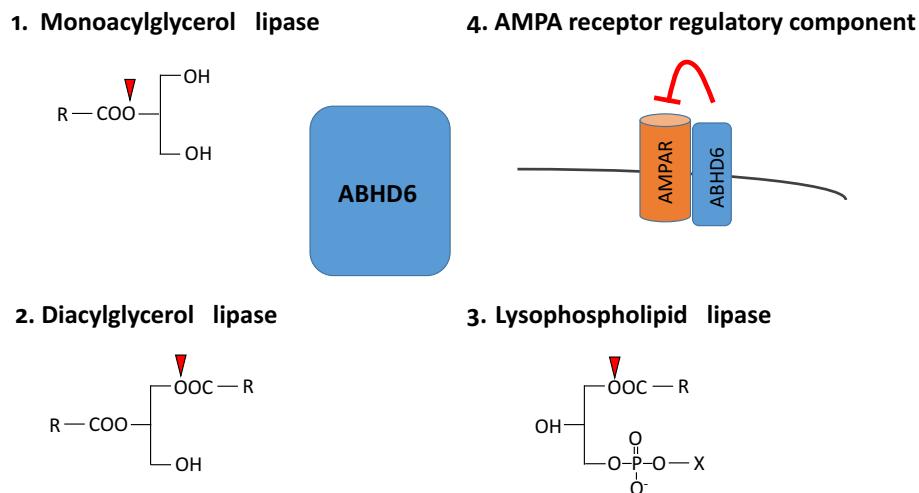
2-AG is one of the major and critical lipid species in the eCB signaling. Soon after its discovery, 2-AG was found to be biosynthesized “on demand” in the postsynaptic domain and to activate CB1 receptor expressed on the presynaptic membrane (Alger & Kim, 2011). This receptor activation modulates neurotransmission and synapse plasticity including depolarization-induced suppression of inhibition (DSI) or depolarization-induced suppression of excitation (DSE) (Lu & Mackie, 2016). Accordingly, 2-AG metabolism is tightly regulated by several enzymes of its biosynthesis and degradation. 2-AG is synthesized by diacylglycerol lipase (DAGL)  $\alpha$  and  $\beta$  and hydrolyzed primarily by monoacylglycerol lipase (MAGL) (Alger & Kim, 2011). However, other enzymes including fatty acid amide hydrolase (FAAH), carboxylesterases, microsomal epoxide hydrolase, neuropathy target esterase, ABHD12, ABHD16A, and ABHD6 are also involved in the hydrolysis of 2-AG in either physiological or pathological conditions (Blankman et al., 2007; Marrs et al., 2010).

ABHD6 is a 38 kDa integral membrane protein that belongs to the serine hydrolase superfamily. This family member has the canonical  $\alpha/\beta$  hydrolase fold structure with a catalytic triad composed of three amino acids S148-D278-H306 in ABHD6 (Navia-Paldanis et al., 2012). Besides its expression in brain, ABHD6 is ubiquitously expressed in peripheral organs including the kidney, spleen, liver, small intestine, testis, and islets (Drehmer et al., 2019; Zhao et al., 2014). In high-fat diet mice, this gene expression was found to increase specifically in the kidney, small intestine, and liver (Grabner et al., 2019; Thomas et al., 2013). The increased expression of ABHD6 was also observed in European patients with systemic lupus erythematosus (Oparina et al., 2015). In association with this female-dominant disease, ABHD6 expression was significantly upregulated by female hormones, estrogen or progesterone in human leukocytes specifically derived from female but not male (Drehmer et al., 2019). In the brain, ABHD6 is known to contribute to a minor portion of the total 2-AG hydrolytic activity (Blankman et al., 2007), however, due to its localization in the postsynaptic compartment where 2-AG is synthesized, it is believed to be crucial to modulate long-term synaptic activity and plasticity (Jung et al., 2012; Kiritoshi et al., 2016; Marrs et al., 2010). For instance, ABHD6 can modulate the activity of  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub> receptor) that is localized in the postsynaptic membrane (Naydenov et al., 2014). However, the short-term plasticity, DSI, and DSE were not affected (Marrs et al., 2010; Straker & Mackie, 2009). ABHD6 was recently reported to be a component of the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor and affects the translocation and assembly of the receptor independent of the hydrolytic activity (Schwenk et al., 2019; Wei et al., 2016). It was also reported that the AMPA receptor-mediated currents and the surface expression were negatively regulated by ABHD6 (Wei et al., 2017).

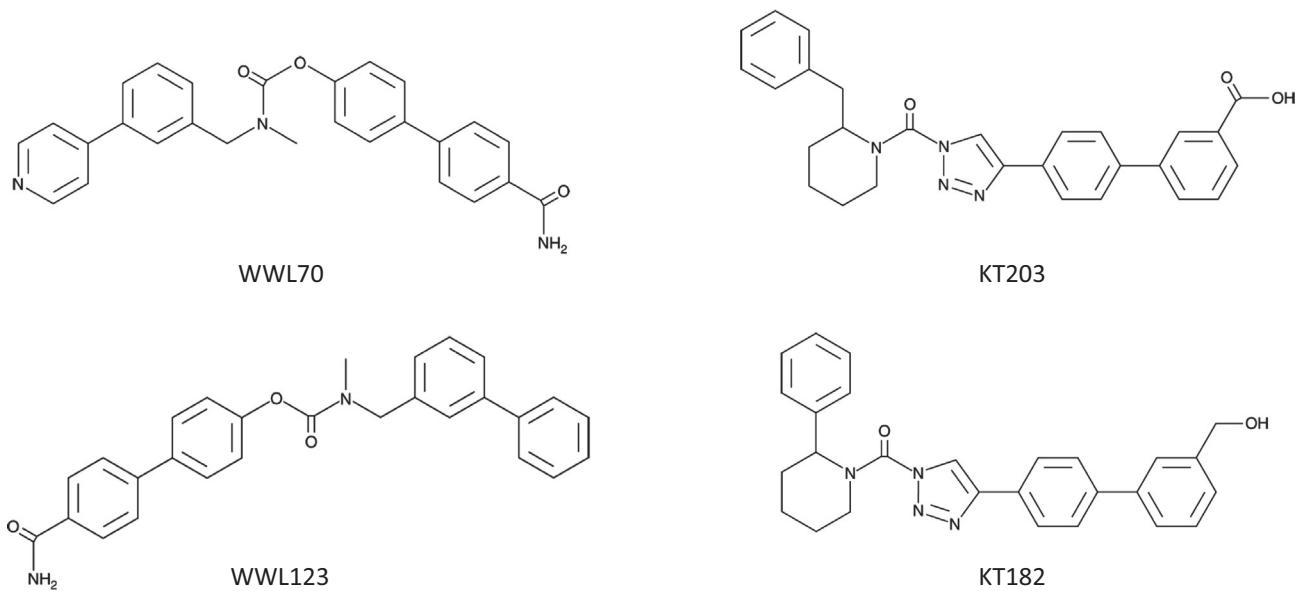
Characterization of ABHD6 enzymology based on glycerol production indicated that the enzyme catalyzes preferably 1-AG, a stereoisomer that also has biological activity, compared with 2-AG (Navia-Paldanis et al., 2012). ABHD6 prefers middle length acyl chain glycerol (C14 to C8) rather than longer acyl chain such as C16, C18, or arachidonoyl chain (C20) in vitro (Navia-Paldanis et al., 2012). With the preference of these substrates, saturated 1-monoacyl chain glycerol (1-MAG) (C12:0, C14:0, C16:0, and C18:0) was significantly increased (Zhao et al., 2014) while the corresponding saturated fatty acids (C16:0, C18:0) were reduced by ABHD6 inhibition in beta cells (Zhao et al., 2014). A recent study reported that knockdown of the 2-AG biosynthetic enzymes DAGL $\alpha$  and DAGL $\beta$  in Neuro2a neuroblastoma cells did not reduce 2-AG levels. Conversely, lipidomic analysis of ABHD6 overexpressing cells showed reduced DAG levels but not 2-AG. Rather, 2-AG levels were slightly increased. In addition, overexpression of wild-type ABHD6 but not mutant ABHD6 showed the DAG lipase activity. Therefore, ABHD6 may play a role in 2-AG biosynthesis, which might contribute to neuronal differentiation (van Esbroeck et al., 2019). Furthermore, ABHD6 has another type of substrate specificity (Thomas et al., 2013). ABHD6 knockdown in mouse liver elevated the levels of several phospholipids, lysophospholipids, and ether-linked glycerophospholipids under high-fat diet. Consistently, in vitro recombinant ABHD6 demonstrated the lipase activity for lysophospholipids such as lysophosphatidylglycerol with high affinity (Thomas et al., 2013). In line with these findings, ABHD6 inhibition increased the levels of lysophospholipid species in mouse lungs after LPS instillation (Bottemanne et al., 2019). ABHD6 can also degrade bis(monoacylglycerol)phosphate (BMP) that is important for the formation of intraluminal vesicles in the late endosomes or lysosomes (Pribasník et al., 2015). The enzyme activity of BMP hydrolysis was distinctly reduced in liver tissue of the ABHD6 knockout mice, whereas several plasma BMP levels were significantly increased in the knockout mice (Grabner et al., 2019). The degradation activity and  $K_m$  for BMP were comparable to those for MAG suggesting that ABHD6 is an important enzyme for the endosomal lipid catabolism and sorting (Pribasník et al., 2015). These studies demonstrate that ABHD6 has a broad substrate specificity and different catalytic activities and a nonenzymatic function (Fig. 1).

## Therapeutic efficacy of WWL70

Given that inhibition of the 2-AG hydrolytic enzymes enhances eCB signaling, which possibly leads to beneficial outcomes in various diseases, several inhibitors of ABHD6 have been developed. To date, there are mainly two structurally distinct ABHD6 inhibitors based on the derivatives of triazole urea and carbamate (Bononi et al., 2021). Fig. 2 shows the ABHD6 inhibitors that have been utilized in several animal disease models. WWL70 and WWL123 are carbamate derivatives, while KT182 and KT203 are piperidyl-1,2,3-triazole urea derivatives. WWL70 was originally reported to be a ABHD6 inhibitor using the membrane proteome of the ABHD6 overexpressing cells (Li et al., 2007). Activity-based protein profiling (ABPP) was performed to characterize the inhibitory profile to the serine hydrolases expressed in the membrane, which showed that in addition to ABHD6, WWL70 also partially inhibits acetylcholinesterase, FAAH, ABHD10, and ABHD16A (Li et al., 2007). A recent study showed that WWL70 can inhibit FAAH in the avian macrophage cells ( $IC_{50}$ : 0.3  $\mu$ M)



**FIG. 1** Functions of ABHD6. ABHD6 was originally identified as a monoacylglycerol (MAG) lipase to hydrolyze 2-AG. Recently, it has been shown to function as diacylglycerol (DAG) lipase and lysophospholipids lipase. In addition, ABHD6 is a component of AMPA receptor complex to negatively regulate the transport and synaptic current. Cleavage sites are shown as arrow heads. R: acyl chain, X: head group, i.e., serine, glycerol, ethanolamine.



**FIG. 2** Chemical structure of ABHD6 inhibitors. WWL70 and WWL123 are carbamate derivative, while KT182 and KT203 are urea-based chemicals. These inhibitors were investigated for therapeutic efficacy in several animal models described in [table 1](#).

**TABLE 1** Summary of pathophysiological effects of the ABHD6 inhibitors in animal disease models.

ABHD6 inhibitors	Animal models	Symptomatic changes	Pathophysiological changes	eCB related results	References
WWL70	TBI (controlled cortical impact)	1. Improved motor function and working memory	2. Reduced lesion volume, 3. Reduced neurodegeneration, 4. Attenuated BBB breakdown, 5. Reduced inflammatory genes, i.e., iNOS, COX-2, 6. Increased phospho-ERK, phospho-AKT, Arg-1	Increased CB1 and CB2	<a href="#">Tchantchou and Zhang (2013)</a>
WWL70	Multiple sclerosis (EAE)	1. Reduced clinical score	2. Reduced CB2, 3. Reduced F4/80, CD4, iNOS, COX-2, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , NF- $\kappa$ B, 4. Attenuated demyelination	1. Reversed by CB2 antagonist, 2. No effects in CB2 KO	<a href="#">Wen et al. (2015)</a>
WWL70	Neuropathic pain (chronic constriction injury)	1. Attenuated thermal hyperalgesia, 2. Attenuated tactile allodynia on hind paw	1. Reduced microglia/macrophage markers Iba1 and F4/80, 2. Reduced astrocyte marker GFAP, 3. Reduced CCL2, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , NGF, NF- $\kappa$ B, 4. No change in 2-AG and AA levels	Not reversed by CB1 or CB2 antagonists	<a href="#">Wen et al. (2018)</a>

**TABLE 1** Summary of pathophysiological effects of the ABHD6 inhibitors in animal disease models—cont'd

ABHD6 inhibitors	Animal models	Symptomatic changes	Pathophysiological changes	eCB related results	References
WWL70	Acute lung inflammation	<ol style="list-style-type: none"> <li>1. Reduced recruitment of leukocytes, macrophages, neutrophils, lymphocytes</li> </ol>	<ol style="list-style-type: none"> <li>2. Reduced lung capillary leak,</li> <li>3. Reduced KC, CCL2, IL-1<math>\beta</math>, IL-6, TNF-<math>\alpha</math>, MIP2<math>\alpha</math>,</li> <li>4. Increased LysoPI in BAL</li> </ol>	Increased 2-AG in lung,	Bottemanne et al. (2019)
WWL70	Systemic anaphylaxis	<ol style="list-style-type: none"> <li>1. Reduced spleen weight,</li> <li>2. No change in locomotor function</li> </ol>	1. Reduced IL-1 $\beta$ , IL-6	2-AG increased in lung but not in cerebellum	Alhouayek et al. (2013)
WWL70	High-fat diet induced diabetes	<ol style="list-style-type: none"> <li>1. Body weight loss,</li> <li>2. Reduced white adipose,</li> <li>3. Reduced plasma glucose tolerance,</li> <li>4. No change triglyceride,</li> <li>5. Reduced food intake after 4 h fasting</li> </ol>	1. Increased browning and thermogenesis related genes (i.e., UCP1) in white adipose		Thomas et al. (2013), Zhao et al. (2016), Fisette et al. (2016)
WWL70	Diabetes model (STZ)	<ol style="list-style-type: none"> <li>1. Increased GSIS in STZ mice and WT mice,</li> <li>2. Reduced glycemia after glucose infusion in STZ mice,</li> <li>3. No WWL70 effects on GSIS in ABHD6 KO</li> </ol>	<ol style="list-style-type: none"> <li>1. Reduced free fatty acids in islets,</li> <li>2. Increased MAGs in islets</li> </ol>		Zhao et al. (2014)
WWL123	Seizure (PTZ) and spontaneous model	<ol style="list-style-type: none"> <li>1. Reduced seizure episodes (global tonic-clonic and myoclonic seizures),</li> <li>2. Reduced episode in spontaneous seizure</li> </ol>		Not reversed in CB1 or CB2 KO mice	Naydenov et al. (2014)
KT182	Multiple sclerosis (Cuprizone)		<ol style="list-style-type: none"> <li>1. Reduced CD11b, GFAP, NG2 positive cells,</li> <li>2. Improved demyelination,</li> <li>3. No change MBP levels</li> </ol>		Manterola, Bernal-Chico, Cipriani, Canedo-Antelo, et al. (2018)
KT182 and KT203	Multiple sclerosis (EAE)	<ol style="list-style-type: none"> <li>1. Decreased clinical score from PDI11 to 30 in KT182, not KT203,</li> <li>2. No change in corticospinal conduction latency</li> </ol>	<ol style="list-style-type: none"> <li>1. No change in spinal cord white matter,</li> <li>2. No change in CD11b, Iba1, COX-2, TNF-<math>\alpha</math>, IL-1<math>\beta</math></li> </ol>	KT182 chronic treatment reduced CB1 functionality	Manterola, Bernal-Chico, Cipriani, Ruiz, et al. (2018)

(Lee et al., 2018). Thus, the therapeutic effect of WWL70 in several disease models may not be solely due to its inhibition of ABHD6.

### **Role of WWL70 in anaphylaxis**

Using an acute systemic anaphylaxis mouse model induced by intraperitoneal administration of lipopolysaccharide (LPS) as well as the LPS-induced macrophage cell lines in vitro, Muccioli group showed potent antiinflammatory effects of WWL70 (Alhouayek et al., 2013). IL-1 $\beta$  and IL-6 were drastically reduced in the LPS treated cells and brain tissues after WWL70 treatment. The authors stated that the antiinflammatory effects of WWL70 were due to the production of PGD<sub>2</sub>-G because of its antiinflammatory property; however, the production of PGD<sub>2</sub>-G was not measured in the presence of WWL70 (Alhouayek et al., 2013). In the subsequent paper, the effect of WWL70 was investigated using the acute lung injury mouse model and alveolar macrophages by administration of LPS (Bottemanne et al., 2019). In the mouse lung and bronchoalveolar lavage fluid, instillation of LPS increased the expression of several inflammatory marker genes such as IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and the recruitment of leukocytes, neutrophils, and macrophages. These acute inflammatory responses were significantly reduced by WWL70 treatment. In addition, WWL70 significantly increased the levels of lysophosphatidylglycerol and lysophosphatidylethanolamine in the lung but not in bronchoalveolar lavage fluid. Several lysophospholipids such as lysophosphatidylserine, lysophosphatidylethanolamine were shown to attenuate IL-1 $\beta$  and TNF- $\alpha$  expression in primary alveolar macrophages treated with LPS (Bottemanne et al., 2019).

### **Role of WWL70 in traumatic brain injury**

Traumatic brain injury (TBI) is caused by external forces that injure brain tissues, nerve fibers, and microvascular structure and functions. Since TBI is one of the most heterogeneous neurological disorders, fairly complicated pathological features and outcomes are observed (Saatman et al., 2008). In addition to the primary injury, secondary injury contributes significantly to the development of TBI. Growing evidence points to the subsequent neuroinflammation that can severely deteriorate the neuronal activity and homeostasis (Johnson et al., 2013). Unfortunately, there are still no effective medications available for TBI patients. Recently, a substantial number of studies indicated that modulation of the endocannabinoid system could be one of the promising treatments (Schurman & Lichtman, 2017). Using the controlled cortical impact TBI mouse model (Tchantchou & Zhang, 2013), we found that treatment with WWL70 attenuated motor dysfunction assessed by rotarod test and beam walk test. Learning and memory deficits in TBI animals were also alleviated by WWL70 treatment. The ipsilateral cortical lesion volume and neuronal loss in dentate gyrus of the TBI mouse brain were reduced by WWL70 in CB receptors, particularly in a CB1 receptor-dependent manner. Interestingly, the protein expression of CB1 and CB2 receptors increased following WWL70 treatment. Compromised blood-brain barrier integrity in TBI was restored by WWL70. Quantitative RT-PCR, Western blotting, and immunohistochemistry demonstrated that the increased expression of the inflammatory genes, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in the TBI cortex was returned to basal levels with WWL70 treatment. Under neuroinflammatory conditions such as TBI, microglia and macrophages shift the intracellular environments from homeostatic state (M0) to M1 state that triggers the expression of pro-inflammatory molecules and phagocytosis (Tanaka et al., 2020). We found that WWL70 shifted the metabolic and genetic states from the M1 state to the M2 alternative state in which genes for tissue remodeling or inflammation resolving are expressed. For instance, arginase-1, a commonly used M2 marker, was increased by WWL70 treatment. These results demonstrated that WWL70 has antiinflammatory and regenerative properties in the TBI mouse model (Tchantchou & Zhang, 2013).

### **Role of WWL70 in multiple sclerosis**

Experimental autoimmune encephalopathy (EAE) is a common animal model of multiple sclerosis. EAE model is created by immunization of oligodendrocyte membrane proteins, which is relevant to pathophysiological feature of multiple sclerosis since injury to oligodendrocyte by the immune cell attack is thought to be an important pathology (Procaccini et al., 2015). In the EAE model, we found that ABHD6 gene expression was elevated by more than twofold (Wen et al., 2015). Systemic injections of WWL70 after disease onset significantly increased the 2-AG levels, along with 10% reduction of the total 2-AG hydrolytic enzyme activity in the spinal cord. Measurement by ABPP indicated that ABHD6 was not completely blocked in the WWL70-treated EAE group. Immune cell markers, such as F4/80 for microglia/macrophages and CD4 $^{+}$  T-cells, were distinctly reduced by WWL70, which was reversed by the CB2 receptor antagonist AM630, but not the CB1 receptor antagonist AM281. Together with the fact that the CB2 receptor expression was increased in EAE, activation

of CB2 receptor is believed to be essential for the therapeutic efficacy of WWL70 and other cannabinoid agonists as reported by previous studies (Kong et al., 2014; Lou et al., 2011). Consistent with the reduced myelination and axonal injury, the expression of inflammatory genes such as COX-2, iNOS, TNF- $\alpha$ , and the expression of activated leukocyte cell adhesion molecule and NF- $\kappa$ B were distinctly reduced after the drug treatment. Moreover, WWL70 treatment did not affect EAE-induced neuroinflammation and demyelination in the CB2 receptor knockout mice. Taken together, our study demonstrated that the therapeutic effects of WWL70 were mediated mainly by 2-AG mediated CB2 receptor signaling (Wen et al., 2015).

### **Role of WWL70 in neuropathic pain**

We also investigated the WWL70 effects in a neuropathic pain model induced by chronic constriction injury of the mouse sciatic nerve (Challa, 2015). WWL70 was systemically administered to the animals, and the allodynic behaviors and the biomarker expressions were examined (Wen et al., 2018). Injury-stimulated thermal and mechanical allodynia was alleviated by the treatment. The therapeutic effect of WWL70 was independent on the activation of CB1 and CB2 receptors since co-administration of the cannabinoid receptor antagonists did not affect the analgesic behaviors. WWL70 did not alter 2-AG and arachidonic acid levels in the injured sciatic nerve, suggesting that the therapeutic effects of WWL70 may not be mediated by enhancement of the eCB signaling. The pathological analyses in the WWL70-treated animals showed the reduction of both the microglia marker, ionized calcium binding adapter molecule1 (IBA1), and the activated astrocyte marker, glial fibrillary acidic protein (GFAP) in the spinal cord dorsal horn. The expression of inflammatory cytokines chemokine (C-C motif) ligand 2 (CCL2), IL-1 $\beta$ , and IL-6 were decreased in the ipsilateral sciatic nerve and the lumbar dorsal root ganglion. NF- $\kappa$ B, the transcription factor that regulates the inflammatory genes, was also reversed by WWL70 treatment in the sciatic nerve and dorsal root ganglion. In addition, the expression of COX-2, PGE<sub>2</sub> synthase 2 (PGES2), and the production of PGE<sub>2</sub> were attenuated by WWL70. Thus, in the sciatic nerve neuropathic pain model, WWL70 treatment alleviated thermal and mechanical allodynia, along with reversed gene expression of several inflammatory markers and PGE<sub>2</sub> synthesis independent of the CB receptor activation (Wen et al., 2018).

### **Role of WWL70 in metabolic disorders**

It is well known that the eCB system plays a crucial role in energy homeostasis. In the previous clinical trial, treatment with a CB1 receptor antagonist such as rimonabant led to a loss of body weight (Silvestri & Di Marzo, 2013). Both ABHD6 and the CB receptors are expressed in the peripheral nerve system including the enteric nerve ending such as small intestine and the brown adipose (Thomas et al., 2013). Several studies have shown that pharmacological and genetic manipulation of ABHD6 causes dramatic alterations in organismal energy homeostasis. High-fat diet increases the risk for metabolic disorders such as diabetes. Thomas et al. reported that the ABHD6 knockdown in peripheral tissues including the liver and white adipose but not in the brain protected against high-fat diet-induced hyperglycemia, hyperinsulinemia, and obesity. However, the involvement of the eCB system seemed to be unlikely (Thomas et al., 2013). Systemic administration of WWL70 resulted in loss of body weight and white adipose tissues, and an improvement of glucose intolerance in mice fed with high-fat diet. Overall treatment with WWL70 and ABHD6 knockdown produced similar outcomes, though the ABHD6 knockdown but not WWL70-treated animals showed significantly reduced hepatic triglyceride levels. In addition, since hepatic steatosis was improved only by ABHD6 knockdown but not by WWL70 inhibition, the therapeutic mechanism of WWL70 may not be due to modulation of hepatic lipogenesis by eCB signaling (Thomas et al., 2013). Zhao et al. reported that WWL70 treatment increased glucose-induced insulin secretion (GSIS) along with increased MAG levels in beta cells (Zhao et al., 2014). In fact, 1-palmitoylglycerol enhanced the GSIS in islets, suggesting that ABHD6 inhibition by WWL70 increased MAG to enhance insulin secretion. Interestingly, overexpression or knockdown of MAGL did not alter GSIS, suggesting that the glucose tolerance was specific for ABHD6. Under the mild diabetic conditions, both ABHD6 knockout and WWL70-treated mice showed consistent improvements of GSIS and glucose tolerance along with an increase in MAG levels in islets (Zhao et al., 2014). Subsequent study indicated that the antiobesity effects of ABHD6 inhibition are mediated by inducing the adipose tissue browning. It was found that WWL70 treatment upregulated UCP1 and genes related to adipose browning in adipocytes, which was consistent with the results by 1-MAG (i.e., 1-oleoylglycerol) treatment or ABHD6 knockout (Zhao et al., 2016). Thus, these studies indicated that the therapeutic effects of WWL70 on high-fat diet-induced diabetes are mediated by inhibition of the MAG lipase activity of ABHD6 to increase 1-MAG levels, which in turn induce GSIS and adipose tissue browning to increase energy expenditure.

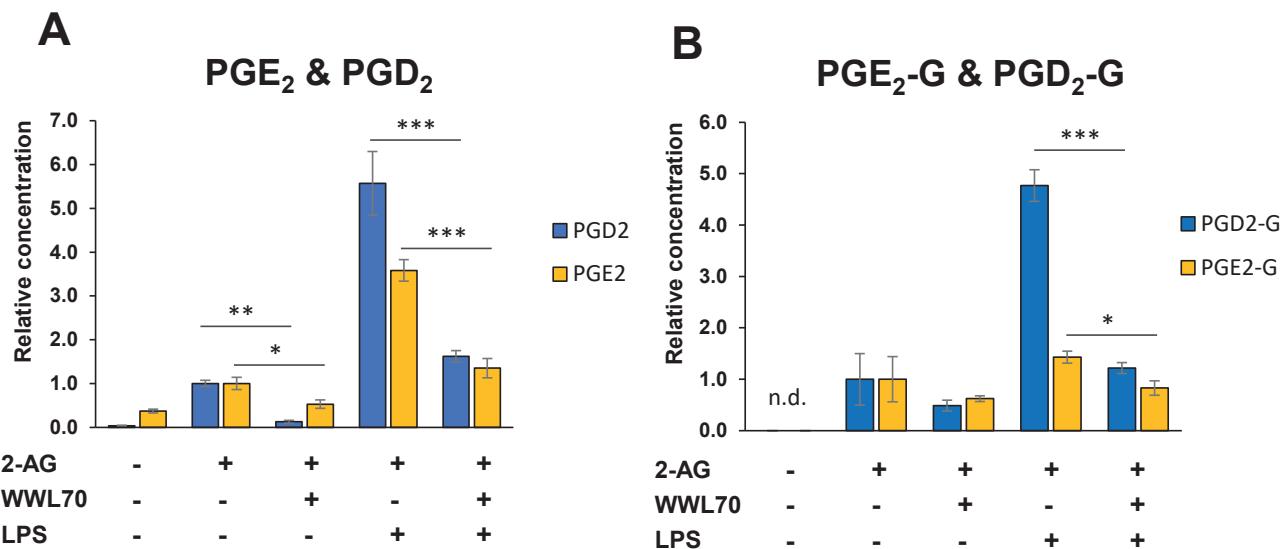
## Pharmacological characterization of WWL70

As ABHD6 is localized in postsynaptic domain, the enzymatic inhibition by WWL70 is expected to directly modulate synaptic activity through eCB signaling. Application of WWL70 in the mouse prefrontal cortex slice induced a robust long-term depression (LTD) under the subthreshold stimulatory conditions, which was mediated by CB1 receptor activation (Marrs et al., 2011). Consistently, LTD in the ventral striatum slice, which was hindered by fragile X mental retardation protein deficiency, was rescued by WWL70 application, suggesting that the lessened 2-AG at the glutamatergic synapse in the knockout mice was restored by ABHD6 inhibition (Jung et al., 2012). Together with the therapeutic efficacy in the previous section, it is clearly indicated that WWL70 can modulate synaptic plasticity through 2-AG-mediated CB receptor signaling in neurons.

WWL70 has also been shown to possess antiinflammatory effects in several disease models. To investigate if the anti-inflammatory effect was mediated by eCB signaling, we examined the inflammatory PGE<sub>2</sub> production in the presence of 2-AG and/or WWL70 in BV2 microglial cells that were known to substantially express ABHD6 (Muccioli et al., 2007). Ten μM of WWL70 completely blocked ABHD6 activity, resulting in a reduction of the total 2-AG hydrolytic activity by 40%, and the increase of 2-AG levels by 20%. Despite a marginal increase of 2-AG, LPS-induced PGE<sub>2</sub> production was abolished to the basal levels by WWL70. This PGE<sub>2</sub> reduction was not mediated by CB receptor activation due to the increase of 2-AG. Instead, addition of exogenous 2-AG increased the production of PGE<sub>2</sub>, which supports the notion that 2-AG is hydrolyzed to AA and then converted to PGE<sub>2</sub>. The production of PGE<sub>2</sub> was blocked in the presence of WWL70 due to its blockage of the 2-AG hydrolysis. Contrary to the previous premise that cytosolic phospholipase A2 activation is the dominant step for AA production, 2-AG is now known to be a significant precursor of AA and PGs synthesis. The surge of AA and PG production by LPS-induced neuroinflammation was dependent on 2-AG hydrolytic activity, as MAGL knockout mice or mice treated with MAGL inhibitors had drastically reduced AA and PGs levels (Nomura et al., 2011). This phenomenon was also observed in several peripheral tissues including the kidney, liver, spleen, and lung (Nomura et al., 2008).

Importantly, PGE<sub>2</sub> reduction by WWL70 was observed after treatment with not only 2-AG but also AA (Tanaka et al., 2017). Since reduction of AA-induced PG production was a bypass to ABHD6-mediated 2-AG hydrolysis, we used a biochemical approach to characterize the enzymatic inhibition of WWL70 using microsomal fraction of BV2 cells. Under the conditions to block endogenous production of AA, WWL70 significantly suppressed PGE<sub>2</sub> production from exogenously added AA. WWL70 was found to inhibit COX activity by measuring the total PGs production including PGH<sub>2</sub>, with an efficient inhibitory kinetics ( $IC_{50}$ : <0.5 μM). However, of note, 60% of the COX activity was still remained in the presence of 10 μM of WWL70 (Tanaka et al., 2017), suggesting that WWL70 only partially inhibited the COXs activity. Nevertheless, our results suggested that inhibition of COX activity is the principal mechanism of the antiinflammatory effects of WWL70 in BV2 cells. This notion was supported by the findings that either a selective ABHD6 inhibitor KT182 or the ABHD6 knockdown did not reduce the PGE<sub>2</sub> production. As shown in Fig. 3A, the reduction of both PGE<sub>2</sub> and PGD<sub>2</sub> further supports the notion that WWL70 inhibits COXs.

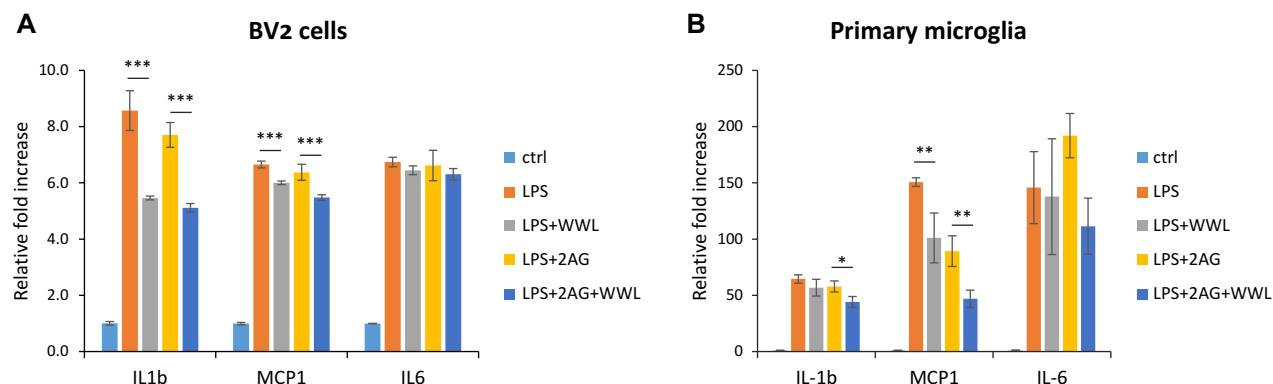
As mentioned early, COX-2 metabolizes 2-AG. Oxygenation of 2-AG is only catalyzed by COX-2 but not COX-1 (Kozak et al., 2000), possibly due to a structural difference in the catalytic site (Smith et al., 2000). The oxygenation product of 2-AG by COX-2 is PGH<sub>2</sub>-glycerol ester, which is subsequently converted to several prostaglandins-glycerol esters (PG-Gs) by the corresponding synthases. As shown in Fig. 3B, the production of both PGE<sub>2</sub>-glycerol ester (PGE<sub>2</sub>-G) and PGD<sub>2</sub>-glycerol ester (PGD<sub>2</sub>-G) in BV2 cells was significantly decreased by WWL70 in the absence or presence of LPS. These data indicate that WWL70 inhibits PG-Gs biosynthesis through COX-2 rather than COX-1 or PGs synthases, although a possibility that both PGE<sub>2</sub> and PGD<sub>2</sub> synthases are the targets of WWL70 cannot be excluded. Previous studies indicated that PG-Gs have specific biological activities; PGE<sub>2</sub>-G triggered calcium mobilization that initiates the signal transduction in macrophages (Richie-Jannetta et al., 2010) and increased excitatory synaptic activity to cause neurotoxicity (Sang et al., 2007). PGE<sub>2</sub>-G was reported to be a nociceptive molecule to induce hyperalgesia in the pain model (Hu et al., 2008). In contrast, PGD<sub>2</sub>-G showed antiinflammatory responses such as suppression of proinflammatory mediators in macrophages (Alhouayek et al., 2013) and alleviated inflammatory pain in the carrageenan model (Buisseret et al., 2019). It is likely that PG-G isomers have different biological functions as PG isomers. Although it was suggested that PG-Gs activate their own receptors, which are different from PG receptors or CB1 receptor (Hu et al., 2008; Sang et al., 2006), the receptors and the specific downstream signaling pathways have not yet been identified. Marnett group has discovered that certain nonsteroidal antiinflammatory drugs (NSAIDs), for instance, R-flurbiprofen, which was previously thought to be inactive as COX2 inhibitor, turned out to be a substrate-selective COX-2 inhibitor using 2-AG as a substrate (Duggan et al., 2011). Mice administered a substrate-selective COX2 inhibitor LM-4131 had increased 2-AG and AEA levels, whereas PGs and AA levels were not changed. The treated animals exhibited anxiolytic behaviors that were mediated by the CB1 receptor (Hermanson et al., 2013). Our laboratory investigated the therapeutic efficacy of LM-4131 in a neuropathic pain model and



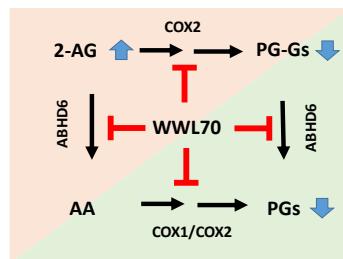
**FIG. 3** Production of PGE<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub>-G, and PGD<sub>2</sub>-G in BV2 cells. BV2 cell culture pretreated with or without 0.1 µg/ml of LPS for 8h was rinsed once with prewarmed serum-free medium (Opti-MEM, Thermo Fisher Scientific), then incubated with Opti-MEM containing 2-AG (10 µM) with or without WWL70 (10 µM) for 30 min in a CO<sub>2</sub> incubator. The medium was collected and loaded into the Solid-Phase Extraction column (Oasis HLB 1cc, Waters Corporation) to purify and concentrate the lipids. The eluted lipids fraction was applied to LC-MS/MS as described in our previous paper (Tanaka et al., 2017). Relative concentrations to the BV2 cells incubated with 2-AG are shown for PGE<sub>2</sub> and PGD<sub>2</sub> in (A) and for PGE<sub>2</sub>-G and PGD<sub>2</sub>-G in (B) ( $n=3$  or 4, represented as average  $\pm$  S.D. \*:  $P<0.05$ , \*\*:  $P<0.005$ , \*\*\*:  $P<0.001$ ). n.d. denotes not detected.

found that the mechanical allodynia in the hind paw was reduced, along with the reduction of all inflammatory markers (Jones et al., 2018). PG-Gs are further hydrolyzed to PGs. Several enzymes including 2-AG hydrolyzing enzymes were reported to catalyze PG-Gs. A recent report showed that the hydrolytic activity for PGE<sub>2</sub>-G was reduced by WWL70 to more than 50% in monocytes but not significantly reduced in neutrophils, eosinophils, and lymphocytes (Turcotte et al., 2019). In contrast, 2-AG hydrolysis was not significantly reduced by WWL70, which suggests that ABHD6 plays a major role in PG-G hydrolysis but not in 2-AG hydrolysis in monocyte. Thus, it is likely that the PG-G hydrolysis pathway, which may be catalyzed by ABHD6, is inhibited by WWL70.

It is known that PGs possess both pro- and anti-inflammatory properties that are partly due to the receptor subtype expression in the relevant tissues or cells (Ricciotti & Fitzgerald, 2011). Consistent with the studies in previous disease models, WWL70 treatment reduced the gene expression of the proinflammatory cytokines and chemokine in LPS-treated BV2 cells (Fig. 4A) and rat primary microglia (Fig. 4B). Thus, our data indicated the unexpected property of WWL70, which turns into strong antiinflammatory effects by COX inhibition. Taken together with our data and others, we proposed



**FIG. 4** Effects of WWL70 on cytokine gene expression in microglial cells. BV2 cells (A) and rat primary microglia cells (B) were incubated with WWL70 (10 µM) for 15 min, then 2-AG (10 µM) was added and incubated for 15 min. LPS (100 ng/ml for BV2, 2 ng/ml for primary microglia) was added and incubated for 16 h. The cells were collected and total RNA was isolated from the cells to perform qRT-PCR. The expression levels of each gene were determined after normalizing with GAPDH and shown as relative to the control condition ( $n = 3$ , represented as average  $\pm$  S.D. \*:  $P<0.05$ , \*\*:  $P<0.005$ , \*\*\*:  $P<0.001$ ).



**FIG. 5** Schematic diagram of inhibitory action points of WWL70. Based on previous studies, WWL70 is an inhibitor for ABHD6, which hydrolyzes 2-AG and PG-Gs to AA and PGs, respectively. In addition, WWL70 inhibits PGs and PG-Gs biosynthesis pathways, possibly including COX-2 oxygenation. Basically the upper left part (orange zone) is involved in eCB, while the lower right part (green zone) is involved in PG; however, some 2-AG metabolic enzyme(s) and PGs biosynthesis enzyme(s) are overlapped, for instance, COX-2 and ABHD6. As a result, WWL70 increases 2-AG levels but decreases levels of AA, PGs, and PG-Gs (shown with blue arrows).

that WWL70 has a strong inhibitory potential to dissociate between eCB and PG, which strengthens the eCB signaling but at the same time suppresses PGs synthesis.

## Perspectives

Studies from our laboratory and others have demonstrated that WWL70 has a strong therapeutic efficacy on a number of pathological conditions. In particular, these studies showed potent antiinflammatory effects in microglia cells and other immune cells. Our cell culture study indicated that inhibition of COXs, especially COX2, is the principal action of WWL70. Therefore, WWL70 is believed to be a dual inhibitor of ABHD6 and COX-2 to block the pathways linking the eCB and PG metabolism (Fig. 5). A recent study using the specific ABHD6 inhibitors KT182 and KT203 in the multiple sclerosis models indicated that although preventive treatment significantly alleviated the clinical scores, the therapeutic efficacy and pathological improvement were less pronounced (Manterola, Bernal-Chico, Cipriani, Canedo-Antelo, et al., 2018; Manterola, Bernal-Chico, Cipriani, Ruiz, et al., 2018), in comparison with our WWL70 study (Wen et al., 2015). Although the actions of these pharmacological agents in these model systems are unclear, it is possible that the inhibitory effect of WWL70 on COX-2 could render it more efficient to reduce the pathogenesis of multiple sclerosis. In terms of COX-2 function in the CNS, this enzyme is not just only involved in the inflammatory response by PGs biosynthesis, but also can modulate DSE in glutamatergic neurons (Straiker et al., 2011) and DSI in inhibitory synapses (Kim & Alger, 2004; Sang et al., 2006). Possibly, the synaptic activity and plasticity mediated by CB receptor or PG-Gs in neurons would be modulated by WWL70 through COX-2 inhibition, especially in the pathological conditions. Most classical NSAIDs target COXs or COX-2 selectively. Since COX-1 is constitutively expressed in various tissues to maintain physiological functions such as gastrointestinal mucosal lining and kidney function, inhibition of COX-1 therefore disturbs organ homeostasis. Conversely, COX-2 that is induced by inflammatory stimuli such as bacterial infection or tissue damage is considered an immunomodulatory factor in response to inflammation to produce PGs. Despite that COX-2 is known to play an important role in vascular homeostasis (Yu et al., 2012), the use of COX-2 inhibitors may result in a risk of hypertension, heart attack, and stroke. Therefore, it is not desirable to use the COX-2 inhibitors chronically or with a high-dose treatment (Antman et al., 2007). Our study suggested that WWL70 does not inhibit COX-2 as completely as the classical NSAIDs, but it inhibits about 50% of the COX activity. However, partial inhibition of COX-2 might be more beneficial and can be used sustainably because of its avoidance of the harmful side effects such as cardiovascular complication in the both preclinical and clinical standpoint. WWL70 could be a new tool for study the connection between eCB and PG metabolism and also a therapeutic intervention to inflammation-associated diseases.

## Summary points

- ABHD6 belongs to the serine hydrolase family and hydrolyzes monoacylglycerol including 2-AG.
- WWL70 inhibits ABHD6, which in turn increases 2-AG levels to modulate neuronal eCB signaling.
- Recently, ABHD6 is found to have other catalytic activities functioning as a lipase for diacylglycerol and lysophospholipids.
- WWL70 has been shown to have potent therapeutic efficacy for several disease models.
- WWL70 has been shown to possess strong antiinflammatory effects in immune cells.
- WWL70 blocks PG biosynthesis by inhibiting not only ABHD6 but also COX-2.

- It is proposed that the dual inhibition of WWL70 is a promising candidate for several neurological and immunological diseases.

## Key facts of ABHD6 inhibitor

- 2-AG and AEA bind and activate the CB1 and CB2 receptors and other noncanonical CB receptors. Since their downstream signaling has shown a variety of biological and bio medicinal effects, scientists hypothesized that enhancing the eCB ligand levels by blocking the degrading enzyme(s) should increase the therapeutic efficacy of eCB signaling. Moreover, it is anticipated that the increased eCB tone by inhibition of eCB degrading enzymes could be limited to the regions where eCB degrading enzymes are active. This limitation may avoid the harmful side effects due to whole body activation of eCB system. Based on the hypothesis, several research groups have been engaged in developing inhibitors for the 2-AG and AEA degrading enzymes.
- From previous proteomic analysis, ABHD6 was found to belong to the serine hydrolase family. Although it was not annotated, the enzymatic function back then, Cravatt group tried to synthesize and test several chemical compounds including WWL70 to inhibit the enzyme for the first time. Later, Stella group investigated ABHD6 and found it to hydrolyze 2-AG and to play an important role in the modulation of synaptic activity. Due to the structural similarity in the catalytic site among serine hydrolase superfamily members, many test compounds reportedly turned out to be low selectivity to the specific 2-AG degrading enzyme. However, to date, several inhibitors have been reported to possess a high selectivity to ABHD6. They are mainly derived from two different groups, triazole urea and carbamate.

## Applications to other areas

In this chapter, we have reviewed the pharmacological properties and therapeutic efficacy of WWL70. The studies from our group and others have demonstrated that WWL70 has potent antiinflammatory effects on immune cells ([Alhouayek et al., 2013](#); [Tanaka et al., 2017](#)). These properties are probably attributed by the dual inhibition to ABHD6 and COX-2. Of note, the inhibition to COX-2 was approximately 50% by WWL70, whose carbamate group is a distinct structure from the canonical NSAIDs. Therefore, it is important to further investigate the molecular mechanism of WWL70 on COX-2 inhibition. Importantly, complete inhibition of COX-2 by NSAIDs has been well known to be at risk for vascular complications such as heart attack or stroke, since COX-2 plays an indispensable role in vascular regulation ([Yu et al., 2012](#)). Therefore, chronic administration of the NSAID is not clinically recommended. Since WWL70 might circumvent the toxic side effect due to partial inhibition of COX-2, it could be applicable as an alternative NSAID for relieving pain and headache or reducing a fever. In fact, our previous study using neuropathic pain model ([Wen et al., 2018](#)) supports the idea that WWL70 has antinociceptive effect on pain ([Wen et al., 2018](#)). In future, it is important to examine the aversive effects of WWL70 in preclinical and clinical studies.

## Mini-dictionary of terms

- ABPP.** Activity-based protein profiling (ABPP) is a technology to identify proteins with certain active sites using a small molecule probe. The probe that consists of reactive group, linker site, and reporter group can bind to the target proteins and label them with a reporter group such as fluorophore. With this strategy, target proteins are able to be detected and identified using the gel-based or mass spectrometry-based platform.
- DSI (DSE).** Depolarization-induced suppression of inhibition (or excitation) is the transient suppression to inhibitory (or excitatory) presynaptic activity following the depolarization stimulus. This short-term response is mediated by retrograde signaling of eCB that is released from post synapse to presynaptic domain to activate CB1 receptor. Activation of CB1 receptor exerts suppression of the neurotransmission at the presynaptic terminal.
- Macrophage/microglia activation.** Macrophage and microglia are innate immune cells to control inflammation. Exogenous pathogen infection triggers macrophage and microglia to the M1 state and induces the classical inflammatory response such as secretion of pro-inflammatory cytokines and chemokines, recruitment to the infected areas, and release of cytotoxic substances including ROS/RNS. These responses are the processes required to defend against pathogen infection and clearance of infected host cells. After clearance of the pathogen, macrophage/microglia turn to alternative state M2 state to resolve inflammation by secreting antiinflammatory cytokines, remodeling and repairing the impaired host tissues.

- **PG.** Prostaglandins are signaling lipid molecules to activate cells expressing the PG receptors. The first step of PGs biosynthesis is catalyzed by COXs from AA. Several PG synthases that are functionally coupled to COXs subsequently catalyze the intermediate molecule ( $\text{PGH}_2$ ) generated by COXs to give rise to various PGs. Each PG has different immunological functions that are dependent on the cell types and corresponding receptors, for instance,  $\text{PGE}_2$  is involved in fever and pain sensation, and  $\text{PGI}_2$  is involved in vasodilation to prevent thrombosis.
- **Serine hydrolase superfamily.** This superfamily is one of the largest and most widely distributed enzyme classes. All serine hydrolases possess a common catalytic mechanism that hydrolyzes the substrate ester/thioester/amide bond through activation of a conserved nucleophilic serine residue. They are divided into two subfamilies: trypsin/chymotrypsin class of serine proteases, and the metabolic serine hydrolases that catalyze metabolites and peptides, including esterases, lipases, peptidases, and amidases. Most of the enzymes for eCB biosynthesis and degradation are included in this metabolic serine hydrolase subfamily.

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## Chapter 15

# Endocannabinoid catabolic enzyme inhibitors and pain alleviation

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## Abbreviations

2-AG	2-arachidonyl glycerol
AEA	anandamide
CB <sub>1/2</sub> R	cannabinoid 1 and 2 receptors
eCB	endocannabinoid
FAAH	fatty acid amide hydrolase
IASP	International Association for the Study of Pain
IP acid	intraperitoneal acid
MAGL	monoacylglycerol lipase
MOR	μ-opioid receptor
NSAID	nonsteroidal anti-inflammatory drug
THC	Δ9-tetrahydrocannabinol

## General overview of pain and current analgesics

Pain is a leading global health problem and major reason for healthcare utilization (Goldberg & McGee, 2011; St. Sauver et al., 2013). For the first time since 1979, the International Association for the Study of Pain (IASP) updated their definition of pain, describing it as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage.” Despite decades of research, the most commonly used analgesics continue to be nonsteroidal anti-inflammatory drugs (NSAIDs) and μ-opioid receptor (MOR) agonists that were introduced over a century ago (Kassin, 2010; Obeng et al., 2021). Use of these compounds is constrained by limited clinical efficacy for some pain indications and side effects that include gastric ulceration for NSAIDs and abuse liability and potentially lethal respiratory depression for MOR agonists. The opioid crisis in particular has invigorated efforts to discover new, effective, and safe medications for pain treatment (Skolnick & Volkow, 2016).

## General overview of pain-related measures in preclinical research

Experimental evaluation of pain inherently involves delivery of a noxious stimulus with the intent of producing a pain state (primary independent variable) followed by measurement of different behaviors interpreted as evidence of that pain state (dependent variable). In preclinical research, noxious stimuli are often classified as either acute, inflammatory ± evocation with an acute stimulus, neuropathic ± evocation with an acute stimulus, or a more specific disease model (e.g., for bone cancer) ± evocation with an acute stimulus (Negus, 2019). While large strides have been made in preclinical development of noxious stimuli to model clinically relevant pain indications, less progress has been made regarding pain-related behaviors, with one of the largest discrepancies between preclinical and clinical pain assessment being the type of behavioral endpoint used to indicate the presence of a pain state and impact of a drug treatment.

Clinical pain in human medicine is primarily measured via verbal reporting, whereas preclinical pain research has focused almost exclusively on reflexive withdrawal behaviors stimulated by noxious stimuli. This creates a

major discrepancy for translational research because verbal behavior cannot be measured in animals, and suppression of nocifensive withdrawal reflexes is a priority for anesthetic but not analgesic administration in humans. For example, 2016 CDC guidelines recommend the primary goal in clinical pain treatment to be decreasing pain-related functional impairments rather than solely decreasing verbally reported pain intensity (Ballantyne & Sullivan, 2015; Dowell et al., 2016; Kandasamy & Morgan, 2020; Tappe-Theodor et al., 2019). Moreover, pain is a multifaceted experience comprising sensory and affective dimensions that manifest as a constellation of unconditioned and learned behaviors in both humans and animals, with adequate assessment likely requiring evaluation of multiple behaviors relevant to different pain manifestations that are mediated by different neural circuits (Garcia-Larrea & Peyron, 2013; Negus, 2019).

Most pain-related behaviors can be classified into the two following categories: (1) pain-stimulated behaviors (often referred to as reflexive), which increase in rate, frequency, duration, or intensity following delivery of a noxious stimulus (e.g., paw- or tail-withdrawal reflexes); and (2) pain-depressed behaviors, which decrease in rate, frequency, duration, or intensity following delivery of a noxious stimulus (e.g., depression of feeding, locomotion, or operant behavior). While pain-stimulated behaviors possess some clinical face validity (e.g., removing your hand from a hot surface), they do not model the primary clinical goal for functional restoration of behavior. Moreover, pain-stimulated behaviors can be reduced by nonanalgesic drugs that impair motor function (e.g., motor sedatives) to produce false-positive effects in analgesic drug development. Conversely, pain-depressed behaviors model the primary clinical treatment goal for restoring pain-related functional impairment (e.g., alleviation of pain-related decreases in sleep, movement, work), and motor sedatives do not produce false-positive analgesia-like effects on endpoints of pain-depressed behavior (Negus, 2019).

### Pain matrix for profiling drug effects

In addition to the general categories of pain-stimulated and pain-depressed behaviors, this chapter will also use a “pain matrix” to provide a more fine-grained depiction of effects produced by different drug classes and to visualize areas requiring further research (Negus, 2019). The pain matrix classifies drug effects by the pain stimulus being delivered and the pain-related behavior it produces, with pain behaviors separated into the following three main categories: unconditioned, classically conditioned, and operant conditioned (see Tables 1–3). For unconditioned behaviors, the unconditioned stimulus (US) produces an unconditioned response (UR). This can be designated as US→UR. The pain stimulus (PS) can function either as the US that stimulates a UR such as a withdrawal reflex (PS→UR) or as a contextual stimulus that usually depresses other US→UR relationships (PS→[US→UR]). In classical conditioning, a subject learns to express a new conditioned response (CR) after repeated pairing of a US with a second stimulus, called the conditioned stimulus (CS), which initially elicits little or no behavioral response. After this US+CS pairing, the CS comes to elicit the new CR as a response. This can be designated as US+CS; CS→CR. As with unconditioned behaviors, the PS can function as the US (PS+CS; CS→CR) or as a contextual stimulus to modify classical conditioning with other stimuli (PS→[US+CS; CS→CR]). Operant conditioning is also a type of learning in which behavior is shaped by a three-term contingency, where a discriminative stimulus ( $S^D$ ) followed by a response (R) will produce delivery of a consequent stimulus ( $S^C$ ). This can be designated as  $S^D \rightarrow R \rightarrow S^C$ . The PS can function as the  $S^D$  (PS→R→ $S^C$ ), the  $S^C$  ( $S^D \rightarrow R \rightarrow PS$ ), or as a contextual stimulus that modifies operant conditioning with other stimuli (PS→[ $S^D \rightarrow R \rightarrow S^C$ ]).

Pain-stimulated behaviors are predominantly unconditioned behavioral responses to noxious stimuli serving as unconditioned stimuli (i.e. PS→UR, first column of the pain matrix exemplified by pain-stimulated withdrawal reflexes). Pain-depressed behaviors are predominantly unconditioned behaviors where the noxious stimulus is a contextual stimulus (PS→[US→UR]; second column of the pain matrix exemplified by pain-depressed locomotion in a novel environment or consumption of food). Pain-depressed behaviors can also include operant behaviors where the noxious stimulus serves either as a consequent stimulus ( $S^D \rightarrow R \rightarrow PS$ ; e.g., depression of behavior that produces pain) or a contextual stimulus (PS→[ $S^D \rightarrow R \rightarrow S^C$ ]; e.g., depression of work or other activities that produce rewards). It should be noted that the most common pain-related behavior in human medicine is verbal behavior, a type of operant behavior in which the PS serves as the  $S^D$  in operant conditioning (PS→R→ $S^C$ ), but this type of endpoint is exceedingly rare in preclinical research. Finally, care should be taken when interpreting this pain matrix table as it illustrates significant antinociceptive effectiveness but does not include data on whether antinociceptive doses also produced pain-independent side effects such as general behavioral disruption or abuse potential.

## Targeting endocannabinoid catabolic enzyme inhibitors as candidate analgesics

Neurons communicate nociceptive information from the periphery to the CNS through excitatory transmission, with clinical pain states often involving hyperactivity of these pathways (Basbaum et al., 2009; Heinricher & Fields, 2013). Cannabinoid receptors (CBRs) are widely expressed throughout the central and peripheral nervous system, including neural pathways that mediate the sensory detection of and behavioral responses to pain stimuli (Blankman & Cravatt, 2013; Reggio, 2010). The eCB system acts as an on-demand feedback-inhibition mechanism to dampen the hyperactive excitatory signaling seen in both acute and chronic pain states. More specifically, CB<sub>1/2</sub>Rs are G<sub>i/o</sub> G-protein-coupled receptors (GPCRs) that can be activated by exogenous orthosteric agonists or by endogenous lipids, with the two main endocannabinoid (eCB) lipids being 2-arachidonyl glycerol (2-AG) and anandamide (AEA) (Devane et al., 1992; Mechoulam et al., 1995). Delivery of a noxious stimulus has been shown to recruit the eCB system in the periphery, dorsal horn of the spinal cord, and neural pathways associated with acute and chronic pain states, such as the spinal-thalamic-cortical and spinal-bulbar-limbic circuits (Wilkerson et al., 2021; Woodhams et al., 2017). Accordingly, great interest has been placed on targeting the eCB system for development of candidate analgesics.

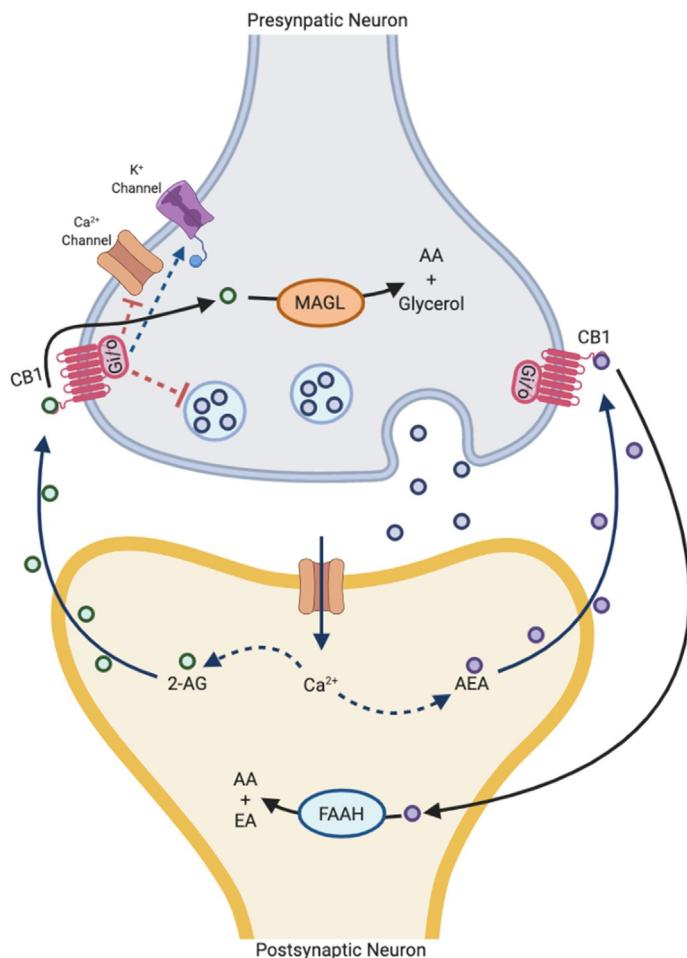
### CB<sub>1/2</sub>R agonists

Clinical evidence has shown unreliable clinical efficacy alongside unwanted psychomimetic and motor-impairing effects with CB<sub>1/2</sub>R agonists as analgesics. The IASP released a statement in 2021 that they "...do not endorse the general use of cannabinoids to treat pain" due to a lack of evidence for efficacy and safety following a systematic review of current clinical evidence by the Presidential Task Force on Cannabis and Cannabinoid Analgesia (Rice et al., 2021). A pain matrix illustrating preclinical antinociceptive effectiveness of CB<sub>1/2</sub>R agonists can be found in Negus (2019), which shows consistent antinociceptive effectiveness in assays of pain-stimulated unconditioned behaviors that are vulnerable to false effects from CB<sub>1/2</sub>R agonist-induced motor impairment (Negus, 2019). The few studies evaluating pain-depressed behaviors in other columns of the matrix usually fail to show antinociceptive effectiveness of CB<sub>1/2</sub>R agonists, suggesting improved preclinical-to-clinical translation as they more accurately reflect the weak or ineffective antinociceptive profile being reported in clinical studies. Accordingly, future research evaluating effectiveness of CB<sub>1/2</sub>R agonists should consider utilizing pain-related behaviors other than unconditioned pain-stimulated endpoints.

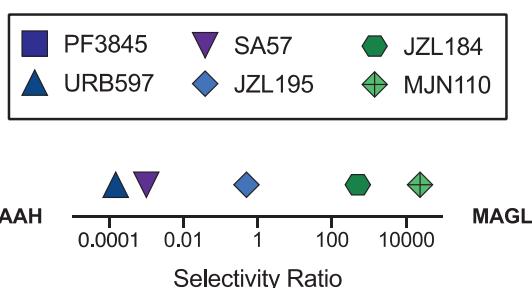
### eCB catabolic enzyme inhibitors

Instead of globally activating all CB<sub>1/2</sub>Rs throughout the central nervous system and periphery via exogenous agonists, increasing evidence suggests analgesic potential for enhanced signaling of the eCB system through inhibition of the main degradative catabolic enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) (Donvito et al., 2018; Wilkerson et al., 2021). While general function of these enzymes is described elsewhere in this textbook, Fig. 1 illustrates their role within the basic components of the eCB system at the neuronal synapse, where MAGL and FAAH act as the primary degradative enzymes for 2-AG and AEA, respectively. Compared with exogenous agonists, development of MAGL- and FAAH-selective inhibitors allows researchers increased temporal and spatial selectivity because enhanced inhibitory signaling of CB<sub>1/2</sub>Rs will only be produced where 2-AG and AEA are being synthesized. Additionally, MAGL and FAAH inhibitors possess dual inhibitory capacity for neuronal signaling and proinflammatory mediators through decreased production of arachidonic acid, suggesting analgesic potential in pain states involving inflammatory mechanisms, particularly for MAGL-selective inhibitors, as 2-AG has been shown as a major precursor of arachidonic acid in the brain (Donvito et al., 2018; Nomura et al., 2011).

Numerous inhibitors have been developed for the main eCB degradative enzymes ranging from MAGL- to FAAH-selective. Fig. 2 illustrates the selectivity of six eCB catabolic enzyme inhibitors, with selectivity calculated based on competitive-binding substrate data and activity-based protein-profiling assays. It should be noted that while selectivities for MAGL and FAAH are denoted, many eCB catabolic enzyme inhibitors also possess activity at other enzymes, particularly at higher doses. For example, the MAGL-selective inhibitor JZL184 also inhibits serine hydrolase ABHD6, an alternative 2-AG hydrolase, and the most commonly studied FAAH inhibitor URB597 also has activity at several carboxylesterases (Ahn et al., 2009; Niphakis et al., 2013). While the details for off-target effects are outside of the scope of this chapter, care should be taken when selecting which eCB catabolic enzyme inhibitor to evaluate for different pain indications.



**FIG. 1** Simplified overview of endocannabinoid system components at the neuronal synapse. Vesicular release of excitatory or inhibitory neurotransmitters from the presynaptic nerve terminal (blue) produces postsynaptic activity (yellow), leading to increased intracellular levels of  $\text{Ca}^{2+}$  and synthesis of 2-AG and AEA. These highly lipophilic molecules travel across the synapse to bind and produce retrograde signaling on the presynaptic nerve terminal via CB<sub>1</sub>Rs. Activation of these G<sub>i/o</sub>-coupled GPCRs inhibits adenylyl cyclase, enhances inwardly rectifying potassium channels, and inhibits calcium channels, effectively dampening further anterograde neurotransmitter release (Lu & Mackie, 2016; Reggio, 2010). 2-AG signaling is terminated through degradation by MAGL into AA and glycerol in the presynaptic nerve terminal, and AEA signaling is terminated through degradation by FAAH into AA and EA in the postsynaptic nerve terminal. Graphic created with BioRender.com (2021). 2-AG: 2-arachidonyl glycerol; AEA: anandamide; CB<sub>1</sub>Rs: cannabinoid 1 receptors; GPCRs: G-protein coupled receptors; MAGL: monoacylglycerol lipase; AA: arachidonic acid; FAAH: fatty acid amide hydrolase; EA: ethanolamine.



**FIG. 2** Selectivity of example test compounds ranging in selectivity for MAGL and FAAH based on competitive substrate binding and activity-based protein profiling (ABPP) assays. Data were obtained from the literature as cited below, with competitive substrate binding used for calculating selectivity if available, and ABPP if no substrate binding data were available. All data are from assays using mouse brain tissue. No data for MAGL binding could be found for either endpoint for PF3845, which is considered to be a highly selective FAAH inhibitor that would fall at the far left of the figure. As a result, no selectivity ratio could be calculated for this inhibitor. PF3845 (Ahn et al., 2009) (main paper denoting FAAH selectivity); URB597 (Kathuria et al., 2003); SA57 (Niphakis et al., 2012); JZL195 (Long, Nomura, et al., 2009); JZL184 (Long, Li, et al., 2009); MJN110 (Niphakis et al., 2013).

## Preclinical pain matrix profiles for eCB catabolic enzyme inhibitors

### MAGL-selective catabolic enzyme inhibitors

Table 1 shows the general antinociceptive effectiveness of MAGL-selective inhibitors in a preclinical pain-matrix profile. JZL184 was the most commonly evaluated compound in preclinical pain assays, followed by MJN110. While MAGL-selective inhibitors have been studied across a wide range of noxious stimuli, the dependent variable has rarely been manipulated, with studies almost exclusively measuring unconditioned paw-withdrawal endpoints as pain-stimulated behaviors. Consistent attenuation of pain-stimulated behaviors in acute, inflammatory, and neuropathic models has been demonstrated, along with efficacy in disease-state models such as cancer-induced bone pain and osteoarthritic pain (Burston et al., 2016; Thompson et al., 2020). Very few studies have evaluated pain-depressed behaviors; however, the current studies available suggest modest antinociceptive potential to alleviate pain-depressed behaviors (Diester et al., 2021; Nass et al., 2021). Regarding side effects, both JZL184 and MJN110 substitute for CB<sub>1</sub>R agonists in drug discrimination assays as a sign of abuse potential; however, MJN110 does not produce hypolocomotion, catalepsy, or hypothermia, suggesting a decreased cannabimimetic profile in comparison with CB<sub>1/2</sub>R agonists and JZL184 (Ignatowska-Jankowska et al., 2015).

Development of tolerance following persistent inhibition of MAGL with repeated JZL184 treatment has been shown to be dose-dependent and likely contingent upon inflammatory mediators recruited within different pain models (Ghosh et al., 2013; Kinsey et al., 2013; Schlosburg et al., 2010). This tolerance profile included functional antagonism of antinociceptive effects, cross-tolerance to CB<sub>1</sub>R agonists and FAAH-selective inhibitors, reduced CB<sub>1</sub>R function and expression, and increased expression of off-target eCB lipids. MJN110 has not been extensively evaluated following repeated administration, but current data in models of cancer-induced bone pain and osteoarthritic pain show sustained antinociception. Interestingly, these studies suggest opposing conclusions regarding dosing parameters, with Thompson et al. (2020) suggesting sensitization following repeated dosing and Burston et al. (2016) suggesting dose-dependent tolerance (Burston et al., 2016; Thompson et al., 2020). This differentiation is likely complex and a result of different noxious stimuli, neural circuitry, and recruitment of inflammatory mediators. Further research is needed to clarify the effects of repeated MJN110 administration across noxious stimuli and pain-related behaviors.

**TABLE 1** Preclinical pain matrix profile for MAGL-selective inhibitors.

Pain stimulus (independent variable)		Pain behavior (dependent variable)					
		Unconditioned US→UR		Classically conditioned CS+US; CS→CR		Operant conditioned S <sup>D</sup> →R→S <sup>C</sup>	
		PS→UR	PS→[US→UR]	CS+PS; CS→CR	PS→[CS+US; CS→CR]	PS→R→S <sup>C</sup>	S <sup>D</sup> →R→PS
Acute	Thermal	✓					
	Mechanical	X					
	Chemical	✓		–			
	+ Thermal	✓					
Inflammatory	+ Mechanical	✓					
	Spontaneous	✓					
	+ Thermal	✓					
Neuropathic	+ Mechanical	✓					
	Spontaneous						
	+ Thermal						
Disease model	+ Mechanical	✓					
	Spontaneous	✓		✓			

General antinociceptive effects in the current preclinical literature produced by JZL184 and MJN110 administration in different types of preclinical pain assays. Effects are categorized by the designated pain stimulus (independent variable, row titles) and the measured pain-related behavior (dependent variable, column titles). Fill color and symbol within each cell of the pain matrix indicate predominant drug effects reported in published studies: green (✓), usually effective; blue (–), drug effects mixed; red (X), drug usually ineffective; no fill indicates drug not tested.

## Dual MAGL and FAAH catabolic enzyme inhibitors

**Table 2** shows the general antinociceptive effectiveness of compounds inhibiting both MAGL and FAAH in a preclinical pain-matrix profile. JZL195 was the most commonly evaluated compound in preclinical pain assays. Similar to MAGL-selective inhibitors, the independent variable (pain stimulus) has been extensively manipulated across acute, inflammatory, neuropathic, and disease-state models such as migraine, but the pain-related behavioral endpoints were almost exclusively within the first column of unconditioned pain-stimulated behaviors that are vulnerable to false-positive effects due to motor impairment. Within these models, dual MAGL/FAAH inhibitors produce consistent antinociceptive effects, with the majority of studies demonstrating decreases of mechanically evoked behaviors in rodent inflammatory or neuropathic pain models. Many additional pain-related behaviors remain to be evaluated.

Very limited work has been conducted evaluating effectiveness of dual MAGL/FAAH inhibitors on pain-depressed behaviors. Work from [Greco et al. \(2021\)](#) using a mouse migraine model suggests antinociceptive effectiveness on pain-stimulated but not pain-depressed behaviors, and work from [Diester et al. \(2021\)](#) using an acute visceral noxious stimulus shows mixed effectiveness across different IP acid-stimulated and IP acid-depressed behaviors ([Diester et al., 2021](#); [Greco et al., 2021](#)). Additionally, regarding side effects, dual MAGL/FAAH inhibitors produce cannabimimetic effects such as catalepsy, memory impairment, hypomotility, and substitution for CB<sub>1/2</sub>R agonists in drug-discrimination assays. The potency for antinociceptive effects versus unwanted cannabimimetic effects is likely dependent upon multiple variables, including the type and intensity of the noxious stimulus and the behavioral endpoint being evaluated. In general, current studies demonstrate antinociceptive effectiveness on pain-stimulated endpoints at doses that also produce moderate cannabimimetic effects, with additional studies showing necessity of both MAGL and FAAH inhibition for CB<sub>1/2</sub>R agonist substitution in drug-discrimination assays ([Hruba et al., 2015](#); [Long, Nomura, et al., 2009](#)). Taken together, the current literature suggests dual MAGL/FAAH inhibitors are less promising as candidate analgesics than more MAGL- or FAAH-selective inhibitors.

## FAAH-selective catabolic enzyme inhibitors

**Table 3** shows the general antinociceptive effectiveness of FAAH-selective inhibitors in a preclinical pain-matrix profile. URB597 was the most commonly evaluated compound, with more pain-related studies conducted using URB597 than any other individual eCB catabolic enzyme inhibitor. Similar to both MAGL-selective and dual MAGL/FAAH inhibitors, FAAH-selective inhibitors were extensively evaluated across multiple noxious stimuli, including acute, inflammatory,

**TABLE 2** Preclinical pain matrix profile for dual MAGL/FAAH inhibitors.

Pain stimulus (independent variable)	Pain behavior (dependent variable)											
	Unconditioned		Classically conditioned		Operant conditioned							
	US→UR	PS→[US→UR]	CS+US; CS→CR	PS→[CS+US; CS→CR]	S <sup>D</sup> →R→S <sup>C</sup>	S <sup>D</sup> →R→PS	PS→[S <sup>D</sup> →R→S <sup>C</sup> ]					
Acute	Thermal	✓	-	-	-	-	-					
	Mechanical	✓										
	Chemical	✓										
	+ Thermal	✓										
Inflammatory	+ Mechanical	✓	-	-	-	-	-					
	Spontaneous	✓										
	+ Thermal	✓										
Neuropathic	+ Mechanical	✓	-	-	-	-	-					
	Spontaneous	-										
	+ Thermal											
Disease model	+ Mechanical	-	-	-	-	-	-					
	Spontaneous	✓	✗	✗	✗	✗	✗					

General antinociceptive effects in the current preclinical literature produced by JZL195 and SA57 administration in different types of preclinical pain assays. Effects are categorized by the designated pain stimulus (independent variable, row titles) and the measured pain-related behavior (dependent variable, column titles). Fill color and symbol within each cell of the pain matrix indicate predominant drug effects reported in published studies: green (✓), usually effective; blue (–), drug effects mixed; red (✗), drug usually ineffective; no fill indicates drug not tested.

**TABLE 3** Preclinical pain matrix profile for FAAH-selective inhibitors.

Pain stimulus (independent variable)	Pain behavior (dependent variable)					
	Unconditioned		Classically conditioned		Operant conditioned	
	US→UR	CS+US; CS→CR	PS→[US→UR]	PS→[CS+US; CS→CR]	PS→R→S <sup>C</sup>	S <sup>D</sup> →R→PS
Acute	Thermal	X				
	Mechanical	✓				
	Chemical	–	–			✓
Inflammatory	+ Thermal	✓				
	+ Mechanical	✓				
	Spontaneous	✓				
Neuropathic	+ Thermal	✓				
	+ Mechanical	✓				
	Spontaneous		–			
Disease model	+ Thermal	✓				
	+ Mechanical	✓				
Spontaneous			X			

General antinociceptive effects in the current preclinical literature produced by URB597, PF3845, and PF0457845 administration in different types of preclinical pain assays. Effects are categorized by the designated pain stimulus (independent variable, row titles) and the measured pain-related behavior (dependent variable, column titles). Fill color and symbol within each cell of the pain matrix indicate predominant drug effects reported in published studies: green (✓), usually effective; blue (–), drug effects mixed; red (X), drug usually ineffective; no fill indicates drug not tested.

neuropathic, and disease-state models such as arthritis and migraine, but the dependent variable was almost exclusively within the first column of unconditioned pain-stimulated behaviors.

Within this column, FAAH-selective inhibitors showed consistent attenuation of pain-stimulated behaviors except for unconditioned withdrawal reflexes elicited by acute thermal stimuli. Only a small number of studies have evaluated FAAH-selective inhibitors on pain-depressed endpoints, which report either a weak or nonsignificant antinociceptive effect. URB597, for example, produced partial attenuation of IP acid-depressed feeding, wheel-running, and nesting behavior, did not attenuate IP acid-depressed rearing behavior, and when tested in an IP acid-depressed operant conditioned paradigm produced partial effectiveness with a different rate of onset for antinociception for IP acid-stimulated vs IP acid-depressed behaviors (Diester et al., 2021; Kwilas et al., 2014; Miller et al., 2012). As AEA is expressed at lower levels than 2-AG, differing efficacy requirements for restoring pain-depressed behaviors may explain the partial or nonsignificant effect of FAAH-selective inhibitors currently reported in the literature. More specifically, FAAH-selective inhibitors may be less effective to attenuate behaviors requiring higher levels of receptor activation; however, further research will need to be conducted to clarify the effectiveness of these compounds, particularly in assays measuring pain-depressed behaviors.

Regarding side effects, FAAH-selective inhibitors do not show evidence for producing cannabimimetic effects similar to CB<sub>1/2</sub>R agonists and were also shown to lack abuse potential in monkeys ( Justinova et al., 2008; Schlosburg et al., 2009). Repeated administration of FAAH-selective inhibitors such as PF3845 was directly compared with persistent inhibition of MAGL through pharmacological (JZL184) and genetic approaches, which showed sustained antinociceptive effectiveness of PF3845 that did not produce functional impairment of CB<sub>1</sub>Rs (Schlosburg et al., 2010). Despite this generally noncannabimimetic profile, the lack of robust antinociceptive effects within pain-depressed behavioral endpoints suggests that FAAH-selective inhibitors may not be ideal candidate analgesics for restoration of functional impairment. Further research evaluating pain behaviors other than unconditioned pain-stimulated behaviors needs to be conducted to help elucidate where FAAH-selective inhibitors may be most effective for clinical utility.

## Additional considerations for eCB catabolic enzyme inhibitors and pain

### Sex as a biological variable

Despite increasing evidence reported in the literature showing sex differences in general pain processing, cannabinoid pharmacology, and eCB-mediated antinociception, studies evaluating eCB catabolic enzyme inhibitors were almost exclusively evaluated in male rodents (Blanton et al., 2021; Cooper & Craft, 2018; Diester et al., 2021; Farquhar et al., 2019; Fullerton et al., 2018; Greenspan et al., 2007). This chapter does not cover the extent of sex differences that have been identified

within the cannabinoid system and pharmacological targeting of it; however, it should be noted that the pain matrices shown in Tables 1–3 represent effectiveness for predominantly male subjects. Many studies need to be conducted to determine the effectiveness of eCB catabolic enzyme inhibitors ranging in selectivity for MAGL and FAAH in female subjects across noxious stimuli and pain-related behaviors.

### **Co-administration of eCB catabolic enzyme inhibitors with other analgesic classes**

While not covered extensively within the scope of this review chapter, studies have investigated co-administration of eCB catabolic enzyme inhibitors with other clinically effective analgesics. Particular interest has been placed on co-administration with NSAIDs and MOR agonists as a method for alleviating the unwanted gastric ulceration produced by repeated NSAID administration and abuse liability, gastric immobility, and respiratory depression produced by MOR agonists. Co-administration of low doses of MAGL-selective inhibitors (e.g., JZL184) or FAAH-selective inhibitors (e.g., URB597) with NSAIDs (e.g., diclofenac) has been shown to attenuate unconditioned pain-stimulated behaviors and provide gastroprotective effects (Kinsey et al., 2013; Slivicki et al., 2018). Pharmacophoric elements from FAAH-selective inhibitors are being combined with elements from COX inhibitors, MAGL/cholinesterase inhibitors, and dopamine system modulators to synthesize novel candidate analgesics, with polypharmacological approaches combining other drug classes with eCB catabolic enzyme inhibitor properties (Papa et al., 2022). Additionally, co-administration of MAGL- or FAAH-selective inhibitors with low doses of MOR agonists has shown opioid-sparing effects and attenuation of unconditioned pain-stimulated behaviors (Slivicki et al., 2018). While very few preclinical pain studies have evaluated polypharmacological approaches with eCB catabolic enzyme inhibitors in assays of pain-depressed behaviors, current studies support continued investigation of their antinociceptive effectiveness across different noxious stimuli and pain-related behaviors.

### **Applications to other areas**

In this chapter, we have reviewed the antinociceptive effectiveness of eCB catabolic enzyme inhibitors ranging in selectivity for MAGL and FAAH on pain-related behavioral endpoints. Enhancement of endogenous cannabinoid tone via MAGL or FAAH inhibition has a wide variety of applications due to the broad expression of CB<sub>1/2</sub>Rs, MAGL, FAAH, and their associated lipids. Additional research areas investigating the utility of eCB catabolic enzyme inhibitors include anxiety, neurodegeneration, mood disorders, metabolic diseases, and inflammatory diseases (Wilkerson et al., 2021). Interestingly, pain and anxiety/depression often overlap, with pain states often producing increased levels of anxiety and distress. While it has been well established that the eCB system plays an important role in stress and anxiety, the data in this chapter and the literature specifically evaluating anxiolytic effects of eCB catabolic enzyme inhibitors suggest potential clinical utility for FAAH-selective inhibitors. In particular, AEA signaling within the basolateral amygdala has been shown as a key mediator in preclinical studies, with FAAH-selective inhibitors effectively increasing AEA levels and producing reductions in stress and anxiety-like behaviors (Gunduz-Cinar et al., 2013). Finally, while this review illustrated multiple pain-matrix tables specified for MAGL and FAAH inhibitors, this method can be used to characterize the antinociceptive effectiveness of any candidate analgesic and identify areas needing additional research (Negus, 2019).

### **Mini-dictionary of terms**

- *Classically conditioned behavior:* A type of learned behavior involving repeated pairing of an unconditioned stimulus (US) and a second stimulus, called a conditioned stimulus (CS), which initially elicits little or no behavioral response. After repeated US+CS pairing, the CS comes to elicit a conditioned response (CR).
- *Operant conditioned behavior:* A type of learned behavior dependent on its consequences and structured by a three-term contingency, where a discriminative stimulus (S<sup>D</sup>) signals that a response (R) will produce delivery of a consequent stimulus (S<sup>C</sup>). Delivery of the S<sup>C</sup> may either increase/reinforce or decrease/punish rates of the preceding behavior.
- *Pain-stimulated behaviors:* Behavioral endpoints that *increases* in rate, frequency, or intensity following delivery of a noxious stimulus.
- *Pain-depressed behaviors:* Behavioral endpoints that *decreases* in rate, frequency, or intensity following delivery of a noxious stimulus.
- *Pain matrix:* A tabular method for visualizing antinociceptive effectiveness of candidate analgesics across classes of noxious stimuli and categories of pain-related behaviors.
- *Unconditioned behavior:* Unlearned reflexive behaviors in which delivery of an unconditioned stimulus elicits an unconditioned response.

## Key facts of endocannabinoid catabolic enzyme inhibitors and pain

- Compounds have been synthesized that cover a range of selectivity for inhibiting the main eCB catabolic enzymes MAGL and FAAH.
- Noxious stimuli (primary independent variable in pain research) are often classified as either acute, inflammatory ± evocation with an acute stimulus, neuropathic ± evocation with an acute stimulus, or a disease model (e.g. bone cancer) ± evocation with an acute stimulus.
- Pain-related behaviors (primary dependent variable in pain research) can be classified as unconditioned, classically conditioned, and operant conditioned.
- eCB catabolic enzyme inhibitors have been evaluated across a wide range of noxious stimuli, but have been predominantly measured with unconditioned pain-stimulated behaviors.
- Pain-stimulated behaviors *do not* model the primary clinical goal for functional restoration of pain-impaired behavior and are also highly susceptible to false-positive effects by nonanalgesic drugs that impair motor function.
- Pain-depressed behaviors *do* model the primary clinical treatment goal for restoring pain-related functional impairment and are not susceptible to false-positive effects by nonanalgesic drugs that impair motor function.
- Very little preclinical research has been conducted evaluating possible sex differences in the antinociceptive effects of eCB catabolic enzyme inhibitors.

## Summary points

- The eCB system acts as an on-demand feedback-inhibition mechanism to dampen hyperactive excitatory signaling seen in acute and chronic pain states.
- Compared with exogenous CB<sub>1/2</sub>R agonists, MAGL- and FAAH-selective inhibitors enhance inhibitory signaling of CB<sub>1/2</sub>Rs only where 2-AG and AEA are actively synthesized.
- Visualization of current preclinical pain research of eCB catabolic enzyme inhibitors through pain matrices provides clear comparisons between drug classes and visualization of areas requiring further research.
- While eCB catabolic enzyme inhibitors have been evaluated across a wide range of noxious stimuli, antinociceptive effects have been almost exclusively measured using unconditioned pain-stimulated behaviors that are vulnerable to false-positive effects with nonanalgesic drugs that impair motor function.
- Current evidence evaluating pain-depressed behaviors suggests that MAGL-selective inhibitors may be more effective than FAAH-selective inhibitors; however, the pain matrices for all categories of eCB catabolic enzyme inhibitors demonstrate the need for additional investigation of these compounds on behaviors other than unconditioned pain-stimulated endpoints.

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## Chapter 16

# The endocannabinoid system in health and disease: Features in epilepsy

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### Abbreviations

[ <sup>35</sup> S]-GTPyS	guanosine triphosphate labeled on the gamma phosphate group with <sup>35</sup> S
2-AG	2-arachidonoylglycerol
Δ <sup>9</sup> -THCV	Δ <sup>9</sup> -tetrahydrocannabivarin
AEA	anandamide
BBB	blood-brain barrier
CB <sub>1</sub>	cannabinoid receptor type 1
CB <sub>2</sub>	cannabinoid receptor type 1
CNS	central nervous system
DAGL $\alpha$	diacylglycerol lipase alpha
DAGL $\beta$	diacylglycerol lipase beta
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
GABA	gamma-aminobutyric acid
iNOS	inducible nitric oxide synthase
IL-1 $\beta$	interleukin 1-beta
JNK	c-Jun N-terminal kinases
kDa	kilodaltons
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
NAPE-PLD	N-acylphosphatidylethanolamine-specific phospholipase D-like hydrolase
NAADP	nicotinic acid adenine dinucleotide phosphate
NVU	neurovascular unit
PEA	palmitoylethanolamide
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptors
TLE	temporal lobe epilepsy
TRPV1	vanilloid receptor type 1 channels
TNF- $\alpha$	tumor necrosis factor-alpha
TNF-R1	tumor necrosis factor receptor 1
VEGF	vascular Endothelial Growth Factor
ZO	zonula occludens

### Endocannabinoid system

The endocannabinoid system (ECS) consists of lipid signaling molecules modulating retrograde synaptic communication. The main endocannabinoids described to date are N-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). The synthesis of endocannabinoids occurs through the hydrolysis of membrane lipid precursors. AEA is synthesized by N-acyl phosphatidylethanolamine-specific phospholipase D-like hydrolase (NAPE-PLD), while 2-AG is synthesized by phospholipase C (PLC) and diacylglycerol lipase alpha (DAGL $\alpha$ ) and beta (DAGL $\beta$ ). After synthesis, AEA is rapidly hydrolyzed and degraded by fatty acid amide hydrolase (FAAH), while

monoacylglycerol lipase (MAGL) degrades 2-AG. Endocannabinoids are released on demand in response to various physiological and pathological stimuli.

In 1990, the first endocannabinoid receptor was identified in the brain tissue and named cannabinoid receptor type 1 (CB<sub>1</sub>). Shortly after that, another receptor for cannabinoids was discovered at the peripheral level and identified as cannabinoid receptor type 2 (CB<sub>2</sub>). Both receptors are composed of seven  $\alpha$ -helix transmembrane domains and a C-terminal domain of 73 amino acids (CB<sub>1</sub>) or 59 amino acids (CB<sub>2</sub>). CB<sub>1</sub> and CB<sub>2</sub> receptors are conventionally coupled to G<sub>αi/o</sub> proteins, and their activation leads to adenylate cyclase (AC) inhibition and mitogen-activated kinases (MAPK) and Rho proteins activation. In addition, evidence indicates that endocannabinoids interact with other G-protein-coupled receptors and ion channels. These channels include vanilloid receptor type 1 (TRPV1), peroxisome proliferator-activated receptors (PPARs), and the orphan receptors GPR55 and GPR18 (Zou & Kumar, 2018).

The ECS is involved in several physiological and pathological processes, including nociception, appetite, lipid metabolism, gastrointestinal motility, cardiovascular regulation, motor activity, mood, and memory. This ECS is also involved in angiogenesis, inflammation, cancer, blood-brain barrier (BBB) regulation, and brain disorders such as epilepsy.

## Endocannabinoids in human epilepsy

Epilepsy is a neurological disease characterized by a permanent predisposition to spontaneous and recurrent epileptic seizures and associated with neurobiological, cognitive, psychological, and social alterations. Among the different types of epilepsy, temporal lobe epilepsy (TLE) is the most prevalent, affecting approximately 40% of patients. In TLE, epileptic activity originates in limbic structures, including the hippocampus, amygdala, entorhinal cortex, and lateral temporal neocortex. A high percentage of patients with TLE exhibit resistance to pharmacological treatment, characterized by failure to control seizures despite using two or more adequate antiseizure medications, delivered as monotherapy or polytherapy.

Experimental evidence supports that epilepsy modifies the ECS in the human brain. In the cerebrospinal fluid of patients with TLE, 2-AG levels remain similar to those of control subjects, while AEA levels are low (Romigi et al., 2010). In contrast, in the hippocampus and cerebral cortex of patients with drug-resistant TLE, the tissue content of AEA is high, while that of 2-AG is decreased (Rocha et al., 2020). The last change is associated with reduced expression of DAGL $\alpha$ , responsible for 2-AG synthesis (Ludányi et al., 2008).

Regarding receptors of the ECS, experimental evidence indicates that CB<sub>1</sub> receptor mRNA and protein expression are decreased in TLE patients' hippocampus (Ludányi et al., 2008). However, other evidence from in vivo studies indicates that CB<sub>1</sub> receptor availability is increased both in the epileptic focus and the surrounding brain neocortex of patients with epilepsy (Goffin et al., 2011). In terms of their functionality, CB<sub>1</sub> and CB<sub>2</sub> receptors expressed in the brain tissue of patients with TLE have an increased efficacy for the stimulation of G<sub>αi/o</sub> proteins when exposed to their agonists (Rocha et al., 2020). This finding indicates that CB<sub>1</sub> and CB<sub>2</sub> receptors have increased functionality due to their greater efficiency in activating transductional cascades in the brain parenchyma of subjects with epilepsy. These changes correlate with the clinical characteristics of patients with epilepsy. In this regard, it has been reported that patients with a history of febrile seizures during childhood have a higher expression of CB<sub>1</sub> receptors. Furthermore, CB<sub>1</sub> receptors of patients with TLE and without psychiatric comorbidities have higher efficacy for the stimulation of G<sub>αi/o</sub> proteins (Rocha et al., 2020).

## Effects of endocannabinoids in preclinical models of seizures, epileptogenesis, and epilepsy

Based on preclinical studies, it is suggested that activation of the ECS induces anticonvulsant effects. In this regard, increased cellular levels of AEA and 2-AG reduce the severity of pentylenetetrazol-induced seizures or electroshock-induced maximal seizures in rats (Wallace et al., 2003, 2002). The  $\Delta^9$ -tetrahydrocannabinol extract obtained from cannabis reduces the seizure activity induced by the lithium-pilocarpine model via activation of the CB<sub>1</sub> receptor (Wallace et al., 2003). The phytocannabinoid  $\Delta^9$ -tetrahydrocannabivarin ( $\Delta^9$ -THCV) decreases in vitro epileptiform activity and acute seizure activity induced by pentylenetetrazol in rats (Hill et al., 2010). Activation of CB<sub>1</sub> and CB<sub>2</sub> receptors by palmitoyl-lethanolamide (PEA) decreases in mice the acute seizure activity induced by different chemoconvulsants, such as pentylenetetrazol, 3-mercaptopropionic acid, bicuculline, and kainic acid (Post et al., 2018).

Concerning the epileptogenesis process, experimental evidence indicates that CB<sub>1</sub> receptor mRNA and protein expression are decreased (Falenski et al., 2009). Activation of the ECS slows the progression of the amygdala kindling but does not modify the expression of fully kindled seizures (Wendt et al., 2011). Similarly, upregulation of 2-AG by MAGL inhibition slows the kindling development but does not modify epileptic activity in fully kindled mice (von Rüden et al., 2015). In itself, amygdala kindling induces emotional disturbances associated with AEA-mediated

neurotransmitter dysfunction, restored by FAAH inhibition (Colangeli et al., 2020). PEA increases latency and decreases seizure duration during chemical kindling with pentylenetetrazol (Aghaei et al., 2015). On the other hand, results obtained from preclinical studies focused to determine the role of activation of ECS in the expression of seizures of animals with epilepsy are controversial.

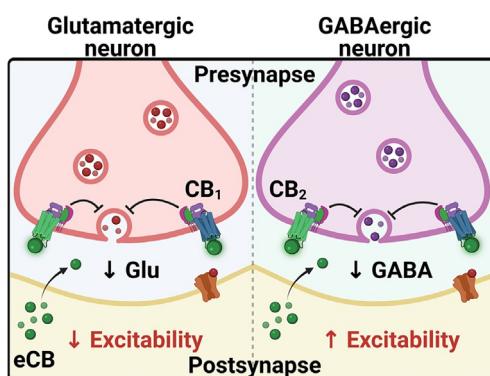
## Endocannabinoids: Pro- or anticonvulsant effects

According to the group of evidences presented in the previous sections, it is possible to suggest that increased endocannabinoid-mediated neurotransmission plays a neuroprotective role in the brain with epilepsy. However, it is important to notice that patients with epilepsy show cerebral deterioration despite the increased expression and functionality of CB<sub>1</sub> and CB<sub>2</sub> receptors at the brain level. It is possible that CB<sub>1</sub> and CB<sub>2</sub> receptors in TLE patients do not mediate protective effects due to low levels of 2-AG in the brain parenchyma (Rocha et al., 2020). Other possibility is that endocannabinoids induce excitatory and harmful effects in the brain parenchyma of patients with epilepsy. Experimental evidence supports the proconvulsant effects of cannabinoids and endocannabinoids. In this regard, chronic administration of cannabis extracts has been described as facilitating the induction of generalized seizures (Whalley et al., 2019). High-dose administration of WIN 55,212, an agonist for CB<sub>1</sub> and CB<sub>2</sub> receptors, does not prevent kainic acid-induced seizure activity and neuronal damage in immature rats (Rudenko et al., 2012). Mice with increased AEA tissue levels due to FAAH deficiency show increased seizure activity and neuronal damage when subjected to bicuculline- or kainic acid-induced seizures (Clement et al., 2003). Several experimental evidences suggest that acute and subchronic activation of CB<sub>1</sub> receptors can lead to a proconvulsant effect in different types of seizures. As for CB<sub>2</sub> receptors, their acute activation by AM1241 administration facilitates the pentylenetetrazol proconvulsant effect (de Carvalho et al., 2016).

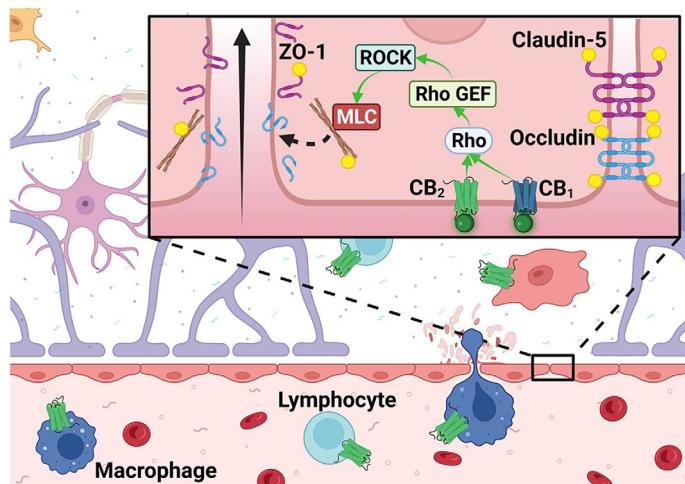
The contradictions between anti- and proconvulsant effects resulting from activation of the ECS may be explained by the induction of dual outcomes. For example, AEA activates the CB<sub>1</sub> receptor at low levels and induces anticonvulsant effects, while at high levels, it activates the TRPV1 receptor and produces proconvulsant results (Manna & Umathe, 2012). Activation of the CB<sub>1</sub> receptor delays the development of epileptogenesis but decreases the degree of brain excitability (Wendt et al., 2011). Based on this body of evidence, it is possible to postulate that the effects of endocannabinoids depend on different circumstances, mainly on the degree of neuronal excitability and the localization of their receptors (Fig. 1).

## Epilepsy and blood-brain barrier

The BBB is a structure that plays a vital role in the homeostasis of the central nervous system (CNS). The BBB consists of endothelial cells that form the cerebral microvessels, surrounded by pericytes, the basal lamina, and the feet of astrocytes and neurons. The whole of this structure is called the neurovascular unit (NVU). The interaction of the endothelium with the cellular and acellular components of the NVU is crucial for the maintenance of the BBB properties.



**FIG. 1** Retrograde signaling of the endocannabinoid system. When a postsynaptic neuron is activated, endocannabinoids (eCB) are synthesized on-demand and released into the synaptic cleft, where they can activate presynaptic receptors. Activation of cannabinoid receptors type 1 (CB<sub>1</sub>) and type 2 (CB<sub>2</sub>) located at the presynapse inhibits vesicular neurotransmitter release from either excitatory or inhibitory terminals. If the synaptic terminal is from a glutamatergic neuron, the reduction of glutamate release leads to a reduction of post-synaptic excitability (left). On the contrary, if the synaptic terminal corresponds to a GABAergic neuron, the excitability increases (right).



**FIG. 2** Role of cannabinoid receptors in the integrity of the blood-brain barrier. The protective functions of the blood-brain barrier (BBB) depend on the stability of tight junctions. These tight junctions are composed of proteins such as claudin-5, occludin, and zonula occludens (ZO-1), which regulate the paracellular flow of small molecules and other elements from the blood. Activation of cannabinoid receptors ( $\text{CB}_1$  and  $\text{CB}_2$ ) located on BBB endothelial cells leads to phosphorylation of Rho-GEF, which activates the Rho-associated protein kinase (ROCK). In turn, ROCK mediates myosin light chain (MLC) phosphorylation. Subsequently, phosphorylation of MLC leads to contraction of actin fibers of the cytoskeleton, impairing the integrity of the BBB. In addition,  $\text{CB}_2$  expressed on immune cells mediates migration from the blood to the brain parenchyma via endothelial cells.

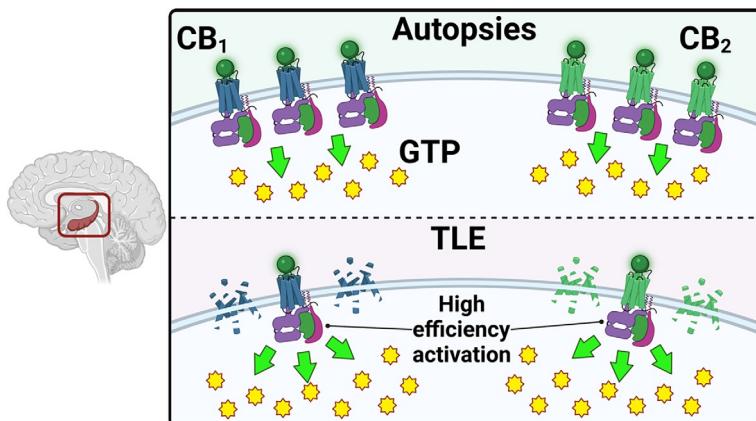
The BBB protects the brain parenchyma by regulating the entry of molecules, xenobiotics, neurotoxins, and pathogens. The protective functions of the BBB depend on tight junction proteins. These are transmembrane proteins that regulate the paracellular passage of solutes. Claudin-5 is a 20–24 kDa protein that controls the migration of small molecules by interacting with its counterpart expressed in adjacent cells. Occludin is another integral membrane protein that assembles into 55–60 kDa oligomers and regulates paracellular movement, mainly of immune system cells. These proteins are anchored to the cytoskeleton by accessory proteins such as the zonula occludens (ZO) family.

Several neurological disorders are associated with BBB changes. Processes such as excitotoxicity, inflammation, and oxidative stress in neurodegenerative diseases promote the release of factors that decrease the expression of tight junction proteins, facilitating BBB dysfunction. In epilepsy, BBB dysfunction contributes to disease progression and drug resistance. In this regard, epileptic activity is related to excessive glutamate release, which causes damage to the cells that constitute the NVU and increases BBB permeability. Studies revealed that the microvasculature of TLE patients shows a decreased expression of occludin and ZO-1 associated with high expression of Vascular Endothelial Growth Factor (VEGF)-A and its receptor 2 (VEGFR-2) and claudin-5 proteins, and increased protein expression of the proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$ , and its receptor TNF-R1, as well as inducible nitric oxide synthase (iNOS), a marker of oxidative stress (Castañeda-Cabral et al., 2020). These changes are associated with damage to the BBB, which facilitates plasma protein extravasation, promotes the passage of leukocytes into the brain parenchyma, and activates inflammatory processes (Fig. 2). When these events are generated chronically, conditions such as gliosis, neuroinflammation, excitotoxicity, neuronal reorganization, and aberrant vascularization are facilitated, favoring the epileptic activity (Marchi et al., 2012).

## Endocannabinoid receptors, blood-brain barrier, and epilepsy

Under normal conditions,  $\text{CB}_1$  and  $\text{CB}_2$  receptors are expressed on different components of the NVU. Experimental evidence indicates that  $\text{CB}_1$  and  $\text{CB}_2$  receptors induce protective effects on NVU integrity and BBB function. In this regard, studies revealed that  $\text{CB}_1$  receptor activation in co-cultures of human brain endothelial cells and astrocytes prevents BBB dysfunction by inhibiting the downregulation of ZO-1, claudin-5, and junctional adhesion molecule-1 (JAM-1) protein expression. Activation of  $\text{CB}_1$  and  $\text{CB}_2$  receptors by 2-AG also prevents BBB dysfunction resulting from brain insult (Lu et al., 2008).

$\text{CB}_2$  receptor activation induces antiinflammatory effects and protects the BBB. In particular,  $\text{CB}_2$  receptor activation has been reported to decrease BBB damage in a murine model of severe head trauma (Amenta et al., 2012). Chronic inactive plaques in the blood vessels of multiple sclerosis patients have also been shown to overexpress the  $\text{CB}_2$  receptor, supporting its protective effect (Zhang et al., 2011). These effects are associated with the fact that  $\text{CB}_2$  receptor activation prevents the inflammation-induced low expression of ZO-1, JAM-1, and claudin-5 proteins in BBB endothelial cells (Lu et al., 2008). Furthermore, activation of  $\text{CB}_1$  and  $\text{CB}_2$  receptors prevents neoangiogenesis by decreasing VEGF signaling and



**FIG. 3** Cannabinoid receptor expression and functionality in the neurovascular unit of hippocampus under epileptic conditions. In hippocampal neurovascular units from autopsies (above), the expression and activation profile of cannabinoid receptors (CB<sub>1</sub> and CB<sub>2</sub>) maintain cellular functions mediated by the endocannabinoid system. In the hippocampal tissue from patients with temporal lobe epilepsy (TLE), the expression of CB<sub>1</sub> and CB<sub>2</sub> is decreased. Regardless of the reduced expression of CB<sub>1</sub> and CB<sub>2</sub> receptors, they present a high efficiency to activate G<sub>αi/o</sub> proteins. This situation suggests a higher neurotransmission mediated by CB<sub>1</sub> and CB<sub>2</sub> receptors.

attenuating chronic inflammation of the vascular endothelium. This set of findings supports the protective role of CB<sub>1</sub> and CB<sub>2</sub> receptors at the BBB level.

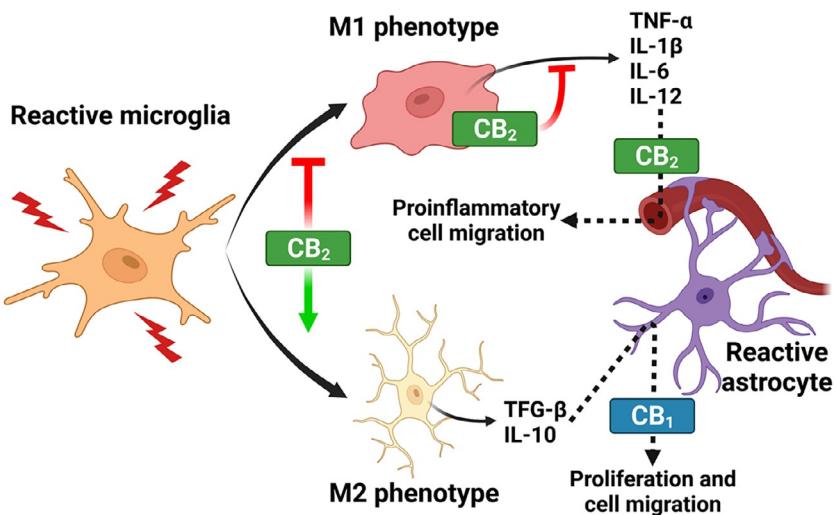
Moreover, recent studies in microvessels of patients with TLE revealed decreased CB<sub>1</sub> and CB<sub>2</sub> receptors in the epileptic hippocampus and increased expression in the temporal cortex (Nuñez-Lumbreras et al., 2021). However, assessment of their functionality by [<sup>35</sup>S]-GTPγS binding assays in NVU cell membranes from patients with TLE revealed that CB<sub>1</sub> and CB<sub>2</sub> receptors exhibit increased efficacy for G<sub>αi/o</sub> protein activation in the epileptic hippocampus and the temporal lobe cortex compared with autopsy brain tissues. These changes were more evident in NVU from patients with a longer duration of epilepsy (CB<sub>1</sub> receptors) or with a high frequency of epileptic seizures (CB<sub>2</sub> receptors). These findings indicate that CB<sub>1</sub> and CB<sub>2</sub> receptors in the NVU of the epileptic hippocampus and temporal cortex of TLE patients have increased functionality (Nuñez-Lumbreras et al., 2021) (Fig. 3).

It is known that CB<sub>2</sub> receptor expression may be positively regulated in neuroinflammatory processes in diseases such as multiple sclerosis and Alzheimer's disease. Activation of CB<sub>1</sub> and CB<sub>2</sub> receptors increases leukocyte recruitment, inflammation, neovascularization, oxidative stress, and BBB cell disruption (Guabiraba et al., 2013). Additionally, activation of CB<sub>1</sub> and CB<sub>2</sub> receptors can affect the expression of tight junction proteins and impair the integrity of the BBB. This deleterious effect may depend on the time of activation and the characteristics of ligand exposure (Di Marzo, 2008). Based on this evidence, it is possible to suggest that the high efficacy of signal transduction mediated by CB<sub>1</sub> and CB<sub>2</sub> receptors activation found in the NVU of epileptic hippocampus and cortex of patients with TLE may facilitate neurotoxicity, inflammation, and aberrant neovascularization. Further studies are needed to determine whether the highly efficient coupling of CB<sub>1</sub> and CB<sub>2</sub> receptor-mediated signal transduction mechanisms results in NVU impairment in patients with TLE.

## Endocannabinoids and antiinflammatory effects in epilepsy

Inflammation is a process that is activated in response to events such as exposure to toxic agents, damage, or infection. It develops acutely to prevent cell damage and promotes tissue repair. In CNS, mast cells play an essential role in activating proinflammatory processes. These cells are attached to the BBB and their activation results in the release of factors that facilitate BBB damage, entry of blood elements into the brain parenchyma, and facilitation of neuroinflammatory processes. The release of factors from mast cells also causes microglial activation, which in turn releases cytokines that increase neuronal excitability and modify synaptic activity (Qin et al., 2021). Additionally, cytokines released by mast cells can facilitate neuronal death by increasing the expression of connexin-43 hemichannel. Indeed, persistent mast cell activation may increase cellular damage in chronic neurological disorders (Sandhu & Kulka, 2021).

The inflammation process is associated with the activation of the ECS. Evidence suggests that activation of the ECS at the peripheral level can induce antiinflammatory effects. Increased AEA concentration protects against cell damage and down-modulates mast cells at the central level, thus preventing microglial activation (Skaper et al., 1996). Activation of the ECS reduces neuroinflammation through reduced expression of the connexin-43 hemichannel (Froger et al., 2009). Increased CB<sub>2</sub> receptor expression under brain injury conditions decreases cell damage and neuroinflammation



**FIG. 4** Cannabinoid receptors in neuroinflammation processes. After an insult, reactive microglia differentiate into the M1 or M2 phenotype. Cannabinoid receptor type 2 (CB<sub>2</sub>) favors the M2 phenotype, which has reparative functions and releases proliferation factors from reactive astrocytes, leading to cannabinoid receptor type 1 (CB<sub>1</sub>)-mediated cell migration. Alternatively, CB<sub>2</sub> activation in the M1 phenotype inhibits the release of proinflammatory cytokines. These cytokines promote the migration of immune cells from blood vessels in a process partially mediated by CB<sub>2</sub>.

(Mangiatordi et al., 2020). The ECS also modulates the effect of cytokines on synaptic activity, which depends on the cellular environment and the degree of inflammation (Rossi et al., 2015) (Fig. 4). Based on this evidence, activating the ECS is suggested as a therapeutic strategy to reduce inflammation.

Studies support that the antiinflammatory effects of ECS could be effective for the control of epilepsy. PEA is an endocannabinoid that acts at the level of PPAR $\alpha$ , GPR55, GPR119 receptors, increases AEA levels, and induces antiinflammatory and anticonvulsant effects. PEA has been considered a drug with therapeutic potential to reduce neuroinflammation related to different brain disorders. Evidence in preclinical models of acute seizures and epilepsy indicates that PEA reduces the frequency and severity of ictal activity more effectively than antiseizure medications. In addition, PEA induces neuroprotection (Bortolotto et al., 2022). It is important to note that, at present, no studies have evaluated the effect of PEA in patients with epilepsy. Therefore, its impact on patients may differ from that observed in animal models.

## Endocannabinoids and proinflammatory effects in epilepsy

It is known that the immune system is activated in response to acute ictal activity, that there is a positive correlation between the inflammatory process and recurrent ictal activity, and that increased proinflammatory cytokines facilitate epileptic activity (Vila Verde et al., 2021). In addition, the epileptic activity involves significant mast cell activation, leading to cell damage and increased neuronal activity (Valle-Dorado et al., 2015).

On the other hand, evidence indicates that activation of the ECS is related to proinflammatory processes in diseases such as obesity, diabetes, and pulmonary or metabolic disorders, while blockade of its receptors produces antiinflammatory effects. In CNS, AEA through the activation of CB<sub>1</sub> receptors modifies and promotes an enhanced proinflammatory glial response (Vázquez et al., 2015). Activation of CB<sub>2</sub> receptors has also been shown to increase microglia activation and angiogenesis associated with neuroinflammation. Furthermore, the administration of CB<sub>2</sub> receptor inverse agonists, such as raloxifene, decreases the inflammatory process by preserving the M2 phenotype of microglia.

Evidence supports that the activation of ECS is associated with proinflammatory effects that favor epileptic activity. In TLE associated with hippocampal sclerosis, CB<sub>1</sub> receptor expression in astrocytes is increased (Meng et al., 2014). Similarly, CB<sub>1</sub> (astrocytes) and CB<sub>2</sub> (microglia) receptors expression is increased in processes such as cortical malformations (Zurolo et al., 2010). Febrile seizures are events associated with the activation of the immune system in response to a central or systemic infectious process. When the immune system is activated at the central level, and due to febrile seizures, macrophages release cytokines, damaging the BBB and brain parenchyma and inducing neuronal hyperexcitability (Mosili et al., 2020). As a consequence of febrile seizures, there is an increase in CB<sub>1</sub> receptor-mediated signaling, which in the long term induces a decrease in GABAergic neurotransmission and an increase in neuronal excitability, facilitating the process of epileptogenesis (Chen et al., 2003).

The controversy between the proinflammatory and antiinflammatory effects of the ECS is explained because the outcome depends on the cellular environment in which they occur (Rossi et al., 2015). Therefore, it is relevant to consider whether the effects of endocannabinoids are obtained under basal conditions or conditions of increased neuroinflammation, excitotoxicity, and oxidative stress, characteristics of neurodegenerative disorders such as epilepsy.

## When do endocannabinoids induce harmful effects?

Several studies support that the acute or subchronic activation of ECS can produce inhibitory and neuroprotective effects. However, controversial evidence reveals that the ECS facilitates neurotoxicity and neuronal death by increasing glutamatergic neurotransmission and decreasing GABAergic neurotransmission. An increase in glutamatergic neurotransmission may result from different conditions such as the activation of extracellular-signal-regulated kinase (ERK)-dependent pathways by chronic stimulation of CB<sub>2</sub> receptors (Kim & Li, 2015). On the other hand, the activation of CB<sub>1</sub> or CB<sub>2</sub> receptors results in decreased GABAergic neurotransmission (Morgan et al., 2009; Ohno-Shosaku et al., 2001). Additionally, chronic blockade of CB<sub>2</sub> receptors produces changes in the expression of the GABA $\alpha$ 2 and GABA $\gamma$ 2 subunits of the GABA complex with consequent anxiolytic-like effects (García-Gutiérrez et al., 2012).

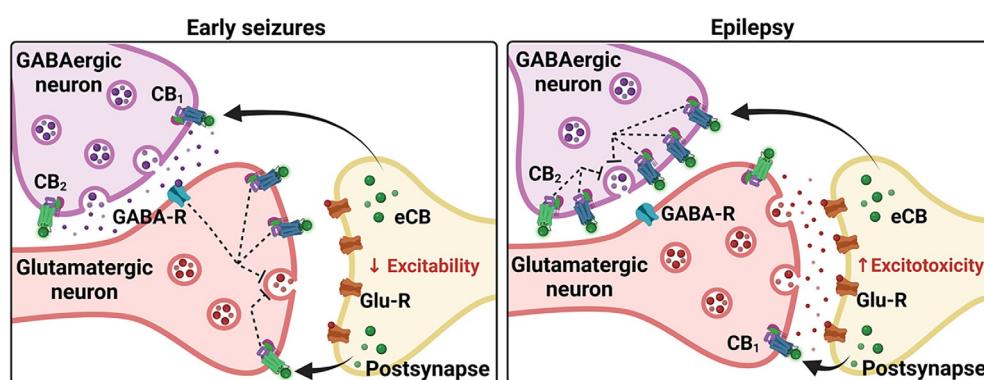
The effects of the ECS may depend on the clinical conditions of the subject (Di Marzo, 2008). This idea is supported with the notion that the effects of endocannabinoids depend on the subject's age: In adult stages, they can induce excitation, while in immature subjects, they produce inhibition (Bernard et al., 2005). It has also been observed that the endocannabinoid-induced proapoptotic effects are more evident in the immature brain (Fernández-Ruiz et al., 1999).

The chronicity and/or severity of the disorder may also influence the effects of ECS activation. CB<sub>1</sub> receptor agonism produces anticonvulsant effects in acute seizure models by inhibiting signaling in glutamatergic neurons (Monory et al., 2006). In contrast, CB<sub>1</sub> expression is increased in GABAergic neurons and decreased in glutamatergic neurons in the epileptic hippocampus of patients with TLE and chronic TLE models (Maglóczky et al., 2010). These changes in TLE are associated with increased retrograde inhibition of GABA release and increased glutamatergic neurotransmission (Chen et al., 2003) (Fig. 5).

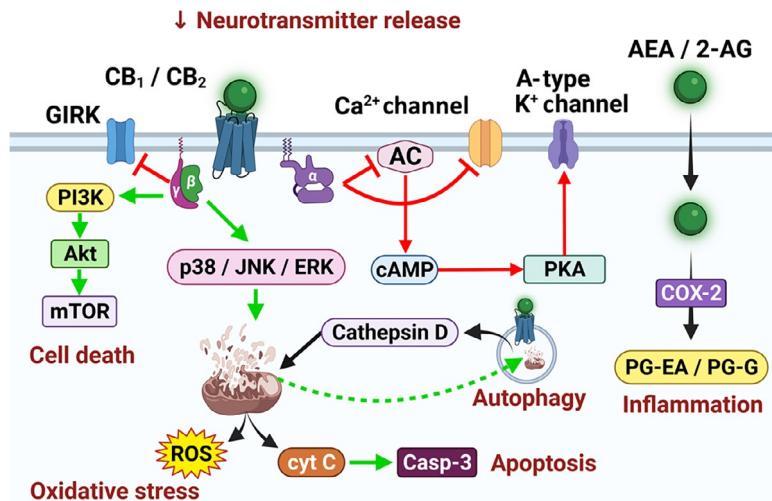
The effects of endocannabinoids may also depend on the histopathological changes associated with epilepsy. Endocannabinoids released from neurons increase calcium in astrocytes, resulting in increased glial glutamate release and neuronal activity. This situation may be more evident in patients with TLE, in which there is hippocampal sclerosis with overexpression of CB<sub>1</sub> receptors at the level of reactive astrogliosis.

## Endocannabinoids may underly harmful effects through different mechanisms

Studies support several mechanisms by which the ECS may facilitate cell damage. AEA promotes cell death by increasing intracellular calcium with a subsequent increase in caspase activity and cytochrome c release. In turn, the release of mitochondrial cytochrome c into the cytosol is facilitated by the activation of Bax resulting from the activation of Jun N-terminal



**FIG. 5** Changes in the endocannabinoid system through the epileptogenic process. Activation of cannabinoid receptors type 1 (CB<sub>1</sub>) and type 2 (CB<sub>2</sub>) in early seizures (left) mainly reduces the excitatory signals by inhibiting glutamate release, with poor effect on GABAergic tone. However, the progression of the epileptogenic process involves changes in the density and localization of cannabinoid receptors. For example, in developed epilepsy (right), the GABAergic transmission is highly susceptible to the inhibitory effects of the endocannabinoid system, favoring glutamate release and excitotoxicity processes.



**FIG. 6** Mechanisms by which endocannabinoids may induce cell damage. Activation of cannabinoid receptors (CB<sub>1</sub> and CB<sub>2</sub>) cleaves the G<sub>αi/o</sub>-protein into an α-subunit and a βγ-complex. The α-subunit inhibits the Ca<sup>2+</sup>-channels and the enzyme adenylate cyclase (AC), consequently reducing the production of cyclic adenosine monophosphate (cAMP). cAMP mediates the activation of the protein kinase A (PKA), thereby reducing the flow of potassium ions through A-type K<sup>+</sup>-channels, the primary regulators of neuronal excitability. In turn, the βγ-complex inhibits the G-protein-coupled inwardly rectifying potassium channels (GIRK) and activates the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt)-mammalian target of rapamycin (mTOR) pathway that is involved in cell death. The βγ-complex also activates the p38/c-Jun N-terminal kinases (JNK)/ERK pathway, leading to mitochondrial damage. Activation of CB<sub>1</sub> receptors located in endolysosomes regulates the release of cathepsin D and Ca<sup>++</sup>, affecting mitochondrial viability and driving the mitochondria to autophagy processes. Mitochondrial dysfunction entails the overproduction of reactive oxygen species (ROS), responsible for oxidative stress, and the release of cytochrome c (Cyt C), which activates the intrinsic pathway of apoptosis. In addition, the metabolism of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) through cyclooxygenase 2 (COX-2) produces prostaglandin-ethanolamides (PG-EA) or prostaglandin glycerol (PG-G), respectively, which participate in inflammatory processes.

kinases 1 and 2 (JNK1 and JNK2) as a consequence of CB<sub>1</sub> activation. These events result in the activation of the cysteine protease caspase-3 and DNA fragmentation (Downer et al., 2003).

Sustained activation of JNKs through the CB<sub>1</sub> receptor in the brain tissue with epilepsy may contribute to neuronal death and mossy fiber sprouting due to the involvement of these kinases in cell death pathways. Activating CB<sub>1</sub> receptors by AEA increases ceramide levels and p38 phosphorylation, conditions that facilitate apoptosis processes, mitochondrial and endoplasmic reticulum stress, and the production of reactive oxygen species (Fig. 6). AEA facilitates edema and cognitive alterations through calpain-dependent mechanisms (Cernak et al., 2004).

Activation of CB<sub>1</sub> receptors can damage the BBB through activation of the Rho protein, which induces contraction and disassembly of the actin cytoskeleton and reduces the expression of the ZO-1, claudin-5, and occludin proteins, which maintain tight junctions (Mai et al., 2015).

Moreover, activating intracellular CB<sub>1</sub> receptors coupled to G<sub>q/11</sub> proteins and phospholipase C facilitates excitotoxicity and cell damage due to increased calcium release from the endoplasmic reticulum and endolysosomes (Brailioiu et al., 2011). Additionally, intracellular CB<sub>1</sub> receptor activation facilitates membrane depolarization due to its action on nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent calcium pathways (Brailioiu et al., 2009).

Lipid rafts are microdomains composed of cholesterol, sphingolipids, plasmenylethanolamine, and arachidonic acid at the plasma membrane level. Alterations in lipid raft expression can affect endocannabinoid-mediated neurotransmission, AEA-induced cell death, and neurodegenerative disorders (Maccarrone et al., 2011).

In neurodegenerative processes, evidence suggests that activated microglia induces the formation of CB<sub>1</sub>-CB<sub>2</sub> heteromers as a mechanism to counteract neuroinflammation. The assembly of CB<sub>1</sub>-CB<sub>2</sub> heteromers generates negative interactions between CB receptors. In these heteromers, CB<sub>2</sub> activation antagonizes the inhibitory effect of CB<sub>1</sub> and facilitates excitation (Callén et al., 2012). In contrast, exposure to a CB<sub>2</sub> antagonist enhances the inhibitory effects of a CB<sub>1</sub> agonist (Rizzo et al., 2014).

Although different mechanisms can explain the side effects induced by endocannabinoids, no studies exist in epilepsy. Future studies are essential to determine the mechanisms by which activation of ECS may facilitate seizure activity.

## Endocannabinoids in epilepsy: To increase or not to increase

The decrease of glutamatergic neurotransmission by activating the ECS is considered a strategy for controlling epilepsy. However, it is essential to point out that such condition may cause adverse effects and increase the prevalence of psychiatric and cognitive disorders characteristic of epilepsy. In this regard, decreased glutamatergic neurotransmission resulting from CB<sub>1</sub> receptor activation in the hippocampus is known to be associated with cognitive impairment (Misner & Sullivan, 1999). Furthermore, endocannabinoid-mediated neurotransmission correlates with the expression of comorbidities in neurodegenerative disorders. For example, activation of CB<sub>1</sub> receptors at the hippocampal level can alter the process of memory formation, mainly short-term memory (Robbe et al., 2006). Moreover, blockade of the CB<sub>1</sub> receptor in animal models has been shown to reduce amnesia produced by β-amyloid fragments (Mazzola et al., 2003), while upregulation of this receptor in the hippocampus induces cognitive impairment (Nawata et al., 2010).

Increased neurotransmission of the ECS can induce antidepressant and anxiolytic effects, which may be beneficial in reducing psychiatric comorbidities in patients with epilepsy. However, evidence has shown that CB<sub>1</sub> receptor activation can induce anxiogenic effects. In addition, schizophrenia is associated with increased CB<sub>1</sub> receptor expression in the cingulate and dorsolateral prefrontal cortices (Zavitsanou et al., 2004). Furthermore, augmented endocannabinoid neurotransmission facilitates schizophrenia-like responses (Kruk-Slomka et al., 2019), whereas blockade of CB<sub>1</sub> receptors induces antipsychotic effects.

The group of evidences presented throughout this chapter indicates that the ECS induces different effects according to the circumstances. This knowledge is relevant for the appropriate application of therapeutic strategies associated with the modulation of the ECS resulting in beneficial effects and preventing adverse outcomes in patients with epilepsy. For this purpose, it is essential to use preclinical models that reproduce specific types of epilepsy and their comorbidities at different ages and different degrees of cell damage, chronicity, excitotoxicity, and neuroinflammation. It is also necessary to determine the effects of cannabinoids and endocannabinoids in the brain tissue obtained from patients with drug-resistant epilepsy undergoing surgery.

## Applications to other areas

In this chapter, we have reviewed the dynamic nature of the ECS components associated with seizures and epilepsy. Studies support that the effects of ECS can be pro- or antiepileptic, depending on the brain maturation, the rate of epileptogenesis, and other factors (Goffin et al., 2011). The dual effects of ECS could be replicated in other pathologies that share pathological conditions, such as high excitotoxicity, neuroinflammation, oxidative stress, and BBB leakage. For example, evidence indicates that ECS enhancement exerts neuroprotective effects in Parkinson's disease. In contrast, other evidence shows that increased endocannabinoid concentrations are associated with the disease progression whereas CB<sub>1</sub> inverse agonism has antiparkinsonian actions. In Alzheimer's disease, CB receptors agonism induces protective effects against β-amyloid neurotoxicity. Conversely, CB<sub>1</sub> blockade counteracts memory impairment caused by β-amyloid fragments (Mazzola et al., 2003). Overall, this evidence supports the role of ECS in modulating the pathological course of several neurological diseases. However, the complexity of ECS requires consideration of the timing and direction of manipulation (agonism, antagonism, and inverse agonism).

Other important issue is that the study of the ECS effects in preclinical studies has to involve experimental models that replicate most of the characteristics of the human brain with epilepsy, such as a longer duration of the disease, comorbid disorders, and the chronic administration of antiseizure medications.

## Mini-dictionary of terms

- **Epilepsy:** A neurological disease in which there is a permanent predisposition to develop seizures.
- **Seizures:** Manifestation of signs and symptoms due to neuronal hyper synchronization.
- **Drug resistance in epilepsy:** Inability to control seizures regardless of adequate antiseizure medication.
- **Excitotoxicity:** Cell death due to overactivation of excitatory receptors.
- **Blood-brain barrier:** Endothelial cell module that regulates molecular exchange between blood and brain interstitial fluid.
- **Neurovascular unit:** A group of cellular (neurons, astrocytes, microglia, pericytes, and endothelial cells) and noncellular (basement membrane) components responsible for homeostasis and regulation of cerebral blood flow.

## Key facts of epilepsy

- Hyperexcitable neuronal circuits characterize epilepsy.
- Increased excitatory transmission and reduced inhibitory tone lead to seizure expression.
- Temporal lobe epilepsy is the most frequent type of focal epilepsy.
- Recurrent seizures result in the accentuation of excitotoxic, neuroinflammatory, and oxidative stress processes.
- Damaging processes promote an irritative environment that may compromise the integrity of the BBB.

## Summary points

- ECS modulates excitatory and inhibitory synaptic transmission.
- Activation of ECS is considered a strategy for treating neurological disorders.
- The ECS induces antiepileptic and protective effects.
- Activation of ECS in the brain may exert harmful effects, depending on the local environment within the CNS.
- ECS may facilitate seizure activity through different mechanisms.

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## Chapter 17

# The endocannabinoid system and autoimmune demyelination: A focus on multiple sclerosis

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## Abbreviations

2-AG	2-arachidonoylglycerol
AA	arachidonic acid
ABHD12	$\alpha/\beta$ -hydrolase domain containing 12
ABHD6	$\alpha/\beta$ -hydrolase domain containing 6
AEA	<i>N</i> -arachidonoyl-ethanolamide
CB <sub>1</sub> R	type 1 cannabinoid receptor
CB <sub>2</sub> R	type 2 cannabinoid receptor
CBD	cannabidiol
CNS	central nervous system
DGL	diacylglycerol lipase
EAE	experimental autoimmune encephalomyelitis
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
FABP	fatty acid binding proteins
MAGL	monoacylglycerol lipase
MS	multiple sclerosis
NAPE	<i>N</i> -arachidonoyl phosphatidylethanolamine
NMO	neuromyelitis optica
OEA	<i>N</i> -oleoylethanolamine
PE	phosphatidylethanolamine
PEA	<i>N</i> -palmitoylethanolamine
POMS	pediatric-onset multiple sclerosis
TMEV-IDD	Theiler's murine encephalomyelitis virus-induced demyelinating disease
$\Delta^9$ -THC	delta-9-tetrahydrocannabinol

## Introduction

Autoimmune demyelinating diseases are pathological conditions in which immune-mediated attacks damage the myelin sheath and compromise axonal function in the nervous system. Autoimmune demyelination is the hallmark of etiologically heterogeneous syndromes such as acute disseminated encephalomyelitis, neuromyelitis optica (NMO), transverse myelitis, and multiple sclerosis (MS), which stands out as the most common neurological disease that typically affects young adults, and causes irreversible physical and mental disability (Dendrou et al., 2015). The endocannabinoid system (ECS) has been deeply investigated in MS based on early reports of therapeutic benefits by patients self-medicating cannabis derivatives.

Research in the field was further fueled by the discovery and characterization of cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors (CB<sub>1</sub>Rs, CB<sub>2</sub>Rs) as the main pharmacological targets of the plant-derived cannabinoid Δ<sup>9</sup>-tetrahydrocannabinol (Δ<sup>9</sup>-THC) in the late 1980s and early 1990s (Pertwee et al., 2010). Nowadays, available evidence demonstrates that (endo)cannabinoids exert symptom control and engage neuroprotective, antiinflammatory, and regenerating effects in experimental MS (Chiurchiù et al., 2018). It is also well established that most therapeutic effects of (endo)cannabinoids in MS rely on the activation of cannabinoid CB<sub>1</sub>Rs and CB<sub>2</sub>Rs highly expressed in neurons and immune cells, respectively (Pertwee et al., 2010). At the clinical level, a number of studies have been conducted to assess the efficacy of different cannabinoid formulations on MS symptoms, mainly spasticity and pain, with mixed results, and conclusive data on the benefits of cannabinoids in MS patients are nowadays still sparse. Despite controversy, research on the role of the ECS in MS has successfully culminated in the approval and commercialization of nabiximols as the first botanical medicine for symptom control in MS (Kmietowicz, 2010). A significant limitation of nabiximols and other cannabinoid preparations as demonstrated by clinical assessments in MS patients is that positive effects are often paralleled by adverse responses, such as psychoactivity or memory impairments, which limit their therapeutic potential. At present, endocannabinoid research in MS aims at firmly establishing the potential of targeting endocannabinoid hydrolysis as potential strategy to engage CB<sub>1</sub>R/CB<sub>2</sub>R dependent and independent benefits while limiting adverse effects.

## Autoimmune demyelination: Focus in multiple sclerosis

### Definition, epidemiology, and clinical classification

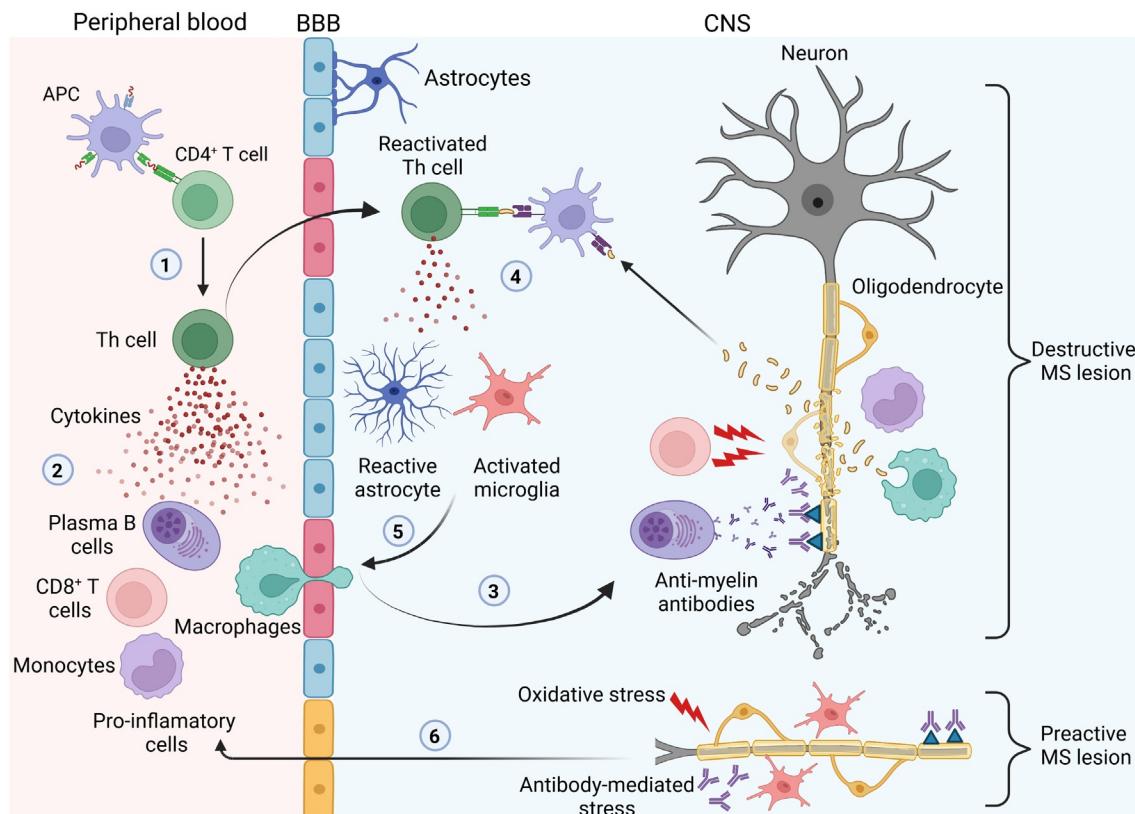
MS is a chronic demyelinating, inflammatory disease affecting 2.8 million people worldwide, and about two-thirds of those affected are women. The causes of MS remain elusive though there is evidence that both genetic and environmental factors contribute to the pathophysiology of this illness. Its principal features are focal lesions with inflammatory reaction to myelin leading to oligodendrocyte death and demyelination and axonal damage (Lassmann, 2018). Demyelination may occur within any area of the central nervous system (CNS) and results in slower axon potential propagation or complete failure of transmission, which causes neuronal dysfunction and a variety of neurological symptoms that depend on the location of the lesions.

MS is a heterogeneous disease clinically and pathologically. Clinical phenotypes are classified into four main groups taking into account magnetic resonance imaging of lesion activity, progression of disability, and the disease symptomatology: clinically isolated syndrome, relapsing-remitting, secondary progressive, and primary progressive. Relapsing-remitting is the most common form, affecting approximately 85%–90% of patients and in most instances (80%) develops into a progressive stage with irreversible neurological decline. Relapses coincide with focal CNS inflammation and demyelination along with mechanisms that remodel and compensate the damage. In turn, primary progressive MS affects 10%–15% of the patients, and the disease course progresses readily from the onset.

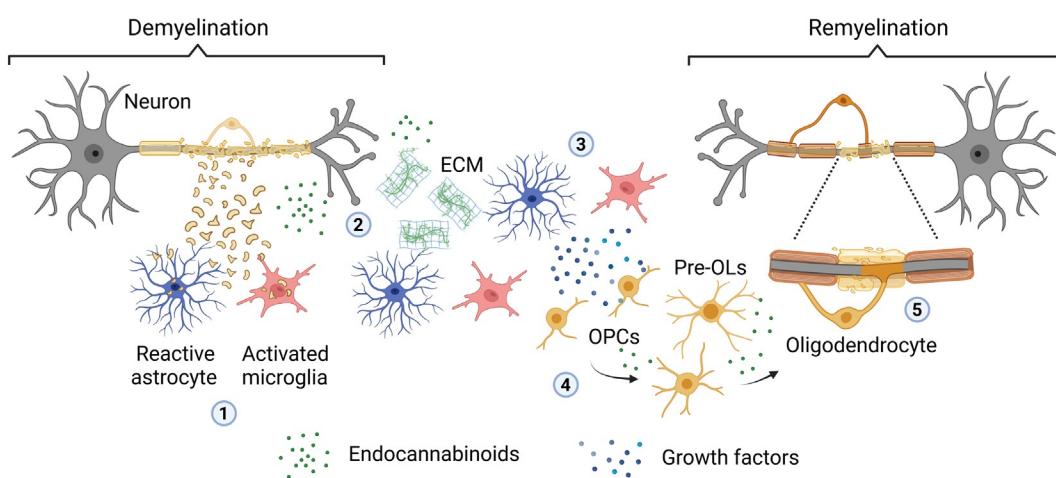
### Mechanisms of myelin damage and repair

The pathological hallmark in MS is the presence of focal areas of inflammatory-mediated demyelination in the brain and spinal cord, indicative of myelin and oligodendrocyte loss, which are called demyelinating plaques or lesions. Initially, axons and neurons are preserved; however, disease progression results in gradual neuroaxonal loss that correlates with patient disability and brain atrophy. Inflammation is present at all stages of the disease being more prominent in the acute, relapsing-remitting stage than in the chronic or progressive phase.

At the beginning of the disease, autoreactive T cells that are activated through molecular mimicry at peripheral sites penetrate the blood-brain barrier, infiltrate the CNS, and attack the oligodendrocytes and myelin. Predominant infiltrating cells in MS lesions include macrophages, CD8<sup>+</sup> and CD4<sup>+</sup> T cells, B cells, and plasma cells (Lassmann, 2018) (Fig. 1). These infiltrates are present not only in white and gray matter lesions but also in normally appearing tissue. Moreover, they express immune-associated molecules, such as major histocompatibility antigens and adhesion molecules, and release inflammatory cytokines that ultimately cause myelin demise and subsequent tissue damage. Experimental evidence also supports the alternative hypothesis that MS can be initiated by oligodendrocyte-related defects or antibody deposition by B cells in myelin-producing glial cells, the latter of which has been consistently related to infection with Epstein-Barr virus (Lanz et al., 2022). Microglia and astrocytes, CNS-resident brain cells that sense homeostatic disturbances, are also recruited at early MS stages and contribute to the neurotoxic inflammatory cascade by releasing cytokines, chemokines, and reactive oxygen species (Scheiblrich et al., 2020). In turn, microglia/macrophage and astrocyte activation can also counteract pathological processes by providing neurotrophic and immunosuppressive factors that favor oligodendrocyte



**FIG. 1** Pathophysiology of multiple sclerosis. (1) Autoreactive CD4<sup>+</sup> T cells are activated in the periphery by oligodendrocyte/myelin antigen-presenting cells (APCs). (2) Differentiated CD4<sup>+</sup> T helper cells (Th) produce cytokines that recruit B cells, CD8<sup>+</sup> T cells, monocytes, and macrophages into the central nervous system. (3) B cells activated by molecular mimicry or in response to autoreactive T cells produce auto-antibodies against CNS self-antigens and cytotoxic CD8<sup>+</sup> T cells attack oligodendrocytes leading to cell death while monocytes and macrophages phagocytose myelin causing a destructive multiple sclerosis lesion. (4) Inflammatory responses by peripheral immune cells favor activation of astrocytes and microglia to different phenotypes. (5) Pro-inflammatory mediators and disrupted glutamate handling by glial cells act to recruit immune cells, aggravate axonal/oligodendrocyte toxicity, and impair remyelination. (6) The recruitment of peripheral immune cells can be initiated in normal appearing, preactive lesions that involve clusters of oligodendrocytes undergoing oxidative or antibody-mediated stress and activated microglia. BBB, blood-brain barrier; CNS, central nervous system; MS, multiple sclerosis. (Created with BioRender.com.)



**FIG. 2** Remyelination in multiple sclerosis: Regulation by endocannabinoids. Activated microglia and/or astrocytes can support remyelination via (1) clearance of myelin debris, (2) secretion of growth factors, and (3) modulation of the extracellular matrix. (4) Oligodendrocyte precursor cells recruited under the influence of neuroglial factors differentiate into preoligodendrocytes and myelin producing, mature oligodendrocytes that engage with demyelinated axons enabling lesion repair (5). Endocannabinoids act on glial cells to facilitate remyelination. ECM, extracellular matrix; OPC, oligodendrocyte precursor cell; Pre-OL, preoligodendrocyte. (Created with BioRender.com.)

differentiation from their progenitors and recovery (das Neves et al., 2021; Lampron et al., 2015) (Fig. 2). Thus, demyelinated areas can be partially repaired by remyelination, but at the chronic phase, these repairing mechanisms are not as efficient as in the initial phases of the disease. Indeed, a failure in the repairing mechanisms and an exhaustion of the neurological reserves determine the transition from relapsing-remitting to secondary progressive MS (Franklin et al., 2012).

## Current therapeutic strategies in multiple sclerosis

Several immunomodulatory therapies are currently available to treat efficiently relapsing-remitting MS. These disease-modifying treatments target T and B cells activation and immune cell infiltration in the CNS and efficiently reduce relapses and their severity. However, they do not halt disease progression, and neuroaxonal damage continues to accumulate resulting in an inevitable chronic phase of the disease (Callegari et al., 2021). There are no effective treatments available for primary progressive MS, supporting the idea that there could be mechanisms involved in driving overt relapses different from those underlying chronic progression. There is thus a clear need for developing new neuroprotective and regenerative agents that efficaciously slow down MS progression, thus acting as disease-modifying therapies. In this regard, exploiting the homeostatic and reparative roles of astrocytes and microglia, not yet targeted with current medications, has the potential to develop novel treatments for MS (Bernal-Chico et al., 2022). A goal for future treatment of MS and other autoimmune demyelinating diseases should be a combination of modulation of peripheral immune cells and neuroglial cells, along with the provision of neuroprotective or neuroregenerative drugs.

## The endocannabinoid system

### (Endo)cannabinoid receptors

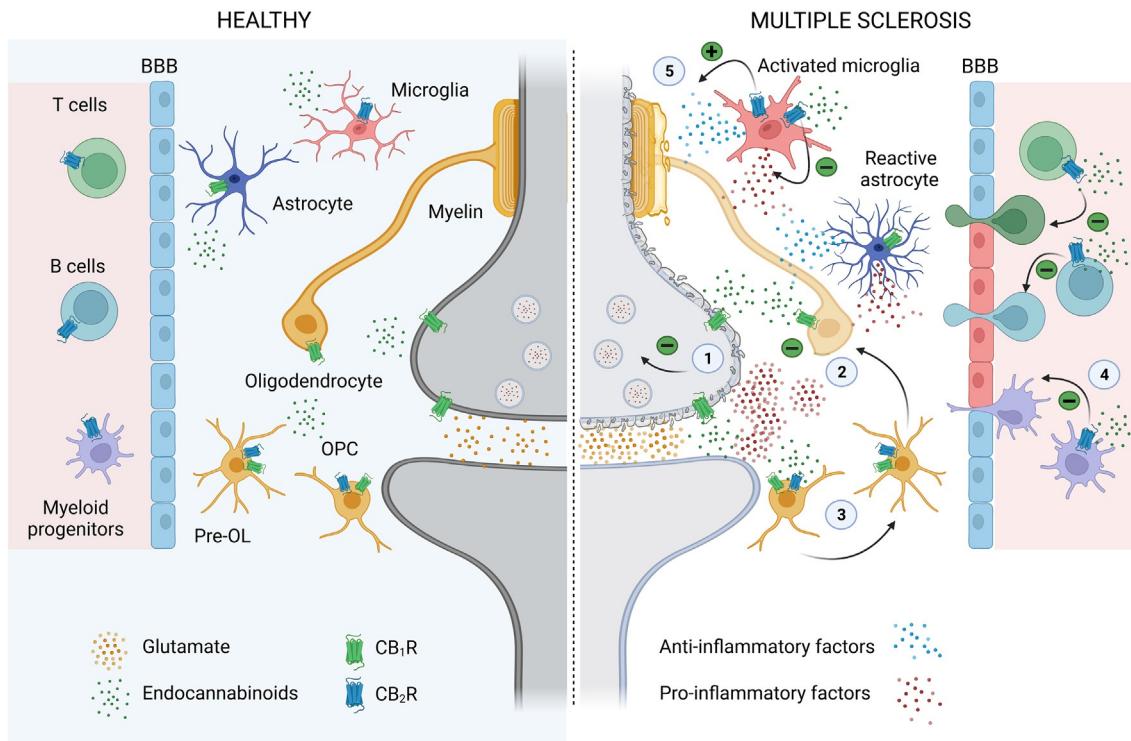
Discovery of  $\Delta^9$ -THC as the main psychoactive compound in *Cannabis sativa* preparations was soon thereafter followed by the characterization of two main receptor proteins mediating their pharmacological effects, namely cannabinoid receptors type 1 (CB<sub>1</sub>R) and type 2 (CB<sub>2</sub>R) (Pertwee et al., 2010). CB<sub>1</sub>Rs have been classically ascribed to the presynaptic compartment of neuronal populations through the brain, including both excitatory and inhibitory cells (Katona & Freund, 2012). Nowadays, there is consistent evidence supporting the expression of CB<sub>1</sub>Rs in glial cells, mainly astrocytes and oligodendroglia (Busquets-Garcia et al., 2018; Ilyasov et al., 2018) (Fig. 3). Conversely, CB<sub>2</sub>Rs are predominantly expressed in peripheral immune cells and tissues and present at very low levels in CNS-resident cells in physiological conditions (Atwood & Mackie, 2010). It is however well established that the CB<sub>2</sub>R is highly inducible in neuroglial cells, mainly microglia and to a lesser extent, astrocytes, under certain pathological conditions, which sustains the potential of targeting this receptor protein for the therapeutic control of neuroinflammation (Fig. 3).

## Endocannabinoids: Biosynthesis and catabolism

Endocannabinoids are fatty acid derivatives that bind and activate cannabinoid receptors. The compound *N*-arachidonoyl-ethanolamide (anandamide or AEA) is a partial CB<sub>1</sub>R and CB<sub>2</sub>R agonist and a full agonist of the transient receptor potential vanilloid subfamily member 1, with complex pharmacological activity in the CNS. Conversely, the endocannabinoid 2-arachidonoylglycerol (2-AG) is a full CB<sub>1</sub>R and CB<sub>2</sub>R agonist and found at higher levels in the brain tissue than AEA (nmol vs pmol/g tissue). The classical view is that these small messengers are synthetized and released “on-demand,” yet, a tonic signaling and the existence of distinct pools have been also proposed (Katona & Freund, 2012).

### Biosynthesis

Neurons and glial cells synthesize endocannabinoids in a process normally initiated by cytosolic calcium rises. Neuronal synthesis of endocannabinoids occurs postsynaptically following stimulation glutamatergic (mGluR<sub>1/5</sub>), muscarinic (M1/M3), or dopaminergic (D2) receptors coupled to G<sub>q</sub> proteins (Katona & Freund, 2012). The main pathway for AEA biosynthesis involves transfer of arachidonic acid (AA) to phosphatidylethanolamine by the enzyme *N*-acyltransferase to form *N*-arachidonoyl phosphatidylethanolamine (NAPE), which is hydrolyzed to AEA by a specific phospholipase D (NAPE-PDL) (Di Marzo, 2008). 2-AG is synthesized through hydrolysis of membrane phospholipids by PLC into diacylglycerol, which subsequently is degraded into 2-AG by the postsynaptic-integral membrane protein diacylglycerol lipase (DGL $\alpha/\beta$ ) (Di Marzo, 2008). The  $\alpha$  isoform of the enzyme is primarily expressed in neurons and astroglia, whereas 2-AG synthesis in microglia/macrophages relies on DGL $\beta$  (Viader et al., 2016).



**FIG. 3** The endocannabinoid system in multiple sclerosis. The left panel depicts the location of cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors in different cell types involved in multiple sclerosis. During disease onset and progression, (1) neuronal CB<sub>1</sub> receptors inhibit glutamate release and attenuate axonal degeneration, (2) CB<sub>1</sub> receptors in mature oligodendrocytes engage protection from excitotoxicity, (3) CB<sub>1</sub>/CB<sub>2</sub> receptors in oligodendrocyte precursor cells facilitate remyelination by promoting proliferation and differentiation to preoligodendrocytes (pre-OLs) and mature cells. (4) CB<sub>2</sub> receptors suppress the infiltration of T cells and myeloid progenitors into the CNS. (5) CB<sub>2</sub> receptors upregulate their expression in microglial cells and tune their activation toward antiinflammatory phenotypes that facilitate myelin repair. BBB, blood-brain barrier; OPC, oligodendrocyte precursor cell. (Created with BioRender.com.)

### Catabolism

The size and duration of endocannabinoid signaling are strictly regulated through hydrolysis by two principal catabolic enzymes, fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MAGL) for 2-AG. Several fatty acid binding proteins (FABPs) that deliver endocannabinoids to their catabolic enzymes have also been recently identified (Kaczocha et al., 2009). Concerning enzymatic hydrolysis, postsynaptically located FAAH converts AEA into AA and ethanolamine and is also involved in the metabolism of other bioactive *N*-acylethanolamines, such as *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA). Alternatively, *N*-acylethanolamines can also be hydrolyzed by lysosomal *N*-acylethanolamine-hydrolyzing acid amidase, whose activity is optimal at a pH of 4.5–5. The enzyme MAGL is predominantly found on presynaptic terminals and responsible for the 85% of 2-AG hydrolysis in the brain, producing AA and glycerol (Blankman et al., 2007). Two additional serine hydrolases capable of metabolizing 2-AG have been identified, namely  $\alpha/\beta$ -hydrolase domain containing 6 and 12 (ABHD6 and ABHD12) (Blankman et al., 2007). ABHD12 accounts for approximately 9% of total 2-AG hydrolase activity in the brain, whereas ABHD6 hydrolyzes only 4% of 2-AG in physiological conditions and is mainly expressed postsynaptically in principal glutamatergic neurons as well as in microglial cells (Marrs et al., 2010). As an alternative to hydrolysis, the activity of both AEA and 2-AG can be terminated through oxidation by some enzymes of the arachidonate cascade, in particular the 12- and 15-lipoxygenases, cytochrome p450 oxygenases, and cyclooxygenase-2, leading to the formation of prostaglandins (Di Marzo, 2008). However, the biological significance of these reactions *in vivo* remains to be firmly established.

Although the ECS has been mainly studied as a neuroregulatory system, glial cells express the enzymes for endocannabinoid synthesis, transport, and catabolism and thus the potential to modulate ECS signaling in physiological and pathological conditions (Bernal-Chico et al., 2022). Astrocytes and microglia *in vitro* synthesize AEA and 2-AG in response to increases in intracellular calcium induced by different stimuli such as ATP or endothelin-1. Cultured oligodendrocytes and oligodendrocyte precursor cells (OPCs) display constitutive generation of endocannabinoids, which seem to act as

autocrine regulators for survival and maturation (Ilyasov et al., 2018). Regarding catabolism, astrocytes, microglia, and oligodendrocytes predominantly express MAGL and ABHD6 activities. Remarkably, astrocytic MAGL is crucially involved in the termination of neuronal 2-AG signaling in vivo (Viader et al., 2015). On the other hand, microglia display significant ABHD6 activity whose inhibition promotes cell migration in vitro, and it is postulated that this enzyme may acquire important roles in 2-AG modulation of microglial responses during CNS inflammatory processes (Marrs et al., 2010).

## Physiological roles

The ECS modulates a variety of physiological activities such as learning and memory, feeding, anxiety, movement, development, pain control, and metabolism, among others. Regulation of CNS functions by endocannabinoids classically involves the neuronal population of CB<sub>1</sub>Rs that inhibits neurotransmission and mediates several forms of synaptic plasticity through the CNS (Araque et al., 2017). Conversely, astrocytic CB<sub>1</sub>Rs promote intracellular calcium elevations leading to the release of gliotransmitters such as glutamate or ATP that in turn modulate synaptic communication. The mitochondrial population of CB<sub>1</sub>Rs inhibits respiration in neurons and astrocytes and participates in the regulation of memory and social behaviors by endocannabinoids (Araque et al., 2017; Jimenez-Blasco et al., 2020). CB<sub>1</sub>Rs/CB<sub>2</sub>Rs in oligodendrocyte lineage cells promote proliferation, survival, and differentiation in vitro and likely mediate the myelination and remyelination promoting effects of cannabinoids in vivo (Huerga-Gómez et al., 2021; Ilyasov et al., 2018). Cannabinoids also modulate cell motility, phagocytosis, proliferation, and migration of microglia cells in vitro acting mainly through CB<sub>2</sub>Rs (Komorowska-Müller & Schmöle, 2020). However, while the ability of microglial CB<sub>2</sub>Rs to attenuate inflammation is well recognized, the role of endocannabinoids in regulating microglia functions in the nonpathological CNS remains elusive. Finally, CB<sub>1</sub>Rs modulate the survival, proliferation, migration, and maturation of neural stem cells, and endocannabinoids are nowadays regarded as important players in neurodevelopment and adult neurogenesis (Oddi et al., 2020).

## Role and therapeutic potential of (endo)cannabinoids in demyelination

### Deregulation of the endocannabinoid system in multiple sclerosis

A role for the ECS in the development of autoimmune demyelination is supported by preclinical and clinical evidence showing deregulation of endocannabinoid signaling in MS. Yet, there is no current consensus on whether the ECS over-expressed or impaired in MS and the prevailing hypothesis is that limited changes take place in a disease-stage dependent manner. Hypertrophic astrocytes in demyelinating lesions display augmented FAAH expression, and experimental evidence shows unaltered or even reduced levels of AEA and 2-AG, these findings suggesting the presence of an impaired ECB system in MS (Benito et al., 2003; Di Filippo et al., 2008). Conversely, increased AEA concentrations have also been demonstrated in MS patients and autoimmune disease models and associated to imbalanced NAPE-PLD/FAAH activities affecting immune and neuroglial cells (Centonze et al., 2007; Jean-Gilles et al., 2009; Moreno-García et al., 2020). Interestingly, available evidence suggests that the levels of AEA increase during relapses, suggesting its potential to limit ongoing inflammation (Di Filippo et al., 2008). On the other hand, NMO patients display elevated 2-AG plasma levels that negatively correlate to pain sensitivity (Pellkofer et al., 2013). Concerning cannabinoid receptors, the most consistent observation is the upregulation of CB<sub>2</sub>R expression in immune cells and activated microglia within MS lesions (Benito et al., 2003; Maresz et al., 2007) (Fig. 3). At the light of the immunomodulatory and antiinflammatory pathways effects of endocannabinoids in MS, it is suggested that deregulation of endocannabinoid signaling is a homeostatic adaptive response aimed to neutralize symptoms and progression.

### Targeting demyelination with (endo)cannabinoids

The discovery of CB<sub>1</sub>Rs as main targets for cannabinoid compounds in the CNS prompted studies on the therapeutic effects and mechanisms of action of these compounds in MS. Research in this field has been continuously encouraged by reports affirming reduced relapses and attenuated symptomatology in patients self-medicating cannabis preparations (Clark et al., 2004; Consroe et al., 1997; Nielsen et al., 2018) and has culminated in the recent approval of nabiximols (Sativex), an oromucosal spray containing a 1:1 mixture of Δ<sup>9</sup>-THC and cannabidiol (CBD) in several countries (Kmietowicz, 2010). Studies addressing the effects of cannabis derivatives and cannabinoids in MS patients demonstrate relief from spasticity and pain, decreased tremor/ataxia, and better quality of sleep, associated to the perception of an improved quality of life, and cannot be disregarded. However, the use of cannabinoid therapies in MS is not devoid of limitations and the

cellular/molecular mechanisms underlying the benefits and unwanted effects of cannabinoid therapies in MS patients and is still under debate.

Initial studies using plant-derived and synthetic cannabinoids, as well as endogenous cannabinoid compounds, demonstrated neuroprotective effects in a variety of rodent MS models such as experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) (Chiurchiù et al., 2018). Neuroprotection by (endo)cannabinoids was soon thereafter associated to the activation of neuronal CB<sub>1</sub>Rs based on results on constitutive and conditional null-mice (Baker et al., 2001; Maresz et al., 2007) (Fig. 3). Early pharmacological and genetic studies also put forward antiinflammatory effects mediated by CB<sub>2</sub>Rs expressed by T cells, myeloid progenitors, and microglia (Arévalo-Martín et al., 2003; Maresz et al., 2007; Palazuelos et al., 2008) (Fig. 3). More recently, a number of in vitro and in vivo evidences have suggested that cannabinoids may limit MS progression by promoting oligodendrocyte differentiation and (re)myelination through the activation of CB<sub>1</sub>Rs and CB<sub>2</sub>Rs present in these cells (Aguado et al., 2021; Ilyasov et al., 2018) (Fig. 3). On the other hand, the nonpsychoactive phytocannabinoid CBD and the endocannabinoid PEA have also been investigated for potential effects in MS. These compounds attenuate immune responses by T cells and microglia and preserve neurological function during EAE despite lacking affinity for CB<sub>1</sub>Rs (Rahimi et al., 2015). In all, there is robust preclinical evidence supporting that exogenous cannabinoids and endocannabinoids attenuate MS symptomatology and limit disease progression. However, clinical experience with cannabis-based medicines has also evidenced that symptom relief is limited to the use of high doses, which engages a number of unwanted side effects associated to the bulk activation of cannabinoid receptor populations. These side effects include psychoactivity, anxiety, disorientation, blood pressure changes, and fainting, among others.

During the last decade, research on the ECS in MS has evolved from the utility of exogenous cannabinoids to the potential of targeting endocannabinoid hydrolysis as strategy to promote ECS signaling and symptom relief with minimal side effects. Both 2-AG and AEA inhibit spasticity and attenuate neurodegeneration in murine models of immune-dependent demyelination (Baker et al., 2001). In addition, the endocannabinoid 2-AG, but not AEA, protects oligodendrocytes from excitotoxicity in vitro (Bernal-Chico et al., 2015) (Fig. 3). Treatment with MAGL inhibitors attenuates neurological disability, demyelination, and immune responses in most preclinical models of MS including EAE, TMEV-IDD, and cuprizone feeding (Bernal-Chico et al., 2015; Hernández-Torres et al., 2014). Additional studies also show that promoting 2-AG signaling facilitates remyelination by (1) improving the phagocytic capacity of microglial cells and their phenotypic activation to an antiinflammatory state, (2) attenuating astrocytic production of extracellular matrix components inhibitory to remyelination, and (3) promoting OPC differentiation (Fig. 2) (Feliú et al., 2017; Ilyasov et al., 2018; Mecha et al., 2016). Mechanistically, the benefits of 2-AG inhibitors may be related both to the activation of CB<sub>1</sub>Rs and CB<sub>2</sub>Rs (Fig. 3) and to the inhibition of prostaglandin production (Nomura et al., 2011), although studies specifically addressing this possibility in MS are still lacking. However, irreversible MAGL inhibitors rapidly desensitize CB<sub>1</sub>R leading to functional tolerance to potential therapeutic benefits that rely on the activation of these receptor population (Schlosburg et al., 2010). The utility of targeting ABHD6 to promote protective effects in MS has also recently been evaluated in pre-clinical models with conflicting results (Manterola, Bernal-Chico, Cipriani, Canedo-Antelo, et al., 2018; Manterola, Bernal-Chico, Cipriani, Ruiz, et al., 2018). Finally, pharmacological and genetic studies have also put forward antiinflammatory and neuroprotective effects of FAAH inhibitors in MS mouse models (Pryce et al., 2013; Webb et al., 2008). However, FAAH knockout mice do not exhibit improved EAE severity during the acute inflammatory phase of the disease despite possessing 15-fold augmented endogenous brain levels of AEA and normal CB<sub>1</sub>R function. These results suggest a limited therapeutic potential for FAAH inhibitors in MS patients. Finally, inhibition of FABPs seems to harness therapeutic effects in MS models via immune inhibition and oligodendrocyte protection, but the possible involvement of endocannabinoids and CB<sub>1</sub>Rs/CB<sub>2</sub>Rs has not been demonstrated (Cheng et al., 2021; Reynolds et al., 2007).

## Clinical studies with cannabinoids in demyelinating disorders

Cannabis-based medications have been extensively investigated for MS symptomatology in patients. Most clinical trials have been performed using nabiximols, nowadays available in 25 countries, including most of Europe, Mexico, and Canada. Although only a minority of studies has reported beneficial effects, cannabinoids have been found to be safe and effective.

A number of metaanalyses report that nabiximols is an effective option for the treatment of spasticity, neuropathic pain, and urinary infections in MS (Conte & Vila Silván, 2021; Torres-Moreno et al., 2018). Clinical evidence also supports an improved clinical outcome following the combination of Sativex with other antispastic agents (Meuth et al., 2020). From a mechanistic perspective, specific cortical and spinal circuits presumably concerning both excitatory and inhibitory pathways have been associated to the clinical benefits of cannabis medications in MS (Russo et al.,

2016). Studies on the immunomodulatory effects of cannabinoids in blood samples are conflicting, but downregulation of several immune-related pathways has been recently reported in MS patients treated with Sativex (Sorosina et al., 2018).

A significant caveat of MS management with cannabis-based medicines is that therapeutic efficacy is restricted to high doses usually accompanied undesired effects, and many therapeutic effects observed in animal models of the disease are not transferable to patients (Baker et al., 2012). Current research on the therapeutic potential of the ECS in MS aims at developing strategies to target specific CB<sub>1</sub>R and CB<sub>2</sub>R populations while diminishing the adverse effects of exogenous cannabinoids, by targeting endocannabinoid hydrolysis. In this context, MAGL inhibitors are nowadays in the spotlight for the treatment of a number of neurodegenerative conditions including MS. Despite rapidly growing interest during the last decade, most of the patented compounds still belong to the large group of irreversible MAGL inhibitors (Bononi et al., 2021). A second line of research supports the therapeutic possibilities of CBD in autoimmune demyelination (Navarrete et al., 2021). This compound stands out as promising drug with disease-modifying potential in MS due to its neuroprotective, immunomodulatory, and safety profile and the lack of psychoactivity (Furgiuele et al., 2021). However, the cellular and molecular targets of CBD remain largely unexplored, and few clinical trials have been conducted, most of them with negative results due mainly to deficient therapeutic management. The recent approval of CBD oral solution (Epidiolex) by the Food and Drug Administration as therapy for severe childhood epilepsy may pave the way for future clinical research in MS and other autoimmune demyelinating conditions.

## Applications to other areas

Research on the role and therapeutic potential of the ECS in autoimmune demyelinating disorders is mostly restricted to studies in MS. The extent to which the benefits of (endo)cannabinoids in MS patients, and the underlying mechanisms, can be extended to additional immune-related demyelinating diseases has been poorly investigated. It is worth mentioning that anecdotal evidence supports promise for cannabinoid treatments in the symptomatic management of NMO and transverse myelitis (Pellkofer et al., 2013; Tisavipat et al., 2020). An additional question to be addressed is the utility of (endo)cannabinoid therapies in pediatric-onset multiple sclerosis (POMS). This form of the disease begins in children or teens with around 98% displaying relapsing-remitting MS. Pediatric MS accounts for 3%–10% of all MS cases and presents a more aggressive onset and disease course with higher relapse rates and greater tissue damage than adult-onset MS. As for other pediatric diseases, current therapeutic agents to treat POMS derive mostly from studies on adult populations. Large well-controlled studies with cannabinoid therapies in POMS have not been conducted and are discouraged by the psychoactive adverse effects of targeting CB<sub>1</sub>Rs with compounds such as Δ<sup>9</sup>-THC, which limit their utility in pediatric populations. Assessments of the efficacy of cannabinoids in ameliorating pediatric spasticity associated to other conditions (e.g., cerebral palsy) in small populations have reported limited beneficial effects. An avenue of future research in this field may be addressing the utility of nonpsychoactive CBD, recently approved for otherwise unmanageable pediatric epilepsy, in POMS.

## Mini-dictionary terms

**Myelin.** Specialized multilayer membrane that wraps around axons and enables fast and efficient nerve conduction while providing protection and metabolic support to sustain neuronal activity. CNS myelin is generated by oligodendrocytes, which in turn generate from oligodendrocyte precursor cells both during development and adulthood.

**Demyelination.** Damage and degeneration of the myelin sheath. It is the pathological hallmark of demyelinating disorders and can occur as a consequence of immune-mediated attacks to oligodendrocytes, primary oligodendrocyte defects, or axonal disturbances.

**Remyelination.** Repair process by which the damaged myelin is partially restored. Remyelination is driven by oligodendrocyte precursor cells that differentiate to mature, myelin-producing oligodendrocytes that enable repair and is associated to neurological improvement in MS.

**Spasticity.** Pathological condition characterized by abnormal muscle tightness that impedes movement. It can be caused by neurological pathologies of diverse origin including MS, cerebral palsy, stroke, or traumatic injury.

**(Endo)cannabinoids.** Compounds that bind and activate cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors. This term englobes endogenous lipid mediators termed endocannabinoids, mainly 2-AG and AEA, and exogenous (plant-derived or synthetic) compounds.

## Key facts of multiple sclerosis

1. Multiple sclerosis is the most prevalent demyelinating disease affecting young adults.
2. Genetic and environmental factors contribute to multiple sclerosis pathophysiology.
3. Multiple sclerosis is characterized by focal areas of inflammatory reaction leading to demyelination and axonal damage.
4. Autoreactive T and B cells activated by molecular mimicry are crucially involved as pathogenic mechanism in multiple sclerosis.
5. Demyelinated areas can be partially repaired by remyelination.
6. Astrocytes and microglial cells contribute to both the neurotoxic inflammation and the repair of damaged myelin in multiple sclerosis.
7. Immunomodulatory therapies targeting T and B reduce relapses but do not halt progressive neurodegeneration.
8. Current research aims at providing neuroprotective and neuroregenerative drugs with the potential to slow down disease progression.

## Summary points

1. Endocannabinoid signaling is dysregulated in MS.
2. Endocannabinoids and cannabinoids exert protective effects in experimental MS acting through CB<sub>1</sub>Rs and CB<sub>2</sub>Rs.
3. Therapeutic mechanisms of (endo)cannabinoids in MS include neuroprotection, antiinflammatory activity, and remyelination promoting effects.
4. MS patients self-medicating cannabis preparations report symptom relief and better quality of life.
5. Nabiximols is an oromucosal spray containing a 1:1 mixture of Δ<sup>9</sup>-THC and CBD approved as treatment for spasticity in MS in several countries.
6. Symptom relief by cannabinoid therapies is limited to the use of high doses that engages a number of unwanted side effects.
7. Blockade of endocannabinoid hydrolysis is a promising therapeutic strategy in multiple sclerosis.

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## Chapter 18

# Endocannabinoid signaling in the lateral habenula regulates opioid addiction

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>ABHD4</b>	$\alpha,\beta$ -hydrolase-4
<b>AC</b>	adenylyl cyclase
<b>AEA</b>	anandamide
<b>aLH</b>	acute learned helplessness
<b>CAMKII</b>	calcium-dependent calmodulin 2
<b>CB<sub>1</sub></b>	cannabinoid-1
<b>CB<sub>2</sub></b>	cannabinoid-2
<b>cLH</b>	congenital learned helplessness
<b>CUS</b>	chronic unpredictable stress
<b>DA</b>	dopamine
<b>DALN</b>	diacetyl-levonantradol (DALN)
<b>DBS</b>	deep brain stimulation
<b>DRN</b>	dorsal raphe nucleus
<b>ECB</b>	endocannabinoid
<b>ECBS</b>	endocannabinoid system
<b>EPM</b>	elevated plus maze
<b>EPSC</b>	excitatory postsynaptic currents
<b>Hb</b>	habenula
<b>FAAH</b>	fatty acid amide hydrolase
<b>FISH</b>	fluorescent in situ hybridization
<b>GDE1</b>	glycerophosphodiesterase-1
<b>GPCR</b>	G-protein-coupled receptors
<b>GPi</b>	globus pallidus
<b>LFS</b>	low frequency stimulation
<b>LH</b>	learned helplessness
<b>LHA</b>	lateral hypothalamus
<b>LHb</b>	lateral habenula
<b>LTD</b>	long-term depression
<b>MAGL</b>	monoacylglycerol lipase
<b>MHb</b>	medial habenula
<b>MORs</b>	mu-opioid receptors
<b>mPFC</b>	medial prefrontal cortex
<b>MRN</b>	medial raphe nucleus
<b>NADA</b>	N-arachidonoyl dopamine
<b>NArPE</b>	N-arachidonoyl-phosphatidylethanolamines
<b>NAPE-PLD</b>	N-acyl-phosphatidylethanolamine specific phospholipase D
<b>PLC</b>	phospholipase C

<b>PKA</b>	protein kinase A
<b>REM</b>	rapid eye movement
<b>RMTg</b>	rostral medial tegmental area
<b>RTS</b>	restraint tail shock
<b>SDS</b>	social defeat stress
<b>SNC</b>	substantia nigra pars
<b>VTA</b>	ventral tegmental area

## Introduction

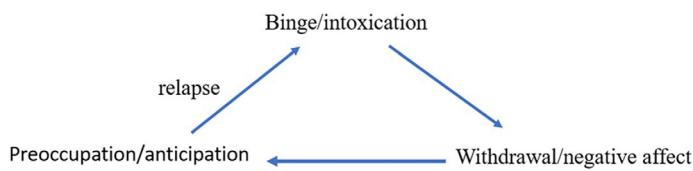
Despite decreases in opioid prescriptions, opioid overdose rates continued to rise in recent years. As of 2016, opioid addiction and overdose are the leading causes of preventable deaths across the nation, causing an average of 116 deaths daily ([National Institute on Drug Abuse, 2018](#)). Additionally, opioid dependency and tolerance affect over 2.1 million Americans. Misused prescription opioids impose over \$78.5 billion on the economy when considering factors such as healthcare costs, loss of productivity, addiction treatment, and criminal justice involvement ([National Institute on Drug Abuse, 2018](#)).

Opioids exert effects on the body by interacting with opioid receptors. Among the four types of opioid receptors,  $\mu$ -opioid receptors (MORs) are responsible for the rewarding properties opioid agonists such as morphine and fentanyl ([Lutz & Kieffer, 2013](#)). Although MORs are mainly known for producing an analgesic effect, MORs also regulate mood and appetite ([Peciña et al., 2019](#)), which points to their role as a key player in opioid addiction. Addiction results from repeated exposure to a drug. Although excessive intake of drugs interferes with daily activities, these negative consequences don't reduce the individual's drug-seeking behavior ([Befort, 2015](#)). There are three stages to addiction ([Fig. 1](#)). During the binge/intoxication stage, the drug activates reward pathways, which motivate one to continue using the drug. However, chronic exposure to a drug can cause neurobiological changes such as decreased dopaminergic and serotonergic transmissions. This leads to the second stage of addiction, the withdrawal/negative affect stage ([Koob & Volkow, 2016](#)). A person in withdrawal can exhibit an array of physical and emotional indicators such as increased pain, nausea and vomiting, flu-like symptoms, depression/anhedonia, dysphoria, and anxiety ([Scavone et al., 2013](#)). During this stage, one seeks drugs to avoid these negative emotional states. Generally, stronger addiction means a more complex withdrawal process and quicker onset of symptoms ([Meye et al., 2017](#)). A third stage, the preoccupation/anticipation stage, contributes to relapse ([Koob & Volkow, 2016](#)). In this chapter, we will focus on discussing systems involved in the withdrawal/negative affect stage and how that contributes to opioid addiction.

In recent years, the lateral habenula (LHb) received increasing attention for its emerging role in addiction and other biological functions. A well-conserved structure across almost all vertebrates, the LHb holds a lengthy ancestral background with a robust evolutionary significance ([Hikosaka, 2010](#)). Positioned at the center of both serotonergic and dopaminergic pathways, the LHb is essential in regulating and encoding negative emotional states ([Hikosaka, 2010](#)). This implies that it may contribute to drug-seeking behavior through negative reinforcement by promoting aversive states.

The endocannabinoid system (ECBS) is also involved in drug addiction. The ECBS is an essential mediator of stress and depression, and its dysfunction is associated with various medical conditions such as mood disorders and suicide. Most research studies how ECBS interacts with marijuana, which contains cannabinoids that activate the ECBS. Growing evidence of marijuana's therapeutic properties points to the potential benefit of modulating ECBS activities ([Dwivedi, 2012](#)).

Since both the LHb and the ECBS modulate negative emotional states, we hypothesize that endocannabinoid signaling in the LHb can regulate opioid addiction by interacting with MORs. This chapter will outline the individual roles of the ECBS and the LHb in the addiction processes. We will then focus on how the ECBS functions in the LHb, *in vivo* and *in vitro*, and their correlations to the development of different phenotypes.



**FIG. 1** Three addiction stages.

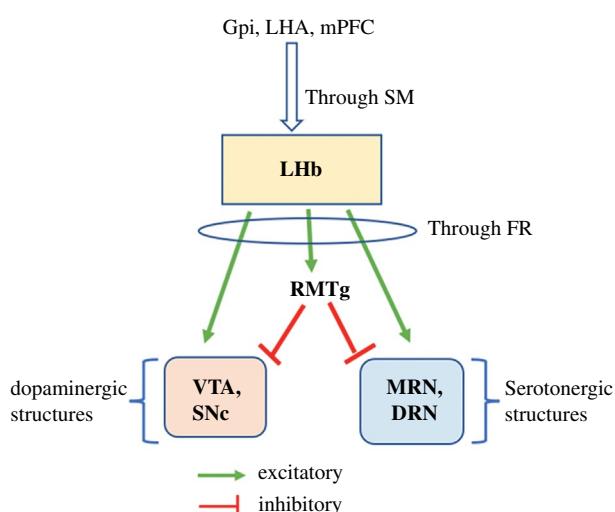
## The lateral habenula

Together with the medial habenula, pineal gland, and the stria medullaris, the lateral habenula (LHb) forms the epithalamus at the posterior-dorsal-medial end thalamus, located adjacent to the third ventricle (Shelton et al., 2012). The LHb interacts with several pathways, which points to its biological importance in several systems. The globus pallidus (GPi), lateral hypothalamus (LHA), and medial prefrontal cortex (mPFC) supply the LHb with afferent neurons through the stria medullaris (Hikosaka, 2010). The LHb's activity directly or indirectly affects downstream areas, such as the ventral tegmental area (VTA), substantia nigra pars (SNc), dorsal raphe nucleus (DRN), and median raphe nucleus (MRN) (Hikosaka, 2010). Outgoing signals from the LHb are mediated by the rostral medial tegmental area (RMTg) through the fasciculus retroflexus, also known as the habenula-interpeduncular tract. These signals ultimately inhibit the activity of dopaminergic structures, such as the VTA and the SNc, and the activity of serotonergic structures, such as the MRN and DRN (Hikosaka, 2010). However, a subsection of lateral habenular neurons can also excite the VTA, SNc, MRN, and DRN directly (Fig. 2) (Proulx et al., 2014).

The LHb is involved in rapid eye movement (REM) sleep, motor processes, and decision-making (Baker et al., 2016). When a lesion on the fasciculus retroflexes in rats blocks lateral habenular activity, there is a significant reduction in the amount of time spent in REM sleep and the atonia accompanying REM sleep (Hikosaka, 2010). Implanting fetal tissue containing the LHb in these rats restored normal sleep patterns (Hikosaka, 2010). Several studies also suggest that the LHb suppresses motor activity in awake animals, further supporting its connection to the SNc and the VTA (Hikosaka, 2010). A lesion on the LHb caused hyperactivity, increased distractibility, and reduced reaction time on time-oriented tasks, probably due to the disinhibition of dopamine release (Hikosaka, 2010).

In another report by Bromberg-Martin and Hikosaka (2011), a study with rhesus monkeys demonstrated that the LHb plays a critical role in prediction error and reinforcement learning. Upon the monkey receiving either a larger-than-expected reward or a smaller-than-expected reward, neurons in the LHb were either inhibited or excited. Such processes are hypothesized to be linked with learning, as the activation or suppression of the dopaminergic neurons downstream from the LHb may reinforce or inhibit the particular action that leads to the reward.

There is little doubt that the LHb is a key player in the neural network. Aversive stimuli and situations, such as pain, excite the LHb (Baker et al., 2016). Thus, the LHb is also heavily involved in pain and stress processes, the dysregulation of which can lead to various illnesses (Li et al., 2017). This is consistent with other studies showing that lateral habenular malfunction is associated with multiple major diseases and disorders, such as anxiety, ADHD, schizophrenia (Boulos et al., 2017), and Parkinson's disease (Han et al., 2014), among many others. LHb dysregulation is implicated in addiction's withdrawal/negative affect stage.



**FIG. 2** Afferent and efferent neuronal projections of the LHb.

### Lateral habenula modulates opioid addiction through the negative emotional states and mu-opioid receptors

In rats and humans, abnormal potentiation and hyperactivity of the LHb are directly associated with depression and depression-like symptoms, including helplessness, anhedonia, and excessive negative focus (Yang et al., 2018). When researchers promoted depressive symptoms by reducing human serotonin (5-HT) levels, PET scans revealed that the LHb was one of a few structures that had increased activity (Morris et al., 1999). Additionally, increasing LHb neuronal activities using optogenetics can generate a negative state (Velasquez et al., 2014). In rats, learned helplessness (LH), a model used to study depression-like symptoms, reduced serotonin levels in the dorsal raphe nucleus (DRN). Lesion of LHb restored serotonin levels and decreased the duration of depression-like symptoms (Yang et al., 2008). Deep brain stimulation (DBS) experiments revealed that a presynaptic mechanism is responsible for producing a negative state. When the study used DBS to minimize excitatory signal transmission from LHb neurons to VTA neurons, the rats' immobility time during forced swim tests decreased (Salvatore et al., 2014). Another study proposed a postsynaptic mechanism. Over-expressing the  $\beta$  form of calcium/calmodulin-dependent protein kinase type II ( $\beta$ CaMKII) results in a longer duration of depression-like symptoms in rats (Li et al., 2013). While these studies established LHb mechanisms involved in depression, further research is necessary to understand how to ameliorate negative states, as neither DBS application (Salvatore et al., 2014) nor pharmacological inhibition of LHb alleviates depression symptoms immediately (Winter et al., 2011).

Opioids can modulate the activity of the LHb (Salvatore et al., 2014). Systematically administered opioids, such as morphine, inhibit pain-induced LHb neural activity, and local injection of morphine into the LHb produces analgesia (Margolis & Fields, 2016). Abstinence from opioids causes LHb neuronal activity to become hyperactive, resulting in increased GABA release indirectly through activating the rostromedial tegmental nucleus (RMTg) in monoaminergic brain centers (Velasquez et al., 2014). This inhibits the release of dopamine, serotonin, and many other monoaminergic neurotransmitters, which can lead to aversive states.

Opioids may regulate LHb by interacting with mu-opioid receptors (MORs). Recent studies in mice reported that one of the densest MOR expression sites lies in the habenula complex (Hb), in a unique neuronal population bridging the boundaries between the medial habenula (MHb) and LHb, which is characterized by the expression of opioid receptors, the Hb-MOR neurons (Boulos et al., 2020; Gardon et al., 2014), which is in keeping with previous studies in rats (Learn et al., 2001; McBride et al., 1998). MORs are G-protein-coupled receptors (GPCRs) (Svíženská et al., 2008). This family of receptors threads through the cell membrane and contains an N-terminal extracellular domain with the ability for ligand binding. MOR's intracellular C terminal is coupled to a G protein complex. Upon activation,  $G_{\alpha i}$  subunit dissociates and inhibits adenylyl cyclase (AC), thus inhibiting cyclic adenosine monophosphate (cAMP) production and cAMP's downstream signaling cascades. G protein also activates G-protein regulated inwardly rectifying potassium channel ( $K_{ir3}$ ) and deactivates calcium channels (Al-Hasani & Bruchas, 2011). Taken together, activated MORs inhibit neurons. A previous study used DAMGO, a MOR selective agonist, to illustrate mechanisms behind MORs' inhibition of specific subpopulations of LHb neurons (Margolis & Fields, 2016). Evidence suggests that DAMGO inhibits MORs by postsynaptic hyperpolarization. Interestingly, while DAMGO inhibits presynaptic glutamate release in one subset of LHb neurons, it also inhibits GABA release in a more significant subset of LHb neurons. It is unclear how these mechanisms contribute to pain alleviation and opioid addiction (Margolis & Fields, 2016). Consistent administration of MOR agonists may induce tolerance (Christie, 2008). To compensate for opioids's action, cells chronically exposed to opioids either upregulate cAMP/PKA activity or downregulate receptor sensitivity to MOR agonists (Christie, 2008).

### The endocannabinoid system (ECBS)

Endocannabinoids, enzymes responsible for their biosynthesis and degradation, and endocannabinoid receptors, CB<sub>1</sub> receptors, and CB<sub>2</sub> receptors form the endocannabinoid system (Scavone et al., 2013). The activation and suppression of the endocannabinoid system play a role in complex interactions, including regulating metabolism and processing memory (Mouro et al., 2018), moderating immune response, and mediating drug addiction (Svíženská et al., 2008). There are currently five known endogenous endocannabinoid ligands that bind to the cannabinoid receptors: anandamide (AEA), 2-arachidonoylglycerol (2-AG), noladin ether, virodhamine, and N-arachidonoyldopamine (NADA) (Svíženská et al., 2008).

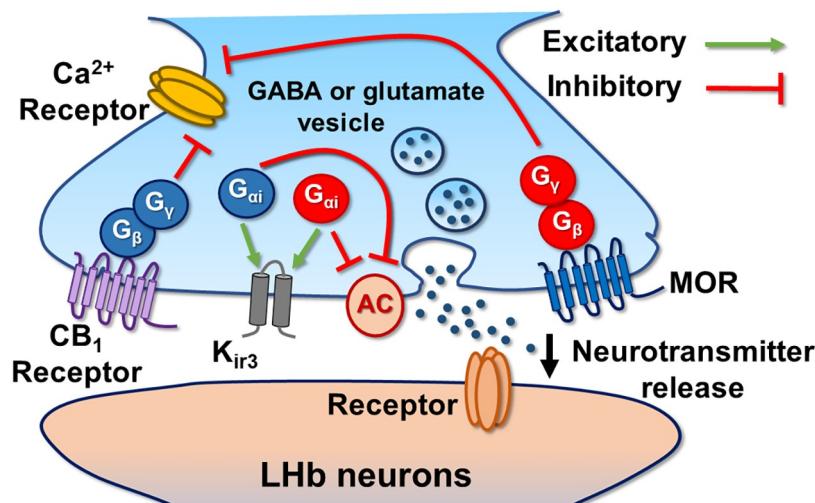
Of these five compounds, AEA and 2-AG are the most well-studied (Scavone et al., 2013). AEA can be synthesized through various routes. AEA can be made from hydrolytic cleavage of N-arachidonoyl-phosphatidylethanolamines (NArPE) by the enzyme N-acyl-phosphatidylethanolamine specific phospholipase D (NAPE-PLD). Alternatively,  $\alpha,\beta$ -hydrolase-4 (ABHD4) converts NArPE into lyso-NArPE before glycerophosphodiesterase-1 (GDE1) catalyzes the

conversion of lyso-NArPE into anandamide. A third synthetic route involves an unidentified phospholipase C (PLC), which converts NArPE into phospho-anandamide. Various phosphatases then remove the phosphate and convert the phospho-anandamide compound into anandamide (Fig. 2) (Di Marzo & De Petrocellis, 2012). The fatty acid amide hydrolase (FAAH) enzyme degrades AEA by converting it into ethanolamine and arachidonic acid. Compared with AEA, the bio-synthesis of 2-AG is much simpler (Fig. 3). Two calcium ion-sensitive DAG lipases, DAGL- $\alpha$  and DAGL- $\beta$ , convert diacylglycerols (DAG) into 2-AG (Di Marzo & De Petrocellis, 2012). Experiments inhibiting each enzyme showed that DAGL- $\alpha$  contributes to most of the 2-AG content in the central nervous system. 2-AG is 200 times more concentrated in the brain than AEA (Di Marzo & De Petrocellis, 2012). An explanation is that 2-AG can serve as either an end-product neurotransmitter in the brain or as a precursor for other lipid substances. This is further supported by the fact that significant amounts of 2-AG are responsible for maintaining neuronal homeostasis rather than interneuronal communication (Di Marzo & De Petrocellis, 2012). Several enzymes contribute to 2-AG degradation, including monoacylglycerol lipase (MAGL), FAAH,  $\alpha/\beta$ -hydrolase-6 (ABHD6), and  $\alpha/\beta$ -hydrolase-12 (ABHD12) (Murataeva et al., 2014). MAGL is the enzyme that contributes the most to 2-AG breakdown, catalyzing 85% of total brain 2-AG hydrolysis.

In the central nervous system, CB<sub>1</sub> receptors remain the most abundant GPCR. Like MORs, activated CB<sub>1</sub> receptors also cause the release of an inhibitory G $\alpha$  subunit that inhibits AC, closes Ca<sup>2+</sup> channels, and opens K<sup>+</sup> channels. These effects lead to a reduced probability of neurotransmitter release (Hernandez & Cheer, 2015). CB<sub>1</sub> receptors have been seen in various species in the Animalia kingdom, from mice to humans. Initially, CB<sub>1</sub> receptors were believed to be only located in the central nervous system, but recent reports indicate otherwise. The inactivation of CB<sub>1</sub> receptors may be a possible therapeutic target for chemotherapy-induced allodynia (Mulpuri et al., 2018).

### ECBS modulates opioid addiction through negative emotional states and by interacting with mu-opioid receptors

The ECBS plays a role in emotional management and mood (Morena et al., 2016). Since opioid negative emotional states contribute to substance abuse and relapse, the ECBS indirectly regulates opioid addiction and withdrawal (Morena et al., 2016). Anecdotal reports attest to its efficacy as an antidepressant and anxiolytic compound for humans (Micale et al., 2013). This is consistent with the findings that activation of the CB<sub>1</sub> receptors, whether through exogenous CB<sub>1</sub> receptor agonists or uptake/degradation enzyme inhibitors, can lead to anxiolytic and antidepressant phenotypes in animal models (Lutz, 2009). CB<sub>1</sub> receptor activation may achieve its effects through the monoaminergic pathway. Exogenous cannabinoid and FAAH inhibitor, URB597, can increase the firing of serotonergic and noradrenergic neurons (Dwivedi, 2012). Supporting this hypothesis,  $\Delta$ 9-THC, the active ingredient in marijuana, can increase dopamine levels in the prefrontal cortex of rats (Pistis et al., 2002). Interestingly, a few groups also reported that chronic intermittent exposure to CB<sub>1</sub> receptor agonists, WIN 55,212-2, leads to depressive-like behaviors, recognition memory impairments, psychiatric disorders such as schizophrenia-spectrum disorders, acute psychosis, and mania, and an unmotivated syndrome



**FIG. 3** Mechanisms of CB1 receptors and MORs' regulation of LHB neurons.

(Mouro et al., 2018). Additionally, in patients who died of suicide, there are higher levels of CB<sub>1</sub> receptors in various brain regions, such as the prefrontal cortex and the ventral striatum of patients (Dwivedi, 2012). The higher density of CB<sub>1</sub> receptors in these brain regions could be a response denoting lower levels of endocannabinoid signaling in the brain. However, because AEA and 2-AG levels drastically change in the hours' postmortem, more research closer to the time of passing or in living patients would be necessary to uncover the underlying mechanisms of CB<sub>1</sub> receptors in humans fully.

In vitro studies explored the relationship between MOR and CB<sub>1</sub> receptors. As detailed earlier, both receptors inhibit cAMP production through G-protein pathways (Hernandez & Cheer, 2015), as concluded by Viganò et al. through experiments done in N18TG2 cells (Viganò et al., 2005). By measuring [35S]GTPγS binding to cell membranes, Shapira et al. (1998) activated MOR and CB<sub>1</sub> receptors by applying endorphins and diacetyl-levonantradol (DALN) respectively. They founded that the effect was similar to the arithmetic sum of each separate impact. (Befort, 2015) In addition to molecular cross-talk between the two receptors, CB<sub>1</sub> receptors and MORs co-express in many brain regions, such as the caudate-putamen and dorsal hippocampus, and substantia nigra (Befort, 2015). These receptors' impact may converge on a downstream process, such as the G-protein regulation of AC (Fig. 3) (Befort, 2015).

Evidence also suggests that the agonists of CB<sub>1</sub> receptors and opioid receptors interact with each other's respective receptors. However, there is controversy about how they interact, as results differ depending on the experimental setup. For instance, while chronic CB<sub>1</sub> receptor activation did not decrease opioid efficacy in N18TG2 cells, it did in COS-7 cells (Viganò et al., 2005). Interestingly, while opioid agonists downregulated CB<sub>1</sub> receptors' binding efficacy to CB<sub>1</sub> receptor agonists, CB<sub>1</sub> receptor agonists did not impact opioid receptors' binding efficacy (Viganò et al., 2005). Numerous in vivo studies also noted how CB<sub>1</sub> receptor agonists could replace morphine and suppress opioid withdrawal symptoms in morphine-dependent mice, highlighting the role of cannabinoids as potential treatments for opioid withdrawal (Viganò et al., 2005). Furthermore, the vice versa proves accurate in rats; various reports show that morphine-dependent rodents will precipitate withdrawal symptoms upon the administration of CB<sub>1</sub> receptor antagonists (Viganò et al., 2005). Additional study shows that chronic administration of CB<sub>1</sub> receptor antagonists during the development of opioid dependence reduced the withdrawal effects on abstinence (Viganò et al., 2005).

As shown, either directly or indirectly, both the ECB system and the LHB have been independently associated with opioid addiction and withdrawal. Because the interactions between the ECB system and opioid addiction are particular to both the area of the brain and the cell type, a deeper look at the endocannabinoid system in the LHB will provide a clearer understanding of opioid addiction.

## Endocannabinoids in the lateral habenula

### CB<sub>1</sub> receptor expression in the lateral habenula

Because the role of CB<sub>1</sub> receptors in the LHB remains elusive, it remains critical to report where they are expressed. In most regions of the thalamus, the expression of CB<sub>1</sub> receptors is deficient. However, the anterior dorsal thalamic nucleus, the reticular thalamic nucleus, and the LHB are exceptions (Pertwee, 2015). Even within the LHB, the expression of CB<sub>1</sub> receptors is unevenly dispersed throughout presynaptic neurons, postsynaptic neurons, mitochondria, and glial cells (Berger et al., 2018). In C57BL/6J mice, presynaptic CB<sub>1</sub> receptors were reported to be present on both excitatory and inhibitory neurons at  $8.82\% \pm 0.60\%$  of the total amount of expression and  $9.76\% \pm 0.66\%$  of the total amount of expression, respectively. Lower levels of CB<sub>1</sub> receptors were also found on postsynaptic dendritic membranes that received excitatory and inhibitory signals,  $5.51\% \pm 0.54\%$  and  $8.86 \pm 0.87\%$  of all CB<sub>1</sub> receptor particles, respectively. Most of the CB<sub>1</sub> receptors in the LHB were expressed in glial processes at  $31.68\% \pm 1.76\%$ . The remaining  $26.46\% \pm 1.69\%$  of CB<sub>1</sub> receptors were identified on mitochondrial membranes. Interestingly, one group using male mice of the same background found no CB<sub>1</sub> receptor mRNA expression in the LHB using fluorescent in situ hybridization (FISH) techniques (Berger et al., 2018). These findings will be dismissed because of the overwhelming evidence supporting their expression in the LHB.

### Stress affects the endocannabinoid system in the lateral habenula

Since stress is one symptom one may experience during the withdrawal/negative affect stage, it is relevant to study how stress affects the LHB and the ECBS. Because there is a high expression of CB<sub>1</sub> receptors in the LHB, the ECBS may regulate and mediate lateral habenular activity during stress. As seen in other brain regions, stress can facilitate the potentiation of neuronal activity and alter endocannabinoid functionality (Morena et al., 2016). However, there is a lack of research on the mechanisms explaining the underlying relationship between the two. Recent reports illustrate that synaptic plasticity in

the LHb is dependent on the endocannabinoid system, which is impaired in the presence of stress (Park et al., 2017). Researchers harvested brain slices from rats that experienced restrained tail shock stress (RTS) and induced long-term depression (LTD) by applying *in vitro* low-frequency stimulation (LFS) in stria medullaris. Surprisingly, there was minimum LTD in LHb neurons. A lack of change in paired-pulse-ratio suggests that this phenomenon has an activity-dependent presynaptic cause. Consistent with these observations, acute stress post-transcriptionally upregulated in RTS models of Sprague Dawley rats. These CaMKII are inhibitors of DAGL, the enzyme responsible for 2-AG synthesis. Moreover, inhibiting 2-AG synthesis blocked LFS-induced LTD in naïve rats (Park et al., 2017). Park et al. thus hypothesized stress decreased LTD in the LHb by downregulating 2-AG. However, this contradicts the findings of another group using the same species of rats in chronic unpredictable stress (CUS) and social defeat stress (SDS) and control conditions (Berger et al., 2018). *In vivo* microdialysis and blood samples obtained from the tails revealed increased levels of 2-AG and corticosterone in CUS compared with the unstressed rats. This was consistent with the general trend found in other brain regions, such as the hypothalamus, amygdala, mPFC, and hippocampus (Morena et al., 2016). A decrease in MAGL hydrolysis activity may explain the higher levels of 2-AG. Also, consistent with most other brain regions, CB<sub>1</sub> receptor binding decreased due to the reduction of CB<sub>1</sub> receptor density from either the desensitization or internalization of CB<sub>1</sub> receptors because of the elevated levels of 2-AG. Overall, though, there was an increase in LHb activity in CUS models compared with control rats. However, in the SDS model, both 2-AG and AEA levels were upregulated, although there was no change in MAGL activity, and CB1 receptor binding sites were intact. Despite these changes in ECB content, LHb neuronal firing rates did not change (Berger et al., 2018).

### Possible explanations for the observed difference in how stress affects the ECB system

Generally, an increase in ECB/CB<sub>1</sub> receptor signaling results in a decrease in adenylate cyclase activity and Ca<sup>2+</sup> influx through N-, P/Q-, and L-type Ca<sup>2+</sup> channels, thus inhibiting neuronal firing (Pertwee, 2006), with a few exceptions such as if the CB<sub>1</sub> receptor is paired with a G<sub>q</sub> protein (Turu & Hunyady, 2010). However, depending on whether the type of experiment and the stress model used, Berger et al. found stress impacted LHb neuronal activity and the ECBS differently (Table 1). One explanation for these phenomena is that different stress models cause various physiological and molecular changes. This is the case in research studying other brain regions. Forced swim stress increased AEA levels in the mPFC while restraint stress and SDS did not (Morena et al., 2016). This is further supported even within the *in vivo* studies in the second group's data. SDS elevated both AEA and 2-AG, whereas CUS only raised 2-AG levels and did not change MAGL activity in the SDS model compared with the CUS model.

Another explanation for the difference between the two stress models lies in the difference between *in vivo* and *in vitro* experiments. *In vitro*, LFS of the stria medullaris would not account for synaptic plasticity in other brain regions. Regions of the brain sensitive to negative states and supply the LHb with neuronal signalings, such as the mPFC or GPi, can be hyperpotentiated in stressful situations. While these changes are reflected in *in vivo* studies, *in vitro* experiments do not provide information on how stress impacts the LHb indirectly by modulating other brain areas.

A third explanation is the difference in time exposed to stress. Rats that faced 7 days of SDS experienced no overall change in LHb activity, and rats that experienced CUS for 6 weeks had the highest amounts of ECB. This implies that, in *in vivo* experiments, the longer stress exposure time may correlate with more significant changes in ECB levels. It is also possible that all three explanations contribute to the observed differences. More research is necessary to understand the underlying mechanisms behind these complex phenomena.

**TABLE 1** Stress's varied effect on the LHb and the ECBS.

Time exposed to stress	Type of stress	Type of experiment	LHb activity	ECB changes	Reference
1 and 24 h before sample extraction	Restrained tail Stress (RTS)	<i>In vitro</i>	LFS failed to induce LTD	Decreased	Park et al. (2017)
7 days	CUS	<i>In vivo</i>	Increased	Increased	Berger et al. (2018)
6 weeks	SDS	<i>In vivo</i>	No change	Increased	Berger et al. (2018)

## Conclusion

Although both the LHb and the ECB system directly influence opioid addiction through modulating negative emotional states, their effects together in the LHb remain elusive. While there are a plethora of therapeutic potentials for CB<sub>1</sub> receptors in the LHb, more research is necessary to unveil the underlying mechanisms of CB<sub>1</sub> receptors in the LHb. As discussed, *in vivo* and *in vitro* experimentations through different stress models may account for the observed difference between phenotypes and neuronal activity. However, many unknown pieces remain, and we still do not entirely understand their interactions.

## Applications to other areas

In this chapter, we reviewed how the endocannabinoid system affects opioid abuse. We speculate that similar effects may also occur in alcohol use disorders, as those suffering from alcohol use disorders often experience similar adverse symptoms during withdrawal, including mood swings (Weiss & Porrino, 2002). Both involve reward and reinforcement pathways, including sensitization and withdrawal, which often drive relapse (Robinson & Berridge, 1993). Like opioids, alcohol interacts with various dopaminergic pathways in the brain, most notably the mesolimbic reward system. Ethanol increases the activity of dopamine neurons in the VTA (Weiss & Porrino, 2002). During the absence of alcohol, dopamine activity diminishes, and adverse withdrawal symptoms develop, suggesting that users potentially seek alcohol to mitigate these symptoms by increasing dopamine levels. The endocannabinoid system might prevent alcohol relapse, as CB<sub>1</sub> receptors may mediate the appetitive and consummatory aspects of alcohol ingestion and disrupt the cycle of alcohol abuse. It is tempting to evaluate the similarities between opioid addiction and alcohol use disorders as both are based on the foundations of drug dependency. However, continued research is necessary to specify the involvement of the endocannabinoid system in alcohol and opioid epidemics.

## Mini-dictionary of terms

**AEA**—a type of lipid neurotransmitter involved in the endocannabinoid system. Binds to the same cannabinoid receptors as compounds found in cannabis. AEA is one of the two most studied cannabinoid receptor ligands.

**Dopamine**—a neurotransmitter found in the brain involved in multiple neurologic pathways, including those pertaining to memory, movement, motivation, attention, and mood.

**Endocannabinoid system**—a neurotransmission network that includes endocannabinoid and their receptors, CB<sub>1</sub> and CB<sub>2</sub> receptors. The ECBS plays a role in regulating metabolism, processing memory, moderating immune response, and mediating drug addiction.

**Learned helplessness**—the belief that one cannot control or change the outcome of a situation, even if opportunities are available, after experiencing a stressful situation repeatedly.

**Lateral habenula**—a brain region located in the epithalamus. The LHb plays a role in learning, REM sleep, and decision-making. Dysregulation of LHb is associated with depression.

**μ-opioid receptors**—a G-protein-coupled receptor activated by opioids and is responsible for the effects associated with opioid consumption.

**Opioids**—a class of substances that interact with opioid receptors to produce pain-relieving effects. Drugs vary in potency and have other medicinal effects other than pain. Naturally synthesized from poppy plants, however, can be manufactured.

**2-AG**—a type of lipid neurotransmitter involved in the endocannabinoid system. One of the two most studied cannabinoid receptor ligands. Primarily binds to CB<sub>2</sub> receptors.

## Key facts

### Key facts of the LHb

- The LHb locates in the epithalamus and acts as a communicator between the hindbrain's forebrain and pain regulatory regions (Shelton et al., 2012).
- The most well-known effect of LHb activation is the suppression of dopamine signaling (Sosa et al., 2021).
- To suppress dopamine release, LHb activation can directly excite GABAergic neurons in VTA and RMTg, ultimately suppress dopamine release (Sosa et al., 2021).

- Although most research discusses the LHb in terms of its regulatory effects on dopamine release, evidence also suggests that dopamine excites a subpopulation of LHb neurons, leading to an excitatory feedback loop (Webster et al., 2021).
- Interestingly, in addition to interacting with MORs, other opioid receptors, such as the kappa opioid receptor, can also regulate LHb neuronal activity (Webster et al., 2021).

## Key facts about opioid addiction

- Opioid addiction is a chronic neurological condition characterized by excessive opioid use.
- This excessive use is often accompanied by tolerance, whereby individuals using opioids must take greater doses of the substance to achieve the same desired results.
- Abstinence is often challenging for individuals suffering from opioid addiction due to numerous physiological and psychological challenges, such as depression, anxiety, nausea, and cravings.
- These symptoms may lead to a relapse, posing significant overdose risks and death.
- Together, these factors contribute to the current opioid epidemic and overdose crisis in North America.

## Summary points

- The LHb and ECB systems play significant roles in developing negative emotional states, especially in the context of opioid addiction.
- CB<sub>1</sub> receptor agonists could potentially replace certain opioids and suppress their associated withdrawal symptoms, much like CB<sub>1</sub> receptor antagonists can precipitate withdrawal in opioid-addicted mice.
- MORs may interact heavily with CB<sub>1</sub> receptors since chronic treatment with MOR agonists downregulates the CB<sub>1</sub> receptor sensitivity.
- Stress can affect the synaptic plasticity of LHb neurons. It does this by modulating the ECB system, which expresses CB<sub>1</sub> receptors, through regulating the levels of CaMKII, MAGL, and 2-AG.
- Interestingly, ECB activation in the LHb increases depression-like symptoms and other negative emotional states associated with relapse, whereas ECB blockade decreases anxiety-like symptoms.
- The complexity of the relationship between the ECB, opioid addiction, and LHb dramatically emphasizes the need for further research.

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## Chapter 19

# On the interplay among endocannabinoid, noradrenergic, and glucocorticoid systems: Evidence from aversive memory studies

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## Abbreviations

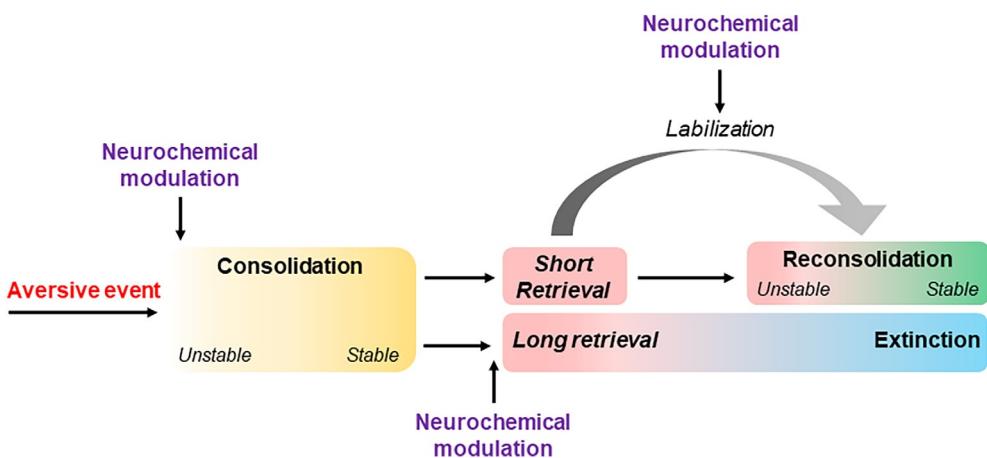
2-AG	2-arachidonoylglycerol
AEA	anandamide
BLA	basolateral nucleus of the amygdala
CB1R	cannabinoid type-1 receptor
CeA	central nucleus of the amygdala
CFC	contextual fear conditioning
CRF	corticotropin-releasing factor
DS	dorsal striatum
GABA	gamma-aminobutyric acid
GR	glucocorticoid receptor
HPA	hypothalamus-pituitary-adrenal axis
IA	inhibitory avoidance
LC	locus coeruleus
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
PFC	prefrontal cortex
PTSD	posttraumatic stress disorder
PVN	periventricular nucleus of the hypothalamus

## Introduction

Understanding mnemonic processes helps uncover the neural mechanisms underlying the development and maintenance of psychiatric conditions, such as posttraumatic stress disorder (PTSD), as long-lasting memories of stressful events often generate maladaptive behavioral outcomes, including pathological anxiety and generalized fear responses.

As depicted in Fig. 1, consolidation refers to the dynamic process stabilizing the memory engram upon acquisition. Based on animal studies, it is time-dependent, requires protein synthesis to support related synaptic plasticity in recruited brain areas, including the dorsal hippocampus and amygdala nuclei (McGaugh, 2015), and may vary according to the training intensity, for instance (Dos Santos Corrêa et al., 2019; Gazarini et al., 2014, 2022).

Consolidated memories can be retrieved and expressed. Memory retrieval can trigger two opposite and dissociable processes: extinction and reconsolidation (Fig. 1). Extinction is a form of inhibitory learning that competes with the original memory and “superposes” it, reducing fear expression (VanElzakker et al., 2014). It takes place after prolonged retrieval



**FIG. 1** The process of memory consolidation, reconsolidation, and extinction. The incidence of an aversive event triggers the acquisition of a fear memory that requires subsequent consolidation to become stable and long-lasting. Its retrieval may allow different outcomes: short retrievals induce labilization of the memory, requiring a new stabilization phase (reconsolidation) to be restabilized and maintained; long or repeated retrievals favor extinction, resulting in suppression of fear responses. These post-retrieval memory phases can be studied and modulated by neurochemical and pharmacological interventions at specific time points.

sessions and is the base of exposure therapy (Mueller & Cahill, 2010). Fear extinction also depends on the hippocampus but highly relies on the medial prefrontal cortex (mPFC) activity, mainly the infralimbic area, which activates inhibitory interneurons of the amygdala to suppress fear expression (Milad & Quirk, 2002; Sohn et al., 2020). The search for new PTSD treatments has long focused on discovering or developing drugs that facilitate fear extinction. However, a limitation of this approach is that extinction does not erase the original memory. Therefore, fear may emerge over time (spontaneous recovery), while stress or context exposures can elicit reinstatement or renewal of the extinguished memory (VanElzakker et al., 2014).

On the other hand, short retrieval periods favor reconsolidation, a restabilization phase requiring prior labilization. Memory labilization is a destabilization process involving proteasome activation and protein degradation, glutamatergic and cannabinoid type-1 receptors (CB1R) recruitment, the activation of L-type voltage-gated calcium channels, among others (Kida, 2020). Although reconsolidation and consolidation share some molecular and neural mechanisms, reconsolidation differs from initial consolidation. It allows a new opportunity to strengthen and maintain the memory besides making it stable again (Lee, 2008). Uncovering the mechanisms underpinning memory reconsolidation can advance treatments that permanently adjust the original fear memory content. Impairing fear memory reconsolidation presumably produces more enduring effects than facilitating fear extinction.

Accumulating evidence indicates that noradrenaline and corticosterone influence aversive memories. Noradrenergic signaling enhances memory consolidation, reconsolidation and modulates extinction (Giustino & Maren, 2018; Roozendaal & McGaugh, 2011). The recruitment of mineralocorticoid (MR) and glucocorticoid (GR) receptors has also been shown during those memory phases (Roozendaal, Hui, et al., 2006; Roozendaal, Okuda, et al., 2006; Taubenfeld et al., 2009). The endocannabinoid system can buffer the activation of the hypothalamic-pituitary-adrenal (HPA) system induced by emotionally relevant stimuli by reducing glucocorticoid release (Hill & McEwen, 2010). Consequently, it is associated with fine-tuning aversive memory consolidation, reconsolidation, and extinction (Gazarini et al., 2022; Stern et al., 2012; Warren et al., 2022). Noteworthy, these three neurotransmitter systems appear to be somehow dysfunctional in PTSD. Overactive noradrenergic system and increased GR sensitivity have been shown, while the opposite (i.e., reduced tonus) often occurs with the endocannabinoid system (Hill et al., 2013; Yehuda et al., 1998, 2004). The unbalance among those stress-related transmitters contributes to fear memories developing abnormal features, such as fear overgeneralization, a typical PTSD symptom (Bahtiyar et al., 2020; Gazarini et al., 2022). The increased resistance to fear memory extinction may also be related to their dysfunctions (Gazarini et al., 2014).

Over decades of animal research, studies using genetic and pharmacological manipulations of noradrenergic, glucocorticoid, and endocannabinoid systems have shed light on the role of each one during fear memory, consolidation, reconsolidation, and extinction. Studies have recently advanced how these systems' interplay influences the aversive learning and memory process.

## Noradrenergic modulation of fear memories and mechanisms underlying the endocannabinoid interplay

The noradrenergic system's contribution to fear memory has long been suggested (Roozendaal & McGaugh, 2011). Increases in peripheral adrenaline release (through sympathoadrenomedullary axis activation) and central noradrenaline release happen during emotionally relevant stimuli (Swenson & Vogel, 1983). The encephalic noradrenaline release depends on locus coeruleus (LC), a brainstem nucleus mainly composed of noradrenergic neurons that project diffusely throughout the brain and are activated by peripheral adrenergic action via vagal afferents (Ross & Van Bockstaele, 2021).

Several studies have shown noradrenergic participation in memory consolidation, reconsolidation, and extinction (van Stegeren, 2008). Overall, noradrenergic activation enhances the consolidation, reconsolidation, and extinction of aversive memories, while pharmacological antagonism produces impairments (Likhtik & Johansen, 2019; Mueller & Cahill, 2010; Roozendaal & McGaugh, 2011), although mixed findings have also been reported. Contrasting results may be attributable to the inverted U-shape function of noradrenergic recruitment due to the level of arousal elicited by the aversive event and/or the concentration of adrenergic agents (Giustino & Maren, 2018).

Some studies have reported a cannabinoid-noradrenergic interplay (Kirilly et al., 2013; Mendiguren et al., 2021; Wyrofsky et al., 2019). The activation of adrenoceptors activates calcium-induced mechanisms in post-synaptic membranes, triggering retrograde cannabinoid-mediated neurotransmission (Pfizer et al., 2005; for a review, see Gyombolai et al., 2012). CB1R is widely expressed in the rodent brain, including at the noradrenergic terminals (Oropeza et al., 2007; Wyrofsky et al., 2018) and LC (Herkenham et al., 1991; Marsicano & Lutz, 1999), allowing the fine-tuning of noradrenaline release. The noradrenergic-cannabinoid cross talk seems to be involved in complex feedback mechanisms, especially during stress-related situations and possible pathological conditions (Carvalho & Van Bockstaele, 2012; Warren et al., 2022; Wyrofsky et al., 2019). Such cross talk, however, is not straightforward. CB1R is coupled to inhibitory mechanisms; thus, their negative feedback on noradrenaline release would be expected and easily extrapolated. The inhibitory effects of endocannabinoid signaling on sympathetic activity and noradrenaline release have been shown in the periphery, mainly when associated with the modulation of cardiovascular functions (e.g., Pfizer et al., 2005). In the central nervous system, the inhibitory effects of cannabinoid agonists over noradrenaline release have been described in the mPFC (Reyes et al., 2012) and hippocampus (Jergas et al., 2014), while the blockade of CB1R produced the opposite outcome in the mPFC (Tzavara et al., 2003).

CB1R present in noradrenergic terminals is associated with inhibitory control over noradrenaline release, but its activation in complex neural circuitries may yield different outcomes (Kirilly et al., 2013). Reduced noradrenergic tonus can result from cannabinoid-induced inhibitory effects on excitatory neurons, as CB1R is expressed in glutamatergic neurons, thus acting as a buffer to excitatory activity (Katona et al., 2006; Kawamura et al., 2006). The glutamatergic-associated increase in noradrenaline release was inhibited by CB1R activation in the cortex or the LC (Kathmann et al., 1999; Mendiguren & Pineda, 2006). Based on the above, the endocannabinoids modulatory actions are bidirectional (i.e., direct presynaptic CB1R activation on noradrenergic terminals is not the only possibility). Indeed, studies have reported stimulatory effects of cannabinoid modulation on LC excitability (Mendiguren & Pineda, 2006). That could be explained by the presence of CB1R on inhibitory interneurons, such as the GABAergic ones (Hájos & Freund, 2002), resulting in attenuated inhibitory control over the noradrenergic activity. Other studies have demonstrated similar cannabinoid-induced enhancement on noradrenergic signaling in brain regions controlling defensive behaviors and aversive memory processing, such as the mPFC (Oropeza et al., 2007).

More recently, another layer of complexity was added to the endocannabinoid-noradrenergic interplay, as endogenous peptides that present negative allosteric activity on CB1R were described (Heimann et al., 2021). These peptides are produced and released mainly by noradrenergic neurons in LC and chromaffin cells of the adrenal medulla (Hofer et al., 2015), allowing their co-release with noradrenaline. Considering that, the endocannabinoid modulation on the noradrenergic transmission results from both agonistic and antagonistic actions on CB1R.

## Evidence for the cannabinoid-noradrenergic cross talk on fear memory processing

**Table 1** summarizes rodent studies assessing the effects of combining cannabinoid and noradrenergic drugs on aversive memory. In an inhibitory avoidance (IA) task, the infusion of WIN55,212-2, a nonselective synthetic cannabinoid receptor agonist, into the central nucleus of the amygdala (CeA) induced dose-dependent consolidation impairments, an effect counteracted by concomitant infusion of subeffective doses of isoproterenol, a nonselective  $\beta$ -adrenoceptor agonist. On the other hand, the blockade of  $\beta$ 1-adrenoceptors potentiated cannabinoid-induced impairment and prevented the isoproterenol action (Zarrindast et al., 2012). Another study found that the local infusion of WIN55,212-2 or clenbuterol, a selective

**TABLE 1** Effects of associating cannabinoid with noradrenergic drugs on consolidation, reconsolidation, and extinction of aversive memories in rats or mice (local infusion doses described as total per animal)

Paradigm	Animal			Intervention (drug, dose, route/site of administration)			Memory phase			Reference
Task, US number, intensity and duration	Species, strain	Sex	Age in days	Cannabinoid	Noradrenergic	Moment(s) of treatment	Consolidation	Reconsolidation	Extinction	
STIA, 1 × 1.0mA/3s	Wistar rats	♂	ND	WIN55,212-2, 0.25 µg, CeA	Isoprenaline, 0.01–0.05 µg, CeA	Both IAT	↓☒			Zarrindast et al. (2012)
STIA, 1 × 1.0mA/3s	Wistar rats	♂	ND	WIN55,212-2, 0.25 µg, CeA	Atenolol, 0.1 µg, CeA	both IAT	↑☒			Zarrindast et al. (2012)
STIA, 1 × 1.0mA/3s	Wistar rats	♂	ND	WIN55,212-2, 0.05 µg, CeA	Atenolol, 0.05–0.1 µg, CeA	both IAT	↑☒			Zarrindast et al. (2012)
STIA, 1 × 1.0mA/3s	Wistar rats	♂	ND	WIN55,212-2, 0.25 µg, CeA	Atenolol, 0.01–0.025 µg + isoprenaline, 0.025 µg, CeA	both IAT	↓☒			Zarrindast et al. (2012)
STIA, 1 × 0.6mA/1s	Sprague-Dawley rats	♂	ND	WIN55,212-2, 20 ng, BLA	Propranolol, 1.0 µg, BLA	both IAT	↓☒			Atsak et al. (2015)
STIA, 1 × 0.6mA/1s	Sprague-Dawley rats	♂	ND	WIN55,212-2, 2.0 ng, BLA	Clenbuterol, 2.0 µg, BLA	both IAT	=☒			Atsak et al. (2015)
STIA, 1 × 0.6mA/1s	Sprague-Dawley rats	♂	ND	AM251, 0.28 ng, BLA	Clenbuterol, 2.0 µg, BLA	both IAT	↓☒			Atsak et al. (2015)
STIA, 1 × 0.6mA/1s	Sprague-Dawley rats	♂	ND	AM251, 0.28 ng, BLA	Clenbuterol, 200 µg, BLA	both IAT	↑☒			Atsak et al. (2015)
CFC, 1 × 0.7mA/3s	Wistar rats	♂	90	AM251, 1.0 mg/kg, i.p.	Adrenaline, 0.05 mg/kg, i.p.	both IAT	☒			Gazarini et al. (2022)
CFC, 1 × 0.7mA/3s	Wistar rats	♂	90	AM251, 1.0 mg/kg, i.p.	Adrenaline, 0.05 mg/kg, i.p.	Both IAR		☒		Gazarini et al. (2022)

US, unconditioned stimulus (shock); ND, not described; ♂, male; ↑, increase; ↔, no effects; ↓, reduce/prevent; ☒, potentiation/facilitation; ☗, impairment; BLA, basolateral amygdala; CeA, central amygdala; CFC, contextual fear conditioning; IAR, immediately after retrieval; IAT, immediately after training; i.p., intraperitoneal; STIA, step-through inhibitory avoidance; AM251, CB1R inverse agonist/antagonist; atenolol, β1-adrenoreceptor antagonist; clenbuterol, β2-adrenoreceptor agonist; isoprenaline, nonselective β-adrenoreceptor agonist; propranolol, nonselective β-adrenoreceptor antagonist; WIN55,212-2, CB1/CB2 receptors agonist.

$\beta$ 2-adrenoceptor agonist, into the basolateral nucleus of the amygdala (BLA) just after IA training increased memory retention, suggesting potentiated memory consolidation. While the blockade of  $\beta$ -adrenoceptors prevented the WIN55,212-2-induced enhancing effects, the local infusion of AM251, a CB1R inverse agonist/antagonist, made higher doses of clenbuterol necessary to enhance consolidation (i.e., reduced the local sensitivity to noradrenergic signaling) (Atsak et al., 2015). Interestingly, both studies performed amygdalar interventions, but the consequences of the noradrenergic-cannabinoid interplay were opposite (i.e., impairing vs. potentiating effects), probably reflecting the different target nuclei. More recently, such interplay was evaluated using systemic injections during a contextual fear conditioning (CFC) protocol. Coadministration of subeffective doses of AM251 and adrenaline enhanced contextual fear memory consolidation and reconsolidation using weak training (Gazarini et al., 2022). Altogether, such results suggest a functional consequence of the modulatory control of endocannabinoid signaling on noradrenergic impact, reinforcing the interplay between both systems on aversive memories' stabilization. Potential discrepancies could be related to animal strain, the memory task adopted, training intensity, drug doses tested, the administration route (local vs. systemic), and the distinct functional connectivity of specific brain structures assessed with local infusions.

To our knowledge, there have been no studies investigating the interplay of those systems on memory extinction, although it is anticipated based on the current neurobiological knowledge and the individual recruitment of noradrenergic and cannabinoid systems (Warren et al., 2022). Moreover, as retrieval is necessary for memory extinction, and previous results highlight the noradrenergic-cannabinoid cross talk at this memory step (see Atsak, Hauer, et al., 2012), future studies are required to examine this question as well.

## Glucocorticoid effects on fear memories and mechanisms underlying the endocannabinoid interplay

Several studies have shown that glucocorticoids (cortisol in humans and corticosterone in rodents) released during emotional or stressful situations modulate different memory processes (de Kloet et al., 1999; Roozendaal & McGaugh, 2011; Sandi & Pinelo-Navar, 2007). When stronger aversive training is adopted (i.e., higher shock intensity or greater number of footshocks), increased post-training plasma corticosterone levels and fear responses during retrieval tests are observed (Cordero et al., 1998; Dos Santos Corrêa et al., 2019). Pharmacological studies have also shown that acute post-training systemic administration of corticosterone or dexamethasone (a synthetic glucocorticoid) enhances the memory of aversively motivated tasks, while the blockade of glucocorticoid synthesis with metyrapone impaired memory. These findings suggest that endogenous glucocorticoids released during an arousing training enhance memory consolidation for such emotional experience (Atsak, Roozendaal, & Campolongo, 2012). Additional evidence suggests that glucocorticoids can similarly enhance memory reconsolidation and extinction but impair memory retrieval (de Quervain et al., 2017).

Glucocorticoids are released from the adrenal cortex in response to the HPA axis activation. In addition to the various peripheral physiological functions that prepare the individual for fight-or-flight reactions, they cross the blood-brain barrier and bind to two types of receptors: MR, with a high affinity for glucocorticoids and occupied in basal conditions, and GR, with a low affinity for glucocorticoids and thus mainly occupied after emotional or stressful situations (Oitzl et al., 1997). Studies with selective agonists and antagonists have shown that glucocorticoids' effects on different memory processes are mediated, at least in part, by GR (Nikzad et al., 2011; Oitzl & de Kloet, 1992; Roozendaal et al., 2003; Sandi & Rose, 1994; Yang et al., 2006).

Glucocorticoids act in several brain regions to influence memory processes, including the hippocampus, BLA, dorsal and ventral striatum, and cortical regions such as the mPFC and the insular cortex (Barsegian et al., 2010; Fornari et al., 2012; Quirarte et al., 2009; Roozendaal, Hui, et al., 2006; Roozendaal, Okuda, et al., 2006; Wichmann et al., 2012). Further studies have suggested that glucocorticoid effects on memory in each of these regions depend on arousal-induced noradrenergic activation, mainly in the BLA, and interactions of the BLA with other brain regions, especially the hippocampus and cortex (de Quervain et al., 2017).

The endocannabinoid system is now well recognized as a crucial stress-response system and is involved in some of the major effects of glucocorticoids, including memory modulation (Akirav, 2013; Atsak, Roozendaal, & Campolongo, 2012; de Bitencourt et al., 2013; Hill & McEwen, 2010; Morena & Campolongo, 2014). Several rodent studies suggest that acute stress elevates 2-AG levels in the PFC, hippocampus, and hypothalamus while reducing anandamide (AEA) levels in the PFC, hippocampus, and amygdala but not in the hypothalamus. Moreover, the blockade of CB1R potentiates stress-induced glucocorticoid release and neuronal activation within the periventricular nucleus of the hypothalamus (PVN), suggesting that the endocannabinoid signaling in this region is essential for limiting the HPA axis response to stress (Hill & McEwen, 2010). That effect seems to be related to the rapid feedback inhibition of the HPA axis by glucocorticoids, as there is

evidence that glucocorticoids released after stress may activate membrane G-protein-coupled receptors in the PVN, which induces the release of endocannabinoids, which in turn act as retrograde messenger inhibiting the release of glutamate onto the PVN neurons (Di et al., 2003; Hill & McEwen, 2010). On the other hand, single systemic administration of corticosterone rapidly (within 10 min) elevated levels of AEA within the amygdala, hippocampus, and hypothalamus but increased 2-AG concentrations only in the hypothalamus (Hill et al., 2010). Based on these findings, glucocorticoids may be necessary for the stress-induced elevations in 2-AG levels and to compensate for or normalize the stress-induced decrease in AEA levels in limbic structures (Hill & McEwen, 2010).

### **Glucocorticoid-endocannabinoid interaction in the modulation of fear memory consolidation, reconsolidation, and extinction**

Rodent studies have suggested that the endocannabinoid system is also involved in the modulation of several fear memory phases and may play an important role in mediating the effects of glucocorticoids in regulating memory of emotionally arousing experiences (Table 2). Most of these studies investigated the effects of this interaction during consolidation by treating animals immediately after memory acquisition. CB1R blockade in the BLA immediately after IA training reverted the fear memory enhancement promoted by post-training systemic or intra-BLA corticosterone treatment (Atsak et al., 2015; Campolongo et al., 2009). Atsak and colleagues also observed that BLA CB1R activation with WIN55,212-2 enhanced aversive memory consolidation, and this effect was not reversed by a simultaneous infusion of mifepristone, a GR antagonist, suggesting that, at least for memory modulation in the BLA, the endocannabinoid signaling is downstream to the GR activation (Atsak et al., 2015). A similar reversal effect of glucocorticoid-induced memory enhancement was observed when the CB1R inverse agonist/antagonist AM251 was infused into the dorsal hippocampus following CFC training (de Oliveira Alvares et al., 2010) or into the dorsal striatum (DS) after IA training (Siller-Pérez et al., 2019). Atsak et al. also showed that post-training intra-BLA blockade of CB1R with AM251 co-infused with a membrane-impermeable corticosterone conjugate (corticosterone:BSA) reverted the memory-enhancing effects seen when the conjugate was infused alone (Atsak et al., 2015). These findings indicate that glucocorticoid and endocannabinoid systems interact to modulate memory consolidation. Such interaction depends, at least in part, on nongenomic, rapid actions of glucocorticoids, possibly via activation of membrane-associated variants of the steroid receptor, which promotes the release of endocannabinoids. Comparable results implicating rapid glucocorticoid-endocannabinoid interaction were observed in the DS (Siller-Pérez et al., 2019). Notably, inhibiting glucocorticoid synthesis prior to IA training prevented the memory-enhancing effects induced by CB1R activation in the DS, suggesting an interaction between glucocorticoid and endocannabinoid systems in modulating memory consolidation (Siller-Pérez et al., 2019).

Divergent results were observed, however, when highly aversive training was used. Shoshan and Akirav (2017) reported that acute infusion of the CB1R agonist WIN55,212-2 into the dorsal hippocampus immediately after a severely strong shock impaired consolidation and facilitated the extinction of the IA memory. The blockade of GR did not revert such effects. Instead, post-training infusions of the GR antagonist RU486 into the dorsal hippocampus had no effects per se but potentiated the memory impairment induced by the CB1R agonist. The same study also showed that, in the BLA, separate infusions of the CB1R agonist, the GR antagonist, or both severely impair memory consolidation (Shoshan & Akirav, 2017). Such conflicting results, especially with the endocannabinoid system (i.e., memory consolidation enhancement vs. impairment after CB1R activation), may be associated with methodological differences, mainly the footshock intensity/duration and the drug doses used (see Table 2). There is evidence that the level of arousal and/or stress at the time of training may influence the effects of cannabinoids on memory (de Oliveira Alvares et al., 2010; Morena & Campolongo, 2014). In agreement, a recent study reported that systemic post-training injection of subeffective doses of corticosterone and the CB1R inverse agonist/antagonist AM251 enhanced memory after weak CFC training only when coinjected with adrenaline, suggesting that increased noradrenergic tonus is necessary for glucocorticoid-endocannabinoid modulation on memory (Gazarini et al., 2022).

Few studies have investigated the interaction of these two systems in extinction learning and reconsolidation. CB1R blockade reversed the facilitation of CFC extinction seen after dexamethasone treatment. Moreover, either direct activation of CB1R or indirect activation of the system with AM404, an endocannabinoid uptake inhibitor, facilitated extinction learning. The facilitation induced by the elevation of endocannabinoid levels was reverted by the GR but not MR blockade or when glucocorticoid synthesis was inhibited with previous systemic metyrapone treatment. On the other hand, the effects of directly activating CB1R were not affected by metyrapone (Bitencourt et al., 2014). These results suggest an essential interplay between glucocorticoids and endocannabinoids during memory extinction, where the release of glucocorticoids and GR activation would precede endocannabinoid actions. Regarding reconsolidation, Gazarini et al. (2022) reported that

**TABLE 2** Effects of associating cannabinoid with glucocorticoid drugs on consolidation, reconsolidation, and extinction of aversive memories in rats or mice (local infusion doses described as total per animal).

Paradigm	Animal			Intervention (drug, dose, route/site of administration)			Memory phase			Reference
	Species, strain	Sex	Age in days	Cannabinoid	Glucocorticoid	Moment(s) of treatment	Consolidation	Reconsolidation	Extinction	
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	AM251, 0.28 ng, BLA	Corticosterone, 3.0mg/kg, s.c.	both IAT	↓☒			Campolongo et al. (2009)
CFC, 1 × 0.3 mA/2 s	Wistar rats	♂	60–90	AM251, 11 ng, DH	Dexamethasone, 0.01 mg/kg, i.p.	Just BT and IAT	↓☒			de Oliveira Alvares et al. (2010)
CFC, 1 × 1.5 mA/1 s	Wistar rats	♂	90	SR141716, 0.2 mg/kg, i.p.	Dexamethasone, 0.5 µg, i.c.v.	25 and 5 min BET			↓☒	Bitencourt et al. (2014)
CFC, 1 × 1.5 mA/1 s	Wistar rats	♂	90	AM404, 0.5 µg, i.c.v.	Metyrapone, 25 mg/kg, s.c.	5 and 95 min BET			↓☒	Bitencourt et al. (2014)
CFC, 1 × 1.5 mA/1 s	Wistar rats	♂	90	WIN55,212-2, 0.1 µg, i.c.v.	Metyrapone, 25 mg/kg, s.c.	5 and 95 min BET			↔☒	Bitencourt et al. (2014)
CFC, 1 × 1.5 mA/1 s	Wistar rats	♂	90	AM404, 0.5 µg, i.c.v.	Mifepristone, 100 ng, i.c.v.	5 and 10 min BET			↓☒	Bitencourt et al. (2014)
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	AM251, 0.28 ng, BLA	RU 28362, 2.0 ng, BLA	both IAT	↓☒			Atsak et al. (2015)
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	AM251, 0.28 ng, BLA	Corticosterone, 6.0 ng, BLA	both IAT	↓☒			Atsak et al. (2015)
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	WIN55,212-2, 20 ng, BLA	Mifepristone, 2.0 ng, BLA	both IAT	↔☒			Atsak et al. (2015)
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	SR141716, 1.0 mg/kg, s.c.	Corticosterone, 3.0 mg/kg, s.c.	both IAT	↓☒			Atsak et al. (2015)
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	WIN55,212-2, 2.0 ng, BLA	CRF (6–33), 0.2 µg + RU38486, 2.0 ng, BLA	both IAT	↓☒			Atsak et al. (2015)
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	AM251, 0.28 ng, BLA	CRF (6–33), 0.2 µg, BLA	both IAT	↓☒			Atsak et al. (2015)

*Continued*

**TABLE 2** Effects of associating cannabinoid with glucocorticoid drugs on consolidation, reconsolidation, and extinction of aversive memories in rats or mice (local infusion doses described as total per animal)—cont'd

Paradigm	Animal			Intervention (drug, dose, route/site of administration)			Memory phase			Reference
	Task, US number, intensity and duration	Species, strain	Sex	Age in days	Cannabinoid	Glucocorticoid	Moment(s) of treatment	Consolidation	Reconsolidation	
STIA, 1 × 1.5 mA/10s	Sprague-Dawley rats	♂	ND	WIN55,212-2, 10 µg, DH	Mifepristone, 20 ng, CA1	both IAT	↑☒			Shoshan and Akirav (2017)
STIA, 1 × 1.5 mA/10s	Sprague-Dawley rats	♂	ND	WIN55,212-2, 10 µg, BLA	Mifepristone, 20 ng, BLA	both IAT	↔☒			Shoshan and Akirav (2017)
STIA, 1 × 0.45 mA/1 s	Wistar rats	♂	ND	AM251, 0.56 ng, DS	Corticosterone, 20 ng, DS	both IAT	↓☒			Siller-Pérez et al. (2019)
STIA, 1 × 0.45 mA/1 s	Wistar rats	♂	ND	AM251, 0.56 ng, DS	Corticosterone 20 ng, DS	both IAT	↓☒			Siller-Pérez et al. (2019)
STIA, 1 × 0.45 mA/1 s	Wistar rats	♂	ND	AM251, 0.56 ng, DS	Corticosterone, 3.0 mg/kg, i.p.	both IAT	↓☒			Siller-Pérez et al. (2019)
STIA, 1 × 0.45 mA/1 s	Wistar rats	♂	ND	WIN55,212-2, 200 ng, DS	Metyrapone, 50 mg/kg, i.p.	IAT and 90 min BT	↓☒			Siller-Pérez et al. (2019)
CFC, 1 × 0.7 mA/3 s	Wistar rats	♂	90	AM251, 1.0 mg/kg, i.p.	Corticosterone, 1.0 mg/kg, i.p.	both IAT	↔			Gazarini et al. (2022)
CFC, 1 × 0.7 mA/3 s	Wistar rats	♂	90	AM251, 1.0 mg/kg, i.p.	Corticosterone, 1.0 mg/kg, i.p.	both IAR		↔		Gazarini et al. (2022)

US, unconditioned stimulus (shock); ND, not described; ♂, male; ↑, increase; ↔, no effects; ↓, reduce/prevent; ☒, potentiation/facilitation; ☓, impairment; AFC, auditory fear conditioning; BET, before extinction training; BLA, basolateral amygdala; BT, before training; CeA, central amygdala; CFC, contextual fear conditioning; DH, dorsal hippocampus (CA1 area); DS, dorsal striatum; IAIE, immediately after extinction; IAR, immediately after retrieval; IAT, immediately after training; i.c.v., intracerebroventricular; i.p., intraperitoneal; s.c., subcutaneous; STIA, step-through inhibitory avoidance; AM251, CB1R inverse agonist/antagonist; AM404, anandamide transport inhibitor; CRF (6–33), corticotropin-releasing factor binding protein inhibitor peptide; dexamethasone, synthetic glucocorticoid agonist; metyrapone, glucocorticoid synthesis inhibitor; mifepristone (alternative names: RU 486, RU 38486), glucocorticoid receptor antagonist; propranolol, nonselective β-adrenoreceptor antagonist; SR141716, CB1R antagonist/inverse agonist; RU 28362, GR agonist; WIN55,212-2, CB1/CB2 receptors agonist.

systemic coadministration of subeffective doses of AM251 with corticosterone after memory reactivation did not affect memory reconsolidation, even though higher doses of each drug separately did facilitate reconsolidation.

In conclusion, current evidence points to a significant interplay between these two systems in modulating fear memories, which may be relevant to PTSD since patients have dysregulated glucocorticoids and endocannabinoid profiles (Hauer et al., 2014).

## The cannabinoid-noradrenergic-glucocorticoid interplay in fear memories

The glucocorticoid-noradrenergic cross talk has been described during memory processing (Bahtiyar et al., 2020; Rozendaal & McGaugh, 2011). Despite the endocannabinoid involvement with both systems, only some studies have addressed their potential interaction directly. The endocannabinoid signaling as an intermediary link between the glucocorticoid and noradrenergic systems was suggested since increased endocannabinoid transmission induced by CB1R agonists, endocannabinoid uptake- or degradation inhibitors prevented the corticotropin-releasing factor (CRF)-induced elevation of plasma noradrenaline/adrenaline (Shimizu et al., 2010). That result supports the “buffer” role of endocannabinoid signaling on stress-evoked responses. In contrast, a recent study points to a permissive function of the endocannabinoids, as the CRF-induced increase in LC excitability was absent in CB1R-knockout mice (Wyrofsky et al., 2018). However, another study showed the recruitment of the HPA axis and peripheral corticosterone secretion induced by systemic injection of a CB1R agonist, an effect reduced by antagonism of adrenoceptors (McLaughlin et al., 2009). Altogether, these results suggest a complex endocannabinoid-glucocorticoid-noradrenergic interplay that can extend to memory modulation, even though its outcome varies according to the particularities of each study.

Two studies have combined drugs acting in the endocannabinoid, noradrenergic, and glucocorticoid systems during memory consolidation or reconsolidation (Table 3). The reduced sensitivity of BLA  $\beta 2$ -adrenoceptor activation on enhancing the IA memory consolidation after the blockade of CB1R was also induced by local infusion of a GR antagonist, positioning both the endocannabinoid and glucocorticoid signaling as upstream steps for noradrenergic activity (Atsak et al., 2015). Interestingly, a subeffective dose of WIN55,212-2 was sufficient to prevent this shift in the noradrenergic sensitivity induced by the blockade of GR (Atsak et al., 2015). According to that complex set of results, a circuit model for such interplay into the amygdala was proposed, in which glucocorticoid activation would recruit the endocannabinoid system that, in turn, would disinhibit noradrenergic terminals by acting on CB1R of inhibitory interneurons (Fig. 2A). That model further supports the permissive role of endocannabinoids as an intermediary step for glucocorticoid-noradrenergic cross talk.

This interplay was also described more recently for CFC but yielded different outcomes. Using weak training, subeffective doses of corticosterone or AM251 combined with adrenaline induced enhancing effects on consolidation and reconsolidation (Gazarini et al., 2022). More importantly, the combination of subeffective doses of all those drugs enhanced fear memory consolidation and reconsolidation and induced a loss of memory specificity (i.e., fear overgeneralization), stressing the noradrenergic signaling relevance in that case. Such results agree with the previously described glucocorticoid and noradrenergic involvement in the specificity of fear-related memories (Bahtiyar et al., 2020; Gazarini et al., 2014) and subside the endocannabinoid-mediated buffering role of stress-related impacts on memory processing. According to that premise, an additional circuit model could be proposed in which glucocorticoid activation would recruit the endocannabinoid system that, in turn, would inhibit noradrenergic terminals either directly in the presynaptic membrane or by acting on CB1R of excitatory interneurons (Fig. 2B).

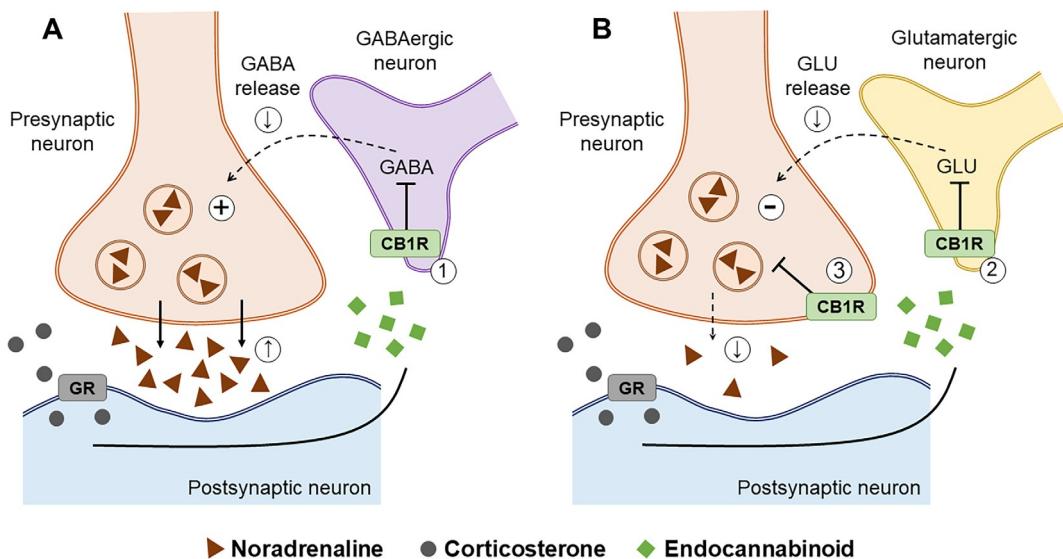
## Conclusions

Varying results like those reviewed here may create noise and challenge the hypothesis that endocannabinoid, noradrenergic, and glucocorticoid systems interact. Several aspects may account for the apparent divergences observed, namely: (i) variability of outcomes in different species (Schultheiss et al., 2005) or specific tissues/organs in the same species (Schlicker et al., 2003); (ii) divergent effects of systemic vs. local administration (Mendiguren & Pineda, 2006), probably owing to heterogeneous expression of receptors and additional peripheral mechanisms; (iii) biphasic effects of drugs, reflecting the well-known inverted-U shaped curve (Akirav, 2011; Calabrese & Rubio-Casillas, 2018; Poddar & Dewey, 1980); (iv) the often opposed/divergent effects induced by different endocannabinoids, such as those mediated by anandamide vs. 2-AG on noradrenaline release (Kurihara et al., 2001) and stress-induced modulation of brain sites (Hill et al., 2010), suggesting complex modulatory pathways; and (v) the level of stress-evoked recruitment of endocannabinoid system, which may influence its final effects on the release of noradrenaline and/or glucocorticoids, as higher stress levels could overcome such modulatory action (Reyes et al., 2012). These aspects add layers of complexity that

**TABLE 3** Effects of associating cannabinoid, noradrenergic, and glucocorticoid drugs on consolidation, reconsolidation, and extinction of aversive memories in rats or mice (local infusion doses described as total per animal).

Paradigm	Animal			Intervention (drug, dose, route/site of administration)				Memory phase			Reference
	Species, strain	Sex	Age in days	Cannabinoid	Noradrenergic	Glucocorticoid	Moment(s) of treatment	Consolidation	Reconsolidation	Extinction	
STIA, 1 × 0.6 mA/1 s	Sprague-Dawley rats	♂	ND	WIN55,212-2, 2.0ng, BLA	Clenbuterol, 2.0µg, BLA	Mifepristone, 2.0ng, BLA	All IAT	☒			Atsak et al. (2015)
CFC, 1 × 0.7 mA/3 s	Wistar rats	♂	90	AM251, 2.0mg/kg, i.p.	Adrenaline, 0.05mg/kg, i.p.	Corticosterone, 1.0mg/kg, i.p.	All IAT	☒			Gazarini et al. (2022)
CFC, 1 × 0.7 mA/3 s	Wistar rats	♂	90	AM251, 1.0mg/kg, i.p.	Adrenaline, 0.05mg/kg, i.p.	Corticosterone, 1.0mg/kg, i.p.	All IAR		☒		Gazarini et al. (2022)

US, unconditioned stimulus (shock); ND, not described; ♂, male; ☒, potentiation/facilitation; BLA, basolateral amygdala; CFC, contextual fear conditioning; IAR, immediately after retrieval; IAT, immediately after training; i.p., intraperitoneal; STIA, step-through inhibitory avoidance; AM251, CB1R inverse agonist/antagonist; clenbuterol, β2-adrenoreceptor agonist; mifepristone (alternative names: RU 486, RU 38486), GR antagonist; WIN55,212-2, CB1/CB2 receptor agonist.



**FIG. 2** A proposed model to explain opposite outcomes of the endocannabinoid-glucocorticoid-noradrenergic interplay. (A) The glucocorticoid-induced increase in the endocannabinoid levels would result in the inhibition of inhibitory neurons (1), allowing disinhibition of noradrenergic neurons and increasing noradrenaline release; (B) the glucocorticoid-induced increase in the endocannabinoid levels would result in reduced noradrenaline release either by inhibitory action on excitatory neurons (2) or direct inhibition via presynaptic receptors (3). GABA, gamma-aminobutyric acid; GLU, glutamate.

may explain most discrepancies reported. However, future studies are needed to investigate still-open questions systematically. For instance, are there sex-dependent differences among the endocannabinoid-noradrenergic-glucocorticoid interplay? Can the association of drugs acting on these stress-related neurotransmitters turn traumatic memories into typical aversive memories that become malleable and prone to mitigation? Can such a synergistic approach represent a translational “next step” for managing traumatic memories?

## Applications to other areas

This chapter reviews evidence from animal studies investigating the endocannabinoid-noradrenergic-glucocorticoid interplay in the brain and its potential implications for fear memory processing, focusing on the modulatory role of cannabinoid transmission. The interplay among these neurotransmitters is evident for memory processing, although the topic was addressed only recently. Most evidence supports such neurochemical cross talk but indicates different outcomes according to the dose used, route of administration, brain structure studied, and memory task adopted. Besides being clear for memory consolidation, reconsolidation, and extinction, these results also point to the need for further studying such complex interplay in other memory phases and events, such as acquisition, retrieval, and persistence. From a translational perspective, understanding such interplay contributes to the further characterization of the physiopathology of memory-related mental disorders, such as posttraumatic stress disorder (PTSD), which is associated with impaired function of all the neurotransmitters mentioned above (Hauer et al., 2014; Hill et al., 2013; Yehuda et al., 1998, 2004), and associated with the abnormal, aberrant formation of trauma-related memories. Indeed, they often are exaggerated and less prone to therapeutic interventions, either psychological or pharmacological (Gazarini et al., 2014). A better understanding of interactions among stress-related mediators may explain clinical particularities of the traumatic memory and guide future research exploring such interplay as a therapeutic approach to optimize the currently available pharmacological arsenal to manage PTSD and other memory-related disorders. The evidence for such interplay on fear memory processing also offers a mechanistic basis for its extension to other biological functions that are notably related to those neurotransmitters.

## Mini-dictionary of terms

- **Fear conditioning.** A memory task in which the animal expresses fear responses when confronted with a conditioned stimulus (a context, for contextual fear conditioning, or a sound for auditory fear conditioning) previously paired with footshocks.

- **Fear extinction.** Induction of a neutral associative memory that suppresses a previously established one, leading to reduced fear expression.
- **Fear overgeneralization.** The loss of fear memory specificity, leading to the expression of fear-related behaviors when confronted even with neutral stimuli.
- **Step-through inhibitory avoidance.** A memory task in which the animal learns to avoid the urge to enter a chamber after pairing such behavior with a footshock.
- **Long-term potentiation.** A plastic synaptic event related to changes in neural communication efficiency, which is interpreted as the physiological mechanism of memory maintenance over time.
- **Memory consolidation.** Post-acquisition time-dependent stabilization phase of the memory sensitive to experimental interventions that can influence its strength or specificity;
- **Memory engram.** The neural representation of a memory in neuron circuits and the brain.
- **Memory reconsolidation.** A new time-dependent restabilization phase elicited after short retrieval of a previously established memory sensitive to interventions influencing its strength, specificity, and maintenance.

## Key facts of the noradrenergic system

- It uses adrenaline and noradrenaline as neurotransmitters.
- They act on membrane G protein-coupled receptors, mainly  $\alpha$ 1-,  $\alpha$ 2-,  $\beta$ 1, and  $\beta$ 2-adrenoceptors.
- Adrenergic receptors expressed in pre- and post-synaptic neurons allow the feedback control on noradrenergic activity and modulation in neuronal communication in general, respectively.
- Locus coeruleus concentrates the noradrenergic-producing neurons, which send projections to most central nervous system regions.
- Stress-induced peripheral release of adrenaline can increase brain noradrenaline through vagal afferents' activation and indirect excitation of locus coeruleus.

## Key facts of the glucocorticoid system

- Human cortisol has comparable actions to rodents' corticosterone.
- Glucocorticoids are released peripherally by the adrenal cortex after the stress-induced activation of the hypothalamus-pituitary-adrenal axis.
- They readily cross the blood-brain barrier and modulate brain responses to stress and emotional stimuli.
- Glucocorticoids released after stress activate membrane-associated variants of the steroid receptors, promoting the release of endocannabinoids.
- Glucocorticoids enhance physiological and cognitive functions following an inverted U-curve dose-effect relation.

## Summary points

- Stress-related neurotransmitters, such as endocannabinoids, noradrenaline, and glucocorticoids, modulate fear memory formation and maintenance.
- Dysfunctions in all these neurochemical signalings are associated with posttraumatic stress disorder physiopathology, including its development and behavioral outcomes.
- Endocannabinoids modulate the release of noradrenaline in the periphery and nervous system, supporting their interplay on memory processing.
- Bidirectional cross talk between the endocannabinoid and glucocorticoid systems underlies their effects on aversive memories.
- The endocannabinoid signaling also plays a role in linking the well-known noradrenergic-glucocorticoid interplay on memory processing.
- Such stress-related mediators can act synergistically or antagonistically to support emotional memory processing and different behavioral outcomes.
- Divergent results are associated with differences in the memory task adopted, animal strain/species used, drug doses, and local vs. systemic modulation.

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## Chapter 20

# Adenosine A<sub>2A</sub>-cannabinoid CB<sub>1</sub> receptor heteromers in the brain: From trans-inhibition to trans-activation

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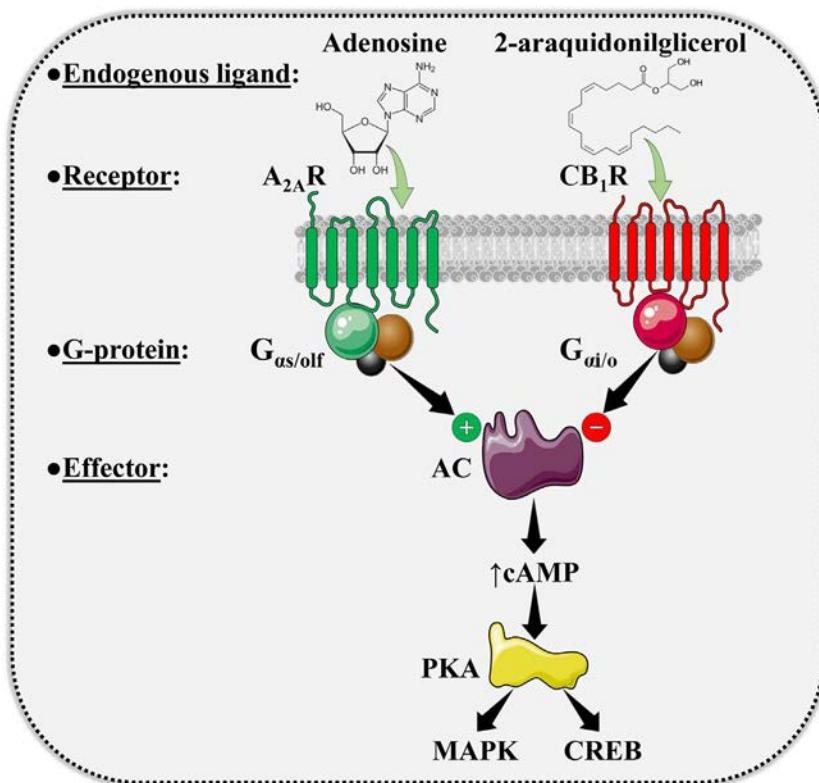
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## Abbreviations

A <sub>2A</sub> R	adenosine A <sub>2A</sub> receptor
AC	adenylyl cyclase
ALPHA	amplified luminescent proximity homogeneous assay
BiFC	bimolecular fluorescence complementation
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CB <sub>1</sub> R	cannabinoid CB <sub>1</sub> receptor
CNS	central nervous system
CREB	cAMP response element-binding
D <sub>2</sub> R	dopamine 2 receptor
FRET	fluorescence resonance energy transfer
GPCR	G-protein-coupled receptor
MAPK	mitogen-activated protein kinase
PKA	protein kinase A
PLISA	proximity ligation in situ assay
R <sub>luc</sub>	<i>Renilla</i> luciferase
SRET	sequential BRET-FRET
THC	Δ <sup>9</sup> -tetrahydrocannabinol
YFP	yellow fluorescent protein

## Introduction

Adenosine A<sub>2A</sub> and cannabinoid CB<sub>1</sub> receptors (i.e., A<sub>2A</sub>Rs and CB<sub>1</sub>Rs, respectively) are G-protein-coupled receptors (GPCRs) highly expressed in the brain. While the A<sub>2A</sub>R is mostly concentrated in the dorsal and ventral striatum as well as in the olfactory tubercle, and significantly lower levels of expression are found at other brain regions including hippocampus and cerebral cortex (Svenningsson et al., 1999), the CB<sub>1</sub>R shows a more widespread distribution (Herkenham et al., 1991; Svenningsson et al., 1999). The A<sub>2A</sub>R couples to G<sub>αs/olf</sub> protein, which leads to adenylyl cyclase (AC) activation and intracellular cAMP accumulation upon adenosine binding, thus triggering a signaling cascade involving PKC activation and Ca<sup>2+</sup> ion channels modulation (Fig. 1) (Cunha, 2001). Conversely, CB<sub>1</sub>R is coupled to G<sub>αi/o</sub> proteins that inhibit AC activity, activate mitogen-activated protein kinase (MAPK) signaling pathways, and regulate K<sup>+</sup> and Ca<sup>2+</sup> ion channels in response to neuronal depolarization or pharmacological stimulation (Fig. 1) (Howlett, 2005). Hence, both receptors may play opposed roles in synaptic transmission regulation, thus while A<sub>2A</sub>R is mostly facilitatory, the CB<sub>1</sub>R inhibits neurotransmitter release. Accordingly, it is widely accepted that A<sub>2A</sub>R and CB<sub>1</sub>R are key modulators of brain homeostasis and are considered interesting targets to treat a plethora of disorders affecting the central nervous system (CNS) (Cristino et al., 2020; Domenici et al., 2019).



**FIG. 1** Schematic representation of A<sub>2A</sub>R and CB<sub>1</sub>R G-protein coupling. A<sub>2A</sub>R and CB<sub>1</sub>R differentially modulate adenylate cyclase (AC) through G<sub>αs/o/olf</sub> and G<sub>αi/o</sub> protein, respectively. The cAMP activates protein kinase A (PKA), which subsequently modulates mitogen-activated protein kinase (MAPK) and cAMP response element-binding (CREB). Figure designed using image templates from Servier Medical Art (<https://smart.servier.com/image-set-download/>).

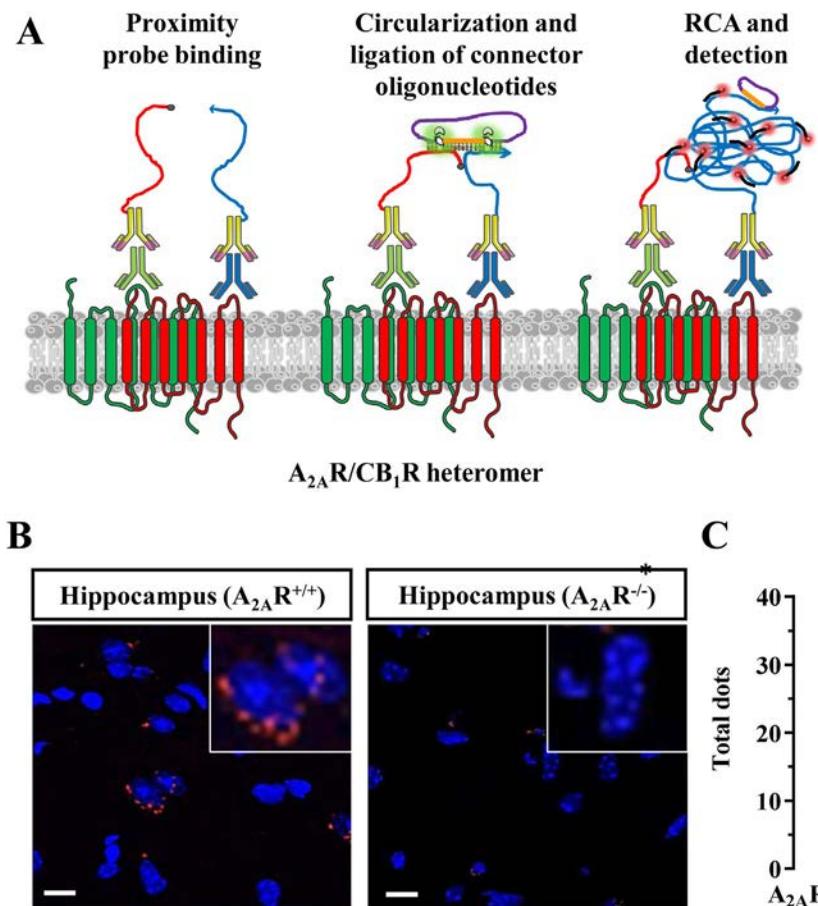
Growing evidence suggests that A<sub>2A</sub>R and CB<sub>1</sub>R functionally and physically interact through the formation of heteromeric complexes with functional consequences to neuronal activity. In the brain, A<sub>2A</sub>R is found predominantly at post-synaptic neurons within the striatum, but they are also detected at significantly lower levels at presynaptic sites in cortico-striatal terminals and in the hippocampus (Rebola et al., 2005). Most of the findings about A<sub>2A</sub>R/CB<sub>1</sub>R heteromer formation have been obtained by studying its expression at the striatum (Bonaventura et al., 2014; Carriba et al., 2007; Chiodi et al., 2016; Köfalvi et al., 2020; Moreno et al., 2018; Navarro et al., 2010), although there is also evidence for its occurrence at the hippocampus (Figs. 2 and 3) (Aso et al., 2019). In this chapter, we review the current knowledge about A<sub>2A</sub>R-CB<sub>1</sub>R interactions and discuss the future perspectives in this field.

## Evidence of A<sub>2A</sub>R and CB<sub>1</sub>R functional interactions

### In vivo and ex vivo experiments

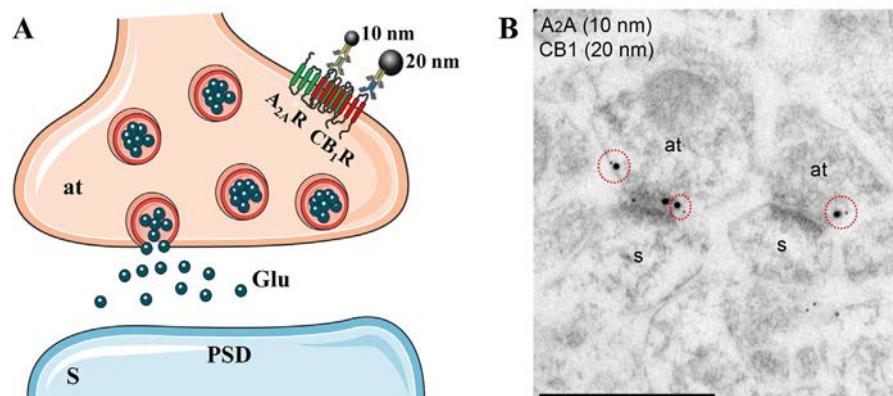
First evidence suggests a functional interaction between A<sub>2A</sub>R and CB<sub>1</sub>R derived from behavioral studies assessing the effects of CB<sub>1</sub>R agonists in A<sub>2A</sub>R knockout mice. Precisely, it was demonstrated that A<sub>2A</sub>R deletion attenuated the somatic manifestations of both the withdrawal and the rewarding effects of Δ<sup>9</sup>-tetrahydrocannabinol (THC), the main psychoactive compound of cannabis, in mice (Soria et al., 2004). In line with these observations, it was shown that the hypolocomotion induced by CP55,940, a synthetic cannabinoid with higher potency over CB<sub>1</sub>R than THC, was reduced in A<sub>2A</sub>R knockout mice (Andersson et al., 2005), an effect that was further corroborated by using an A<sub>2A</sub>R antagonist in behaving animals. In addition, the motor depressant effects produced by the intrastriatal administration of WIN55,212-2, a potent non-selective CB<sub>1</sub>R agonist, were completely abolished by the administration of a low systemic dose of the A<sub>2A</sub>R antagonist MSX-3, which did not significantly modify motor activity by itself (Carriba et al., 2007).

Aside from those pioneering studies, some additional findings supported the idea of the existence of a facilitatory A<sub>2A</sub>R-CB<sub>1</sub>R functional interaction within the striatum, thus suggesting that an endogenous tonic A<sub>2A</sub>R activation is



**FIG. 2** Detection of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in the hippocampus through PLISA. (A) Schematic representation of Proximity Ligation in situ Assay (PLISA). Primary antibodies are used to detect A<sub>2A</sub>R and CB<sub>1</sub>R (left image). Next, the dual binding of a pair of proximity probes (secondary species-antibodies with attached DNA strands, illustrated in red and blue) targeting the primary antibodies serves to template the hybridization of circularization oligonucleotides, which are then joined by ligation by T4 DNA ligase (illustrated as green oval) into a circular ssDNA molecule (purple curvy circle) (middle image). The closed circular ssDNA may serve as a template for the phi29 DNA polymerase, which extends the 3'-OH ends of one of the PLA probes (blue) acting as a primer for rolling circle amplification (RCA). Finally, the generated concatemeric product is hybridized with fluorescent oligonucleotide probes (red spot) (right image). Figure designed using image templates from Servier Medical Art (<https://smart.servier.com/image-set-download/>). (B) Representative images of dual recognition of A<sub>2A</sub>R and CB<sub>1</sub>R by PLISA in mouse hippocampus. Hippocampus from A<sub>2A</sub>R<sup>-/-</sup> mice was used as negative control. Scale bar: 100 μm. (C) Quantification of PLISA signals for A<sub>2A</sub>R and CB<sub>1</sub>R proximity confirmed the significant difference in PLISA signal density between A<sub>2A</sub>R<sup>+/+</sup> and A<sub>2A</sub>R<sup>-/-</sup> mice. Values correspond to the mean ± SEM (total dots). \*\*P < 0.01, Student's t-test. (Panel A: Adapted from Taura, J., Fernández-Dueñas, V., & Ciruela, F. (2015). Visualizing G protein-coupled receptor-receptor interactions in brain using proximity ligation in situ assay. Current Protocols in Cell Biology, 67, 17.17.1–17.17.16. doi:10.1002/0471143030.cb1717s67; Panel C: Adapted from Aso, E., Fernández-Dueñas, V., López-Cano, M., Taura, J., Watanabe, M., Ferrer, I., ... Ciruela, F. (2019). Adenosine A<sub>2A</sub>-cannabinoid CB<sub>1</sub> receptor heteromers in the hippocampus: Cannabidiol blunts Δ9-tetrahydrocannabinol-induced cognitive impairment. Molecular Neurobiology, 56(8), 5382–5391. <https://doi.org/10.1007/s12035-018-1456-3>.)

required for proper CB<sub>1</sub>R signaling. Hence, the ability of WIN55,212-2 to depress synaptic transmission in corticostriatal slices was prevented by the pharmacological or genetic blockade of A<sub>2A</sub>R (Tebano et al., 2009). Similarly, a submaximal dose of MSX-3 reduced the reinforcing effects of THC and anandamide, one of the major endogenous cannabinoids, in squirrel monkeys ( Justinová et al., 2011). However, many other studies suggested that A<sub>2A</sub>R and CB<sub>1</sub>R exhibit a reciprocal antagonistic functional interaction. Specifically, while A<sub>2A</sub>R blockade boosted both CB<sub>1</sub>R-mediated presynaptic effects at GABAergic synapses (Rossi et al., 2009) and the CB<sub>1</sub>R-dependent long-term depression in the dorsal striatum (Lerner et al., 2010; Lerner & Kreitzer, 2012), its activation precluded both CB<sub>1</sub>R-mediated depression of synaptic transmission and inhibition of glutamate release within the same brain area (Ferreira et al., 2015; Köfalvi et al., 2020; Martire et al., 2011). Accordingly, striatal A<sub>2A</sub>R overexpression resulted in a significant reduction of CB<sub>1</sub>R-mediated effects on synaptic transmission in corticostriatal slices, glutamate outflow and on locomotor activity (Chioldi et al., 2016). Nevertheless, a recent study suggested that CB<sub>1</sub>R-mediated inhibition of striatal glutamate release could be explained by the existence



**FIG. 3** Detection of A<sub>2A</sub>R and CB<sub>1</sub>R in the hippocampus by immunogold EM. (A) Schematic diagram showing the immunogold/EM-based approach for the detection of A<sub>2A</sub>R and CB<sub>1</sub>R within a hippocampal synapse. The axon terminal (at), spine (S), postsynaptic density (PSD), and glutamate (Glu) are indicated. Figure designed using image templates from Servier Medical Art (<https://smart.servier.com/image-set-download/>). (B) Electron micrographs revealing A<sub>2A</sub>R-CB<sub>1</sub>R co-clustering in presynaptic terminals in the CA1 area of the hippocampus using a double-labeling, post-embedding immunogold technique. Immunoparticles specifically recognizing A<sub>2A</sub>R (10 nm size) and CB<sub>1</sub>R (20 nm size) were detected along the extrasynaptic and perisynaptic plasma membranes of the same presynaptic axon terminals (dotted red circles), establishing synaptic contact with spines (s). Scale bar: 0.2 μm. (Adapted from Aso, E., Fernández-Dueñas, V., López-Cano, M., Taura, J., Watanabe, M., Ferrer, I., ... Ciruela, F. (2019). Adenosine A2A-cannabinoid CB1 receptor heteromers in the hippocampus: Cannabidiol blunts Δ9-tetrahydrocannabinol-induced cognitive impairment. Molecular Neurobiology, 56 (8), 5382–5391. <https://doi.org/10.1007/s12035-018-1456-3>.)

of an A<sub>2A</sub>R/CB<sub>1</sub>R heteromer-dependent CB<sub>1</sub>R transinhibition of A<sub>2A</sub>R-mediated AC constitutive activation (Kőfalvi et al., 2020). Finally, a comparable A<sub>2A</sub>R-CB<sub>1</sub>R transinhibitory functional interaction was also suggested at the hippocampus, thus a subeffective dose of THC resulted in memory impairment in mice previously pretreated with the preferentially pre-synaptic A<sub>2A</sub>R antagonist SCH442416 (Aso et al., 2019).

Overall, this apparent controversy about the facilitatory or inhibitory nature of A<sub>2A</sub>R-CB<sub>1</sub>R functional interaction could be explained by (i) the convergence of adenosine and endocannabinoid signaling through the same/different downstream pathways, thus independently of the A<sub>2A</sub>R/CB<sub>1</sub>R heteromer formation; or (ii) the different subcellular localization (i.e., pre- vs. postsynaptic) of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers within the same/different neuronal populations. Finally, it should be considered that both receptors also interact differentially with a plethora of partners in each specific brain area implicated in such effects. In this sense, previous studies demonstrated that both A<sub>2A</sub>R and CB<sub>1</sub>R may form heteromers with other GPCRs, including adenosine A<sub>1</sub> receptor (A<sub>1</sub>R) (Ciruela et al., 2006), dopamine D<sub>2</sub> (Bonaventura et al., 2014; Ferré et al., 1991; Marcellino et al., 2008; Pinna et al., 2014), or glutamate mGlu<sub>5</sub> receptor (Cabello et al., 2009; Nishi et al., 2003).

## In vitro experiments

In parallel, in vitro functional interactions were also described, thus deciphering the detailed signaling engagement between A<sub>2A</sub>R and CB<sub>1</sub>R in cultured cells. To this end, signaling experiments performed in cultured human neuroblastoma cells (i.e., SH-SY5Y) endogenously expressing A<sub>2A</sub>R and CB<sub>1</sub>R were reported (Carriba et al., 2007). As already stated, A<sub>2A</sub>R stimulates AC through coupling to G<sub>αs/olf</sub>, whereas CB<sub>1</sub>R inhibits it by means of G<sub>αi/o</sub> proteins (Fig. 1), thus classical cAMP accumulation experiments were used to monitor functional cross talk between these receptors. Assuming a canonical interaction between G<sub>αs/olf</sub> and G<sub>αi/o</sub>-protein-coupled receptors at the AC level, CB<sub>1</sub>R activation will counteract A<sub>2A</sub>R agonist-induced AC activation. Indeed, treating SH-SY5Y cells with ACEA, a selective CB<sub>1</sub>R agonist, was able to counteract forskolin-induced cAMP accumulation, only when tonic activation of A<sub>2A</sub>R was achieved (Carriba et al., 2007). Accordingly, the CB<sub>1</sub>R-G<sub>αi/o</sub> protein signaling was abolished when A<sub>2A</sub>R was blocked. These results indicate that in human neuroblastoma SH-SY5Y cells, a basal activation of A<sub>2A</sub>R is necessary for CB<sub>1</sub>R signaling, thus suggesting that CB<sub>1</sub>R functioning is entirely dependent on A<sub>2A</sub>R signaling.

Also, the functional interplay between A<sub>2A</sub>R and CB<sub>1</sub>R was evaluated in conditionally immortalized striatal neuroblasts obtained from wild-type mice (STHdh<sup>Q7/Q7</sup> cells) by assessing the global cellular response using dynamic mass redistribution (DMR) label-free assays (Moreno et al., 2018). Interestingly, despite STHdh<sup>Q7/Q7</sup> cells endogenously expressing A<sub>2A</sub>R and CB<sub>1</sub>R, these receptors do not significantly couple to G<sub>αs/olf</sub> or G<sub>αi/o</sub> proteins, respectively. Thus, neither the A<sub>2A</sub>R nor the CB<sub>1</sub>R agonists were able to modify basal or forskolin-induced cAMP accumulation (Moreno et al., 2018). Instead, a coupling to G<sub>αq</sub> protein was observed as the preincubation with the G<sub>αq</sub> protein inhibitor YM-254890

precluded both A<sub>2A</sub>R and CB<sub>1</sub>R signaling. Importantly, the A<sub>2A</sub>R and CB<sub>1</sub>R canonical G<sub>αs/olf</sub>/G<sub>αi/o</sub> proteins coupling was rescued upon incubation of the cells with transmembrane A<sub>2A</sub>R and CB<sub>1</sub>R peptides disrupting the putative A<sub>2A</sub>R/CB<sub>1</sub>R heteromer (Moreno et al., 2018). These data indicate that A<sub>2A</sub>R and CB<sub>1</sub>R co-expression facilitates G<sub>αq</sub> rather than G<sub>αs/olf</sub>/G<sub>αi/o</sub> proteins coupling in mouse striatal neuroblasts.

Apparently, the discrepancies shown for the precise A<sub>2A</sub>R-CB<sub>1</sub>R functional interplay in different cellular systems may reflect the complexity of the biochemical properties behind the potential physical interactions that might be established between these two receptors. Thus, the existence of unique structural poses for A<sub>2A</sub>R/CB<sub>1</sub>R heteromers defining the coupling to different transducers (i.e., G<sub>αs/olf</sub>, G<sub>αi/o</sub>, and/or G<sub>αq</sub>) may be outlined by the cellular environment (i.e., conditionally immortalized striatal neuroblasts vs. SH-SY5Y cells) and/or the subcellular location (i.e., pre- vs. postsynaptic).

## Molecular confirmation of the existence of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers

### In vitro evidence

The ability of A<sub>2A</sub>R and CB<sub>1</sub>R to physically interact forming heteromers was initially suggested from results obtained in heterologous expression systems using biophysical techniques (Carriba et al., 2007). Thus, bioluminescence resonance energy transfer (BRET) experiments were performed in HEK-293T cells transiently expressing A<sub>2A</sub>R and CB<sub>1</sub>R tagged with *Renilla* luciferase (*Rluc*) and yellow fluorescent protein (YFP), respectively. Interestingly, these BRET experiments demonstrated that A<sub>2A</sub>R and CB<sub>1</sub>R form constitutive heteromers in living cells (Carriba et al., 2007; Navarro et al., 2008). In addition, the formation of higher-ordered oligomers containing A<sub>2A</sub>R and CB<sub>1</sub>R plus D<sub>2</sub>R in cultured cells was also demonstrated by implementing either a sequential BRET-FRET (SRET) technique (Carriba et al., 2008) or by combining bimolecular fluorescence complementation (BiFC) with BRET (Navarro et al., 2008). In any case, the in vitro demonstration of the existence of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers relied in cells ectopically overexpressing these receptors, which engaged in energy transfer-based processes, thus making difficult any biological interpretation. Overall, these results add a layer of complexity to understand the signaling of these receptors.

### Ex vivo evidence

Subsequently, the existence of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in native systems was further corroborated. Initially, double immunofluorescence staining of coronal sections of rat striatum showed that A<sub>2A</sub>R and CB<sub>1</sub>R co-distributed within the same striatal neurons (Carriba et al., 2007). Also, the existence of potential A<sub>2A</sub>R-CB<sub>1</sub>R physical interactions in striatal membrane extracts was demonstrated by co-immunoprecipitation experiments (Carriba et al., 2007). Despite these results being compatible with the existence of striatal A<sub>2A</sub>R/CB<sub>1</sub>R heteromers, the presence of intermediate proteins bridging both receptors can't be ruled out. Interestingly, for detecting GPCR oligomerization in the brain, several experimental approaches have been implemented, for review see (Fernández-Dueñas et al., 2021). Precisely, the ability of A<sub>2A</sub>R and CB<sub>1</sub>R to heteromerize in the monkey striatum (Bonaventura et al., 2014), mouse dorsal striatum (Moreno et al., 2018), and CA1 area of the mouse hippocampus (Aso et al., 2019) was demonstrated by Proximity Ligation in situ Assay (PLISA) experiments, thus successfully allowing the visualization of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in these animal brain regions. Indeed, while PLISA was originally designed to detect protein-protein interactions in general, it also provided enough sensitivity to quantify GPCR oligomers in native tissues with subcellular resolution (Taura et al., 2015). Hence, through PLISA experiments, it was possible to determine that while A<sub>2A</sub>R/CB<sub>1</sub>R heteromers were absent at presynaptic corticostriatal projections and striatonigral neurons of the mouse dorsal striatum, these heteromers were abundantly expressed on GABAergic striatopallidal neurons, not only on their somatodendritic compartment but also likely on their terminals (Moreno et al., 2018). These results may sound ambiguous according to the proposed functional role of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in the control of glutamate release from corticostriatal projections at the striatum (Kőfalvi et al., 2020). Consequently, the pre- vs. postsynaptic location of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers at the striatum still is a matter of debate, thus more precise experiments would be needed to answer to this apparent conundrum. Conversely, at the CA1 region of the hippocampus, PLISA experiments revealed that A<sub>2A</sub>R/CB<sub>1</sub>R heteromers were mostly expressed presynaptically (Fig. 2) (Aso et al., 2019). Importantly, the presynaptic localization of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers at the hippocampus was demonstrated by: (i) co-distribution of the PLISA signal (i.e., A<sub>2A</sub>R/CB<sub>1</sub>R heteromer) with vGlut1 (i.e., a presynaptic glutamate transporter) (Fig. 2); and (ii) double-labeling immunogold electron microscopy experiments showing the co-distribution of immunoparticles for A<sub>2A</sub>R and CB<sub>1</sub>R in axon terminals projecting to dendritic spines (Fig. 3) (Aso et al., 2019). Overall, this compiling evidence demonstrated the existence of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in the brain, thus showing a unique presynaptic expression at the hippocampus and a likely pre- and postsynaptic distribution at the striatum.

## Pharmacological consequences of A<sub>2A</sub>R/CB<sub>1</sub>R heteromerization

A common feature of GPCR heteromer formation consists of the appearance of pharmacological alterations at the heteromeric receptor: the ligand binding to one receptor can modulate ligand binding to the partner receptor, thus constituting a pharmacological fingerprint of the named receptor heteromer (Ferre et al., 2009). As for the A<sub>2A</sub>R and CB<sub>1</sub>R, the pharmacological information regarding the consequences of its heteromerization is limited when compared with other GPCR oligomers studied (i.e., A<sub>2A</sub>R/D<sub>2</sub>R heteromer). Yet, it has been reported that the A<sub>2A</sub>R and CB<sub>1</sub>R heteromerization generates a pharmacological cross talk between these receptors (Ferreira et al., 2015). Thus, radioligand-binding experiments in the striatal synaptosomal membranes, where the endogenous adenosine and 2-arachidonoyl-glycerol were removed, revealed that CGS21680, an A<sub>2A</sub>R agonist, significantly reduced (~17%) the binding of the radiolabeled CB<sub>1</sub>R antagonist SR141716A (Ferreira et al., 2015). Indeed, this limited piece of information will be compatible with the existence of an A<sub>2A</sub>R/CB<sub>1</sub>R heteromer-dependent negative allosteric interaction within the context of macromolecular membrane assemblies containing these two receptors (Ferré et al., 2022). However, further pharmacological studies will be required to demonstrate this contention. Overall, these scarce results point toward the existence of a transinhibitory A<sub>2A</sub>R- CB<sub>1</sub>R interaction, now at the pharmacological level.

Nevertheless, in view of the previously mentioned functional results suggesting that A<sub>2A</sub>R antagonists behave as CB<sub>1</sub>R antagonists both *in vitro* and *in vivo* models (Carriba et al., 2007), the ability of the former (i.e., ZM241385, MSX-2, and MSX-3) to selectively bind to the CB<sub>1</sub>R was assessed in rat striatal membranes. Importantly, A<sub>2A</sub>R antagonists were unable to displace a low concentration (0.1 nM) of [<sup>3</sup>H]CP55940, a CB<sub>1</sub>R radioligand agonist (Carriba et al., 2007). These results indicate that A<sub>2A</sub>R antagonists do not bind to CB<sub>1</sub>R, thus suggesting that CB<sub>1</sub>R antagonist-like behavior of A<sub>2A</sub>R receptor antagonists is due to a functional A<sub>2A</sub>R-CB<sub>1</sub>R interplay.

## Future perspectives and challenges in the field

As stated above, there is a significant body of evidence, summarized in Tables 1 and 2, supporting the existence of functional A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in the brain, mainly in the striatum but also in the hippocampus, although the precise sub-cellular expression of these heteromers still is under debate. Indeed, both A<sub>2A</sub>Rs and CB<sub>1</sub>Rs play a key role in adenosine- and cannabinoid-mediated fine-tune modulation of neurotransmission (i.e., glutamatergic, and dopaminergic) in many neurological functions including cognition, emotion, motor activity, or pain. Accordingly, knowing the precise molecular and functional interplay of these two receptors through the formation of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers at different neural levels will be required to better understand the neurobiology of these neuromodulators in health and disease.

One of the challenges within the GPCR oligomerization field in general, and in the study of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in particular, consists of having robust, reliable, and sensitive experimental approaches to monitor the dynamics of heteromer formation in native conditions. Indeed, the detection and relative quantification of GPCR oligomers in the brain tissues from animal and human origin can be assessed through different experimental approaches, for review see Fernández-Dueñas et al. (2021). From these methodologies, some will be more prone to be used routinely in conventional research laboratories (i.e., PLISA) (Fig. 2), but others requiring more sophisticated and specialized devices (i.e., Immunogold electron microscopy) (Fig. 3). In addition, the results throughput from each technique constitutes an important question to be considered, as to at least a medium throughput would be desirable if robust semi-quantitative experiments are aimed

**TABLE 1** Summary of the evidence of A<sub>2A</sub>R and CB<sub>1</sub>R functional interaction.

Experimental model	Test	Results	References
In vitro			
Human neuroblastoma cells (SH-SY5Y)	cAMP accumulation	A basal A <sub>2A</sub> R activation was needed for CB <sub>1</sub> R signaling	Carriba et al. (2007)
Immortalized striatal neuroblasts (STHdh <sup>Q7/Q7</sup> )	DMR label-free assay	A <sub>2A</sub> R and CB <sub>1</sub> R co-expression facilitated G <sub>αq</sub> rather than G <sub>αs/olf</sub> /G <sub>αi/o</sub> proteins coupling	Moreno et al. (2018)
		Disruption of A <sub>2A</sub> R/CB <sub>1</sub> R heteromer rescued the A <sub>2A</sub> R and CB <sub>1</sub> R canonical G <sub>αs/olf</sub> /G <sub>αi/o</sub> proteins coupling	

**TABLE 1** Summary of the evidence of A<sub>2A</sub>R and CB<sub>1</sub>R functional interaction—cont'd

Experimental model	Test	Results	References
Ex vivo			
Corticostriatal rat and mice slices	Extracellular FP recordings	A <sub>2A</sub> R blockade prevented the WIN55,212-2-induced depression of synaptic transmission	Tebano et al. (2009)
Corticostriatal mice slices	Whole-cell patch clamp recordings	A <sub>2A</sub> R blockade enhanced CB <sub>1</sub> R-mediated presynaptic effects at GABAergic synapses	Rossi et al. (2009)
Corticostriatal Drd2-GFP mice slices	Whole-cell voltage-clamp recordings	A <sub>2A</sub> R blockade enhanced CB <sub>1</sub> R-dependent LTD in striatum	Lerner et al. (2010)
Corticostriatal rat slices	Extracellular FP recordings	A <sub>2A</sub> R activation inhibited CB <sub>1</sub> R-mediated PPF and inhibition of glutamate release	Martire et al. (2011)
Corticostriatal rat nerve terminals	Radioligand binding	A <sub>2A</sub> R activation decreased CB <sub>1</sub> R binding	Ferreira et al. (2015)
	[ <sup>14</sup> C]-Glu release	A <sub>2A</sub> R activation decreased CB <sub>1</sub> R inhibition of evoked Glu release	
Corticostriatal rat slices	Whole-cell patch clamp recordings	A <sub>2A</sub> R activation prevented CB <sub>1</sub> R-mediated PPF and inhibition of Glu transmission	Ferreira et al. (2015)
Corticostriatal NSE <sub>A<sub>2A</sub></sub> rats slices	Extracellular FP recordings	A <sub>2A</sub> R overexpression reduced CB <sub>1</sub> R-mediated synaptic depression	Chiodi et al. (2016)
Corticostriatal NSE <sub>A<sub>2A</sub></sub> rat nerve terminals	Glu levels by HPLC	A <sub>2A</sub> R overexpression reduced CB <sub>1</sub> R-mediated inhibition of evoked Glu release	Chiodi et al. (2016)
Corticostriatal rat nerve terminals	[ <sup>14</sup> C]-Glu release	A <sub>2A</sub> R agonist or an antagonist allosterically counteracted CB <sub>1</sub> R agonist-induced inhibition of evoked Glu release	Köfalvi et al. (2020)
		CB <sub>1</sub> R agonist inhibited Glu release dependent on the activity of A <sub>2A</sub> R by a canonical G <sub>s</sub> -G <sub>i</sub> antagonistic interaction at the AC level	
In vivo			
A <sub>2A</sub> R KO mice	THC withdrawal and CPP	Attenuation of withdrawal signs and rewarding effects of THC	Soria et al. (2004)
A <sub>2A</sub> R KO mice	Locomotor activity	Reduction of the CP55,940-induced hypolocomotion	Andersson et al. (2005)
Sprague-Dawley rats	Locomotor activity	MSX-3 blocks the intrastriatal WIN55,212-2-induced hypolocomotion	Carriba et al. (2007)
C57BL/6 and CB <sub>1</sub> R KO mice	Locomotor activity	CB <sub>1</sub> R blockade attenuates the hyperlocomotion induced by an A <sub>2A</sub> R antagonist	Lerner et al. (2010)
Squirrel monkeys	I.V. THC and anandamide self-administration	MSX-3 reduces cannabinoids reinforcing effects	Justinová et al. (2011)
NSE <sub>A<sub>2A</sub></sub> rats	Locomotor activity	A <sub>2A</sub> R overexpression reduced the WIN55,212-2-induced hypolocomotion	Chiodi et al. (2016)
C57BL/6J mice	Novel object recognition test	A subeffective dose of THC resulted in memory impairment after A <sub>2A</sub> R blockade	Aso et al. (2019)

AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; CP55,940, potent non-selective CB<sub>1</sub>R agonist; CPP, conditioned place preference; DMR, dynamic mass redistribution; Drd2-GFP, transgenic mice expressing the reporter green fluorescent protein under the control of the D<sub>2</sub> dopamine receptor promoter; FP, field potentials; Glu, glutamate; KO, knockout; LTD, long-term depression; MSX-3, A<sub>2A</sub>R antagonist; NSE<sub>A<sub>2A</sub></sub>, a transgenic rat strain over-expressing A<sub>2A</sub>R under the control of the neural-specific enolase promoter; PPF, pair-pulse facilitation; THC, Δ<sup>9</sup>-tetrahydrocannabinol; WIN55,212-2, potent non-selective CB<sub>1</sub>R agonist; See text for details.

**TABLE 2** Summary of the evidence of A<sub>2A</sub>R and CB<sub>1</sub>R heteromerization.

Experimental model	Test	Results	References
In vitro			
HEK-293T cells expressing A <sub>2A</sub> R-Rluc and CB <sub>1</sub> R-YFP	BRET	A <sub>2A</sub> R and CB <sub>1</sub> R form constitutive heteromers	Carriba et al. (2007) Navarro et al. (2008)
HEK-293T cells expressing A <sub>2A</sub> R-Rluc, D <sub>2</sub> R-GFP and CB <sub>1</sub> R-YFP	SRET	A <sub>2A</sub> R, CB <sub>1</sub> R and D <sub>2</sub> R form constitutive heteromers	Carriba et al. (2008)
HEK-293T cells expressing A <sub>2A</sub> R-cYFP, CB <sub>1</sub> R-nYFP and D <sub>2</sub> R-Rluc	BiFC-BRET	A <sub>2A</sub> R, CB <sub>1</sub> R and D <sub>2</sub> R form constitutive heteromers	Navarro et al. (2008)
Ex vivo			
Striatal membrane extracts	Double IF staining and co-IP	Co-distribution and physical interactions between A <sub>2A</sub> R and CB <sub>1</sub> R in striatum	Carriba et al. (2007)
<i>Macaca fascicularis</i> brain tissue	PLISA	A <sub>2A</sub> R-CB <sub>1</sub> R heteromers existence in dorsal striatum	Bonaventura et al. (2014)
Mice brain tissue	PLISA	A <sub>2A</sub> R-CB <sub>1</sub> R heteromers existence in GABAergic striatopallidal neurons	Moreno et al. (2018)
Mice brain tissue	PLISA	A <sub>2A</sub> R-CB <sub>1</sub> R heteromers existence in CA1 area of the hippocampus at the pre-synaptic level	Aso et al. (2019)

*BiFC*, bimolecular fluorescence complementation; *BRET*, bioluminescence resonance energy transfer; *cYFP*, C-terminal truncated version of YFP; *Co-IP*, co-immunoprecipitation; *GFP*, green fluorescent protein; *IF*, immunofluorescence; *nYFP*, N-terminal truncated version of YFP; *PLISA*, proximity ligation in situ assay; *Rluc*, *Renilla* luciferase; *SRET*, sequential BRET-fluorescent resonance energy transfer (FRET); *YFP*, yellow fluorescent protein. See text for details.

to be performed. In that sense, recently an Amplified Luminescent Proximity Homogeneous Assay (ALPHA), which is an assay based on Luminescent Oxygen Channeling Immunoassay (LOCI) (Ullman et al., 1996), was specifically adapted for the study of GPCR oligomerization in native tissue, precisely for the study of A<sub>2A</sub>R/D<sub>2</sub>R heteromers in mouse models of disease and in human samples (Fernández-Dueñas et al., 2019; Valle-León et al., 2021). Accordingly, engineering a specific ALPHA approach for assessing A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in native tissue will be a key endeavor that would allow to study these heteromers in certain mental diseases and to evaluate the effects of potential therapeutic strategies.

Currently, another hot topic within the GPCR oligomerization field revolves around the capacity to ectopically manipulate the formation of endogenous heteromeric complexes, thus driving their function toward a specific signaling pathway and avoiding others (i.e., heteromer-based bias signaling). Accordingly, one more challenge in the study of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers consists of the design of specific tools promoting or disrupting the formation of these molecular complexes in vivo. Importantly, two of the abovementioned studies reported the use of transmembrane A<sub>2A</sub>R and CB<sub>1</sub>R interfering-peptides disrupting the putative A<sub>2A</sub>R/CB<sub>1</sub>R heteromer both in living cells and brain slices (Köfalvi et al., 2020; Moreno et al., 2018). Interestingly, these studies allowed to identify the key domains of A<sub>2A</sub>R and CB<sub>1</sub>R sustaining their heteromerization, precisely the transmembrane domain 5 and 6 of each receptor (Köfalvi et al., 2020; Moreno et al., 2018). However, certain specificity and safety concerns raise about their use in vivo, thus further research in this sense is warranted. Overall, once these technical challenges would be overcome, the knowledge about the expression and manipulation of A<sub>2A</sub>R/CB<sub>1</sub>R heteromer content in the brain will grow rapidly, thus being able to generate many pharmacotherapeutic expectancies.

## Applications to other areas

In this chapter, we have reviewed the current knowledge revolving the existence of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in the brain and its potential role in CNS function. In that sense, these A<sub>2A</sub>R/CB<sub>1</sub>R heteromers emerge as potential targets for the management of certain neurological diseases. Indeed, this notion can be illustrated with a couple of examples. The first of them is related with the putative role of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in the regulation of dopaminergic signaling in mesocortical and mesolimbic pathways, which are clearly altered in psychotic disorders such as schizophrenia (McCutcheon et al., 2019).

According to the adenosine hypothesis of schizophrenia, the classically associated hyperdopaminergic state in those brain circuitries may be secondary to a loss of function of the adenosinergic system (Boison et al., 2012). Precisely, a decline in extracellular adenosine levels or a loss of A<sub>2A</sub>R function would reduce the well-established tonic A<sub>2A</sub>R trans-inhibition of D<sub>2</sub>R signaling, thus exacerbating the psychotic symptomatology (Ferré et al., 2018). Moreover, CB<sub>1</sub>R activity is known to potentiate dopamine release in the striatum, what explains at least in part both the reinforcing effects and the psychotic-like states induced by certain cannabinoids (Bloomfield et al., 2016; Kuepper et al., 2010). Thus, considering all the evidence suggesting an A<sub>2A</sub>R-CB<sub>1</sub>R functional interaction, it seems plausible than targeting these heteromers in a way that, at the same time, facilitates the A<sub>2A</sub>R-mediated inhibition of D<sub>2</sub>R and decreases CB<sub>1</sub>R-mediated dopamine release might represent a promising strategy for schizophrenia management. Another example of the therapeutic potential of targeting A<sub>2A</sub>R/CB<sub>1</sub>R heteromers might be controlling of the imbalance within the excitatory/inhibitory neurotransmission occurring in pathological disorders with recurring episodes of excessive neural activity, such as the enhanced excitability in response to A<sub>β</sub> peptide production observed in the hippocampus of Alzheimer's disease patients (Irizarry et al., 2012; Palop et al., 2007) or the repeated seizures in epilepsy patients (Pitkänen & Sutula, 2002). Previous findings demonstrate that A<sub>2A</sub>R activation leads to glutamate release in the hippocampus (Temido-Ferreira et al., 2020), while CB<sub>1</sub>R activation produces the opposite effect protecting neurons from excitotoxicity (Marsicano et al., 2003), thus allowing the control of neuronal excitability in this brain area relevant for main cognitive functions (Lutz, 2004; Monory et al., 2006). Consequently, and considering the existence of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers at the pre-synaptic level in the hippocampus, promoting the activity of CB<sub>1</sub>R in parallel to inhibiting A<sub>2A</sub>R facilitation of glutamate release by targeting these heteromers might offer a novel therapeutic approach to mitigate the excessive neural activity occurring in Alzheimer's disease, epilepsy, or other pathologies with similar aberrant neural activity patterns.

## Mini-dictionary of terms

- **Allosteric interaction in the receptor heteromer:** Intermolecular interaction by which binding of a ligand to one of the receptor protomer within the receptor heteromer changes the binding properties of another receptor protomer.
- **Pharmacological fingerprint of the receptor heteromer:** Pharmacological characteristic of a receptor heteromer, which can be used for its identification in a native tissue.
- **Receptor heteromer:** Macromolecular complex composed of at least two (functional) receptor protomers with biochemical properties that are clearly different from those observed for its individual components.
- **Receptors trans-activation:** Facilitatory functional interaction between the receptors forming an heteromer, in which the activation of one of the heteromer protomers facilitates the activity of the other receptor protomer.
- **Receptors trans-inhibition:** Reciprocal antagonistic functional interaction between the receptors forming an heteromer, in which the activation of one of the heteromers protomer inhibits the activity of the other receptor protomer.

## Key facts of A<sub>2A</sub>-CB<sub>1</sub> receptor heteromers in the brain

- A<sub>2A</sub> and CB<sub>1</sub> receptors are G-protein-coupled receptors (GPCRs) highly expressed in the brain.
- A<sub>2A</sub>R and CB<sub>1</sub>R are key modulators of neurotransmission and brain homeostasis.
- A<sub>2A</sub>R and CB<sub>1</sub>R are interesting targets to treat a plethora of mental and neurological diseases.
- GPCRs, including A<sub>2A</sub>R and CB<sub>1</sub>R, may form functional heteromers with pharmacological properties different from those of each individual component.
- Targeting A<sub>2A</sub>R/CB<sub>1</sub>R heteromers may represent a promising and new strategy to treat certain brain disorders.

## Summary points

- Several in vitro and in vivo findings suggest an A<sub>2A</sub>R-CB<sub>1</sub>R functional interaction in the brain.
- To date, the existence of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers has been demonstrated at least in striatum and hippocampus of murine brains.
- The facilitatory or inhibitory nature of A<sub>2A</sub>R-CB<sub>1</sub>R functional interaction might depend on the exact location of these heteromers at the post- or presynaptic level and/or on the neural populations where they express.
- Further research is needed to better know the precise molecular and functional interplay of A<sub>2A</sub>R/CB<sub>1</sub>R heteromers in brain in health and disease.
- Targeting A<sub>2A</sub>R/CB<sub>1</sub>R heteromers may represent a promising and new strategy to treat certain brain disorders such as schizophrenia, Alzheimer's disease, or epilepsy.

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## Chapter 21

# Role of hippocampal CB1 and CB2 receptors in fear memory consolidation, extinction, and reconsolidation

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## Abbreviations

2-AG	2-arachidonoylglycerol
BLA	basolateral amygdala
CB1	cannabinoid type-1
CB2	cannabinoid type-2
FAAH	fatty acid amide hydrolase
KO	knockout
mPFC	medial prefrontal cortex
NMDA	<i>N</i> -methyl-D-aspartate
PTSD	posttraumatic stress disorder

## Introduction

The endocannabinoid system is highly engaged in the modulation of defensive reactions counterbalancing the consequences of a stressful stimulus (Viveros et al., 2007). Patients with posttraumatic stress disorder (PTSD) present dysfunction and low “endocannabinoid tonus,” evidenced by lower circulating levels of anandamide and 2-arachidonoylglycerol (2-AG), and reduced hair concentration of palmitoylethanolamide, oleoylethanolamide, and stearoylethanolamide compared with healthy controls (Hill et al., 2013; Wilker et al., 2016). These alterations are accompanied by a decreased hippocampal volume (Bonne et al., 2008), whereas the cannabinoid type-1 (CB1) receptor is upregulated, which seems to be a compensatory mechanism, more pronounced in women than in men (Hill et al., 2013).

A requirement of endocannabinoids, as well as other neurotransmitters and hormones, regulates the dynamic process of fear memory consolidation (Costanzi et al., 2004; Gazarini et al., 2021), in which the hippocampus plays a pivotal role (McGaugh, 2015). The interaction of the hippocampus with the basolateral amygdala (BLA) integrates sensory information from cortical regions following an aversive event, processing the adequate reaction to environmental threats. Also, the interaction of the medial prefrontal cortex (mPFC) and the hippocampus takes place to differentiate cues divergent in their meanings and contexts, playing a fundamental role in memory encoding and retrieval (Maren et al., 2013).

Systems consolidation is the most currently accepted theory of memory consolidation, which states that acquiring new and relevant information requires a time-dependent stabilization process for “permanent” information storage (Dudai, 2012). It was extensively described in preclinical studies that a time up to 6 h is necessary to fear memory consolidation (Gazarini et al., 2013; Lynch, 2004; Stern et al., 2017) and its molecular mechanisms of synaptic strength and connections among neurons, a form of Hebbian learning called long-term potentiation (Bliss & Lomo, 1973), supports the memory stabilization.

After decades of acceptance that a consolidated memory trace was not susceptible to further modifications, Przybylski and Sara (1997) suggested that memories may become again labile and consequently susceptible to interferences, evidenced by administering an antagonist of glutamatergic *N*-Methyl-D-aspartate (NMDA) receptors after short retrieval of a consolidated radial maze task. This finding was referred to as reconsolidation and in the last years has been

suggested as a mechanism for memory maintenance and updating and was observed in several animal species and types of memories, including the fearful ones (Besnard et al., 2012; Nader et al., 2000; Stern et al., 2012).

While a short retrieval induces the reconsolidation process, on the other hand, the prolonged or repeated retrieval of a fear memory may induce its extinction, a form of inhibitory learning that competes with the original one and leads to reduced fear expression. Although fear extinction depends highly on the mPFC activity, mainly the infralimbic area (Milad & Quirk, 2002; Sohn et al., 2020), the hippocampus is also required for the extinction of contextual fear memory (Lacagnina et al., 2019). Since extinction does not erase the original memory, fear may emerge over time in a process called spontaneous recovery, and also, stress or context exposures may elicit reinstatement or renewal of fear memory (VanElzakker et al., 2014). Regarding that, impairing fear memory reconsolidation has been shown to have more long-lasting effects than facilitating fear extinction.

The first evidence linking emotional memories and the endocannabinoid system derived from studies showing that knockout (KO) mice for CB1 receptor present impaired fear memory extinction (Marsicano et al., 2002). Thus, over the last two decades, several works using genetic or pharmacological manipulations in rodents exposed to protocols of associative memory, such as Pavlovian fear conditioning, or to passive or active avoidance tasks, which combines Pavlovian contextual fear conditioning and an instrumental response, have been of great value, providing new insights into the role of hippocampal cannabinoid receptors in fear memory acquisition, consolidation, reconsolidation, and extinction (Bienvenu et al., 2021).

## The hippocampus and cannabinoid receptor location

In rodents, the hippocampus is subdivided along its dorsal-ventral axis and the dorsal part is primarily related to memory consolidation, recent memory storage, and retrieval (Fanselow & Dong, 2010). It is a well-known structure with different regions, subregional differences, and diverse cell populations (Lothmann et al., 2021).

The CB1 receptor was first described and is widely expressed in several brain regions including the hippocampus (Herkenham et al., 1991). This receptor is typically localized in axon terminals, and its activation is mediated by retrograde signaling by endocannabinoids such as anandamide and 2-AG (Castillo et al., 2012). Much less is known about cannabinoid type-2 (CB2) signaling in the central nervous system; however, it has been detected in the hippocampus, cortex, cerebellum, brainstem, and midbrain (Li & Kim, 2015).

In the hippocampus, CB1 and CB2 receptors are extensively distributed along CA1, CA2, and CA3 regions and dentate gyrus (Brusco et al., 2008; Onaivi et al., 2006). The CB2 receptor is found in the microglia, principal neurons, and interneurons, and its main expression in the CA1 region is in the principal neurons, the excitatory pyramidal cells (Li & Kim, 2015; Onaivi et al., 2006), primarily on the postsynapsis. They can also be observed in dendrites near the plasma membrane, in the rough endoplasmic reticulum, Golgi apparatus, and neuronal cytoplasm, but not on axon terminals (Brusco et al., 2008). Whereas the hippocampal CB1 receptors are mainly expressed presynaptically, primarily in GABAergic interneurons (Castillo et al., 2012), but are also present in excitatory neurons and glial cells, mostly in astrocytes but also in microglia (Monory et al., 2015). CB1 receptors are also found in different cell compartments, such as endosomes and mitochondria, which increase the physiological repertoire of CB1 receptors' actions (Bénard et al., 2012), although their activation leads mostly to a reduced neurotransmitter release by protein-Gi  $\beta\gamma$  subunit, which inhibits voltage-dependent calcium channels (N and P/Q types) present in the axon terminal (Herkenham et al., 1991). Besides that, there are suggested functional differences between CB1 and CB2 receptors.

## Effects of selective blockade or activation of hippocampal CB1 receptors in fear memory consolidation and acquisition

CB1 receptor manipulations are comprehensively explored on the hippocampus in several fear memory processes, and its role in memory consolidation was primarily studied. Table 1 summarizes the effects of manipulating CB1 receptors in the dorsal hippocampus during fear memory consolidation and acquisition. Most of the studies have used the CB1 receptor antagonist AM251 into the CA1 posttraining. This drug impaired inhibitory avoidance and contextual fear conditioning consolidation in male rats (de Oliveira Alvares et al., 2005, 2006, 2010; de Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008). However, the effect of AM251 was dependent on training intensity in contextual fear conditioning, previous stress presentation, or i.p. administration of dexamethasone. When a mild footshock was used, no effect of AM251 was detected (de Oliveira Alvares et al., 2010). This finding is in line with the evidence that CB1 KO mice present enhanced fear memory consolidation only when mice are conditioned under strong training (Jacob et al., 2012), evidencing the

**TABLE 1** Effects of blockade or activation of hippocampal CB1 and CB2 receptors on fear memory consolidation.

Receptor	Drug or genetic manipulation	Site of manipulation, time-point, and animal	Behavioral test	Main results	Reference
CB1	AM251 (0.55, 5.5, and 55 ng/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (2–3 months, 210–300g)	Step-down Passive Inhibitory Avoidance	5.5 ng impaired fear memory consolidation	de Oliveira Alvares et al. (2005)
CB1	AM251 (0.55 and 5.5 ng/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (2–3 months, 210–300g)	Step-down Passive Inhibitory Avoidance	5.5 ng impaired fear memory consolidation	de Oliveira Alvares et al. (2006)
CB1	AM251 (5.5 ng/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (2–3 months, 210–300g)	Step-down Passive Inhibitory Avoidance	Impaired fear memory consolidation	de Oliveira Alvares, Genro, Diehl, and Quillfeldt (2008)
CB1	AM251 (5.5 ng/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (2–3 months, 250–320g)	Contextual fear conditioning	Impaired fear memory consolidation of a strong training (0.7 mA). In a weak training (0.3 mA) impaired fear memory consolidation only when associated with an immediate stress or a dexamethasone (0.01 mg/kg) i.p administration.	de Oliveira Alvares et al. (2010)
CB1	AM251 (5.5 ng/side)	Intra-CA1 of the dorsal hippocampus, pretraining, 24 h after a 180 s preexposure, male Wistar rats (2–3 months old, 290–350g)	Context preexposure facilitation effect	Impaired fear memory consolidation	Lunardi et al. (2020)
CB1	AM281 (0.05 ng or 0.05 µg)	Intra-CA1 of the dorsal hippocampus pretraining, male mice C57BL/6s (6–8 weeks of age, 19–26 g)	Contextual fear conditioning	0.05 µg enhanced fear memory consolidation; 0.05 ng alone had no effect, but prevented the AM404 impairing effect	Q. S. Lin et al. (2011)
CB1	CP55,940 (CB1 agonist; 1, 5, or 10 µg/µL)	Intra-CA1 of the dorsal hippocampus, posttraining, Wistar rats (270–320g)	Contextual fear conditioning	5 µg impaired fear memory consolidation	Santana et al. (2016)
CB1	AM251 (0.2 µM)	Intra-CA1 of the dorsal hippocampus, posttraining, Wistar rats (270–320g)	Contextual fear conditioning	Alone had no effect, but prevented the CP55,940 impairing effect	Santana et al. (2016)
CB2	AM630 (CB2 antagonist, 50, 75, or 100 ng/rat)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (200–240g)	Step-through Passive Inhibitory Avoidance	75 and 100 ng impaired fear memory consolidation; 50 ng restored the fear memory impairment induced by D-AP5 (NMDA antagonist)	Nasehi et al. (2017)

*Continued*

**TABLE 1** Effects of blockade or activation of hippocampal CB1 and CB2 receptors on fear memory consolidation—cont'd

Receptor	Drug or genetic manipulation	Site of manipulation, time-point, and animal	Behavioral test	Main results	Reference
CB2	AM630 (CB2 agonist, 1, 10, or 100 µg/mouse)	Intra-CA1 of the dorsal hippocampus, posttraining, male NMRI mice (25–30g, 5–8 weeks old)	Step-down Passive Inhibitory Avoidance	Alone had no effect; 1 and 10 µg restored the fear memory impairment induced by muscimol	Nasehi et al. (2018)
CB2	AM630 (1, 10, or 100 µg/mouse)	Intra-CA3 of the ventral hippocampus, posttraining, male NMRI mice (25–30g)	Step-down Passive Inhibitory Avoidance	Alone had no effect; 10 and 100 µg restored the fear memory impairment induced by scopolamine	Nasehi et al. (2020)
CB2	AM630 (0.1 nmol/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (3 months, 270–320g)	Contextual fear conditioning	Alone had no effect, but prevented the impairing effect on consolidation induced by cannabidiol i.p. administration	Stern et al. (2017)
CB2	AM630 (0.1 nmol/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (2–3 months, 270–320g)	Contextual fear conditioning	Alone had no effect, but prevent the impairing effect on consolidation induced by cannabidiol intra-dorsal hippocampus administration	Raymundi et al. (2020)
CB2	GP1a (CB2 agonist, 50 or 150 ng/rat)	Intra-CA1 of the dorsal hippocampus posttraining, male Wistar rats (200–240g)	Step-through Passive Inhibitory Avoidance	150 ng impaired fear memory consolidation; 50 ng intensified the fear memory impairment induced by D-AP5 (NMDA antagonist)	Nasehi et al. (2017)
CB2	GP1a (CB2 agonist, 1, 10, or 100 µg/mouse)	Intra-CA1 of the dorsal hippocampus, posttraining, male NMRI mice (25–30g, 5–8 weeks old)	Step-down Passive Inhibitory Avoidance	100 µg impaired fear memory consolidation; 10 µg intensified the fear memory impairment induced by muscimol	Nasehi et al. (2018)
CB2	GP1a (10 or 100 µg/mouse)	Intra-CA3 of the ventral hippocampus, posttraining, male NMRI mice (25–30g)	Step-down Passive Inhibitory Avoidance	10 and 100 µg impaired fear memory consolidation; 100 µg intensified the fear memory impairment induced by scopolamine	Nasehi et al. (2020)
CB2	Overexpression in pyramidal neurons	CA1 of the dorsal hippocampus, Camk2aCas9 mice (2–3 months)	Tone fear conditioning	No effect on contextual or cued fear memory	Li and Kim (2017)
CB2	Overexpression in interneurons	CA1 of the dorsal hippocampus, Gad2-Cas9 mice (2–3 months)	Tone fear conditioning	No effect on contextual or cued fear memory	Li and Kim (2017)
CB2	Overexpression in microglia	CA1 of the dorsal hippocampus, Cx3cr1-Cas9 mice (2–3 months)	Tone fear conditioning	Enhanced contextual but not cued fear memory	Li and Kim (2017)
CB2	Disruption of Cnr2 gene expression in pyramidal neurons	CA1 of the dorsal hippocampus, Camk2aCas9 mice (2–3 months)	Tone fear conditioning	No effect on contextual or cued fear memory	Li and Kim (2017)
CB2	Disruption of Cnr2 gene expression in interneurons	CA1 of the dorsal hippocampus, Gad2-Cas9 mice (2–3 months)	Tone fear conditioning	No effect on contextual or cued fear memory	Li and Kim (2017)
CB2	Disruption of Cnr2 gene expression in microglia	CA1 of the dorsal hippocampus, Cx3cr1-Cas9 mice (2–3 months)	Tone fear conditioning	Impaired contextual but not cued fear memory	Li and Kim (2017)

CB1/CB2	WIN55,212-2 (0.25, 0.5, or 1 µg/mouse)	Intra-CA1 of the dorsal hippocampus posttraining, male albino NMRI mice (25–30g)	Step-down Passive Inhibitory Avoidance	0.5 and 1 µg dose-dependent impaired fear memory consolidation. The effect of 1 µg was blocked dose-dependent by pretest administration of L-arginine (1 and 3 µg/mouse)	Nasehi et al. (2010)
CB1/CB2	WIN55,212-2 (0.1, 0.25, or 0.5 µg/mouse)	Intra-CA1 of the dorsal hippocampus posttraining, male Wistar rats (220–250g)	Step-through Passive Inhibitory Avoidance	0.25 and 0.5 µg dose-dependent impaired fear memory consolidation. The effect of 0.5 µg was blocked dose-dependent by a pretest administration of scopolamine (0.25 and 0.5 µg/mouse)	Jamali-Raeufy, Nasehi, Ebrahimi-ghiri, et al. (2011)
CB1/CB2	WIN55,212-2 (0.1, 0.25, or 0.5 µg/rat)	Intra-CA1 of the dorsal hippocampus, pretraining or posttraining, male Wistar rats (220–250g)	Step-through Passive Inhibitory Avoidance	Pretraining and posttraining: 0.25 and 0.5 µg dose-dependent impaired fear memory acquisition/consolidation. The effect of 0.5 µg was blocked dose-dependent by a pretest administration of MK801 (2 and 4 µg)	Jamali-Raeufy, Nasehi, and Zarrindast (2011)
CB1/CB2	WIN55,212-2 (5 µg/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Sprague-Dawley rats (~300g)	Step-through Passive Inhibitory Avoidance	Impaired fear memory consolidation. The association with a subeffective dose of a GR antagonist (RU486 10 ng/side) enhanced the impairment.	Shoshan and Akirav (2017)
CB1	AM251 (0.3 ng/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Sprague-Dawley rats (~300g)	Step-through Passive Inhibitory Avoidance	Alone had no effect, but prevented the WIN55,212-2 effect in fear memory consolidation	Shoshan and Akirav (2017)
CB1/CB2	WIN55,212-2 (5 µg/side)	Intra-CA1 of the dorsal hippocampus, preacquisition, male Sprague-Dawley rats (60 days old, 250–300g)	Step-through Passive Inhibitory Avoidance	No effect in fear memory consolidation	Abush and Akirav (2010)
CB1	AM251 (CB1 antagonist; 6 ng/side)	Intra-CA1 of the dorsal hippocampus, preacquisition, male Sprague-Dawley rats (60 days old, 250–300g)	Step-through Passive Inhibitory Avoidance	No effect in fear memory consolidation	Abush and Akirav (2010)
CB1/CB2	WIN55,212-2 (10 nmol/side)	Intra-CA1 of the dorsal hippocampus, 24 h pretraining, male Wistar rats (3-month old, 250–280g)	Step-down Passive Inhibitory Avoidance	No effect in fear memory acquisition	Clarke et al. (2008)
CB1	AM251 (0.5 nmol/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (3 months, 270–320g)	Contextual fear conditioning	Alone had no effect, but prevented the impairing effect in consolidation induced by cannabidiol i.p. administration (10 mg/kg)	Stern et al. (2017)
CB1	AM251 (0.5 nmol/side)	Intra-CA1 of the dorsal hippocampus, posttraining, male Wistar rats (3 months, 270–320g)	Contextual fear conditioning	Alone had no effect, but prevented the impairing effect in consolidation induced by cannabidiol intra-dorsal hippocampus administration (30 pmol/side)	Raymundi et al. (2020)

involvement of the endocannabinoid system in immediate stressful situations or modulation of glucocorticoid-mediated effects. Similar results are observed when AM251 was infused into the mPFC (Kuhnert et al., 2013).

The effects of AM251 administration into the CA1 region before training on acquisition seem to be dependent on context preexposure. While context preexposure leads to an enhancement in fear memory, the blockade of CB1 receptors leads to an impairment (Lunardi et al., 2020). A similar pattern of result is observed with the infusion of AM251 intra-mPFC or intra-BLA before olfactory fear conditioning in which the acquisition of fear memory is disrupted (Laviolette & Grace, 2006; Tan et al., 2011). Controversially with these findings, infusing AM281 into CA1 of the dorsal hippocampus of mice, pretraining of contextual fear conditioning increases fear memory acquisition (Q. S. Lin et al., 2011). This difference could be attributed to different drugs used. However, in a subeffective dose, AM281 prevented the impairing effect on fear memory acquisition induced by AM404, an inhibitor of anandamide reuptake (Q. S. Lin et al., 2011).

Regarding direct agonism of CB1 receptors, only one study evaluated the administration of a selective CB1 agonist, CP55,940, intra-CA1 of the dorsal hippocampus posttraining of contextual fear conditioning. CP55,940 impaired fear memory consolidation, whereas a subeffective dose of AM251 prevented the effect of CP55,940 on memory consolidation (Santana et al., 2016).

In summary, either blockade or activation of hippocampal CB1 receptors modulates aversive memory consolidation and acquisition, an effect comparable across species and in brain areas interconnected with the dorsal hippocampus, such as the mPFC or the BLA (Laviolette & Grace, 2006; Tan et al., 2011). Primarily, these studies used male rats and mice.

## Effects of selective blockade or activation of hippocampal CB2 receptors in fear memory consolidation

In contrast to CB1 receptors, studies exploring the effect of modulation on hippocampal CB2 receptors in fear memory processes are more recent and scarcer and all of them evaluate the memory consolidation process. Table 1 summarizes the effects of manipulating CB2 receptors in the dorsal hippocampus during fear memory consolidation. A study has used the CB2 receptor antagonist AM630 into the CA1 posttraining of inhibitory avoidance (Nasehi et al., 2017). Infusing AM630 intra-CA1 region dose-dependently impaired fear memory consolidation (Nasehi et al., 2017). Although in other studies, administration of AM630 alone did not affect memory consolidation, in all of them the subeffective doses were able to prevent D-AP5 (NMDA antagonist), muscimol (GABA<sub>A</sub> agonist), scopolamine (muscarinic antagonist), or cannabidiol (multiple mechanisms of action) impairment effect (Nasehi et al., 2017, 2018, 2020; Raymundi et al., 2020; Stern et al., 2017). In addition, a subeffective dose of SR144528 (antagonist of CB2 receptors) but not SR141716 (CB1 antagonist) prevented the effects of systemic administration of 2-AG on memory consolidation, an effect accompanied by a decrease in mTOR phosphorylation in the hippocampus but not in the amygdala (Ratano et al., 2018), suggesting selective participation of hippocampal CB2 receptors in 2-AG signaling.

The CB2 receptor agonist, GP1a infusion into the CA1 region dose-dependently impaired fear memory consolidation (Nasehi et al., 2017, 2018), and a similar effect was observed after infusing GP1a into the CA3 region of the ventral hippocampus (Nasehi et al., 2020). Also, the administration of a subeffective dose of GP1a before D-AP5 (Nasehi et al., 2017), muscimol (Nasehi et al., 2018), or scopolamine (Nasehi et al., 2020) intra-hippocampal microinfusion potentiates the consolidation impairment effect induced by the drugs alone. Altogether, these results evidenced not only that CB2 receptors play a role in fear memory consolidation, but that they interact with other neurotransmission systems.

Since CB2 receptors are expressed in pyramidal cells, interneurons, and microglia, a study evaluated the effect of overexpression and disruption of the CB2 gene on specific hippocampal cells on tone fear conditioning (Li & Kim, 2017). Both overexpression and disruption of CB2 receptors in pyramidal cells and interneurons did not affect fear memory consolidation. However, the overexpression and disruption of CB2 receptors on microglia, increased and decreased, respectively, contextual but not cued fear memory. Global disruption of the CB2 gene (CB2 KO mice), independent of cell type, also caused an impairment in contextual but not in tone fear conditioning consolidation (Li & Kim, 2016), sustaining the importance of CB2 receptors in hippocampal-dependent tasks regarding its main expression on this region in the brain.

In summary, the studies suggest that CB2 receptors located on microglia are mainly responsible for the effects induced by the pharmacological manipulation of hippocampal CB2 or in CB2 KO mice. However, different from the effect of an agonist infusion reported by Nasehi et al. (2017, 2018, 2020), the overexpression enhanced contextual fear memory. Such differences can be explained by models (tone fear conditioning vs inhibitory avoidance), the manipulation of specific cell types vs a global manipulation, and also the neuronal rearrangement that is possible to occur in animals with genetic manipulations. Also, it cannot be excluded an interference with the acquisition and not only consolidation process on these animals, once their basal learning could be affected by genetic manipulation.

## Effects of unselective blockade or activation of hippocampal CB1 and CB2 receptors in memory consolidation and acquisition

The effects of direct or indirect activation of both CB1 and CB2 receptors in memory consolidation are also under investigation. Table 1 summarizes the effects of the activation of both receptors in memory consolidation. Most of the studies have used the agonist WIN55,512-2 into the CA1 injected posttraining of step-through or step-down inhibitory avoidance. Four studies demonstrated impairment in memory consolidation (Jamali-Raeufy, Nasehi, Ebrahimi-ghiri, et al., 2011; Jamali-Raeufy, Nasehi, & Zarrindast, 2011; Nasehi et al., 2010; Shoshan & Akirav, 2017). Similar effects are observed intra-BLA infusion in the same model (Shoshan & Akirav, 2017) and after tone fear conditioning (Kuhnert et al., 2013). In contrast, the systemic posttraining administration of the same drug enhances the fear memory consolidation of inhibitory avoidance (Ratano et al., 2017), suggesting that region-specific manipulation may differ from systemic manipulation of cannabinoid receptors, which probably is associated with activation of other brain areas. An enhancement in memory consolidation of inhibitory avoidance was also observed after infusing anandamide into the CA1 region (de Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008).

The effects of WIN55,212-2 or anandamide infused into CA1 region pretraining of inhibitory avoidance are contradictory, and both impairment (Jamali-Raeufy, Nasehi, & Zarrindast, 2011) or no effect (Abush & Akirav, 2010; Clarke et al., 2008; de Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008) were observed.

The activation of CB1 and CB2 receptors seems to influence glutamatergic, cholinergic, nitrergic, and glucocorticoid neurotransmission, since the pretest administration of MK801 (Jamali-Raeufy, Nasehi, & Zarrindast, 2011), scopolamine (Jamali-Raeufy, Nasehi, Ebrahimi-ghiri, et al., 2011), or L-arginine (Nasehi et al., 2010) intra-CA1 was able to reverse the WIN55,212-2-induced effect. In summary, activation of hippocampal CB1 and CB2 receptors seems to impair memory consolidation and interact with other neurotransmitter systems, but its contribution to fear memory acquisition requires further investigation.

The effects of drugs that inhibit fatty acid amide hydrolase (FAAH) enzyme and consequently increase the levels of anandamide on memory consolidation mostly agree with the effects of WIN55,212-2. Posttraining administration of URB597 into the CA1 region impaired contextual fear memory consolidation (Raymundi et al., 2020; Stern et al., 2017). In addition, posttraining infusion of AM251 or AM630 abolished the effect of cannabidiol on memory consolidation only when the antagonists were infused immediately after fear conditioning, but not 1 h later, suggesting a time-dependent contribution of hippocampal CB1 and CB2 receptors on fear memory consolidation (Raymundi et al., 2020).

## Effects of blockade or activation of hippocampal CB1 and CB2 receptors in fear memory reconsolidation

In contrast to the involvement of hippocampal cannabinoid receptors in fear memory consolidation, the participation of CB1 and CB2 receptors in fear memory reconsolidation is less explored. Table 2 summarizes the effects of manipulating CB1 and CB2 receptors in the dorsal hippocampus during fear memory reconsolidation. Most of the studies have used the antagonist AM251 into the CA1 posttraining of contextual fear conditioning. AM251 impaired fear memory reconsolidation and abolished anandamide reconsolidation impairing effect (de Oliveira Alvares, Genro, Diehl, Molina, et al., 2008). Similar to the anandamide-induced impairment on memory reconsolidation, the selective CB1 agonist CP55,940 infused intra-CA1 region immediately after contextual fear memory retrieval impaired fear memory reconsolidation (Santana et al., 2016). No effect was observed infusing the WIN55,212-2 intra-CA1 region after memory retrieval of inhibitory avoidance (Zubedat & Akirav, 2017). These differences could be related to the models or drugs used. In addition, all studies were conducted in rats, and only one used the inhibitory avoidance task.

In summary, blockade or activation of CB1 receptors in the dorsal hippocampus potentiates or impairs fear memory reconsolidation, respectively. These results are comparable with the effects observed administering drugs systemically or intra-mPFC (Bayer et al., 2021; Stern et al., 2012, 2015) but are discrepant with the effects observed in BLA (H. C. Lin et al., 2006), suggesting a region-specific role of cannabinoid receptors in fear memory reconsolidation.

## Effects of blockade or activation of hippocampal CB1 and CB2 receptors in fear memory extinction

Table 2 summarizes the effects of manipulating CB1 and CB2 receptors in the dorsal hippocampus during fear memory extinction. Most of the studies have used the antagonist AM251 into the CA1 pre- or postextinction of contextual fear

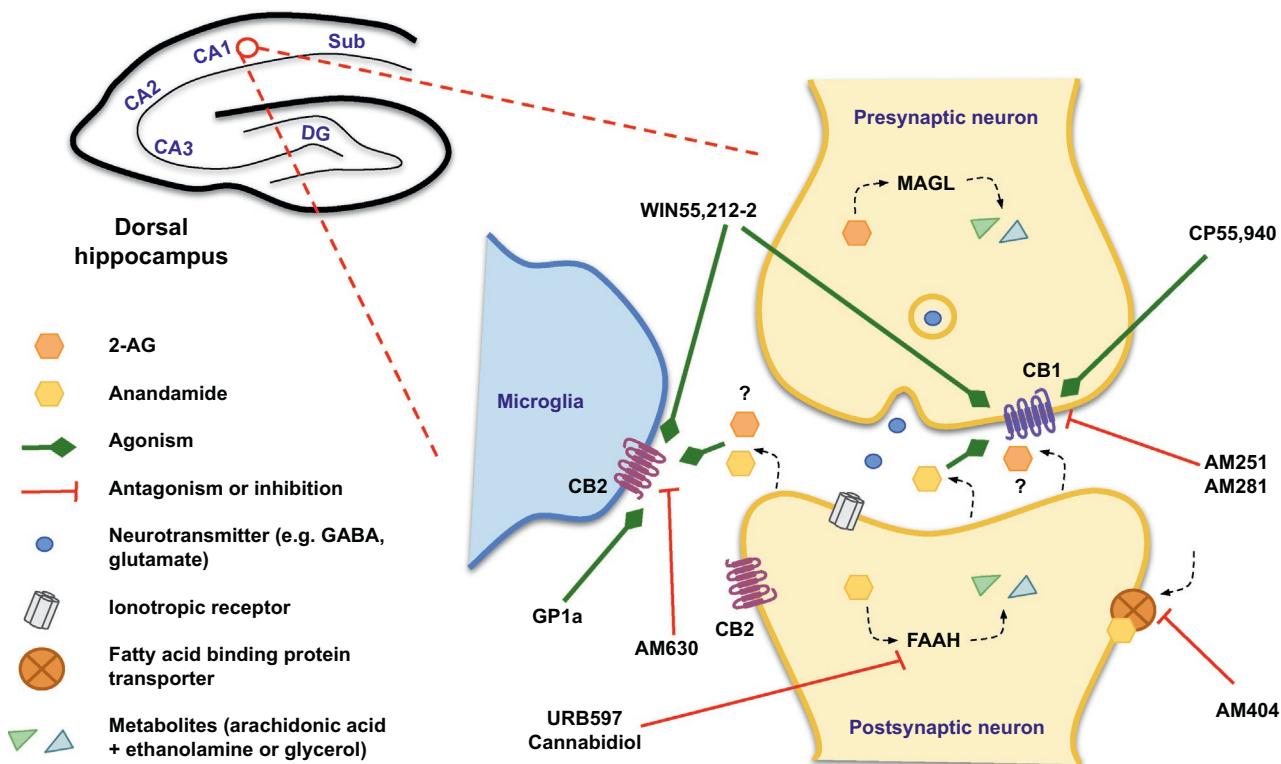
**TABLE 2** Effects of blockade or activation of hippocampal CB1 and CB2 receptors on fear memory reconsolidation and extinction.

Receptor	Drug or genetic manipulation	Site of manipulation, time-point, and animal	Behavioral test	Main results	Reference
CB1	AM251 (5.5 or 0.27 ng/side)	Intra-CA1 of the dorsal hippocampus, postretrieval, male Wistar rats (2–3 months, 210–300 g)	Contextual fear conditioning	Enhanced fear memory reconsolidation and prevented anandamide impairing effect	<a href="#">de Oliveira Alvares, Genro, Diehl, Molina, et al. (2008)</a>
CB1	CP55,940 (5 µg/µL)	Intra-CA1 of the dorsal hippocampus postretrieval, Wistar rats (270–320 g)	Contextual fear conditioning	Impaired fear memory reconsolidation	<a href="#">Santana et al. (2016)</a>
CB1/CB2	WIN55,212-2 (CB1 and CB2 receptor agonist; 5 µg/side)	Intra-CA1 of the dorsal hippocampus, postretrieval, male Sprague-Dawley rats (60 days old, ~220 g)	Step-through Passive Inhibitory Avoidance	No effect in fear memory reconsolidation	<a href="#">Zubedat and Akirav (2017)</a>
CB1/CB2	WIN55,212-2 (5 µg/side)	Intra-CA1 of the dorsal hippocampus, preextinction, male Sprague-Dawley rats (60 days old, 250–300 g)	Step-through Passive Inhibitory Avoidance	Facilitated fear memory extinction	<a href="#">Abush and Akirav (2010)</a>
CB1	AM251 (CB1 antagonist; 6 ng/side)	Intra-CA1 of the dorsal hippocampus, preextinction, male Sprague-Dawley rats (60 days old, 250–300 g)	Step-through Passive Inhibitory Avoidance	Impaired fear memory extinction	<a href="#">Abush and Akirav (2010)</a>
CB1	AM251 (0.01 µg/0.5 µL)	Intra-CA1 of the dorsal hippocampus, preextinction, male Sprague-Dawley rats (60 days old, ~250 g)	Step-through Passive Inhibitory Avoidance	Alone had no effect, but prevented the facilitatory effect of URB597 on fear memory extinction	<a href="#">Segev et al. (2018)</a>
CB1	AM251 (5.5 or 0.27 ng/side)	Intra-CA1 of the dorsal hippocampus, postextinction, male Wistar rats (2–3 months, 210–300 g)	Contextual fear conditioning	Impaired fear memory extinction and prevented anandamide facilitatory effect	<a href="#">de Oliveira Alvares, Genro, Diehl, Molina, et al. (2008)</a>

conditioning or step-through passive avoidance. Postextinction infusion of AM251 impaired the extinction of contextual and passive avoidance memories ([Abush & Akirav, 2010](#); [de Oliveira Alvares, Genro, Diehl, Molina, et al., 2008](#)), whereas preextinction infusion of AM251 did not affect the extinction of passive avoidance ([Segev et al., 2018](#)).

The infusion of WIN55,212-2, as well as the infusion of anandamide or URB597 into the CA1 region preextinction, facilitated contextual and passive avoidance fear memories, respectively, and both effects were blocked by AM251 ([de Oliveira Alvares, Genro, Diehl, Molina, et al., 2008](#); [Segev et al., 2018](#)). Similar effects are observed in URB597 systemic and intra-BLA administration ([Segev et al., 2018](#)). The infusion of URB597 or WIN55,212-2 intra-mPFC also facilitates fear memory extinction ([H. C. Lin et al., 2009](#)).

Together, these findings are in line with the observation that CB1 KO mice presented impaired extinction of auditory fear memory compared with wild-type mice ([Marsicano et al., 2002](#)). In summary, the blockade or activation of hippocampal CB1 receptors modulates aversive memory extinction similarly to systemic manipulations of the CB1 receptors ([Pamplona et al., 2006](#); [Shoshan et al., 2017](#)), suggesting an important role of hippocampal cannabinoid receptors in fear extinction. Furthermore, the effects are comparable across species ([Fig. 1](#)).



**FIG. 1** Schematic representation of endocannabinoid transmission and main pharmacological manipulations in the CA1 region of dorsal hippocampus on fear memory processes. The CB1 receptors are mostly located in presynaptic terminals of interneurons or pyramidal cells, whereas the CB2 receptors are mostly located in postsynapses of pyramidal cells and microglia. The results obtained from studies administering antagonists of CB1 receptors mostly used AM251, and observed impairments in fear memory consolidation. AM281 is another antagonist of CB1 receptors. These drugs may also enhance memory acquisition or reconsolidation and impair fear extinction. CP55,940 is a selective agonist of CB1 receptors and WIN55,212-2 is an agonist of both CB1 and CB2 receptors, their administration leads to impairment in memory consolidation and reconsolidation, and facilitates fear extinction. The use of anandamide, FAAH inhibitors such as URB597 or cannabidiol, or inhibitors of anandamide uptake by fatty-acid-binding protein transporter mostly agree with these results. The selective activation of CB2 receptors by GP1a also leads to impairment in memory consolidation, but its effects on memory reconsolidation and extinction require further investigation. Studies evaluating genetic manipulation of CB2 receptors suggest that only microglial CB2 receptors modulate fear memory consolidation. Some studies suggest that signaling of 2-AG in the CB2 receptor modulates fear memory consolidation, but their hippocampal participation are still uncertain. 2-AG, 2-arachidonoylglycerol; DG, dentate gyrus; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; Sub, subiculum.

## Applications to other areas

Evidence from studies with healthy volunteers has shown that  $\Delta^9$ -tetrahydrocannabinol or cannabidiol facilitatory effects on fear memory extinction are accompanied by increased activity of the hippocampus (Das et al., 2013; Rabinak et al., 2014). Polymorphisms in the gene of CB1 receptors and the FAAH enzyme have shown corresponding findings. Healthy volunteers expressing a FAAH variant with a lower activity present a downregulation of CB1 receptors in the hippocampus after repeated use of FAAH inhibitor (Rivera et al., 2015). It can influence stress reactivity and fear extinction, being protective against other anxiety disorders (Dincheva et al., 2015; Lazar et al., 2016). In contrast, a variant that results in high expression of the CB1 receptor in the hippocampus is related to a high risk to develop anxiety-related disorders (Gee et al., 2016; Hariri et al., 2009). These observations encourage future studies to better understand the role of hippocampal cannabinoid receptors in fear and stress-related protocols.

## Concluding remarks

At present, numerous studies indicate that cannabinoid receptors located in the dorsal hippocampus are recruited during emotionally relevant learning and memory. Pavlovian fear conditioning and active and passive avoidance paradigm have been extensively used in research focusing to investigate the role of CB1 and CB2 receptors in fear-related learning and memory processes and the potential drugs targeting this system for the treatment of PTSD. Preclinical research focusing on

the role of CB2 receptors in the reconsolidation and extinction of fear memories is still scarce and requires future investigations. In addition, the data on the CB2 receptor role in consolidation support a role of microglial CB2 receptors instead of the neuronal receptors, suggesting that different cell types in the hippocampus mediate the effects of cannabinoid drugs during fear memory processing. The data reviewed herein also indicate that disruption of consolidation and reconsolidation and facilitation of extinction can be achieved by direct or indirect activation of brain CB1 receptors. Finally, in light of differences in the endocannabinoid system and plasticity in the dorsal hippocampus between sex, future studies are necessary to elucidate whether hippocampal CB1 and CB2 receptors in females have a similar role in fear memory processing than males.

## Mini-dictionary of terms

**Active avoidance:** A type of avoidance task that requires the induction of responses to avoid the aversive stimuli (e.g., transiting from a side chamber to another to “avoid” a foot shock after presenting a cue).

**CA1 region:** The region superior of Cajal with distinct morphological properties, composed mainly of small pyramidal neurons in contrast to large pyramidal neurons in CA3 (region inferior of Cajal, with mossy fibers) and CA2 (without mossy fibers). It represents the “ending points” of the hippocampal “trisynaptic circuit” (dentate gyrus > CA3 > CA1 > subiculum) and primary sources of “extrinsic” hippocampal-subicular projections. It is a highly engaged area on spatial and contextual tasks since its place cells fire action potentials in particular locations (place fields).

**Consolidation:** Refers to the progressive postacquisition stabilization phase of a memory trace.

**Dorsal hippocampus:** The portion of the hippocampus that corresponds to the posterior hippocampus in primates, performing primarily cognitive functions.

**Extinction:** Type of inhibitory learning that suppresses the original one.

**Fear memory:** A memory from a stressful experience.

**Memory:** The retention of internal representations created by one or several experiences that can subsequently change the behavior.

**Passive or inhibitory avoidance:** Avoidance task that requires the suppression of expected response after learning (e.g., staying on the platform or in the light compartment of a box to avoid a foot shock).

**Pavlovian conditioning:** A type of associative learning in which a previously neutral stimulus (CS) is paired in space and time with a motivationally relevant unconditioned stimulus (footshock; US). The behavior of the individual does not affect the contingency between the presentations of both stimuli.

**Reconsolidation:** A restabilization phase of a memory that may occur after its retrieval and reactivation.

**Ventral hippocampus:** The portion of the hippocampus that corresponds to the anterior hippocampus in primates and is related to stress, emotion, and affect.

## Key facts of memory and hippocampal function

In 1953, H.M., a patient incapacitated by his seizures, had bilateral medial temporal lobe resection. Then, he was evaluated by Brenda Milner, who observed his epilepsy was under control; however, he was presenting an important memory impairment. Furthermore, it was observed that his memory impairment was mainly attributed to the bilateral damage in the hippocampus and dentate gyrus ([Scoville & Milner, 1957](#)).

In Milner's observations, she states that H.M.'s daily events were forgotten as fast as day occurred, with the apparent absence of intellectual or perception loss. In addition, the findings from H.M. established the fundamental principle that memory is a distinct cerebral function, separable from other perceptual and cognitive abilities, and identified the medial part of the temporal lobe is important for memory.

The case of H.M. also contributed to the discovery that the brain organizes memory into short-term and long-term memory and that multiple memory systems support the learning of different tasks, suggesting that memory is not a single faculty of the mind.

Thereafter, many works identified in laboratory animals that the hippocampus mediates a neural representation of the physical space, doing a cognitive map and that place cells increase their firing rate when animals find a specific location. The hippocampus is very sensitive to stress effects, and the atrophy of this region is frequently observed in animal models of stress, along with cognitive impairments.

## Summary points

- Blockade or activation of hippocampal CB1 receptors mostly impairs fear memory consolidation
- Activation of hippocampal CB2 receptors impairs fear memory consolidation
- Genetic manipulation of hippocampal CB2 receptors specifically in microglia modulates fear memory consolidation
- The role of hippocampal CB1 and CB2 receptors on fear memory acquisition is uncertain regarding contradictory results observed
- Whereas blockade of hippocampal CB1 receptors potentiates fear memory reconsolidation, their activation impairs it
- Activation of hippocampal CB1 receptors facilitates fear memory extinction and their blockade mostly leads to an opposite effect
- The role of hippocampal CB2 receptors in fear memory reconsolidation and extinction is still scarce and requires future investigations

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## Chapter 22

# Possible use of beta-caryophyllene as an agent to facilitate the recovery from COVID-19-induced tissue and organ damage

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## Abbreviations

<i>Adams</i>	a disintegrin and metalloprotease with thrombospondin type I motifs
<b>BCP</b>	beta-caryophyllene
<b>BrdU</b>	bromodeoxyuridine
<b>CB</b>	cannabinoid receptor
<b>CNS</b>	central nervous system
<b>COX</b>	cyclooxygenase
<i>Dlx3</i>	distal-less homeobox 3
<b>FGF</b>	fibroblast growth factor
<i>Hoxc13</i>	homeobox C13
<b>IL-1beta</b>	interleukin-1β
<b>IL6</b>	interleukin 6
<b>IPA</b>	ingenuity pathway analysis
<i>Msx2</i>	Msh homeobox 2
<b>MW</b>	molecular weight
<b>NF-kB</b>	nuclear factor kappa-light-chain-enhancer of activated B
<i>Padi1</i>	peptidyl arginine deiminase 1
<b>PASC</b>	postacute sequelae of SARS-CoV-2
<b>PCP</b>	planar cell polarity
<b>PPAR</b>	peroxisome proliferator-activated receptor
<i>S100a</i>	S100 calcium-binding protein A1
<b>TNF-α</b>	tumor necrosis factor α
<b>TREM1</b>	triggering receptor expressed on myeloid cells 1

## Introduction

The outbreak of a coronavirus, SARS-CoV-2, has escalated into a worldwide pandemic. Over 492 million people in the world have contracted the virus as of April 5, 2022. Although the symptoms of SARS-CoV-2–induced disease, COVID-19, are broad (Koyama, Ueha, et al., 2021), COVID-19 has been known as a respiratory disease, hence the disease was named SARS (Severe Acute Respiratory Syndrome).

Studies in the field of natural products have revealed that many of the chemical constituents of essential oils have bioactive properties, which, for example, suppress neuropathic pain, suppress inflammation, antiviral effects, and anxiolytic effects (Koyama & Heinbockel, 2020; Koyama, Kondo, et al., 2021). Utilization of the chemical compounds of natural products' origin has a high possibility of facilitating the recovery from wounds and diseases by suppressing chronic inflammation and excessive inflammation (cytokine storm), through disrupting the proinflammatory pathways, and through

binding with the exogenous biological substances (bacteria and viruses) including the SARS-CoV-2. It will be especially beneficial to select those chemical compounds for which the routes of action (receptors and pathways) are known, so that it is possible to determine the most effective delivery methods for the molecular target. Although there are many phytochemicals with bioactive properties,  $\beta$ -caryophyllene (BCP) received a lot of attention as a ligand of cannabinoid receptor 2 (CB2) (Gertsch et al., 2008). Activation of CB2 was found to control pain and inflammation. Therefore, BCP, as a ligand of CB2, has been considered for therapies to treat painful conditions (Bie et al., 2018). BCP also activates peroxisome proliferator-activated receptor (PPAR)  $\alpha$  and  $\gamma$  (Galaj et al., 2021), and importantly, it does not activate CB1 (Gertsch et al., 2008). Activation of CB2 without activating CB1 is important because activation of CB1 is known to be involved in the psychotropic conditions caused by inhaling or consuming marijuana (*Cannabis sativa*).

BCP is a FDA-approved food additive based on its safety and because it does not carry the risk of addiction nor the risk of psychotropic effects (<https://www.cfsanappexternal.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&id=CARYOPHYLLENE>). In our previous study, we have shown that it suppresses inflammation and improves cutaneous re-epithelialization (Koyama et al., 2019). BCP stimulates the release of the endogenous opioid  $\beta$ -endorphin and exerts an analgesic effect (Klauke et al., 2014), attenuates neuropathic pain, and has anxiolytic and antidepressant effects (for example, Youssef et al., 2019a, 2019b). Studies have also shown that BCP suppresses muscle soreness (Amalraj et al., 2020), suppresses cocaine addiction-related behaviors (Galaj et al., 2021), suppresses mycoplasmal pneumonia by suppressing the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signal transduction (M. Liu et al., 2021). BCP possesses antioxidant activity (Calleja et al., 2013), while reducing the expression of stress-related genes (Pant et al., 2014), enhances testosterone levels in women's saliva (Tarumi & Shinohara, 2020), and suppresses lipopolysaccharide-induced lung injury (Y. Zhang et al., 2021). These studies indicate the significance and the broad range of applicability of BCP in improving various problematic health conditions.

In this review, we briefly summarize COVID-19 symptoms and discuss studies of BCP. First, we review the plants that contain BCP as major chemical components. Then, we present human studies using BCP, although there are no human studies using BCP related to COVID-19 or other diseases. Lastly, we summarize the factors that require consideration in using BCP, i.e., oxidation of BCP into caryophyllene oxide, which has a higher risk of causing allergic reactions and the hydrophobic nature of BCP, which limits the bioavailability of BCP. We show here the generation of an inclusion complex as a method that can overcome both problems.

## Cannabinoid receptor 2 signaling pathway

Cannabinoids form a group of chemical substances that are found in the cannabis plant with  $\Delta 9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) as the main cannabinoids. THC acts in the human body like the chemical substances that our body produces, the endogenous cannabinoids or endocannabinoids (eCBs). The two main eCBs involved in cannabinoid signaling are 2-arachidonoyl glycerol (2-AG) (Sugiura et al., 1995) and arachidonoyl ethanolamine (AEA, anandamide) (Devane et al., 1992). These lipid molecules are derived from membrane lipids upon cellular activation and are broken down enzymatically, extracellularly after receptor activation. Endocannabinoids activate the two cannabinoid receptors, CB1 and CB2. The two cannabinoid receptors together with the eCBs transport proteins and enzymes that synthesize or degrade the endocannabinoids, form the endocannabinoid system (eCB system) (Vučković et al., 2018). The eCB system can be activated also by plant-derived compounds, phytocannabinoids, such as THC, the bioactive ingredient of cannabis (Ameri, 1999), as well as synthetic cannabinoids. The eCB system has widespread effects to regulate many functions in the body, including neural signaling, learning and memory, mood and anxiety, drug addiction, feeding behavior, perception, and the modulation of pain and cardiovascular functions (Vučković et al., 2018).

The two types of cannabinoid receptors, CB1 and CB2, share 44% amino acid sequence homology (Munro et al., 1993). They are not homogeneously expressed throughout the body. CB1 is abundantly expressed in the brain (Herkenham et al., 1990), whereas CB2 is found mainly in immune cells such as macrophages and microglia and peripheral tissues to regulate immune responses and inflammatory pathways (Munro et al., 1993). Mice that lack CB<sub>2</sub> have been shown to exhibit an exacerbated inflammatory phenotype (Turcotte et al., 2016).

The antiinflammatory effects of BCP are thought to be mediated through CB<sub>2</sub> receptor activation and the peroxisome proliferator-activated receptor (PPAR)  $\gamma$  pathway (reviewed in (Rahimi & Askari, 2022)). The immunomodulatory effects of BCP work through several mechanisms such as inhibiting microglial cells and CD<sup>4+</sup> and CD<sup>8+</sup> T lymphocytes. Relevant to cannabinoid signaling, BCP diminishes axonal demyelination and modulates Th1/Treg immune balance through the activation of CB2 in a murine model of multiple sclerosis (Alberti et al., 2017). Distinct subsets of CD<sup>4+</sup> T cells exist, including Th1, Th2, Th17, and T regulatory cells, which are activated by a specific set of cytokines and transcription factors. The subsets are characterized by the cytokines they secrete and effector functions they perform. T helper (Th) cells

provide helper functions to other immune cells and are critical for their activation and maturation (Dong, 2021). Regulatory T cells (Tregs) terminate and suppress immune responses to maintain homeostasis and self-tolerance by inhibiting T cell proliferation and cytokine production, thereby preventing autoimmunity (Kondělková et al., 2010).

CB2 was initially considered as the peripheral cannabinoid receptor, but expression in the brain microglia suggests a role for CB<sub>2</sub> in neuroinflammation (Ashton & Glass, 2007). Other studies indicate that CB2 is present in the brainstem, cortex, and cerebellar neurons and microglia (Núñez et al., 2004). A growing body of evidence suggests that CB2 is expressed in the central nervous system, plays a relevant role in modulating several neural processes, and contributes to neuronal plasticity, which potentially expands the role of CB2 in the brain (Stempel et al., 2016).

CB1 and CB2 are seven-transmembrane G-protein-coupled receptors (GPCRs). Their signal transduction pathway is mediated by inhibiting adenylyl cyclase, subsequent decreased cAMP formation, and an increase in the activity of mitogen-activated protein kinases (MAPKs) (Ibsen et al., 2017). CB2 inhibits the activity of adenylyl cyclase through its Gi/G<sub>o</sub> subunits. However, other studies found that CB2 can be coupled to G<sub>o</sub>s activation, e.g., in human leukocytes, thereby increasing cAMP (Börner et al., 2009).

CB2 is localized at the cell surface and at endosomal compartments (reviewed in L. Zhang et al., 2016). As such, CB2 can modulate signaling cascades from receptors localized at the cell surface and in endosomal compartments to modulate calcium levels, reduce cAMP levels, activate ion channels, and recruit beta-arrestin. Ligand binding can initiate CB2 endocytic trafficking such that CB2 is recycled to the cell surface via Rab4-, Rab5-, and Rab11-dependent pathways (Atwood et al., 2012). Rab4, Rab5, and Rab11 are three regulatory components of the transport machinery involved in the organization of the recycling pathway.

New studies suggest that different ligands acting on the same GPCR, in the same tissue, can give rise to markedly different cellular responses, a concept named agonist bias or biased signaling (Ibsen et al., 2017). Most likely, this is due to each ligand stabilizing different receptor conformations. Differential signaling pathway activation by different agonists could also be a consequence of kinetics. In this case, more slowly dissociating ligands may allow receptor conformations that favor low-affinity interactions for a particular receptor/signaling molecule pair to persist long enough for productive coupling (Ibsen et al., 2017). Such biased signaling has been proposed for CB1 and CB2, which would make them clinically more relevant and flexible. Based on its location and roles in immune responses, CB2 has emerged as prominent therapeutic target to alleviate pain, tissue injury, and inflammatory diseases among other pathological states. However, its therapeutic usefulness has been hampered by its sophisticated pharmacology, functional selectivity, biased signaling, and ability to activate or inhibit different subsets of signaling pathways with variable potencies and efficacies depending on the type of ligands, receptor species, and localization (Atwood et al., 2012).

## **Impact of COVID-19 on tissues/organs**

### **Recent studies on COVID-19**

Since the beginning of the COVID-19 pandemic more than 2 years ago, studies have found a broad range of damage in tissues and organs caused by COVID-19. The uniquely found chemosensory dysfunction of both taste and smell due to COVID-19 stimulated studies on the causation. Animal studies have found damage of the olfactory epithelium. Studies on diseased patients of COVID-19 have found damage in the lung (Peiris et al., 2021), olfactory bulb (Aragao et al., 2020), reproductive system (Kopanska et al., 2022), liver, heart and cardiovascular system, kidney, CNS, and gastrointestinal system (Peiris et al., 2021). These damages in many organs also indicate that the disease is causing inflammation in this broad range of organs as well. Many of the symptoms suggest that inflammation is one of the key reasons that is causing the symptoms in various tissues and organs.

### **Postacute sequelae from COVID-19 (PASC)**

It is becoming now well known that many COVID-19 patients experience symptoms long-term after the acute phase is over and even after the results of COVID-19 tests have turned negative. The ratio of COVID-19 patients who experienced PASC ranged from one-third to almost 70% depending on studies, showing there are rather large differences in the ratio for some unknown reasons (Dennis et al., 2021). About 10% have died after being discharged (Ayoubkhani et al., 2021). There are many ongoing study projects conducting longer-term follow-ups of the COVID-19 patients to determine the progress of PASC symptoms (Routen et al., 2022). Sex differences were negative in some studies (Ayoubkhani et al., 2021) and positive in some studies (more in women than in men). The severity of the first acute phase does not seem to correlate with whether the patient will experience PASC (LaVergne et al., 2021). There are COVID-19 patients who were asymptomatic

at first or mild to moderate level at first and then experience PASC. Studies have found some differences by age (more in younger patients) (Ayoubkhani et al., 2021) and ethnicity (more in nonwhite than in white) (Ayoubkhani et al., 2021). Examples of the symptoms are fatigue, shortness of breath (breathlessness), headaches and muscle aches, chemosensory dysfunction, cough, nausea, diarrhea, diabetes mellitus, myalgia, and cognitive dysfunction (Dennis et al., 2021), although the combination of the symptoms differs depending on the person, and there are various other symptoms other than these symptoms. Multiorgan dysfunction was found in the lungs, heart, kidneys, liver, pancreas, and spleen (Dennis et al., 2021).

## Possible utilization of BCP to facilitate the recovery from COVID-19-induced damage of tissues and organs

### Sources of BCP

BCP is included in various plants but at different percentages. Table 1 shows examples of the plants that contain BCP at rather high percentage and the effects of BCP using the plants that contain BCP (Table 1). These percentages can vary by multiple factors, for example, the geographical locations, methods and procedures used in the extraction, depending on whether they were harvested early or late in the season, and the ripeness in case of fruits (for review on the differences in black pepper, *Piper nigrum* L., see Dosoky et al., 2019). The chemical constituents other than BCP also vary not only depending on the plant species but also often in the same plant species depending on the geographical locations and other factors such as those listed above. It will need to be determined which other chemical constituents are included with BCP in the extracts to consider synergistic influences as well as the roles of other chemical constituents themselves, if extracts will be used rather than using BCP alone.

**TABLE 1** Examples of the percentages of BCP in plants.

Scientific name	Common name	Part of the plants	Percentages of BCP in the extraction	Effects	References
<i>Spiranthera odoratissima</i>		Leaves	20.64%	Headaches, renal and hepatic diseases, rheumatism, anxiety, depression	Galdino et al. (2012)
<i>Ayapana triplinervis</i>		Leaves	45.93%	Control the larvae of <i>Aedes aegypti</i> , which is an insect carrier of viruses that cause Dengue, chikungunya, Zika, and yellow fever	Rodrigues et al. (2021)
<i>Myrica rubra</i>	Chinese bayberry, Japanese bayberry, red bayberry	Leaves	89.9% (Sharma et al., 2016); 43% (Ambroz et al., 2015)	Suppressed proliferation of cancer cells (Ambroz et al., 2015)	Sharma et al. (2016), Ambroz et al. (2015)
<i>Piper nigrum</i>	Black pepper	Fruits	70.4% (Sharma et al., 2016); 20.3%–34.7%, 24.4%–30.8%, 23.0%–38.4%, 7.6%–21.3% depending on the location (Menon et al., 2003)		Sharma et al. (2016), Menon et al. (2003)
<i>Copaifera multijuga Hayne</i>	Copaiba	Trunks	57.5% (Sharma et al., 2016); 36.0% (Kobayashi et al., 2011)	Antiinflammatory (Kobayashi et al., 2011)	Sharma et al. (2016), Kobayashi et al. (2011)

**TABLE 1** Examples of the percentages of BCP in plants—cont'd

Scientific name	Common name	Part of the plants	Percentages of BCP in the extraction	Effects	References
<i>Melampodium divaricatum</i>	Melampodium, butter daisy	Aerial parts	56.0%	Antibacterial ( <a href="#">Moreira et al., 2014</a> )	<a href="#">Sharma et al. (2016)</a> , <a href="#">Moreira et al. (2014)</a>
<i>Tabernaemontana catharinensis</i>		Leaves	57.0% ( <a href="#">Sharma et al., 2016</a> ); 56.87% ( <a href="#">Boligon et al., 2013</a> )	Antioxidant effects	<a href="#">Sharma et al. (2016)</a> , <a href="#">Boligon et al. (2013)</a> , <a href="#">Maffei (2020)</a>

## Possible use of BCP as a therapeutic agent

### Effects of BCP on facilitating recovery from tissue damage

In our study ([Koyama et al., 2019](#)), we tested the effects of BCP on experimentally induced cutaneous wounds using mouse as an animal model system ([Koyama et al., 2019](#)). Immunofluorescence staining with epidermal cell markers ([Braiman-Wiksman et al., 2007](#)) revealed that mice treated with BCP displayed more epidermal migration (greater wound closure) than mice treated with oil alone ([Koyama et al., 2019](#)). Cell proliferation around the wound, measured by BrdU, was significantly higher in the epidermis, hair follicles, and dermis excluding hair follicles in the skin of mice exposed to BCP. Transcriptome analyses of the skin, exposed to BCP or to oil as control, showed large differences between BCP and oil control groups. Many genes related to cell proliferation and migration and embryonic growth were upregulated in the BCP group compared with the oil control group, for example, *Adams*, *Bambi*, *Msx2*, *Dlx3*, *Pad1*, *Hoxc13*, *S100a*, suggesting that BCP enhances skin regeneration rather than simply facilitating wound healing by suppressing inflammation. The upregulation of genes related to embryonic growth also suggested that BCP may induce full regeneration without scar formation, which has significant meaning in the quality of regeneration of tissues and organs. Proinflammatory cytokines genes, IL-1 $\beta$  and IL6, were significantly downregulated in the BCP group compared with the oil control group, and an ingenuity pathway analysis (IPA) showed that the signaling pathways related to inflammation and immune system (TREM1 signaling) were suppressed in the BCP group. The pathways related to cell proliferation and migration (for example, Sonic Hedgehog signaling, the planar cell polarity (PCP) pathway, fibroblast growth factor (FGF) signaling) were activated in the BCP group. This was the first study showing at the gene expression level that an aromatic odorant included in herbs and spices and various essential oils made from them improves wound healing.

### Effects of BCP on inflammation

As written above, transcriptome analyses of the skin with experimentally induced small excision and exposure to BCP showed that the expression of proinflammatory cytokines, for example, IL-1 $\beta$  and IL6, was downregulated, and TREM1 signaling was suppressed ([Koyama et al., 2019](#)). Preceding to these studies, the effects of BCP on inflammation were found in as early as 2008 when BCP was found to be a CB2 ligand ([Gertsch et al., 2008](#)). In the in vitro studies by [Gertsch et al. \(2008\)](#), whole blood cells infused with lipopolysaccharide with 1 h of preexposure to BCP showed significantly less expression of TNF- $\alpha$  and IL-1 $\beta$ , the markers of inflammation ([Gertsch et al., 2008](#)). In their in vivo studies, orally administered BCP (5 and 10 mg/kgbw) significantly facilitated the recovery from the edema caused by an injection of 30  $\mu$ L of 2% carrageenan into the hind right paw (saline was injected on the hind left paw as control). The mice that did not receive BCP oral administration showed increasing edema during the 4 h postinjection time, whereas BCP group showed decrease in the swell from 2 h postinjection. When they used CB2 receptor knockout mice, these effects of BCP were not observed, indicating the roles of CB2 receptors in the antiinflammatory effects by BCP ([Gertsch et al., 2008](#)).

Several recent review papers on BCP provide a detailed summary about the antiinflammatory effects by BCP (for example, Scandiffio et al., 2020). Scandiffio et al. (2020) have described the mechanisms by which BCP suppresses inflammation as follows: “the effectiveness of BCP as an anti-inflammatory agent is at least partly due to its ability to inhibit the main inflammatory mediators, e.g., inducible nitric oxide synthase (iNOS), IL-1 $\beta$ , IL-6, TNF- $\alpha$ , NF-kB, cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2)” (Scandiffio et al., 2020).

These effects of BCP may not be only mediated by the activation of CB2 receptors. After it was found that BCP is a ligand of CB2 (Gertsch et al., 2008), and because the activation of CB2 is known to have antiinflammatory effects (Ashton & Glass, 2007), the image of BCP has become “a CB2 ligand.” BCP has a smell, which indicates that it activates olfactory receptors, and thus, there is a route to the brain from the olfactory sensory neurons. Olfactory sensation can cause changes in hormone secretion and in the emotional status (Prete et al., 2003). Our recent study has also shown that the influences of BCP on facilitating the recovery of cutaneous wound could be “not solely” mediated by CB2 (Koyama et al., 2019). The effects of BCP on facilitating cutaneous re-epithelialization were not mediated by the olfactory system, but exposure to BCP through olfactory pathway had influences on the behaviors of mice (Koyama et al., 2019). In vitro assays using primary cells from CB2 knockout mice indicated the involvement of other pathways in the enhanced re-epithelialization of cutaneous by the exposure to BCP (Koyama et al., 2019). There are studies showing that BCP activates peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) (Wu et al., 2014). PPARs are nuclear transcription factors that regulate the gene expressions related to cell development and metabolism. PPAR $\alpha$  is one of the three forms of PPAR (others are PPAR $\gamma$  and PPAR $\beta/\delta$ ), and it is considered to regulate the expression of genes related to lipid metabolism (Rigano et al., 2017). In addition, the activation of PPAR $\alpha$  during inflammation functions to promote inactivation of NF-kB, by direct binding and inactivation of p65 NF-kB or indirectly interfering with NF-kB (Korbecki et al., 2019). Although the function of NF-kB is not completely understood, it is considered to be a regulator of innate immunity and involved in the induction of proinflammatory responses. These studies show that BCP can activate at least three receptors, the olfactory receptors, CB2 receptor, and PPAR $\alpha$ . Each route may generate independent influences, e.g., the pathways through the olfactory system affect behavior but not cutaneous re-epithelialization, and the pathways through CB2 receptors and PPAR $\alpha$  affect cutaneous re-epithelialization. The multiple routes causing influences on the same target (re-epithelialization) may involve synergistic influences because of the activation of multiple different types of receptors and routes.

### *Preclinical studies with human subjects using BCP*

There are a couple of studies with human subjects so far, although none of them are related to the effects of BCP on suppressing inflammation and enhancing regeneration. In one study, the subjects were all male between 19 and 29 years of age in good health condition, and the effect of BCP was examined on muscle soreness after exercise (Amalraj et al., 2020). They received one encapsulated BCP loaded in liposome, went through exercise 1 day later, and tested the soreness on the day of exercise, 24 h later, 48 h later, and 72 h later. The capsule of BCP/liposome was 500 mg, which included approximately 48 mg of BCP (less than 10% of the capsule weight), estimated from the MW of liposome (MW, 938.1) and BCP (MW, 204.36) and the ratio of each chemical compound (2:1) added in generating the encapsulated BCP/liposome structure (details of the encapsulation will be described in the section on formulation). The control group received a placebo capsule, which contained starch, and not a nonencapsulated BCP, and it was visually the same as the experimental group capsule with BCP/liposome. The results showed statistically significant differences especially on the third and fourth day, showing less self-reported soreness in the BCP/liposome group, suggesting that BCP/liposome facilitated the recovery from the soreness due to exercise (Amalraj et al., 2020).

Another study with human subjects used olfactory exposure to BCP (Tarumi & Shinohara, 2020). They used 3% BCP, and the subjects (19 women with average age of 23.47) were exposed to the smell for 20 min. Saliva was collected, and it was found that the exposure to BCP significantly enhanced the concentration of testosterone in the saliva, whereas the concentration of estrogen was not different (Tarumi & Shinohara, 2020). They also reported that the concentration of 3% was selected because it did not cause allergic responses.

These studies show that, although there are still few preclinical studies with humans using BCP, the results so far are showing that BCP has positive effects on recovery from soreness caused by exercise and that BCP can affect hormone secretions.

## **Enhancing the effects of BCP**

The studies on BCP on its antiinflammatory effects and its effects on facilitating regeneration indicate the high possibility that BCP can be used in facilitating recoveries from COVID-19 and PASC. The preclinical studies shown above bring up

the questions of “what is the best way to deliver”: the delivery routes (ingestion and inhalation as in the preclinical studies, as well other routes, such as transdermal route) and the form of BCP (formulation and nonformulation). The best delivery methods perhaps depend on the goal as well, i.e., the target organs (lung, olfactory system, gustatory system, brain, skin, and so on) and the target symptoms (inflammation, regeneration of damaged organs, anxiety, and so on).

### Chemical properties of BCP that affect the effects

There are studies also mentioning the chemically unstable nature of BCP (Sköld et al., 2006), which can affect the effects of BCP. BCP was found to start its oxidation immediately when it is exposed to air and reduced to about 50% in 5 weeks, turning into caryophyllene oxide as a major oxidized product (Sköld et al., 2006). Importantly, when BCP or caryophyllene oxide was applied on the shaved skin of guinea pigs, caryophyllene oxide caused confluent erythema, although BCP did not (Sköld et al., 2006). These results indicate the importance of enhancing the stability of BCP.

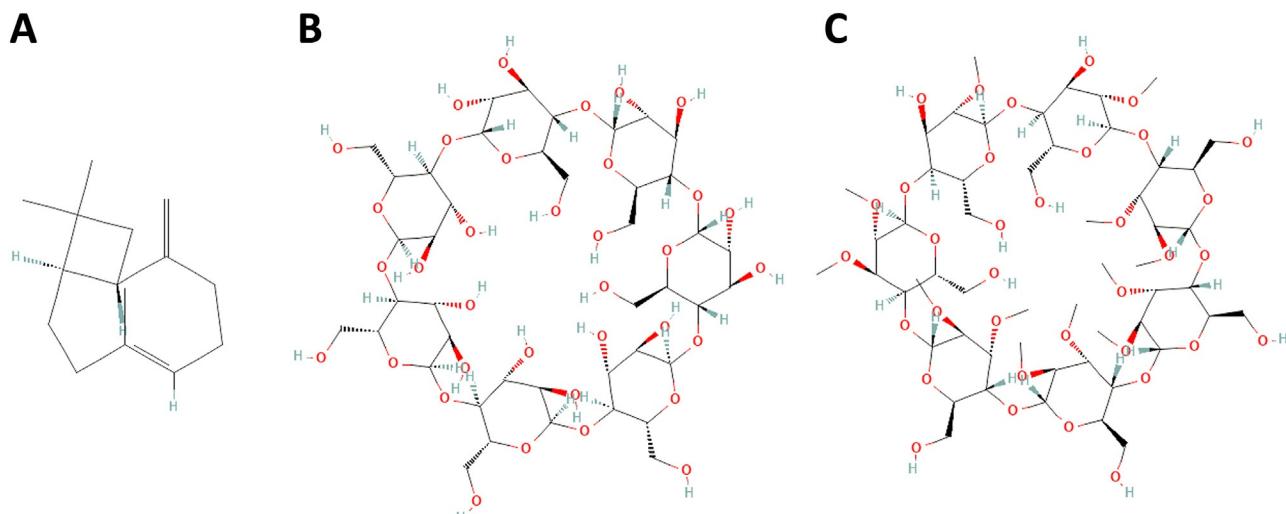
Another important chemical characteristic is that BCP is hydrophobic, and this hydrophobic nature of BCP affects the amount of BCP that can be bioactively available when it is used. Studies have shown that formulation of an inclusion complex to convert BCP hydrophilic enhances the effects of BCP, most likely by increasing the bioavailability. There is also a possibility that an inclusion complex may enhance stability and suppress oxidation because of less exposure to oxygen. Hence, there is a strong need to develop the best formulation method in utilizing BCP for its bioactive properties.

Several materials and techniques have been used for the formulation of BCP (Fig. 1A), for example, inclusion complex using  $\beta$ -cyclodextrin (Koyama et al., unpublished data; H. Liu et al., 2013) (Fig. 1B), or hydroxypropyl- $\beta$ -cyclodextrin (Santos et al., 2017), or methyl- $\beta$ -cyclodextrin (Santos et al., 2017) (Fig. 1C), and a nanofiber weaving technology using turmeric nanofiber and using high pressure to homogenize and spray drying to remove water (Amalraj et al., 2020).

### Formulation using cyclodextrins

There are three types in the natural cyclodextrins, i.e.,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrin. The inner dimension sizes of the ring structure are different; 0.57 nm in case of  $\alpha$ -cyclodextrin, 0.78 nm in case of  $\beta$ -cyclodextrin, and 0.95 nm in case of  $\gamma$ -cyclodextrin (Cid-Samamed et al., 2022). The inclusion complexes using BCP were made using the  $\beta$ -cyclodextrin and its derivatives, methyl- $\beta$ -cyclodextrin (Santos et al., 2017), or hydroxypropyl- $\beta$ -cyclodextrin (Santos et al., 2017), which is most likely based on the fit of it to the inner dimension size of  $\beta$ -cyclodextrin.

In a study comparing an inclusion complex of BCP made by using hydroxypropyl- $\beta$ -cyclodextrin and methyl- $\beta$ -cyclodextrin, the solubility of BCP increased 7 times and 10 times, respectively, and the stability constants ( $K_s$ ) were higher for these made by using methyl- $\beta$ -cyclodextrin than hydroxypropyl- $\beta$ -cyclodextrin (Santos et al., 2017). These results suggest that methyl- $\beta$ -cyclodextrin could be a better fit for the formulation of an inclusion complex with BCP.



**FIG. 1** Structures of BCP (A), beta-cyclodextrin (B), and methyl-beta-cyclodextrin (C). All from PubChem database, PubChem ID, 5281515, 444041, 51051622, respectively.

The antiinflammatory effects, antioxidant effects, and the effects on preventing ethanol-induced gastric damage were higher when mice were treated with BCP/methyl- $\beta$ -cyclodextrin than BCP (Santos et al., 2017), which are due to the higher bioavailability by the complexation.

## Delivery routes

In the studies using animal model system, intra-peritoneal injections and oral administration (gavage) are often used. Another delivery route that can be developed for future clinical use would be using the transdermal patches. Although the method itself has been around for decades, the rapid development in the chemical engineering has contributed to the emergence of the far more sophisticated technology (Bird & Ravindra, 2020). Utilization of transdermal patches eliminates the need for taking the drugs daily because of the slower, controlled longer-term release of the drugs.

## Conclusion

Utilization of phytochemicals with antiinflammatory effects, analgesic effects, antioxidant effects, and the effects to facilitate regeneration may be very beneficial in the tragic pandemic years. How to use them in the most effective way and at the same time avoid any side effects is the question. BCP has a strong potential as a candidate phytochemical chemical compound as a CB2 ligand. The oxidated product caryophyllene oxide has higher possibility of causing allergic reaction, therefore it is important to suppress exposure to oxygen, which is one of the reasons that the formulation of an inclusion complex is beneficial. Another beneficial point of the formulation of an inclusion complex is the enhancement of bioavailability. Enhanced bioavailability will also contribute to reducing the concentration required to expect sufficient effects, which will also reduce the possibility of the risk of allergic reactions. Understanding of the nature of the chemical compound and improving the nature by utilizing chemical engineering techniques are important steps in pushing forward a possible candidate chemical compound and bring it into the shape of a clinically testable drug.

## Applications to other areas

Based on the antiinflammatory effects it has, there is a high possibility of utilizing BCP in clinical use as a drug or as a supplementary treatment to facilitate recovery. The possible problem it has is its hydrophobic nature and that the oxidated product caryophyllene oxide has higher possibility of causing allergic responses. As one of the possible methods to solve both of these problems is formulation of inclusion complex. Delivery methods can be as a pill, inhalation, and using transdermal patches.

## Key facts of $\beta$ -caryophyllene

- B-caryophyllene is a CB2 ligand.
- B-caryophyllene oxidates to caryophyllene oxide.
- B-caryophyllene is hydrophobic.
- Activation of CB2 does not have psychotropic influences.
- Activation of CB2 has antiinflammatory, antioxidant, analgesic effects, and the effects to facilitate regeneration.
- Exposure to  $\beta$ -caryophyllene facilitated cutaneous re-epithelialization, downregulated the genes related to inflammation, and upregulated the genes related to cell proliferation/cell migration and embryonic growth.

## Mini-dictionary of terms

B-caryophyllene: A phytochemical, which is included in various herbs and spices. It is a CB2 ligand.

Phytochemicals: Chemical compounds produced by plants.

Cannabinoid receptor 2: A G-protein-coupled receptor encoded by CNR2 in case of human one. Abbreviation is CB2.

Inclusion complex: In the host-guest chemistry, chemical compounds with cavity structure (host) can hold a guest chemical compound with van der Waals bonding.

Trans-dermal patch: A medicated patch that can be attached to the skin to deliver medication.

## Summary points

- B-caryophyllene is CB2 ligand and has antiinflammatory effects.
- Utilization of phytochemicals with antiinflammatory effects can have beneficial effects in facilitating recovery from injuries and diseases.
- Suppressing oxidation of β-caryophyllene is important because oxidated product caryophyllene oxide causes allergic responses.
- Hydrophobic nature of β-caryophyllene hinders the effects of it.
- Formulation of inclusion complex increases bioavailability of β-caryophyllene and considered to suppress oxidation.
- Understanding the nature of the chemical compound and improving the nature, if necessary, using chemical engineering techniques will enhance the possibility of utilizing it as drugs.

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## Chapter 23

# CB1 agonism on mesolimbic and nigrostriatal dopaminergic neurotransmission

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## Abbreviations

<b>Δ<sup>9</sup>-THC</b>	delta-9-tetrahydrocannabinol
<b>CB1-R</b>	cannabinoid receptor type 1
<b>CB2-R</b>	cannabinoid receptor type 2
<b>DA</b>	dopamine
<b>DAT</b>	dopamine transporter
<b>DLS</b>	dorsolateral striatum
<b>DS</b>	dorsal striatum
<b>GABA</b>	<i>Gamma</i> -aminobutyric acid
<b>Ki</b>	inhibitory constant
<b>MSN</b>	medium spiny neurons
<b>NAc</b>	nucleus accumbens
<b>OVX</b>	ovariectomized
<b>PFC</b>	prefrontal cortex
<b>SNpc</b>	substantia pars compacta
<b>TH</b>	tyrosine hydroxylase
<b>VTA</b>	ventral tegmental area

## Introduction

Cannabis is the third more popular substance of abuse, after nicotine and alcohol, with an estimated 192.2 million people that have used cannabis at least once in the last year (UNODC, 2018). Currently, around 545 natural compounds from Cannabis Sativa L. have been identified, of which 104 correspond to the chemical group of “phytocannabinoids.” One of these phytocannabinoids, the delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC), is considered the primary psychoactive molecule and responsible for the addictive effects of this plant (Pertwee, 2014). The physiological effects of acute exposure to  $\Delta^9$ -THC have been mainly attributed to the agonism of CB1 and CB2 receptors. Interestingly, the CB1 receptor has shown a regulatory role in the homeostasis of a wide range of neurotransmitters. Dopamine is highly interesting given its crucial role in rewarding processing and impulsive behaviors.

## Dopaminergic pathway: Role and regulation

Midbrain dopamine (DA) neurons represent around 0.03% of the total number of cells in the central nervous system in rodents (Steiner & Tseng, 2017). Interestingly, this small fraction of cells modulates essential behaviors to survive, such as reward-based learning, motor control, and cognition. The use of immunocytochemical approaches with antibodies against molecular markers of DA neurons as tyrosine hydroxylase (TH), vesicular monoamine transporter 2, and DA

transporter (DAT), has allowed distinguishing three main groups of DA neurons in the midbrain: substantia nigra *par compacta* (SNpc), ventral tegmental area (VTA), and retrorubral field (Fuxe et al., 2010). The DA neurons from VTA project to limbic forebrain areas such as the amygdala, ventral pallidum (VP), prefrontal cortex (PFC), and nucleus accumbens (NAc) (Björklund & Dunnett, 2007; Root et al., 2015), while DA neurons from SNpc and retrorubral field project send their axons to the striatum (Björklund & Dunnett, 2007).

These differences in the projections have been associated with a specificity in the physiological role of DA neurons in the VTA that project to PFC and NAc (Mesocorticolimbic pathway) and the DA neurons in the SNpc that project to the dorsal striatum (Nigrostriatal pathway). The mesocorticolimbic dopaminergic pathway modulates working memory, decision-making, motivation, and reward signaling (Baik, 2013; Floresco & Magyar, 2006). On the other hand, the nigrostriatal dopaminergic pathway regulates habit learning (Everitt & Robbins, 2013) and impulsive choice (Moreno et al., 2021).

Impulsivity is typically defined as acting without forethought to the possible negative consequences. It has been proposed that impulsivity is a trait showing different dimensions: impulsive action and impulsive choice. The impulsive action is characterized by a deficit in motor inhibition, and the impulsive choice is the intolerance to delay of rewards or delay aversion (Pattij & Vanderschuren, 2008).

Evidence from different experimental approaches supports the idea that impulsive choice is closely modulated by the mesolimbic and nigrostriatal dopaminergic pathways. Clinical studies using genetic, positron emission tomography, functional magnetic resonance imaging, and preclinical approaches in rodents have shown that an overactivation and deficit of the dopaminergic transmission in the mesolimbic pathways are associated with higher impulsive choice (Adriani et al., 2009; Joutsa et al., 2015; Pine et al., 2010), suggesting an inverted-U shape pattern in the relation between mesolimbic DA signaling and impulsivity (Petzold et al., 2019). With regard to nigrostriatal pathway, recent evidence has shown a direct correlation between dopamine release in the dorsolateral striatum and impulsive choice: rats classified as high impulsive show an increase DA release compared with low-impulsive rats. This evidence suggests that an hyperactive nigrostriatal pathway contributes to delay aversion (Moreno et al., 2021).

A psychiatric disorder that shows high levels of impulsive choice and altered dopaminergic transmission is drug addiction. The mesolimbic and nigrostriatal dopaminergic pathways are strongly associated with the development of addictive behavior (Everitt & Robbins, 2013). An increase in the mesolimbic dopaminergic activity accompanies the rewarding effects of natural reinforcers and drugs of abuse (Ito et al., 2002). On the other hand, the transition from an initial intake of drug of abuse to habitual consumption has been associated with a progressive increase in the neurotransmission of the nigrostriatal dopaminergic pathway (Willuhn et al., 2012).

Due to the critical role of DA transmission in the dorsolateral striatum (DLS) in the habitual seeking behavior of drugs and impulsive choice, it is fundamental to understand the regulation of this neurotransmission in physiological and pathological conditions.

Interestingly, the endocannabinoid system and cannabinoid agonists have shown a modulatory effect on the dopaminergic transmission in both pathways (Covey et al., 2017). For this reason, there is a growing interest to study how the modulation of DA signaling by cannabinoids impacts in neuro-psychiatric disorders associated with dopaminergic dysfunction, from both an ontogenetic and individual differences perspective, including sexual differences.

## CB1 agonism in dopaminergic transmission

CB1 and CB2 receptors are fundamental in different physiological processes, such as appetite, pain sensation, mood, memory, and motivation (Pertwee, 2014). The CB1 receptors (CB1-R) are mainly localized in axon terminals in the central nervous system, where their activation inhibits the release of different neurotransmitters such as *gamma*-aminobutyric acid (GABA), glutamate, histamine, serotonin, acetylcholine, and DA (Kano et al., 2009). On the other hand, CB2 receptors (CB2-R) expression is higher in the immune system cells than in brain neurons (Grotenhermen, 2005). However, recent evidence has shown an active role of CB2 agonism in reward/aversion demonstrating a functional effect in the central nervous system (Jordan & Xi, 2019).

The CB1-R is widely distributed across the brain, highlighting the prefrontal cortex, hippocampus, striatum, and midbrain (Egertová & Elphick, 2000; Matsuda et al., 1993). Notably, evidence in rodent and human has shown a high expression of CB1-R in the VTA and SNpc, the two main dopaminergic nuclei in the brain (Matsuda et al., 1993). Despite the presence in these nuclei, CB1-R is not expressed in dopaminergic neurons (Herkenham, 1991), indicating that the effects of CB1 agonists on DA transmission depend on an indirect mechanism.

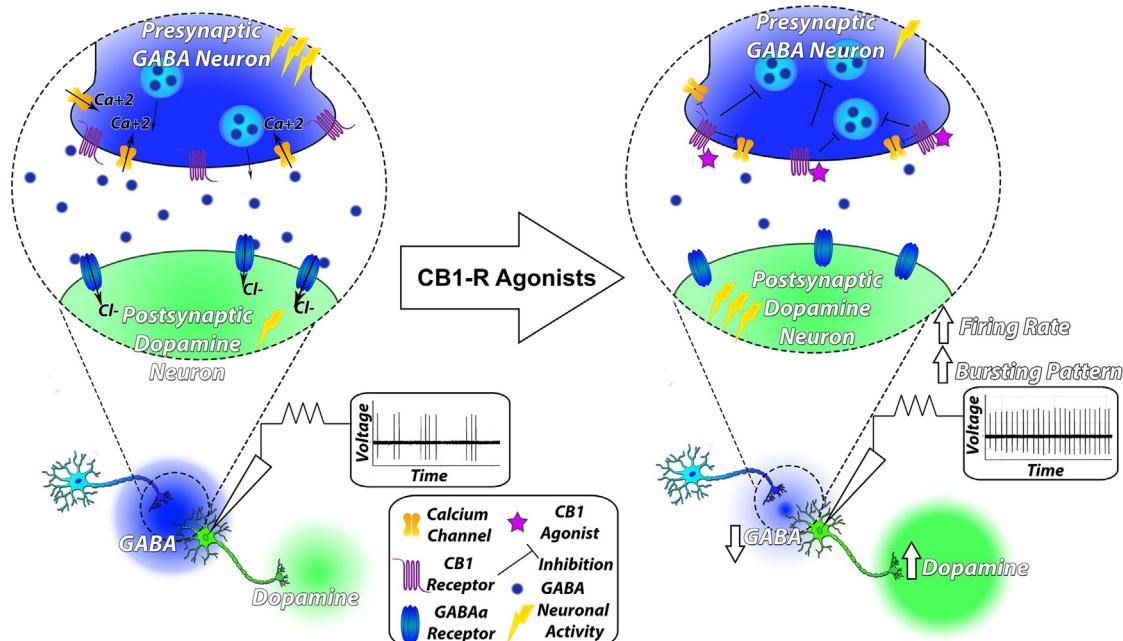
## Acute and long-lasting effects of CB1 agonists in adult dopaminergic transmission

Previous evidence has shown the effects of CB1-R agonism in the dopaminergic transmission in the mesolimbic and nigrostriatal pathways. Bosier et al. showed that acute CB1 agonism increases the DA content in the striatum and increases the

expression of TH, the critical enzyme in the synthesis of DA (Bosier et al., 2012). Preclinical studies using microdialysis have shown that an acute systemic administration of the CB1 receptor agonist, WIN55212-2, increases DA extracellular levels in the NAc and DLS of adult rats (Polissidis et al., 2014; Tanda et al., 1997). Interestingly, the increase DA extracellular level induced by WIN55212-2 is associated with an increase in DA release in terminal of DLS (Pérez-Valenzuela et al., 2019). Other experimental approaches also support this evidence. Fast scan voltammetry experiments have shown that acute injection of WIN55212-2 increases the frequency and amplitude of the nonstimulated transient signal of DA suggesting an increase in phasic transmission (Cheer et al., 2004; Gomez et al., 2021). In addition, acute injection of CB1 agonists increases firing rate of DA neurons in VTA and SNpc (French et al., 1997). The effects on DA release and firing rate are attenuated after a dose of CB1 antagonist, indicating CB1-R dependency (Tanda et al., 1997).

Since CB1-R is not expressed in DA neurons (Herkenham, 1991), an indirect mechanism has been proposed to explain the cannabinoid modulation of dopaminergic transmission. The activation of CB1-R on presynaptic GABAergic neurons of the VTA and SNpc attenuates the inhibitory GABAergic, disinhibiting DA neurons (See figure 1) (Sperlágh et al., 2009; Yanovsky et al., 2003).

In contrast with the consistency of the activating acute effects of CB1 agonists on dopaminergic transmission, the consequences of chronic cannabinoid exposure on dopamine homeostasis depend on the withdrawal period and the dopaminergic pathway. Morphological studies of the dopaminergic pathway have shown a decrease in the area, perimeter, major length, and minor axis length of DA neurons of VTA without changes in DA neurons of SNpc, one day after chronic treatment of CB1 agonist (Spiga et al., 2010). These anatomical changes were accompanied by a lower density of dendritic spine in medium spiny neurons (MSN) of NAc Shell, suggesting that repeated activation of CB1-R selectively modifies areas of the mesolimbic pathway (Carvalho et al., 2016; Spiga et al., 2010). In this way, functional studies of dopaminergic release have shown that repeated CB1 agonism reduces the increasing of DA release induced by WIN55212-2 in NAc (Gomez et al., 2021). However, molecular approaches have shown differential effects of CB1 agonists in the expression of proteins associated with dopaminergic homeostasis in the mesolimbic and nigrostriatal pathways. Fanarioti et al., using chronic treatment of WIN55212-2 and testing on the last day of the treatment, showed a decreased binding and expression of DAT in the SNpc and VTA without changes in the striatum. Furthermore, the repeated treatment with the CB1 agonist exclusively diminished the binding of the D2 receptor in the dorsal striatum (DS) and the expression of mRNA of D2 pre-synaptic isoform in the SNpc (Fanarioti et al., 2014). Then it is tempting to suggest that the decrease in DAT and D2 expression is a compensatory change in the face of the attenuation in DA release after chronic exposure to WIN55212-2. Recent studies have suggested that the consequences of repeated activation of CB1-R in dopaminergic transmission



**FIG. 1** Cellular mechanism of the effect of CB1 agonists on dopaminergic activity. Since CB1-R is not expressed in DA neurons, an indirect mechanism has been proposed to explain the cannabinoid modulation of dopaminergic transmission. The activation of CB1-R on presynaptic GABAergic neurons of the VTA and SNpc disinhibits the DA neurons (Sperlágh et al., 2009).

may be reversed after prolonged withdrawal. A study of Perdikaris et al. shows transitory changes in DAT expression during the withdrawal after chronic WIN55212-2 treatment. While the DAT expression and binding were reduced after 0-day withdrawal in the SNpc, VTA, and striatum, on day 20 of withdrawal, the DAT expression and binding were normalized (Perdikaris et al., 2018). In this sense, electrophysiological experiments have shown that the activity of DA neurons from VTA is unaltered after a prolonged withdrawal following a chronic treatment with THC (Renard et al., 2016), supporting the hypothesis that the consequences of chronic exposure to CB1 agonists in the dopaminergic pathway are reversible in adult subjects.

Despite the vast knowledge about the acute and chronic effects of CB1 agonism in DA transmission during adulthood (French et al., 1997; Polissidis et al., 2013; Tanda et al., 1997; Yanovsky et al., 2003), the effects of cannabinoids on the dopaminergic transmission during adolescence and the long-lasting consequences have begun to be studied.

### **Acute and long-lasting effects of CB1 agonists on the dopaminergic transmission during adolescence**

Adolescence is a life period characterized by gradual physiological and behavioral changes from childhood to adulthood (Spear, 2000). This period is triggered by the first surge of sex hormones, which are responsible for beginning the reproductive stage, sexual maturity, and the development of the characteristic behavior of adulthood.

Adolescents are more sensitive to rewarding stimuli such as social peer interactions, novelty-seeking, and palatable food than adults (Doremus-Fitzwater & Spear, 2016). In adolescents, this “sensitive reward” phenotype has been associated with differential changes in dopaminergic pathways (Doremus-Fitzwater & Spear, 2016; Robinson et al., 2016). Preclinical evidence has demonstrated that DA activity shows an age-dependent inverted U-shaped trajectory, which means a maximum during middle adolescence and then a decrease during adulthood (McCutcheon et al., 2012).

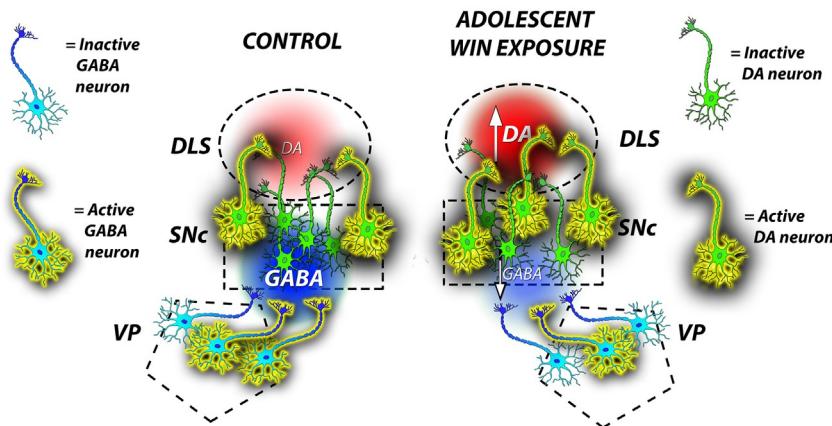
Furthermore, CB1-R expression also shows transitory changes through central nervous system development between adolescence and adulthood. In the striatal areas in male rats, the expression of the CB1-R increases gradually until a maximum at PD40, then decreases until it reaches adult values (PD70) (Rodríguez de Fonseca et al., 1993). Conversely, in midbrain regions, it has been shown that the expression of the CB1-R is lower in adolescent rats (PD 37) compared with adult rats (PD72) (Verdurand et al., 2011). Considering that both DA system and CB1-R are not fully developed during adolescence, it is tempting to propose that CB1 agonism may induce differential effects in the adolescent population.

Wiley et al. compared the acute and repeated effects of low doses of  $\Delta^9$ -THC in adolescent and adult rats on locomotor activity, behavior highly associated with dopaminergic transmission. The adolescent rats showed greater locomotor activity induced by low doses of  $\Delta^9$ -THC compared with adult rats (Wiley et al., 2008). Recently, a quantitative microdialysis study determined a differential action mechanism of WIN55212-2 on striatal DA release when comparing adolescent and adult rats. While WIN55212-2 increased the dopamine transmission in both groups, the increase in adult rats was dependent on the DA release, and in adolescent rats, it was associated with a decrease in the DA clearance (Pérez-Valenzuela et al., 2019).

The above evidence supports the idea of an age-dependent effect of CB1 agonism in nigrostriatal dopaminergic transmission. Furthermore, since age-dependent changes in CB1-R expression occur in parallel with the development of dopaminergic pathways (McCutcheon et al., 2012), it is possible to suggest that repeated activation of CB1-R during adolescence could modify the development of the dopaminergic system.

Preclinical evidence indicates that the repeated activation of the CB1-R during adolescence modifies dopaminergic signaling in mesolimbic and nigrostriatal pathways. Gomes et al., 2015 showed that exposure to WIN55212-2 during adolescence increases the spontaneous activity of dopaminergic neurons of the VTA in adult rats and increases hyperlocomotion induced by amphetamine (Gomes et al., 2015). Furthermore, Pistis et al. (2004) observed that exposure to WIN55212-2 during adolescence produces long-term cross-tolerance to the effect on the firing rate of DA neurons of VTA of other drugs of abuse such as morphine, cocaine, and amphetamine (Pistis et al., 2004). Similarly, Renard et al. (2016) showed that adolescent exposure to  $\Delta^9$ -THC increases the firing rate, the bursting activity, and the population activity of DA neurons in the VTA of adult rats (Renard et al., 2016).

Regarding the nigrostriatal pathway, the repeated treatment with WIN55212-2 during adolescence increased DA turnover in the DLS of adult rats (Bortolato et al., 2014). In addition, the repeated treatment of WIN55212-2 during adolescence increased the release of DA in the DLS of the adult rats associated with a disinhibition of the DA neurons in the SNpc. Interestingly, this disinhibition was associated with a decrease in the activity of GABAergic input from the VP (see Fig. 2) (Pérez-Valenzuela et al., 2021). These evidences suggest long-term consequences of adolescent CB1-R repeated activation on dopaminergic neurotransmission. Whether these changes are reversible, as observed after CB1-R repeated activation during adulthood, should be addressed. As mentioned before, recent neurochemical evidence suggests that an hyperactive nigrostriatal pathway accompanies higher levels of impulsive choice (Moreno et al., 2021). Then, the



**FIG. 2** Adolescent WIN 55212-2 activates adult nigrostriatal dopamine neurons. A significant increase in population activity of DA neurons associated with a significant decrease in GABA concentration in SNpc and an increase in DA extracellular concentration in the DLS were observed in adult rats after an adolescent exposure to WIN 55212-2. An attenuation of GABA inhibitory tone on DA neurons in SNpc driven by VP neurons could contribute to the nigrostriatal pathway activation during adulthood after an adolescent exposure to WIN 55212-2 (Pérez-Valenzuela et al., 2021).

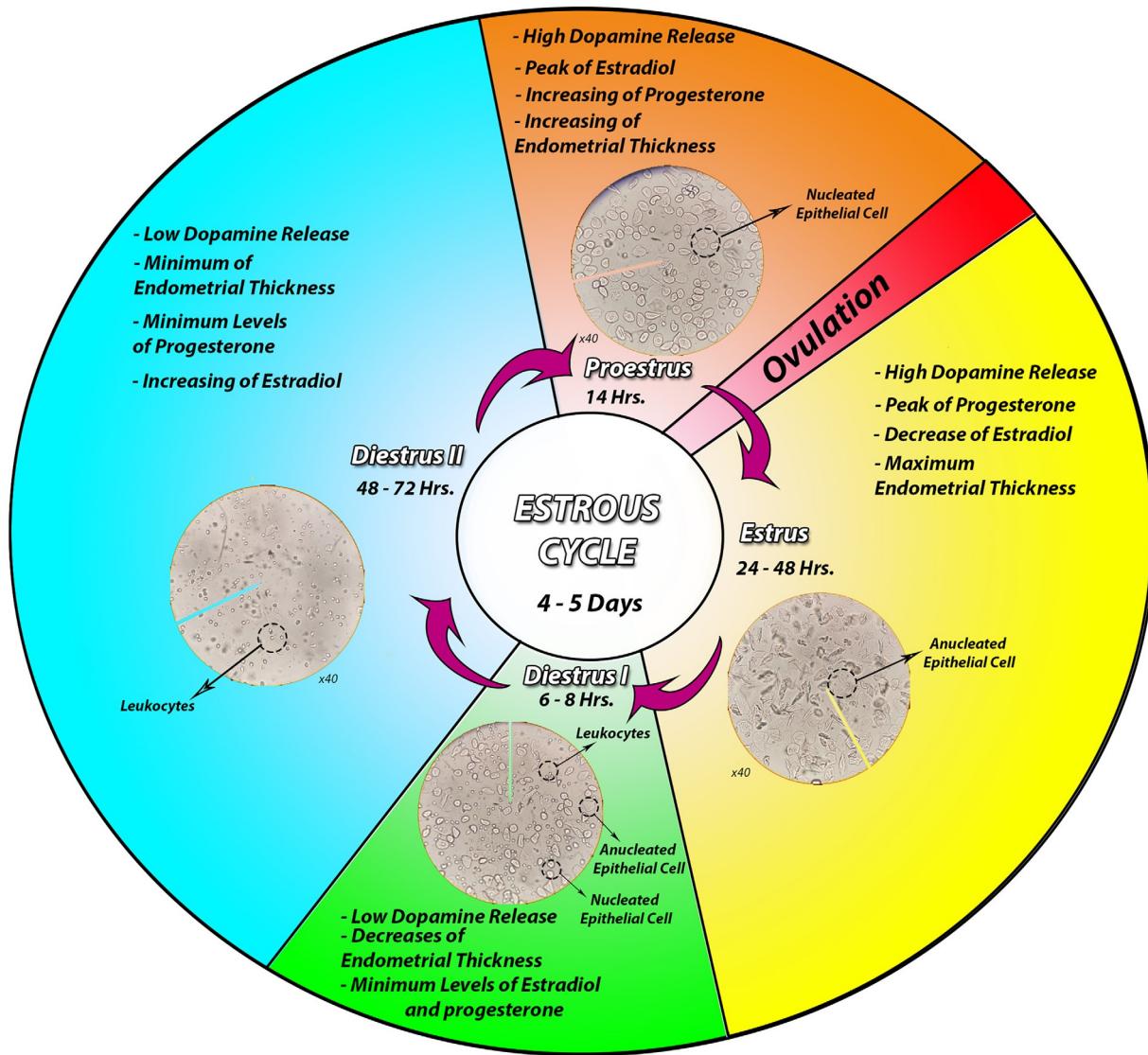
disinhibition of the nigrostriatal pathway observed after adolescent exposure to cannabinoids (Pérez-Valenzuela et al., 2021) could predispose to impulsivity during the adulthood.

### Sex-dependent effect of CB1 agonists in dopaminergic transmission

There is a consensus in the clinical literature that patterns of drug intake show sex differences. Regarding cannabis, the increase in drug use observed in women during the last time has diminished the gap compared with men's usage (Greaves & Heming, 2020). Importantly, since mood fluctuates during the menstrual cycle, it has been suggested that compulsive drug intake may increase during the premenstrual and menstrual phases to reduce negative moods. In contrast, ovulation has been associated with an increased sensitivity to reward (Joyce, Good, et al., 2021). In this sense, a higher cannabis consumption during menstruation has been observed in women with the premenstrual dysphoric disorder compared with healthy women (Joyce, Thompson, et al., 2021). In general terms, women appear more vulnerable to the deleterious effects of cannabis consumption on cognition. For example, a greater deficit in working memory is observed in women than in men after heavy cannabis use (Bassir Nia et al., 2018). Also, women show an accelerated progression toward cannabis use disorder, a phenomenon termed the "telescoping effect" (Khan et al., 2013).

Both clinical and preclinical evidences show that the greater rewarding effects of  $\Delta^9$ THC reported by women when compared with men (Cooper & Haney, 2014) coincides with the faster acquisition of a compulsive-like pattern of cannabinoid self-administration observed in female rats when compared with male rats (Fattore et al., 2007). Interestingly, this pattern is attenuated after ovariectomy, suggesting that ovarian hormones contribute to the reinforcing properties of cannabinoids (Fattore et al., 2007). Because compulsive drug-seeking largely depends on dopamine neurotransmission (Everitt & Robbins, 2013), it is tempting to suggest that sex differences in DA control underlie the telescoping effect observed in cannabis women users. As mentioned before, we recently showed that adolescent exposure to the synthetic cannabinoid WIN 55212-2 induces sensitization of nigrostriatal dopamine neurotransmission in adult male rats, characterized by an increase in dopamine release in DS (Pérez-Valenzuela et al., 2021). However, the consequences of adolescent exposure to cannabinoids on nigrostriatal dopamine neurotransmission in female rats have not been studied yet. Early evidence shows transient changes in extracellular DA levels in DS during the estrous cycle and suggests a sensitizing effect of estrogens on nigrostriatal dopaminergic neurotransmission (see figure 3). Quantitative microdialysis studies show that the lowest extracellular concentration of DA in DS is observed during the diestrus phase (I and II), comparable with the levels observed in ovariectomized (OVX) female rats. The proestrus and estrus phases show higher DA levels in the DLS, similar to those observed in males. Importantly, castration in males does not modify extracellular DA concentration, strongly suggesting that only ovarian hormones regulate extracellular DA levels in the DS of female rats (Xiao & Becker, 1994). In this sense, repeated estrogens administration restores DA's extracellular concentration in the DLS in female OVX rats to levels comparable with those observed in control females (Ohtani et al., 2001). Then, compelling neurochemical evidence demonstrates that estrogens exert rapid, nongenomic effects to facilitate DA release and increase the extracellular concentration of the neurotransmitter (Yoest et al., 2018).

Because nigrostriatal dopamine release depends on the electrical activity of dopaminergic neurons at the SNpc (Goto et al., 2007), it is also essential to determine the effect of ovarian hormones on both firing patterns and number of



**FIG. 3** Summary of Estrous Cycle of rats. The estrous cycle in rats is short compared with humans, with around 4–5 days. Experimentally, the estrous cycle may be divided into four main stages according to the type and the proportion of cells of vaginal secretion: proestrus (orange), estrus (yellow), diestrus I (green), and diestrus II (light blue). The proestrus, characterized by a high proportion of nucleated epithelial cells in vaginal secretion, shows an increase in progesterone levels and endometrial thickness, accompanied by a peak in estradiol levels. The dopamine release during proestrus is higher compared to male rats. During estrus is observed in a high proportion of anucleated epithelial cells in vaginal secretion, a peak of progesterone and endometrial thickness, and the estradiol concentration decreases. The dopamine release during estrus is higher compared to male rats. After that, during diestrus I, it is observed a similar proportion of leukocytes, nucleated, and anucleated epithelial cells in the vaginal secretion. In this stage, progesterone and estradiol levels are minimum, and the endometrial thickness decreases. The dopamine release during diestrus I is lower than in female rats in estrus/proestrus. Finally, during diestrus II, the proportion of leukocytes is predominant in the vaginal secretion, the levels of progesterone and endometrial thickness are minimum, and the levels of estradiol are increasing. The dopamine release during diestrus II is lower than in female rats in estrus/proestrus.

spontaneously active neurons in this region. Unfortunately, there is no electrophysiological evidence regarding the influence of the estrous cycle on neuronal activity in the SNpc. Only early evidence showing a heterogeneous effect of systemic estradiol administration on the basal firing rate of SN dopamine neurons of anesthetized female rats has been reported (Chiodo & Caggiula, 1983). In contrast, cutting-edge experiments using innovative techniques have shown that the activity of VTA dopaminergic neurons is modified during the estrous cycle of the rat, opening the possibility to speculate about the effect of ovarian hormones on SNpc dopaminergic neurons (Calipari et al., 2017). Electrophysiological experiments show more spontaneously active dopaminergic neurons in female rats in the estrus phase than in females in the

diestrus phase and male rats. Regarding the firing mode of VTA dopaminergic neurons, a higher burst firing is observed during the estrus phase. Moreover, during this phase, the reinforcing properties of cocaine also increase, leading the authors to suggest that sensitization of mesolimbic dopaminergic neurotransmission associated with increased estradiol levels during the estrous cycle is a vulnerability factor for the development of addiction (Calipari et al., 2017). Given that nigrostriatal dopaminergic neurotransmission is also sensitized after repeated adolescent exposure to cannabinoids (Pérez-Valenzuela et al., 2021), it is plausible to propose an accelerated hyper-excitability of nigrostriatal pathway after adolescent cannabinoid exposure in female rats compared with male rats.

Finally, we believe that further studies are needed to determine whether these estrogen-dependent changes in dopaminergic neurotransmission of the nigrostriatal pathway contribute to more complex DA-dependent behaviors. For example, during the proestrus phase, a significant decrease in reward-based decision-making is observed, suggesting an attenuation of the impact of positive feedback on behavior (Verharen et al., 2019). We recently showed in our laboratory that greater DA release in the DS correlates with greater impulsive decision-making (Moreno et al., 2021). Because during the estrous cycle, the release of dopamine in the DS shows transient increases, it is also possible to suggest that an accelerated hyper-excitability of nigrostriatal pathway after adolescent cannabinoid exposure in female rats impacts the ability of females to control impulsive behaviors, contributing to the sex differences observed in DA-dependent neuropsychiatric disorders that involve higher levels of impulsive decision-making.

## Applications to other areas

This chapter reviews the effects of the CB1 agonism in the two main dopaminergic pathways: the mesocorticolimbic and the nigrostriatal pathways. Both pathways play a key role in the regulation of rewarding processing and impulsive behavior. Currently, with the new regulations on cannabis consumption worldwide, it is necessary to research the short and long-term consequences of CB1-R activation on vulnerable populations in order to apply evidence-based THC consumption control policies. Despite the vast amount of evidence about acute and chronic effects of CB1 agonism in DA transmission in male adult subjects (French et al., 1997; Polissidis et al., 2013; Tanda et al., 1997; Yanovsky et al., 2003), the effects of cannabinoids on the dopaminergic transmission during adolescence and in females remain to be addressed. This chapter discusses the differential effects of CB1 agonists among adolescents, adults, male and female subjects. Interestingly, clinical evidence has shown that the progression of cannabis use disorder is more accelerated among women than men (Khan et al., 2013), which suggests a differential effect of CB1 agonism on dopaminergic transmission. However, there is no experimental evidence supporting this hypothesis, and future studies are required to determine sex differences in the effect of CB1 agonism exposure on dopaminergic signaling and their long-term consequences, such as rewarding processing and impulsive behavior.

## Mini-dictionary of terms

- **Adolescence:** The adolescence is a life period characterized by gradual physiological and behavioral changes during the transition from childhood to adulthood. This period is triggered by the first surge of sex hormones, which are responsible for beginning the reproductive stage, sexual maturity, and the development of the characteristic behavior of adulthood.
- **Substantia nigra pars compacta:** Basal ganglia structure in the midbrain, which is made up predominantly of large densely packed dopaminergic neurons. This area forms a clearly distinguishable structure in humans because of their high content in neuromelanin, a by-product of dopamine autooxidation. The substantia nigra pars compacta plays a key role in the motor control and rewarding processing.
- **Ventral tegmental area:** This area is localized in the midline between the cerebral peduncles and at the depth of the interpeduncular fossa. It is continuous with the substantia nigra pars compacta to each side and with the lateral hypothalamus rostrally. The VTA is the source of the mesolimbic dopamine system that acts as a regulator of reward processing.
- **Telescoping effect:** In cannabis use disorder context, the telescoping effect is defined as the phenomenon that women show an accelerated progression across the landmark stages of substance use disorder.
- **WIN55212-2:** Synthetic aminoalkylindole derivate cannabinoid with similar physiological properties that the main psychoactive compound of cannabis, Δ<sup>9</sup>-THC. This synthetic cannabinoid presents high affinity to both CB1-R and CB2 receptor. Whereas Δ<sup>9</sup>-THC acts as a partial agonist of CB1-R, WIN55212-2 is full agonist.

## Key facts of dopaminergic transmission

- Dopamine transmission modulates essential behaviors to survive, such as reward-based learning, motor control, and cognition.
- Alterations of the dopaminergic neurotransmission in the brain have been associated to several neuropsychiatric diseases, such as: drug addiction, Parkinson, depression, schizophrenia, and attentional deficit.
- The homeostasis of DA depends mainly by two processes: the release and the uptake. The release is controlled by the synthesis of DA and the activity of the dopamine neuron. While the uptake is controlled by the activity of the dopamine transporter.
- The DA is mainly synthesized from L-tyrosine, which is hydroxylated to L-Dopa, and finally L-Dopa is decarboxylated to dopamine. This biosynthesis is mainly regulated in the first step by tyrosine hydroxylase enzyme.
- The DA neurons present three different states of activity: no-firing state, tonic firing state, and phasic firing state. Tonic firing is characterized by a slow frequency of spike between four and eight spikes per second. Phasic firing state is the transition into a rapid burst-firing mode, which can reach an intra-burst frequency of 30 spikes per second.
- Dopaminergic transmission changes depending on the ontogenetic and the sex of the subject. For instance, it has been observed that teenagers show higher dopamine release than adults. Also, female rodents show higher dopamine release during estrus than female rodents in diestrus and male rodents.

## Summary points

- Delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC), is considered the primary psychoactive molecule and responsible for the addictive effects of cannabis, and the physiological effects of acute exposure of  $\Delta^9$ -THC have been mainly attributed to the agonism of CB1 and CB2 receptors.
- Mesocorticolimbic and nigrostriatal dopaminergic pathways have been associated with the development of addictive behavior.
- Despite that CB1 receptor are not expressed in dopamine neurons, the CB1-R agonist increases the dopaminergic transmission in both mesocorticolimbic and nigrostriatal pathways.
- The effects induced by chronic cannabinoid exposure on dopamine homeostasis are dependent of the withdrawal period and the dopaminergic pathway.
- Both dopaminergic system and CB1-R are not fully developed during adolescence, then it is tempting to propose that CB1 agonism may induce differential effects in adolescent population.
- The repeated activation of the CB1-R during adolescence induces a long-term increase in the dopaminergic transmission of mesolimbic and nigrostriatal pathways.
- Women appear more vulnerable to deleterious effects of cannabis consumption on cognition and show an accelerated progression toward cannabis use disorder.
- Neurochemistry and electrophysiological data show that estrogens exert rapid, nongenomic effects to facilitate dopaminergic neurotransmission.
- However, the consequences of adolescent exposure to cannabinoids on dopamine neurotransmission in female have not been studied yet.

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## Chapter 24

# Peripheral cannabinoid receptor 1 antagonists and impact on adipocytes

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## Abbreviations

T2D	type 2 diabetes
CB1R	cannabinoid receptors1
ECS	endocannabinoid system
eCB	endocannabinoid ligands
WAT	white adipose tissue
BAT	brown adipose tissue
SAT	subcutaneous adipose tissue
VAT	visceral adipose tissue
AEA	anandamide
2-AG	2-arachidonoylglycerol
RIM	rimonabant
KO	knockout
β3R	β3-adrenergic receptors
NPs	natriuretic peptides
HSL	hormone-sensitive lipase
ATGL	adipose triglyceride lipase
UCP1	uncoupling protein 1
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
NRF-1	nuclear respiratory factor-1
Tfam	transcription factor A
OCR	oxygen consumption rate
Prdm16	PR domain containing 16
Cidea	cell death inducing DFFA like effector A
Elovl3	ELOVL fatty acid elongase 3
Tmem26	transmembrane protein
Tbx1	T-Box transcription factor 1
Cited1	Cbp/P300 interacting transactivator with Glu/Asp Rich carboxy-terminal domain

## Introduction

Obesity is known to increase the risk for multiple pathologies, including type 2 diabetes (T2D), cardiovascular disease, and some cancers (Flegal et al., 2010; Gallagher & LeRoith, 2015; National Institutes of Health, 1998). The prevalence of obesity in the USA will rise by 2030 to one in two adults (Ward et al., 2019). In addition, obesity has been associated with an increase in the rate of hospitalizations and death in Covid patients (Hendren et al., 2021; Sanchis-Gomar et al., 2020). Current therapies that emphasize lifestyle changes such as caloric restriction and physical exercise have been largely unsuccessful for achieving substantial long-term weight loss. Long-term body weight maintenance with pharmacological therapies with appropriate tolerability and safety remained largely unavailable (Afshin et al., 2017). But some of the new medicines, such as glucagon-like peptide 1 receptor (GLP1R) agonism, semiglutide, showed substantial efficacy, in

addition to diet and exercise. However, only a small number of subjects can achieve and maintain >10% weight loss at tolerable doses (Bray et al., 2016). The best approach to fight obesity would be targeting multiple mechanisms to accomplish optimal management. One of the therapeutic approaches to fight obesity is targeting the endocannabinoid system (ECS). CB1R is involved in the feeding and peripheral metabolic regulation of different organs, including white adipose tissue (WAT). This chapter focuses on how peripheral and central CB1R antagonists influence WAT function.

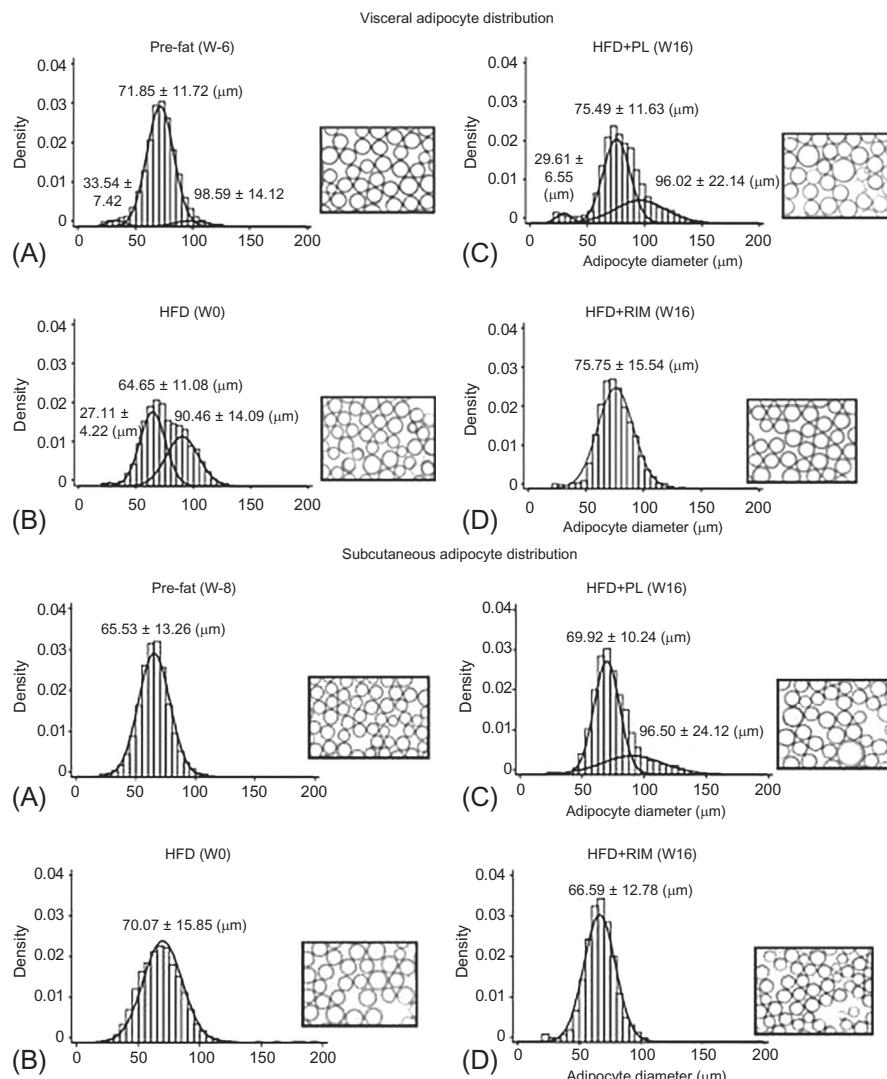
## Endocannabinoid system

The Endocannabinoid system (ECS) has garnered great attention as a potential therapeutic target for a broad range of diseases, including obesity and T2D. It includes cannabinoid receptors type 1 and 2 (CB1R and CB2R) and their major endogenous endocannabinoid ligands (eCBs): arachidonoyl ethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al., 1992; Sugiura et al., 1995). The ECS regulates several metabolic processes, such as food intake, energy expenditure, fat accumulation, and thermogenesis (Horn et al., 2018). CB1R is expressed in the brain but also in the WAT, brown adipose tissue (BAT), liver, muscle, gastrointestinal tract, and heart (Abate et al., 1997; Bonz et al., 2003; Pacher et al., 2018). CB1R is highly expressed in WAT both in mice (Tang et al., 2012; Vida et al., 2014) and humans (Ge et al., 2013) and also in dogs (Iyer et al., 2019; Paszkiewicz et al., 2020). These data confirmed that ECS is a conserved system across vertebrate species (Elphick, 2012). CB1R expression increases in obesity (Rossi et al., 2018) especially in adipose tissue (Bensaid et al., 2003) and liver (Osei-Hyiaman et al., 2005). Of particular interest, studies in humans and rodents demonstrated that adipocytes not only express CB1R, but they possess the ability to synthesize and degrade the endogenous ligands (Gasperi et al., 2007; Matias et al., 2006). The presence of CB1R in WAT suggests that ECS could possibly control WAT function (Cota et al., 2003). CB1R stimulation induces adipogenesis by promoting lipogenesis and the formation of lipid droplets (Silvestri & Di Marzo, 2013). Levels of eCB increase in the epididymal fat of diet-induced obese mice (Pagano et al., 2007) and in the visceral adipose tissue (VAT) of obese patients. Consistent with these data, an elevation of 2-AG levels was observed in the VAT, but not subcutaneous adipose tissue in overweight and obese subjects (Matias et al., 2006). Alterations of eCB levels in human obesity occur in both genders and in WAT depot-specific and insulin-dependent manner (Silvestri & Di Marzo, 2013). It's well established that Rimonabant (RIM), the first selective CB1 antagonist/inverse agonist, crosses the blood-brain barrier, this drug would be expected to exert both central and peripheral effects, and its central action has been linked to adverse effects that restricted the drug's clinical use (Christensen et al., 2007; Meye et al., 2013). However, the peripheral CB1R antagonist AM6545 is a nonbrain-penetrant compound that is a specific antagonist of peripheral CB1Rs (Tam et al., 2010), thus providing a potential tool to examine the role of peripheral vs. central CB1R antagonism in the regulation of WAT.

## CB1 antagonist Rimonabant and adipose tissue function

The history of CBR inverse agonist rimonabant (SR141716A) (RIM) goes back to the early 1990s (Rinaldi-Carmona et al., 1994). The drug was developed for smoking cessation. The first study with RIM showed that not only was the drug was showing efficacy for smoking cessation, but surprisingly the compound promoted weight loss (Le Foll et al., 2008). Since then, studies have shown that CB1R is involved in feeding, energy expenditure, reward pathways, and metabolic homeostasis (Silvestri & Di Marzo, 2013). RIM became the most recognized and systematically studied CB1R inverse agonists developed for obesity management. Several studies showed that chronic treatment with RIM leads to weight loss and improved insulin sensitivity in obese rodents (Gary-Bobo et al., 2007; Ravinet et al., 2003), canines (Kabir et al., 2015; Richey et al., 2009), and humans (Van Gaal et al., 2005). In clinical studies, RIM improved cardiometabolic risk factors, when given to patients with abdominal obesity by significantly reducing intra-abdominal fat mass and liver fat (Despres et al., 2009). ECS exists in WAT (Bluher et al., 2006; Gonthier et al., 2007; Roche et al., 2006). The specific "knock-out" of CB1R in WAT normalized weight gain of high-fat-fed mice. This latter result provides strong evidence that CB1R acts on WAT (Ruiz de Azua et al., 2017). CB1R agonists stimulated lipogenesis in primary adipocyte culture (Cota et al., 2003). In contrast, RIM reduces pro-inflammatory cytokines and chemokines in WAT (Iyer et al., 2019; Jourdan et al., 2010; Wang et al., 2011). RIM reduces diet-induced obesity-associated inflammation through alterations in macrophage miR expression that promote M2 polarization from adipose tissue (Mehrpooya-Bahrami et al., 2019).

Studies suggest that the lowering of WAT mass by RIM in diet-induced obesity is due to increased lipolysis leading to the elevation of fatty acid oxidation and the reduction of fat cell size and consequently the reduction in fat mass (Iyer et al., 2019; Jbilo et al., 2005). Our laboratory showed that after 16 weeks of RIM treatment in fat-fed dogs, the average cell size decreased in the SC and VIS isolated adipocytes. Additionally, the cell size distribution shifted to the left, consistent with



**FIG. 1** Visceral and subcutaneous adipocyte distributions (A) in prefat fed dogs, (B) after 6 weeks of fay diet, (C) after 16 weeks of fat diet + PL, (D) after 16 weeks of fat diet + RIM. (From Kabir, M., Stefanovski, D., Hsu, I. R., Iyer, M., Woolcott, O. O., Zheng, D., Richey, J. M. (2011). Large size cells in the visceral adipose depot predict insulin resistance in the canine model. *Obesity* (Silver Spring), 19(11), 2121–2129.)

reduced cell diameters (Fig. 1) (Kabir et al., 2011). Consequently, the reduced fat mass is partly due to a decrease in adipocyte size compared with adipocyte size in control animals, despite maintenance on an HFD (Richey et al., 2009).

Consistent with these results, it has been demonstrated that RIM results in WAT reshaping by increasing the number of adipocyte nuclei rather than improving energy loss (Jbilo et al., 2005). RIM could also reduce cell proliferation in cultured 3T3 F442A preadipocytes (Gary-Bobo et al., 2006). However, increased eCB levels promote preadipocyte differentiation similar to chronic activation of CB1R (Matias et al., 2006; Matias & Di Marzo, 2007).

The ECS is not only involved in WAT proliferation and adipocyte size but also plays a role in regulating lipolysis. The mechanisms by which RIM enhances lipolysis remained controversial. Some studies showed acute or chronic effects at the adipose tissue or isolated adipocytes in different species such as mice, humans, dogs, and primates (Backhouse et al., 2012; Buch et al., 2021; Chen et al., 2013; Iyer et al., 2019; Paszkiewicz et al., 2020; Vaidyanathan et al., 2012). In fact, chronic administration of RIM has been shown to increase the expression of lipolytic enzymes, such as carnitine-acylCoA transferase, carnitine palmitoyltransferase 2, and crotinase as well as  $\beta$ 3-adrenergic receptors ( $\beta$ 3R) in the WAT to diet-induced obese mice (Jbilo et al., 2005). One of our studies performed in a fat-fed insulin-resistant canine model showed that chronically RIM increased lipolysis (Iyer et al., 2019). It has been found that RIM can potentiate the release of cardiac natriuretic peptides (NPs) (Ruginsk et al., 2012), which can also promote beiging or browning of WAT and increase lipolysis (Hankir, 2018). NPs are able to activate lipolysis via the activation of their own receptors (Lafontan, 2008). We showed that lipolysis

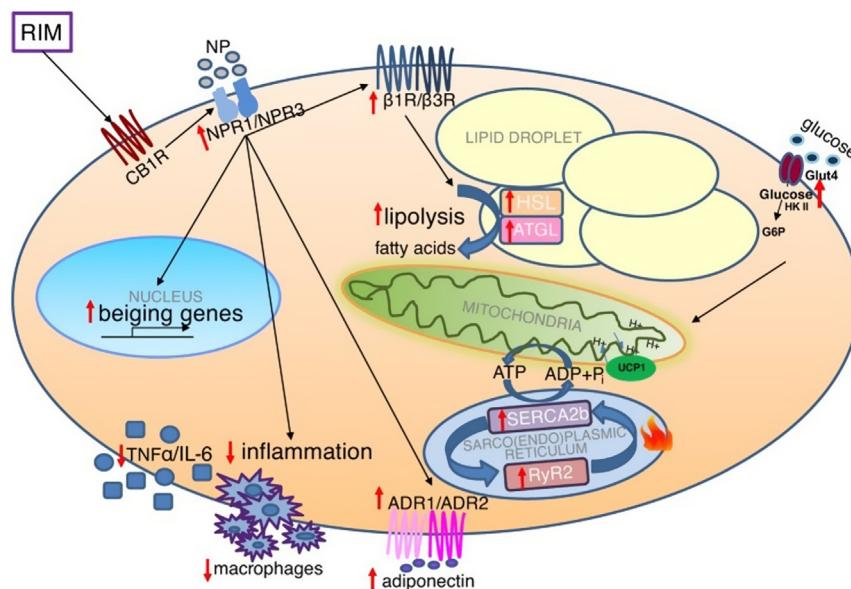
increased in the SAT and VAT fat depots by upregulation of  $\beta 1R$ ,  $\beta 3R$ , adipose triglyceride lipase (ATLG) expressions as well as increased hormone-sensitive lipase HSL phosphorylation (Iyer et al., 2019).

Acute studies in isolated adipocytes or 3T3-L1 cells showed that RIM increased lipolysis directly independent of the central effects (Müller et al., 2020; Paszkiewicz et al., 2020). A study in Wistar rats showed that acute administration of RIM stimulated lipolysis. In the cited study, the interaction of RIM with both the lipid droplet surface and the CB1R each led to the increased access of HSL to lipid droplets in a phosphorylation-independent and dependent manner, consequently stimulating lipolysis (Müller et al., 2020). Our study in 3T3-L1 showed that RIM increased lipolysis at 4 and 48 h of adipocyte culture (Paszkiewicz et al., 2020).

Recent studies suggested the possibility of treating obesity by converting WAT into brown BAT or BAT-like “beige” WAT, which is more metabolically active. These beige adipocytes contain greater mitochondria number and activity, which promote thermogenesis (Wu et al., 2013). While the thermogenic potential of BAT in rodents is several times higher than that of beige WAT, the clear difference between brown and beige fat does not exist in humans (Herz & Kiefer, 2019). By contrast, the excess of WAT in humans could be a potential target to convert to metabolic active beige fat. Therefore, the study of beige adipocytes and the beiging of WAT has become an area of substantial research focus. Beige cells enhance energy expenditure by consuming stored lipids through fatty acid oxidation within the adipose tissue (Thyagarajan & Foster, 2017). It is crucial to identify novel pharmacological targets that can activate WAT beiging and lead to the resultant increase in mitochondrial function and energy expenditure.

The first study on the effects of CB1R antagonist on beiging showed that RIM increased uncoupling protein 1 (UCP1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 $\alpha$ ) expressions and mitochondrial biogenesis in immortalized murine WAT (Perwitz et al., 2010). The latter suggests that beiging could be induced by CB1R antagonist in WAT. In addition, CB1R knockout mice were protected from HFD-induced obesity and had instead activated macrophages and increased sympathetic tone in WAT (Ruiz de Azua et al., 2017). Recently, we found that the increases of natriuretic peptides (NPRs) lead to increased beiging and lipolysis of WAT. This latter study showed the mechanisms by which CB1 antagonist promoted beiging and improved WAT function, mediated through NPRs and a UCP1-independent pathway (Iyer et al., 2019). The study showed that the upregulation of markers of WAT beiging and lipolysis might be the main mechanisms by which the CB1R antagonist RIM promotes the improvement of metabolic homeostasis (Fig. 2).

Although research showed a remarkable potential for RIM to promote weight loss in obese patients, it was finally removed from the market due to the high adverse psychiatric side effects, including anxiety, depression, and suicidal thoughts (Moreira & Crippa, 2009).



**FIG. 2** Proposed mechanism by which rimonabant promotes beiging in adipose tissue. (From Iyer, M. S., Paszkiewicz, R. L., Bergman, R. N., Richey, J. M., Woolcott, O. O., Asare-Bediako, I., ... Kabir, M. (2019). Activation of NPRs and UCP1-independent pathway following CB1R antagonist treatment is associated with adipose tissue beiging in fat-fed male dogs. American Journal of Physiology. Endocrinology and Metabolism, 317(3), E535-e547.)

The antiobesity potential therapeutic of RIM has led to the development of a new CB1R antagonist with limited brain penetrance, such as a “peripheral antagonist” to interact with the CB1R.

## Peripheral CB1R antagonist and adipose tissue function

Numerous peripheral CB1Rs have been discovered over the past few years, Dr. Makriyannis and his colleagues have done outstanding work discovering different types of agonists and antagonists of CB1R. They developed an efficient peripheral neutral CB1R antagonist, AM6545, which has very limited brain penetrance and has been shown to reduce food intake, body weight, and adiposity (Cluny et al., 2010; Tam et al., 2010). Other compounds such as TXX-522 and peripheral inverse agonists TM38837 and JD-5037 showed antiobesity effects in rodents (Chen et al., 2017; Chorvat et al., 2012; Klumpers et al., 2013). Acute and chronic administration of JD5037 did not displace a CB1R PET ligand showing limited brain penetrance of this compound (Tam et al., 2012). Administration of JD5037 in DIO mice restored their sensitivity to leptin mainly by blocking the adipose tissue CB1R and peripheral sympathetic terminals (Tam et al., 2012).

Most of the studies in adipose tissue function were observed using AM6545. First *in vivo* study showed that AM6545 increased total energy expenditure due to increased fat burning in diet-induced obesity mice. In addition, AM6545 treatment normalized the diet-induced suppression of plasma adiponectin. By contrast, AM6545 decreased lipogenesis enzymes gene expressions such as fatty acid synthase (FAS) and stearoyl coenzyme-A desaturase 1 (Scd1) in WAT. AM6545 improved obesity-related glucose intolerance and insulin resistance as much as RIM (Tam et al., 2010).

In other types of insulin-resistant rodent models such as high-fructose high-salt rats, AM6545 increased adiponectin, displayed antidiabetic, antihyperuricemic, and antiinflammatory effects in insulin resistance (Eid et al., 2020). Peripheral CB1R blockade activates brown fat tissue and diminishes dyslipidemia and obesity in diet-induced obesity (Boon et al., 2014). In addition, AM6545 decreased fat mass, adipocyte size, and leptin level as well as increasing adiponectin was reduced markedly. Most interestingly, AM6545 showed significant improvement on levels of circulating adipokines including lowering leptin and TNF $\alpha$  and increasing adiponectin. Additionally, dysregulated gene expression including lipogenesis, lipolysis, and adipokines in the WAT was normalized by AM6545 treatment in monosodium-glutamate-induced hypometabolic and hypothalamic obesity in mice (Ma et al., 2018).

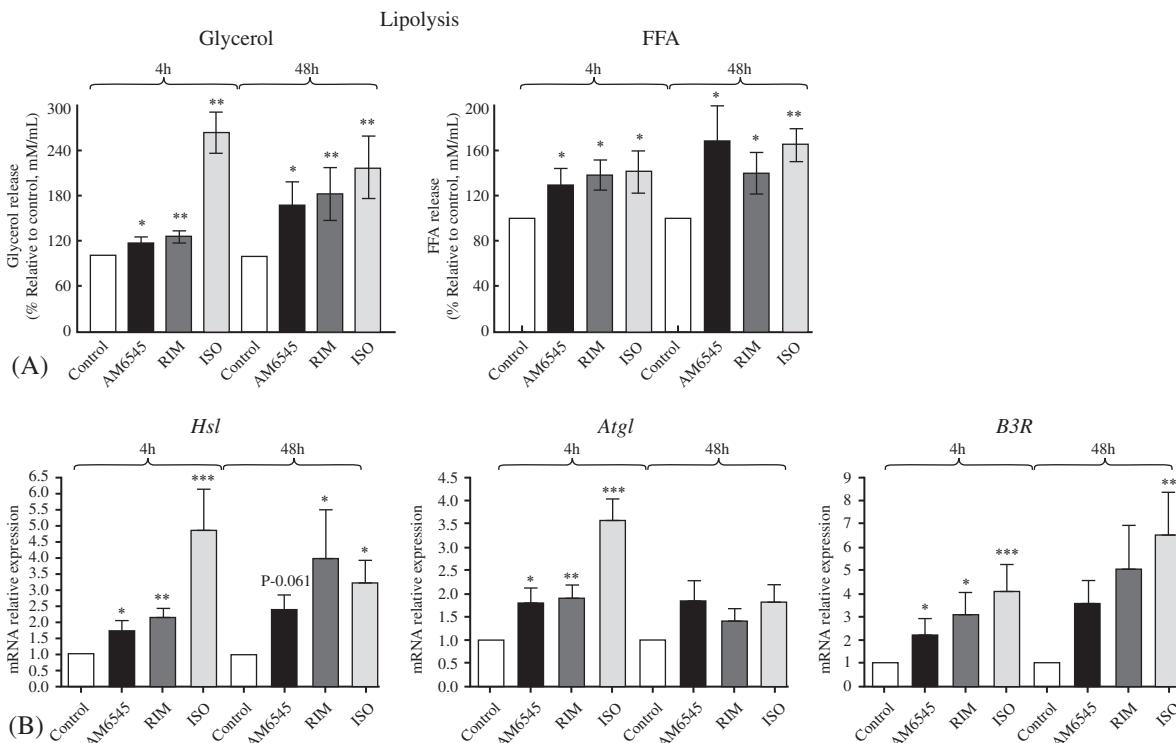
It has been shown that adipose tissue CB1R genetic deletion increased mitochondrial biogenes and increased energy expenditure resulting in browning of white fat and increased thermogenesis (Ruiz de Azua et al., 2017; Tedesco et al., 2010). By contrast, CB1R agonist arachidonyl2'-chloroethylamide hydrate decreased mitochondrial biogenesis, mtDNA, and mitochondrial mass, the PGC-1 $\alpha$ , nuclear respiratory factor-1 (NRF-1), and transcription factor A (Tfam) in mouse and human primary WAT (Tedesco et al., 2008, 2010). The direct effects of peripheral CB1R antagonists on adipocytes were not clear. Recently, our laboratory has explored the effects of AM6545 on adipocyte function. The aim of the study was to determine the cellular and molecular mechanisms by which peripheral CB1R antagonism AM6545 acts on beiging, lipolysis, mitochondrial biogenesis, and oxygen consumption rate (OCR) in adipocytes when the central and sympathetic nervous system effects could be avoided (Paszkiewicz et al., 2020).

We showed that AM6545 promotes upregulation of beiging markers and improves mitochondrial function in 3T3-L1 adipocytes similar to what we observed following *in vivo* administration of RIM (Iyer et al., 2019). The key beiging genes such as PGC1 $\alpha$ , PR domain containing 16 (Prdm16), Cell Death Inducing DFFA-Like Effector A (Cidea), (ELOVL Fatty Acid Elongase 3) Elovl3, Cd137, transmembrane protein (Tmem26), T-box transcription factor (Tbx1), and Cbp/P300 Interacting Transactivator with Glu/Asp Rich Carboxy-Terminal Domain 1 (Cited1) were increased following treatment with AM6545 and RIM, with larger increases in UCP1 seen with AM6545 than was seen with *in vivo* or *in vitro* treatment with RIM, suggesting that mitochondrial uncoupling may be greater following treatment with the peripheral antagonist.

It has been demonstrated that when the beiging process increases, mitochondria's thermogenic action requires increased fuel such as FFA (Bartelt et al., 2011; Seale & Lazar, 2009). Consistent with that, we showed that AM6545 increased lipolysis by releasing more glycerol and FFA into the culture media after 4 and 48 h of cell culture. Similarly, AM6545 upregulated the expression of HSL, ATGL, and  $\beta$ 3R and decreased TG storage (Fig. 3).

Also, our data suggest that AM6545, similar to RIM, increases PGC1 $\alpha$  expression via  $\beta$ 3R activation. In addition, mtDNA and genes related to mitochondrial biogenesis, such as Tfam and NRF-1, were significantly upregulated.

The increased mitochondrial biogenesis was confirmed by an increased in oxygen consumption and proton leak of adipocytes treated with AM6545 (Paszkiewicz et al., 2020). Treatment with the peripheral CB1R antagonist AM6545 significantly improved oxygen consumption rate (OCR) by increasing basal and maximal respiration as well as proton leak and ATP production at 4 and 48 h. Unexpectedly, AM6545 and RIM showed a transient decrease of real-time OCR during the first 45 min after initiation of treatment (Paszkiewicz et al., 2020). This effect was not seen in Isoproterenol-treated cells, which were used in these experiments as a positive control. This transient dip in oxygen consumption was due to the CB1R



**FIG. 3** Peripheral CB1R antagonist AM6545 increased lipolysis in 3T3-L1 adipocytes. (A) Glycerol and FFA release in culture media after 4 and 48 of culture. (B) HSL, ATGL, and b3R gene expression. (From Paszkiewicz, R. L., Bergman, R. N., Santos, R. S., Frank, A. P., Woolcott, O. O., Iyer, M. S., Kabir, M. (2020). A peripheral CB1R antagonist increases lipolysis, oxygen consumption rate, and markers of beiging in 3T3-L1 adipocytes similar to RIM, suggesting that central effects can be avoided. International Journal of Molecular Sciences, 21(18), 6639.)

antagonist induced by lipolysis. The treatment with the lipolysis blocker Atglinstatin reversed the effects of AM6545 and RIM on OCR during the first 45 min of treatment. In the presence of Atglinstatin, OCR for cells treated with AM6545 and RIM remained no different than vehicle control for the entire study duration. Atglinstatin also decreased basal respiration, maximal respiration, proton leak, and ATP production, most importantly after 4 h treatment with CB1R antagonists, further suggesting that the impact of CB1R antagonism on adipocytes is driven by increased lipolysis (Fig. 4).

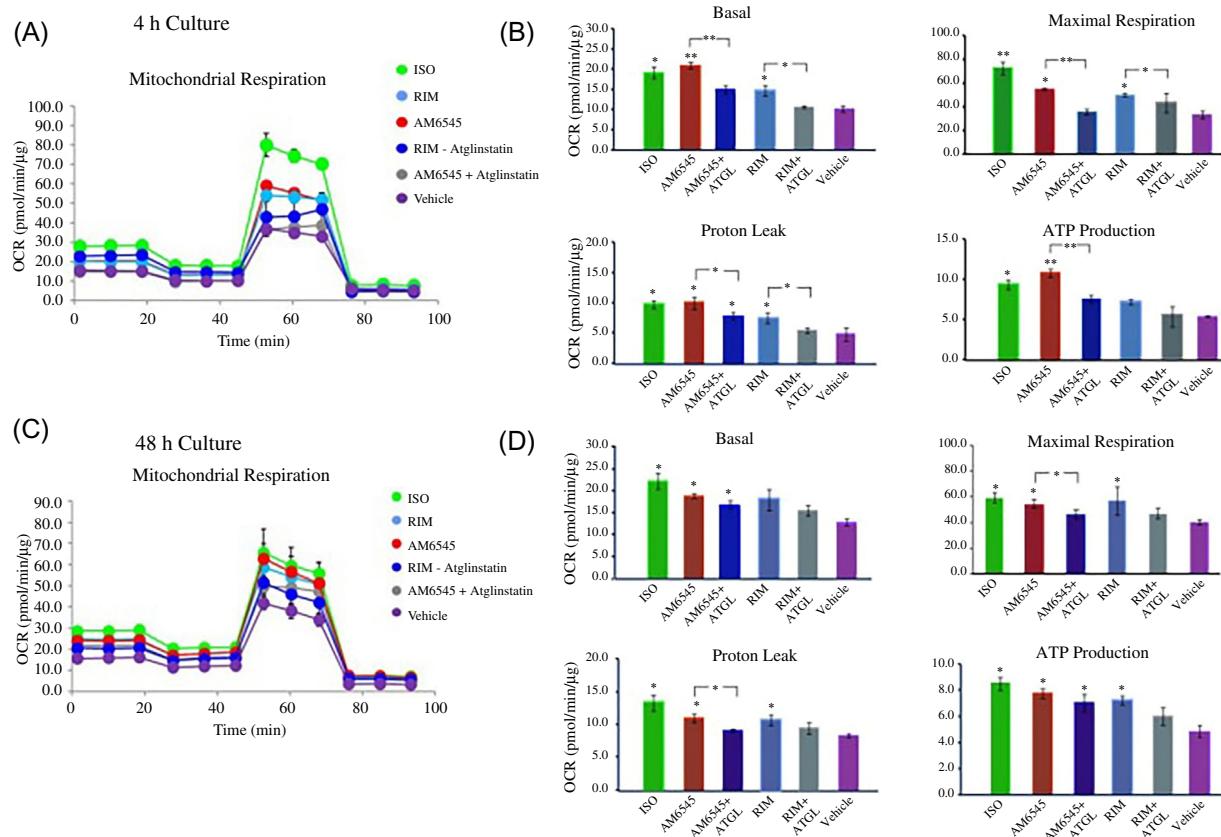
Our data exclusively focus on an in vitro model, but provide a beginning point to understand the mechanism by which this peripheral CB1R antagonist may improve adipose tissue function.

## Applications to other areas

In this chapter, we have reviewed the impact of central vs. peripheral CB1R antagonists on adipose tissue function. Here we provide evidence that the direct role of CB1R antagonism on adipocytes does not require brain penetrance. The beneficial effects of CB1R antagonists are not limited in adipose tissue; preclinical studies showed potential efficacy of different types of peripheral CB1 antagonists on fatty liver disease and NASH and insulin resistance, targeting the liver and muscle. T2D and obesity are heterogeneous and multifactorial diseases and require combination therapies. Future in vivo studies in the large animal models, followed by studies in humans, are required to elucidate the potential applications of peripheral CB1R antagonism as a therapeutic agent for T2D and obesity.

## Mini-dictionary of terms

- CB1R antagonist: cannabinoid CB1 receptor antagonist is a type of cannabinoidergic drug that binds to cannabinoid receptors 1 and prevents their activation by endocannabinoids
- Adipose tissue beiging: beiging/browning of white adipose tissue is the activation of the brown fat-specific genes in response to cold,  $\beta$ -adrenergic receptor agonists, or CB1R antagonists
- Lipolysis: the breakdown of lipid triglycerides is hydrolyzed into a glycerol and three fatty acids.
- Oxygen consumption rate (OCR): Assessing mitochondrial function in physiology and pathophysiology.



**FIG. 4** Peripheral CB1R antagonist AM6545 increased OCR inhibited by lipolysis blocker. 3T3-L1 adipocytes treated with AM6545 and RIM with or without Atglinstatin and isoproterenol at 4 and 48h. (A) OCR after 4h treatments, (B) parameters calculated from OCR at 4h, (C) OCR at 48h, (D) parameters calculated from OCR at 48h. (From Paszkiewicz, R. L., Bergman, R. N., Santos, R. S., Frank, A. P., Woolcott, O. O., Iyer, M. S., Kabir, M. (2020). A peripheral CB1R antagonist increases lipolysis, oxygen consumption rate, and markers of beiging in 3T3-L1 adipocytes similar to RIM, suggesting that central effects can be avoided. International Journal of Molecular Sciences, 21(18), 6639.)

## Key facts of peripheral cannabinoid receptor 1 antagonists

The first generation of CB1R antagonists targeted several tissues including the brain and peripheral tissues such as the liver, adipose tissue, gut, and muscle. However, it was withdrawn from the market because of adverse neuropsychiatric side effects. In the last 10 years, new peripherally restricted CB1 blockers, characterized by limited-brain penetrance, have been developed. Animal studies showed that the peripheral CB1R antagonists presented limited brain penetrance without any neuropsychiatric effects, while inducing weight loss and other metabolic beneficial effects.

## Summary points

- Pharmacological treatment with the CB1R antagonists such as rimonabant induced weight loss by decreasing fat mass.
- RIM crosses the blood–brain barrier, this drug was able to exert both central and peripheral effects and the central action has been linked to neuropsychiatric side effects.
- Peripheral CB1R antagonist, with limited-brain penetrance, exerts beneficial metabolic effects and provides a powerful tool to study the role of peripheral vs. central CB1R antagonism in the regulation of adipose tissue.
- Peripheral CB1R antagonist improved adipose tissue function by increasing the beiging, lipolysis, and oxygen consumption rate in adipocytes
- The direct role of CB1R antagonism on adipocytes does not require brain penetrance, supporting the importance of focusing on peripheral CB1R antagonist pharmacology for reducing the incidence of obesity and T2D.

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## Chapter 25

# Plasma membrane localization of endocannabinoids system receptors

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## Abbreviations

AC	adenylyl cyclase
AEA	anandamide
CB	cannabinoid receptor
CRAC	cholesterol interaction/recognition amino acid sequence consensus
DRM	detergent resistant membrane
EGF	epidermal growth factor
FAAH	fatty acid amide hydrolase
GPI	glycosylphosphatidylinositol
GPCRs	G-protein-coupled receptors
HEK	human embryonic kidney
MCD	methyl-β-cyclodextrin
MAPK	mitogen-activated protein kinases
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
TMH	transmembrane helices

## Introduction

The endocannabinoids are defined as the endogenous ligand of the cannabinoid (CB1 and CB2) receptors. Endocannabinoids and their target receptors form a signaling mechanism that has yet to be fully understood. In the last decade, there has been a great progress in our knowledge on the endocannabinoid signaling, and experimental evidence is mounting that pharmacological augmentation of endocannabinoid tone might be linked to treatment of variety of diseases. Study of the localization of CB receptors in the plasma membrane helps to better understand the details of the CB signaling pathway. It would be critical to identify a possible differential regulation and localization of CB1 and CB2 receptors, especially given that these receptor subtypes have been identified as distinct drug discovery targets for a variety of potential therapeutic applications, including food intake, neurological disorders, cancer, and immune suppression (An et al., 2020; Cristina et al., 2020).

Recent studies have discovered that detergent-insoluble membrane regions known as lipid rafts are involved in signal transduction and trafficking by the endocannabinoid anandamide and receptors. The cellular accumulation of anandamide has been attributed to a caveola-related process. Caveolae/lipid raft disruption, as well as treatment with agents known to inhibit caveola-related endocytic processes, decreased [<sup>3</sup>H] anandamide uptake in RBL-2H3 cells (McFarland et al., 2004). Furthermore, after uptake, fluorescence from a fluorescently labeled anandamide analog co-localized with caveolae markers (McFarland et al., 2004).

Not surprisingly, lipid rafts have been suggested as a potential regulator of CB receptors activity (Zou & Kumar, 2018). Moreover, intact detergent membrane domain appears to play a significant role in anandamide-induced signaling cascade. Detergent-insoluble membrane domains regulate cannabinoid receptors localization, trafficking, and endocannabinoid signaling (Wickert et al., 2018).

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## Lipid rafts: Detergent-insoluble membrane domains

In 1972, Singer and Nicolson proposed their fluid mosaic model of plasma membrane, which served as the foundation for our understanding of the structure of cellular membranes (Singer & Nicolson, 1972). In this model, membrane proteins were viewed as icebergs floating in a sea of lipids. However, research over the last three decades has shown that the plasma membrane is not a random sea of lipids. Instead, structures exist within this sea of lipids, which imposes organization on the distribution of proteins in the bilayer. Lipid raft domains are nanoscale lipid “structures” within the membrane ocean (Lingwood & Simons, 2010; Pike, 2003).

Different lipid species with a variety of physicochemical properties are found in cell membranes (Casares et al., 2019). Lipid-lipid immiscibility causes lateral heterogeneities in the membrane plane, of which lipid rafts are a subset. Initially identified biochemically as detergent-resistant membrane (DRM) fractions, lipid rafts are thought to be extremely dynamic, submicroscopic (10–200 nm) structures that float freely inside the liquid disorganized bilayer of cell membranes and coalesce when their constituents are clustered. Lipid rafts are involved in many functions including intracellular signaling, cellular polarity, molecular sorting, membrane transport, and endocytosis (Levental et al., 2020). These nanodomains are located in both the inner and outer leaflets of an asymmetric plasma membrane (Rivel et al., 2019). The outer exoplasmic leaflet’s sphingolipids and cholesterol play a key role in the formation of these nanodomains. Lipid rafts are liquid-ordered domains in the lipid bilayer that are more ordered and highly packed than the nonraft phase (Allen et al., 2007; Rajendran & Simons, 2005). These liquid-ordered regions of the lipid rafts are thicker than the liquid-disordered bulk of the plasma membrane, and cholesterol molecules in this region diffuse at a slower rate of lateral and interlayer (flip-flop) movement (Dainese et al., 2010).

The saturated hydrocarbon chains of raft sphingolipids and phospholipids, as opposed to the unsaturated fatty acids of phospholipids in the nonraft phase, cause the tighter packing. Caveolin-1, one of the first proteins shown to be abundant in rafts, oligomers to create the proteinaceous coat of caveolae flask-shaped plasma membrane invaginations, which constitutes a subset of organized raft domains. In addition to their known role in endocytosis, caveolae have been linked to a variety of functions, including the assembly of important signaling proteins, cholesterol transport, and potocytosis (Lajoie & Nabi, 2010). They have been proposed as platforms for signaling molecule accumulation and may provide a physical location for the initiation of downstream signaling events (Fridolfsson et al., 2014).

Rafts are too tiny to participate in raft-associated processes at steady state. Researchers believe that these domains contain only a few proteins, regardless of their size. They normally have to cluster together in order to engage in membrane function. In raft clusters, there is growing evidence that the outer leaflet domains and the interior leaflet domains are linked (Sezgin et al., 2017).

Rafts are dynamic structures, which implies that proteins and lipids can migrate in and out of them at varying rates. Despite a great body of evidence and published literature supporting the existence of lipid rafts domains, the size and functions of these domains are still up for debate. The debate is mainly raised by the fact that these domains are too tiny to be optically resolved. Recent progress in imaging is now revealing insights into their behavior (Lozano et al., 2016).

The classical method to biochemically define lipid rafts was based on their resistance against being solubilized or extracted by ice-cold Triton X-100 (Taghibiglou et al., 2009). Rafts are isolated by flotation in a linear sucrose density gradient ranging from 5% to 40%, where they are distributed in the top few fractions of the gradient. This method produces a relatively consistent product that is high in cholesterol and raft structural marker proteins such as flotillin, caveolin-1, and glycosylphosphatidylinositol (GPI)-linked proteins. However, differences can occur if the extent of physical manipulation of the detergent lysates is varied. For example, if the lysate is placed in a tube and simply inverted several times prior to centrifugation and flotation of rafts, epidermal growth factor (EGF) receptors are retained. EGF receptors, on the other hand, are lost from detergent-resistant membranes if the original detergent lysate is homogenized prior to centrifugation. To obtain preparations that are comparable across experiments, care must be taken to be consistent in all aspects of the isolation procedure (Pike, 2003). These DRM fractions are raft domain aggregates and do not reflect the natural condition of lipid rafts in cell membranes. Several novel methods for studying rafts in cells are being launched, and improved methodology will definitely assist us to better understand these mysterious membrane regions (Bieberich, 2018; Klymchenko & Kreder, 2014).

## A simplified method for the preparation of lipid rafts

There are a number of complementary approaches to studying lipid rafts and their possible role in signal transduction. In order to prepare lipid rafts from cells or tissue, a cold nonionic detergent using a discontinuous sucrose gradient could be used. A raft-associated protein is the one that cannot be extracted using cold nonionic detergents. The disruption of lipid

rafts can be used to evaluate the physiological significance of protein localization in these domains. The preparation of membranes resistant to cold Triton X-100 solubilization and subsequent sucrose density gradient centrifugation is a simple and straightforward method for assessing a protein's potential raft association. Agents that sequester, chelate, or prevent cholesterol biosynthesis are commonly utilized to disrupt lipid rafts and being used to study the effects of lipid rafts disruption on biochemical and signaling pathways.

## Sample preparation

1. Prepare cells grown to confluence in 10 cm culture plates (four to five plates).
2. Wash twice with ice-cold PBS buffer and place the plates on ice. (CRITICAL STEP: Unless stated otherwise, all extraction and subsequent isolation processes are carried out at 4°C. This is best done in a cold room.)
3. Scrap cells in cold homogenization buffer including 150mM NaCl, 20mM Na<sub>2</sub>HPO<sub>4</sub>, 2mM NaH<sub>2</sub>PO<sub>4</sub>, 20% (v/v) glycerol, 2mM sodium orthovanadate, and protease inhibitors.
4. A Dounce homogenizer with the tight-fitting pestle on ice is used to homogenize (5 ml of buffer per brain or 4–5 dishes).
5. Frozen brain tissue samples were thawed on ice and homogenized in cold homogenization buffer by 30 strokes in a Dounce homogenizer, followed by 20 runs through a 22-gauge needle.
6. When particulate membrane fragments no longer are observed (~35 strokes), nuclei are pelleted at 12,000×g in 4°C for 11 min (Beckman Coulter Avanti J25 centrifuge, SW55 rotor), then membranes are centrifuged 124,000×g (SW55 rotor) for 90 min at 4°C to pellet the total PM.

## Sucrose step density gradient

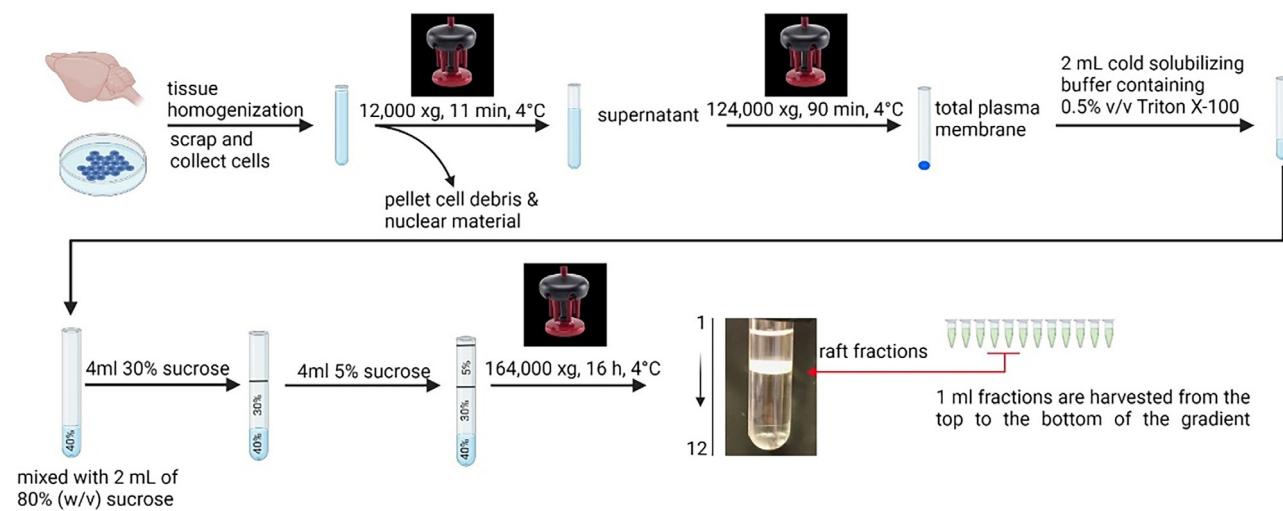
6. The pellet is resuspended in 2mL cold solubilizing buffer containing 0.5% v/v Triton X-100 in Mes-Buffered Saline (MBS, 25mM MES, pH 6.5, 150mM NaCl), protease inhibitors, and 2mM sodium orthovanadate and incubated for 30 min at 4°C. This procedure requires a long incubation time to avoid contamination from other subcellular organelles such as the endoplasmic reticulum and mitochondria (Kim et al., 2008).
7. Two mL of solubilized plasma membrane was mixed with 2 mL of 80% (w/v) sucrose with gentle pipetting to prevent the formation of bubbles and placed to the bottom precooled of a 12mL ultracentrifuge tube. To this, 4ml 30% sucrose was layered on top, followed by 4mL of MBS buffer containing 5% sucrose.
8. The tubes are centrifuged at 164,000×g (SW41Ti rotor) for 16 h at 4°C to isolate the lipid raft and nonraft compartments.
9. Twelve equal fractions (1mL each) were collected from the top of gradient to the bottom.

The floating opaque band corresponding to the detergent-resistant membrane fraction can be found at the interface between the 30% and 5% sucrose gradients after centrifugation (Miranzadeh Mahabadi et al., 2021) (see Fig. 1).

## Plasma membrane localization of CB1 receptor

Lipid rafts regulate cellular metabolism and responsiveness to external stimuli at a different level, effecting a variety of physiologic and pathologic processes (Sviridov & Miller, 2020). Rather than simply functioning as a medium through which membrane proteins diffuse, lipid rafts have now been proven to create compartmentalized domain with various biophysical properties (rafts/caveolae) (Bieberich, 2018). Both lipid rafts and caveolae are enriched in cholesterol, and chemical interventions that remove cholesterol from the plasma membrane, such as methyl-β-cyclodextrin (MCD), disrupt the integrity of lipid raft structures (Huang et al., 2007).

Although the liquid disordered regions contain a high proportion of cell surface proteins, some proteins predominantly partition into the ordered raft domains. Four, not mutually exclusive, mechanisms for targeting transmembrane proteins to the raft domains have been suggested: (i) interaction with components of the lipid raft such as cholesterol and glycosphingolipids (Eroglu et al., 2003; Pucadil & Chattopadhyay, 2004); (ii) protein association with the scaffolding domain of caveolin (Okamoto et al., 1998); (iii) association with hydrophobic amino acids, especially in the transmembrane domains near the exoplasmic leaflets of the plasma membrane (Anderson & Jacobson, 2002; Yamabhai & Anderson, 2002); and (iv) the covalent attachment of a hydrophobic myristoyl group or palmitate (Moffett et al., 2000). In both central and peripheral cells, lipid rafts are well-known modulators of G-protein-coupled receptors (GPCRs)-dependent signaling and membrane trafficking (Barnett-Norris et al., 2005). A raft domain provides platform for the assembly of signaling complexes and prevents cross talk between pathways in membrane proteins such as GPCRs. The conformational activity of GPCRs is also



**FIG. 1** An overview of the protocol and a flow diagram for isolating lipid rafts using the nonionic detergent Triton X-100 and discontinuous sucrose gradient centrifugation. DRM fractions are obtained from purified plasma membrane fractions. The tubes are placed in an ultracentrifuge rotor with a swinging bucket. The tubes are swung at 90 degrees during ultracentrifugation so that the different macromolecules migrate horizontally and are separated from one another. The lipid raft fraction can be seen as a creamy white layer at the interface of the 5% and 30% sucrose layers. Further information can be found in the text.

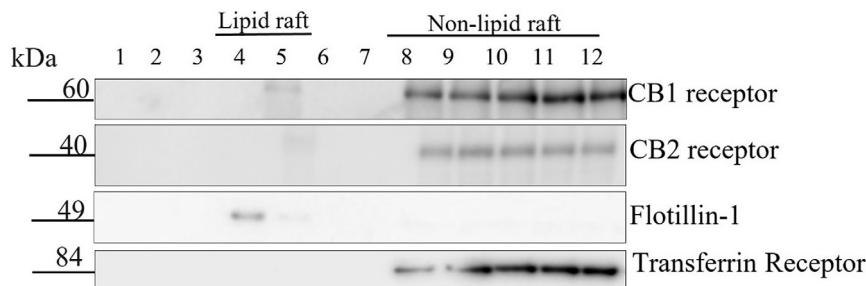
influenced by lipid composition (Bari, Battista, et al., 2005; Oddi et al., 2011). The lipid bilayer is especially important for particular GPCRs, such as CB receptors. CB receptors constitutively recycled between the plasma membrane and endosome, mediated by GTPase Rab5 and Rab4 (Leterrier et al., 2004).

It has been previously shown that CB1 receptor interacted directly with caveolin-1 (Bari et al., 2008). A cholesterol interaction/recognition amino acid sequence consensus (CRAC) (L/V-X[1–5]–YX[1–5]–R/K) was also identified in the transmembrane helix 7 of CB receptors. Replacement of Lys402 of the CB1 receptor with Gly in the corresponding position of CB2 receptors revealed the important role of the CRAC sequence in the cholesterol sensitivity of CB1 receptors, when compared with the cholesterol insensitivity of CB2 receptors (Oddi et al., 2011). Moreover, GPCRs could be targeted to lipid domains through palmitoylation of particular cysteine residues (Greaves et al., 2009). Palmitoylation promotes the protein association with cell membrane, supporting the so-called “kinetic trapping” theory (Schroeder et al., 1997). This theory suggests that palmitoylation tightens modified protein association with plasma membrane and thus restricts desorbing ability of the protein (Qanbar & Bouvier, 2003).

CB1 receptor palmitoylation has been shown to impact the G-alpha protein subtype with which it interacts. However, this palmitoylated modification is not necessary for targeting proteins to the raft domains. For example, the transferrin receptor despite being palmitoylated is localized in nonraft region of the plasma membrane (Jing & Trowbridge, 1990).

The cysteine residue (C7.71(414)) in the C-terminal domain of CB1 receptors is >90% palmitoylated (Oddi et al., 2017). This cysteine residue is evolutionarily conserved in almost all members of class A GPCRs and seems to be critical for receptor activity and regulation (Dainese et al., 2010). G-alpha-0 and G-alpha-i3 have been demonstrated to interact with the C-terminal domain of CB1 receptor between transmembrane helices 7 (TMH7) and the palmitoylated cysteine (401–417) (Mukhopadhyay & Howlett, 2001). The third intracellular loop, on the other hand, has been demonstrated to interact with G-alpha-i1 and G-alpha-i2 (Barnett-Norris et al., 2005; Mukhopadhyay & Howlett, 2001). Chemical depalmitoylation with hydroxylamine followed by coimmunoprecipitation experiments in rat brain and N18TG2 neuronal cell membrane showed that depalmitoylation destroyed CB1/G-alpha-i3 and CB1/G-alpha-o interaction irreversibly, as it could not be recovered after repalmitoylation with palmitoyl-CoA (Howlett et al., 2002). After depalmitoylation, no changes in CB1/G-alpha-i1 or G-alpha-i2 interaction were found. These findings show that palmitoylation is important for Gi/o protein selectivity, and that palmitoylation may be required to direct CB1 receptors to certain lipid bilayer subdomains.

It has been also demonstrated that cellular accumulation of CB endogenous ligand (anandamide; AEA) was mediated by caveolae-mediated endocytosis in RBL-2H3 cells (McFarland et al., 2004), and depletion of cholesterol from the plasma membrane by methyl- $\beta$ -cyclodextrin (MCD) fully blocked AEA-induced cell death in a variety of cells, including PC12, C6, HEK, and HL-60 cells (Biswas et al., 2003). There was a 50% reduction in AEA uptake when caveolae or lipid rafts were disturbed by cholesterol depletion through pretreatment with nystatin or progesterone or by pretreatment with agonists known to inhibit caveolae endocytosis such as genistein or N-ethylmaleimide (NEM), compared with untreated cells



**FIG. 2** Purification of CB1 and CB2 receptor in cold Triton X-100 and sucrose density gradients. The C57BL mouse cortical tissue was lysed in cold Triton X-100 solubilizing buffer and then run through sucrose gradient (5%–40%). An aliquot of each fraction (15 µl/lane) was loaded in parallel on 10% gel and analyzed by Western blotting and ECL (Amersham Biosciences). A representative Western blot for fractionated proteins probed with CB1 and CB2 receptors. Flotillin-1 and transferrin receptor are rafts and nonrafts markers, respectively. Fractions 4–5 represent lipid raft-containing fractions as indicated by the presence of raft resident protein Flotillin-1, and fractions 8–12 include nonlipid raft fractions. Protein migration matched their predicted molecular mass.

(McFarland et al., 2004). All of these findings suggest that caveolae and cholesterol-rich membrane regions are involved in CB1R trafficking and activity. Moreover, McFarland et al. also employed SKM4-45-1, a fluorescent AEA derivative, to track AEA following cellular uptake. Upon delivering SKM4-45-1 into RBL-2H3 cells, it was cleaved/metabolized by the enzyme fatty acid amide hydrolase (FAAH), and anandamide metabolites were rapidly enriched in caveolae and visualized through confocal microscopy. After being fixed, SKM4-45-1 cells were immunolabeled with caveolae/lipid raft markers flotillin-1 and caveolin-1, and co-localization was found. These findings imply that caveolae/lipid rafts are involved in AEA entrance into cells (McFarland et al., 2004).

Bari et al. discovered that rat C6 glioma cells CB1 receptors were regulated by lipid raft domains, so that raft disruption by cholesterol depletion (MCD for 30 min) doubled the binding efficiency of CB1 receptor and signaling (Bari, Battista, et al., 2005), while raft alteration by cholesterol enrichment affected CB1 receptors adversely (Bari, Paradisi, et al., 2005). Shortly afterward, an independent research group reported the localization of CB1 receptors in lipid raft region of human breast cancer cell line, MDA-MB231 cells (Sarnataro et al., 2005, 2006). Sarnataro et al. using confocal analysis of indirect immunofluorescence confirmed the plasma membrane expression of CB1 receptor. They showed that MCD-induced cholesterol depletion led to a significant change in CB1 receptor cell surface distribution. Consequently, the receptor's clustered distribution at the cell surface was lost, and it turned on a more diffuse staining, demonstrating that CB1 receptor clustering in the plasma membrane was dependent on cholesterol-rich microdomains (lipid rafts or DRMs) (Sarnataro et al., 2005, 2006). Moreover, internalization of the CB1 receptor occurs via caveolae and clathrin-coated pits in human embryonic kidney (HEK) 293 cells-CB1 transfected cells (Wu et al., 2008).

In contrast, there are several lines of evidence that demonstrated the association of CB1 receptor with nonlipid raft fractions. Rimmerman et al. characterized the membrane compartmentalization of CB1 receptor into the nonlipid raft compartment in the BV-2 microglia cell line, which was confirmed to be caveolin-1-free, and devoid of the caveolar-membrane/lipid raft subtype. The lipid raft fractions from BV2 cells were distributed differently in comparison with other cell lines and nonraft fractions were not well separated from lipid rafts fractions. Nonlipid raft and lipid raft fractions in BV-2 cells showed a much closer density (Rimmerman et al., 2008). In addition, Miranzadeh et al. recently reported the enrichment of CB1 receptor in nonlipid rafts fractions in C57BL mouse cortical tissue (Fig. 2) (Miranzadeh Mahabadi et al., 2021). Because mature neurons express less caveolin-1, CB1 receptors association with lipid raft compartments of the cortical cell membrane may be reduced. Caveolin-1, which is required for the formation of caveolae, was reported to modulate cholesterol transport. This may explain apparent differences in lipid raft localization of CB1 receptor between various cell types.

## Plasma membrane localization of CB2 receptor

AEA binding to CB2 receptors modulates two key signaling pathways: adenylyl cyclase (AC) suppression and (Mitogen-activated protein kinases) MAPK activation, both of which are mediated by Gi/o proteins (Howlett et al., 2004). The CB2 receptor in neurons has been mostly localized in the postsynaptic region of the hippocampus (Brusco et al., 2008).

CB2 receptor study in human DAUDI leukemia cells reveals that CB2 receptor unlike CB1 receptor is not changed by perturbation of lipid rafts: (1) binding of [<sup>3</sup>H]CP55.940 to CB2 receptor and kinetic constants of saturation curves of this binding were not changed when MCD applied at a concentration range while these MCD concentrations increased CB1

receptors in glioma cells (Bari, Battista, et al., 2005) and in breast cancer cell line (Sarnataro et al., 2005); (2) AEA-induced stimulation of the binding of GTP $\gamma$ S to DAUDI cells was not changed by pretreatment with MCD; (3) CB2 receptor-mediated signaling via AC and MAPK; (4) MCD treatment had no effect on the antiapoptotic effect of CB2 receptor activation mediated by the MAPK pathway; and (5) lipid raft perturbation by membrane cholesterol enhancement did not affect CB2 receptor. This data confirmed that lipid raft regulates CB1 receptor, but not CB2 receptor and endocannabinoid transport in neuronal and immune cells (Bari et al., 2006).

The CB2 receptor has been found in the nonraft region of the dorsal root ganglion X neuroblastoma cell lines (F11) (Rimmerman et al., 2008). Similar to this finding, Miranzadeh et al. confirmed previous study for CB2 receptor localization that could be recovered in the higher density protein fractions (nonlipid raft) of the sucrose gradient in mouse cortical tissue (Miranzadeh Mahabadi et al., 2021).

## Different distribution of CB1 and CB2 receptor in the plasma membrane

There are discrepancies among reported studies on the PM distribution of CB1 and CB2 receptors (Table 1), requiring a thorough analysis of the lipid environment of the receptors as well as analysis of the three-dimensional structures of the two receptor classes in plasma membrane. Despite the inherent similarity between CB1 and CB2 receptors (40% similarity in amino acid sequence), these receptors are localized in a different cell environment with important consequences on their bioactivity (Ramos et al., 2011). While CB1 receptor seems to distort lipid bilayer regularity, particularly in the extracellular moiety, CB2 receptor has a minimal impact on lipid distribution along the bilayer plane (Ramos et al., 2011).

The lipid rafts contain a variety of components including specific lipids with saturated fatty acid chains, such as sphingomyelin and sphingolipids, as well as cholesterol and proteins such as caveolins (Pike et al., 2002). These compositions are intended to produce more liquid order domains, which can result in, for example, thicker lipid bilayer. This elongation may have the effect of reducing the hydrophobic mismatch between the hydrophobic transmembrane helices package of large proteins and the aliphatic lipid chains of the bilayer, resulting in system stabilization.

CB receptors are mainly formed by seven transmembrane helices (TMH1–TMH7) in which the hydrophobic residues dominate. These hydrophobic residues can interact with the acyl tail of phospholipid molecules, whereas polar residues preferentially interact with the lipid polar heads or water molecules on the membrane surface. The so-called hydrophobic mismatch is a key factor in the interaction of a transmembrane protein and a lipid bilayer. The length difference between the lipid bilayer's nonpolar interior and the hydrophobic  $\alpha$ -helix package can cause distortions in the lipids surrounding the

**TABLE 1** Dissimilar interaction of CB1/CB2 receptor with lipid raft fractions.

Sample	CB receptor localization	Method	Reference(s)
MDA-MB-231 cells	CB1 receptor in lipid raft domains	Confocal analysis, Membrane fractionation	Miranzadeh Mahabadi et al. (2021), Sarnataro et al. (2005, 2006)
C6 glioma cell	CB1 receptor in lipid raft domains	Cholesterol Quantitation and Analysis of Cell Membrane Fluidity, Membrane fractionation, Confocal analysis	Bari, Battista, et al. (2005), Bari et al. (2008), Biswas et al. (2003)
BV2	Nonlipid raft	Membrane fractionation	Rimmerman et al. (2008)
HEK293	CB1 receptor in lipid raft domains	Confocal analysis	Wu et al. (2008)
Human DAUDI leukemia cell	CB2 receptor in lipid raft	Cholesterol quantitation, Analysis of cell membrane fluidity	Bari et al. (2006)
Mouse cortical tissue	CB1 and CB2 receptors in nonlipid raft	Membrane fractionation	Miranzadeh Mahabadi et al. (2021)

A variety of techniques used in different cell lines and tissue to study CB receptor distribution in lipid raft and nonlipid raft compartments of PM.

protein or in the protein itself (de Planque et al., 2001). The geometry study for CB1 and CB2 receptors along with the distribution of phosphorus atoms of the lipid head in two different orientation shows that more disordered structure of P atoms is observed for CB1 while a larger tilt was observed for CB2 receptor. It was also reported that the presence of the CB1 receptor has a greater impact on the bilayer's extracellular moiety. In fact, there is strong coulombic interaction between the charged amino acids and the polar head in the lipid bilayer; therefore, the high number of charged amino acids in the extracellular side of the bilayer might explain more disorder of the bilayer and reduced tilt for the CB1 receptor. This observation is consistent with the experimental finding that CB1 typically associated with lipid rafts, while CB2 receptor can be found in nonraft membrane. It is possible that a lipid raft structure aids in the stabilization of the CB1 receptor in a lipid environment (Ramos et al., 2011).

Moreover, using combined high-resolution nuclear magnetic resonance (NMR) and computer modeling revealed that CB1 and CB2 receptors have conformational properties and salt bridge differences in the so-called juxtamembrane segment (or helix 8), which is essential for their activity and regulation and, more importantly, is influenced by the surrounding chemical environment (Xie & Chen, 2005). As a result, it is tempting to hypothesis that lipid raft compartments could mediate the CB1 receptor by interacting with specific regions of its three-dimensional structure, including helices 3 and 6 (Tian et al., 2005) or helix 8 (Xie & Chen, 2005). Because of the lack of these interactions, CB2 receptor might be insensitive to lipid raft perturbation. The discovery that CB1 and CB2 receptor subtypes are differentially modulated by lipid raft perturbation is a significant finding, which appears to open a new perspective of research for better understanding of the biological function as well as regulation of cannabinoid receptors, and critical for the development of receptor subtype-specific drugs. The other interesting finding of CB1 receptor is related to the cholesterol-binding behavior. Previous data demonstrated that pregnenolone, a derivative of cholesterol, acts as a CB1 allosteric modulator (Hua et al., 2020; Vallée et al., 2014).

The lack of caveolin-1 or reduction of expression may also affect the association of CB1 receptor with lipid raft fractions (Miranzadeh Mahabadi et al., 2021; Rimmerman et al., 2008). Since mature neurons express less caveoline-1, CB1 receptor association with lipid raft compartments in cortical cell membrane may be reduced (Miranzadeh Mahabadi et al., 2021). More studies are needed to investigate the effects of reduced caveolin-1 expression in mature cortical tissue on interaction of the CB1 receptor with cortical tissue lipid rafts.

It has previously been reported that the average densities of membranes from different cells differ, resulting the raft and other membranes, are restored at lower or higher-density gradient fractions (Macdonald & Pike, 2005). This inconsistency in CB1 receptor localization may arise from differences in protein expression, lipid composition in membranes, and experimental methodologies used in different studies (Table 1). These findings call for more research into the plasma membrane compartmental distributions of CB1 and CB2 receptors in various tissue and cell lines.

## Key facts

- Lipid rafts with high concentration of cholesterol are insoluble in cold nonionic detergent.
- CB1 receptor functions in the context of lipid raft and the integrity of these domains may regulate the signaling pathway.
- Lipid raft disruptor alters the binding efficiency of CB1 receptors to endogenous agonist anandamide.
- CB1 receptor localized in nonlipid raft region in the absence of caveolin-1.
- Lipid raft disruption does not affect AEA binding to CB2 receptor.

## Mini-dictionary

- **Lipid raft:** Lipid rafts are functional dynamic microdomains with high concentration of sphingolipids and cholesterol.
- **Caveolae:** Caveolae are 70–100 nm flask-shaped invaginations in the lipid raft that contain caveolins.
- **Detergent resistant membrane:** One biochemical definition of lipid rafts is their insolubility in cold nonionic detergent, which results in detergent-resistant membranes (DRMs).
- **Flotillin:** Flotillins are lipid-raft-associated proteins that function in a variety of cellular context such as signaling, endocytosis, and cytoskeleton interactions.
- **Transferrin receptor:** Transferrin receptor is a glycoprotein in the plasma membrane that has role in mediating cellular uptake of iron from plasma glycoprotein, transferrin.
- **Methyl- $\beta$ -cyclodextrin:** Methyl cyclodextrin is a cholesterol-removing agent that is primarily used to disrupt lipid rafts.

## Summary points

- This chapter reports that CB1 receptors associate with both lipid raft and nonraft compartment of the plasma membrane.
- The presence of Caveolin-1 and protein modification modulate the localization of CB1 receptor in the lipid raft.
- CB2 receptors are localized in nonlipid raft domain of the plasma membrane.
- Lipid raft alteration could be a way to regulate CB1 receptor-dependent signaling.
- Further investigations are required to clarify the details of CB receptor localization in the cell membrane.

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## Chapter 26

# Applications to wound healing: Cannabinoid receptor 1 antagonism and collagen deposition

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>ACEA</b>	arachidonyl-2'-chloroethylamide
<b>AEA</b>	anandamide, N-arachidonylethanolamine
<b>CB</b>	cannabinoid
<b>CB1</b>	cannabinoid 1 receptor
<b>CB2</b>	cannabinoid 2 receptor
<b>CTGF</b>	connective tissue growth factor
<b>ECM</b>	extracellular matrix
<b>ERK</b>	extracellular signal-regulated kinase
<b>FAAH</b>	fatty acid amide hydrolase
<b>GPCRs</b>	G-protein-coupled receptors
<b>GPR55</b>	G-protein-coupled receptor 55
<b>IL</b>	interleukin
<b>NAEs</b>	N-acyl ethanolamines
<b>OEA</b>	oleoylethanolamide
<b>PDGF</b>	platelet-derived growth factor
<b>PEA</b>	palmitoylethanolamide
<b>PPAR</b>	peroxisome proliferator-activated receptor
<b>PPAR-<math>\alpha</math></b>	peroxisome proliferator-activated receptor alpha
<b>PPAR-<math>\gamma</math></b>	peroxisome proliferator-activated receptor gamma
<b>SSc</b>	systemic sclerosis
<b>TGF-<math>\beta</math></b>	transforming growth factor beta
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor alfa
<b>TRPV</b>	transient receptor potential cation channel subfamily vanilloid member
<b>TSK-1</b>	tight skin 1 mouse model
<b>VEGF</b>	vascular endothelial growth factor

## Introduction

### Wound healing

Wound healing is an unspecific form of tissue repair that occurs in tissues of complex multicellular organisms, after disruption of a normal anatomic structure. This form of healing occurs with the formation of fibrosis and scar and represents the main form of human skin repair (Reinke & Sorg, 2012).

Although unspecific, wound healing is a highly regulated physiological process that begins immediately after wounding and lasts for up to a year. It has conceptually been divided into three subsequent and overlapping phases: hemostasis/inflammation, proliferation, and remodeling phases (Schilling, 1976). Each phase involves different cells, growth factors, and cytokines and implicates different cellular, humoral, and molecular mechanisms (Broughton et al., 2006; P. H. Wang et al., 2018; Werner & Grose, 2003). A detailed description of this process is beyond the scope of this chapter, but a short overview of each phase is described and represented in Fig. 1.

*Hemostasis/Inflammation Phase:* This stage begins immediately after injury and lasts up to 4–6 days. After the injury, the sub-endothelium is exposed, and actions, such as vasoconstriction, platelet aggregation, and activation of the intrinsic and extrinsic pathways of blood coagulation, are taken to control bleeding. Activated platelets release cytokines and growth factors (interleukin (IL)-1, IL-6, tumor necrosis factor alfa (TNF- $\alpha$ ), and transforming grown factor-beta (TGF- $\beta$ )) that initiate the inflammatory response. Neutrophils are immediately recruited and their concentration peaks at 24 h. Neutrophils produce a wide variety of proteases, metalloproteinases, and reactive oxygen species and are involved in cell debris phagocytosis. At 48–96 h post-injury, monocytes enter the wound site and differentiate into macrophages to maintain the ongoing process by performing phagocytosis of pathogens and cell debris. Macrophages acquire an inflammatory phenotype (M1) upon exposure to pro-inflammatory cytokines and secret cytokines that will mobilize immune cells (IL-1, IL-6 and TNF- $\alpha$ ). Macrophages have a pivotal role in the transition from the inflammatory to the proliferative phase of wound healing.

*Proliferative phase:* This phase lasts approximately from day 4 till day 14. It is characterized by epithelialization, angiogenesis, and the formation of a provisional matrix. It begins with the migration and proliferation of keratinocytes and fibroblasts from wound neighborhood and epithelial stem cells from hair follicles or sweat glands. In response to TGF- $\beta$ , fibroblasts are activated into myofibroblasts. Myofibroblasts are responsible for wound contraction and extracellular matrix (ECM) deposition. ECM is composed mainly of collagen, glycosaminoglycan, and fibronectin. At the same time, endothelial cells located at intact venules are induced by vascular endothelial growth factor (VEGF) to form new capillary tubs. The resulting wound connecting tissue is known as granulation tissue.

*Remodeling phase:* This final phase lasts from day 8 till up to 1 year after injury. During the remodeling phase, collagen III, produced in the proliferative phase, is replaced by collagen I. This type of collagen is aligned in small parallel bundles, different from the basket weave collagen in the uninjured dermis. Simultaneously, myofibroblasts contract and decrease the surface of the developing scar, and angiogenesis diminishes.

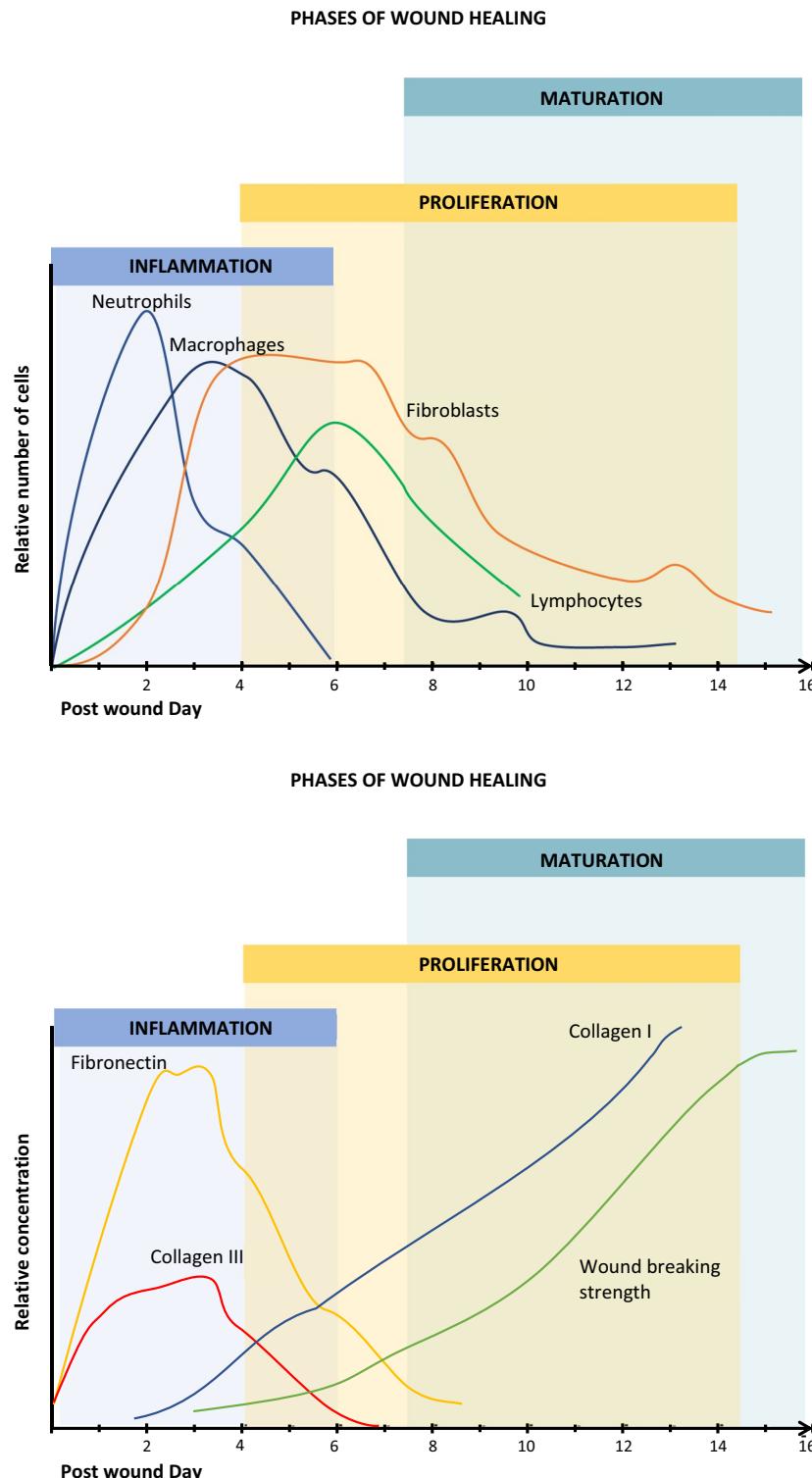
In some instances, wound healing disruption occurs, and healing can be compromised with the persistence of chronic wounds. On other occasions, an exuberant healing response can occur, leading to hypertrophic or keloid scars formation (Reinke & Sorg, 2012). Commonly, an increased macrophage infiltration and amplified inflammatory response at the wound site will disturb wound healing. This shift may prevent the transition from the inflammatory to the proliferative phase, leading to a chronic wound (Larouche et al., 2018). There are also evidences that the underlying mechanism that leads to excessive fibrosis in hypertrophic scars and keloids in a prolonged or excessive inflammatory phase. Thus, pathophysiological, increased inflammation or a prolonged inflammatory phase seems to be, at least in part, behind both pathological conditions. Histologically, however, hypertrophic scars and keloids are characterized by an overabundant accumulation of ECM components, especially collagen (Sephel & Woodward, 2001).

Treatment of both pathological forms of wound healing is of the main interest of clinics and a subject of extensive investigation in the scientific field. Both of these conditions represent an important biological, psychological, and financial burden for the patients and the health system. Besides the obvious cosmetic impact, those wounds are responsible for functional impairment, disfigurement, subjective symptoms such as pruritus, pain, and tenderness, and a decrease in quality of life, physical status, and psychological health (Chiang et al., 2016). Current treatment of these conditions is often unsuccessful, and recurrent rates of unhealing wounds or hypertrophic or keloid scars after treatment are high (Andrews et al., 2016).

A better understanding of the cellular and molecular mechanisms underlying wound healing will allow us to enhance or attenuate the wound repair/regeneration process and eventually identify new molecular drug treatments for these conditions.

## The endocannabinoid system

The endocannabinoid system has been identified in the early 1990s. This system comprises the cannabinoid 1 receptor (CB1) and cannabinoid 2 receptor (CB2), their endogenous ligands, and the enzymes responsible for their metabolism (Howlett, 2005; Howlett et al., 2002). A detailed description of endocannabinoids, their receptors, and pathways of synthesis and degradation can be found in other chapters of this book.



**FIG. 1** Main cellular mediators (A) and wound matrix components (B) over time, during inflammatory, proliferative and remodeling/maturating phases of wound healing. Macrophages and neutrophils are the predominant cells during inflammation, whereas lymphocytes peak later and fibroblasts are the predominant cells during the proliferative phase. Fibronectin and collagen type III constitute the early matrix and collagen type I accumulates later, matching with the increase in wound-breaking strength. (*Adapted from Broughton, G., et al. The basic science of wound healing. Plast Reconstr Surg. 2006;117(7 Suppl):12S–34S.*)

Recently, the endocannabinoid system and its components have been identified in the skin. It has been shown that it is responsible to maintain skin homeostasis processes, such as proliferation, differentiation and apoptosis, barrier formation, regeneration, and release of inflammatory mediators (Sheriff et al., 2019). Dysregulation of this delicate balance was implicated in several highly prevalent skin diseases and disorders such as systemic sclerosis, acne, seborrheic dermatitis, allergic dermatitis, psoriasis and skin tumors as melanoma, and non-melanoma skin cancers (Biro et al., 2009; Eagleston et al., 2018; Milano & Friedman, 2019; Toth et al., 2019). Gathering these data, some authors have proposed a new “C(*ut*)annabinnoid” System (Toth et al., 2019).

In the past, the endocannabinoid system was proven to have a role in fibrotic diseases in different organs such as the liver (Julien et al., 2005), heart (Rajesh et al., 2010), or pancreas (Michalski et al., 2008). In 2009, this system was implicated, for the first time, in the formation of skin fibrosis (Akhetshina et al., 2009). Till now, both CB1 and CB2 receptors, as well as cannabinoid-related receptors, such as adenosine receptors and peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ), have been implicated in wound healing and skin fibrosis formation (I. Correia-Sá, Paiva, et al., 2020). This chapter focuses on the role of the CB1 receptor on skin fibrosis and possible applications in wound healing.

### CB1 receptor and skin wound healing

There are only a few studies published in the literature relating CB1 receptor in skin fibrosis (Marquart et al., 2010; Palumbo-Zerr et al., 2012; Zhao et al., 2010). We have compiled a summary of the available information in Table 1.

In 2010, Marquart et al. (Marquart et al., 2010) reported that CB1 $^{−/−}$  knockout mice were less sensitive to bleomycin-induced skin fibrosis and showed reduced dermal thickness, leucocyte and macrophages infiltration, hydroxyproline content, and myofibroblast count, than CB1 $^{+/+}$  mice. The opposite effect was observed when the CB1 receptor was activated with Arachidonyl-2'-chloroethylamide (ACEA), a highly selective cannabinoid CB1 receptor agonist. Instead, CB1 $^{−/−}$  knockout tight-skin-1 (TSK-1) mice were not protected from fibrosis. While bleomycin local injections are used

**TABLE 1** Published studies regarding CB1 receptor and skin fibrosis.

Study	Experimental model	CB1 receptor activation	Outcome of CB1 receptor activation	CB1 receptor inactivation	Outcome of CB1 receptor inactivation
Marquart et al. (Marquart et al., 2010)	Mice CB1 $^{+/+}$ CB1 $^{−/−}$ TSK-1	ACEA	↑ fibrosis	CB1 $^{−/−}$	↓ fibrosis ↓ inflammation
Zhao et al. (Zhao et al., 2010)	Mice	–	CBr1 is time-dependently expressed during skin wound healing	–	–
Palumbo-Zerr et al. (Palumbo-Zerr et al., 2012)	Mice FAAH deficient mice SSc human fibroblasts Human fibroblasts	JNJ1661010 (FAAH inhibitor)	↑ fibrosis	AM281	↓ fibrosis
Correia-Sá et al. I. B. Correia-Sá et al., 2021)	Primary cultures of human fibroblasts  Human ex vivo skin culture	ACEA	↑ collagen	AM281	↓ fibrosis
Ruhl et al. (Ruhl et al., 2021)	Mice Wildtype CB1 $^{−/−}$	–	–	CB1 $^{−/−}$	↓ wound healing ↑ inflammation

SSc, Systemic sclerosis; CB, Cannabinoid; CB1, Cannabinoid 1 receptor; FAAH, Fatty acid amide hydrolase; ACEA, arachidonyl-2'-chloroethylamide; TSK-1, Tight skin 1 mouse model.

to produce dermal fibrosis, in an animal model of Systemic Sclerosis (SSc), TSK-1 mice are an animal model for later SSc stages. In TSK-1 mice, inflammatory infiltrates are absent, and the increased release of collagen by fibroblasts is caused by endogenous activation (Green et al., 1976). The authors proposed that CB1 receptor effects on skin fibrosis were due to an inflammatory response, rather than an effect of fibroblast activity itself.

In the same year, the CB1 receptor has shown to be time-dependently expressed during skin wound healing in both fibroblasts and immune cells in mice (Zhao et al., 2010).

Palumbo-Zerr et al. (Palumbo-Zerr et al., 2012) later reported that pharmacological or genetic inactivation of fatty acid amide hydrolase (FAAH) significantly aggravated bleomycin-induced fibrosis in mice. Consistent with previous results, the authors showed that blocking the CB1 receptor with AM281, a CB1 selective antagonist, completely abolished the profibrotic effects of FAAH inhibition. In systemic sclerosis human fibroblast, FAAH expression was also reduced. The authors considered that the CB1 receptor was the dominant receptor for endocannabinoid experimental fibrosis and that it would be a promising candidate for targeted treatments in fibrotic skin diseases.

Simultaneously with these reports, Garcia-Gonzalez et al. (Garcia-Gonzalez et al., 2009) and Balistreri et al. (Balistreri et al., 2011) showed in *in vitro* and *in vivo* experimental studies, that WIN55,212-2, a cannabinoid non-selective agonist, reduced fibroblast transdifferentiation into myofibroblasts and ECM deposition in a non-dependent CB1 or CB2 response. It was reported, for the first time, that synthetic cannabinoids could directly influence fibroblast activity, rather than only having an effect on the inflammatory response. It was also shown that WIN55,212-2 strongly inhibited the expression of transforming growth factor-beta (TGF- $\beta$ ), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF) and downregulated SMAD2/3 (Balistreri et al., 2011) and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (Garcia-Gonzalez et al., 2009) in cultured fibroblasts.

Recently, our group has demonstrated that the CB1 receptor is involved in fibrogenesis in humans (I. B. Correia-Sa et al., 2021). More specifically, we have shown that ACEA, a CB1 selective receptor agonist, increased collagen production in a primary culture of adult human fibroblasts and that AM251, a CB1 selective receptor antagonist, inhibited fibroblast differentiation into myofibroblast and collagen deposition induced by TGF- $\beta$ . AM251 inhibitory effect on human fibroblast collagen production was shown to be drug concentration-dependent, with a significant effect at 1  $\mu$ M and reaching a maximum at 10  $\mu$ M with an IC50 of 2.9  $\mu$ M. TGF- $\beta$  is a well-studied fibrotic cytokine (Brunner & Blakytny, 2004) and a crucial regulator of fibroblast phenotype and function. It is responsible for fibroblast chemotaxis and proliferation, myofibroblast differentiation, and collagen (Lawrence & Diegelmann, 1994; Sporn et al., 1986), fibronectin and proteoglycan synthesis (Bassols & Massague, 1988; Ignotz et al., 1987). TGF- $\beta$  can also organize the ECM and is involved in scar remodeling and wound contraction (Fukamizu & Grinnell, 1990). Interestingly, we found that this cytokine, when added to primary cultures of human fibroblast, significantly increased the expression of both CB1 and CB2 receptors.

Furthermore, we tested these compounds in a human *ex vivo* skin culture model (Xu et al., 2012). Although descriptive, this technique allowed us to confirm our results in a more complex model of human wound healing. A full-thickness human skin graft was wounded and cultured in the liquid-air interphase for 9 days. ACEA-treated skin grafts were totally re-epithelialized after the experiments, whereas AM251-treated skin grafts still presented a disruption of the epithelium. This not only confirms our results but also points out that further preclinical and clinical studies should be conducted, regarding the role of CB1 ligands on human skin wound healing (I. B. Correia-Sa et al., 2021).

Recently, Ruhl et al. (Ruhl et al., 2021) also reported delayed wound healing, in CB1 $-/-$  knockout mice, submitted full-thickness cutaneous excisional wounding. It was found that, in this genotype, wound closure was delayed during early phases of healing—between day 1 and day 3—but a similar subsequent time course of wound closure was observed when compared with wild-type or CB2 $-/-$  knockout mice. The experiment ran for 14 days and, in the end, however, CB1 $-/-$  knockout mice were not completely healed. This healing delay was accompanied by elevated tissue levels of IL-6, monocyte chemoattractant protein (MCP)-1, and TNF- $\alpha$ . Moreover, CB1-deficient mesenchymal stromal cells released high levels of MCP-1 upon stimulation with TNF- $\alpha$  and IL-1 $\beta$ . MCP-1 is a chemotactic cytokine that attracts monocytes, macrophages, and further immune cells (Gschwandtner et al., 2019). The authors proposed that CB1 receptor inactivation increases MCP-1 concentration in wounds and that this leads to an amplified and delayed inflammatory phase of wound healing, reinforcing the importance of this receptor in the early phases of wound repair. These findings diverged from those of Marquart et al. (Marquart et al., 2010). This author observed less inflammatory cellular infiltration in CB1 $-/-$  knockout mice, whereas Ruhl et al. (Ruhl et al., 2021) proposed an increased inflammatory phase in this mice genotype. However, both authors proposed that CB1 genetic inactivation reduces fibrosis in these animals. Different experimental models (bleomycin-induced fibrotic skin disease vs wound healing model) and different time set observations (delayed vs early) may explain these differences. Ruhl et al. (Ruhl et al., 2021) results are consistent with those found in our human *ex vivo* skin culture model experiments (I. B. Correia-Sa et al., 2021). CB1 receptor inactivation, either genetic or pharmacological, in early phases of wound healing, seems to delay wound healing in both experimental models.

### Cannabinoids during skin wound healing in humans

Our group also investigated the endocannabinoid system in plasma, in skin and scars during wound healing in humans, in a prospective study involving patients subjected to body counteracting surgery (I. B. Correia-Sa, Carvalho, et al., 2020). In this study, anandamide (N-arachidonylethanolamine, AEA), 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA), and oleoylethanolamide (OEA) were quantified in human skin and scars of patients subjected to surgery. Those endocannabinoids and N-acyl ethanolamines (NAEs) were also quantified on the human plasma of the same patients, before and at different times after surgery. A positive correlation was found between plasma and skin AEA concentrations in patients who later developed normal scars, but this correlation was absent in patients with hypertrophic scars. Also, the AEA concentration was significantly lower in hypertrophic scars than in normal scar tissue. This finding supports the hypothesis of a role for endocannabinoids, essentially AEA, in the pathophysiological process of skin fibrosis. The authors proposed a new role for AEA with cross talk between systemic and local skin endocannabinoid systems during human wound healing. Endocannabinoids are generally considered to be anti-inflammatory agents (Turcotte et al., 2015). More specifically, AEA was shown to suppress the production and release of key Th1- and Th17-polarizing cytokines (IL-12 and IL-23) via CB1-mediated inhibition of the mammalian target of rapamycin (mTOR) in human keratinocytes (Chiarchiu et al., 2016). Hypertrophic scars and keloids, on the other hand, are believed to occur in an increased and prolonged inflammatory environment (Castagnoli et al., 1997; Niessen et al., 2004). It was suggested that reduced AEA levels in hypertrophic scars may be related to increased inflammation or a prolonged inflammatory phase. More studies are needed to confirm this hypothesis, but, if corroborated, topical administration of AEA, other non-psychotropic cannabinoids or FAAH inhibitors, could be an interesting tool to treat or prevent hypertrophic scars or keloids, possibly taking advantage of a dual AEA action: anti-inflammatory and anti-fibrotic.

It was reported, by three patients suffering from epidermolysis bullosa, faster wound healing, less blistering, and improvement of pain after self-administration of topical cannabidiol oil (Chelliah et al., 2018).

### Conclusions

This chapter highlights the CB1 receptor as an interesting target in the treatment of wound healing diseases, such as chronic wounds and hypertrophic or keloid scars, and systemic fibrotic conditions such as SSc. Available data in this setting are still very limited, but it has clearly been shown that CB1 receptor has a role in both fibroblast activity and inflammatory response during wound healing, in mice and human cellular cultures. Shortly, CB1 receptor inactivation reduces fibroblast activation and collagen deposition in human fibroblasts. In animal models, CB1 receptor inactivation reduces dermal thickness, skin fibrosis, and delays wound healing. Data regarding the role of the CB1 receptor on inflammation are conflicting, but this may be explained by different experimental models and different observation time sets.

CB1 receptor inactivation seems to be a promising tool to treat skin fibrotic disease. AM281 showed to reduce fibroblast differentiation and collagen deposition in a concentration-dependent manner, with no cytotoxic effects on fibroblasts. More clarification, regarding its role in delayed wound healing, should be done. The timing seems to be essential in this setting, as a premature application of these drugs may delay healing. On the other hand, CB1 activation may hypothetically also have a role in the treatment of chronic wounds, although investigation in this setting is even more scarce.

Although promising, further preclinical and clinical studies should be conducted to verify the efficiency, security, and tolerance of those compounds in the treatment of these pathological disorders. Topical skin administration will possibly increase tolerance of cannabinoid compounds in this setting.

### Applications to other areas

In this chapter, we have reviewed the effects of CB1 receptor inactivation on skin fibrosis and cellular models of wound healing. We have shown that CB1 receptor activation increases skin fibrosis in animal models of SSc and collagen deposition in cellular models of wound healing. On the other hand, CB1 receptor inactivation has the opposite effect. It decreases skin fibrosis in animal models of fibrosis and decreases collagen deposition and fibroblast activation on TGF- $\beta$  stimulated human fibroblasts.

It has been shown that CB2 receptor activation reduces inflammation and skin fibrosis in experimental models of wound healing and SSc (I. Correia-Sa et al., 2022; Du et al., 2018; Li et al., 2016; Servettaz et al., 2010; L. L. Wang et al., 2016) and increases keratinocyte proliferation, migration, and re-epithelialization (L. L. Wang et al., 2016). Taken together these data suggest that CB2 agonists might also be a novel premise for skin wound therapy.

Hypothetically, this dual effect (increasing keratinocyte migration and reduction of fibroblast activity) could be appropriate to achieve scarless healing.

Other cannabinoid-related receptors have also been involved in wound healing. Adenosine A2A receptor activation increases fibroblast activity, either directly or through a cross talk with the cannabinoid system and the CB1 receptor (Lazzerini et al., 2012). PPAR- $\gamma$  activation, on the other hand, has shown an anti-fibrotic effect in animal and cellular models of skin wound healing (Del Rio et al., 2018, 2016; Garcia-Martin et al., 2019, 2018; Gonzalez et al., 2012).

It was proposed that the endocannabinoid system may be a target to treat or prevent wound healing disorders. We speculate that a multi-target approach, involving CB1, CB2, A2A, or PPAR- $\gamma$ , may be an interesting strategy in this setting. Randomized clinical trials with cannabinoid drugs administration for treatment of various dermatological disorders are already being conducted (Milando & Friedman, 2019; Toth et al., 2019), and a few compounds based on THC, such as Marinol®, Cesamet®, and Sativex®, have already been FDA-approved with restricted indications (Eagleston et al., 2018). Topical administration on the skin of cannabinoids is associated with high tolerance rates and low psychotropic or other side effects (Fraguas-Sanchez & Torres-Suarez, 2018; Grotenhermen & Muller-Vahl, 2012; Pergolizzi et al., 2018). This will certainly allow other preclinical and clinical studies on the use of cannabinoids in the treatment of wound healing and fibrotic diseases.

## Mini-dictionary of terms

**Anandamide:** also known as *N*-arachidonoyl ethanolamine (AEA), is a fatty acid neurotransmitter, and the first endocannabinoid discovered. AEA behaves as a partial agonist for CB1 receptors and exhibits less relative intrinsic activity and affinity for CB2 than CB1 receptors. It also binds and activates non-CB1 and non-CB2 receptors and channels, as the G-protein-coupled receptor GRP55, 5-HT3, opioid receptors, the transient receptor potential cation channel subfamily vanilloid member (TRPV) 4, the TRPV1, and several subtypes of the peroxisome proliferator-activated receptor (PPAR) family. AEA is not stored in vesicles but rather synthesized on-demand, in response to increased concentration of intracellular calcium. It acts mainly as a presynaptic retrograde messenger, with inhibitory activity over the release of other neurotransmitters, preventing the development of excessive neuronal activity in the central nervous system. AEA is then cleared by a process of cellular intake and enzymatic inactivation.

**CB1 receptor:** cannabinoid 1 receptor is a member of the superfamily of G-protein-coupled receptors (GPCRs). It signals through Gi/o proteins (Gi1,2 and 3, and Go1 and 2), inhibiting adenylyl cyclase and activating mitogen-activated protein. CB1 receptors are mainly found in terminals of central and peripheral neurons, where they usually inhibit the release of excitatory and inhibitory neurotransmitters. They are commonly located in central nervous areas responsible for cognition and memory, control of motor function, and analgesia. CB1 receptors are now also known to be present in several peripheral tissues as in the immune system, adipocytes, liver, pancreas, skeletal muscle, and skin.

**FAAH:** fatty acid amide hydrolase (FAAH) is a serine hydrolase with a prominent role in the hydrolysis of endocannabinoids. AEA and other NAEs are catabolized by hydrolysis of the amide bond through the actions of this enzyme.

**N-acylethanolamines (NAEs):** are a group of fatty acid derivatives, which act, in grand part, as agonists of cannabinoid receptors. Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are NAEs that act by influencing AEA metabolism and binding to peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) and to TRPV1.

**Wound healing:** an unspecific form of tissue repair that occurs after disruption of a normal anatomic structure or wounding. It occurs with the formation of fibrosis and scar and represents the main form of human skin repair.

## Key facts of wound healing

- The collagen organization and tensile strength of a scar will never be the same found in uninjured skin: at 1 week, the wound only has 3% of its final strength; at 3 weeks, it is 30%; and at 3 months, it is around 80%.
- Epidermal appendages such as hair follicles or sweat glands have no potential to heal or regrow.
- Excessive scarring was first described in the Smith papyrus about 1700 BC.
- Keloid scars occur in ~15%–20% of individuals of African, Hispanic, and Asian ancestry, are less common in Caucasians, and there are no reported cases in patients with albinism.
- Hypertrophic scars occur in 40%–70% of patients following surgery and up to 91% of patients after burn injury.

## Key facts of cannabinoids effect on wound healing

- Chelliah, Zinn, et al. (Chelliah et al., 2018) reported that three patients suffering from epidermolysis bullosa described faster wound healing following self-administration of cannabidiol.
- CB1 receptor inactivation is associated with an improvement of hepatic fibrosis and steatosis.
- Randomized clinical trials with cannabinoid drugs administration for the treatment of various dermatological disorders are already being conducted.
- In the past, rimonabant, a CB1 selective antagonist, was already approved to treat obesity and metabolic syndrome.
- Due to substantial psychiatric adverse effects, rimonabant was later withdrawn from the market.

## Summary points

- CB1 receptor inactivation reduces skin fibrosis in mice.
- CB1 receptor inactivation reduces fibroblast activation into myofibroblasts and collagen deposition in human fibroblasts cultures.
- CB1 receptor has shown to be time-dependently expressed during skin wound healing in both fibroblasts and immune cells in mice.
- CB1<sup>-/-</sup> knockout mice receptors have delayed early wound healing.
- Endocannabinoids are generally considered anti-inflammatory.
- AEA concentration was found to be significantly lower in hypertrophic scars than in normal scar tissue.
- A positive correlation was found between plasma and skin AEA concentrations in patients who later developed normal scars, but this correlation was absent in patients with hypertrophic scars.

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## Chapter 27

# The role of transient receptor potential vanilloid 1 (TRPV1), a modulator of the endocannabinoid system in anxiety, depression, and cocaine addiction

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## Abbreviations

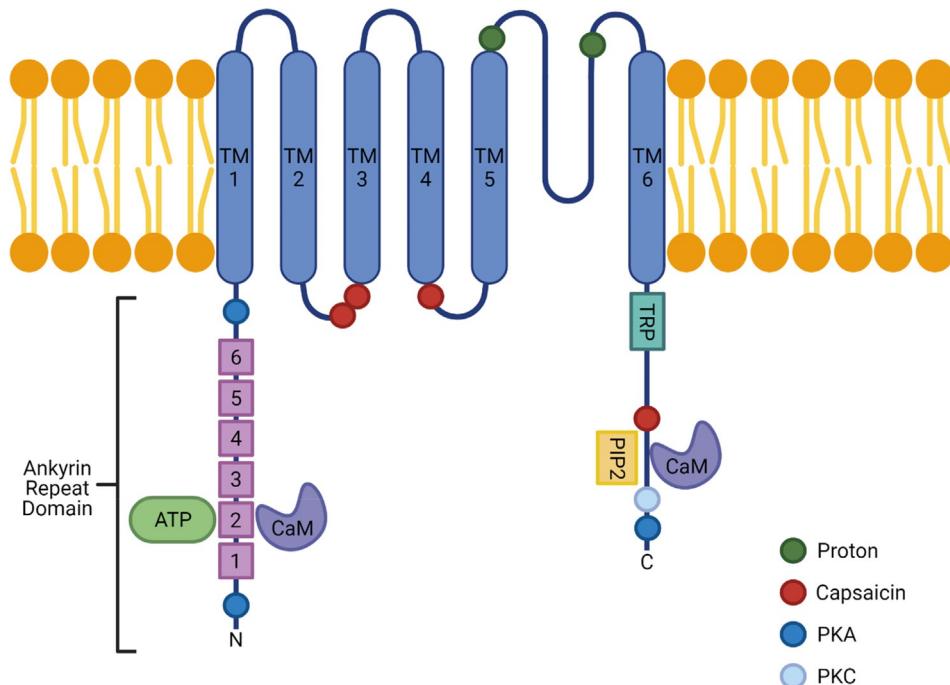
AC	adenylyl cyclase
ACTH	adrenocorticotrophic hormone
AEA	anandamide
2-AG	arachidonoylglycerol
ATP	adenosine triphosphate
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CaN	calcineurin
CamKII	Ca <sup>2+</sup> /calmodulin-dependent kinase II
CB1R	cannabinoid receptors 1
CNS	central nervous system
CRH	corticotrophin-releasing hormone
DAG	diacylglycerol
ER	endoplasmic reticulum
FAAH	fatty acid amide hydrolase
GPCRs	G-protein-coupled receptors
GABA	gamma-aminobutyric acid
HPA	hypothalamic-pituitary-adrenocortical
HPC	hippocampus
IP <sub>3</sub>	inositol 1,4,5-trisphosphate
MAGL	monoacylglycerol lipase
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
NAPE	N-arachidonoyl phosphatidylethanolamine
NAPE-PLD	NAPE-phospholipase D
NAT	N-acyltransferase
NGF	nerve growth factor
PKA	protein kinase A
PKC	protein kinase C
PIP <sub>2</sub>	phosphoinositide 4,5-bisphosphate
PLC	phospholipase C
PVN	paraventricular nucleus

SN	substantia nigra
TM	transmembrane
TrkA	tyrosine kinase A
TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid

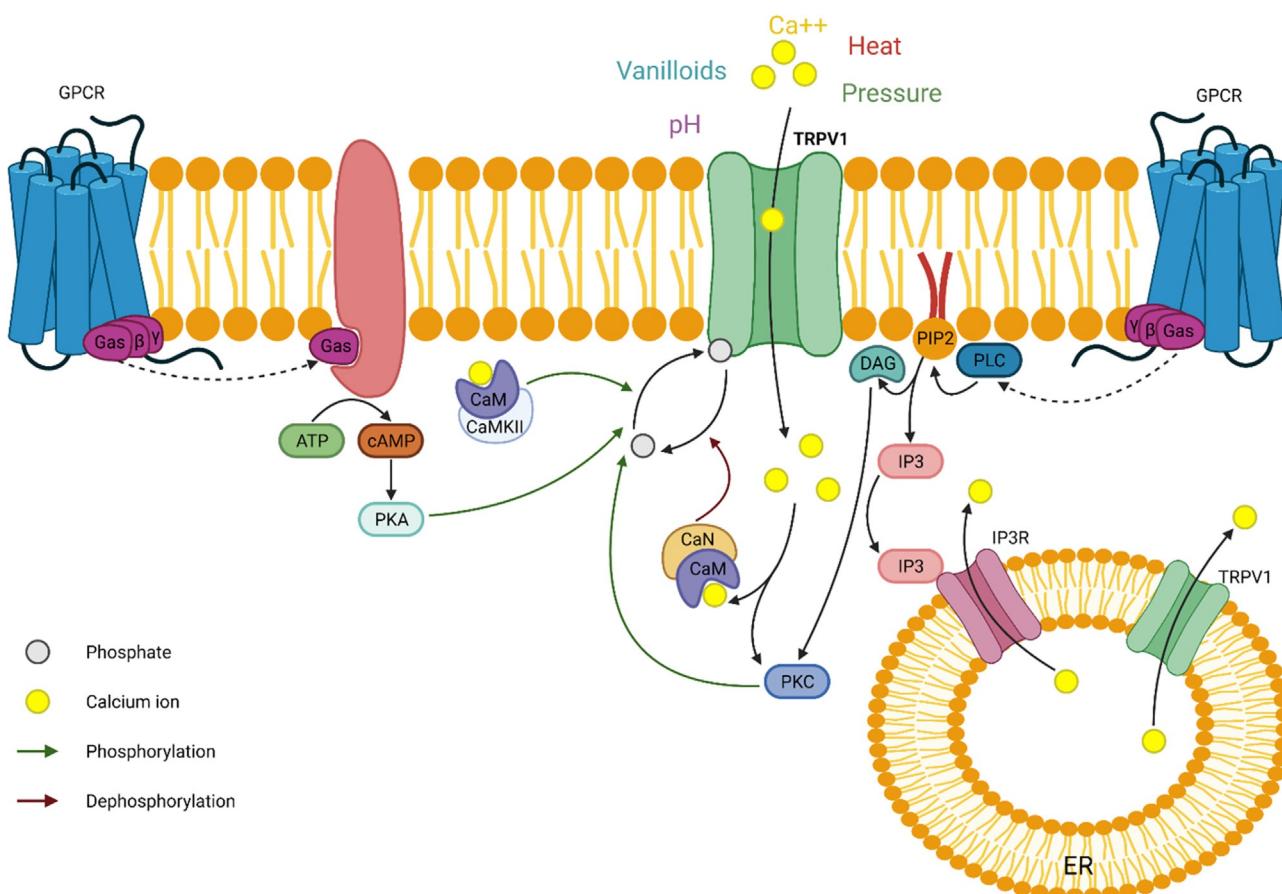
## Introduction

The transient receptor potential (TRP) is a superfamily of non-selective cation channels divided into seven families: canonical (TRPC1–7), no mechanoreceptor potential C (TRPN), melastatin (TRPM1–8), mucolipin (TRPP1–3), polycystin (TRPP1–3), ankyrin (TRPA1), and vanilloid (TRPV1–6), which exist in several species throughout the animal kingdom (Clapham, 2007). All TRP channels are tetramers with high calcium permeability, formed by subunits with six transmembrane domains and selective cation pores (Latorre et al., 2009). The vanilloid family is the best studied of the TRP superfamily, particularly TRPV1, due to its role in nociception (Ho et al., 2012). TRPV1 shows a compact transmembrane region and a large intracellular basket formed by six transmembrane domains, a structural feature common to all TRP and intracellular N- and C-terminal (Chung et al., 2008). The N-terminal tail contains numerous phosphorylation sites and ankyrin repeats that bind calmodulin and ATP (Szallasi et al., 1999). At the bottom of the C-terminal, there is a TRP domain and binding sites for calmodulin and PIP2 (Sanz et al., 2004). TRPV1 antagonist, such as capsaicin, a lipophilic compound, readily crosses the membrane to bind various sites to TRPV1 (Jung et al., 1999) (Fig. 1).

TRPV1 is a polymodal cation channel initially identified as a receptor for capsaicin that can be activated endogenously by various harmful stimuli, such as high voltage, noxious heat ( $>42^{\circ}\text{C}$ ), pressure, low pH, and interact with other receptors, including G-protein-coupled receptors (GPCRs), which contributes to its polymodal nature (Ho et al., 2012) (Fig. 2). The endocannabinoids, including anandamide and N-arachidonoyl dopamine, can activate TRPV1 and the cannabinoid receptors 1 (CB1R), suggesting the importance of cross-talk mechanisms between these receptors (Szallasi et al., 1999;



**FIG. 1** Molecular structure of the TRPV1. TRPV1 contains six transmembrane domains with a pore region between the fifth and sixth domains and long intracellular N- and C-terminal tails. In the N-terminal tail, six ankyrin repeat domains enable the binding of calmodulin and ATP to modulate TRPV1 activation. The C-terminus contains a TRP domain and binding sites for PIP2 and Calmodulin. Several phosphorylation sites exist for PKC, PKA, and CaMKII throughout TRPV1. *TRPV1*, transient receptor potential vanilloid 1; *aa*, amino acid; *TM*, transmembrane; *PIP2*, phosphoinositide 4,5-bisphosphate; *CaM*, calmodulin; *ATP*, adenosine triphosphate; *PKC*, protein kinase C; *PKA*, protein kinase A; *CaMKII*, Ca<sup>2+</sup>/calmodulin-dependent kinase II.



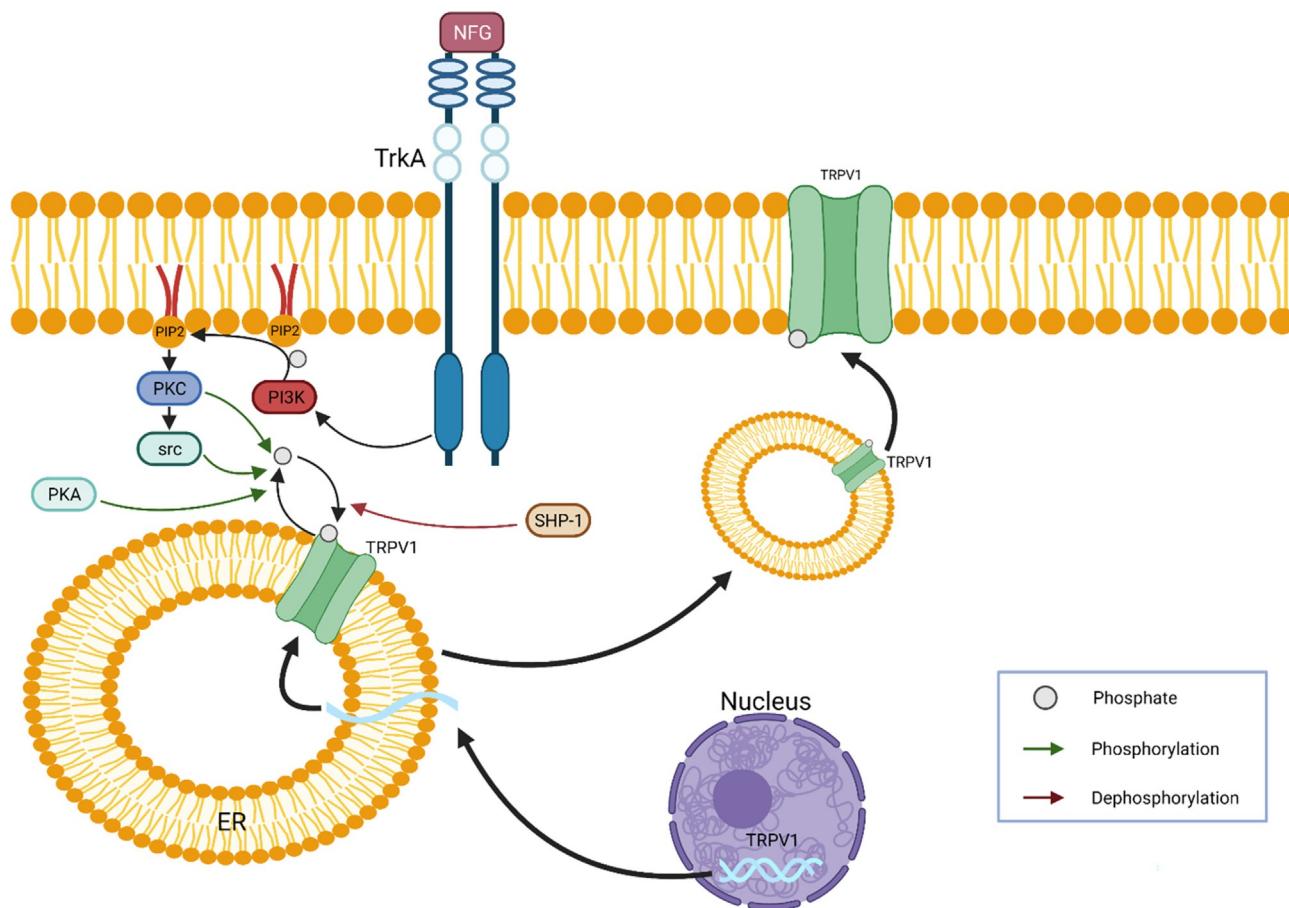
**FIG. 2** Diagram of TRPV1 and its activators. TRPV1 is a polymodal cation channel activated by various harmful stimuli, such as pH, heat, pressure, and vanilloids. When GPCR is activated, it can directly lead to the recruitment of PKC and PKA by phospholipase C and adenylyl cyclase to phosphorylate TRPV1 and sensitize the channel. An increase in intracellular calcium from TRPV1 and GPCR stimulation can activate calcineurin and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) through calmodulin to modulate TRPV1. GPCR, G-protein-coupled receptors; cAMP, cyclic AMP; AC, adenylyl cyclase; CaN, calcineurin; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; PIP2, phosphoinositide 4,5-bisphosphate; PLC, phospholipase C; ER, endoplasmic reticulum.

Szallasi & Di Marzo, 2000). Furthermore, phosphorylation is indispensable for modulating TRPV1, allowing rapid responses to external stimuli or environmental changes. In general, phosphorylation sensitizes, while dephosphorylation desensitizes the channel (Varga et al., 2006).

TRPV1 receptors are also observed in the endoplasmic reticulum and mobilize calcium from intracellular stores (Liu et al., 2003). The activation of signal pathways can translocate TRPV1 from intracellular compartments to the membrane, usually through phosphorylation; PKC activation, for example, can lead TRPV1 to the membrane through SNARE-mediated exocytosis (Morenilla-Palao et al., 2004). The cAMP-dependent activation of PKA can rapidly translocate monomer TRPV1 from intracellular compartments to the plasma membrane. Furthermore, the stimulation of tyrosine kinase Src by nerve growth factors (NGF) can phosphorylate TRPV1 to increase TRPV1 membrane levels (Vetter et al., 2008) (Fig. 3).

## TRPV1 expression within the central nervous system

The presence of TRPV1 within the central nervous system (CNS) was proposed in various studies using different species and several techniques, such as immunohistochemistry (IHC), in situ hybridization (ISH), PCR, (RTPCR) (Mezey et al., 2000), and TRPV1-specific radioactive ligand [<sup>3</sup>H]-resiniferatoxin (RTX) (Roberts et al., 2004). However, some contradictory results reported no TRPV1 expression within the CNS (Caterina et al., 1997). These inconsistent findings have raised questions about the reliability of the methods used to detect TRPV1. The discrepancies regarding TRPV1 expression may be due to different reasons. One explanation is the use of different background strains in previous studies. Another possibility is the increase of TRPV1 compensatory effects during development, leading to age-dependent phenotype



**FIG. 3** Interaction of TRPV1 with other pathways. TrkA stimulation by NGF, for example, can cause src-mediated phosphorylation of TRPV1 and then transport it from the endoplasmic reticulum to the plasma membrane. PKA and PKC-mediated phosphorylation can also increase the translocation of TRPV1 to the membrane. However, dephosphorylation by SHP-1 can inhibit translocation. *NGF*, nerve growth factor; *PIP<sub>3</sub>*, phosphatidylinositol 3,4,5-trisphosphate; *PI3K*, phosphoinositide 3 kinases.

differences, particularly in some TRPV1 isoforms/variables (O'Sullivan et al., 2006). TRPV1 variants and their differential distribution in the CNS complicate the interpretation of results and the conclusion of several expression studies (Sanchez & Krause, 2001). Furthermore, TRPV1 expression levels within the CNS appear to be 20–30 times smaller than the peripheral nervous system, which, along with the limitations of traditional approaches to determine gene expression, including variable sensitivity, lack of specificity, and poor signal, can also be responsible for the inconsistencies in TRPV1 expression within the CNS (Han et al., 2013).

A genetically modified TRPV1 reporter mouse and several complementary strategies, such as *in situ* hybridization, RT-PCR, calcium imaging, and electrophysiological slices, were used to confirm the expression of TRPV1 within the CNS (Cavanaugh et al., 2011). Previous study confirmed the expression of functional TRPV1 in primary afferent neurons. The study showed low TRPV1 expression within the hypothalamus, hippocampus, olfactory bulb, raphe nucleus, and periaqueductal gray (Martins et al., 2014). This expression profile could indicate that TRPV1 could be transiently expressed during embryonic development, and its expression may suffer postnatal restrictions in some brain regions (Martins et al., 2014). Other studies showed a relatively large distribution of TRPV1 in mouse, rat, and primate brains using specific antibodies, mRNA probes, and the TRPV1-specific radioactive ligand [<sup>3</sup>H]RTX (Mezey et al., 2000). These studies detected TRPV1 expression in the prefrontal cortex, dentate gyrus, amygdala, striatum, thalamus, SN pars compacta, cerebellum, locus coeruleus, cochlear nuclei, the nucleus of the trigeminal nerve, NAc shell, and NAc core (Mezey et al., 2000).

## TRPV1 interactions with the endocannabinoid system

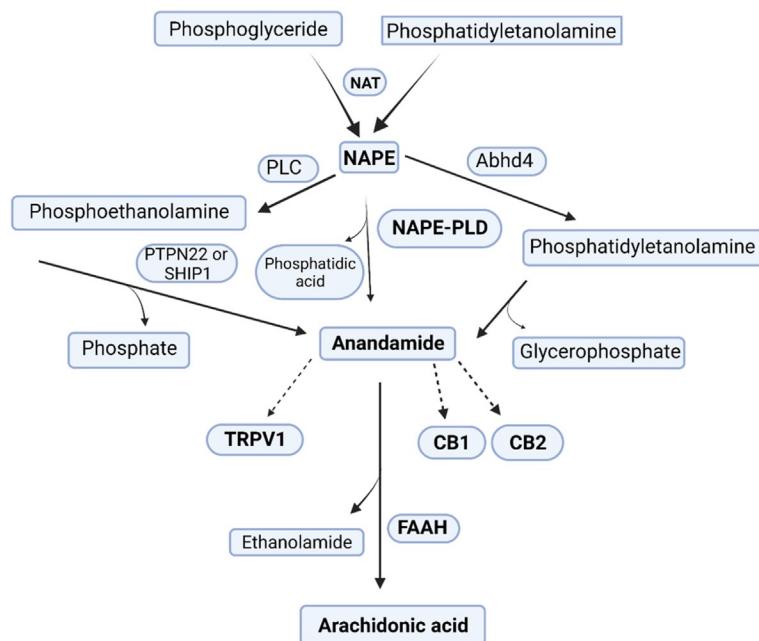
### The endocannabinoid system

Endocannabinoids (ECs) are lipid mediators isolated from the brain and peripheral tissues, including amides, esters, and ethers of long-chain polyunsaturated fatty acids (Battista et al., 2012), and are considered neuromodulators of the nervous

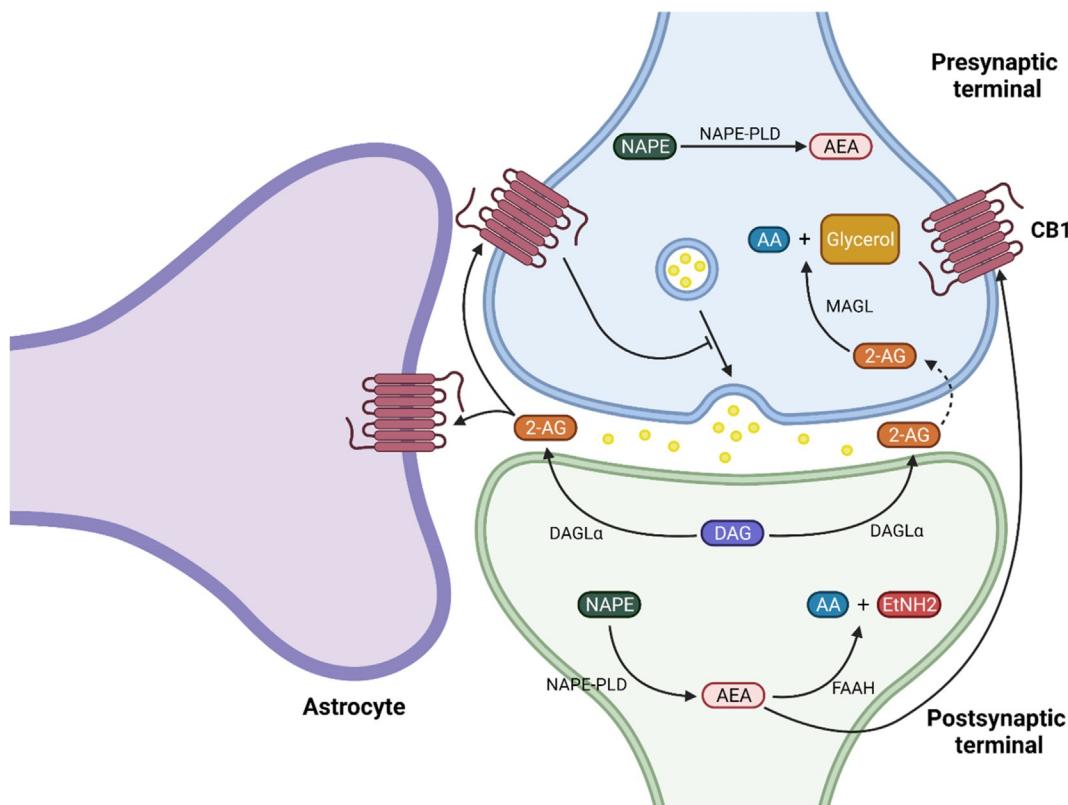
system, which play an essential role in mesolimbic dopamine systems and neuronal plasticity (Heifets & Castillo, 2009). The endocannabinoid system is composed of the endogenous ligands such as arachidonoyl ethanolamide (AEA), also known as anandamide, and 2-arachidonoyl glycerol (2-AG), the cannabinoid receptors (CB1 and CB2), the fatty acid amide hydrolase (FAAH), and monoacylglycerol lipase (MAGL) principal enzymes responsible for their synthesis and degradation (Basavarajappa, 2007). Endocannabinoids are released from cells immediately after biosynthesis on-demand and act as retrograde messengers that regulate the presynaptic release of neurotransmitters, which occurs in response to a physiological and pathological stimulus that increases the intracellular concentration of Calcium (Di Marzo & Petrosino, 2007). AEA is an EC derived from phosphatidylethanolamine and arachidonic acid, which are converted into N-arachidonoyl phosphatidylethanolamine (NAPE) by the enzyme N-acyltransferase (NAT) (Di Marzo et al., 1994). NAPE-phospholipase-D converts NAPE into AEA through a two-step process (Okamoto et al., 2004). One involves sequential deacylation of NAPE by alfa-beta-hydrolase 4 (Abhd4) and the subsequent separation of glycerophosphate to produce anandamide (Simon & Cravatt, 2006). The other proceeds from the hydrolysis of NAPE by phospholipase C to produce phosphoanandamide, which is later dephosphorylated by phosphatases, including tyrosine phosphatase PTPN22 and the inositol 50 phosphatase SHIP1 (Liu et al., 2008). Synthesized AEA can activate cannabinoid receptors (CB1 and CB2) and vanilloid receptor 1 (TRPV1) (Pertwee & Ross, 2002) (Fig. 4). However, the intrinsic efficacy of anandamide at TRPV1 is relatively low compared with that observed at the CB1 receptors (Pertwee & Ross, 2002).

Several lines of evidence indicate that the endocannabinoid system (ECS) is an integral regulator of the stress response. Studies have shown that AEA acts as an anxiolytic agent on the CB1 receptor and as an anxiogenic response when binding to TRPV1 (Rubino et al., 2008). Thus, AEA release has opposite effects on anxiety behavior depending on which receptor is activating. It is reported that CB1 activation results in decreased anxiety, whereas TRPV1 activation by AEA elicits an increase in anxiety-like behaviors, then AEA activates CB1R retrogradely to inhibit calcium channels and excitatory neuronal activity (Vaughan et al., 2000), whereas, in TRPV1, AEA promotes calcium influx and glutamate release (Xing & Li, 2007).

Various studies revealed that the functions of the ECS, at least in mammals, are not limited to the brain, but the whole organism; CB1 receptors, for example, have been found in the CNS and peripheral tissues, such as the hippocampus, pre-frontal cortex, amygdala, nucleus accumbens (NAc), olfactory bulb, basal ganglia, and cerebellum (Mackie, 2005). However, CB2 receptors have been found mainly in immune system cells and hematopoietic cells (Pacher & Mechoulam, 2011), as well as in several areas of the brain, such as the putamen, nucleus accumbens (NAc), hippocampus,



**FIG. 4** Metabolism of anandamide. The step that limits the speed of anandamide synthesis is the calcium ( $\text{Ca}^{2+}$ ) dependent synthesis of N-arachidonylethanolamide (NAPE) from phosphatidylethanolamine and phosphoglyceride catalyzed by N-acyltransferase (NAT). NAPE can be converted into anandamide through three pathways. The most prominent is catalyzed by a selective NAPE-phospholipase D enzyme (NAPE-PLD). Alternatively, NAPE can convert into phosphoethanolamine through phospholipase C (PLC) and to anandamide through tyrosine phosphatase PTPN2 or inositol phosphatase SHIP1. In addition, NAPE can convert into phosphatidylethanolamine by alfa, beta hydrolase 4 (Abhd4), prior metabolism to anandamide. Synthesized anandamide can activate cannabinoid receptors (CB1 and CB2) and vanilloid receptor 1 (TRPV1). The fatty acid amide hydrolase predominantly (FAAH) catalyzes anandamide breakdown to produce arachidonic acid.



**FIG. 5** Retrograde signal of the endocannabinoid (EC). Anandamides are predominantly released from the postsynaptic  $\text{Ca}^{2+}$  membrane after membrane depolarization and influx and interact with the CB1 receptors in the presynaptic terminal by retrograde signaling to inhibit presynaptic activity by modulating neurotransmitters release by primarily coupling to Gi/o proteins to inhibit adenylate cyclase and cyclic AMP signaling.

ventromedial hypothalamic nucleus, thalamus, dorsal raphe, and raphe nucleus (García-Gutiérrez & Manzanares, 2011). CB1 receptors are the most expressed G-protein-coupled receptors (GPCRs) in the brain and target 9-tetrahydrocannabinol (9-THC), the essential psychoactive component of cannabis used for millennia for recreational and therapeutic purposes (Marsicano & Lutz, 1999). The primary neurobiological effects of EC are complex and consist of several psychophysical effects, including emotions, memory, reward, digestion, stress, inflammation, mood, sleep, appetite, pain, sensation, addiction, and relapse (Mackie, 2006). The ECs are released predominantly from the postsynaptic neuron after membrane depolarization and  $\text{Ca}^{2+}$  influx, which interact with the CB1 receptors in the presynaptic terminal by retrograde signaling to inhibit presynaptic activity and modulate neurotransmitters release by coupling primarily to Gi/o proteins to inhibit adenylate cyclase and cyclic AMP signaling (Lauckner et al., 2005) (Fig. 5). The activation of the CB1 receptor has anxiolytic, analgesic, neuroprotective, and anti-nausea effects (Micale et al., 2013).

## The involvement of TRPV1 in anxiety and depression disorders

### TRPV1, endocannabinoids, stress, and anxiety

Stress might be associated with psychiatric disorders such as anxiety, depression symptoms, interpersonal sensitivity, frustration, and substance abuse (Firth et al., 2012). Anxiety and depression disorders are often comorbid with each other, resulting in estimates of lifetime prevalence of 16%–50% (Angold et al., 1999). Previous studies have shown that 70% of persons with major depression have comorbid lifetime anxiety disorders, whereas 27%–77% of those with a principal diagnosis of anxiety disorder develop a lifetime diagnosis of depression (Brown et al., 2001). Compared with those with pure diagnoses, people with comorbid anxiety and depression disorders experience greater chronicity and severity of each diagnosis; poorer work and psychosocial functioning; lower perceived quality of life; and a heightened risk of suicide (Pfeiffer et al., 2009).

Stress is also a common life condition that is significantly involved in the maintenance of health or the development of disease. In response to stress, different regulatory systems in the body are activated to allow the organism the ability to adapt to internal or external challenges. Adaptive responses can be specific to the stressor or can be generalized and nonspecific

(Chrousos & Gold, 1992). The hypothalamic-pituitary-adrenocortical axis (HPA) is one of the essential physiological components of the stress response system. Activation of the HPA axis begins in the paraventricular nucleus (PVN) of the hypothalamus, which is characterized by corticotrophin-releasing hormone (CRH) neurons (Antoni, 1986). Since the basal status of the HPA response is maintained by an inhibitory tone produced by profuse GABAergic receptors in the PVN, the HPA response begins with disinhibition of GABAergic terminals (Park et al., 2007). PVN-released CRH binds to receptors in the pituitary to initiate secretion of adrenocorticotropic hormone (ACTH), which also binds to its receptor in the adrenal cortex and indirectly stimulates the release of glucocorticoids (Slominski et al., 2005). This is a relatively slow stress response, with cortisol the primary glucocorticoid in humans (corticosterone in other mammals like rodents) peaking approximately 30 min after HPA activation (Herman et al., 2016). Glucocorticoids serve multiple adaptive purposes, including stabilizing and neutralizing the stress response by negative feedback to increase energy and prepare the body for a repeated stressful encounter (Sapolsky et al., 2000).

Previous studies showed evidence that the ECS mediates stress responses through the HPA axis (Morena et al., 2016). Other studies showed that the glucocorticoid-driven inhibition of parvocellular (Di et al., 2003) and magnocellular, including the subsequent inhibition of CRH cells, depended on retrograde endocannabinoid signaling (Di et al., 2005). There were also early observations of high co-expression of CRH with CB1 receptors in the limbic system (Hermann & Lutz, 2005). Other studies provided mechanistic evidence of EC mediation of the HPA stress response (Hill & Tasker, 2012). Following acute stress, AEA appears to tonically gate inhibition of the HPA axis, as reduction of AEA in the BLA is necessary for HPA activation (Hill et al., 2009) and occurs through elevated CRH-mediated fatty acid amide hydrolase (FAAH) increase (Natividad et al., 2017). In addition to its role in emotional behavior, the ECS plays a crucial role in HPA-axis regulation. Work conducted by Di et al. (2003) suggested that ECS regulates glucocorticoid feedback at the level of the PVN. Immunohistochemical staining demonstrated that both CB1 receptor and FAAH are present in the PVN (Patel et al., 2004), and electrophysiological studies showed that these receptors are presynaptically located on glutamatergic afferents, which terminate on neurosecretory cells that regulate activation of the HPA axis (Di et al., 2003).

Similar to cannabinoid modulation of anxiety, TRPV1 was also shown to modulate anxiety behavior (Marsch et al., 2007). Previous studies reported that CB1 and TRPV1 receptors are co-expressed in several brain regions (Mezey et al., 2000) and confirmed functional relationships in the central nervous system. Furthermore, previous findings revealed that the endocannabinoid and vanilloid systems interact in the brain to modulate anxiety behavior (Aguiar Jr et al., 2009; Rubino et al., 2007). Additional studies demonstrated that CB1 and TRPV1 receptors' expression within the medial prefrontal cortex area (mPFC) and hippocampus changes when exposed to an anxious stimulus (Hill et al., 2009).

Aguiar Jr et al. (2009) have shown that TRPV1 receptors mediate anxiety-like behaviors through their expression in the medial prefrontal cortex (mPFC), an area implicated in anxiety disorders, and found that inhibition of TRPV1 within the mPFC elicited anxiolytic effects. Other studies suggested that capsaicin treatment (TRPV1 agonist) produces an anxiogenic response in mice, while capsazepine (TRPV1 antagonist) significantly exhibits anxiolytic effects (Manna & Umathe, 2011); this result was confirmed by microinjection of capsazepine within the mPFC of rats (Aguiar Jr et al., 2009). In addition, anandamide synthesis in postsynaptic neurons produced an opposite effect on anxiety-like behavior where CB1 receptor activation inhibited, while TRPV1 receptor activation promoted anxiety-like behaviors in rodents (Marsch et al., 2007). Studies using TRPV1 knockout mice showed that the transgenic mice showed less anxiety-related behaviors when compared with their wild-type littermates (Marsch et al., 2007). In addition, recent studies in our laboratory revealed that the blockade of TRPV1 receptors produced anxiolytic-like effects in rats following exposure to cocaine conditioning (Norzé et al., 2018). This study showed that the blockade of TRPV1 receptors by capsazepine 10 µg/kg/ip significantly increased the total time experimental animals spent in the open arms in the elevated plus-maze (EPM) and decreased locomotor activity elicited by cocaine cues.

Previous data showed that AEA could act differently to the endocannabinoid and endovanilloid systems, acting as an anxiolytic agent on the CB1 receptor and an anxiogenic agent on TRPV1 receptors (Rubino et al., 2008). Other studies have shown that CB1 receptor agonists have a biphasic effect on anxiety behavior, with lower doses acting as anxiolytic and higher doses as anxiogenic (Viveros et al., 2005). Several brain regions are involved in modulating anxious states, such as the mPFC, which plays a crucial role in modulating anxiety behavior in human and animal models (Bystritsky et al., 2001). Increased neuronal activity in mPFC was evident in animal models of anxiety, stress, and anxiety-inducing stimuli that consistently activate mPFC in rats (Rubino et al., 2007) and pharmacological inactivation of mPFC by systemic administration of benzodiazepine midazolam produces anxiolytic effects (Shah & Treit, 2004). Several studies with CB1 receptor knockout mice have reported anxiogenic responses in anxiety paradigms in the EPM and light-dark box test (Haller et al., 2004). Other studies showed that stimulation of CB1 receptors in PFC, hippocampus, and amygdala, with subsequent activation of different signaling pathways, could be the initial event underlying the anxiolytic effect of low doses of

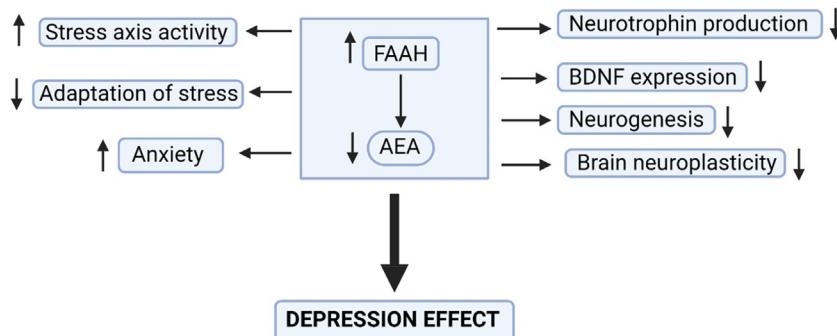
intraperitoneal tetrahydrocannabinol (THC) (Rubino et al., 2007). The activation of CB1 receptor by EC leads to a reduction in neurotransmitters release, such as (GABA, glutamate) through a retrograde mechanism (Wilson & Nicoll, 2002).

## TRPV1, endocannabinoids, and depression

Depression is one of the most common devastating disorders affecting society, and it's one of the most prevalent among neurological and psychiatric disorders. Depression exists in almost 80% of patients and is associated with impaired health-related quality of life, often contributing to high mortality (Hasset et al., 2014). Ample evidence clearly suggests that endocannabinoids (EC) participate in stress-associated neuronal signaling alterations and behavioral changes in the brain that lead to the development of depression (Gorzalka et al., 2008). Stress has been shown to enhance the activity of FAAH, resulting in a significant decline of AEA content in the brain (Gunduz-Cinar et al., 2013). In contrast, the inhibition of FAAH produced antidepressant behavioral responses (Hill et al., 2009). Thus, restoring deficient AEA signaling may be served as a therapeutic mechanism in acutely stressed naïve animals (Hill et al., 2009). In support of this idea, the administration of FAAH inhibitor URB597 or genetic deletion of FAAH alleviated depression-like behavioral responses in the forced swim test and tail suspension tests (Bambico et al., 2010a, 2010b). In addition, it also has been proved that acute and chronic treatments with CB1 receptor agonists elicit antidepressant responses in the forced swim test and chronic mild stress paradigm (Griebel et al., 2005). For example, administration of CB1 receptor agonists has been shown to elicit an antidepressant-like profile in both rat and mouse forced-swim test (Rutkowska & Jachimczuk, 2004), CB1 receptor agonists, HU210 and WIN 55212-2, reduce immobility duration in the forced swim test in male rats at very low doses, consistent with antidepressant efficacy (Bambico et al., 2007).

CB1 receptors contributed to depressive phenotypes in animal and human studies and are widely located in brain structures involved in the pathogenesis of depression (prefrontal cortex, frontal cortex, hippocampus, cerebellum) and are associated with anhedonia (dorsal striatum and nucleus accumbens) (Robinson et al., 2012). At the functional level, CB1 receptors modulate brain neurotransmission, including the 5-HT, dopamine (DA), NA,  $\gamma$ -aminobutyric acid (GABA), and glutamate systems, inhibiting the stress axis and restoring brain neuroplasticity (Micale et al., 2013). The glutaminergic neurons and the GABAergic interneurons play opposite roles in controlling the excitation state of the brain. Interestingly, these cell types express strongly in CB1 receptors, which mediates signaling to maintain the homeostasis of excitatory and inhibitory neurotransmitters (Marsicano & Lutz, 1999). Preclinical studies have shown that depression-like behaviors were associated with an increase in FAAH's brain levels, which reduces AEA (Gunduz-Cinar et al., 2013). In animal models of depression, rats exhibited higher FAAH levels in the mPFC and hippocampus, with unchanged levels of the NAPE-PLD (Vinod et al., 2012). Chronic mild stress (CMS), for example, increases FAAH levels in male and female animals in the dorsal hippocampus (Reich et al., 2009). Studies supported these results in FAAH knockout mice, which showed anxiolytic and antidepressive effects associated with changes in 5-HT transmission and postsynaptic 5-HT1A and 5-HT2A/2C receptor function (Bambico et al., 2010a, 2010b). Furthermore, deficiency in EC signaling is associated with a depressive-like phenotype (Hill et al., 2008). After exposure to several stressors (e.g., social defeat stress and chronic unpredictable stress (CUS)), AEA levels decreased within the hippocampus, hypothalamus, ventral striatum, and prefrontal cortex of rats, as well as in the hippocampus and hypothalamus of mice (Hill et al., 2008) (Fig. 6).

At the clinical level, studies reported that serum 2-AG concentrations are reduced in women with major depression (Hill et al., 2008), and postmortem analysis has revealed that the expression of CB1 receptors on glial cells in the anterior



**FIG. 6** Effect of stimulation of the endocannabinoid system. This figure above shows that a reduction in anandamide can cause several biochemical changes (excitation of the stress axis, reduction of neurotrophin production, and reduction of neurogenesis associated with depression).

cingulate cortex is significantly reduced in depressed individuals (Koethe et al., 2007). Also, several drugs that produce antidepressant effects in humans have increased endocannabinoid/CB1 receptor signaling in the brain, including chronic treatment with the antidepressant desipramine (Hill et al., 2006). Various studies reported that the hypofunctional EC signaling contributes to the etiology or symptom spectrum of depressive illness and that increasing endocannabinoid signaling is associated with antidepressant efficacy (Hill et al., 2008). On the other hand, evidence reported that hyper-functional ECS is associated with depression. The CB1 receptor protein expression, binding site density, and signal transduction are upregulated in the mPFC of depressed individuals who have committed suicide (Hungund et al., 2004). Similarly, chronic mild stress exposure to rats (Bortolato et al., 2007) and CUS exposure to mice increases CB1 receptor mRNA in the prefrontal cortex (Hillard et al., 2006). Studies realized by Hill et al. (2008), reported that CUS produced changes in both CB1 receptor agonist binding site density and the tissue content of the endocannabinoid ligands, in addition, they reported that rats exposure for 21 days to CUS produced a reduction in the tissue content of AEA in (PFC, hippocampus, hypothalamus, ventral striatum, amygdala, and midbrain). Except for the mPFC, chronic stress is associated with a downregulation of CB1 receptor expression (Morena et al., 2016). Rats exposed to chronic stress showed a decrease of CB1 receptor binding site density in the hippocampus (Hill et al., 2006); therefore, there were no effects on CB1 agonist binding in the amygdala.

Behavioral evidence suggested that TRPV1 also contributes to depression-like behaviors. One study revealed that TRPV1 knockout (KO) mice showed antidepression-like effects (You et al., 2012). In addition, TRPV1 knockout (KO) mice exhibit less immobility than their wild-type littermates when tested in the forced swim test (FST) (You et al., 2012). TRPV1 receptor knockout mice also decreased latency times in the novelty-suppressed feeding paradigm compared with wild-type mice demonstrating a decreased depressive response (You et al., 2012). Another study revealed that intracerebroventricular (icv) injection of capsazepine reduced immobility time in mice in the forced swim test (Manna & Umathe, 2011).

## Cocaine addiction and TRPV1

Addiction is a chronic disease that involves the loss of control of substance use and relapse episodes that are critical to the persistence of this condition (Adamczyk et al., 2012). Cocaine addiction is a severe socio-medical issue that affects people of all ages, regardless of their economic status (Hull et al., 2010). Preclinical reports suggested that the ECS plays an essential role in cocaine addiction (Arnold, 2005), particularly in the reinstatement of cocaine-seeking behavior (Adamczyk et al., 2009). As such, it could be a potential signal system that can be used to reduce relapse (Adamczyk et al., 2012). It also reported that the EC action mechanisms depend not only on the activation of CB receptors. In fact, TRPV1 can also mediate EC action (Zygmunt et al., 1999). Many studies have examined the role of TRPV1 in drug addiction (Marsch et al., 2007) and suggest that TRPV1 could be involved in neuronal and behavioral adaptations induced by addictive drugs, such as drug consumption and drug-seeking (Nguyen et al., 2014). Previous studies have examined the mPFC of methamphetamine-addicted rats and observed an increase in TRPV1 levels (Tian et al., 2010). In addition, other studies have shown that TRPV1 expression/activation affects the addictive behavior of ethanol, nicotine, and cocaine-addicted animals (Adamczyk et al., 2012), which supports TRPV1 role in drug addiction. It was reported that the blockade of TRPV1 reduced cocaine-seeking behavior during the reinstatement (Adamczyk et al., 2012) and attenuated morphine tolerance and withdrawal symptoms in mice (Nguyen et al., 2010). Furthermore, studies showed that TRPV1 antagonist (capsazepine) decreased locomotor activity in rats after exposure to cocaine conditioning-related cues (Norzé et al., 2018).

## Applications to other areas

In addition to the role of TRPV1 receptors in anxiety and depression behavior, TRPV1 receptors are also involved in detecting harmful sensations (Caterina & Julius, 2001). TRPV1 is best considered a receptor for capsaicin, an ingredient in chili peppers known to trigger a burning sensation and pain (Caterina et al., 1997). The TRPV1 receptors are highly expressed in the plasma membrane of the nociceptive of dorsal root ganglia neurons (DRG) (Caterina & Julius, 2001). The activation of nociceptive TRPV1 ion channels in sensitive neurons (i.e., dorsal root ganglion, DRG) leads to calcium influx across the plasma membrane, causing membrane depolarization, which in turn could trigger voltage-gated ion channel-dependent action potentials that transmit the information to the spinal cord (Gees et al., 2010). The TRPV1 is the most studied TRP channel due to its role in nociception. TRPV1 antagonists have been shown to effectively attenuate thermal hyperalgesia induced in inflammatory conditions (Tekus et al., 2010). Similar results were found in mice models lacking TRPV1 (Davis et al., 2000). Additional evidence supports the role of TRPV1 in inflammatory pain caused by electroacupuncture in a mouse model of inflammatory pain (Liao et al., 2017). The injection of capsaicin intradermic causes pain and hyperalgesia in humans dose-dependent (Simone et al., 1989). Many pro-inflammatory factors, including the

substance P, nerve growth factor, bradykinin, prostaglandins, and ATP, can also potentiate and sensitize TRPV1 channels (Moriyama et al., 2005). However, oral administration of TRPV1 antagonists reduces capsaicin pain behavior and hyperalgesia in rodents (Cui et al., 2006).

## Mini-dictionary of terms

- **Anxiolytics:** Class of drugs used to prevent or manage anxiety symptoms or disorders.
- **Antidepressants:** Class of drugs used to treat major depressive disorder.
- **Neurotransmitters:** Molecules used by the nervous system (NS) to transmit messages between neurons or muscles that can act on neurons by stimulating, inhibiting, or modulating.
- **Neuromodulators:** Substances that act with neurotransmitters to activate ion-channel receptors to improve the excitatory or inhibitory responses of the receptors.
- **Capsaicin:** Active ingredient of chili peppers and the primary endogenous ligand of TRPV1 receptors.

## Key facts of major depression disorder (MDD)

- Major depressive disorder is a devastating disorder characterized by depressed mood, impaired interests, and cognitive function.
- The etiology of MDD is multifactorial, but the hereditary cause is estimated at around 35%.
- Environmental factors such as physical, emotional, or sexual abuse during childhood are associated with the risk of developing MDD. MDD is associated with changes in the regional brain volume, especially the hippocampus, and functional changes in the brain circuits.
- MDD management consists of psychotherapy and pharmacological treatment.

## Summary points

- The vanilloid family is the best-studied within the TRP channel, particularly TRPV1, due to its role in nociception.
- TRPV1 can be activated endogenously by various harmful stimuli, such as high voltage, noxious heat ( $>42^{\circ}\text{C}$ ) pressure, and low pH.
- Endocannabinoids, including anandamide and N-arachidonoyl dopamine, can activate TRPV1.
- TRPV1 receptors are expressed within the central and peripheral nervous systems.
- TRPV1 receptors can mediate anxiety and depression-like behaviors.

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## Chapter 28

# Org27569, the allosteric modulators and the cannabinoid receptor 1 (CB1)

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### Abbreviations

<b>2-AG</b>	2-arachidonoyl glycerol
<b>AC</b>	adenylyl cyclase
<b>AEA</b>	<i>N</i> -arachidonoyl ethanolamide
<b>AP1</b>	activator protein-1
<b>cAMP</b>	cyclic adenosine monophosphate
<b>CB1</b>	cannabinoid receptor type 1
<b>CB2</b>	cannabinoid receptor type 2
<b>CNS</b>	central nervous system
<b>CREB</b>	cAMP response element-binding protein
<b>CRIP1a</b>	cannabinoid receptor interacting protein 1a
<b>ERK1/2</b>	extracellular signal-regulated kinase ½
<b>FAAH</b>	fatty acid amide hydrolase
<b>FAN</b>	factor associated with neutral sphingomyelinase activation
<b>GIRK</b>	G protein-coupled inwardly rectifying potassium channels
<b>GPCR</b>	G protein-coupled receptor
<b>GRK</b>	G protein-coupled receptor kinase
<b>GTPγS</b>	Guanosine 5'-O-(3-thio)triphosphate
<b>IP<sub>3</sub></b>	inositol 1,4,5 triphosphate
<b>JNK</b>	c-Jun N-terminal kinase
<b>MAGL</b>	monoacylglycerol lipase
<b>MEK</b>	ERK kinase
<b>mTOR</b>	mammalian target of rapamycin
<b>NAM</b>	negative allosteric modulator
<b>NF-κB</b>	nuclear factor κB
<b>p38</b>	mitogen-activated protein kinase (MAPK)
<b>PAM</b>	positive allosteric modulator
<b>PDB</b>	protein data bank
<b>PI3K</b>	phosphoinositide-3 kinase
<b>PKA</b>	protein kinase A
<b>PKB/AKT</b>	protein kinase B
<b>PKC</b>	protein kinase C
<b>PLC</b>	phospholipase C
<b>Raf-1</b>	MEK kinase
<b>Rap-1</b>	Ras-related protein-1
<b>Ras</b>	Rat sarcoma virus-derived GTPase
<b>S1P</b>	sphingosine 1-phosphate
<b>Src</b>	v-src Sarcoma viral oncogene homolog
<b>STAT3</b>	signal transducer and activator of transcription 3
<b>THC</b>	tetrahydrocannabinol

## Introduction

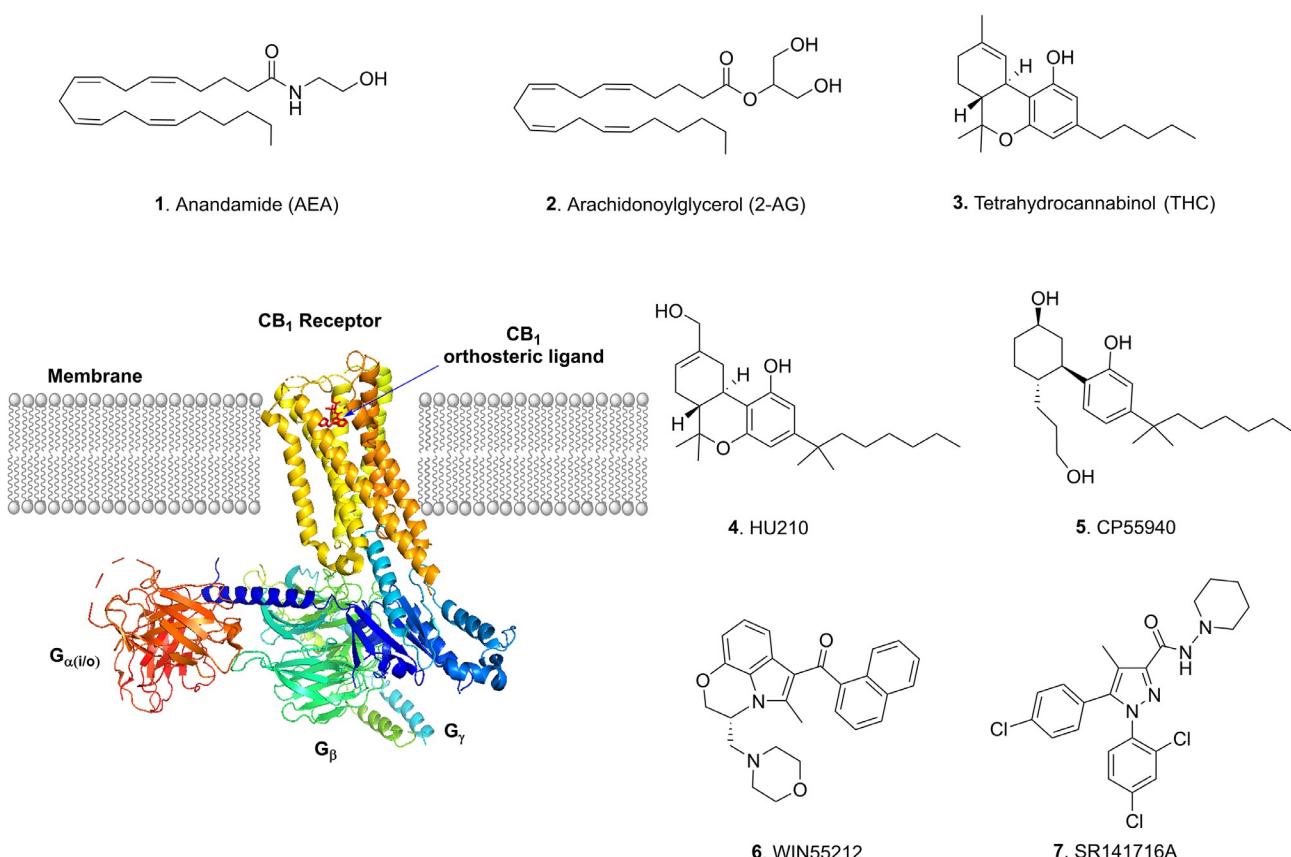
The endocannabinoid system contains at least two G protein-coupled receptors (GPCRs), which include the cannabinoid type-1 ( $\text{CB}_1$ ) and type-2 ( $\text{CB}_2$ ) receptors. The two cannabinoid receptors mediate the pharmacological effects of  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9\text{-THC}$ ) and some other active phytocannabinoids isolated from cannabis (Turner et al., 2017). A group of lipid-derived molecules including *N*-arachidonoyl ethanolamide (AEA) and 2-arachidonoyl glycerol (2-AG) function as the endogenous ligands of the cannabinoid receptors (termed “endocannabinoids” (Lu & Mackie, 2021)). Several catabolic enzymes including fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) can degrade the endocannabinoids promptly to ensure that the cannabinoid receptors are not overly activated by endocannabinoids under physiological conditions (Pacher et al., 2006).  $\text{CB}_1$  is the most abundant GPCR expressed in the central nervous system (Glass et al., 1997; Herkenham et al., 1990) and is also widely distributed, albeit at much lower concentrations, in a variety of peripheral tissues (Pacher et al., 2006). In contrast,  $\text{CB}_2$  is largely found in the periphery with high concentrations in the tonsil, spleen, and immune-related cells (Howlett et al., 2004). Modulating cannabinoid receptors has been implicated as a therapeutic approach for a wide range of neurological, cardiovascular, metabolic, and immune disorders (Pacher et al., 2006). These include neuropathic pain, multiple sclerosis, Parkinson’s and Huntington’s disease, spinal cord injury, atherosclerosis, myocardial infarction, stroke, hypertension, glaucoma, obesity/metabolic syndrome, osteoporosis, and cancer (Pacher et al., 2006). Conventionally, development of cannabinoid-based therapeutics has focused on ligands targeting orthosteric sites on the receptor, i.e., where the endogenous cannabinoids bind. However, ligands targeting  $\text{CB}_1$  orthosteric sites either induce psychotropic adverse effects from  $\text{CB}_1$  agonists or psychiatric adverse effects from globally active  $\text{CB}_1$  antagonists/inverse agonists (Cridge & Rosengren, 2013; Grotenhermen & Müller-Vahl, 2012). These untoward effects have hampered drug discovery efforts aimed at orthosteric modulation of  $\text{CB}_1$  for therapeutic gain. On the other hand,  $\text{CB}_2$  has attracted considerable attention as a therapeutic target for pain, cancer, inflammation-derived neurodegenerative diseases, and osteoporosis (Atwood et al., 2012; Dhopeshwarkar & Mackie, 2014). Some  $\text{CB}_2$  selective agonists have been assessed in clinical trials. Unfortunately, the outcomes were disappointing and failed to meet the primary endpoints (Atwood et al., 2012; Dhopeshwarkar & Mackie, 2014). The failures of candidates targeting  $\text{CB}_2$  may be due to several factors: (1) inadequate preclinical studies to precisely predict clinical efficacies in humans; (2) lack of  $\text{CB}_2$ -specific ligands to validate the target in preclinical investigations; and (3) insufficient engagement of the drug with its target and possible off-target side effects. The disappointment of using orthosteric ligands to modulate cannabinoid receptors for therapeutic benefits has led to pursuit of alternative approaches such as allosteric modulation.

Allosteric modulation is typically achieved from ligand binding to alternative receptor sites that are topologically different from the orthosteric site (Changeux, 2013). Binding of allosteric ligands provokes receptor conformations that are distinct from those induced by orthosteric ligands, thus, novel pharmacological effects can be achieved (May & Christopoulos, 2003). Binding of an allosteric ligand can alter the binding affinity and kinetics and functional efficacy of orthosteric ligands. Frequently, allosteric ligands modulate receptor functions in the absence of any orthosteric ligand (allosteric agonists/inverse agonists). Allosteric modulation of GPCRs offers many advantages over orthosteric regulation of GPCR, such as higher receptor subtype selectivity, maintaining the temporal profile of endogenous signaling, stronger capacity to fine-tune the receptor function through probe or pathway-dependent functional selectivity (also known as biased signaling). Hence, allosteric modulators provide a greater possibility to separate the therapeutic benefits from adverse effects through functional selectivity that preferentially activate certain signaling pathways while others are spared.

In this chapter, we will review orthosteric signaling through  $\text{CB}_1$ , possible functional selectivity (biased signaling) induced by  $\text{CB}_1$  ligands and the evidence supporting the discovery of the first allosteric modulator of  $\text{CB}_1$  (i.e., Org27569). Org27569 is the most intensively investigated  $\text{CB}_1$  allosteric modulator so far. Understanding its novel pharmacological profile will improve the understanding of the ample opportunities offered by  $\text{CB}_1$  allosteric modulators for discovery of safer and more efficacious therapeutics from modulation of  $\text{CB}_1$  functions.

## $\text{CB}_1$ and cannabinoid orthosteric ligands

$\text{CB}_1$  was identified and cloned in the early 1990s. It has been characterized as a class 1A rhodopsin-like GPCR (Howlett et al., 2004). While  $\text{CB}_1$  is expressed primarily in the central nervous system (CNS), they are also found to a lesser yet functional extent in the periphery (Lauckner et al., 2005; Pertwee, 1997).  $\text{CB}_1$  is a GPCR with characteristic protein structure having seven membrane-spanning helices connected with three intracellular loops and an intracellular C-terminal

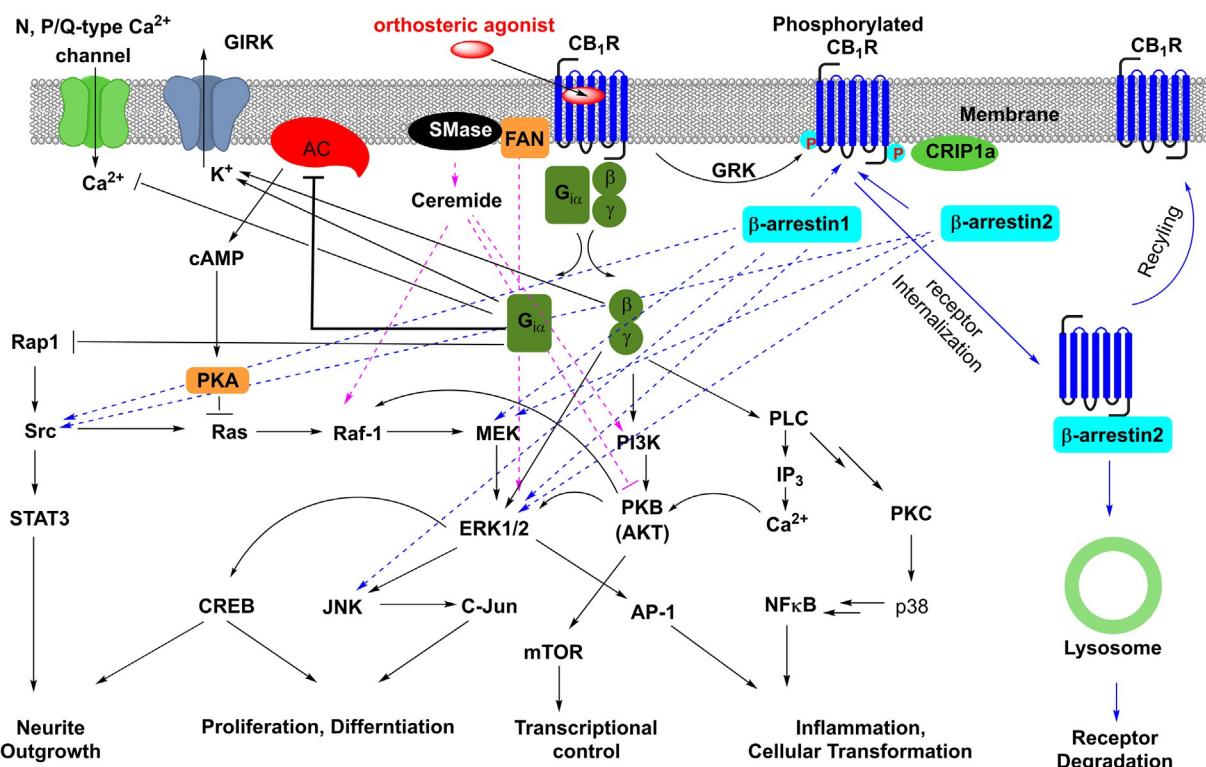


**FIG. 1** The CB<sub>1</sub>-G<sub>i</sub> protein complex structure obtained from Protein Data Bank (PDB code: 6N4B) and structures of representative CB<sub>1</sub> orthosteric ligands. Compounds 1–6 are CB<sub>1</sub> orthosteric agonists and 7 is an inverse agonist/antagonist.

domain. Its intracellular components interact with the coupled G proteins (i.e., the heterotrimer of G<sub>α</sub> and G<sub>βγ</sub> proteins, Fig. 1) (Howlett, 2005). CB<sub>1</sub> receptors are expressed with especially high level in the neocortex, hippocampus, basal ganglia, cerebellum, and brainstem (Glass et al., 1997; Herkenham et al., 1990). Activation of CB<sub>1</sub> regulates a variety of physiological processes, such as neuronal development, neuronal plasticity, food intake and energy balance, perception processes, immune modulation, cell apoptosis, cardiovascular and reproductive functions among others (Di Marzo et al., 2004; Pacher et al., 2006; Rodriguez de Fonseca et al., 2005). The diverse functions of CB<sub>1</sub> receptor are evidently the result of its complex cellular signaling pathways (Hunter et al., 2017; Turu & Hunyady, 2010). Traditionally, the functions of CB<sub>1</sub> are regulated by various endogenous and exogenous ligands that bind to the orthosteric sites. The representative CB<sub>1</sub> orthosteric ligands (Fig. 1) include endogenous agonists such as AEA (1) and 2-AG (2); exogenous agonists such as phytocannabinoid Δ<sup>9</sup>-THC (3); synthetic cannabinomimetics (e.g., HU210, 4; CP55,940, 5; and WIN55,212-2, 6); and synthetic inverse agonists/antagonists (e.g., SR141716A, 7). Typically, an orthosteric inverse agonist binds to the same receptor site as an orthosteric agonist but induces an opposite biological response by reducing the constitutive activity of the receptor (i.e., receptor activity occurring in the absence of agonist) (Howlett, 2005). Recently, the crystallographic structures of CB<sub>1</sub> bound with either an agonist or an antagonist have been reported (Hua et al., 2017, 2016; Shao et al., 2016). These ligand-bound CB<sub>1</sub> structures provide insights for the mechanism of CB<sub>1</sub> activation and serve as critical tools for rational design of new CB<sub>1</sub> ligands.

## CB<sub>1</sub> receptor-mediated cell signaling

Upon ligand binding, CB<sub>1</sub> transduces signals mainly through G protein-dependent pathways and G protein-independent processes to some extent (Howlett, 2005; Ibsen et al., 2017). The complex network of CB<sub>1</sub>-mediated signaling is illustrated in Fig. 2. Upon orthosteric agonist binding, CB<sub>1</sub> interacts with and activates G<sub>αβγ</sub> heterotrimer to release G<sub>α</sub> and the obligate dimer G<sub>βγ</sub>. Though CB<sub>1</sub> predominately couples to the G<sub>i</sub> and G<sub>o</sub> proteins, signal transduction via G<sub>s</sub> (Finlay et al., 2017;



**FIG. 2** Main signaling pathways of cannabinoid CB<sub>1</sub> receptor. Upon agonists binding, CB<sub>1</sub> transduces signals via G proteins and non-G protein partners. Such as β-arrestin1 and β-arrestin2. Agonist binding to the receptor resulted in dissociation of G protein and releasing of G<sub>α</sub> and G<sub>βγ</sub> subunits, of which G<sub>(i/o)α</sub>-mediates inhibition of adenylyl cyclase (AC) to decrease cAMP production, and this in turn inhibits protein kinase A (PKA). Downregulating PKA reduces the Ras protein-mediated signaling to MAPKs including MEK and ERK1/2. The released G<sub>βγ</sub> subunits can activate PI3K, PLC, and ERK1/2 to be part of the signaling events that control cell proliferation, differentiation, and transcription via activation of mTOR, CREB, JNK, etc. Both G<sub>(i/o)α</sub> and G<sub>βγ</sub> subunits can activate GIRK channel and inhibit voltage-gated N, P/Q-type calcium channels. Phosphorylated CB<sub>1</sub> recruits β-arrestin1 and β-arrestin2 to mediate some G protein-independent signaling events through activation of MEK, ERK1/2, JNK, Src, etc. β-arrestin1 is believed to mediate most of the signaling, whereas β-arrestin2 mediates receptor internalization and desensitization. Upon agonist binding, CB<sub>1</sub> promotes the production of ceramide, which functions as a second messenger to mediate CB<sub>1</sub> signaling processes by activation of Raf-1, ERK1/2, and inhibition of PKB. Signaling processes mainly mediated by G proteins are depicted by solid black arrow; signal transductions involving β-arrestin1 and β-arrestin2 are shown in blue dashed arrow; the processes involving only β-arrestin2 are shown by solid blue arrow; other non-G protein-dependent processes are shown by dashed arrows in pink. Arrows indicate stimulation/activation, and blunted arrows indicate inhibition. Abbreviations: AC, adenylyl cyclase; PKB/AKT, protein kinase B; AP1, activator protein-1; CREB, cAMP response element-binding protein; ERK1/2, extracellular signal-regulated kinase 1/2; FAN, factor associated with neutral sphingomyelin activation; GRK, G protein-coupled receptor kinase; IP<sub>3</sub>, inositol 1,4,5-triphosphate; JNK, c-Jun N-terminal kinase; MEK, ERK kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; p38, mitogen-activated protein kinase (MAPK); PI3K, phosphoinositide-3 kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; Raf-1, MEK kinase; Rap-1, Ras related protein-1; Ras, Rat sarcoma virus derived GTPase; S1P, sphingosine 1-phosphate; Src, v-src sarcoma viral oncogene homolog; STAT3, signal transducer and activator of transcription 3.

Glass & Felder, 1997), G<sub>q/11</sub> (Lauckner et al., 2005), and G<sub>12/13</sub> (Roland et al., 2014) has also been observed under some conditions. Upon dissociation, the G<sub>(i/o)α</sub> inhibits adenylyl cyclase (AC) to reduce cAMP production and subsequently downregulates protein kinase A (PKA), which in turn suppresses PKA-mediated signaling events such as regulating gene expression and the phosphorylation of various mitogen-activated protein kinases such as MEK and ERK1/2 (Basavarajappa, 2017; Bosier et al., 2010; Howlett et al., 2010). The dissociated G<sub>βγ</sub> subunits can evoke the phosphatidylserine 3-kinase (PI3K) and protein kinase B (PKB) pathways (Howlett et al., 2010), of which the latter one can induce phosphorylation of MAPKs such as MEK (Basavarajappa, 2017; Howlett et al., 2010; McCoy, 2016). Additionally, CB<sub>1</sub> activation leads to inhibition of voltage-gated N- and P/Q-type calcium channels and activates G protein-coupled inwardly rectifying potassium channels (GIRK) via either G<sub>(i/o)α</sub> or G<sub>βγ</sub> proteins-mediated processes (Herkenham et al., 1990). These acute actions mediated by G proteins occur quickly upon orthosteric agonists binding to CB<sub>1</sub>. This is followed by receptor phosphorylation catalyzed by G protein receptor kinase (GRK) to recruit β-arrestin1 and β-arrestin2 to the receptor and results in β-arrestin1 and/or -2-mediated signaling events via stimulation of MAPKs, including the MEK and ERK1/2. Additionally, recruitment of β-arrestin2 by phosphorylated CB<sub>1</sub> promotes receptor desensitization and internalization (Delgado-Peraza et al., 2016; Ibsen et al., 2019). Generally, orthosteric agonists induce less β-arrestin1

translocation compared with β-arrestin2 (Ibsen et al., 2019). β-Arrestin2 alters CB<sub>1</sub> signaling largely by reducing receptor mediated G protein activity (Nguyen et al., 2012). Along with the G protein-mediated signal transduction, CB<sub>1</sub> can interact with a variety of other non-G protein partners including adaptor protein AP-3, GPCR-associated sorting proteins (GASPs), the factor associated with neutral sphingomyelinase (FAN), and the cannabinoid receptor interacting protein 1a (CRIP1a) to regulate downstream effectors such as MAPKs, multiple receptor tyrosine kinases, and extracellular signal-regulated kinases (ERK1/2) (Blume et al., 2016; Howlett et al., 2010; Smith et al., 2010; Turu & Hunyady, 2010). CRIP1a has also been reported to be involved in CB<sub>1</sub> desensitization and intracellular trafficking (Smith et al., 2015). The CRIP1a may exert its effects through competition with β-arrestins for CB<sub>1</sub> binding sites (Blume et al., 2016). In addition to protein partners, CB<sub>1</sub> can also transduce signals through lipid-derived ceramide as the second messenger through activation of the Raf-1, MEK, and ERK1/2 cascade (Velasco et al., 2005).

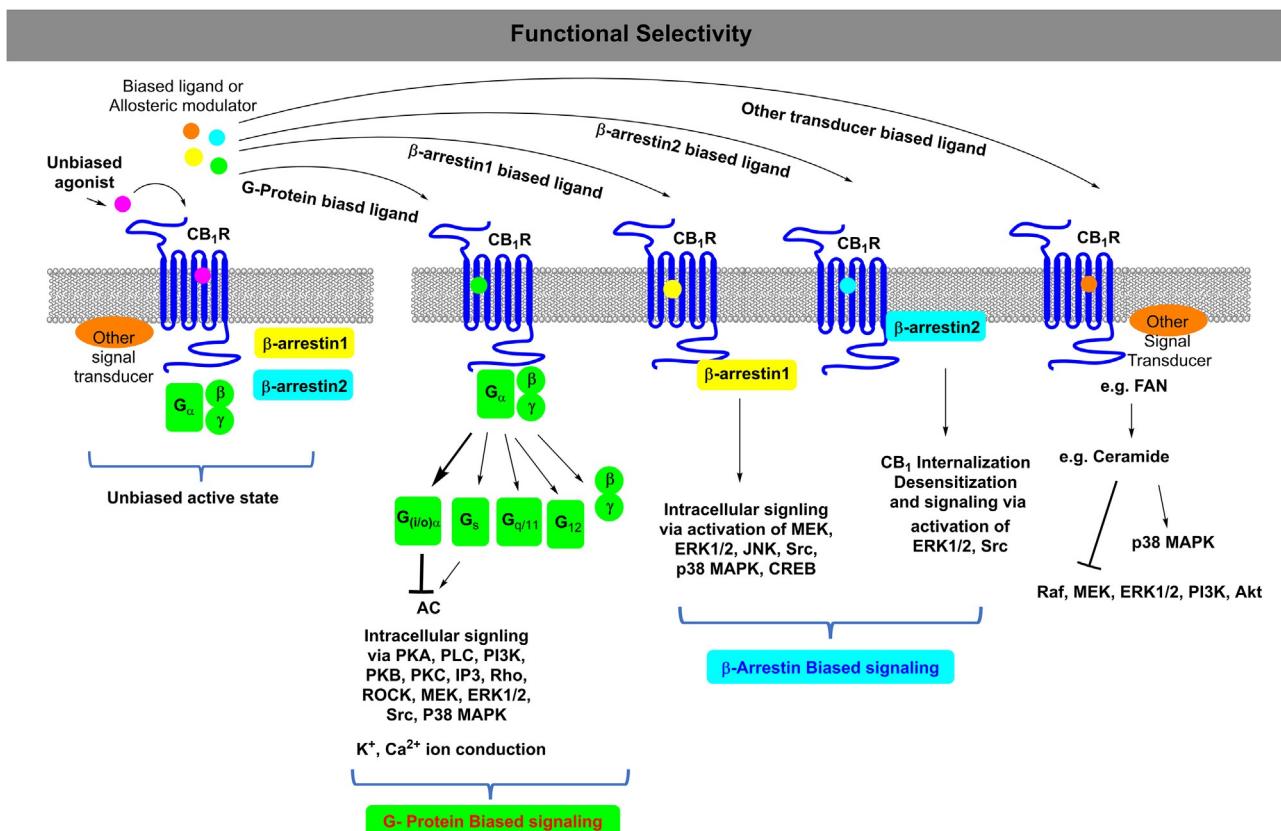
It has been suggested that signal transduction of CB<sub>1</sub> may take place in three sequencing waves (Nogueras-Ortiz & Yudowski, 2016). The first one is transient signal transduction wave commenced by heterotrimeric G proteins (G<sub>αβγ</sub>) in a time course <10 min. Following the G protein-mediated transient signal transduction is the second wave, which is mediated by β-arrestins, and lasts more than 5 min. The third and final wave happens at intracellular compartments and could be mediated either by G proteins or β-arrestins (Nogueras-Ortiz & Yudowski, 2016). The complexity of CB<sub>1</sub> signal transduction implies a finely tuned regulation of the signaling events that control the physiological functions of CB<sub>1</sub>.

### The functional selectivity induced by CB<sub>1</sub> ligands

Growing evidence indicates that GPCRs are pluridimensional proteins, which can take numerous structural conformations resulting in various signaling states (Chang & Bruchas, 2014). Functional selectivity or biased signaling refers to the ability of a ligand to stabilize a GPCR toward a conformation that can preferentially couple to a subset of the possible intracellular signaling proteins and therefore selectively evoke certain responses over others (Gouplil et al., 2012; Kenakin, 2011; Urban et al., 2007; Vaidehi & Kenakin, 2010). There is good evidence to support the conclusion that CB<sub>1</sub> can exhibit ligand-induced biased signaling, which has been recently reviewed by others (Bosier et al., 2010; Ibsen et al., 2017; Patel et al., 2021). For instance, CB<sub>1</sub> orthosteric ligands HU210, CP55,940, WIN55,212-2, 2-AG, and AEA can respectively elicit preferential interactions with different subtypes of G proteins (i.e., G<sub>i</sub>, G<sub>o</sub>, G<sub>s</sub>, or G<sub>q/11</sub>) with varying efficacies and yield ligand-dependent biased signaling (Glass & Northup, 1999; Laprairie et al., 2014; Lauckner et al., 2005). Depending on the ligand, CB<sub>1</sub> has the ability to engage a G protein and a non-G protein partner such as arrestins or favor one over the other (Fig. 3). The complex signaling network of CB<sub>1</sub> indicates a possibility for special ligands that can selectively transduce the signals relevant for desired therapeutic effects while sparing the pathways linked to adverse effects. Although some orthosteric ligands can achieve ligand-dependent biased signaling, allosteric ligands have been widely recognized for offering greater opportunity to produce biased signaling (Grover, 2013; Khouri et al., 2014). Allosteric modulators of a given receptor can induce various receptor conformations distinct from those stabilized by orthosteric ligands (Foster & Conn, 2017; Vaidehi & Kenakin, 2010). Different conformations of a receptor can in turn impart selective interaction with downstream effectors and lead to biased signaling. Over the last 15 years, several class of CB<sub>1</sub> allosteric modulators have been discovered and investigated for their therapeutic usefulness (Lu et al., 2019).

### Allosteric modulators of CB<sub>1</sub> and Org27569

Recently, there have been tremendous advances in the discovery of ligands that can regulate GPCR functions through binding to receptor allosteric sites that are topographically distinct from orthosteric sites (Changeux, 2013; May et al., 2007). Allosteric modulators in general bind to a site outside of the highly conserved orthosteric binding site and therefore allow the receptor to simultaneously bind an orthosteric ligand at the orthosteric binding pocket. This can alter the signaling process of the orthosteric ligand, either by enhancing or attenuating the orthosteric ligand binding or by altering the relative efficacy of the orthosteric ligand in producing pharmacological responses. Additionally, an allosteric ligand may activate or inactivate the receptor in the absence of an orthosteric ligand to elicit pharmacological effects on its own. Characterization of an allosteric ligand for CB<sub>1</sub> (as for any GPCR) is a challenging task. It requires multiple in vitro assays that mostly include: (1) receptor-binding assays such as equilibrium-binding assays and kinetic-binding assays, (2) multiple receptor functional assays to determine G protein activation and β-arrestin1 and β-arrestin2 recruitment, and (3) assays for phosphorylation of ERK1/2 ERK1/2 and other kinase (Jakubík et al., 2020; Scott & Kendall, 2017). As both G protein and non-G protein-mediated ERK1/2 phosphorylation can take place, ERK1/2 phosphorylation (pERK) assays can be challenging to



**FIG. 3** Potential ligand-induced biased signaling of CB<sub>1</sub>. Biased ligands can elicit distinct pathway-specific pharmacology. Unbiased ligands modulate every possible signaling pathway, which is the underlying cause for on-target adverse effects. Biased ligands or allosteric modulators induce biased signaling to preferentially activate certain signaling pathways. G protein-biased ligands can generate enhanced efficacy by avoiding the β-arrestin2-mediated desensitization of G protein signaling seen with unbiased agonists. Additionally, if on-target adverse events are associated with β-arrestin signal transduction, G protein-biased ligands will avoid these effects. Conversely, β-arrestin-biased ligands can induce beneficial pharmacology compared with unbiased agonist if on-target adverse events are linked to the G protein signaling cascades.

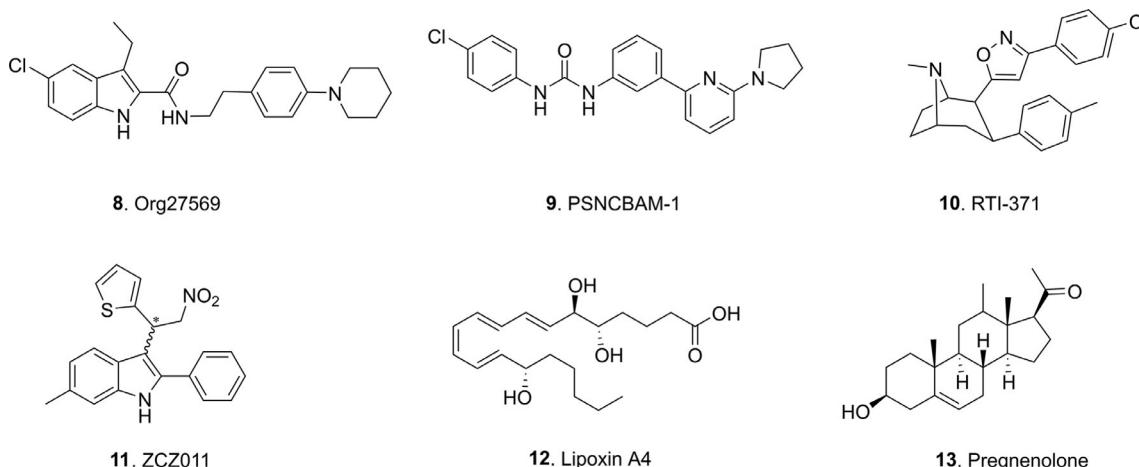
interpret and ascribe to a particular pathway; however, they can add valuable insight into the integrated cellular response and hence are included here.

The parameters obtained from in vitro assays for characterization of allosteric modulators mainly include: (1) the equilibrium dissociation constant of the allosteric ligand (i.e., K<sub>B</sub>); (2) the binding cooperativity between the allosteric ligand and the orthosteric ligand (i.e., α); (3) the factor of cooperativity between binding of orthosteric ligand and receptor activation (i.e., β); (4) the factor of cooperativity between the binding of allosteric modulator and receptor activation (i.e., γ); and (5) the factor of cooperativity between the binding of an allosteric modulator and orthosteric ligand-induced receptor activation (i.e., δ) (Christopoulos, 2002; Jakubík et al., 2020).

During the last 15 years, several structurally distinct molecules that allosterically modulate CB<sub>1</sub> have been discovered. The representative members are shown in Fig. 4. These include the small molecules 5-chloro-3-ethyl-N-(4-(piperidin-1-yl)phenethyl)-1*H*-indole-2-carboxamide (**8**, Org27569) (Price et al., 2005), 1-(4-chlorophenyl)-3-(3-(6-(pyrrolidin-1-yl)pyridin-2-yl)phenyl)urea (**9**, PSNCBAM-1) (Horswill et al., 2007), 3-(4-chlorophenyl)-5-(8-methyl-3-p-tolyl-8-azabicyclo[3.2.1]octan-2-yl)-isoxazole (**10**, RTI-371) (Navarro et al., 2009), 6-methyl-3-(2-nitro-1-(thiophen-2-yl)ethyl)-2-phenyl-1*H*-indole (**11**, ZCZ011) (Ignatowska-Jankowska et al., 2015), and the endogenous molecules lipoxin A<sub>4</sub> (**12**) (Pamplona et al., 2012) and pregnenolone (**13**) (Vallée et al., 2014) as well as some others (Lu et al., 2019).

### Biased signaling of Org27569 and its in vivo pharmacology

Further investigations of Org27569 by several groups revealed that Org27569 showed some preference among different signaling pathways (Ahn et al., 2012; Baillie et al., 2012; Cawston et al., 2013; Gamage et al., 2016; Khajehali et al., 2015). Following the initial characterization of Org27569 as an allosteric modulator of CB<sub>1</sub> (Price et al., 2005), the Kendall group



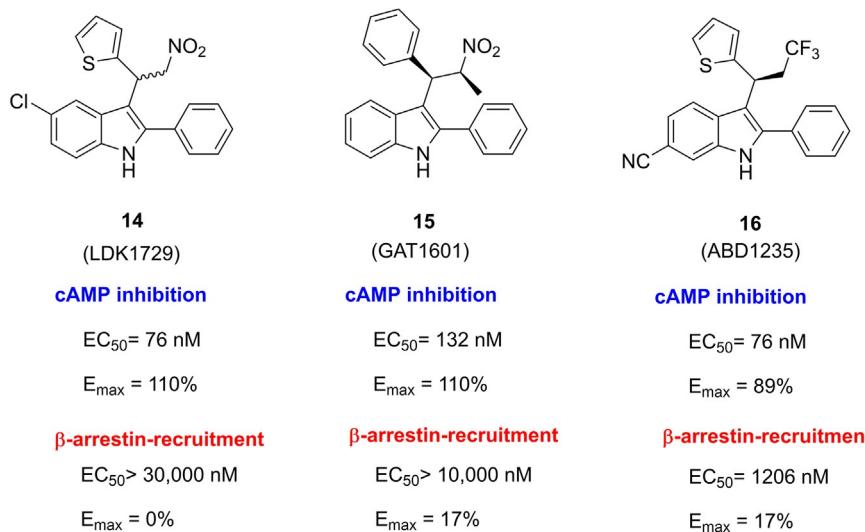
**FIG. 4** Structures of representative allosteric modulators of cannabinoid CB<sub>1</sub> receptor. Compounds **8–12** are exogenous allosteric modulators. Compounds **12** and **14** are endogenous allosteric modulators of CB<sub>1</sub>.

reported that Org27569 potentiated orthosteric agonist-induced receptor internalization in a mutant CB<sub>1</sub> receptor and the recruitment of β-arrestin2, promoting G<sub>(i)</sub>α protein-independent ERK phosphorylation (pERK) through a proposed β-arrestin1 pathway (Ahn et al., 2012, 2013) consistent with some PAM activity of Org27569 (outside of enhanced binding). Meanwhile, Org27569 was confirmed to abrogate a number of agonist-mediated G-protein pathways such as [<sup>35</sup>S]GTPγS binding and cAMP (Ahn et al., 2012). In contrast to the β-arrestin1-mediated pERK activation of Org27569 described above, others have only observed Org27569-mediated inhibition of orthosteric agonist-induced pERK (Gamage et al., 2016; Khajehali et al., 2015); while others have described a decrease in agonist-mediated receptor internalization (Cawston et al., 2013; Gamage et al., 2016). Intriguingly, allosteric effects of Org27569 may produce a different extent of allosteric modulation at different time points (Cawston et al., 2013), with a significant delay in the receptor moving to an inactive conformation following Org27569 binding (Cawston et al., 2013). Along with these findings, Christopoulos and colleagues provided evidences that the allosteric effects of Org27569 display probe dependence and pathway dependence (Khajehali et al., 2015). Thus, Org27569 is an allosteric modulator with complex receptor pharmacology.

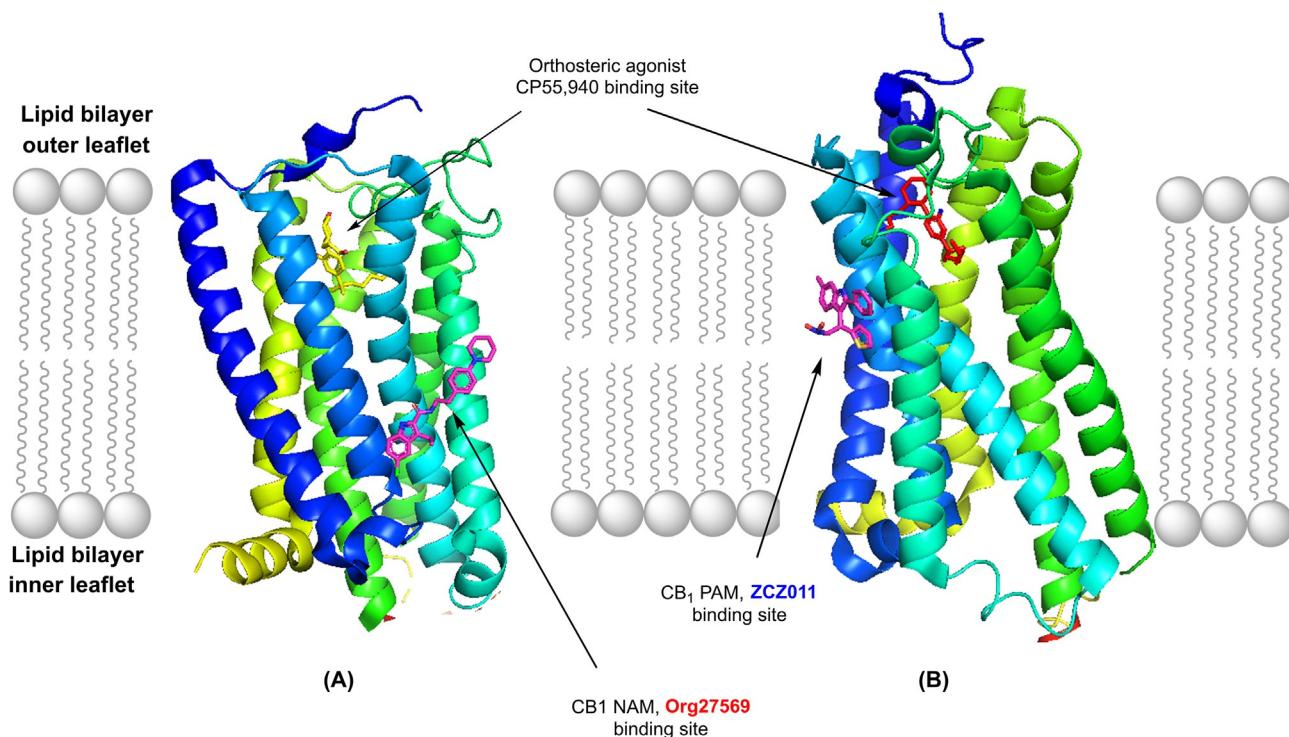
In *in vivo* mice studies, Org27569 was demonstrated to reduce food intake, but did not suppress analgesia, catalepsy, or hypothermia induced by CB<sub>1</sub> orthosteric agonists such as Δ<sup>9</sup>-THC, anandamide, or CP55,940 (Gamage et al., 2014). In rats, Org27569 also decreased food intake and inhibited hypothermia induced by CB<sub>1</sub> orthosteric agonists anandamide and CP55,940, but again, did not attenuate the catalepsy and analgesia induced by CP55,940 (Ding et al., 2014). The hypophagic effect of Org27569 in mice was shown to be CB<sub>1</sub>-independent (Ding et al., 2014; Gamage et al., 2014). Though the *in vitro* allosteric pharmacology of Org27569 was not translated into *in vivo* efficacious pharmacology of CB<sub>1</sub>-dependent effects in these limited animal studies, the discovery of Org27569 paved the way for the development of numerous novel allosteric modulators of CB<sub>1</sub>, which modulate orthosteric agonist activity in both positive and negative manner (Lu et al., 2019). Of these newly developed CB<sub>1</sub> allosteric modulators, several CB<sub>1</sub> PAMs derived from the ZCZ011 scaffold function as G protein-biased CB<sub>1</sub> PAMs, which include LDK1279 (Fig. 5, 14) (Immadi et al., 2019), GAT1601 (Fig. 5, 15) (Garai et al., 2021), and ABD1235 (Fig. 5, 16) (Tseng et al., 2019). These compounds potentiated G protein-mediated cAMP inhibition without stimulating β-arrestins recruitment (data shown in Fig. 5). Notably, LDK1279, GAT1601, and the racemate of ABD1235 showed no cannabimimetic adverse effects in mice. These signaling-biased CB<sub>1</sub> PAMs provide valuable tools for investigation of pharmacology from allosteric modulation of CB<sub>1</sub> and offered new leads for developing novel analgesics (Tseng et al., 2019) and antiglaucoma medications (Garai et al., 2021).

It has been reported that β-arrestin2 knockout mice displayed enhanced antinociceptive responses upon acute administration of Δ<sup>9</sup>-THC and decreased tolerance of the drug's antinociceptive effects but enhanced tolerance to the catalepsy induced by Δ<sup>9</sup>-THC (Nguyen et al., 2012). This evidence suggested that signaling-biased CB<sub>1</sub> PAMs are promising new CB<sub>1</sub> targeting therapeutics that may have reduced adverse effects such as motor function suppression and other drug-induced CNS liabilities.

The recent disclosure of crystal structures of CB<sub>1</sub> receptor bound with either a CB<sub>1</sub> PAM (i.e., ZCZ011) (Yang et al., 2022) or a CB<sub>1</sub> NAM (i.e., Org27569) (Shao et al., 2019) continue to enhance our understanding of novel conformations induced by CB<sub>1</sub> allosteric modulators. The X-ray crystallographic structure of CB<sub>1</sub> bound with Org27569 indicated that the



**FIG. 5** CB<sub>1</sub> PAMs showing biased signaling. The biased signaling can be reflected by the efficacy difference between the G protein-mediated cAMP inhibition and  $\beta$ -arrestins recruitments, which do not involve G proteins.



**FIG. 6** Allosteric modulators bind to the CB<sub>1</sub> receptor in the presence of orthosteric agonist CP55,940. (A) Binding mode of CB<sub>1</sub> negative allosteric modulator Org27569 on the CB<sub>1</sub> receptor bound with orthosteric agonist CP55,940. The image is reproduced using PyMOL 2.5.1 (Schrodinger LLC) and protein data bank (PDB) coordinate accession number 6KQI. (B) Binding mode of CB<sub>1</sub> positive allosteric modulator ZCZ011 on the CB<sub>1</sub> receptor bound with orthosteric agonist CP55,940. The image is reproduced using PyMOL 2.5.1 (Schrodinger LLC) and protein data bank (PDB) coordinate accession number 7FEE.

binding of Org27569 is in an extrahelical site in the TM2-TM3-TM4 surface inner leaflet of the lipid bilayer (Fig. 6A). ZCZ011 in the presence of CP55,940 binds to an extrahelical site in the TM2-TM3-TM4 surface in the upper leaflet of the lipid bilayer (Fig. 6B). These allosteric-binding sites are distinct from the orthosteric agonist binding site that accommodates either an orthosteric agonist (Hua et al., 2017) or an orthosteric antagonist (Hua et al., 2016). The structures of allosteric modulator bound receptors indicate the possibility of inducing pharmacology that is different from orthosteric ligand binding. These findings together with the results from in vitro pharmacological investigations suggest that allosteric modulation is an exciting path toward developing biased ligands, which may have therapeutic advantages over orthosteric agonists. For CB<sub>1</sub>, the question remains open as to which pathways are the most therapeutically desirable, but as new ligand tools are developed, we will be able to begin to decipher the complex pharmacology resulting from activation of CB<sub>1</sub> with signaling biased ligands.

## Conclusion

CB<sub>1</sub> is involved in the pathology of a variety of diseases and has been explored as a therapeutic target. Up- or downregulation of CB<sub>1</sub> function by orthosteric ligands has been pursued for the management of several disorders such as pain and obesity. However, the psychotropic and psychiatric side effects from CB<sub>1</sub> orthosteric ligands reduced the enthusiasm on the orthosteric ligands as potential therapeutic agents. The discovery of Org27569 as an allosteric modulator of CB<sub>1</sub> revealed a novel and perhaps a druggable target based on the pleiotropic pharmacology of CB<sub>1</sub>. The complex signaling pathways of CB<sub>1</sub> and the stronger capacity of allosteric modulators to induce biased signaling offer greater opportunities to preferentially activate signaling pathways that mediate therapeutic effects over the pathways responsible for adverse effects. Along with the discovery and development of more CB<sub>1</sub> allosteric modulators, evidence of signaling bias induced by CB<sub>1</sub> allosteric modulators is growing. It is foreseeable that safer and more efficacious drugs could be developed from targeting the allosteric binding sites of the pharmacologically and physiologically important receptor.

## Applications to other areas

In this chapter, we have reviewed CB<sub>1</sub> receptor and its signaling complexity, which is the underpinning for ligand-induced biased signaling or so-called functional selectivity. The cellular response from activation of CB<sub>1</sub> receptor can be mediated by G protein or non-G protein partners such as β-arrestins. Biased signaling provides a unique opportunity for separation of therapeutic effects from side effects. In addition to the orthosteric ligands, allosteric ligands can also regulate the function of CB<sub>1</sub>. In comparison with orthosteric ligands, allosteric ligands including positive and negative allosteric modulators offer better capacity to induce biased signaling, which provides a mechanism for separation of therapeutic effects from side effects that have been associated with orthosteric activation of CB<sub>1</sub>. At present, allosteric modulators that are G protein-biased have been identified. The biased CB<sub>1</sub> allosteric modulators have been found to produce therapeutic effects in several clinically relevant indications that have unmet medical needs such as pain, neurodegenerative diseases, and glaucoma. Thus, they are speculated to provide novel tools for deciphering novel pharmacology from allosteric modulation of CB<sub>1</sub>. CB<sub>1</sub> allosteric modulators provide lead compounds for the discovery and development of new non-addictive analgesics and antiglaucoma drugs.

## Mini-dictionary of terms

**Allosteric ligands:** Ligands that bind to a receptor site different from where endogenous ligands bind to a receptor.

**Biased signaling (Functional selectivity):** Signaling pathways induced by some ligands that preferentially couple to a subset of the possible intracellular signaling proteins and therefore selectively evoke certain responses over others.

**Constitutive activity:** Impulsive activation of a receptor in the absence of an agonist.

**Cannabinoid Tetrad Tests:** A series of behavioral tests in rodents to assess the drug's capacity to show cannabinoid (like THC) effects such as to induce catalepsy, hypothermia, and antinociception.

**G protein:** Guanosine nucleotide-binding proteins.

**G protein-coupled receptor:** A family of seven transmembrane cell surface proteins, which respond to external stimuli by coupling (primarily) to intracellular G proteins.

**Inverse agonist:** A ligand binds to a receptor and causes a response opposite to what is caused by an agonist.

**Orthosteric ligands:** Ligands that bind to the site where endogenous ligands bind to a receptor.

**Phytocannabinoids:** Cannabinoids isolated from plant cannabis.

## Key facts of cannabinoid receptors

- The cannabinoid receptors CB<sub>1</sub> and CB<sub>2</sub> are the biological targets that mediate most of the pharmacological effects of endocannabinoids (e.g., AEA & 2-AG), phytocannabinoids (e.g., THC) and synthetic cannabinoids (e.g., CP55,940). Activation of the receptors by endogenous or exogenous agonists triggers a series of intracellular signaling responses.
- Though the cannabinoid receptors mainly coupled to G protein to mediate signal transduction, they can interact with non-G protein partners to transduce signals cross cell membrane.
- The CB<sub>1</sub> orthosteric agonists such as THC, CP55,940, and WIN55,212-2 can elicit psychoactive effects once bind to the orthosteric site where the endogenous cannabinoids such as *N*-arachidonoyl ethanolamine and 2-arachidonyl glycerol bind.

## Key facts of allosteric modulators of cannabinoid receptors

- Allosteric modulators bind to the site different from where endocannabinoids bind and can induce pharmacology different from orthosteric ligands.
- Generally, allosteric modulators can induce biased signaling, which activates a subset of signaling pathways while spare other signaling transmissions.
- Allosteric modulators can upregulate or downregulate the functions of CB<sub>1</sub> receptor and therefore can be classified as positive and negative modulators.
- Allosteric modulators can generate pharmacological effects by alternating the binding or efficacy of orthosteric ligands. They can also produce pharmacological effects on their own without the presence of orthosteric ligands.

## Summary points

- Cannabinoid CB<sub>1</sub> receptor is a G protein-coupled receptor, which transduces signals cross cell membrane mainly by G protein-mediated process.
- Orthosteric activation of CB<sub>1</sub> results in a complex of signaling events including inhibition of adenylyl cyclase and activation of various protein kinases and recruitment of β-arrestins.
- The functions of CB<sub>1</sub> can be regulated by orthosteric ligands including agonists and inverse agonists/antagonists.
- Activation of CB<sub>1</sub> by orthosteric agonists leads to psychotropic effects.
- Allosteric modulation of CB<sub>1</sub> can lead to biased signaling, which preferentially regulates a subset of downstream signaling events.
- Characterization of allosteric modulators involves multiple in vitro and in vivo assays.
- Allosteric modulators showed less adverse effects than orthosteric ligands.
- Positive allosteric modulators of CB<sub>1</sub> exhibit therapeutic potentials for the treatment of pain, glaucoma, and neurodegenerative diseases.

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## Chapter 29

# Naturally occurring delta-9-tetrahydrocannabinol derivatives and binding to CB<sub>1</sub> and CB<sub>2</sub> receptors: Linking in the endocannabinoid system

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## Abbreviations

$\Delta^8$ -THC	$\Delta^8$ -tetrahydrocannabinol
$\Delta^9$ -THC	$\Delta^9$ -tetrahydrocannabinol
$\Delta^9$ -THCA	$\Delta^9$ -tetrahydrocannabinolic acid
$\Delta^9$ -THCAA	$\Delta^9$ -tetrahydrocannabinolic acid A
$\Delta^9$ -THCAA-C4	$\Delta^9$ -tetrahydrocannabinolic acid A-C4
$\Delta^9$ -THCAB	$\Delta^9$ -tetrahydrocannabinolic acid B
$\Delta^9$ -THCB	$\Delta^9$ -tetrahydrocannabutol
$\Delta^9$ -THC-C4	$\Delta^9$ -tetrahydrocannabinol-C4
$\Delta^9$ -THCH	$\Delta^9$ -tetrahydrocannabihexol
$\Delta^9$ -THCO	$\Delta^9$ -tetrahydrocannabiorcol ( $\Delta^9$ -THC1)
$\Delta^9$ -THCOAA	$\Delta^9$ -tetrahydrocannabiorcolic acid
$\Delta^9$ -THCP	$\Delta^9$ -tetrahydrocannabiphorol
$\Delta^9$ -THCV	$\Delta^9$ -tetrahydrocannabivarin
$\Delta^9$ -THCVAA	$\Delta^9$ -tetrahydrocannabivarinic acid A
11-OH- $\Delta^9$ -THC	11-hydroxy- $\Delta^9$ -tetrahydrocannabinol
2-AG	2-arachidonoyl glycerol
CB <sub>1</sub> R	cannabinoid receptor 1
CB <sub>2</sub> R	cannabinoid receptor 2
CBC	cannabichromene
CBD	cannabidiol
CBDA	cannabidiolic acid
CBG	cannabigerol
CBGA	cannabigerolic acid
CBN	cannabinol
CNS	central nervous system
FAAH	fatty acid amide hydrolase
FRET	fluorescence resonance energy transfer
GPCR	G-protein-coupled receptor

\* Equal contribution.

<b>HTRF</b>	homogenous time-resolved FRET
<b>NAPE</b>	arachidonoyl phosphatidylethanolamine
<b>NSB</b>	nonspecific binding
<b>PC-PLC</b>	phosphatidylcholine-specific phospholipase C
<b>THCAS</b>	delta-9-tetrahydrocannabinolic acid synthase
<b>TR-FRET</b>	time-resolved FRET

## Introduction

Cannabinoids are among the most used compounds throughout human history due to their wide possibilities in the field of medicine and pharmacology. More than 560 compounds have been extracted from *Cannabis sativa* plant, such as phytocannabinoids (more than 120), terpenes, and phenolic compounds (ElSohly et al., 2017). Delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC) was the first isolated active constituent, in 1960, and it is the responsible of the main psychoactive effects of the plant (Gaoni & Mechoulam, 1964).  $\Delta^9$ -THC is hydroxylated to 11-hydroxy- $\Delta^9$ -THC, the psychoactive compound, and then oxidized to the  $\Delta^9$ -THC-11-oic acid that is not psychoactive (Schurman et al., 2020).  $\Delta^9$ -THC together with cannabidiol (CBD) represents the most studied phytocannabinoids. However, cannabigerol (CBG), cannabichromene (CBC), tetrahydrocannabivarin ( $\Delta^9$ -THCV), and the acid forms of  $\Delta^9$ -THC and CBD,  $\Delta^9$ -THCA and CBDA, represent other predominant constituents.

Cannabinoid compounds activate the endocannabinoid system by binding to cannabinoid receptors, CB<sub>1</sub> and CB<sub>2</sub>. CB<sub>1</sub>R is the most abundant membrane receptor in the Central Nervous System (CNS), being predominantly expressed in presynaptic neurons where it regulates neurotransmitter release through a retrograde activation.

The determination of the crystal structures of this receptor bound to agonist or antagonist has helped to further characterize the binding pocket, revealing the relevance of the transmembrane helices, N-terminal tail, and extracellular loops (Hua et al., 2016; Shao et al., 2016). These receptors are responsible for most of the cannabinoid psychoactive effects. On the other hand, CB<sub>2</sub>R is mainly expressed in immune cells and in few neurons.

The endocannabinoid system is formed by cannabinoid receptors, endocannabinoid ligands, with arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG) being the most abundant and well-described endocannabinoids and the synthesis, transport, and degradation enzymes. Anandamide and 2-AG are eicosanoids synthesized on demand from arachidonic acid-containing phospholipids (Di Marzo & De Petrocellis, 2012). The endocannabinoid metabolic pathways are complex due to the number of metabolites acting on different targets and the enzymes involved in their synthesis and degradations. On the one hand, anandamide is mainly synthesized by phospholipase D from its membrane precursor arachidonoyl phosphatidylethanolamine (NAPE) and degraded by the fatty acid amide hydrolase (FAAH) enzyme (Toczek & Malinowska, 2018). On the other hand, 2-AG, the most abundant endocannabinoid, which plays an important role in CNS development and synaptic plasticity (Murataeva et al., 2014), is mainly synthesized by phospholipase C $\beta$  and a diacylglycerol lipase from phospholipids. 2-AG degradation is complex and implicates more than one enzyme. 2-AG is a full agonist for both cannabinoid receptors while anandamide is a partial agonist.

The endocannabinoid system is implicated in motivation, memory, superior cognitive processes, and movement. It also regulates physiological processes such as pain, appetite, pleasure, and reward.

To date, FDA has approved Marinol and Syndros that contain dronabinol, a synthetic  $\Delta^9$ -THC for nausea associated with cancer chemotherapy and for the treatment of anorexia associated with weight loss in AIDS patients. Also Cesamet, which contains nabilone, with a chemical structure similar to  $\Delta^9$ -THC has been approved for nausea associated with cancer chemotherapy. However, the pharmacological potential of cannabinoid compounds is very high, and new research is required to achieve treatment of new pathologies avoiding the undesired psychoactive effect.

## History of cannabinoids

Cannabis, commonly known as marijuana, has been used for millennia due to its therapeutic potential and its anxiolytic and euphoric properties. The oldest known written document on cannabis use comes from the Chinese Emperor Shen Nung in 2727 BC. The Greek and Roman civilizations also became familiar with the use of this plant, using its seeds in religious ceremonies and being its properties described in religious scriptures (Touw, 1981). Years later, its use spread with nomadic people through the Islamic empire in the North of Africa. They used cannabis to treat different symptoms and pathologies such as rheumatism, inflammation, and malaria (Zuardi, 2006). In the 16th century, Spaniards produced rope, clothing, and

paper from cannabis fiber, and they exported the plant to Chile. However, cannabis therapeutic used was not discovered in these countries until mid-19th century (Di Marzo, 2006). The Irish physician William O'Shaughnessy published the first scientific report on cannabis therapeutic properties and safety in pathologies such as infantile convulsions and cholera (Haspula & Clark, 2020).

The first compound isolated from *C. sativa* was cannabidiol (CBD) in 1940, and its structure was described in 1963 (Burstein, 2015). One year later, due to the fact that CBD is not psychoactive, its interest was eclipsed by the isolation of  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC), which is responsible for the main psychoactive effects of the plant (DeVono & Parker, 2020; Mechoulam & Parker, 2013). Specifically, Raphael Mechoulam, working at Weizmann Institute, used a column to separate different compounds from cannabis that were tested in monkeys and then in volunteers before discovering  $\Delta^9$ -THC as an inducer of psychoactive effects. The structure of  $\Delta^9$ -THC was determined in Israel by Mechoulam and Gaoni in 1964 (Crocq, 2020).

This discovery led to new investigations and to the discovery of the endocannabinoid system. The first cannabinoid receptor, named cannabinoid receptor 1 (CB<sub>1</sub>R), was described and characterized in rat and human brains in 1980 by Howlett's group (Devane et al., 1988). The same group described in 1992 the first endocannabinoid, arachidonoylethanolamide, or anandamide (Devane et al., 1992) from the word *ānanda*, which means bliss, happiness, or pleasure (Crocq, 2020). Soon the existence of a second cannabinoid receptor named cannabinoid receptor 2 (CB<sub>2</sub>R) or the peripheral cannabinoid receptor (due to the low expression of this receptor in the brain) (Munro et al., 1993) was discovered. CB<sub>2</sub>R is mainly expressed in immune cells and glia and at low levels in some neurons. However, its expression is upregulated under some pathological processes, acquiring a relevant role. A singular characteristic of the endocannabinoid system is its retrograde signaling; endocannabinoids are synthesized in the postsynaptic neuron in response to calcium second messenger and released to the synaptic space to bind presynaptic receptors. However, cannabinoid receptors are not only expressed in the presynaptic neurons but also in the postsynaptic.

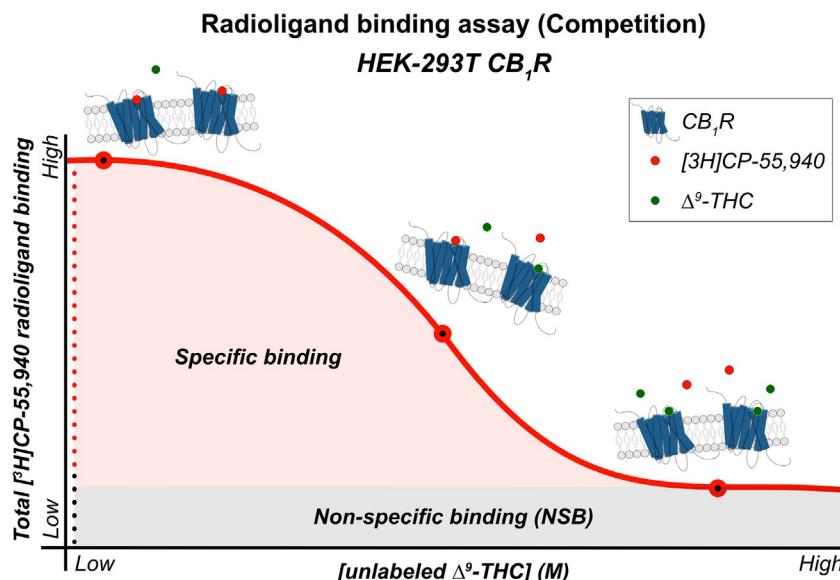
To date, cannabinoid compounds are one of the most consumed drugs worldwide, with their beneficial effects being numerous and undoubted, although their psychoactive effect keeps them illegal in many countries. However, the huge advances in the last years will maintain the debate open for the next few years.

## Radioligand-binding assays

Radioligand-binding assays are the most sensitive experimental procedures, to date, to detect the affinity of a ligand for a receptor (Flanagan, 2016). For this reason, radioligand-binding assays are the main type of assays used to characterize the binding of a ligand to a GPCR, and they can also be used in the case of orphan receptors, i.e., for which no endogenous ligand is known (Al Mahmud et al., 2017), in case a ligand is suspected to bind to these receptors. Radioligand-binding assays are also used to study the binding of new compounds from the pharmaceutical industry in receptors of therapeutic interest, such as the receptors of the cannabinoid system (Finlay et al., 2020). Other reachable purposes using radioligand-binding assays are the characterization of allosteric binding sites, capable of modulating receptor functionality (Martínez-Pinilla et al., 2017) or determining the anatomical distribution of receptors using an autoradiography (Simmons et al., 2021).

There are different types of radioligand-binding assays; saturation assays where the sample containing the receptor of interest is incubated in increasing concentrations of the radiolabeled ligand to determine its affinity; kinetic assays that allow to calculate the rate of association to or dissociation from the receptor of the radiolabeled ligand, competition or indirect assays where a constant concentration of radioligand is incubated with an increasing concentration of unlabeled ligand (Fig. 1) of interest; and the autoradiography assays that allow to determine the distribution of a receptor in a tissue section (Flanagan, 2016; Walker, 2012).

Radioligand-binding assays are performed in cell membrane extracts from transfected cells, tissue sections, or homogenates containing the receptor of interest. Samples are prepared and incubated with the radiolabeled ligand at determined temperature and time conditions to reach equilibrium. The temperature of the assays is usually room temperature (Aronstam & Narayanan, 1988), although there can be exceptions, such as in the case when enzymatic degradation needs to be prevented, where assay temperature would be 4°C. Usual incubation time for labeled ligand is around 120 min (Khoramjouy et al., 2021; Walker, 2012). However, it can change between 20 and 240 min. In addition, bovine serum albumin is typically added in the radioligand-binding assay buffer to reduce the nonspecific binding (NSB) of the radio-labeled ligand used in the experiment (Walker, 2012). At the end of the incubation time, the mixture is filtered to eliminate unbound radioligand, and after several washes, it is possible to measure total radioactivity in the sample coming from the bound ligand.



**FIG. 1** Scheme of a competition radioligand-binding assay in HEK-293T cells transfected with CB<sub>1</sub>R. Cells are incubated with a constant amount of [<sup>3</sup>H]CP-55,940 radioligand and, subsequently, are incubated with an increasing concentration of unlabeled Δ<sup>9</sup>-THC. NSB signal is usually produced for unspecific interactions of labeled ligand with lipids or other membrane proteins.

Most radioligands are labeled with [<sup>3</sup>H] isotopes, which are cheaper and commonly very stable, with a half-life of more than 12 years (Bylund & Toews, 1993), or [<sup>125</sup>I], the latter having higher radioactivity (Luo et al., 2020). However, in certain circumstances, compounds for radioligand-binding assays can be labeled with other isotope species such as <sup>35</sup>S (Strange, 2010) or <sup>32</sup>P (Galiano et al., 2014). One of the most widely used radioligands for the study of cannabinoid receptors is [<sup>3</sup>H]CP-55,940, capable of binding both CB<sub>1</sub> and CB<sub>2</sub> receptors. By using [<sup>3</sup>H]CP-55,940 in a receptor binding experiment, it was possible to resolve the affinity for CB<sub>1</sub>R and CB<sub>2</sub>R of the main phytocannabinoids, including Δ<sup>9</sup>-THC and Δ<sup>9</sup>-THCA, in neural cell cultures (Rosenthaler et al., 2014).

Unfortunately, despite the wide spectrum of applications, radioligand-binding assays have several limitations (Flanagan, 2016) intrinsic to the procedure that must be considered, such as the impossibility in a competition binding assay to distinguish whether the compound has antagonist or agonist activity on receptor signaling, or the challenge to dispose of a labeled ligand for the receptor of interest, either commercially available or laboratory-based labeling. Furthermore, the requirement to dispose of appropriate facilities for the handling of radioactive compounds is another burden.

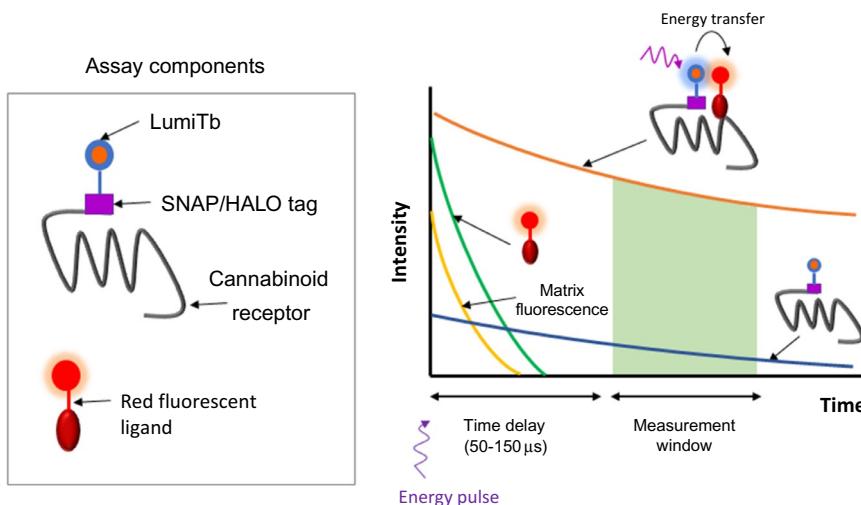
## Homogeneous time-resolved binding assays

The use of radioactive methods to study ligand binding to a receptor has drawbacks as the use of radioactivity or the long and tedious process of isolating cell membrane. In the last decade, a new technique to assess receptor-ligand interaction has emerged, namely Homogenous Time-Resolved FRET (HTRF) (Degorce et al., 2009; Emami-Nemini et al., 2013; Martínez-Pinilla et al., 2016).

Time-resolved FRET (TR-FRET) is based on the fluorescence resonance energy transfer (FRET) between a pair of donor and acceptor fluorophores. When two molecules come close enough to each other, excitation of the donor by an energy source (e.g., a flash lamp or a laser) triggers an energy transfer toward the acceptor, which in turn emits specific fluorescence at a given wavelength.

TR-FRET combines standard FRET technology with time-resolved measurement of fluorescence, which is based on a time delay between the system excitation and the fluorescence measurement, thus eliminating short-lived background fluorescence (Bazin et al., 2001, 2002).

In traditional FRET chemistries, the signal is hampered by background fluorescence from sample components such as buffers, proteins, chemical compounds, and cell lysate. However, this type of background fluorescence is extremely transient (with a lifetime in the nanosecond range) and can therefore be eliminated using time-resolved methodologies. In contrast, TR-FRET acceptors emit long-lived fluorescence when engaged in an FRET process. Introducing a time delay of 50–150 µs between the system excitation and fluorescence measurement allows the signal to be cleared of all nonspecific short-lived emissions as it allows interfering short-lived fluorescence (compounds, proteins, medium, etc.)



**FIG. 2** Scheme of an HTRF-based binding assay. *Left:* Components of an HTRF assay to study ligand binding to cannabinoid receptors. *Right:* Introducing a time delay of 50–150  $\mu$ s between the system excitation and fluorescence measurement allows the signal to be cleared of all nonspecific short-lived emissions as it allows interfering short-lived fluorescence (compounds, proteins, medium) to decay.

to decay (Fig. 2). This is possible thanks to a special kind of compounds acting as FRET donors, called lanthanide cryptates. They consist of a rare earth complex in which the lanthanide ion (Europium or Terbium) is tightly embedded in a macrocycle. Both cryptates show long-lived emission in the range of 1–2 ms, a characteristic that is fundamental for a time-resolved detection (Bazin et al., 2001, 2002).

HTRF combines TR-FRET advantages with the possibility of doing homogeneous assays. Homogeneous assays do not require separation steps such as centrifuging, washing, filtration, or magnetic partitioning, like in classic radioligand-binding assays. It also allows perform the assay on live cells.

The covalent labeling of the receptor with the cryptate is performed through Tag-Lite Technology (Zwier et al., 2010), which is based on click chemistry. The first step consists of expressing on the cell membrane the cannabinoid receptor fused to a tag, such as SNAP-tag or HALO-tag, which are enzymes. On the day of the experiment, cells are incubated with the cryptate bound to a substrate of the SNAP-tag enzyme. By means of an enzymatic reaction between SNAP and its substrate, SNAP will metabolize its substrate and transfer part of this dye-coupled substrate to itself. At the end of this irreversible process, the enzyme is no longer active, and the donor of the TR-FRET will be covalently bound to our receptor (Fig. 2). As FRET acceptor, a cannabinoid agonist/antagonist covalently bound to a red or green fluorescent molecule is used (Cottet et al., 2013).

To perform the assay, live cells expressing the receptor of interest, already labeled with the donor, are loaded in a microplate, immediately followed by addition of the labeled/nonlabeled ligands. After 2 h incubation at room temperature, fluorescence is detected on a plate reader.

## Delta-9-tetrahydrocannabinol and its main derived compounds

So far, more than 100 cannabinoid compounds extracted from *C. sativa* L. have been isolated and can be chemically classified under the category of terpenophenols (Amin & Ali, 2019). These compounds are named as phytocannabinoids (Franco et al., 2020). The effects produced in the human organism by their consumption are mainly because they interact on the receptors of the endocannabinoid system, the G-protein-coupled cannabinoid receptor type 1 (CB<sub>1</sub>R) (Shao et al., 2016) and type 2 (CB<sub>2</sub>R) (Yeliseev, 2019).

Delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC) is the primary psychoactive constituent and one of the main phytocannabinoids of *C. sativa* L. plant, along with the nonpsychoactive cannabidiol (CBD) and cannabinol (CBN). First isolation of pure  $\Delta^9$ -THC took place from an extract of hashish using a column chromatography by Gaoni and Mechoulam in 1964 (Gaoni & Mechoulam, 1964).  $\Delta^9$ -THC has a tri-cyclic 21-carbon structure without nitrogen and with two chiral centers in transconfiguration (Gaoni & Mechoulam, 1964). Although  $\Delta^9$ -THC can also undertake several chemical reactions (Duggan, 2021), the most relevant ones are the isomerization to Delta-8-tetrahydrocannabinol ( $\Delta^8$ -THC) through rearrangement of the  $\Delta^9$ -double bond and the oxidation to the fully aromatic CBN compound.

$\Delta^9$ -THC is a particular phytocannabinoid, as it has been shown to interact not only with CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors but also with many others such as the opioid (Pisanu et al., 2006) or benzodiazepine (Mokler et al., 1986)

receptors, contributing in part to its psychotropic effects. In the human body,  $\Delta^9$ -THC is rapidly absorbed when inhaled through the lungs. In the liver, it is processed producing the psychoactive compound 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH- $\Delta^9$ -THC) and further oxidation generates the inactive form ( $\Delta^9$ -THC-COOH) that it is detectable in plasma for several days.  $\Delta^9$ -THC-COOH is then further processed in the kidneys and excreted in the urine (Sharma et al., 2012).

Delta-9-tetrahydrocannabinolic acid synthase (THCAS) from *C. sativa* L. is an enzyme with flavin-dependent oxidase activity capable of catalyzing the conversion of cannabigerolic acid (CBGA) into  $\Delta^9$ -tetrahydrocannabinolic acid ( $\Delta^9$ -THCA), the direct and principal nonpsychoactive precursor of  $\Delta^9$ -THC by decarboxylation (Lange et al., 2016) (Fig. 3).  $\Delta^9$ -THCA was discovered in 1965 by Korte et al. (1965), and due to its difficulty to crystallize, it has not been possible to accomplish this until recently (Skell et al., 2021). It is important to highlight that the isomer of  $\Delta^9$ -THCA,  $\Delta^9$ -tetrahydrocannabinolic acid B ( $\Delta^9$ -THCAB) is a minoritary phytocannabinoid in which the carboxylic acid group is in the position 3, following the carbon chain.  $\Delta^9$ -THCAB is also able to decarboxylate to  $\Delta^9$ -THC (Filer, 2022).

A few years later, in 1970, Delta-9-tetrahydrcannabivarin ( $\Delta^9$ -THCV), another minor cannabinoid compound, was discovered in *C. sativa* L. extract (Fig. 3). The only chemical difference between  $\Delta^9$ -THC and  $\Delta^9$ -THCV is the presence of a propyl group linked to the aromatic moiety in  $\Delta^9$ -THCV instead of the pentyl group present in  $\Delta^9$ -THC (Gul et al., 2018).

$\Delta^9$ -THC and its derivatives have powerful and very interesting medicinal applications. In general,  $\Delta^9$ -THC and related-cannabinoids such as  $\Delta^9$ -THCA and  $\Delta^9$ -THCV have a significant antioxidant power, similar to vitamin E (Dawidowicz et al., 2021). Also, these THC-related compounds exert an antiinflammatory power (Pattnaik et al., 2022), in fact, the

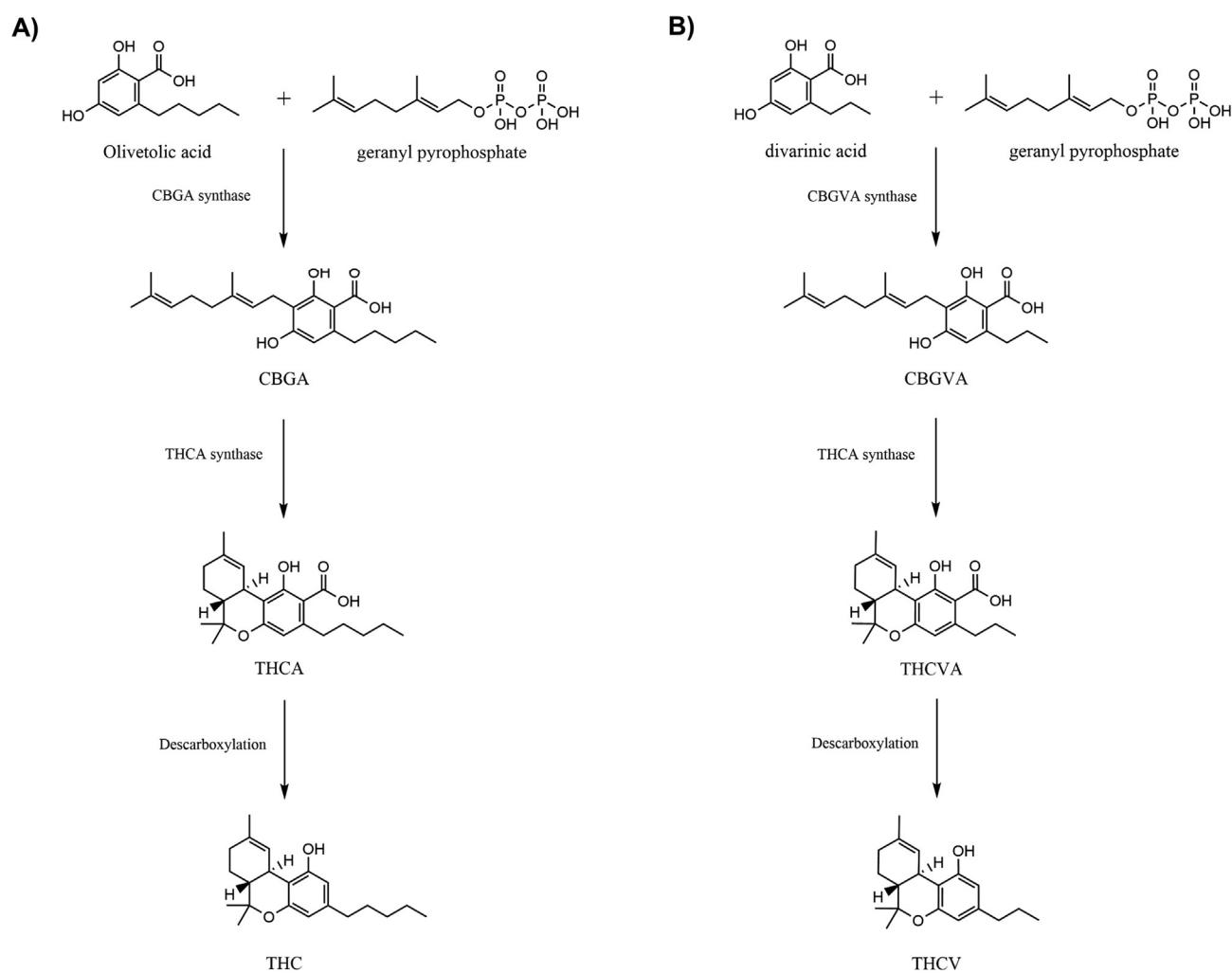


FIG. 3 Biosynthetic pathways of phytocannabinoids  $\Delta^9$ -THC,  $\Delta^9$ -THCA, and  $\Delta^9$ -THCV.

use of cannabinoid compounds for the modulation of the immune response through CB<sub>2</sub>R, naturally present in immune cells, is a hot topic in the scientific community (Becker et al., 2021; Oláh et al., 2017).

Overall, the consideration of Δ<sup>9</sup>-THC-related compounds, which lack unwanted psychotropic effects such as Δ<sup>9</sup>-THCA or Δ<sup>9</sup>-THCV, is one of the most promising avenues of current research to safety treat diseases through interactions with cannabinoid receptors in the human organism.

See Table 1 for facts about the main Δ<sup>9</sup>-THC-related compounds.

## Δ<sup>9</sup>-Tetrahydrocannabinolic acid (Δ<sup>9</sup>-THCA)

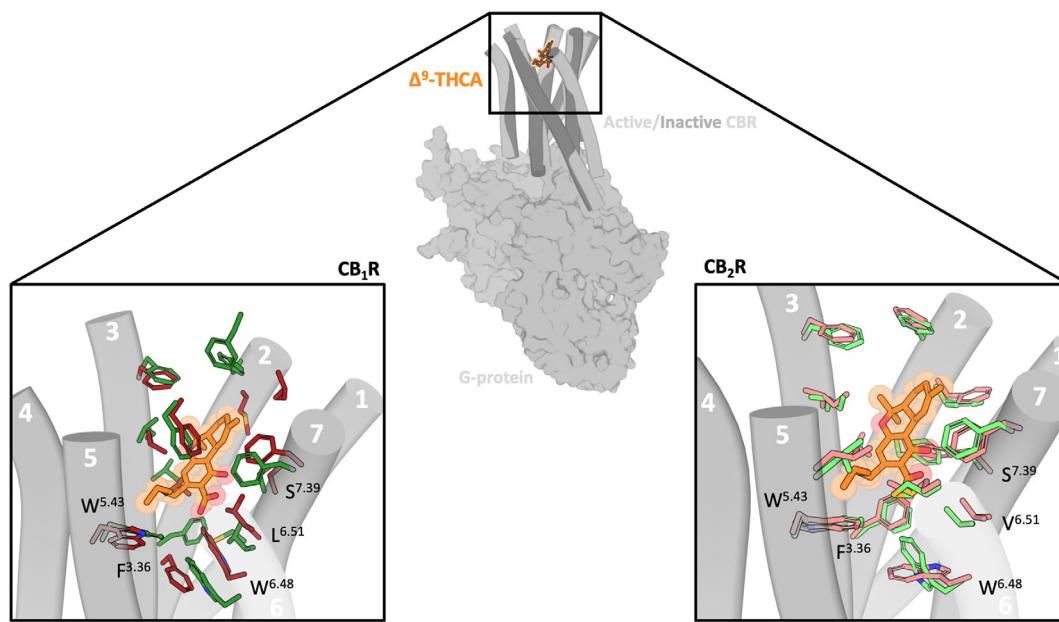
Δ<sup>9</sup>-THCA is a precursor of Δ<sup>9</sup>-THC that lacks psychotropic effects in humans.

This compound is able to bind both CB<sub>1</sub>R and CB<sub>2</sub>R, as well as to the CB<sub>1</sub>R-CB<sub>2</sub>R heteromer (McPartland et al., 2015; Palomares et al., 2020; Raïch et al., 2021; Rosenthaler et al., 2014; Zagzoog et al., 2020). A study using HTRF assays shows affinities of Δ<sup>9</sup>-THCA for CB<sub>1</sub>R and CB<sub>2</sub>R in the low micromolar range. Δ<sup>9</sup>-THCA was able to displace fluorescent CELT-335 from HEK-293T cells expressing SNAP-CB<sub>1</sub>R and fluorescent CM157 from HEK-293T cells expressing SNAP-CB<sub>2</sub>R, with pK<sub>i</sub>s of 5.8 ± 0.6 (*K<sub>i</sub>* = 1.6 μM) and 5.2 ± 0.5 (*K<sub>i</sub>* = 6.3 μM), respectively (Raïch et al., 2021). The fact that affinities of Δ<sup>9</sup>-THCA are lower than those of Δ<sup>9</sup>-THC or Δ<sup>9</sup>-THCV, which are in the nanomolar range, would be consistent with the restraint posed by the charge due to the carboxylic acid in Δ<sup>9</sup>-THCA (Figs. 3 and 4). When using HEK-293T cells expressing SNAP-CB<sub>2</sub>R in the presence of CB<sub>1</sub>R, the competition curves were similar as for SNAP-CB<sub>2</sub>R-expressing cells (Raïch et al., 2021).

Other studies using radiolabeled ligands and HEK-293T cell membranes describe similar results for CB<sub>1</sub>R and CB<sub>2</sub>R, showing pK<sub>i</sub> values that differ by 1.1 from data obtained by HTRF (McPartland et al., 2015; Palomares et al., 2020). A

**TABLE 1** Summary of the most relevant delta-9-tetrahydrocannabinol-related compounds.

Cannabinoids	Psychoactive agent	Key statements	References
Cannabigerolic acid (CBGA)	No	<ul style="list-style-type: none"> <li>Common biosynthetic precursor for all the most abundant phytocannabinoids.</li> <li>Antiinflammatory and antimicrobial effects with analgesic activities.</li> </ul>	Pattnaik et al. (2022)
Δ <sup>9</sup> -Tetrahydrocannabinolic acid (Δ <sup>9</sup> -THCA)	No	<ul style="list-style-type: none"> <li>Derived from CBGA.</li> <li>Direct and principal precursor of Δ<sup>9</sup>-THC.</li> </ul>	Korte et al. (1965), Skell et al. (2021)
Δ <sup>9</sup> -Tetrahydrocannabinolic acid B (Δ <sup>9</sup> -THCAB)	No	<ul style="list-style-type: none"> <li>Isomer of Δ<sup>9</sup>-THCA and precursor of Δ<sup>9</sup>-THC.</li> </ul>	Filer (2022)
Delta-9-tetrahydrocannabinol (Δ <sup>9</sup> -THC)	Yes	<ul style="list-style-type: none"> <li>Main psychoactive component of the Cannabis plant.</li> <li>High potency for CB<sub>1</sub> and CB<sub>2</sub> receptors.</li> </ul>	Franco et al. (2020)
11-Hydroxy-Δ <sup>9</sup> -tetrahydrocannabinol (11-OH-Δ <sup>9</sup> -THC)	Yes	<ul style="list-style-type: none"> <li>Derived from Δ<sup>9</sup>-THC, liable for psychological effects of Cannabis in the human organism.</li> </ul>	Sharma et al. (2012)
11-Nor-9-carboxy-Δ <sup>9</sup> -tetrahydrocannabinol (Δ <sup>9</sup> -THC-COOH)	No	<ul style="list-style-type: none"> <li>Inactive form produced by human metabolism to facilitate excretion of Δ<sup>9</sup>-THC.</li> </ul>	Sharma et al. (2012)
Cannabinol (CBN)	Yes	<ul style="list-style-type: none"> <li>It is produced by oxidation of Δ<sup>9</sup>-THC.</li> <li>Less psychotropic than Δ<sup>9</sup>-THC.</li> <li>Sedative properties, antibacterial and antiinflammatory effects.</li> </ul>	Pattnaik et al. (2022)
Delta-8-tetrahydrocannabinol (Δ <sup>8</sup> -THC)	Yes	<ul style="list-style-type: none"> <li>It is produced by rearrangement of the Δ<sup>9</sup>-double bond of Δ<sup>9</sup>-THC.</li> </ul>	Duggan (2021)
Delta-9-tetrahydrocannabivarin (Δ <sup>9</sup> -THCV)	No	<ul style="list-style-type: none"> <li>Derived compound from Δ<sup>9</sup>-THC.</li> <li>It has a propyl group linked to the aromatic moiety.</li> </ul>	Abioye et al. (2020)



**FIG. 4**  $\Delta^9$ -THCA docking to CB<sub>1</sub> and CB<sub>2</sub> receptors. Central panel shows the general location of the ligand (orange) inside the receptors binding pocket. In light gray, the active state receptor with G-protein bound in its intracellular site. In dark gray, the inactive state receptor. Zoom-in panels show closer details of the possible interactions between the ligand and the residues defining the receptor-binding pocket in the CB<sub>1</sub>R (left, active state—dark green PDB ID 6KPG; and inactive state—dark red PDB ID 5TGZ) and CB<sub>2</sub>R (right, active state—lime PDB ID 6KPF; and inactive state—salmon PDB ID 5ZTY). Some predicted key residues are numbered using Ballesteros numbering.

difference of p*Ki* of about 1 (10-fold difference in *Ki*) is tolerable considering that two different experimental approaches have been undertaken. However, other studies also using radiolabeled ligands and CHO or Sf9 cell membranes describe higher affinities of  $\Delta^9$ -THCA for CB<sub>1</sub>R and CB<sub>2</sub>R, which are in the low nanomolar range (Rosenthaler et al., 2014; Zagzoog et al., 2020). The reasons explaining these discrepancies are not evident.

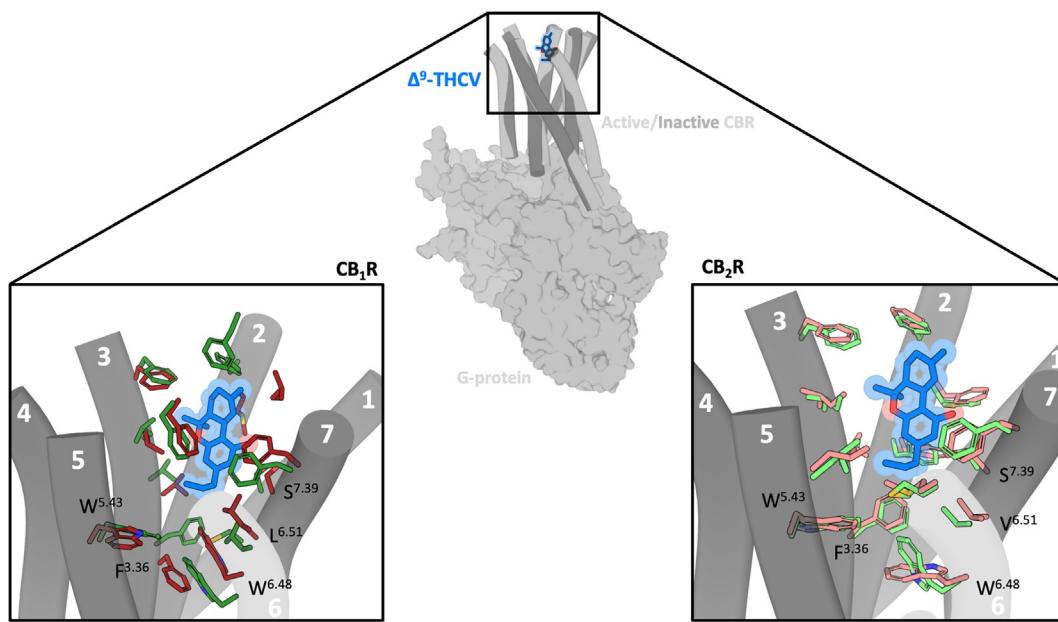
When studying signaling pathway activation upon binding of  $\Delta^9$ -THCA to cannabinoid receptors, Raïch et al. (2021) reported that the compound was able to reduce forskolin-induced cAMP levels in HEK293T expressing CB<sub>2</sub>R or CB<sub>1</sub>R-CB<sub>2</sub>R heteromers, but not in those only expressing CB<sub>1</sub>R. However,  $\Delta^9$ -THCA could activate MAPK pathway in all three types of cells, with potencies in the nanomolar range in all cases. When analyzing  $\beta$ -arrestin II recruitment, again  $\Delta^9$ -THCA was able to activate this pathway in HEK293T cells expressing CB<sub>1</sub>R, CB<sub>2</sub>R, or CB<sub>1</sub>R-CB<sub>2</sub>R heteromers, although to a less extent than  $\Delta^9$ -THC or  $\Delta^9$ -THCV (Raïch et al., 2021).

Raïch et al. (2021) also report that  $\Delta^9$ -THCA was able to revert the effect of a selective CB<sub>2</sub> receptor agonist, but not that of a selective agonist of the CB<sub>1</sub> receptor on Gi-mediated pathway.

$\Delta^9$ -THCA has been described to cause cell death (Morimoto et al., 2007; Nallathambi et al., 2018), but also to have neuroprotective effects in primary neurons used as a Parkinson's disease model (Moldzio et al., 2012). This compound has been shown to behave as a PPAR- $\gamma$  agonist. Effects mediated through this receptor could mediate this neuroprotective action, as they could be related to the role of this compound in glucose regulation and adipocyte metabolism, thus linking the effects of  $\Delta^9$ -THCA to its potential usefulness in treating metabolic syndrome (Nadal et al., 2017; Palomares et al., 2019). Other effects produced by  $\Delta^9$ -THCA are the reduction of nausea, which was antagonized by a selective CB<sub>1</sub>R antagonist (Rock et al., 2013), a decrease of elicited gaping (Rock et al., 2014), a decrease of anticipatory nausea (in co-administration with CBDA), which was blocked by antagonists of both CB<sub>1</sub>R and 5-HT<sub>1A</sub>R (157). This compound has also been shown to have antiinflammatory effects mediated, at least in part, through orphan receptor GPR55 (164). More information on a  $\Delta^9$ -THCA can be found on (Franco et al., 2020; Moreno-Sanz, 2016).

## $\Delta^9$ -Tetrahydrocannabivarin ( $\Delta^9$ -THCV)

$\Delta^9$ -THCV is a natural  $\Delta^9$ -THC analog with a propyl side chain instead of a pentyl group (Fig. 3). It was discovered in 1970 by Edward Gill (Gill et al., 1970), who detected it in tincture of cannabis BPC, which was back then as a licensed medicine in the United Kingdom. This compound was subsequently named  $\Delta^9$ -THCV (Merkus, 1971).



**FIG. 5**  $\Delta^9$ -THCV docking to CB<sub>1</sub> and CB<sub>2</sub> receptors. Central panel shows the general location of the ligand (blue) inside the receptors binding pocket. In light gray, the active state receptor with G-protein bound in its intracellular site. In dark gray, the inactive state receptor. Zoom-in panels show closer details of the possible interactions between the ligand and the residues defining the receptor-binding pocket in the CB<sub>1</sub>R (left, active state—dark green PDB ID 6KPG; and inactive state—dark red PDB ID 5TGZ) and CB<sub>2</sub>R (right, active state—lime PDB ID 6KPF; and inactive state—salmon PDB ID 5ZTY). Some predicted key residues are numbered using Ballesteros numbering.

$\Delta^9$ -THCV is able to bind CB<sub>1</sub>R and CB<sub>2</sub>R (Fig. 5), as well as the CB<sub>1</sub>R-CB<sub>2</sub>R heteromer (Pertwee, 2008; Pertwee et al., 2007; Raich et al., 2021; Thomas et al., 2005). HTRF-based competition assays in cells expressing CB<sub>1</sub>R, CB<sub>2</sub>R showed affinities of  $\Delta^9$ -THCV in the nanomolar range.  $\Delta^9$ -THCV was able to displace fluorescent CELT-335 from HEK-293T cells expressing SNAP-CB<sub>1</sub>R and fluorescent CM157 from HEK-293T cells expressing SNAP-CB<sub>2</sub>R, with *Kis* of  $7.2 \pm 0.3$  (*Ki* = 63 nM) and  $6.5 \pm 0.3$  (*Ki* = 316 nM), respectively (Raich et al., 2021). Other studies using radiolabeled ligands assays described similar results for CB<sub>1</sub>R and CB<sub>2</sub>R, showing *Kis* of 75.4 nM (Thomas et al., 2005) or 46.6 nM (Pertwee, 2007) for CB<sub>1</sub>R and of 62.8 nM for CB<sub>2</sub>R (Thomas et al., 2005) in experiments performed with mouse brain (CB<sub>1</sub>R) or mouse spleen (CB<sub>2</sub>R) membranes. Raich et al. obtained a biphasic curve for the binding of  $\Delta^9$ -THCV to CB<sub>2</sub>R in cells expressing CB<sub>1</sub>-CB<sub>2</sub>Hets, but not in cells expressing one of the receptors, thus “re-identifying” two populations of the CB<sub>2</sub>R displaying different affinities for a given cannabinoid ligand (Martínez-Pinilla et al., 2016; Raich et al., 2021).

When studying signaling pathway activation upon binding of  $\Delta^9$ -THCV to cannabinoid receptors, Raich et al. (2021) reported that the compound was not able to reduce forskolin-induced cAMP levels in HEK293T expressing CB<sub>1</sub>R or CB<sub>2</sub>R, but it was in those expressing both cannabinoid receptors. In the study performed by McPartland et al. (2015),  $\Delta^9$ -THCV is described as a partial agonist of CB<sub>2</sub>R. However,  $\Delta^9$ -THCV could activate MAPK pathway in cells expressing CB<sub>1</sub>R or CB<sub>2</sub>R, with potencies in the nanomolar range. In contrast, in cells expressing the heteromer, the effect of  $\Delta^9$ -THCV was smaller. When analyzing  $\beta$ -arrestin II recruitment, again  $\Delta^9$ -THCV was able to activate this pathway in HEK293T cells expressing CB<sub>1</sub>R, CB<sub>2</sub>R or CB<sub>1</sub>R-CB<sub>2</sub>R heteromer (Raich et al., 2021).

Raich et al. (2021) also report that  $\Delta^9$ -THCV was able to revert the effect of a selective CB<sub>1</sub> receptor selective agonist and the effect of a selective CB<sub>2</sub> receptor agonist on Gi-mediated pathway. Thomas et al. (2005) also described that  $\Delta^9$ -THCV can act as an antagonist in both cannabinoid receptors.

The main advantage of  $\Delta^9$ -THCV over  $\Delta^9$ -THC is the lack of psychoactive effect.  $\Delta^9$ -THCV is a neutral CB<sub>1</sub>R antagonist and likely via this mechanism, it ameliorates insulin sensitivity. In rodent studies, it decreases appetite, increases satiety, and upregulates energy metabolism, making it a clinically useful remedy for weight loss and management of obesity and type 2 diabetic patients (Abioye et al., 2020; Romano et al., 2016). Via CB<sub>2</sub>R receptor,  $\Delta^9$ -THCV, inhibits nitrite production in macrophages. This antioxidant CB<sub>2</sub>R agonist properties of  $\Delta^9$ -tetrahydrocannabivarin ( $\Delta^9$ -THCV) afforded neuroprotection in experimental Parkinson’s disease (PD), whereas its CB<sub>1</sub>R antagonist profile at doses lower than 5 mg/kg caused antihypokinetic effects (Espadas et al., 2020; Romano et al., 2016).

Interestingly, a systematic review from 2015 noted that the in vitro CB<sub>1</sub>R antagonist Δ<sup>9</sup>THCV did not always behave as such in in vivo assays. The lack of correlation between in vivo actions and phytocannabinoid/receptor-binding affinity or other in vitro measures appears to be common. In vitro, ex vivo, and in vivo data show that the effects of Δ<sup>9</sup>THCV are mediated by both cannabinoid receptors and alternative targets (Franco et al., 2020).

For example, Δ<sup>9</sup>THCV can interact with 5-HT<sub>1A</sub> receptors, and its activation it has been shown to decrease psychosis in rat models (Cascio et al., 2015). Other targets for Δ<sup>9</sup>THCV are the TRPV receptors. For example, TRPV1 can be activated by Δ<sup>9</sup>THCV and induce a therapeutic metabolic effect by restoring insulin sensitivity in obese mice models (Abioye et al., 2020). Activation of TRPV3 and TRPV4 channels had antiinflammatory action in the gastrointestinal tract (De Petrocellis et al., 2012). Furthermore, Δ<sup>9</sup>-THCV negatively regulates calcium transport in epithelial cells via the inhibition of TRPV5 and TRPV6 (Janssens et al., 2018).

## Other Δ<sup>9</sup>-THC derivatives

As mentioned above, other compounds related to Δ<sup>9</sup>-THC besides Δ<sup>9</sup>-THCA and Δ<sup>9</sup>-THCV have been isolated from the cannabis plant.

The (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinol-C4 (Δ<sup>9</sup>-THC-C4), also known as Δ<sup>9</sup>-tetrahydrocannabutol or Δ<sup>9</sup>-THCB, is the butyl analog of Δ<sup>9</sup>-THC (Harvey, 1976; Linciano, Citti, Luongo, et al., 2020). Δ<sup>9</sup>-THC-C4 is much less abundant than Δ<sup>9</sup>-THC in the cannabis plant. This compound displaced radiolabeled CP-55940 from human CB<sub>1</sub> and CB<sub>2</sub> receptors, showing affinities in the low nanomolar range (*Ki* = 15 and 51 nM, respectively) (Linciano, Citti, Luongo, et al., 2020). When assessing cannabimimetic action by means of the tetrad test in mice, Δ<sup>9</sup>-THC-C4 showed a partial agonistic activity toward the CB<sub>1</sub> receptor (Linciano, Citti, Luongo, et al., 2020). Also, the (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolic acid A-C4 (Δ<sup>9</sup>-THCAA-C4), the precursor of Δ<sup>9</sup>-THC-C4, has been isolated from the cannabis plant (Harvey, 1976).

Eight cannabinoid esters have been isolated from a high potency variety of the plant, namely α-fenchyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate, β-fenchyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate, epi-bornyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate, bornyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate, terpenyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate, 4-terpenyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate, cadinyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate, and γ-eudesmethyl (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolate (Ahmed et al., 2008). Authors assayed antimicrobial activity of these compounds, but none of them was effective against *Candida albicans* (Ahmed et al., 2008).

Radwan et al. described the isolation of three naturally occurring hydroxylated forms of Δ<sup>9</sup>-THC also from a high potency variety of *C. sativa* L., (i) 8α-hydroxy-(−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinol, (ii) 8β-hydroxy-(−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinol, and (iii) 11-acetoxy-(−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolic acid A (Radwan et al., 2015). Affinity of the three compounds for human CB<sub>1</sub> and CB<sub>2</sub> receptors was determined by radioligand binding assays. 8α-Hydroxy-(−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinol showed *Kis* of 1.9 μM and 3.2 μM for CB<sub>1</sub>R and CB<sub>2</sub>R, respectively, but it did not exhibit typical cannabimimetic activity in the mouse tetrad assay (20 mg/kg). Authors state that further evaluation of the functional activity at the CB<sub>1</sub>R needs to be done for this compound in order to determine if it acts as an agonist or antagonist of CB<sub>1</sub>R, as well as a full characterization of its in vivo dose-response activity. 8β-Hydroxy-(−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinol showed *Kis* of 65 nM and 88 nM for CB<sub>1</sub>R and CB<sub>2</sub>R, respectively. When analyzing the cannabimimetic action by means of the mouse tetrad assay, it lacked typical cannabimimetic-like action at doses up to 20 mg/kg, although it displayed a significant dose-dependent hypolocomotive activity. Binding assays with 11-acetoxy-(−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinolic acid A show *Kis* of 47 nM and 912 nM for CB<sub>1</sub>R and CB<sub>2</sub>R, respectively, although no data regarding the tetrad assay are given for this compound.

Also new heptyl and hexyl homologs of Δ<sup>9</sup>-THC were isolated, known as (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabiphorol (Δ<sup>9</sup>-THCP) (Citti et al., 2019) and (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabihexol (Δ<sup>9</sup>-THCH) (Linciano, Citti, Russo, et al., 2020), respectively. Δ<sup>9</sup>-THCP showed similar binding affinity against human CB<sub>1</sub>R in vitro (*Ki* = 1.2 nM) to that of CP55940. In the cannabinoid tetrad pharmacological test, this compound showed a THC-like cannabimimetic activity, as it induced hypomotility, analgesia, catalepsy, and decreased rectal temperature (Citti et al., 2019).

The isolation of other Δ<sup>9</sup>-THC-related compounds has been reported, for which no binding or functional data are available, such as (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabivarinic acid A (Δ<sup>9</sup>-THCVAA), the precursor of Δ<sup>9</sup>-THCV, whose isolation was reported in 1973 (Shoyama et al., 1977), (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabiorcol (Δ<sup>9</sup>-THCO or Δ<sup>9</sup>-THC1) (Turner et al., 1973) and its precursor (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabiorcolic acid (Δ<sup>9</sup>-THCOAA) (Harvey, 1976), (−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinal (Δ<sup>9</sup>-THC aldehyde), which was isolated in 2015 from a high potency variety of *C. sativa* (Ahmed et al., 2015), 8-oxo-(−)-Δ<sup>9</sup>-*trans*-tetrahydrocannabinol (Ahmed et al., 2015), which was isolated from the same variety of

the plant than the previous compound, or a compound known as cannabisol (Zulfiqar et al., 2012), which was isolated from a group of illicit cannabis samples.

## Applications to other areas

Cannabinoids are implicated in multiple distinct functions such as motivation, memory, superior cognitive processes, and movement in the CNS. They also regulate physiological processes such as pain, appetite pleasure, and reward. Thus, their possible therapeutic actions in multiple pathologies seem to have no ending. However, due to its psychoactive side effect, its use is forbidden in multiple fields. In recent years, a great effort has been made, which continues to grow exponentially, in order to identify cannabinoid compounds that maintain the beneficial effects while avoiding the unwanted psychoactive effect.

In this sense, new synthetic derivatives from  $\Delta^9$ -THC have been synthetized, and further natural compounds such as the acid tetrahydrocannabinol ( $\Delta^9$ -THCA) and the tetrahydrocannabivarin ( $\Delta^9$ -THCV) have been isolated and analyzed. It has been observed that their applications open up new avenues. On the one hand,  $\Delta^9$ -THCA-A represents the 90% of the total THC in *C. sativa* plant (Moreno-Sanz, 2016).  $\Delta^9$ -THCA-A binds to both cannabinoid receptors, showing higher affinity for CB1R than CB2R (Rosenthaler et al., 2014). Furthermore, it induces no psychoactive effects while it shows antiinflammatory, immunomodulatory, neuroprotective, and antineoplastic properties in in vitro studies and pharmacological actions in vivo studies in rodents. A clear example was demonstrated analyzing the enzymatic activity of phosphatidylcholine-specific phospholipase C (PC-PLC) and observing that  $\Delta^9$ -THCA-A induces immunomodulatory effects by inhibiting it, but not  $\Delta^9$ -THC (Verhoeckx et al., 2006). Another study shows  $\Delta^9$ -THCA-A neuroprotective effects in an in vitro model of Parkinson's disease by decreasing 1-methyl-4-phenyl pyridinium (MPP<sup>+</sup>) toxicity and inducing neuronal survival (Moldzio et al., 2012). On the other hand,  $\Delta^9$ -THCV is a potent CB<sub>2</sub>R partial agonist and CB<sub>1</sub>R antagonist (Pertwee, 2008).  $\Delta^9$ -THCV has been investigated in different study areas and shows a high potential to be considered against some pathologies. Among the different examples, we find its capacity to reduce the food intake and body weight in obese and nonobese mice, having a huge potential to combat obesity and diabetes (Abioye et al., 2020). Also, it decreases appetite, increases satiety, and upregulates energy metabolism (Abioye et al., 2020).

The antiepileptiform and anticonvulsant properties of  $\Delta^9$ -THCV, which reduced the burst complex incidence and the amplitude and frequency of paroxysmal depolarizing shifts by acute treatment, have also been analyzed (Hill et al., 2010). Moreover, it is lack of psychoactivity and its safety has been demonstrated across different age groups including adolescent and younger adult populations.

Other cannabinoid derivatives to take into consideration are those from the isobaric isomer of  $\Delta^9$ -THC, the (-)-trans- $\Delta^8$ -THC ( $\Delta^8$ -THC), which show important effects in cancer therapy such as decreasing lung carcinoma (Munson et al., 1975), despite having an important psychoactive effect (Morales et al., 2017; Tagen & Klumpers, 2022).

Altogether, the recent studies demonstrate the capacity of  $\Delta^9$ -THC derivatives to become potent therapeutic targets to combat a wide range of pathologies avoiding or decreasing the psychoactive side effects. However, more studies and effort are required to afford it.

## Key facts

- *C. sativa* plant contains hundreds of different compounds, known as phytocannabinoids, with  $\Delta^9$ -THC and CBD being the most abundant ones.
- Cannabis is by far the most widely cultivated, trafficked, and abused illicit drug.
- About 147 million people, 2.5% of the world population, consume cannabis (annual prevalence) compared with 0.2% consuming cocaine and 0.2% consuming opiates.
- Some of the risks of consuming marijuana are problems with concentration, memory, coordination and judgment, mental health problems, such as anxiety, depression, hallucinations and even psychosis, chronic respiratory or lung problems from smoking, reproductive problems, cannabis dependence, or cannabinoid hyperemesis syndrome (severe nausea and vomiting).
- Cannabinoids can have therapeutic effects for nausea and vomiting in the advanced stages of diseases such as cancer and AIDS. Dronabinol (tetrahydrocannabinol) has been available by prescription for more than a decade in the United States.

## Mini-dictionary of terms

- **$\Delta^9$ -THC:** Delta-9-tetrahydrocannabinol, is the main psychoactive compound of *C. sativa* plant.
- **$\Delta^9$ -THCA:** Delta-9-tetrahydrocannabinolic acid. Also present in the cannabis plant, is the acidic form and precursor of  $\Delta^9$ -THC. It lacks psychoactive effects.
- **$\Delta^9$ -THCV:** Delta-9-tetrahydrocannabivarin, a natural  $\Delta^9$ -THC analog with a propyl side chain instead of a pentyl group. It lacks psychoactive effects.
- **CBD:** Cannabidiol, is the main nonpsychoactive compound of *C. sativa* plant. It has gained high interest nowadays due to its antiinflammatory, antipsychotic, and anxiolytic effects, among others.
- **FAAH:** Fatty acid amide hydrolase, is the enzyme that degrades anandamide.
- **HTRF:** Homogeneous Time-Resolved Fluorescence, a novel method to study ligand binding to a receptor with advantages over classical radioligand-binding assays.
- **Mouse tetrad:** group of four behavioral tests performed in mice to assess the typical cannabimimetic effects of a compound, compared with  $\Delta^9$ -THC: hypolocomotion (measured by spontaneous activity), catalepsy (determined by the bar test), hypothermia (assessed by rectal temperature), and analgesia (measured by the hot plate test).

## Summary points

- Ligand affinity for a desired cannabinoid receptor can be analyzed by radioligand-binding assays or Homogeneous Time Resolved Fluorescence (HTRF).
- Radioligand-binding assays are very useful (i) to characterize affinity of a ligand for a receptor with high sensitivity and (ii) to determine anatomical distribution of receptors in tissue samples.
- Homogeneous Time-Resolved Fluorescence (HTRF) is a robust technology that can determine ligand binding in live cells avoiding handling of radioisotopes and in a homogenous manner. Further advantages of HTRF are reduced (i) background fluorescence, (ii) nonspecific binding, and (iii) dispersion of the data.
- The  $\Delta^9$ -THC derivatives  $\Delta^9$ -THCV and  $\Delta^9$ -THCA show no psychoactive effects.
- $\Delta^9$ -THCV is a potent CB<sub>2</sub>R partial agonist and CB<sub>1</sub>R antagonist.
- $\Delta^9$ -THCA binds to both cannabinoid receptors, showing higher affinity for CB<sub>1</sub>R than CB<sub>2</sub>R.
- $\Delta^8$ -THC is less potent than  $\Delta^9$ -THC, and it also induces psychoactive effects.
- $\Delta^9$ -THC derivatives can be possible therapeutic agents to combat obesity, diabetes, epilepsy, neurodegenerative disorders, or cancer, between others.

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## Chapter 30

# New cannabinoid receptor type 1 and 2 agonists and applications to understanding the endocannabinoid system: A chemical approach

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## Abbreviations

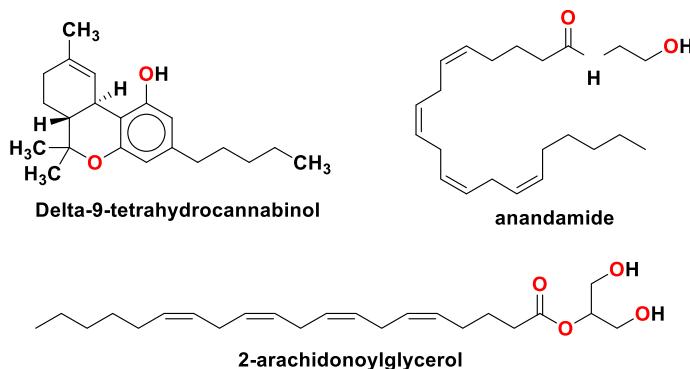
CB1R	cannabinoid receptor type I
CB2R	cannabinoid receptor type II
AAI	aminoalkylindole
ECS	endocannabinoid system
GPCR	G-protein-coupled receptor
$\Delta^9$ -THC	delta-9 tetrahydrocannabinol
AEA	anandamide
2-AG	2-arachidonoylglycerol

## Introduction

The endocannabinoid system (ECS) comprises a complex network of lipid signaling mediators. It is composed of transmembrane proteins belonging to the family of heptameric G-protein-coupled receptors (GPCRs) that are activated by endogenous molecules known as endocannabinoid ligands. Two types of receptors have been identified to date, called cannabinoid receptor type 1 and type 2 (CB1R and CB2R, respectively). It is one of the most abundant GPCRs in the mammalian brain, hence one of its functions is related to the attenuation and release of excitatory and inhibitory neurotransmitters (Kreitzer, 2005). This signaling system is named after the activation generated by delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC) (Fig. 1), phytocannabinoid mimetic that modulates the activity of CB1R and CB2R. The complex signaling network of CB1R and CB2R suggests that the modulation of these receptors is related to numerous physiological and cognitive processes. These receptors are modulated by endogenous ligands called endocannabinoid ligands derived from long-chain fatty acids that comprise a group of natural members of the eicosanoid superfamily structure. These compounds show cannabimimetic activity, i.e., they act as  $\Delta^9$ -THC mimetics main component of *Cannabis sativa*. The main endogenous ligands are arachidonylethanolamide, also called anandamide (AEA) y 2-arachidonoylglycerol (2-AG) (Felder et al., 1995) (Fig. 1). Both act on CB1R and CB2R with dissimilar affinities for these receptors (Reggio, 2010).

The endocannabinoid system is widespread in mammalian tissues and cells and appears to play a pro-homeostasis role by activating following transient or chronic perturbation of homeostasis and by locally regulating the levels and action of other chemical signals. The modulation of the biosynthesis and enzymatic degradation pathways of endogenous cannabinoids, in addition to the activation and deactivation of their receptors, has led to study the pathophysiological responses to the use of different pharmacological strategies applied in the ECS (Pacher et al., 2006; Rodríguez de Fonseca et al., 2005; Walker et al., 1999).

It is now known that the activation of CB1R and CB2R cannabinoid receptors is generated by the interaction of the orthosteric site of the receptor with an endogenous (endocannabinoid) or exogenous agonist ( $\Delta^9$ -THC, synthetic agonists, among others). The effects of modulation of these systems have implicated the ECS in a variety of pathophysiological

**FIG. 1** Endocannabinoid receptor agonists.

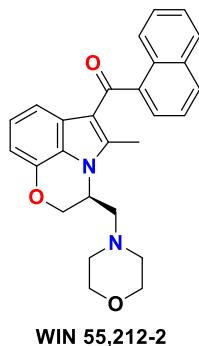
processes, both in the central and peripheral nervous system and in various peripheral organs (Pacher et al., 2006). Such studies have further suggested that modulation of ECS activity may have therapeutic potential in almost all diseases affecting humans, including the obesity/metabolic syndrome (Kunos & Tam, 2011); diabetes and diabetic complications (Horváth et al., 2012); neurodegenerative diseases (Centonze et al., 2007), inflammatory diseases (Klein, 2005), cardiovascular (Montecucco & Di Marzo, 2012), gastrointestinal disorders (Izzo & Camilleri, 2008) pain (Guindon & Hohmann, 2008); psychiatric disorders (Hillard et al., 2013); cancer (Velasco et al., 2012); chemotherapy-induced nausea and vomiting (Parker et al., 2011).

The design of synthetic cannabinomimetics that act as endocannabinoid receptor agonists has been a strategy approached by many synthetic chemists to explore ECS modulation in the development of therapeutic action for a wide range of diseases. One example of molecular design has been the incorporation of heterocycles into chemical structures such as indoles (Adam et al., 2010; Banister et al., 2013; Huffman et al., 1994, 2006; Huffman & Padgett, 2005; Makriyannis & Hongfeng, 2000; Mella-Raipan et al., 2014; Moir et al., 2019; Morrison et al., 2011; Yang et al., 2020), pyrazoles (Lainton et al., 1995; Moir et al., 2021; Pertwee et al., 1995; Wiley et al., 2012), benzimidazoles (Espinosa-Bustos et al., 2015; Gijsen et al., 2012; Mella-Raipán et al., 2013; Nimczick et al., 2014; Pagé et al., 2008; Romero-Parra et al., 2016; Schoeder et al., 2018), quinolines (Capozzi et al., 2018; Lucchesi et al., 2014; Manera et al., 2006, 2007), 2-pyridones (Arena et al., 2020; Chicca et al., 2018; Faúndez-Parraguez et al., 2021; Kusakabe et al., 2013), among others, with molecular templates explored during the last 20 years in the search for selectivity and higher affinity for endocannabinoid receptors.

The following chapter reviews some chemical structures designed as CB1R and CB2R agonists with different heterocycles as molecular templates, with a chemical perspective to generate variability in the design of new structures that can activate the endocannabinoid system emerging as potential therapeutic agents.

## WIN55,212-2 and the design of indole-based cannabinoid agonists

In the early 1990s, the first synthetic molecules were developed that bind to the cannabinoid receptor. WIN55,212-2 is an aminoalkylindole analog (AAI) (Fig. 2) whose structure was initially related to the dual cyclooxygenase inhibitor and non-opioid analgesic, pravadolone (D'Ambra et al., 1992). Studies of AAI's derivatives showed that they possessed

**FIG. 2** Synthetic agonist of the cannabinoid receptor derived from the aminoalkylindole group.

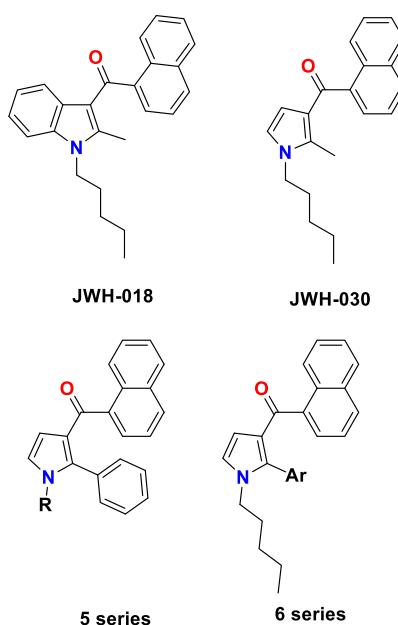
antinociceptive activity; however, they did not appear to inhibit cyclooxygenases, neither inhibit prostaglandin synthesis nor bind to opioid receptors. This class of drugs could be related to the mechanism by which cannabinoids produce nociception blockade.

WIN 55,212-2 produces an effect like cannabinoids such as  $\Delta^9$ -THC but has a completely different structure than classic cannabinoids. It has been reported as a potent analgesic and has been shown to have anticonvulsant effects in the electroshock model of generalized seizures (Wallace et al., 2001). WIN 55,212-2 is a potent CB1R and CB2R full agonist ( $K_i = 1.9 \text{ nM}$  and  $3.3 \text{ nM}$ , respectively). Structural modifications have been carried out on its indole nucleus to improve selectivity for one or the other receptor. John W. Huffman and his collaborators reported the first synthetic structures derived from a structural analysis based on the traditional cannabinoids and indole ring of WIN55,212-2 (Huffman et al., 1994). Several rotational isomers on the carbonyl carbon were incorporated at the 3-position of the heterocycle. By suppressing the phenolic oxygen atom of WIN55,212-2 allowing rotation of the N-1 bonded methylene carbon, it was possible to incorporate different substituents on the nitrogen atom of the indole (Table 1).

Early findings indicated that 3-acylindoles lacking a naphthoyl substituent at C-3 lacked cannabinoid activity, whereas N-alkylindoles with a chain of four to six carbon atoms showed activity, both *in vitro* and *in vivo*, comparable with both THC and WIN55,212-2 (Huffman et al., 1994). With regard to structure JWH-018 (Fig. 3), Lainton and coworkers preliminarily explored the replacement of the indole ring by a pyrrole ring conserving the substituted portions at C-3 and N-1 (molecule JWH-030) (Lainton et al., 1995). Decreased affinity was observed on CB1R ( $K_{i,\text{JWH-030}} = 87 \text{ nM}$ ) relative to

**TABLE 1** Binding affinity results of a series of aminoalkylindole derivatives (4) on CB1R.

Entry	Compound	CB <sub>1</sub> R $K_i$ (nM)	In vivo (ED <sub>50</sub> , $\mu\text{M}/\text{Kg}$ )	
			4	JWH-018
1	4. R = 2-phenylethyl	$1250 \pm 250$	>257	
2	4. R = 2-cyclohexylethyl	$46 \pm 13$	65.8	
3	4. R = n-propyl	$164 \pm 22$	Not determined	
4	4. R = 2-butyl	$22 \pm 1.5$	Not determined	
5	4. R = n-pentyl	$9.5 \pm 4.5$	2.62	
6	4. R = n-hexyl	$48 \pm 13$	14.0	
7	4. R = n-heptyl	>10,000	>189	
8	4. R = RS-2-heptyl	$33 \pm 11$	35.6	
9	4. R = R-2-heptyl	$81 \pm 41$	21.5	
10	4. R = S-2-heptyl	$72 \pm 32$	Not determined	
11	4. R = CH <sub>3</sub>	>10,000	>350	
12	4. R = (CH <sub>3</sub> ) <sub>2</sub> C=CH	>10,000	>103	
13	$\Delta^9$ -THC	41	4.70	
14	CP-55,940	0,924	0.55	
15	WIN-55,212-2	24	6.53	



**FIG. 3** Synthetic cannabinoid receptor agonists. Molecules 5 and 6 are the templates in Tables 2 and 3, respectively.

JWH-007. Huffman and coworkers improved the affinity of pyrrole derivatives by including an aryl segment at C-2 of the heterocycle (Huffman et al., 2006) while keeping the number of carbons of the alkyl group at N-1 (Table 2). The affinity of the resulting compounds was measured in both CB1R and CB2R. The authors reported that the affinities of 2-arylpyrroles (**6** series) with small substituents in the ortho position mostly possess high affinity for CB1R. For JWH-373, whose aromatic portion is constituted by an o-butylphenyl group, a decrease in affinity for CB1R was observed, probably due to the steric effect of the butyl group (Fig. 3). Comparatively, the 2-arylpyrroles of the **6** series have similar affinity for both CB1R and CB2R (Table 3).

Derivatives synthesized with meta-position isomers showed lower affinity for CB1R and CB2R compared with ortho-substituted analogs; however, meta-substituted 6-series derivatives showed higher CB2R selectivity than CB1R. The para-substituted analogs showed relatively lower affinities for CB1R and CB2R compared with those obtained with the ortho- and meta-substituted analogs (Huffman et al., 2006). According to the structures evaluated, the authors point out the importance of C-2 of the indole and that a group longer than methyl destroys the activity on the receptors and that a hydrogen at C-2 generates a structure with slightly higher affinity than a methyl group (Huffman & Padgett, 2005).

**TABLE 2** Affinity of N-1 substituted pyrrole derivatives on CB1R and CB2R.

5 Series	$K_i$ (nM)	
	CB <sub>1</sub> R	CB <sub>2</sub> R
WIN55,212-2	1.9±0.1	0.28±0.16
Δ <sup>9</sup> -THC	41±2	36±10
1-Alkyl Group, R		
Propyl, <b>JWH-156</b>	404±18	104±18
Butyl, <b>JWH-150</b>	60±1	15±2
Pentyl, <b>JWH-145</b>	14±2	6.4±0.4
Hexyl, <b>JWH-147</b>	11±1	7.1±0.2
Heptyl, <b>JWH-146</b>	21±2	62±5

**TABLE 3** Affinity of C-2 substituted pyrrole derivatives on CB1R and CB2R.

6	$K_i$ (nM)	
Aryl Group, Ar	CB <sub>1</sub> R	CB <sub>2</sub> R
Phenyl, JWH-145	14±2	6.4±0.4
<i>o</i> -Methylphenyl, JWH-370	5.6±0.4	4.0±0.5
<i>m</i> -Methylphenyl, JWH-346	67±6	39±2
<i>p</i> -Methylphenyl, JWH-244	130±6	18±1
<i>o</i> -Ethylphenyl, JWH-365	17±1	3.4±0.2
<i>p</i> -Ethylphenyl, JWH-364	34±3	29±1
<i>o</i> -Butylphenyl, JWH-373	60±3	69±2
<i>p</i> -Butylphenyl, JWH-371	42±1	64±2
<i>o</i> -Methoxyphenyl, JWH-292	29±1	20±1
<i>m</i> -Methoxyphenyl, JWH-367	53±2	23±1
<i>p</i> -Methoxyphenyl, JWH-243	285±40	41±3
<i>o</i> -Fluorophenyl, JWH-307	7.7±1.8	3.3±0.2
<i>m</i> -Fluorophenyl, JWH-368	16±1	9.1±0.7
<i>p</i> -Fluorophenyl, JWH-308	41±1	33±2
<i>o</i> -Chlorophenyl, JWH-369	7.9±0.4	5.2±0.3
<i>m</i> -Chlorophenyl, JWH-246	70±4	16±1
<i>p</i> -Chlorophenyl, JWH-245	276±4	25±2
<i>m</i> -Nitrophenyl, JWH-293	100±5	41±4
1-Naphthyl, JWH-309	41±3	49±7
2-Naphthyl, JWH-347	333±17	169±17
3-pyridyl, JWH-366	191±12	24±1

This can be deduced from the results obtained by Huffman and Aung and coworkers (Aung et al., 2000; Huffman et al., 1994) and summarized in the Table 4.

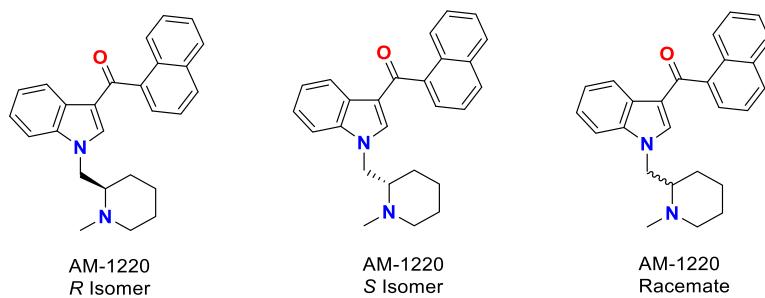
Starting from the indole nucleus, the affinity for CB1R and CB2R is very weak when the side chain length of N-alkyl is one or two carbon atoms. This affinity increases with increasing carbon chain length. There is a small influence on CB1R, but a greater increase in affinity is shown for CB2R. Optimal binding to CB1R was observed with butyl, pentyl, and heptyl side chains, whereas optimal binding to CB2R occurred only with pentyl and hexyl side chains. In the other embodiments, the structures show higher selectivity for CB2R than CB1R, particularly in the C-2 series with a methyl group of the N-propyl analog (JWH-015) that had a high affinity for CB2R resulting in a 24-fold receptor selectivity. Thereby, the authors suggest that there is a tendency for CB2R selectivity through the 2-methyl indole series (Aung et al., 2000).

In 2001, the research group led by Alexandros Makriyannis filed a patent on aminoalkylindoles derivatives that exhibited high affinity for cannabinoid receptors (Makriyannis & Hongfeng, 2000). Based on the research developed so far, Makriyannis and coworkers explored the incorporation of an (*N*-methyl-2-piperidinyl) methyl group at position 1 of the indole ring, retaining at C-3 the naphthoyl group of the series reported by J.W. Huffman. They succeeded in obtaining the AM-1220 molecule and described its activity on the endocannabinoid system (D'Ambra et al., 1996). The AM-1220 molecule showed high affinity and selectivity for CB1R in its racemic mixture ( $K_i=3.88\text{ nM}$ ) versus CB2R ( $K_i=73.4\text{ nM}$ ), while its (R) enantiomer showed even higher affinity for CB1R ( $K_i=0.27\text{ nM}$ ) versus (S) enantiomer (CB1R  $K_i=217\text{ nM}$ ) (Fig. 4).

This allowed the incorporation of different substituents on the indole ring to be explored. Of note are the analogs AM-1218, AM-1219, and AM1224 with a nitro, amino, and azide group, respectively. These molecules have a high affinity for both receptors; however, they are highly selective for CB2R.

**TABLE 4** Affinity and selectivity of a series of N-1 and C-2 substituted indole derivatives on CB1R and CB2R.

Compound	R <sub>1</sub>	R <sub>2</sub>	CB <sub>1</sub> R K <sub>i</sub> (nM)	CB <sub>2</sub> R K <sub>i</sub> (nM)	CB <sub>1</sub> R/CB <sub>2</sub> R ratio
JWH-070	Methyl	H	>10,000	>10,000	ND
JWH-071	Ethyl	H	1340±123	2940±852	0.45
JWH-072	N-propyl	H	1050±55	170±54	6.18
JWH-073	N-butyl	H	8.90±1.80	38.0±24.0	0.23
JWH-018	N-pentyl	H	9.00±5.00	2.94±2.65	3.06
JWH-019	N-hexyl	H	9.80±2.00	5.55±2.00	1.77
JWH-020	N-heptyl	H	128±17.0	205±20.0	0.62
JWH-042	Methyl	CH <sub>3</sub>	>10,000	5050±192	>1.98
JWH-043	Ethyl	CH <sub>3</sub>	1180±44	964±242	1.23
JWH-015	N-propyl	CH <sub>3</sub>	336±36	13.8±4.6	24.3
JWH-016	N-butyl	CH <sub>3</sub>	22±1.5	4.29±1.63	5.13
JWH-007	N-pentyl	CH <sub>3</sub>	9.5±4.5	2.94±2.60	3.23
JWH-004	N-hexyl	CH <sub>3</sub>	48±13	4.02±1.46	11.9
JWH-009	N-heptyl	CH <sub>3</sub>	311±106	141±14.5	2.21
Δ <sup>9</sup> -THC			40.7±1.7	36.4±10	1.12
WIN 55,212-2			1.89±0.09	0.28±0.16	6.75

**FIG. 4** Structure of compound AM-1220. Structural representation of the R and S isomers and of the racemic mixture.

When a methyl group was incorporated at C-2 of the indole ring of these derivatives, molecules with even higher affinities for CB<sub>2</sub>R were obtained while the affinities for CB<sub>1</sub>R decreased markedly, except for AM-1231 whose affinity was similar for CB<sub>1</sub>R and CB<sub>2</sub>R (**Table 5**).

When they replaced the (N-methyl-2-piperidinyl) methyl N-1 bonded segment of the indole ring by alkyl groups with heteroatoms at the end of the alkyl chain, the selectivity of the analog molecules was flipped toward CB<sub>1</sub>R over CB<sub>2</sub>R. By changing the length of the alkyl chain attached to N-1, variable affinities on both receptors were obtained (**Table 6**).

**TABLE 5** Affinity and selectivity of a series of C-2 and C-6 substituted indole derivatives on CB1R and CB2R.

Analog	R <sub>1</sub>	n	R <sub>2</sub>	CB <sub>1</sub> R K <sub>i</sub> (nM)	CB <sub>2</sub> R K <sub>i</sub> (nM)	CB <sub>1</sub> R/CB <sub>2</sub> R ratio
AM-683	H	4	CH <sub>3</sub>	272	281	0.97
AM-669	H	5	CH <sub>3</sub>	47.2	38.6	1.22
AM-682	H	6	CH <sub>3</sub>	332	693	0.48
AM-672	H	7	CH <sub>3</sub>	1603	1511	1.06
AM-690	OH	5	CH <sub>3</sub>	4850	1972	2.46
AM-2225	F	5	CH <sub>3</sub>	5.97	3.8	1.57
AM-2229	I	5	CH <sub>3</sub>	116.5	46.2	2.52
AM-679	H	5	H	13.5	49.5	0.27
AM-693	OH	5	H	835	526	1.59
AM-694	F	5	H	0.08	1.44	0.06
AM-698	I	5	H	135.8	314.7	0.43

**TABLE 6** Affinity and selectivity of a series of N-1 and C-6 substituted indole derivatives on CB1R and CB2R.

Analog	R <sub>1</sub>	n	R <sub>2</sub>	CB <sub>1</sub> R K <sub>i</sub> (nM)	CB <sub>2</sub> R K <sub>i</sub> (nM)	CB <sub>1</sub> R/CB <sub>2</sub> R ratio
AM-1292	I	3	H	3.1	18.1	0.17
AM-1288	I	4	H	1.3	10.5	0.12
AM-2203	I	5	H	7.8	45.8	0.17
AM-1295	F	4	H	2.5	30.7	0.08
AM-2201	F	5	H	1.0	2.6	0.38
AM-1283	OH	4	H	117.2	196.5	0.60
AM-2202	OH	5	H	7.8	45.8	0.17
AM-2232	CN	4	H	0.28	1.48	0.19
AM-2231	CN	4	NO <sub>2</sub>	4.90	23.9	0.21

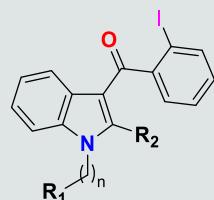
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**TABLE 6** Affinity and selectivity of a series of N-1 and C-6 substituted indole derivatives on CB<sub>1</sub>R and CB<sub>2</sub>R—cont'd

Analog	R <sub>1</sub>	n	R <sub>2</sub>	CB <sub>1</sub> R K <sub>i</sub> (nM)	CB <sub>2</sub> R K <sub>i</sub> (nM)	CB <sub>1</sub> R/CB <sub>2</sub> R ratio
AM-1234	OH	5	NO <sub>2</sub>	77.6	196.8	0.39
AM-1235	F	5	NO <sub>2</sub>	1.5	20.4	0.07
AM-1237	OH	5	NH <sub>2</sub>	836.8	244.4	3.42
AM-1238	OH	5	I	3.1	17.3	0.18
AM-1230	F	5	I	1.1	2.4	0.46

The highest affinity for CB<sub>1</sub>R occurred with AM-2232 with K<sub>i</sub>=0.28 nM with a butyl group attached to N-1 of the indole ring that resulted in a fivefold receptor selectivity than for CB<sub>2</sub>R. The highest selectivity was observed for AM-1295 and AM-1235 molecules. The presence of the nitro group in the synthetic derivatives decreases the affinity for both receptors when an OH group is at the end of the R<sub>1</sub> alkyl chain, unlike the analogs that have an iodine atom at C-7 of the indole ring whose affinity increases considerably for both receptors. Derivatives of the series possessing halogens at the carbon chain end of the N-1 bonded alkyl groups exhibit high affinity for CB<sub>1</sub>R and CB<sub>2</sub>R whose optimum carbon chain length of the alkyl group is between 4 and 5 units.

The series described above with a naphthoyl group attached to C-3 of the indole ring showed mainly high selectivity for CB<sub>1</sub>R. In the search to find higher selectivity for CB<sub>2</sub>R, the authors explored modification of the naphthalene ring and found that incorporation of a 2-iodophenyl group generates new molecules with moderate affinities for both receptors. As in the previous series, it is suggested that the presence of a chain of five carbon atoms linked to N-1 provides greater affinities for both receptors, since the longer the chain length, the greater the affinity decreases markedly. From the data shown in Table 7 the AM-694 molecule presents the highest affinity for CB<sub>1</sub>R and CB<sub>2</sub>R, in addition to the highest selectivity for the first receptor.

**TABLE 7** Affinity and selectivity of a series of N-1 and C-2 substituted 3-(2-benzoyl)indole derivatives on CB<sub>1</sub>R and CB<sub>2</sub>R.

Analog	R <sub>1</sub>	n	R <sub>2</sub>	CB <sub>1</sub> R K <sub>i</sub> (nM)	CB <sub>2</sub> R K <sub>i</sub> (nM)	CB <sub>1</sub> R/CB <sub>2</sub> R ratio
AM-683	H	4	CH <sub>3</sub>	272	281	0.97
AM-669	H	5	CH <sub>3</sub>	47.2	38.6	1.22
AM-682	H	6	CH <sub>3</sub>	332	693	0.48
AM-672	H	7	CH <sub>3</sub>	1603	1511	1.06
AM-690	OH	5	CH <sub>3</sub>	4850	1972	2.46
AM-2225	F	5	CH <sub>3</sub>	5.97	3.8	1.57
AM-2229	I	5	CH <sub>3</sub>	116.5	46.2	2.52
AM-679	H	5	H	13.5	49.5	0.27
AM-693	OH	5	H	835	526	1.59
AM-694	F	5	H	0.08	1.44	0.06
AM-698	I	5	H	135.8	314.7	0.43

The presence of a hydrogen atom in R2 gives the highest selectivity for CB1R while a methyl group in R2 gives the highest selectivity for CB2R or the derivative substituted by a hydroxyl group (AM-693) and an iodine atom (AM-698) at R1 their affinities decrease markedly for both receptors.

## Incorporation of new heterocycles in the search for new CB1R and CB2R agonists

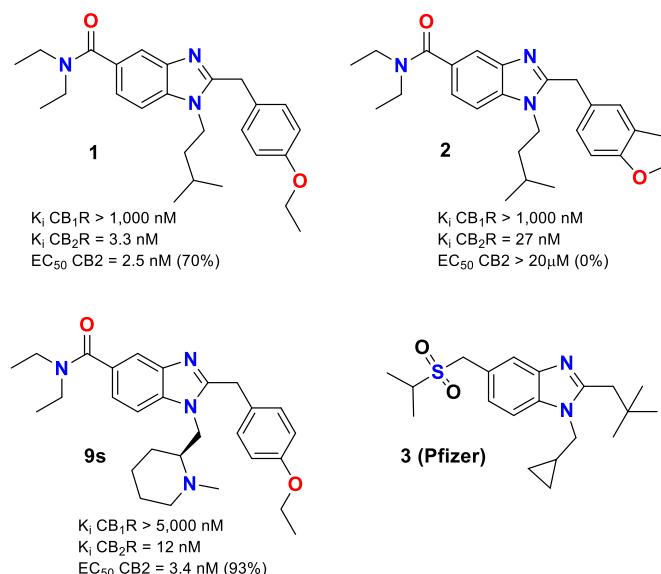
The search for new structures to design new CB1R and CB2R agonists that are more selective and with higher affinity led scientists to explore various bioisosteres of the indole heterocycle to improve their pharmacological and pharmacodynamic characteristics.

Pagé and coworkers initiated the modification of different segments of the benzimidazole ring from a new molecule reported as a CB2R inverse agonist (Pagé et al., 2006). Compound **1** (Fig. 5) presented a high affinity and selectivity for CB2R, and its activity was described as a partial agonist of this receptor. Exploring the structural modification of the ethoxybenzyl group, the structure of compound **2** was evaluated, which conserved its high affinity and selectivity for CB2R; however, its activity was described as an inverse agonist of this receptor. The modifications and structural extensions in the cannabinoid ligands had to meet certain requirements to confer potency on these receptors.

In 2008, Pagé and coworkers synthesized a series of new benzimidazole derivatives by modifying the N-1-bonded substituent segments by different alkyl and cycloalkyl groups obtaining for them excellent CB2 affinity values and maintaining the high selectivity for CB1R (Table 8). These ligands possess a high potency acting most of them as partial agonists of CB2R ( $E_{max}$  63%–93%) while compound **9s** showed an agonist activity (Pagé et al., 2008).

Mella-Raipán and coworkers synthesized a series of benzimidazole derivatives as high-affinity CB1R by modifying the positions 1, 2, 5, and 6 of the heterocycle (Mella-Raipán et al., 2013). Using quantitative structure-activity relationship, three-dimensional models (3D-QSAR) of their synthetic derivatives, they related the stabilization of the ligands to the localization of their molecular segments in three regions of the orthosteric site of the CB1R: **region I** where a hydrogen bond was obtained between the pyridine ring and the Lys192 residue, **region II** where hydrophobic and aromatic interactions were established between the benzimidazole core-bound naphthalene ring and the Phe170, Phe174, and Ala380 residues and **region III** where the benzimidazole ring interacts with the Phe170, Phe200, and Trp356 residues in addition to the hydrogen bond between the basic nitrogen of the benzimidazole and the Thr197 residue of the orthosteric CB1R site (Fig. 6).

The molecules reported by the authors, according to molecular modeling studies, are oriented Y-like conformation at the CB1R active site with the pyridine and the naphthyl and naphthoyl groups as the arms and the benzimidazole scaffold as the body. The 3-pyridyl moiety is oriented to region I, and its stability would be influenced by the interaction of the heterocycle with the side chain of the Lys192 residue. Varying the position of the nitrogen atom of the pyridine ring had a



**FIG. 5** Synthetic cannabinoid receptor agonists derived from the heterocycle benzimidazole. The molecules shown here are agonists with high affinity and selectivity for CB2R.

**TABLE 8** Affinity and potency of a series of N-1 substituted benzimidazole derivatives on CB1R and CB2R.

Molecule	R	<i>h</i> CB <sub>1</sub> R <i>K<sub>i</sub></i> (nM)	<i>h</i> CB <sub>2</sub> R <i>K<sub>i</sub></i> (nM)	<i>h</i> CB <sub>2</sub> R EC <sub>50</sub> (nM)
9a	(CH <sub>3</sub> ) <sub>2</sub> CH-CH <sub>2</sub> -	>5000	4.5±0.9	2.9±0.2 (63%)
9b	Cyclopropyl	>5000	4.1±0.6	2.1±0.7 (68%)
9c	Cyclobutyl	3115±149	1.6±0.1	1.3±0.1 (64%)
9d	Cyclopentyl	491±27	1.0±0.1	0.7±0.2 (79%)
9e	Cyclohexyl	110±1.9	3.7±1.3	0.52±0.04 (79%)
9f	1-adamantyl	406±44	2.8±0.2	1.3±0.2 (81%)
9g	Phenyl	4766±348	12±3	1.1±0.2 (88%)
9h	2-pyridinyl	>5000	39±3	25±5 (77%)
9i	4-pyridinyl	>5000	16±2	5.0±0.7 (78%)
9k	2-furan	>5000	11±3	3.3±0.9 (78%)
9l	4-tetrahydrofuran	1209±53	3.9±0.9	1.0±0.3 (89%)
9r	2-R-(N-Me-piperidine)	4315±270	8.9±1.1	2.3±0.3 (81%)
9s	2-S-(N-Me-piperidine)	>5000	12±2	3.4±0.7 (93%)
Δ <sup>9</sup> -THC	—	2.9±0.3	41±2	1.5±0.1 (100%)
WIN55212-2	—	140±42	20±3	14±3 (64%)

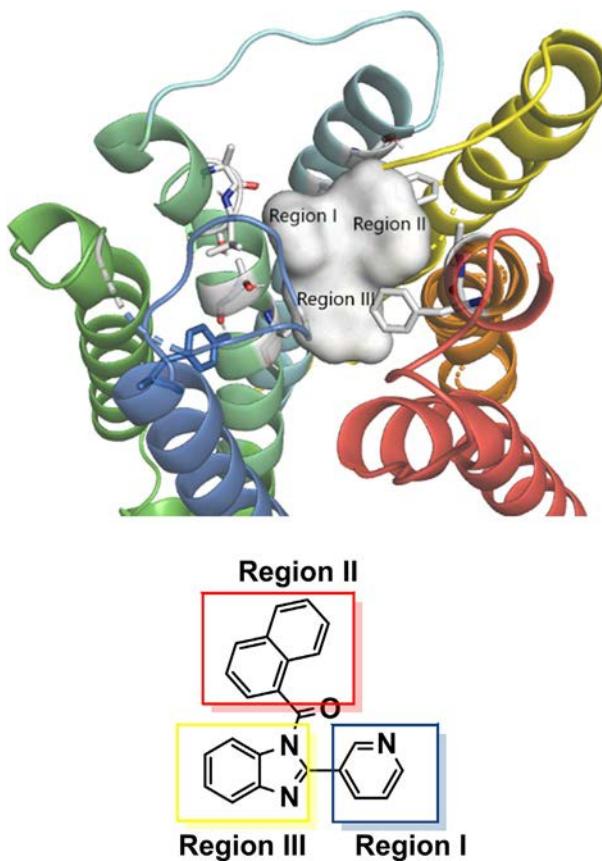
**TABLE 9** Affinity of a series of N-1 substituted benzimidazole derivatives on CB1R with the affinity data of the positional variation of the nitrogen atom of the pyridine ring.

Compound	X	Y	Z	R	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	CB1R <i>K<sub>i</sub></i> (nM)
14	CH	N	CH	C=O	H	H	1-naphthyl	98.24±20
18	CH	CH	N	C=O	H	H	1-naphthyl	37.81±5.3
21	CH	CH	N	C=O	CH <sub>3</sub>	CH <sub>3</sub>	1-naphthyl	3.95±0.8
23	N	CH	CH	C=O	H	F	2-naphthyl	11.41±0.9
24	N	CH	CH	C=O	CH <sub>3</sub>	CH <sub>3</sub>	2-naphthyl	28.08±2.3
26	CH	N	CH	C=O	F	H	2-naphthyl	5700±200
27	CH	N	CH	C=O	H	F	2-naphthyl	963±155

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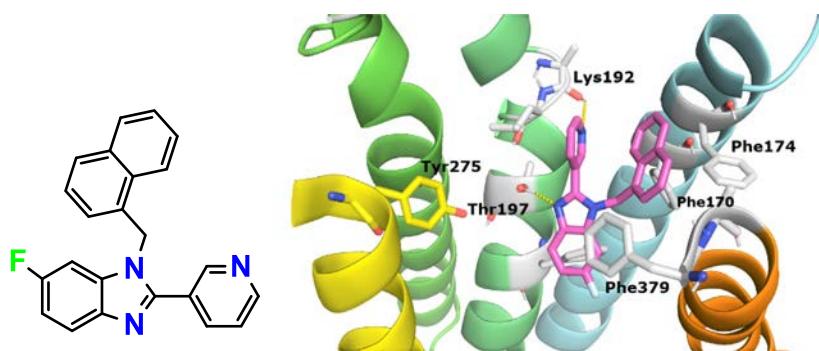
**TABLE 9** Affinity of a series of N-1 substituted benzimidazole derivatives on CB1R with the affinity data of the positional variation of the nitrogen atom of the pyridine ring—cont'd

Compound	X	Y	Z	R	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	CB1R Ki (nM)
28	CH	N	CH	C=O	CH <sub>3</sub>	CH <sub>3</sub>	2-naphthyl	567±27.8
29	CH	CH	N	C=O	H	H	2-naphthyl	4487±133
30	CH	CH	N	C=O	F	H	2-naphthyl	1028±57.9
31	CH	CH	N	C=O	H	F	2-naphthyl	5.55±0.9
37	CH	N	CH	CH <sub>2</sub>	H	H	1-naphthyl	75.87±16.6
38	CH	N	CH	CH <sub>2</sub>	F	H	1-naphthyl	98.18±33.5
39	CH	N	CH	CH <sub>2</sub>	H	F	1-naphthyl	0.53±0.9

**FIG. 6** On the left is a model of CB1R showing the orthosteric ligand binding site cavity. The regions where the agonist molecular segments interact with the amino acid residues are indicated. The proposed orientation of the molecular segments of the molecules synthesized by Mella-Raipán et al. is shown on the right.

dramatic effect on the affinity for CB1R (Table 8). According to the authors, this could be due to the variation in the distance of the nitrogen atom to establish a hydrogen bridge with Lys192 (Fig. 7).

The importance of the naphthyl or naphthoyl group at N-1 of the benzimidazole ring would be explained in relation to the hydrophobic and aromatic interactions with the side chains of the hydrophobic residues. As this molecular portion has been conserved relative to other synthetic ligands reported in this abstract, presumably this aromatic ring is stabilized in the active site by interactions with Phe170 and Phe174, mainly. On the other hand, the authors point out a possible hydrogen



**FIG. 7** On the left is the structure of compound 39 of Table 9. On the right is a representation of the molecular docking of molecule 9. The orientations of the substituents at the orthosteric site of CB1R and the respective interactions with the amino acid residues are shown.

bond interaction between the N-3 nitrogen of the benzimidazole ring and the side chain of Thr197, which would influence a high stability of this heterocycle at the CB1R binding site.

### 3-Carboxamide derivatives: Quinolones and 2-pyridones as cannabinoid agonists

Clementina Manera et al. reported in 2006 a series of compounds derived from quinolone-3-carboxamides as selective CB2R agonists based on a CB1R and CB2R selectivity study by Tuccinardi et al. (2006), which described structural data from WIN55,212-2 for the design of cannabinoid ligands from naphthyridine derivatives.

The analysis performed by the authors suggested that preserving good selectivity between CB1R/CB2R and improving the affinity for CB2R would require the presence of a non-aromatic substituent on R2 capable of interacting in CB2R with the non-conserved residue Phe197 and a lipophilic substituent on R1 with a hydrogen bond acceptor atom capable of interacting in CB2R with the non-conserved residue Ser112 (Tuccinardi et al., 2006). Most of the reported compounds showed good affinity for CB2R (Table 10). Particularly compounds 7–11 and 39 and 40 were found to have very high affinity, with  $K_i$  value less than 1.0 nM and especially the last two, which possess high selectivity for CB2R.

As the authors pointed out that 4-quinolone-3-carboxamide derivatives with non-aromatic substituents at R2 could improve the affinity for CB2R and its selectivity, Pasquini and coworkers, starting from compound 10 (Table 10), introduced an adamantyl group to the nitrogen atom of the carboxamide group and an n-pentyl group at the N-1 position (Pasquini et al., 2010). The latter incorporation was considered based on the results of research conducted by Stern et al. on 1,4-dihydroquinoline-3-carboxamide derivatives (Stern et al., 2006) (Fig. 8).

The compounds obtained with the incorporation of the above modifications and the addition of a substituent at C7 of the heterocycle presented a new series of molecules with high affinity and selectivity for CB2R with agonist activity. Cascio and coworkers analyzed some derivatives obtained by Pasquini and observed that the incorporation of bulky and long substituents (Fig. 9) could affect the activity on cannabinoid receptors (Cascio et al., 2010).

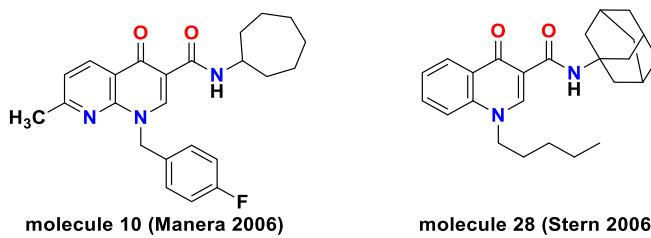
Subsequent work by Pasquini et al. showed that structural modification and extension in the length of the molecular segments resulted in obtaining a series of 4-quinolone-3-carboxamide derivatives as cannabinoid receptor ligands that behaved as partial agonists and CB2R inverse agonists/antagonists (Pasquini et al., 2010, 2011, 2012).

The heterocycle 2-pyridone is a bioisostere of a portion of the quinolone heterocycle. Kusakabe and coworkers in 2003 used this heterocycle to design new chemical structures with potential activity on cannabinoid receptors (Kusakabe et al., 2013). From the structural analysis of the CB2R-selective agonist they proposed the molecular template derived from thiopyridones in search of important segments to achieve optimal CB2R affinity. To decrease the lipophilicity generated by the isoquinolone heterocycle, the aromatic segment was truncated and the piperidin-2-one was aromatized generating the molecular template. The sulfur atom connecting both heterocycles was replaced by an oxygen atom to prevent the thioether portion from having metabolic concerns. From the molecular template, the study presented a series of molecules to analyze the optimal length of the substituent at N-1 of the 2-pyridone (Kusakabe et al., 2013) (Table 11).

In relation to the substituents incorporated in N-1 of the pyridone, it is suggested that the alkyl groups favor the binding affinity to the receptor; however, the length of the carbon chain would be limited in length to avoid decreasing its activity. Considering that the authors found that the n-butyl group would be the optimal segment for this template, they found that the n-butyl group would be the optimal segment for this template. In this study, the benzoxazole heterocycle of the molecular template was also replaced by the amide bioisosteric group incorporating an aromatic segment at its carbon atom

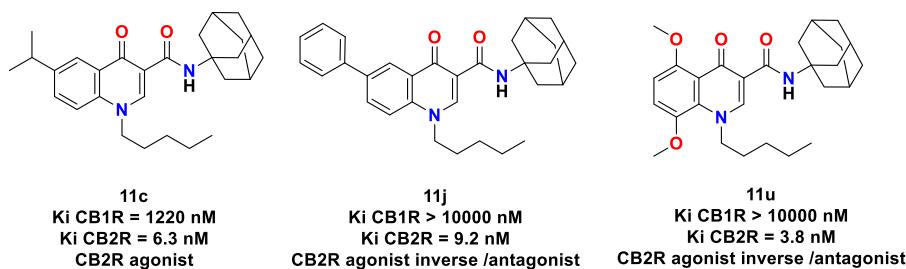
**TABLE 10** Affinity and selectivity of a series of 1,8-naphthyridin-4(1*H*)-on-3-carboxamide and 4-quinolone-4-carboxamide derivatives on CB1R and CB2R.

Compound	molecule 7 - 20		molecule 39 - 40		CB2R Ki (nM)	Ki (CB1R) /Ki (CB2R)
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	CB1R Ki (nM)		
7	p-fluorobenzyl	4-methylcyclohexyl	Methyl	8.7±1.6	1.4±0.1	6
8	o-fluorobenzyl	4-methylcyclohexyl	Methyl	37.5±5.4	8.4±0.3	4
9	Benzyl	Cycloheptyl	Methyl	143.2±9.1	5.1±1.3	28
10	p-fluorobenzyl	Cycloheptyl	Methyl	4.3±0.6	1.0±0.1	4.3
11	o-fluorobenzyl	Cycloheptyl	Methyl	149.4±1.8	13.4±4.7	11
12	Benzyl	Cyclohexyl	Cloro	463.6±1.1	24.6±4.7	19
13	p-fluorobenzyl	Cyclohexyl	Cloro	495.0±39.4	21.4±1.0	23
14	o-fluorobenzyl	Cyclohexyl	Cloro	171.2±12.3	18.1±2.7	9.5
15	1-ethyl-4-phenylpip	Cyclohexyl	Methyl	>1000	>1000	
16	Phenethyl	Cyclohexyl	Methyl	>1000	16.3±1.2	>62
17	p-metoxibenzyl	Cyclohexyl	Methyl	>1000	35.8±2.1	>28
18	p-fluorobenzyl	Cyclohexyl	H	384.1±25.3	13.0±1.4	29
19	Benzyl	Cyclohexyl	H	>1000	48.6±12.0	>21
20	Ethylmorph	Cyclohexyl	H	>1000	67.2±11.3	>15
39	Benzyl	Cyclohexyl	H	>1000	4.8±0.4	>210
40	Ethylmorph	Cyclohexyl	Cloro	>1000	3.3±0.4	>303
JWH-133				458.0±15.1	65±8.7	7.0

**FIG. 8** Comparison of structures reported by Manera and Stern and the incorporation of an adamantyl ring in the N-carboxamide group.

(Kusakabe et al., 2013). Based on the proposed modifications, the activity of a series of 2-pyridone-3-(N-carboxamide) derivatives with substituted aromatic portions was evaluated (Table 12).

The incorporation of an aromatic ring attached to the carbon atom of the carboxamide group allowed the generation of molecules active on cannabinoid receptors. From the phenyl derivative, the compounds obtained achieved binding affinities at concentrations lower than 100nM. When substituents were included in the aromatic portion, the binding affinities for both CB1R and CB2R were improved. However, with the incorporation of a chlorine atom and a methyl group in



**FIG. 9** Structural differences in Pasquini molecules that could relate changes in functionality on CB2R from agonist to inverse agonist/antagonist ligands.

**TABLE 11** Binding affinity of N-1 substituted thiopyridone series as CB2R ligands.

Compound	X	R	CB1R
			Ki (nM)
15a	O	N-butyl	976
18a	S	N-butyl	101
18b	S	Ethyl	3855
18c	S	N-propyl	276
18d	S	N-pentyl	214
<b>18e</b>	S	N-hexyl	609

**TABLE 12** Binding affinity of a series of 2-pyridone-3-(N-carboxamide) derivatives on cannabinoid receptors substituted with an aromatic moiety.

Compound	R	hCB1R Ki (nM)	hCB2R Ki (nM)
39a	Methyl	>5000	>5000
39b	Benzyl	>5000	1310
39c	Phenyl	1376	89
39d	2-Fluorophenyl	617	88
39e	2-chlorophenyl	391	16
<b>39f</b>	2-methylphenyl	390	14

*Continued*

**TABLE 12** Binding affinity of a series of 2-pyridone-3-(N-carboxamide) derivatives on cannabinoid receptors substituted with an aromatic moiety—cont'd

Compound	R	hCB1R Ki (nM)	hCB2R Ki (nM)
39g	3-methylphenyl	343	42
39h	4-methylphenyl	854	72
39i	1-naphthyl	19	12
39j	2-naphthyl	>5000	768

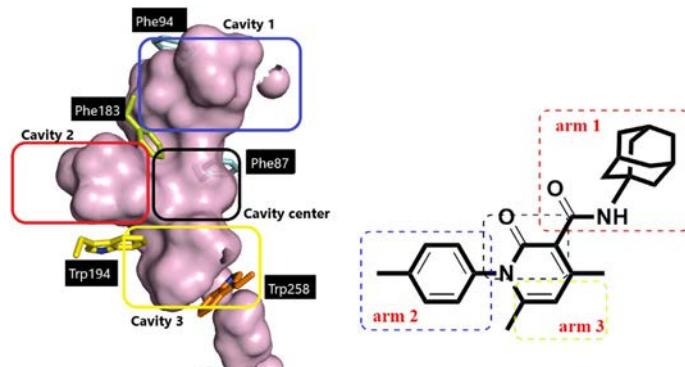
position 2 of the phenyl group, high affinities for and selectivity for CB2R were obtained. The introduction of lipophilic groups such as alkyl groups in position 2 would be important to contribute to the affinity for this receptor. Considering the naphthyl group that has been used in numerous structures derived from cannabinoid ligands, it is observed that when it is attached through its carbon 1 to the carboxamide group, it generates high affinity for both receptors and its selectivity is considerably low compared with derivatives of phenyl groups, while the naphthyl group attached through its carbon 2 considerably decreases the affinity for both CB1R and CB2R (Kusakabe et al., 2013).

Recently, our research group also explored the use of 2-pyridone heterocycle by attaching the carboxamide group through the carbon atom of the carboxylic group at position 3 of the pyridone (Faúndez-Parraguez et al., 2021). In addition, a series of molecules were synthesized by replacing the N-butyl group of the heterocycle with phenyl group and cycloalkane groups were included to the nitrogen atom of the carboxamide. The percentages of CB2R agonist response were analyzed with the synthesized compounds.

The incorporation of an adamantyl group was relevant on the nitrogen atom of the carboxamide group. This cycloalkane appears to favor the activity of the derivatives on CB2R compared with cyclohexyl and cycloheptyl. It seems that a bulky molecular portion attached to the carboxamide is necessary to favor interactions with the orthosteric site of CB2R. In addition, the presence of the phenyl group would increase the response generated by the receptor due to the interaction of this substituent, the best result was obtained with a tolyl group in N-1 of the pyridone, ruling out the existence of polar amino acid residues in the region of binding with the active site, which would explain the low percentage of CB2R agonist response of the phenolic derivatives **8g** and **8h** (Table 13). According to the functional assay performed on the compounds, the **8d** molecule resulted to act as a CB2R full agonist with EC<sub>50</sub> = 112 nM, allowing through computational assays to

**TABLE 13** Evaluation of the percentage CB2R agonist response at a concentration 10 μM of a series of 2-pyridone-3-carboxamide with N-phenyl derivatives and cycloalkanes linked to carboxamide.

Compound	Molecular template		% CB2R Agonist response	CB2R EC <sub>50</sub> (μM)
	R <sub>1</sub>	R <sub>2</sub>		
8a	Cyclohexyl	H	12	–
8b	Cycloheptyl	H	31	–
8c	Adamantyl	H	51	–
8d	Adamantyl	CH <sub>3</sub>	95	0,11
8e	Cycloheptyl	CH <sub>3</sub>	0	–
8f	Cycloheptyl	H	31	–
8g	Cycloheptyl	OH	0	–
8h	Cyclohexyl	OH	4	–



**FIG. 10** Left: structural representation of the orthosteric CB2R site. Three cavities oriented in the shape of a Y are depicted. According to the literature, for a ligand to exert agonist action on CB2R, it must occupy part of these cavities and interact with the amino acid residues in that area. Right: Representation of the agonist 8d whose computational model suggests the orientation of arm 1 in cavity 1, arm 2 toward cavity 2, the methyl groups of the pyridone in cavity 3 and the heterocycle with the carboxamide group in the cavity center.

support the design, propose a binding model, and work on future modifications to the substituents of the molecular template. In the same study, a description of the orthosteric site was made according to computational modeling studies of the receptor, pointing out that for a molecule to act on this receptor, it must locate the three-group scaffold in what is described as a “three-arm pose” (Faúndez-Parraguez et al., 2021; Li et al., 2019; Xing et al., 2020). For the interaction of ligands with the orthosteric site of CB2R, three major contact cavities were described (Fig. 10): the adamantyl group (arm 1) portion would be located toward hydrophobic **cavity 1** interacting with Phe87, Phe91, and Phe94. The tolyl group (arm 2) directed toward **cavity 2** where Tyr190, Leu191, and Trp194 are located. The methyl groups attached to the pyridone ring oriented toward hydrophobic **cavity 3**. The pyridone heterocycle would be in the **cavity center** through interactions with aromatic residues such as Phe117 and Phe183. According to the information published with the crystal structure and Cryo-EM of CB2R (Li et al., 2019; Xing et al., 2020), residue Trp258 would be key to change the activity and functionality of the receptor. The ligand must occupy cavity 3 to generate an agonist effect on CB2; however, its arm 3 must have an adequate length so that the structural segment of the ligand must not generate interaction or twisting of the amino acid residue, since its activation would be related to the change in the functional activity of CB2R turning the molecule into an inverse agonist/antagonist of CB2R.

## Summary points

- The modulation of the endocannabinoid system by synthetic ligands has allowed mediating several pathophysiological processes in the control of various diseases.
- The work of many chemists in designing drugs that activate cannabinoid receptors type I (CB1R) and type II (CB2R) has allowed exploring the structural requirements of new ligands to improve affinity and selectivity at both receptors.
- The functionality of these receptors remains poorly understood and the efforts of medicinal chemists over the last 20 years have allowed the study and creation of new molecular templates to diversify the construction of new CB1R and CB2R agonists with potential therapeutic action.
- From the classical agonists derived from tetrahydrocannabinol and the agonist WIN-55,212-2, new compounds that modulate the signaling pathway of these receptors have been developed, and this review focuses on an analysis of various cannabinoid receptor agonists.
- A chemical description of the structural requirements that allowed new molecules with higher affinity and selectivity for these systems is provided.

## Key factors

1. The endocannabinoid system is a cell signaling system that regulates biological processes such as pain, appetite, and mood by means of endogenous ligands. It consists of enzymes that synthesize and degrade endogenous ligands and receptors that are activated by their action.
2. Activation of cannabinoid receptors can inhibit or trigger neurotransmitter release, decrease neuronal excitability, regulate gene expression, and modulate cytokine release by cells of the immune system. The response may also depend on the type of receptor activated and the concentration of cannabinoid agonist used.

3. Research on the activation of cannabinoid receptors has shown that they may have therapeutic applications in a variety of diseases including cancer, epilepsy, multiple sclerosis, and chronic pain.
4. New CB1 and CB2 receptor ligands with higher affinity and selectivity than endogenous ligands of the endocannabinoid system and phytocannabinoids such as THC and cannabidiol have been designed and obtained.
5. Structural challenges for the design of new cannabinoid ligands have focused on the use of isosteric structures to improve the bioavailability, pharmacokinetics, and pharmacodynamics of the drugs and their therapeutic approach.
6. Several drugs based on cannabinoid receptor agonism have been shown to be potential new drug candidates for the control of various diseases and many of these are in clinical trials.

## Mini dictionary of terms

**Endocannabinoid system:** Cell signaling system found in the human body and other animals. This system is composed of receptors, ligands, and enzymes that work together to regulate a variety of physiological processes. Cannabinoid ligands, such as anandamide and 2-arachidonoylglycerol (2-AG), are naturally produced by the body and bind to CB1 and CB2 receptors to regulate a variety of functions, including pain regulation, appetite, mood, and memory. The endocannabinoid system is also involved in the body's response to stress and inflammation and has been shown to play an important role in regulating the immune system and protecting the brain against oxidative damage. Exogenous cannabinoids, such as tetrahydrocannabinol (THC) and cannabidiol (CBD), found in the cannabis plant, may also interact with the endocannabinoid system, and have potential therapeutic effects.

**Tetrahydrocannabinol:** Mainly referring to delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC) or simply THC. This is an organic molecule and the primary psychoactive compound found in cannabis plants. It binds specific receptors in the brain and body to produce a range of effects, including euphoria, relaxation, altered perception, and pain relief. Those receptors are called cannabinoid receptors by for being activated by this family of compounds.

**Cannabinoid receptor agonist:** A cannabinoid receptor agonist is a substance that binds to and activates cannabinoid receptors in the central and peripheral nervous system, mimicking the effects of compounds produced naturally by the human body, known as endocannabinoids. THC, the main psychoactive component present in the cannabis plant, is an example of a cannabinoid receptor agonist. THC binds to cannabinoid receptors in the brain and produces a euphoric effect, which can have a variety of unwanted side effects. Other examples of cannabinoid receptor agonists are synthetic compounds designed to interact with cannabinoid receptors.

**Synthetic cannabinoid receptor ligands:** They are man-made chemicals that mimic the effects of cannabinoids binding to the same receptors in the brain and body as THC, the primary psychoactive component in marijuana.

**Cannabinoid receptor selectivity:** It refers to the ability of a chemical compound to interact selectively with cannabinoid receptors in the human body. The CB1 receptor is found primarily in the brain and central nervous system, while the CB2 receptor is found primarily in the immune system and peripheral tissues. Compounds that interact selectively with one or the other receptors are known as selective agonists. The selectivity for cannabinoid receptors is important for the development of drugs that act specifically on these receptors and can be used in the treatment of diseases such as epilepsy, chronic pain, multiple sclerosis, and cancer, among others.

**Bioisosteres of cannabinoid ligands:** Bioisosteres are molecules that have a similar structure but with different atoms, which allows them to maintain a similar biological function. In the case of cannabinoid ligands, bioisosteres have been developed to improve their activity and selectivity. For example, THC bioisosteres have been developed that contain a methyl group in place of the hydroxyl group at position 11, which increases their affinity for CB1 and CB2 cannabinoid receptors and decreases their psychoactive activity. CBD bioisosteres containing a pyrazole ring instead of the benzene ring have also been developed, which increases their antioxidant and antiinflammatory activity.

**Orthosteric site of cannabinoid receptors:** The orthosteric site is the primary binding site on a receptor for its natural ligand or agonist. In the case of cannabinoid receptors, the orthosteric site is where the endogenous cannabinoid anandamide and exogenous cannabinoids such as THC found in marijuana bind. The orthosteric site is also important in the design of drugs that target cannabinoid receptors. By understanding the structure and function of the orthosteric site, scientists can design molecules that selectively bind to the orthosteric site to modulate receptor activity.

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## Chapter 31

# Fatty acid amide hydrolase, anandamide, and neurological diseases

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### Abbreviations

ABPP	activity-based protein profiling
AD	alzheimer’s disease
AEA	N-arachidonylethanolamine
2-AG	2-arachidonoylglycerol
ARDS	acute respiratory distress syndrome
AS	amidase signature
CBD	cannabidiol
CB <sub>1</sub> R	cannabinoid receptor type 1
CB <sub>2</sub> R	cannabinoid receptor type 2
CNS	central nervous system
COX-2	cyclooxygenase-2
CSF	cerebrospinal fluid
eCBs	endocannabinoids
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
GPCR	G-protein-coupled receptor
HD	Huntington’s disease
HTT	huntingtin
NAAA	<i>N</i> -acylethanolamine-hydrolyzing acid amidase
NADA	<i>N</i> -arachidonoyldopamine
NAPE-PLD	<i>N</i> -acyl-phosphatidylethanolamines-specific phospholipase D
NArPE	<i>N</i> -arachidonyl-phosphatidylethanolamine
NAT	<i>N</i> -acyltransferase
3NP	3-nitropropionic acid
MAGL	monoacylglycerol lipase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	multiple sclerosis
PD	Parkinson’s disease
PPARs	peroxisome proliferator-activated receptors
THC	Δ <sup>9</sup> -tetrahydrocannabinol
TM	transmembrane domain
TRPV1	transient receptor potential vanilloid 1

## Introduction

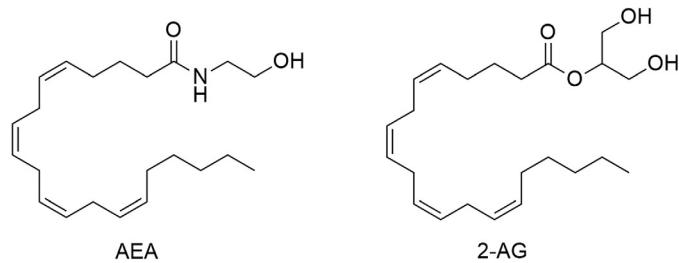
The endocannabinoid system (ECS) is a complex cell-signaling system identified in the early 1990s by researchers who were interrogating the mechanism(s) of action of cannabinoids. The ECS is an evolutionarily conserved lipid-based network, which comprises endocannabinoids (eCBs) such as anandamide (*N*-arachidonylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) (Fig. 1), along with their receptor targets and metabolic enzymes.

In addition to AEA and 2-AG, eCBs include other important  $\omega$ -6 (n-6) fatty acid compounds, such as 2-arachidonoylglycerylether (noladin ether), *O*-arachidonylethanolamine (virodhamine) and *N*-arachidonoyldopamine (NADA), as well as  $\omega$ -3 (n-3) fatty acid derivatives like *N*-eicosapentaenylethanolamine and *N*-docosahexaenylethanolamine (Fezza et al., 2014). Furthermore, “eCB-like” compounds such as *N*-palmitoylethanolamine, *N*-oleoylethanolamine, and *N*-stearoylethanolamine have been shown to exert a receptor-independent “entourage effect” that prolongs the half-life of eCBs, thus potentiating their biological activity (Fezza et al., 2014).

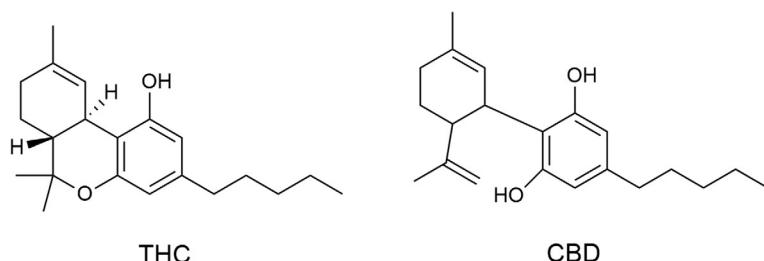
The best-known molecular targets for eCBs are two G-protein-coupled receptors (GPCR), termed type 1 (CB<sub>1</sub>R) and type 2 (CB<sub>2</sub>R) cannabinoid receptors (Maccarrone, 2020). Yet, accumulated evidence suggests that additional targets for eCBs (and notably also for plant-derived cannabinoids) do exist, such as the transient receptor potential vanilloid type 1 (TRPV1) channel (Fenwick et al., 2017), the peroxisome proliferator-activated receptors (PPARs)  $\alpha$ ,  $\delta$ , and  $\gamma$ , and the orphan G-protein-coupled receptors GPR55, GPR119, and GPR18 (Iannotti & Vitale, 2021; Maccarrone, 2020; Ramírez-Orozco et al., 2019). In particular, AEA shares many properties with  $\Delta^9$ -tetrahydrocannabinol (THC) (Fig. 2), the major psychoactive component of marijuana that acts as a partial agonist of CB<sub>1</sub>R and as a weak partial agonist/antagonist of CB<sub>2</sub>R (Mechoulam & Parker, 2013).

The biological functions of eCBs are finely tuned through the control of their endogenous tone, achieved by controlling both biosynthesis and degradation. AEA can be produced by several biosynthetic routes (Fezza et al., 2014), of which the best known is a *N*-acylation catalyzed by *N*-acyltransferase (NAT) to generate the AEA precursor, *N*-arachidonyl-phosphatidylethanolamines (NArPE), followed by the activity of a *N*-acyl phosphatidylethanolamines-specific phospholipase D (NAPE-PLD) that generates AEA. To date, several enzymes and metabolites involved in the NAPE-PLD-independent biosynthesis of AEA have been demonstrated through knockout mice (Fezza et al., 2014). These alternative pathways of AEA metabolism are shown in Fig. 3.

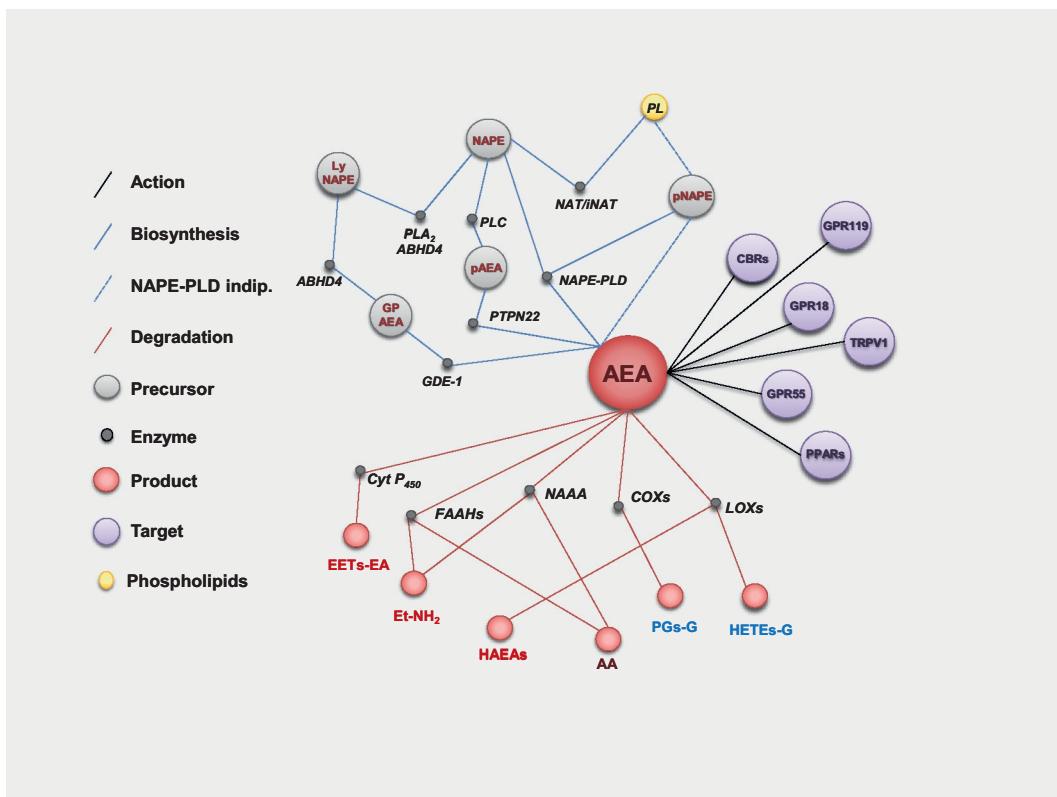
The ECS also includes a number of catabolic enzymes responsible for the termination of eCBs signaling, among which the main responsible for AEA hydrolysis is fatty acid amide hydrolase (FAAH) (Maccarrone, 2017). More recently, alternative hydrolytic enzymes of AEA have been reported, e.g., a new FAAH-2 that was discovered in humans (Wei et al., 2006) and a lysosomal hydrolase termed *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Ueda et al., 2010) (Fig. 3).



**FIG. 1** Chemical structure of main endocannabinoids. Structure of *N*-arachidonylethanolamine, AEA and 2-Arachidonoylglycerol, 2-AG.



**FIG. 2** Chemical structure of the most important phytocannabinoids. Structure of  $\Delta^9$ -tetrahydrocannabinol, THC, and Cannabidiol, CBD.



**FIG. 3** Figure shows the alternative metabolic pathways and molecular targets of AEA. AA, arachidonic acid; ABHD4, alpha/beta-hydrolase 4; CBRs, cannabinoid receptors; COXs, cyclooxygenases; Cyt P<sub>450</sub>, cytochrome P<sub>450</sub>; EETs-EA, epoxyeicosatrienoyl ethanolamides; EtNH<sub>2</sub>, ethanolamine; FAAHs, fatty acid amide hydrolases; GDE1, glycerophosphodiester phosphodiesterase 1; GP-AEA, glycerophospho-AEA; HAEAs, hydroxyanandamides; LOXs, lipoxygenases; ly-NAPE, lyso-N-arachidonoylphosphatidylethanolamine; NAAA, N-acylethanolamine-hydrolyzing acid amidase; NAPE-PLD, N-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D; NArPE, N-arachidonoylphosphatidylethanolamine; NAT, N-acyltransferase; pAEA, phospho-AEA; PL, phospholipids; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PLC, phospholipase C; PMs, prostamides; pNAPE, N-arachidonoylethanolamine plasmalogens; PPARs, peroxisome proliferator-activated receptors; PTPN22, protein tyrosine phosphatase, nonreceptor type 22; TRPV1, Transient Receptor Potential Vanilloid 1.

In addition, AEA can be oxygenated by well-known enzymes able to produce biologically active lipids, among which cyclooxygenase-2 (COX-2), various lipoxygenase isozymes and cytochrome P450 monooxygenases (Fazio et al., 2020) (Fig. 3).

Despite biosynthesis and breakdown of AEA are quite well understood, the extracellular, transmembrane, and intracellular transport (trafficking) of this eCB remains largely elusive. AEA internalization has been studied for years in multiple cellular models, and a variety of mechanisms has been suggested to explain it. The first suggested mechanism has been facilitated transport, yet no transporter protein has been ever cloned (Maccarrone, 2017). Then, a passive diffusion driven by FAAH has been put forward, along with an intracellular sequestration and a caveolae-dependent endocytosis (Kaczocha et al., 2012).

Overall, it can be stated that FAAH is the most promising target to modulate biological activity (and therapeutic potential) of AEA in human health and disease (Maccarrone, 2017).

FAAH has been found in the human central nervous system (CNS), where it is localized in postsynaptic neurons, and in many peripheral tissues and organs. In particular, the distribution of FAAH in the CNS, which often overlaps on that of CB<sub>1</sub>R, is fairly uniform within the gray matter of cerebral cortex, cerebellum, basal ganglia, and thalamus (Egertová et al., 2003). Instead, AEA has been found in regions that poorly express CB<sub>1</sub>R, and it seems to correlate with the expression of eCB targets other than CBRs (Leishman et al., 2016).

Altogether, eCBs, their metabolic enzymes, and receptors form a complex network called “expanded” ECS or endo-cannabinoidome (Cristino et al., 2020).

Accumulated data suggest that enhancing the eCBs tone, in particular that of AEA, has therapeutic potential for many human diseases. It is also apparent that FAAH is the key player in the control of AEA signaling, thus serving as an attractive therapeutic target for the treatment of human pathologies, neurological disorders included.

## Fatty acid amide hydrolase

Fatty acid amide hydrolase (FAAH) is a membrane-bound enzymes belonging to the serine hydrolase family. It is a homodimer enzyme with a primary sequence rich in Ser and Gly residues, showing a molecular mass of ~63 kDa per subunit. FAAH is a member of the amidase signature (AS) superfamily and is endowed with an unusual Ser241-Ser217-Lys142 catalytic triad with a strong preference for hydrophobic substrates (Fazio et al., 2020) (Fig. 4).

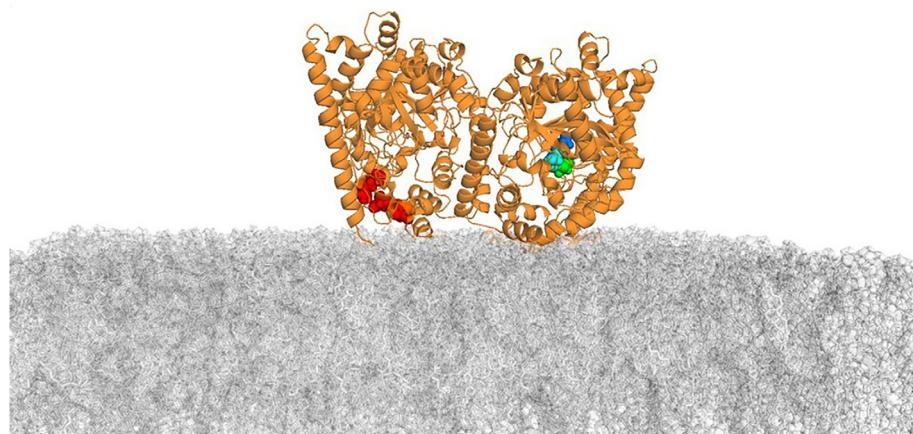
It plays a significant role in termination of fatty acid amides signaling, both in the CNS and at the periphery (Tripathi, 2020) and is widely distributed throughout the body. In rats, it is expressed in large amounts not only in the nervous system but also in the liver, small intestine, testes, uterus, kidney, ocular tissues, and spleen. Furthermore, abundant human FAAH mRNA is localized in the pancreas, kidney, brain, and skeletal muscle while smaller amounts were found in the placenta and liver (Giang & Cravatt, 1997).

FAAH not only hydrolyzes AEA to arachidonic acid (AA) and ethanolamine, but it also hydrolyzes 2-AG to AA and glycerol, although the main 2-AG hydrolytic enzyme remains monoacylglycerol lipase (MAGL). A polymorphism in the *faah* gene, whereby Thr replaces Pro at position 129, makes the protein more susceptible to proteolysis and leads to reduced enzymatic activity (Rafiei & Kolla, 2021). Structurally, FAAH homodimer is characterized by a core structure of a twisted  $\beta$ -sheet surrounded by 24  $\alpha$ -helices (Cravatt et al., 1996). In particular,  $\alpha$ -18 and  $\alpha$ -19 helices are very important, because they allow attachment to the membrane also in the case of a  $\Delta$ TM-rat FAAH where part of the transmembrane (TM) domain has been deleted; indeed, also this truncated form shows a remaining TM of 20 amino acids (residues 9–29), so that the AEA substrate can reach the active site via a membrane channel (Criscuolo et al., 2020). Furthermore, FAAH presents four cavities: (i) a membrane access channel, through which the substrates reach the catalytic site; (ii) an acyl-chain binding cavity; (iii) an oxyanion hole close to the catalytic triad; and (iv) a cytosolic port that allows the exit of hydrolyzed substrate.

Of note, recently, it has been demonstrated that both rat and human FAAH have an unprecedented allosterism, due to a fine communication between the two enzyme subunits that is seemingly controlled by a single amino acid at the dimer interface (Dainese et al., 2020).

## AEA and neurological disorders

Accumulated evidence suggests that cannabis (*Cannabis sativa* or *C. indica*) extracts can afford symptomatic relief in diverse neurodegenerative disorders (Chiurchiù et al., 2018). This observation supports the hypothesis that AEA signaling dysregulation could be implicated in the symptomatology of these diseases (Table 1). In particular, alterations in the ECS have been extensively investigated in a range of neurodegenerative disorders that include Alzheimer's (AD), Parkinson's (PD), Huntington's (HD) diseases, and multiple sclerosis (MS).



**FIG. 4** FAAH structure. Overview of the FAAH embedded in a lipid bilayer. The membrane lipids are shown in gray. The catalytic triad (Ser241-Ser217-Lys142) residues are represented in sticks green, blue, and cyan, respectively. The so-called “membrane access” is shown in red molecular surface representation.

**TABLE 1** Selected patented FAAH inhibitors.

Compound	IC <sub>50</sub> value	Patent
URB597	19 nM versus rFAAH 5 nM versus hFAAH	US20130150346; 2013
URB937	27 nM versus peripheral FAAH	WO2012015704; 2012
ARN2508	31 nM versus rFAAH	WO2014023643; 2014
JNJ-42119779	70 nM versus hFAAH	WO2010141817; 2010
JNJ-40413269	5.3 nM versus hFAAH	WO2009105220; 2009
PF-04457845 derivative	7.4 nM versus rFAAH 7.2 nM versus hFAAH	WO2009127944; 2009

## Alzheimer's disease

Affecting up to 55 million people worldwide, Alzheimer's disease (AD) is the most common form of dementia among the elderly. Extracellular neuritic plaques, caused by the accumulation of a toxic form of amyloid  $\beta$ -protein (A $\beta$ ), are AD pathological hallmarks (Iannotti et al., 2016). These features lead to synaptic dysfunction and degeneration of neurons in cortical and subcortical regions, as well as to a chronic inflammatory response (due to activated microglia and astrocytes) and cellular energy deficits.

Growing evidence supports a correlation between AD and ECS alterations. CB<sub>1</sub>R has been reported to be downregulated, upregulated, or unchanged in AD models, whereas CB<sub>2</sub>R appears to be always upregulated, and so does FAAH. Increased AEA hydrolysis by FAAH results in elevated AA concentration, which is notably a precursor of proinflammatory molecules such as eicosanoids, and thus promotes neuroinflammation (Chiuchiù et al., 2018; Maccarrone et al., 2007).

Unsurprisingly, in experimental animal models, FAAH inhibition is neuroprotective through mechanism(s) involving PPAR $\alpha$ , PPAR $\gamma$ , and TRPV1, which are all activated by enhanced content of AEA, and possibly of other FAAH substrates such as PEA, overall triggering A $\beta$  reduction. Accordingly, in FAAH knockout mice, depletion in amyloid concentration and improvement in memory deficit have been observed (Bajaj et al., 2021; Cristino et al., 2020).

Consistent with these findings, in human AD brain, increased FAAH expression has been detected in astrocytes and activated microglial cells embracing neuritic plaques, confirming that FAAH could be a key modulator of pathogenic AD microglial cells function (Ren et al., 2020). Furthermore, FAAH activity appears also enhanced in CNS and in peripheral blood mononuclear cells in human. Accordingly, in a postmortem study a reduction in AEA concentration and a negative correlation between AEA levels and A $\beta$  accumulation in midfrontal and temporal cortex (Jung et al., 2012) have been reported.

More recently, studies on AD human brain reported a decrease FAAH activity in the frontal cortex and no alteration in FAAH expression in the hippocampus (Berry et al., 2020; Cristino et al., 2020). Although AEA levels in AD serum appear unaltered, the reduced oleamide concentration in serum confirms the increased FAAH activity peripherally (Berry et al., 2020).

## Parkinson's disease

Parkinson's disease (PD) is the second most common, slowly progressing, neurodegenerative disease, characterized by dyskinesia, postural instability, and muscular rigidity. Neuropathologically, the main PD feature, is the progressive loss and degeneration of dopaminergic neurons in the substantia nigra pars compacta and in the striatum, which causes typical motor activity disorders (Drui et al., 2014).

Since many evidences highlight ECS's key role in modulating dopaminergic neurotransmission its function in PD pathogenesis and treatment has been investigated.

In particular, high CB<sub>1</sub>R distribution in basal ganglia, area involved in motor activity, has been reported, suggesting that AEA concentration can be crucial to ameliorate motor dysfunction in PD, such as the ability of the well-known eCB receptors to reduce neuroinflammation (Ren et al., 2020; Wang et al., 2022).

Research and clinical observations suggest ECS hyper-functionality in both PD patients and experimental animal models. In particular, AEA levels in the cerebrospinal fluid (CSF) appear more than double in untreated PD patients compared with normal controls, regardless of disease stage and symptoms (Pisani et al., 2005). Experimental models, mainly obtained treating animals (rats and nonhuman primates) with neurotoxins able to reproduce dopaminergic neurons degradation, show similar pattern of results. In fact, in PD rat models, increased striatal AEA levels, correlated with decreasing AEA transport and FAAH activity, were reported; reserpine treated rats and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned monkeys exhibit augmented AEA levels in the globus pallidus (Cristino et al., 2020; More & Choi, 2015).

Furthermore, parkinsonian rats chronically treated with FAAH inhibitor showed ameliorate motor dysfunction, due to decreased AEA degradation, enhancing CB<sub>1</sub>/CB<sub>2</sub>R activation. However, contradictory data are reported on FAAH inhibition neuroprotective effect, exerted by preventing dopaminergic neurons loss or reducing glial activation (Soti et al., 2022). In particular, chronic FAAH inhibitor treatment on PD rat models showed to prevent motor dysfunction but not dopaminergic neurons loss, and therefore, lacking a neuroprotective effect. Conversely, in a different study, a decrease in dopaminergic cell loss and a reduction in glial activation following FAAH inhibition were reported (Viveros-Paredes et al., 2019).

Interestingly, in PD animals treated with L-DOPA, thus exhibiting typical dyskinesia (L-DOPA induced dyskinesia, LID), AEA levels reduction was observed (van der Stelt et al., 2005). LID improvement with URB597, a potent FAAH inhibitor, treatment was only detected in coadministration with a TRPV1 antagonist, which prevented AEA binding to TRPV1 receptor (More & Choi, 2015).

Altogether these findings suggest that increased AEA levels could represent a compensatory mechanism to counterbalance dopamine deficit and motor dysfunction.

## Huntington's disease

Huntington's disease (HD), also known as Huntington's chorea, is a rare, autosomal dominant, neurodegenerative disorder. The main pathologic HD peculiarity is the abnormal aggregation of huntingtin protein (HTT), due to a mutation of its encoding gene (i.e., an expansion of the CAG triplet repeat, resulting in polyglutamine expansion), which leads to dopaminergic neurons death in the globus pallidus. Characteristic symptoms, proportional to the CAG triplet expansion rate, are cognitive decline, uncontrolled dancing-like movements, psychiatric disturbances, and premature death (Cristino et al., 2020; Iannotti et al., 2016; Maccarrone et al., 2007).

In vivo positron emission tomography of HD patients highlights CB<sub>1</sub>R loss, suggesting an ECS degeneration in this disease and a possible role for eCBs in neurodegenerative progression of HD (Van Laere et al., 2010). Consistent with this, alteration in AEA levels and FAAH activity was detected in postmortem human brain and in animal models, such as transgenic mice HTT mutated form and rats treated with toxins (e.g., 3-nitropropionic acid, 3NP), able to reproduce the characteristic HD neurodegeneration. In particular, 3NP lesioned-induced rats exhibit low levels of AEA in the corpus striatum and increased AEA in substantia nigra; in R6/1 transgenic mice, decreased AEA levels in hippocampus were reported, while in R6/2 change in eCB tone seems to be region-specific and disease-phase related (Bisogno et al., 2008; Maccarrone et al., 2007).

Furthermore, in HD patients, FAAH peripheral activity strongly decreased, causing an AEA sixfold increase in lymphocytes. Interestingly, HD patients in a presymptoms phase already show a depression of FAAH activity, suggesting its potential role as early peripheral marker (Battista et al., 2007).

Overall, in light of ECS hypo-functionality in HD, compounds able to modulate eCBs tone have been taking into account as neuron protectors. Consistent with this, in 3NP-injected rats treated with AM404, an AEA transport inhibitor and TRPV1 agonist, a reduction in motor dysfunction in early phase of hyperkinetic activity and in neurochemical deficit (Lastres-Becker et al., 2003) has been observed. Administration of UCM707, a potent and selective AEA transport inhibitor, to 3NP and malonate lesioned rats corroborated the antihyperkinetic activities, relating with AEA enhanced levels, although, in this case without protection against neurodegeneration (de Lago et al., 2006).

Furthermore, no antihyperactivity effect was observed in HD experimental models treated with AM347 or URB597, two potent FAAH inhibitors. Nonetheless, AM347 gave rise to ambulation improvement, while URB597 administration prevented CB<sub>1</sub>R depletion in the striatum (Lastres-Becker et al., 2003; Maccarrone et al., 2007; Maya-López et al., 2017).

## Multiple sclerosis

Multiple sclerosis (MS) is a chronic, immune-mediated disease, and it is the most common inflammatory demyelinating disease of the CNS. Nowadays, no effective cure is available in fact there is a need for new treatment options, especially for

its progressive forms. The ECS could help with the identification of novel therapeutic route interacting in three key pathomechanisms of MS: inflammation, neurodegeneration, and oxidative stress. In a preclinical study, mice treated with THC, methanandamide, and synthetic CB<sub>1</sub>R or CB<sub>2</sub>R agonists have shown an amelioration of tremor and spasticity, demonstrating that ECS may participate in the control of MS (Baker et al., 2000). In different studies, MS patients have shown increased eCBs levels in the blood; otherwise CSF levels were decreased, instead other studies have reported increased AEA levels in the CSF, in the peripheral lymphocytes and brain (Cristino et al., 2020). Furthermore, in animal models, PEA levels were increased in CNS but decreased in the CSF (Baker et al., 2000; Di Filippo et al., 2008).

In order to evaluate the beneficial effects of enhanced AEA level, selective inhibitors of the cellular AEA reuptake were used. These compounds were able to alleviate both motor deficits and neuroinflammation, and these effects were partially reversed by CB<sub>1</sub>R or CB<sub>2</sub>R antagonists (Maramai & Brindisi, 2020).

Moreover, Malfitano and coworkers demonstrated that selective activation of CB<sub>2</sub>R on isolated T lymphocytes from MS patients inhibits cell proliferation and immune responses without inducing cell death (Malfitano et al., 2008). The involvement of CBRs was also confirmed by their genetic deletion. In fact, CB<sub>1</sub>R ablation in mice led to poorly tolerated inflammatory and excitotoxic insults and neurodegeneration development (Musella et al., 2014; Rossi et al., 2015). Whereas, specific brain CB<sub>2</sub>R deletion is correlated with T cells increased proliferation, reduced apoptosis, and elevated production of proinflammatory cytokines. All together, these results suggest that pharmacological ECS manipulation might also result in novel pharmaceuticals for MS.

## Clinical trials

In the last two decades, several studies were conducted to discover small molecules able to interact with the ECS. Initially, CB<sub>1</sub>R was the most relevant target to better understand its involvement in several pathophysiological conditions and how to take advantage of its modulation. Unfortunately, there were substantial difficulties in translating CB<sub>1</sub>R ligands into druggable candidates, mainly due to the adverse effects associated with CB<sub>1</sub>R activation.

For that reason, it has been thought to inhibit AEA degradation rather than directly CB<sub>1</sub>R activation, and specific FAAH inhibitors have been developed and reported in literature. In Table 2, selected FAAH inhibitors (Fazio et al., 2020) are reported.

Over the years, many clinical trials have been performed with FAAH inhibitors to assess their therapeutic potential. In this context, the tragic use of FAAH inhibitor BIA10–2474 in phase I clinical trials has to be recalled. In 2016, one volunteer died and four others were hospitalized because of serious adverse neurological events. Newsworthy, activity-based protein profiling (ABPP) analysis demonstrated that BIA10–2474 interacts with many serine hydrolases in addition to FAAH (van Esbroeck et al., 2017). Furthermore, integrated approach allowed to disclose differences in species-specific sensitivity of FAAH inhibitors (Criscuolo et al., 2020).

**TABLE 2** Changes of AEA and FAAH in the major neurological disorders.

Neurological disorders	AEA and/or FAAH alteration	Reference
Parkinson's disease	Increased AEA level in the external globus pallidus	van der Stelt et al. (2005)
	Elevated AEA level in cerebrospinal fluid of patients	Pisani et al. (2005)
Alzheimer's disease	In midfrontal and temporal cortex AD patients decreased AEA level	Jung et al. (2012)
	Low FAAH in human frontal cortex	Pascual et al. (2014)
Amyotrophic lateral sclerosis	Increased AEA level in the spinal cord of SOD1 transgenic mice	Bilsland et al. (2006)
Multiple sclerosis	Decrease AEA level in the striatum and midbrain	Cabranes et al. (2005)
	Increased AEA level in human lymphocytes subsets	Sánchez López et al. (2015)
Epilepsy	Elevated AEA level in the hippocampus	Marsicano et al. (2003)
Schizophrenia	Elevated AEA in human blood	De Marchi et al. (2003)
Huntington's disease	Increase AEA in human lymphocytes Low FAAH activity	Battista et al. (2007)

Beyond these poor results, several trials are conducted using all the ECS proteins as targets because their involvement in several diseases is indisputable.

## Neurological disorders

eCBs are involved in different neurological disorders, so during the last years, many studies and clinical trials were conducted.

Anxiety disorders are the most common mental health problem, which are one of the principal disability worldwide. Furthermore, existing treatments are partially effective for most patients (Paulus et al., 2021).

Mice FAAH knockout are resistant to stress-induced changes in structure and function (Hill et al., 2013), and other studies show how the CB<sub>1</sub>R activation is involved in fear reduction (Gunduz-Cinar et al., 2013; Lisboa et al., 2015). Therefore, FAAH inhibition and the resulting accumulation of fatty acid amides may have anxiolytic effect in humans, caused by accumulation of eCBs acting on the CB<sub>1</sub>R. Paulus and coworkers used JNJ-42165279 (potent and selective FAAH inhibitor, with an IC<sub>50</sub> of 70 nM) in a 4-day administration study with healthy male volunteers. They reported that JNJ-42165279 has attenuated the activation in the amygdala, anterior cingulate, and bilateral insula during the face emotion processing task. Taken together, their results are consistent with the hypothesis that JNJ-42165279 shares some effects with existing anxiolytic agents such as benzodiazepines but does not affect fear conditioning.

Further, FAAH seems to be correlated with posttraumatic stress as well, disorder characterized by persistence of fear memories. In particular, Mayo and coworkers recently reported that FAAH inhibition, in human, can improve the recall of fear extinction memories and decrease the effects of stress. In this study, they used PF-04457845, a potent FAAH inhibitor, for 10 days, and it produced a large increase of AEA peripheral levels. Individuals reported enhanced recall of fear extinction when tested 24 h after extinction training. Moreover, FAAH inhibition attenuated specific components of the stress response and reduced negative affect assessed via facial electromyography. The authors reported no serious adverse events and few no serious adverse events. Together, these data provide evidence that FAAH inhibition has beneficial effects on fear extinction and stress reactivity providing a rationale for developing this drug class for posttraumatic stress disorder (Mayo et al., 2020).

Aran and coworkers studied cannabinoids treatment for autism, supported by animal models data, 150 children and adolescents entered the trial (Aran et al., 2021). Participants received placebo or cannabinoids for 12 weeks, testing efficacy, followed by a 4-week washout and predetermined crossover for another 12 weeks to evaluate tolerability. In particular, they tested BOL-DP-O-01-W, a whole-plant cannabis extract containing two major cannabinoids: cannabidiol (CBD) and THC (Fig. 2) at a 20:1 ratio and BOL-DP-O-01, purified CBD and THC at the same ratio. The authors reported no serious adverse events; however, common adverse events were somnolence and decreased appetite, reported for 28% and 25% on whole-plant extract, respectively; 23% and 21% on pure-cannabinoids, and 8% and 15% on placebo. In this study, they have demonstrated that cannabinoids treatment has the potential to decrease disruptive behaviors associated with autism spectrum disorder, with acceptable tolerability. This treatment could be important specifically for individuals with autism spectrum disorder who are overweight, because cannabinoids treatment was associated with net weight loss in contrast to the substantial weight gain typically produced by antipsychotics.

Leweke and coworkers previously reported that an elevation of AEA levels in CSF inversely correlated to psychotic symptoms (Leweke et al., 2012). They performed a double-blind, randomized clinical trial of CBD vs amisulpride, a potent antipsychotic, in acute schizophrenia to evaluate the clinical relevance of their initial findings (Leweke et al., 1999). CBD was selected because at 10 μM reduces FAAH activity (roughly 50%) but does not significantly interact with common signaling systems involved in schizophrenia such as dopamine, glutamate, and serotonin. To determine whether treatment with CBD reduces FAAH activity, they evaluated AEA levels in serum before and after exposure to CBD or amisulpride. They reported that AEA levels were higher in subjects exposed to CBD than in those exposed to amisulpride. Furthermore, they found out that the serum levels of PEA and OEA (FAAH substrates) were also elevated in schizophrenic patients treated with CBD, compared with those treated with amisulpride. They also reported a statistically significant association between increase in AEA levels and decrease in psychotic symptoms in patients treated with CBD; meanwhile, no such association was found in patients treated with amisulpride. These results suggest that FAAH inhibition may contribute to the antipsychotic effects representing a completely new route in the treatment of schizophrenia.

## Applications to other area 1

Typically, all studies about FAAH, in preclinical and clinical phase, involve its inactivation. However, it could be possible to investigate FAAH activation in order to develop new therapeutic strategy. About that, it well known that arachidonic acid and its metabolism are correlated with vasodilation effects (Miller et al., 2003). A novel approach, it could be discovering a

class of molecules acting as FAAH activator to get up AEA degradation and so high arachidonic acid concentration. Miller and coworkers figured out that arachidonic-acid-induced vasodilation is lipoxygenase-dependent; in fact this effect is abolished by lipoxygenase inhibitors (Miller et al., 2003).

Furthermore, aspirin's pharmacological effects are attributed to the inhibition of COX-1 and COX-2 and interference with arachidonic acid metabolism (Patrono et al., 2005). Its mechanism of action occurs through permanent inactivation of the COX enzymes in concentration manner dependent, low dose for COX-1 and high dose for COX-2 (Patrono et al., 2005). Blocking COXs activity could lead at high arachidonic acid degrading via LOXs with consequent vasodilation effect. It is thus possible that, FAAH activator, increasing arachidonic acid level, could be important to find out new strategy to treat hypertension condition.

## Applications to other area 2

The ECS is a complex network of enzymes and receptors, widely expressed in the human body, including several members of the innate and adaptive immune system. In general, eCBs, as well as several cannabinoids, have showed to affect immune functions, thereby regulating inflammation, autoimmunity as well as antipathogen immune responses. It is important to emphasize that the arachidonic acid product by FAAH activity can be further converted into a wide range of eicosanoids by oxidative enzymes.

Noteworthy, CBD administration has shown to downregulate the level of proinflammatory cytokines and ameliorated the clinical symptoms of Poly I:C-induced acute respiratory distress syndrome (ARDS) in a murine model (Khodadadi et al., 2020). The Poly(I:C) in a murine system may be a practical model to investigate the mechanisms responsible for SARS-CoV-2 and other respiratory-virus-induced ARDS-like symptoms (Khodadadi et al., 2020).

Considering all CBD therapeutic effects and the large distribution of ECS in the human body, it is plausible that CBD could be used as a protective agent in the treatment of various inflammatory conditions including COVID-19 (van Breemen et al., 2022). CBD is a nonpsychoactive phytocannabinoid, which is able to bind a series of receptors, including GPR55, TRPV, and PPAR $\gamma$ . Further, CBD was shown to influence eCB balance via FAAH inhibition (Criscuolo et al., 2020).

All together, these findings suggest to evaluate the potential protective role for CBD as part of the treatment of COVID-19 by reducing the cytokine storm and reestablishing inflammatory homeostasis via FAAH inhibition blocking arachidonic acid cascade.

## Mini-dictionary of terms

- **AS superfamily.** Enzymatic class composed of hydrolytic enzyme that catalyzes amide bond cleavage. Members of this family share the presence of 130 aminoacids, called “amidase signature,” a high conserved C-terminal region, rich in Ser and Gly, and a unique catalytic triad composed of two Ser and one Lys.
- **Allostery.** Biological phenomenon referred to the transmission of the binding effect at one site to another distant functional site. Biological activity regulation often occurs through this type of mechanism.
- **Knockout animals.** Laboratory animals genetically modified by suppressing a targeted gene, with the aim of investigating gene function, genetic causes of human diseases and validating new drugs and treatment.
- **Methanandamide.** Synthetic AEA analogous, agonist of CBRs, with a  $K_i$  of 20 and 815 nM for CB<sub>1</sub>R and CB<sub>2</sub>R, respectively. The only structural difference, with respect to natural AEA, is the methyl substituent on one carbon, which confers improved stability and resistance to FAAH hydrolysis.
- **Poly I:C.** Abbreviation of Polyinosinic:polycytidylic acid that is an immunostimulant. Its sodic salt is useful to simulate virus infection.
- **Positron Emission Tomography.** This functional imaging technique uses radioactive substances, allows to measure metabolic and/or biochemical activity (function). It is particularly helpful for cancer, PD, and HD diagnosis.
- **R1/6 and R2/6 mice.** Two HD transgenic mice models. The first one presents 115 CAG repeats, able to reproduce a slow disease progression; on the contrary, the latter model has 150 CAG repeats and is characterized by cognitive disorders and motor dysfunction in premature age.

## Key facts of FAAH

- FAAH was purified and cloned from rat and later on from mouse, porcine, and human tissues in 1996.
- The 3D structure of rat FAAH was reported in 2003.
- The 3D structure of human FAAH has not yet been reported, due to solubility problems and tendency of this enzyme to aggregate.

- The central role of FAAH was confirmed by the analysis of *faah*  $-/-$  mice, which show a 50–100-fold reduction in FAAH activity and a more than 10-fold increase in brain levels of many fatty acid amides, including AEA.
- A functional genetic polymorphism of FAAH has been related to heavier use of alcohol in youth, prior to the onset of chronic drinking problems.

## Key facts of AEA

- The name anandamide derived from “ananda,” the Sanskrit word for “bliss.”
- AEA is also considered an endovanilloid.
- The low AEA levels reflect the amounts of arachidonic acid esterified on the *sn*-1 position of phospholipids.
- The identity of the putative “endocannabinoid membrane transporter” still remains elusive.
- In 2002, a compound where the arachidonic acid and ethanolamine joined together by an ester bond and was called virohodamine was reported.

## Summary points

- AEA regulates several pathophysiological conditions
- AEA targets are CBRs and other receptors, such as TRPV1 and PPARs
- AEA biological activity finely modulates by its in vivo concentration
- FAAH is a key regulatory enzyme of fatty acid amide levels
- AEA levels and FAAH activity/expression are altered in many neurodegenerative diseases
- FAAH is an important pharmacological target

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## Chapter 32

# The endocannabinoid system in the immunobiology of dendritic cells

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## Abbreviations

2-AG	2-arachidonylglycerol
AEA	anandamide
CB	cannabinoid receptor
CDC	conventional dendritic cell
DC	dendritic cell
IFN	interferon
PDC	plasmacytoid dendritic cell
TLR	toll-like receptor

## Introduction

Dendritic cells (DCs) are very important innate immune cells, not only due to their function as professional antigen-presenting cells, but also for provision of the critical second and third signals to interacting lymphocytes for an effective immune response, namely critical cytokine production and co-stimulatory capacity for antigen-specific lymphocyte activation, respectively. Thus, a number of regulatory pathways, both cell-intrinsic and extrinsic, are operative in these cells. Among such regulatory receptors, expression of cannabinoid receptors on DCs has been documented, which opens up the diverse potential for regulation by the endogenous ligands of these receptors, i.e., the endocannabinoids, in diverse clinical contexts. We first briefly introduce the present knowledge on the endocannabinoid system that will be essential to appreciate their potential role in dendritic cells. Then we briefly look up the present domain knowledge on dendritic cell immunobiology. These are followed by critical assessment of the diverse insights gathered on the potential role of endocannabinoid system in regulating the functions of the two major DC subsets.

## The key components of endocannabinoid system

### The endocannabinoids

The endocannabinoid system comprises the endocannabinoids (or endogenous cannabinoids) that are small bioactive lipid molecules, enzymes that are responsible for either biosynthesis or degradation of the endocannabinoids, and finally, the receptors that bind the endocannabinoids and drive a downstream signaling cascade influencing the functional responses in the cells that express them (Lu & Mackie, 2016; Rahaman & Ganguly, 2021). Arachidonic acid is the initial substrate for the biosynthesis of the endocannabinoids anandamide (or *N*-arachidonylethanolamine or AEA) and 2-arachidonyl glycerol (2-AG) (Chiurchiù et al., 2015). Oleamide another metabolite of arachidonic acid metabolism also is considered to be a member of the endocannabinoid family, although it does not seem to signal through the bona fide cannabinoid receptors (Kita et al., 2019). Enzymes that synthesize and degrade endocannabinoids play key roles in their physiological functions (Ramer et al., 2019), namely fatty acid-amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), and alpha/beta-hydrolase domain containing 6 (ABHD6) (Rahaman & Ganguly, 2021; Zurier & Burstein, 2016). There is

evidence that endocannabinoids may also undergo oxidative catabolism through other enzymes such as lipoxygenase, cyclooxygenase 2 (COX-2), and cytochrome P450 isoenzymes (Pandey et al., 2009). Some of the metabolites derived from endocannabinoids on enzymatic degradation are also bioactive and warrant further exploration (Turcotte et al., 2017).

## Cannabinoid receptors

The endocannabinoids play critical physiological roles in pain sensation, fear response, memory formation as well as inflammation. Their roles in inflammation are mediated plausibly by both neural regulations and receptor signaling in immune cells. There are two established receptors for endocannabinoids, cannabinoid receptor 1 (CB1, coded by the gene CNR1) and cannabinoid receptor 2 (CB2, coded by the gene CNR2), originally discovered as receptors for the naturally occurring cannabinoid molecules, especially  $\Delta$ -9-tetrahydrocannabinol (THC), the major bioactive molecule found in marijuana plant (Matsuda et al., 1990; Rahaman & Ganguly, 2021).

Cannabinoid receptors are G-protein-coupled receptors with characteristic seven transmembrane domains, activated by three major categories of ligands, namely the endocannabinoids, plant cannabinoids such as THC, and a variety of synthetic cannabinoids. CB1 receptor is expressed mainly in the central nervous system (CNS) apart from its documented expression in the lungs, liver, and kidneys. CB2 receptor is known to get expressed in the immune cells, with some evidence for their expression in the CNS as well. There are also evidences for other cannabinoid receptors, in addition to CB1 and CB2, expressed in endothelial cells as well as in the CNS (Di Marzo & Piscitelli, 2015). For example, a G-protein-coupled receptor GPR55 expressed in the CNS has been shown to bind several cannabinoid molecules (Johns et al., 2007). In the microglia, P2Y family receptors have been shown to be activated by the endocannabinoid oleamide (Kita et al., 2019).

## Dendritic cells and their role in the immune system

Dendritic cells (DCs) are the innate antigen-presenting cells (APCs) in the immune system and so named because of their surface morphology consisting of cellular extensions presumably facilitating their antigen presentation function (Banchereau et al., 2000; Ganguly et al., 2013; Steinman, 2012). DCs are released from the bone marrow, on being differentiated through common dendritic cells precursors (CDPs) that have shared contribution from both myeloid (major) and lymphoid (minor) lineages (Reizis, 2019).

## Conventional dendritic cells

The classically described DCs (called conventional DCs or cDCs), first conclusively established in the late 70s of the last century, are the professional APCs, which patrol the body through circulation as well as through brief sojourns in the tissue spaces sampling antigens and experiencing different danger signals or pathogen-associated molecular patterns (DAMPs or PAMPs) using the diverse array of pattern recognition receptors (PRRs), e.g., toll-like receptors (TLRs) (Ganguly et al., 2013; Steinman, 2012). In inflammatory contexts, circulating monocytes also may assume a functional phenotype mimicking cDCs forming the so-called monocytes-derived inflammatory DCs (moDCs) (Tang-Huau & Segura, 2019). In vitro such monocytes-derived DCs are generated by culturing human monocytes in the presence of the cytokines GM-CSF and IL-4, and this has been a major source of human dendritic cells for experimental probing (Steinman, 2012).

On encountering an event of PRR activation in the tissue, cDCs undergo a phenotypic differentiation (so-called “maturation”) and respond to chemotactic cues driving a migration to secondary lymphoid organs, such as the draining lymph nodes for the tissues cDCs are traveling through (Steinman, 2012). In the lymph nodes, the cDCs interact with the T cells, presenting peptides, processed from the antigens they have sampled in the tissues, in the context of MHC class I and class II molecules. The “maturation” process also upregulates a plethora of surface molecules, called the costimulatory molecules (namely CD80, CD86, and CD83), which can interact with cognate receptors on the T cells sending an activating “second signal.” The MHC-peptide complex (pMHC) recognition by the T cell receptors (TCRs) in the context of concomitant costimulatory molecule interactions drive activation, clonal expansion, and cytokine production in T cells and initiates the adaptive immune response. While several other cells, especially of the myeloid lineage, namely macrophages, can also perform the antigen presentation function, the key ability that distinguishes cDCs from them is the high density surface expression of MHC molecules on the cell membrane, enabling them to present antigen to relatively rare antigen-inexperienced naïve T cells, in addition to the lymph node-resident clones of antigen-experienced effector T cells (Banchereau et al., 2000; Ganguly et al., 2013).

## Plasmacytoid dendritic cells

Another subset of DCs, established much later in late 90s of the last century, are called the plasmacytoid dendritic cells (pDCs) (Gilliet et al., 2008; Liu, 2005; Reizis, Bunin, et al., 2011; Reizis, Colonna, et al., 2011; Reizis, 2019). PDCs do have the ability to present antigens as well, but the key functional attribute of these cells is rapid and massive production of type I interferons (IFNs) and at times type III interferons, especially in response to viruses, and also to other pathogens. The activation of pDCs driving type I IFN induction ensues from activation of specialized TLRs in pDCs, which reside in the endolysosomal compartments and recognize nucleic acids—TLR9 recognizing unmethylated CpG motifs in DNA molecules and TLR7 recognizing single-stranded RNA molecules (Ganguly, 2018; Gilliet et al., 2008). The nucleic acids recognized by TLR9 and TLR7 frequently are of pathogenic origins. Nevertheless, release of extracellular nucleic acids from host cells in various contexts of aberrant tissue damage and cell death can also be recognized by TLRs, driving type I IFN induction and fueling a cascade of inflammation (Ganguly, 2018; Ganguly et al., 2009, 2013; Lande et al., 2007).

Apart from their role as key initiators of immune response, DCs have also been demonstrated to have the potential to thwart an impending immune response, through their so-called tolerogenic functions (Steinman et al., 2003). In the steady state, self-antigen sampling by cDCs in the tissues in the absence of PRR activation has been shown to generate tolerogenic DCs, which presenting antigens to T cells drive them into states of quiescence, anergy, or regulatory. The T cells thus primed may generate Foxp3-expressing T regulatory cells (Tregs) or IL-10 producing Tr1 cells, which in turn can thwart activation of effector T cells (Ganguly et al., 2013). This tolerogenic function of cDCs can also play a role in an ongoing inflammation, thereby providing a breaking mechanism. Like cDCs, pDCs have also been demonstrated to drive expansion of Tregs as well as Tr1 cells through various mechanisms, again shown to be operative in the steady state as well as during ongoing inflammation, largely determined by microenvironmental cues (Gilliet & Liu, 2002; Ito et al., 2007; Nestle et al., 2005).

## Endocannabinoid system in conventional dendritic cells

### Expression in conventional dendritic cells

Perhaps the first demonstration of cannabinoid receptors in human DCs was done almost 20 years back, in a study that used LC-APCI-MS on lipids extracted from immature human dendritic cells and identified 2-AG, AEA, and an anti-inflammatory AEA congener, *N*-palmitoylethanolamine (PalEtn). In this study, the immature DCs were generated from peripheral blood monocytes using GM-CSF and IL-4, and they were driven to maturation using the TLR4 agonist bacterial lipopolysaccharide (LPS). These DCs were shown express transcripts of both CB1 and CB2 receptors. Mature DCs were found to have higher abundance of 2-AG, with no significant change in the other endocannabinoids (Matias et al., 2002). Source of the endocannabinoids in the body may be diverse and context-specific. Notably, peripheral nerve endings are known to lie in the vicinity of immune cells both in the secondary lymphoid organs and in the tissues, and they can also be a source of endocannabinoids, providing a micro-niche neuro-immune regulation (Ahluwalia et al., 2003).

A more recent study attempted at mapping the expression of CB2 receptors in different immune cell subsets, as this receptor is known to be expressed more widely in hematopoietic cells, using a transgenic mouse, which expressed the green fluorescent protein controlled by the CB2 promoter (Schmöle et al., 2015). On flow cytometry CB2 expression was documented in CD11b+ cells, which notably represent both macrophages and a significant subset of cDCs in mouse. On immunohistochemistry, a significant co-expression was documented with CD11c, the bona fide cell surface marker of all DCs, as well as with B220, a cell surface molecule expressed on both B cells and pDCs.

### Early studies on putative function of endocannabinoids in cDCs

In one of the early studies in this domain, the endocannabinoid 2-AG, which has potent ligand action on CB2 receptors, was shown to function as a chemotactic cue for mouse cDCs, in both their immature and mature phenotypic states (Maestroni, 2004). In a delayed-type hypersensitivity model sensitization of mice by keyhole limpet hemocyanin (KLH) in the presence of the TLR2 agonist peptidoglycan from *S. aureus*, in the presence or absence of 2-AG, revealed that sensitization in the presence of 2-AG drove an enhanced DC migration and Th1 memory response in the lymph nodes in subsequent exposure to the soluble KLH antigen. This effect could be inhibited by the CB2 antagonist SR-144528. In another study, similar enhancement of Th1 polarized immune response was documented when AEA was administered prior to sensitization using ovalbumin. AEA also drove cDCs on ex vivo stimulation toward higher co-stimulatory molecule expression (CD80, CD86) and cytokine induction (IL-12p40). This study also demonstrated a differential effect of AEA on ex vivo splenocytes when compared between nano-molar (pro-inflammatory) and micro-molar (anti-inflammatory) concentrations (Ribeiro et al., 2010). Of note here, in another

delayed-type hypersensitivity model in mice using methylated BSA as the antigen, it was shown that endocannabinoids produced by T and B cells themselves attenuate inflammation (Sido et al., 2016).

In contrast to this pro-inflammatory potential for endocannabinoids acting on cDCs, AEA has been shown to inhibit voltage-gated K<sup>+</sup> channels in mouse bone-marrow-derived cDCs and abrogate co-stimulatory molecule expression as well as antigen presentation, as demonstrated in allogeneic mixed lymphocyte reaction (Svensson et al., 2010; Wacnik et al., 2008). In mice, cannabinoid receptor activation with high doses of endocannabinoids also has been shown to induce apoptosis of cDCs (Do et al., 2004). Mice deficient in both the cannabinoid receptors when infected with influenza virus showed more severe pulmonary disease associated with enhanced cDC maturation and T cell activation (Karmaus et al., 2011).

### A dominant anti-inflammatory function for endocannabinoids

These contradictory reports of role of CB receptor signaling in cDCs with time have been replaced with more skewed data toward an anti-inflammatory effect. For example, AEA has been shown to inhibit TNF- $\alpha$  induction in cDCs and expansion of TH1 and Th17 cells (Chiurchiù et al., 2013). In a murine cardiac allograft model, using a fully MHC-mismatched transplant, it was shown that allograft rejection was significantly accelerated in mice genetically deficient in CB2 receptor (Kemter et al., 2015). This robust graft rejection reaction in these CB2-deficient mice was associated with higher activation of cDCs. Bone-marrow-derived cDCs in these mice produced higher amounts of proinflammatory cytokines and drove T cells more toward producing IFN- $\gamma$  and IL-17 in vitro.

In another study, supporting the anti-inflammatory effect of endocannabinoid signaling, stimulation of bone-marrow-derived cDCs by the TLR4 agonist bacterial LPS was shown to induce production of 2-AG (Dotsey et al., 2017). In this study, administration of a cannabinoid receptor antagonist concomitant to immunization with a custom vaccine led to significantly enhanced immune response in both young and aged (usually shown to drive an attenuated response in response to the vaccine) mice. This study thus demonstrated a critical role of endocannabinoids in immune response to vaccines.

In a more recent study, using both type 1 (LPS-induced sepsis) and type 2 (allergy) immune contexts, cannabinoid receptor signaling was shown to modulate cDCs to promote generation of FoxP3<sup>+</sup> Tregs (Angelina et al., 2022). While this study explored the cDC phenotype using synthetic cannabinoid agonist, the functional outcomes should be comparable for endocannabinoids as well. CB1 signaling in human cDCs was shown by this study to inhibit NF- $\kappa$ B, MAPK, and mTOR signaling. It rather induced AMPK and autophagy. A role of PPAR $\alpha$  also was shown to contribute to a metabolic switch to oxidative phosphorylation. These drove a tolerogenic phenotype in the cDCs, which was demonstrated by comparative protection in both the LPS-induced sepsis and anaphylaxis models in mice.

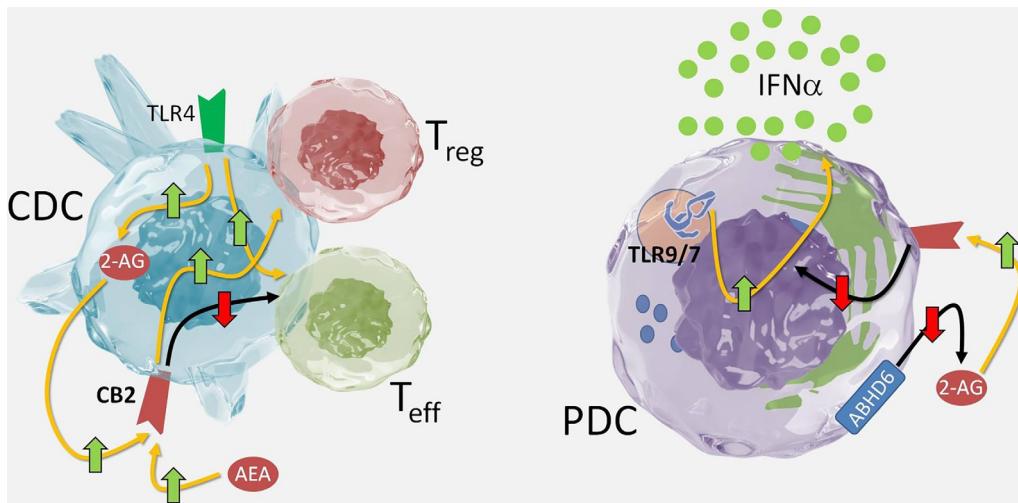
### Endocannabinoids and cDCs in anti-cancer immunity

Cannabinoid receptors are expressed by a variety of cancer cells and a plethora of cancer-cell-intrinsic effects of cannabinoid receptor agonists, most frequently anti-tumor effects, are reported (Iozzo et al., 2021). On the other hand, cDCs play a critical role in the so-called cancer-immunity cycle, namely tumor antigen sampling and tumor-antigen-specific T cell priming as well as tuning the inflammatory milieu in tumor microenvironment by expressing relevant cytokines (Chen & Mellman, 2013). Thus, whether the endocannabinoid system has any role in regulating cDCs in the context of anti-tumor immunity is an intriguing question, more so because in most contexts, an anti-inflammatory role of them is envisaged, which should be detrimental in case of cancer. Interestingly, in a study with orthotopic pancreatic ductal adenocarcinomas in mice, a more equivocal effect of 2-AG was demonstrated. While 2-AG inhibited growth of the cancer cells and rather drove a maturation program in cDCs, it was shown to expand a population of myeloid-derived suppressor cells, thus counteracting its anti-tumor roles. Further exploration is thus warranted to chart the cDC-intrinsic effects of endocannabinoids and cannabinoid receptor signaling in anti-cancer immunity.

Thus, although majority of studies with cDCs demonstrate an anti-inflammatory effect of endocannabinoids and cannabinoid receptor signaling, diversification of response in specific contexts and at different systemic and local concentrations of endocannabinoids cannot be ruled out and warrants deeper exploration.

### Endocannabinoid system in plasmacytoid dendritic cells

Endocannabinoid system and its role in plasmacytoid dendritic cells are rather less explored. Nevertheless, an inhibitory effect on type I IFN induction in pDCs was reported in an early study that looked at them in parallel to cDCs (Chiurchiù et al., 2013).



**FIG. 1** Regulatory effect of endocannabinoids in conventional and plasmacytoid dendritic cells.

More recently, expression of ABHD6, an enzyme that degrades endocannabinoids, was found to have enriched expression in human pDCs (Rahaman et al., 2019). ABHD6 has been genetically linked to systemic lupus erythematosus (SLE), the systemic autoimmune disease in which pDC-derived type I IFNs are known to play a critical role (Ganguly, 2018; Lande et al., 2011; Oparina et al., 2015; Sisirak et al., 2014).

Rahaman et al. reported that in a subset of SLE patients, there is higher expression of ABHD6, which is correlated with the interferon signature gene expression in the peripheral blood of these patients. 2-AG when added to pDC cultures did not inhibit type I IFN induction in response to TLR9 stimulation. But with concomitant inhibition of ABHD6, an inhibitory role of 2-AG on type I IFN induction was demonstrated, revealing a pDC-intrinsic function of endocannabinoid degradation. Thus, the level of ABHD6 expression in pDCs fine-tuned a resistance mechanism of the endocannabinoid for type I IFN induction, which is deranged in some patients with SLE leading to aberrant type I IFN induction, which fuels the disease activity. This inhibitory role of 2-AG was mediated through CB2 receptors as a specific antagonist for CB2 or RNA interference of CB2 in pDCs abrogated the functional effects of 2-AG (Rahaman et al., 2019; Rahaman & Ganguly, 2021).

pDCs are very critically involved in a number of autoimmune diseases as well as in metabolic disorders (namely type 2 diabetes and atherosclerosis), wherein the type I IFN induction in response to TLR activation plays a major role (Ganguly, 2018; Ganguly et al., 2013; Ghosh et al., 2016). On the other hand, an intra-tumoral rewiring of pDCs to a tolerogenic phenotype, which is devoid of efficient type I IFN induction but leads to priming of Tregs *in situ*, has also been described (Conrad et al., 2012; Labidi-Galy et al., 2011; Raychaudhuri et al., 2019; Sisirak et al., 2012). This intra-tumoral anti-inflammatory rewiring has been shown to be mediated by cancer-cell-derived cytokines as well as the metabolite lactate. Whether dysregulated abundance of endocannabinoids in the tumor microenvironment or in tissues involved in an autoimmune response play a role in pDC regulation in these contexts, making pDCs dysfunctional in the context of cancer or allowing hyperactivation of type I IFN production in an autoimmune context, remains an interesting question left to be explored (Fig. 1).

## Conclusion

The two major subsets of DCs, namely conventional DCs (cDCs) and plasmacytoid DCs (pDCs), are specialized in their functions and thus play critical and non-redundant roles in orchestrating both innate and adaptive immune responses, with distinctive immuno-cellular cross talks specific for discreet pathophysiological contexts. Thus, regulatory mechanisms operative in these cells, involving endocannabinoids and the cannabinoid receptors, add very critical dimensions in our knowledge about immunity in particular and on human pathophysiology in general. Knowledge in this domain is still in its adolescence, but concerted efforts worldwide in understanding this novel regulatory pathway in DCs are gradually establishing endocannabinoids and the cannabinoid receptors as novel therapeutic targets in DCs for different clinical contexts.

## Summary points

- Dendritic cells are critical innate immune cells
- Endocannabinoids and their receptors are documented in dendritic cells
- Most literature points to an anti-inflammatory role of endocannabinoids in dendritic cells
- Endocannabinoids drive tolerogenic polarization of conventional dendritic cells
- Endocannabinoid dysregulation may underlie activation of plasmacytoid dendritic cells in autoimmunity

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## Further reading

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## Chapter 33

# Palmitoylethanolamide and other anandamide congeners in neuroinflammation-based disorders: Linking in the endocannabinoid system

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>Abn-CBD</b>	abnormal cannabidiol
<b>AEA</b>	arachidonylethanolamide or anandamide
<b>AMT</b>	anandamide membrane transporter
<b>BBB</b>	blood-brain barrier
<b>BDNF</b>	brain-derived neurotrophic factor
<b>CB1</b>	cannabinoid receptor 1
<b>CB2</b>	cannabinoid receptor 2
<b>CNS</b>	central nervous system
<b>DAGL<sub>α/β</sub></b>	Diacylglycerol lipase $\alpha$ and $\beta$
<b>FAAH</b>	Fatty acid amide hydrolase
<b>GDNF</b>	glial cell-derived neurotrophic factor
<b>GPR</b>	G protein-receptor
<b>IL</b>	interleukin
<b>LPS</b>	lipopolysaccharide
<b>MAGL</b>	monoacylglycerol lipase
<b>NAPE-PLD</b>	<i>N</i> -acylphosphatidylethanolamine phospholipase D
<b>NEAs</b>	<i>N</i> -acylethanolamines
<b>NO</b>	nitric oxide
<b>PEA</b>	palmitoylethanolamide
<b>PEA-OXA</b>	<i>N</i> -palmitoylethanolamine-oxazoline
<b>PNS</b>	peripheral nervous system
<b>PPAR-α</b>	peroxisome proliferator-activated receptor $\alpha$
<b>ROS</b>	reactive oxygen species
<b>TBI</b>	traumatic brain injury
<b>TNF-α</b>	tumor necrosis factor alpha
<b>TRPV1</b>	transient receptor potential vanilloid type 1

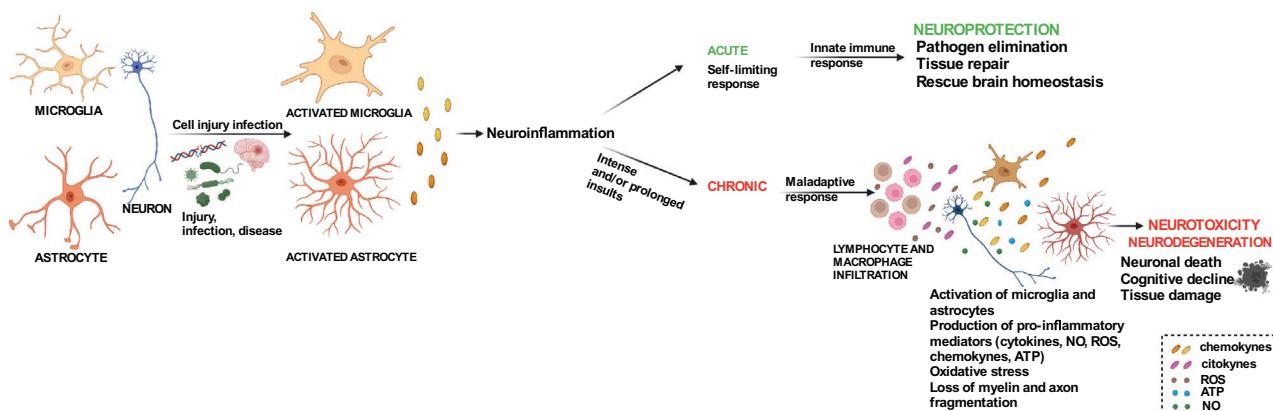
## Introduction

Neuroinflammation is a common mechanism in the pathogenesis of many, if not all, diseases of the central and peripheral nervous system (CNS and PNS) including anxiodepressive and cognitive disorders, psychosis, neurodegenerative diseases, traumatic brain injury (TBI), stroke, multiple sclerosis, epilepsy, chronic pain, and autism spectrum disorders. Neuroinflammation represents the response of the CNS to injury, infection, and disease aimed at protecting and preserving the integrity of the CNS. If prolonged, intense, or unbalanced, the process loses its physiological defensive role and promotes

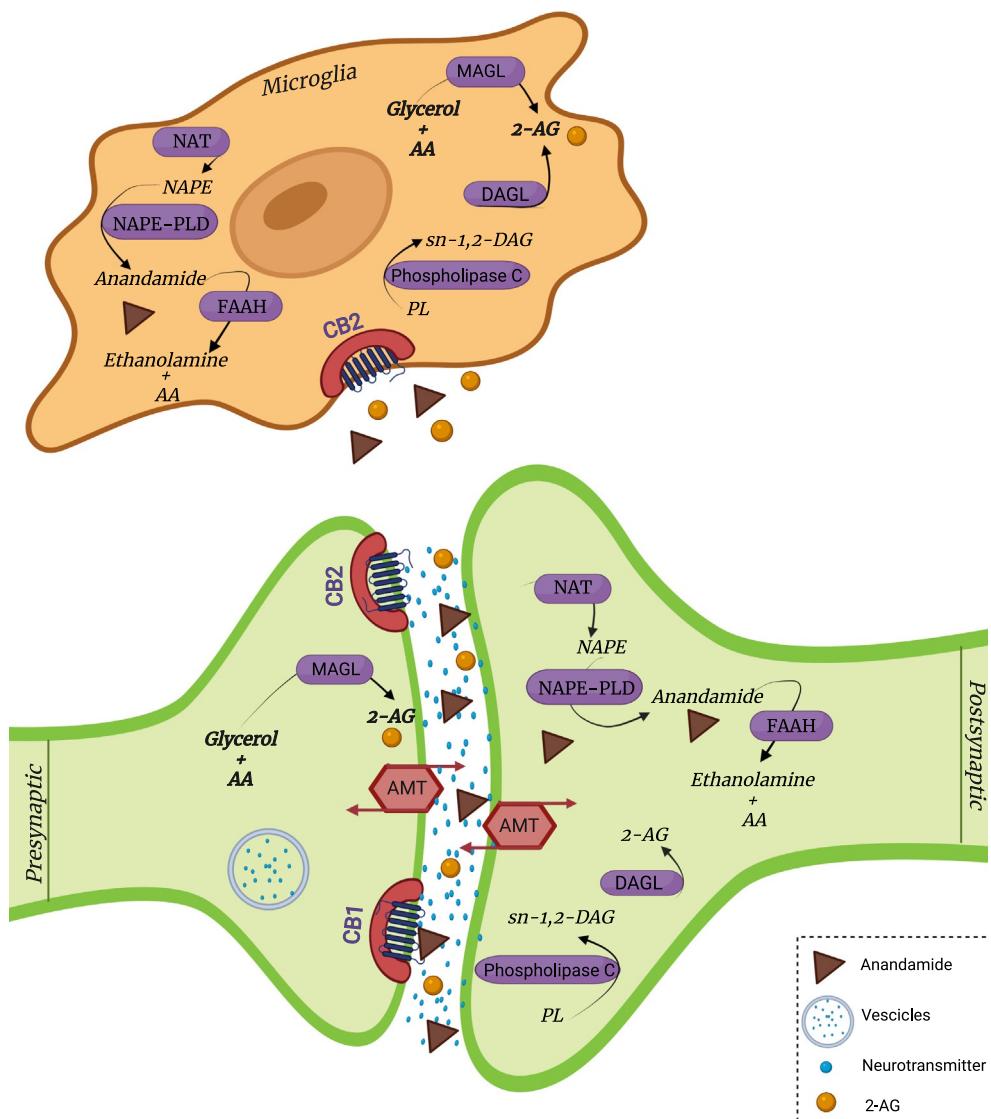
aberrant and maladaptive responses with harmful rather than protective consequences. Neuroinflammation consists of a cascade of events carried out by neurons and non-neuronal cells. Microglia are the main cell population involved in primary immune surveillance serving as the CNS-resident macrophages. In a resting state, microglia guarantee the homeostasis controlling cell development, survival, and synaptogenesis in the CNS. Under brain injury, infection, stroke, and disease microglia become “activated,” undergo a cytoskeletal rearrangement, produce cytokines and chemokines promoting the recruitment of leukocytes in the brain, thus causing neuroinflammation. The main mediators involved in neuroinflammation produced by activated microglia and astrocytes are the pro-inflammatory cytokines interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), the chemokines CCL2, CCL5, and CXCL1, nitric oxide, prostaglandins, and reactive oxygen species (ROS). Other cell populations involved in neuroinflammation are astrocytes, endothelial cells, perivascular macrophages, and peripherally migrated T cells. This alteration of the surrounding microenvironment causes demyelination and loss of axons on neurons. The pathological consequences of neuroinflammation are edema, increased permeability or breakdown of the blood-brain barrier (BBB), vascular occlusion, ischemia, tissue damage, loss of neuronal functions driving to cognitive impairment, and cell death at the base of neurodegenerative diseases (DiSabato et al., 2016) (Fig. 1). It appears evident that the crucial point in the development of diseases based on neuroinflammation is the transition from a beneficial/protective role toward a harmful mechanism. Given the common “neuroinflammatory” nature of neuropsychiatric and neurodegenerative diseases, the enhancement of neuroprotective mechanisms at the expense of harmful ones could represent the turning point for effective therapy (Brambilla, 2019).

## The “Good Actors” in Neuroinflammation

A crucial role in the protective response to neuroinflammation is played by the endocannabinoid system, consisting of the two main endogenous ligands, the *N*-arachidonoyl ethanolamine or anandamide (AEA) and 2-arachidonylglycerol (2-AG), the cannabinoid receptors 1 and 2 (CB1 and CB2), the synthesizing and degrading enzymes for AEA (the anabolic N-acylphosphatidylethanolamine phospholipase D, NAPE-PLD, and catabolic fatty acid amide hydrolase, FAAH) and 2-AG (the anabolic diacylglycerol lipase  $\alpha$  and  $\beta$ , DAGL $\alpha/\beta$ , and catabolic monoacylglycerol lipase, MAGL), and the anandamide membrane transporter (AMT) (Fig. 2). This system is activated by the direct CB1/2 receptor ligands and by cannabimimetics that indirectly enhance the activity of endocannabinoids. Endocannabinoid congeners of the AEA, such as *N*-acylethanolamines (NAEs), lipoaminoacids, and neurotransmitter acyl conjugates, which act also on different receptors such as the peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ), the transient receptor potential vanilloid type 1 (TRPV1), and non-cannabinoid G-protein-coupled receptors (GPRs) 18, 55, and 119 play a fundamental role in the homeostatic response to harmful endogenous and exogenous stimuli. This involvement in intrinsic protective responses against neuroinflammation offers a target, whose enhancement could have a relevant therapeutic impact (Di Marzo, 2009).



**FIG. 1** Neuroinflammation mechanisms. The scheme illustrates the main cell types involved, the causes, the underlying mechanisms, and the pathophysiological consequences that underlie neuroinflammation. Neuroinflammation is a protective mechanism, but if intense, protracted, or unresolved, it loses its protective significance and becomes harmful.



**FIG. 2** Endocannabinoid system. Representative image of the cannabinoid system in which the CB1 and CB2 receptors, the endogenous endocannabinoids AEA and 2-AG, the respective anabolic and catabolic pathways, precursors of anabolism and products of catabolism and AMT are reported.

## Endocannabinoids in Neuroinflammation

Endocannabinoids and related NAEs play a crucial role in the regulation of metabolic processes, behavior, and immunity. Normally these lipid mediators, which are produced “on-demand,” are abundant in the brain and are found in traces in the periphery and the blood. In pathological conditions affecting the CNS, the system is activated, carrying out an anti-inflammatory, analgesic, and neuroprotective action (Kasatkina et al., 2021).

## Endocannabinoids and Microglia

Microglia can produce AEA and 2-AG in both healthy and neuroinflammatory conditions. In damaged tissue, the release of ATP stimulates microglia to produce 2-AG. The 2-AG and PEA, in turn, modulate microglia reducing the number of excitotoxic lesions and damaged neurons in the hippocampus via the activation of abnormal cannabidiol (abn-CBD), GPR18, and PPAR- $\alpha$  receptors (Hohmann et al., 2019). 2-AG also increases the chemokinesis and chemotaxis enhancing the migration of microglia at the site of injury in a way dependent on CB2 receptors, which result upregulated on microglia by pro-inflammatory cytokines. Since microglia can switch to pro-inflammatory M1 and anti-inflammatory M2 phenotypes, the CB2 receptor stimulation attenuates the microglia M1 phenotype promoting the M2 phenotype (Ma et al.,

**TABLE 1** Endocannabinoids and neuroinflammation.

Cell types	Ligand	Receptor	Outcome	References
Microglia	2-AG	abn-CBD, GPR18, PPAR $\alpha$	Reduction of excitotoxic lesions and damaged neurons in the hippocampus	Hohmann et al. (2019)
	PEA	CB2		Ma et al. (2015)
	AM1241	abn-CBD	Inhibition of pro-inflammatory M1 and enhancement of anti-inflammatory M2 phenotype	Kreutz et al. (2009)
	(CB2 agonist)		Decrease in inflammatory cytokines	
	2-AG		Inhibition of NMDA-induced excitotoxicity	
Astrocytes	$\Delta^9$ -THC	CB1	Protection against oxidative stress and apoptosis	Carracedo et al. (2004)
	2-AG			Wang et al. (2016, 2018)
	2-AG		Protection against LPS exposure and oxygen-glucose deprivation	Feliu et al. (2020)
	2-AG, PEA	PPAR $\alpha$	Protection against multiple sclerosis lesions	Scuderi et al. (2011)
	PEA, OEA		Attenuate amyloid $\beta$ -induced astrocyte activation	Gajardo-Gómez et al. (2017)
			Protection against astrogliosis and neural death	Scuderi et al. (2012); Luo et al. (2019)
	2-AG	CB1/CB2	Inhibition of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, inducible nitric oxide synthase, and prostaglandins	Li et al. (2017)
	FAAH inhibitors		Inhibition of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, Iba-1, MCP-1	Henry et al. (2017)
	PEA	CB2	Inhibition of inflammatory cytokines	Impellizzeri et al. (2017)
	OEA, PEA	CB1/CB2 (inverse agonism)	Reduction of TNF- $\alpha$ , PGE2, NF-kB, iNOS, COX2 protection against LPS	Sayd et al. (2014)
	SR141716A, SR144528	CB1	Reduction of TNF- $\alpha$ , protection against LPS	Roche et al. (2006)
	SR141716A	(inverse agonism)	Increase pro-inflammatory cytokines	Lou et al. (2012)

The table shows some of the main actions performed by cannabinoids on the mechanisms underlying neuroinflammation. The cell type, the cannabinoid ligand, the receptor involved, the mediated effect, and the respective reference are indicated for each evidence.

2015) (Table 1). This evidence indicates a reciprocal interaction between endocannabinoids and microglia, which conveys in a neuroprotective effect mainly mediated by the CB2 receptor (Kasatkina et al., 2021).

## Endocannabinoids and Astrocytes

In addition to microglia, astrocytes also play an important neuroprotective role in the CNS and express the CB1 and CB2 receptors. By being close to the blood vessels, they maintain the functionality of the BBB and modulate the innate immune response by controlling the release of cytokines, chemokines, and ROS. Astrocytes, such as microglia, produce 2-AG in response to ATP, or following the exposure to endothelin and/or the stimulation of the CB1 receptor. 2-AG plays, in turn, a protective role on astrocytes by being able to protect them against LPS (Wang et al., 2018), chondroitin sulfate proteoglycan, which accumulates in multiple sclerosis (Feliu et al., 2020), oxygen and/or glucose deprivation (Wang et al.,

2016), and  $\beta$ -amyloid exposure (Scuderi et al., 2011). Stimulation of CB1 and CB2 receptors on astrocytes prevents apoptosis and oxidative stress induced by ceramide or the LPS-induced release of NO (Carracedo et al., 2004; Kasatkina et al., 2021). NAEs such as PEA and oleylethanolamide (OEA) counteract astrocyte activation via activation of PPAR $\alpha$  (Luo et al., 2019; Scuderi et al., 2011, 2012) (Table 1).

## Endocannabinoids and Pro-inflammatory Mediators

Many of the neuroprotective effects of endocannabinoids are based on the regulation of cytokine release. 2-AG is able to reduce the overexpression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, inducible nitric oxide synthase, and prostaglandins following several inflammatory stimuli in the brain (Li et al., 2017). Similarly, the increase in AEA levels, by inhibition of its degradation, counteracts the overexpression of TNF- $\alpha$ , IL-1 $\beta$ , Iba-1, IL-6, and monocyte chemo-attracting protein-1 caused by aging or excessive exposure to ethanol (Henry et al., 2017). Intriguingly, the CB1 receptor inverse agonist, SR141719A or rimonabant, reduced the LPS-induced increase in IL-1 $\beta$  levels and exhibits neuroprotective effects in models of retinal degeneration and brain ischemia (Roche et al., 2006), although it is also capable of increasing pro-inflammatory cytokines levels in an experimental autoimmune encephalomyelitis model (Lou et al., 2012). The CB2 receptor indirectly activated by OEA and PEA seems particularly involved in the inhibitory modulation of pro-inflammatory cytokines following pathological states such as LPS exposure, intracerebral hemorrhages, surgery, and in a Parkinson's disease model (Sayd et al., 2014). As observed for rimonabant, also the CB2 inverse agonist, SR144528, decreased the LPS-induced increase in IL-1 $\beta$  levels (Kasatkina et al., 2021; Roche et al., 2006) (Table 1).

## Endocannabinoids and Neurodegeneration

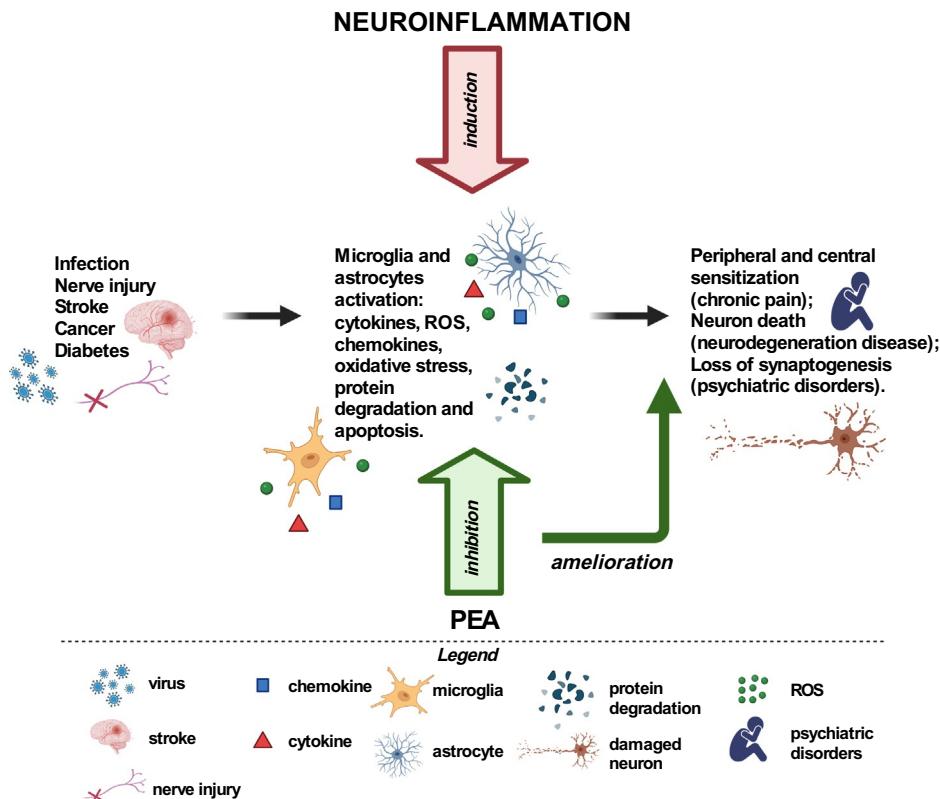
Endocannabinoids deeply reduce the progression of neurodegeneration. AEA, 2-AG, and PEA and the stimulation of CB1 and CB2 receptors have shown neuroprotective effects in cerebral ischemia, TBI, spinal cord injury, Parkinson's, and Alzheimer's disease models. The SR141716A also proved to be neuroprotective in Parkinson's and Alzheimer's disease models. Interestingly, the inhibition of FAAH and MAGL enzymes, able to increase the levels of AEA and 2-AG respectively, played anti-inflammatory, anxiolytic, and antidepressant actions and reduced the amyloid  $\beta$  deposition. These encouraging outcomes lead to clinical trials testing the efficacy of SR141716A in Parkinson's disease and several FAAH and MAGL inhibitors in chronic pain, cannabis withdrawal, anxiety, and Tourette syndromes (Ren et al., 2020). Unfortunately, the SR141716A, which entered the market for the treatment of obesity in 2006, was then withdrawn in 2008, due to severe psychiatric side effects such as mood disorders in 10% of cases up to suicide in 1% of cases. The FAAH inhibitor, BIA 10-2474, was also suspended from the phase I clinical trial as it was associated with a serious adverse reaction, i.e., the lethal brain toxic syndrome (Kerbrat et al., 2016). This serious adverse reaction interrupted clinical studies also targeting other FAAH and MAGL inhibitors by soliciting pharmacological approaches aimed at enhancing the endocannabinoid system with adequate safety profiles (Seghetti et al., 2021).

## Endocannabinoids and BBB

Neuroinflammation is also associated with BBB increased permeability and/or disruption and immune cell infiltration. 2-AG, AEA, NAEs, FAAH, and MAGL inhibitors and CB2 receptor agonists decrease BBB permeability in several brain pathological states. Interestingly, one of the most abundant NAEs, the stearoyl ethanolamide prevents the leukocyte infiltration in the brain in a systemic inflammation model in mice (Kasatkina et al., 2020). It is also interesting that the cytokines IL-6 and TNF- $\alpha$ , key mediators in neuroinflammation, increase the expression of the CB2 receptor on macrophages, making them more sensitive to endocannabinoid control (Jean-Gilles et al., 2015).

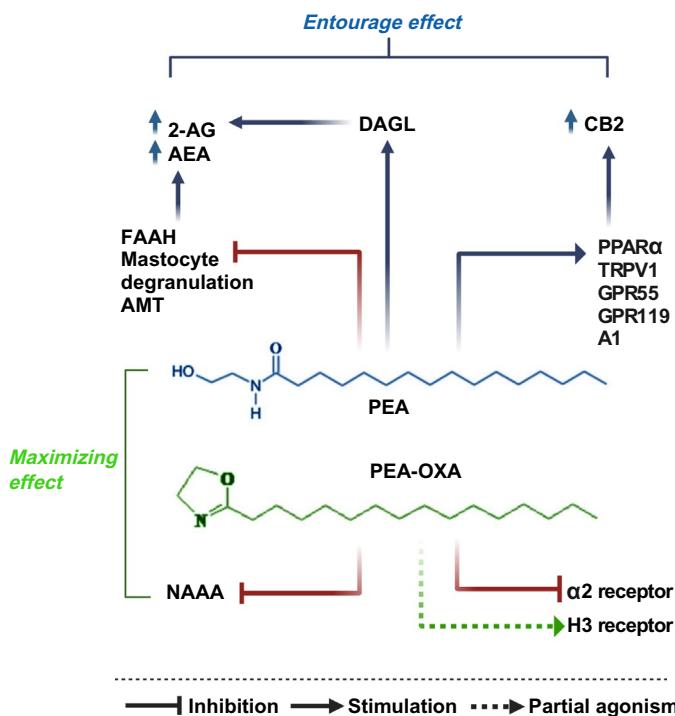
## Palmitoylethanolamide in Neuroinflammation-Based Disorders: Preclinical Trials

Among the NAEs, PEA is produced on demand in all tissues including the brain. Its levels increase following cellular insults or under pathological states as a pro-homeostatic protective response promoting the resolution of neuroinflammation. PEA plays an immunomodulatory action being able to enhance microglia and macrophage migration and phagocytic activity through the increase of the CB2 receptor expression occurring in a PPAR $\alpha$  receptor-dependent manner (Guida, Luongo, et al., 2017). Other neuroprotective actions of PEA include the inhibition of the pro-inflammatory cytokine release, local inflammatory cascades, apoptosis, autophagy, glutamate excitotoxicity, and necrotic processes while enhancing synaptic homeostasis and neurogenesis (Fig. 3). These actions associated with the analgesic,



**FIG. 3** Effect of PEA on neuroinflammation. Schematic representation of the effects of nervous system insults and PEA on the main cellular and molecular mechanisms underlying neuroinflammation. Infection, injury, or diseases induce microglia and astrocytes activation that in turn release cytokines and chemokines. Throughout the activation of their own receptors, these pro-inflammatory mediators trigger intracellular mechanisms conveying in protein degradation, oxidative stress, axonal transport impairments, and apoptosis driving to peripheral and central sensitization, loss of synaptogenesis, neuron death, at the basis of chronic pain, psychiatric disorders, and neurodegenerative disorders. PEA exhibits an opposite action (almost an equal and opposite force, green arrow) to the main mechanisms underlying neuroinflammation such as the inhibition of microglia and astrocytes, the inhibition of cytokines and chemokine release, and the infiltration of immune cells, thus playing a neuroprotective effect.

anti-inflammatory, antiepileptic, anti-infective, and neuroprotective effects depend on the multitarget pharmacodynamic profile of PEA. Although it is identified as an endocannabinoid, it does not bind CB<sub>1</sub> and CB<sub>2</sub> receptors but activates them indirectly through the FAAH inhibition and the increase of AEA levels (entourage effect). PEA acts as an agonist on PPAR $\alpha$ , TRPV1, and adenosine A1 receptors and as an inhibitor of AMT. PEA also activates GPR55 and GPR119, identified as novel cannabinoid receptors. Finally, PEA inhibits mast cells activation (De Filippis et al., 2013), an effect defined as an autacoid local inflammation antagonism (Fig. 4). Being highly lipophilic, insoluble in water, rapidly metabolized, and excreted, formulations of PEA with improved bioavailability and half-life have been developed. Micronized and ultra-micronized formulations have improved the pharmacokinetic profile of PEA for both preclinical and clinical studies. The beneficial effects of PEA have been demonstrated in numerous neuroinflammation-based disorders including chronic pain, neuropsychiatric, neurodegenerative, and autoimmune diseases. In animal models of neuropathic pain, PEA has been shown to improve sensory symptoms and anxiodepressive consequences, restoring the homeostasis of the highly dysregulated glutamate synaptic activity following peripheral nerve injury (Di Cesare Mannelli et al., 2015; Guida et al., 2015). PEA also restored the loss of neuronal plasticity and cognitive performance associated with neuropathic pain in a metabotropic glutamate receptor 5 and 8-dependent way (Boccella et al., 2019a,b). PEA ameliorated also the sensory and affective/cognitive impairments associated with a mouse model of TBI. In this model, PEA also rescued the IL-1 $\beta$  levels and neural activity in the medial prefrontal cortex, a brain area that plays a critical role in neuropathy- or TBI-related affective and cognitive disorders (Guida, Boccella, et al., 2017). The efficacy toward affective and cognitive deficits could be associated with the capability of PEA to increase the levels of brain and glial-cell-derived neurotrophic factors (BDNF and GDNF) enhancing neurogenesis and synaptic plasticity (Caltagirone et al., 2016). It is also interesting that endocannabinoid and PEA levels change in neuropsychiatric conditions such as anxiety, depression, post-traumatic stress, and autism spectrum disorders.



**FIG. 4** Targets of PEA and PEA-OXA. The figure shows the multitarget action of the PEA and PEA-OXA. Although defined as an endocannabinoid, PEA does not directly stimulate the CB1 and CB2 receptors but increases the levels of AEA through the inhibition of FAAH, the enzyme responsible for its catabolism, and the membrane transporter responsible of its reuptake. Other PEA targets include the intracellular PPAR $\alpha$  receptor, which mediates mainly the analgesic, anti-inflammatory, and neuroprotective effect, the TRPV1 receptor, which functions as a pain transducer but easily desensitizes upon stimulation, and the adenosine A1 receptor. PEA inhibitory action on mast cells is also reported. PEA-OXA was developed as an oxazoline derivative and prodrug of PEA, but exhibited a completely different pharmacodynamics, acting via adrenergic  $\alpha$ 2 receptor antagonism and partial histamine H3 receptor antagonist. Inhibition of NAAA (NAE-hydrolyzing acid amidase) increases the availability of PEA and indirectly stimulates the endocannabinoid system.

## Palmitoylethanolamide in Neuroinflammation-Based Disorders: Clinical Trials

Most clinical trials on PEA have been focused on chronic pain management. Altogether these studies used doses of 300–1300 mg per day and treatment periods of 14–120 days. PEA has shown good efficacy in the low back and lumbosciatic pain. In combination with trans-polydatin, PEA reduced pelvic and endometriosis pain. Case reports also documented the efficacy of PEA in chronic idiopathic axonal neuropathy pain, central neuropathic pain, multiple sclerosis, pudendal neuralgia, and chronic regional pain syndrome type 1 (Marchesi et al., 2022). Combined treatment of PEA and levoDOPA improved motor symptoms and mood, sleep, and cognition impairments in patients suffering from Parkinson's disease (Brotini et al., 2017). The combination of PEA and luteolin has been tested in patients suffering from a stroke. In combination with neurorehabilitation therapy, the treatment with PEA and luteolin improved cognitive deficits, pain, spasticity, and overall neurological status (Caltagirone et al., 2016). The beneficial effect of PEA has also been reported in patients with major depressive disorder. Combined treatment with citalopram and PEA showed a better response compared with the treatment with citalopram and placebo (Ghazizadeh-Hashemi et al., 2018). When administered in combination with riluzole, PEA improved also respiratory function and general conditions in amyotrophic lateral sclerosis patients (Palma et al., 2016). PEA has also been shown to improve sociability, cognition, and behavior in autistic children (Antonucci et al., 2015). Noteworthy is the fact that in all clinical studies, PEA showed good tolerability and no significant adverse reactions (Table 2).

## PEA Derivatives: The 2-Pentadecyl-2-oxazoline (PEA-OXA)

One of the attempts to improve the metabolic stability of PEA has produced the oxazoline derivative, 2-pentadecyl-2-oxazoline (PEA-OXA), which is also a natural compound found in the green and toasted coffee beans (Impellizzeri et al., 2016). Developed as a prodrug of PEA capable of increasing PEA bioavailability, PEA-OXA has instead shown a different pharmacodynamic profile, behaving as a histamine H3 receptor partial agonist and an α<sub>2</sub> adrenoceptor

**TABLE 2** Clinical trials on PEA effects towards neuroinflammatory-based disorders.

Disease	Treatment	Outcome	References
Low back lumbosciatic pain	PEA	Analgesia	Marchesi et al. (2022)
Pelvic and endometriosis pain	PEA/trans-polydatin	Analgesia	
Idiopathic axonal neuropathy pain, central neuropathic pain, multiple sclerosis, pudendal neuralgia, and chronic regional pain syndrome type 1 (case reports)	PEA	Analgesia	
Parkinson's disease	PEA/levodopa	Improvement in motor symptoms, mood, sleep, and cognition impairments	Brotini et al. (2017)
Stroke	PEA/luteolin/rehabilitation	Improvement in cognitive deficits, pain, spasticity, and overall neurological status	Caltagirone et al. (2016)
Major depressive disorder	PEA/citalopram	Improvement of remission compared to placebo/citalopram	Ghazizadeh-Hashemi et al. (2018)
Amyotrophic lateral sclerosis	PEA/riluzole	Improvement of respiratory function and general conditions	Palma et al. (2016)
Autism spectrum disorder	PEA	Improvement in sociability, cognition, and behavioral response	Antonucci et al. (2015)

The table shows the main evidence on the use of PEA, alone or in add-on therapy, on neuroinflammatory-based diseases. For each evidence of the pathology, the treatment including PEA or PEA-combined treatments, the observed effects, and the relative reference are reported.

antagonist (Boccella et al., 2020, 2021). The neuroprotective action of PEA-OXA has been shown in different animal models of neuropathic pain (Boccella et al., 2021; Campolo et al., 2021; Gugliandolo et al., 2018). PEA-OXA not only improved the sensory component but also the affective and cognitive one that is often associated with neuropathic pain (Boccella et al., 2021). The same beneficial action of PEA-OXA on pain perception and affective/cognitive dysfunctions has emerged in TBI, spinal injury (Boccella et al., 2020; Impellizzeri et al., 2017), intestinal inflammation (Cordaro et al., 2020), Parkinson's disease (Cordaro et al., 2018; Fusco et al., 2019), bilateral carotid occlusion (Impellizzeri et al., 2019), and prolonged stress caused by social isolation. In the latter condition, PEA-OXA improved also the metabolic component, reducing body weight and reverting the inflammatory phenotype of adipocytes (Belardo et al., 2022). Like PEA, PEA-OXA is also found in food sources, and this suggests that it may be well tolerated in future clinical trials.

## Conclusions

Neuroinflammation is the fundamental component of many, if not all, diseases of the nervous system. Although it is a protective mechanism aimed at preserving the functional integrity of the nervous system, the slightest imbalance of some of its components can trigger harmful processes that worsen the damage. The knowledge of the mechanisms that promote the passage of neuroinflammation from beneficial to destructive and the modulation of these mechanisms could represent the druggable target for hitherto intractable neuropsychiatric and neurodegenerative pathologies. One of the systems that potentiate the beneficial aspects and the resolution of neuroinflammation is the endocannabinoid system, whose enhancement, although it has shown encouraging results in preclinical studies, has not shown an adequate safety profile in the clinic. In this regard, the endocannabinoids present in some food sources could represent the turning point. The pre-clinical and clinical results on the use of PEA and its oxazoline analog, PEA-OXA, in neuropsychiatric and neurodegenerative pathologies (also in add-on therapies) are promising. These compounds, being associated with a multitarget pharmacodynamic profile, show good efficacy even in pathologies with high drug resistance and present good tolerability.

## Application to Other Areas

The chapter deals with a possible therapeutic strategy applicable to the management of neuropsychiatric and neurodegenerative pathologies. Having these at the base maladaptive neuroinflammatory process, the inhibition of the harmful component of neuroinflammation could represent the turning point for the treatment of these disorders characterized by high resistance to traditional drugs. The endocannabinoid system inhibits deleterious neuroinflammatory processes, but its modulation in the clinic has proved inapplicable due to an unfavorable adverse reaction profile. Natural products found in some foods such as PEA and its congener PEA-OXA not only show greater safety but also a large spectrum efficacy probably associated with a multitarget pharmacodynamic profile. The neuroprotective effect of PEA and PEA-OXA is associated with the inhibition of inflammatory cytokines, activated microglia, immune cell proliferation, BBB permeability and disruption, excitotoxicity, apoptosis, oxidative stress, and the enhancement of BDNF levels and neurogenesis. An important study on PEA-OXA highlights its ability to reverse the neuropsychiatric, sensory, and metabolic alterations associated with prolonged stress from social isolation. Given that the Covid-19/20 pandemic has caused enormous restrictions on social relationships with consequences on affectivity, behavior, and metabolism, the potential of this molecule in actuality is decisive. Apart from the current pandemic, metabolic diseases are on the rise and are often the cause and/or the effect of neuropsychiatric pathologies. The possibility of counteracting these disorders with drugs active on both components appears to be relevant. Like the new multitarget molecules recently developed in neuropsychiatry, the broader spectrum of efficacy does not correspond to an increased risk of adverse reactions, as demonstrated by the good tolerability of PEA in clinical studies.

## Mini-dictionary of Terms

*Neuroinflammation:* the inflammatory response of the central and/or peripheral nervous system.

*Cytokines:* proteins promoting the growth and activity of the immune system cells.

*Microglia:* resident immune cells of the central nervous system similar to macrophages.

*N-acylethanolamines:* a group of fatty acid derivatives interacting with endocannabinoid system.

*Brain-derived neurotrophic factor:* neurotrophin supporting the survival of existing neurons and promoting synaptogenesis.

*Endocannabinoid system:* neurotransmitter system activated by anandamide, 2-arachidonoyl glycerol, and Cannabis Sativa plant-derived cannabinoids such as THC and CBD. Includes also anabolic and catabolic enzymes, receptors, and membrane transporters.

*Neuropathic pain:* irreversible chronic pain type associated with central and/or peripheral nervous system damage.

*PEA:* N-acylamine lipid mediator found naturally in foods such as egg yolks and peanuts and in the human body. It interacts with the endocannabinoid system by inhibiting the catabolism and membrane transporter of anandamide. PEA stimulates also PPAR $\alpha$ , TRPV1, adenosine A1, GPR55, and GPR119 receptors. PEA down-modulates also mast cell behavior.

*PEA-OXA:* Oxazoline derivative of PEA. It is found in food products such as green and roasted coffee beans. Born as a prodrug of PEA to improve its bioavailability, it has instead shown a different pharmacodynamic action by acting as a partial agonist of the H3 histaminergic receptor and antagonist of the  $\alpha$ 2 adrenergic receptor.

## Key Facts of *N*-Acylethanolamine in Neuroinflammation

Neuroinflammation is the underlying component of nervous system pathologies

Endocannabinoids promote the beneficial mechanisms of neuroinflammation at the expense of the harmful ones

PEA and PEA-OXA interact with the endocannabinoid system but have numerous other targets

The multitarget property of PEA and PEA-OXA increases the spectrum of action without increasing adverse reactions

PEA and PEA-OXA are particularly relevant for the treatment of neuropsychiatric pathologies characterized by a high degree of drug resistance

## Summary Points

Neuropsychiatric and neurodegenerative diseases share a basic neuroinflammatory process

Prolonged neuroinflammation loses its protective role and promotes aberrant responses with harmful consequences

Endocannabinoids promote homeostatic and protective aspects of neuroinflammation  
 N-acylethanolamines such as anandamide, PEA, and its congener PEA-OXA interact with the endocannabinoid system and other targets  
 The multitarget action of PEA and PEA-OXA broadens the spectrum of action in neuropsychiatric, neurodegenerative, and metabolic disorders  
 PEA and PEA-OXA show a good safety profile

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## Chapter 34

# Endocannabinoid modulation of synaptic function and behavior in the dorsomedial hypothalamus

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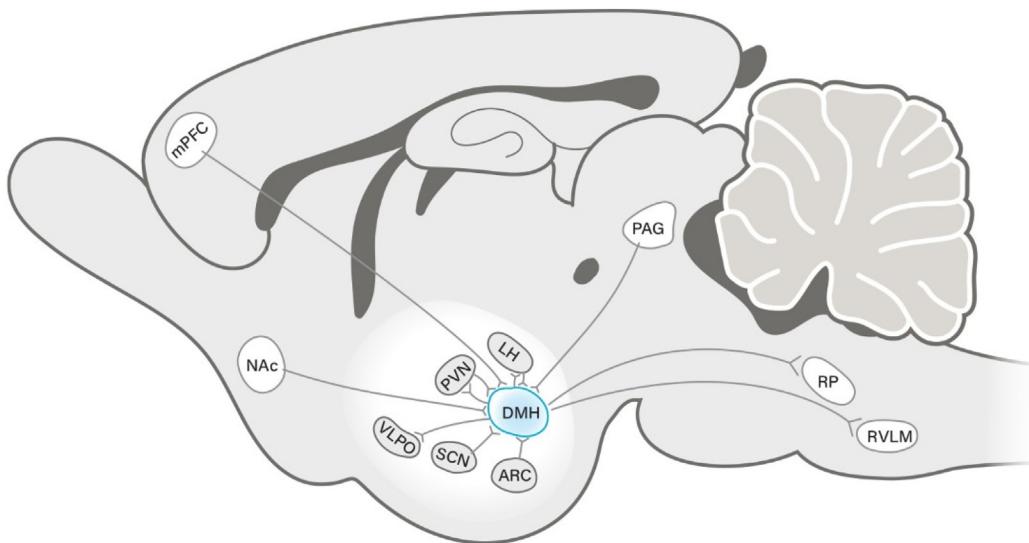
### Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>ARC</b>	arcuate nucleus
<b>BAT</b>	brown adipose tissue
<b>CART</b>	cocaine and amphetamine-related transcript
<b>CB1R</b>	type I cannabinoid receptor
<b>CB2R</b>	type II cannabinoid receptor
<b>CNS</b>	central nervous system
<b>DMH</b>	dorsomedial nucleus of the hypothalamus
<b>eCB</b>	endocannabinoid
<b>FAAH</b>	fatty acid amide hydrolase
<b>LH</b>	lateral hypothalamus
<b>mPFC</b>	medial prefrontal cortex
<b>MCH</b>	melanin-concentrating hormone
<b>NAc</b>	nucleus accumbens
<b>NO</b>	nitric oxide
<b>PAG</b>	periaqueductal gray
<b>PVN</b>	paraventricular nucleus
<b>RP</b>	raphe pallidus
<b>RVLM</b>	rostral ventrolateral medulla
<b>SCN</b>	suprachiasmatic nucleus
<b>VLPO</b>	ventrolateral preoptic nucleus

### Introduction

It's 6:00 am. You awaken from a deep sleep, stressed about your overwhelming work schedule today. Your thoughts are interrupted by your stomach growling, signaling hunger after a night without food. The idea of breakfast motivates you to get out of bed and start your day. These physiological phenomena—the control of food intake, the response to stress, and the regulation of sleep and wake cycles—are mediated, in part, by an area of the brain called the dorsomedial nucleus of the hypothalamus (DMH).

The DMH is a discrete collection of cells located in the dorsal region of the hypothalamus on each side of the third ventricle. It is subdivided into dorsal and ventral regions, separated by a compact zone rich in neurons. Collectively, neurons in the DMH play an important role in maintaining homeostasis across a wide range of vertebrates. Numerous studies have implicated the DMH in basic physiological functions such as regulating food intake and metabolism, the stress response, and the sleep–wake cycle. The DMH carries out these and other homeostatic functions through complex and



**FIG. 1** The DMH and some of its major afferent and efferent projections. *ARC*, arcuate nucleus; *DMH*, dorsomedial hypothalamus; *LH*, lateral hypothalamus; *mPFC*, medial prefrontal cortex; *NAc*, nucleus accumbens; *PAG*, periaqueductal grey; *PVN*, paraventricular nucleus; *RP*, raphe pallidus; *RVLM*, rostral ventrolateral medulla; *SCN*, suprachiasmatic nucleus; *VLPO*, ventrolateral preoptic nucleus.

coordinated interactions with other brain regions (see Fig. 1 for some of the important projections to and from the DMH that are relevant to this chapter).

The majority of projections to and from the DMH are intrahypothalamic. Hypothalamic nuclei including the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamus (LH), and suprachiasmatic nucleus (SCN) communicate with the DMH and play a role in the regulation of food intake, the response to stress, and the sleep–wake cycle, among other functions (Gautron et al., 2010; Lee et al., 2013; Myers et al., 2014; Thompson & Swanson, 1998). Beyond the hypothalamus, various cortical regions including the prefrontal cortex send extensive projections to the DMH to coordinate the response to stress. In the midbrain, the raphe pallidus (RP) and periaqueductal gray (PAG) are important downstream targets of the DMH that contribute to thermogenesis and the regulation of metabolism (de Git et al., 2018; Houtz et al., 2021; Piñol et al., 2018). Various brainstem nuclei also project to and receive input from the DMH. For example, the rostroventral lateral medulla (RVLM) receives information from the DMH that is involved in the cardiovascular response to stress (Kono et al., 2020). Importantly, many of these projections appear to be conserved across species (Dai et al., 1998). While not an exhaustive list of areas that communicate with the DMH (see Thompson et al., 1996 and Thompson & Swanson, 1998 for extensive tracing studies), it is clear that coordination is required to carry out these critical homeostatic functions.

## Endocannabinoids and the DMH

To carry out homeostatic functions effectively, DMH neurons release and respond to a multitude of classical and retrograde transmitters. Like many neurons throughout the central nervous system (CNS), DMH neurons contain endogenous cannabinoids (eCBs), retrograde neurotransmitters that have been implicated in virtually all of the functions carried out by the DMH. Evidence of the eCB system in the DMH stems from both rodent and human studies. Early immunohistochemical work revealed cannabinoid receptor mRNA distribution throughout the rat brain, including low to moderate intensity signals in the DMH (Matsuda et al., 1993). It has since been suggested that despite the relatively low density of cannabinoid receptors in the hypothalamus demonstrated in early studies, the high level of activation of the associated G-proteins makes for efficient eCB-mediated signaling in this region (Breivogel et al., 1997). This enhanced G-protein activity downstream of cannabinoid receptor activation could help to explain the robust responses observed following activation of these receptors in the DMH in rodents.

Subsequent studies have confirmed that cannabinoid receptors are expressed in the DMH in both mice and rats. Type I cannabinoid receptor (CB1R)-immunoreactive axons densely innervate the DMH in mice (Wittmann et al., 2007), and modest staining of CB1Rs has been reported in the rat DMH (Jelsing et al., 2008). Further immunohistochemical data have indicated that the majority of CB1Rs are present on axonal fibers (Dos Anjos-Garcia & Coimbra, 2019).

Pharmacological studies have corroborated the early mRNA and immunohistochemical data. Intraperitoneal administration of HU-580, an analog of cannabidiolic acid, activates neurons in the dorsal, ventral, and compact parts of the DMH, as evidenced by increases in c-Fos and NeuN expression in these regions (Murillo-Rodríguez et al., 2021). Type II cannabinoid receptors (CB2Rs) also appear to be expressed in the DMH (Viana et al., 2019).

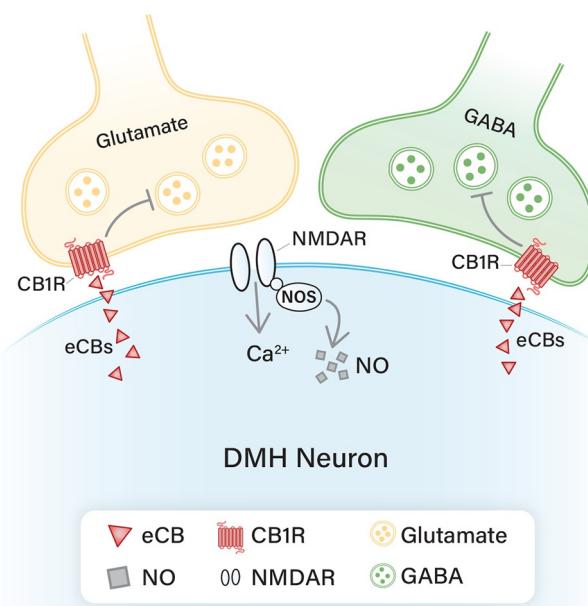
There is also evidence that the machinery involved in the synthesis of eCBs is found in the DMH. Diacylglycerol lipase- $\alpha$ , the enzyme that synthesizes the endocannabinoid 2-arachidonyl-glycerol (2-AG), exhibits intense immunostaining in the rat DMH, indicating that 2-AG is synthesized in this region (Suárez et al., 2011). Anandamide is also known to be active here; fatty acid amide hydrolase (FAAH), the enzyme responsible for the breakdown of anandamide, has been previously identified in the DMH (Tsou et al., 1998). While many studies have examined eCBs in the rodent brain, the presence of the eCB system in the DMH appears to be conserved across species, including humans (Palkovits et al., 2008).

In the DMH, eCBs appear to be important players in a wide range of functions including the regulation of appetite, the stress response, and sleep-wake cycles. Although the exact mechanisms by which eCBs modulate these important functions are not fully understood, they likely involve changes in synaptic transmission and potential interactions with other neurotransmitters. This chapter will focus on the role of eCBs in modulating synaptic transmission, regulating various homeostatic behaviors in the DMH, and interacting with other neurotransmitters. Questions to stimulate further thought will be included at the end of each section.

*Future Questions:* Are CB1Rs expressed in all subregions of the DMH? Are postsynaptic, astrocytic, or mitochondrial CB1Rs important in the DMH? What is the role of CB2Rs in the DMH?

## Synaptic effects of eCBs

Electrophysiological studies provide further support for the presence of the eCB system in the DMH. Activation of CB1Rs by the agonist, WIN 55,212-2, in acute rat brain slices containing the DMH results in a decrease in glutamate (Sukkar, Bobbitt, From, & Crosby, unpublished observations) and GABA (Crosby et al., 2011) release onto DMH neurons. These findings suggest that CB1Rs are expressed on both glutamate and GABA terminals that impinge upon DMH neurons (see Fig. 1). The effects of the *endogenous* cannabinoids on glutamate and GABA release are not as clear-cut as those observed with agonist-induced CB1R activation. At GABA synapses, high frequency stimulation of afferents leads to eCB synthesis and subsequent activation of CB1Rs on GABA terminals, resulting in a long-lasting depression in GABA release (Crosby et al., 2011). Pharmacological blockade or genetic knockdown of CB1Rs abolishes this long-term depression, confirming that it is indeed mediated by eCBs. Interestingly, this eCB-mediated long-term depression at GABA synapses also requires nitric oxide (NO) signaling (NO is also produced in DMH neurons; see Fig. 2); blockade of NO synthesis prevents the



**FIG. 2** DMH neurons synthesize endocannabinoids (eCBs) and nitric oxide (NO) and receive input from glutamate and GABA terminals that express CB1Rs. *CB1R*, type I cannabinoid receptor; *eCB*, endocannabinoid; *NMDAR*, *N*-methyl-D-aspartate receptor; *NO*, nitric oxide; *NOS*, nitric oxide synthase.

depression in GABA release (Crosby et al., 2011). Blocking NO synthesis also completely abolishes the decrease in GABA release triggered by WIN 55,212-2. However, the same high-frequency stimulus at glutamate terminals fails to trigger long-term depression of glutamate transmission, unless NO synthesis/signaling is prevented (Sukkar, Bobbitt, From, & Crosby, unpublished observations). Collectively, these data suggest that NO plays an important role in eCB signaling in the DMH. The interactions between eCBs and NO will be discussed in more detail later in the chapter.

Although substantial progress has been made in investigating the synaptic effects of eCBs in the DMH, the functional implications of these effects remain largely unknown. The remainder of this chapter will focus on the role of eCBs in modulating important homeostatic functions in the DMH.

*Future Questions: Under what conditions is eCB signaling at glutamate or GABA terminals dominant? What are the functional implications of eCB-mediated synaptic plasticity in the DMH?*

## Appetite regulation and metabolism

There is growing evidence for a link between eCBs, the DMH, and appetite regulation. The DMH is a key center for regulating food intake as demonstrated by a wide range of studies, from early lesioning experiments (Bellinger & Bernardis, 2002) to more recent optogenetic and chemogenetic reports (Jeong et al., 2017; Otgon-Uul et al., 2016). The exact role of the DMH in regulating appetite, however, is not entirely clear. There appear to be numerous distinct populations of DMH neurons that can have differential effects on food intake, with some stimulating (Jeong et al., 2017; Otgon-Uul et al., 2016) and others suppressing appetite (Houtz et al., 2021; Imoto et al., 2021). The eCB system is also strongly linked to appetite, and emerging evidence suggests that eCBs may act in the DMH to modulate food intake. The effect of microinjection of 2-AG or anandamide in the DMH on food intake has been studied in rats, with varying effects depending on the composition of the food and the presence or absence of NO signaling (see Table 1). Microinjection of either eCB alone does not affect the consumption of regular or high-fat food, but when 2-AG is administered with the NO precursor, L-arginine, consumption of regular chow is significantly elevated (with no effect observed with L-arginine alone; McGavin et al., 2019; Poole et al., 2020). In contrast, L-arginine alone increases *high-fat* food intake when administered into the DMH, and when 2-AG and L-arginine are co-administered, rats eat significantly less high fat food, suggesting that 2-AG interferes with the L-arginine-induced increase in appetite. These findings are summarized in Table 1.

These data suggest that 2-AG plays a complex role in appetite regulation in the DMH. It's possible that the role for eCBs in the DMH is primarily homeostatic, rather than hedonic, as 2-AG contributes to stimulating regular food intake while it seems to prevent high-fat food intake that is stimulated by L-arginine alone. This is in contrast to eCB actions in the parabrachial nucleus (DiPatrizio & Simansky, 2008) and nucleus accumbens (NAc; Parker et al., 2015) where 2-AG has been shown to stimulate feeding of palatable foods.

It is currently unknown exactly how eCBs (and NO) act in the DMH to alter food intake. Specifically, two main questions emerge from this data: (1) How do eCBs and NO interact with one another (see the end of this chapter for a discussion on interactions)? (2) How exactly do eCBs modulate food intake? With respect to the latter, one possibility is that eCBs alter the release of glutamate or GABA onto “hunger” or “satiety” neurons in the DMH. Such changes in synaptic transmission could ultimately control the excitability of these neurons. As described above, CB1Rs are expressed on both glutamate and GABA terminals in the DMH, and activation of these receptors by synthetic agonists decreases the release of both neurotransmitters onto DMH neurons.

In addition to the potential effects of eCBs on appetite regulation through glutamate and GABA signaling, there is accumulating evidence that eCBs can regulate appetite by modulating the release of appetite-regulatory peptides. Systemic

**TABLE 1** Effect of intra-DMH administration of eCBs and nitric oxide on food intake in rats.

Endocannabinoid (with and without nitric oxide)	Regular chow consumption	High-fat chow consumption
2-AG	No change	No change
2-AG + L-arginine	Increase	No change
L-arginine	No change	Increase
Anandamide	No change	Data not available
Anandamide + L-arginine	No change	Data not available

administration of the CB1R antagonist rimonabant increases c-Fos expression in DMH neurons that specifically express melanin-concentrating hormone (MCH) and cocaine and amphetamine-related transcript (CART; [Verte et al., 2009](#)). Other findings suggest that CART is localized in CB1R-expressing terminals in the DMH ([Jelsing et al., 2008](#)), and the expression and release of CART are altered by eCB signaling. For example, in FAAH-deficient mice with increased anandamide levels, decreased CART levels have been observed ([Osei-Hyiaman et al., 2005](#)). There are also reports that eCBs can influence appetite by acting in brain regions that project to the DMH. Administration of anandamide or an anandamide reuptake inhibitor into the NAc leads to an increase in both DMH c-Fos expression and food intake. This could be the result of disinhibition, whereby GABA release from NAc medium spiny neurons into the DMH is inhibited by eCB activity ([Soria-Gómez et al., 2007](#)).

The DMH is also important in regulating metabolism. Optogenetics research has revealed that a population of cholinergic neurons in the DMH controls brown adipose tissue metabolism. An increase in ambient temperature activates these neurons, resulting in a subsequent decrease in BAT activity that is mediated by DMH output to the raphe pallidus ([Jeong et al., 2015](#)). Endocannabinoids have also been linked to BAT metabolism, with potential direct and indirect effects triggering changes in BAT activity. In support of the latter, pharmacological or genetic disruption in eCB signaling in the CNS, particularly the hypothalamus, has been shown to increase thermogenesis, BAT activity, and overall leanness ([Cardinal et al., 2012; Jung et al., 2012](#)). CB1Rs are also expressed directly on brown adipocytes, suggesting a potential direct mechanism through which eCBs can also modulate BAT activity ([Matias et al., 2016; Perwitz et al., 2006](#)). Although both the eCB system and the DMH have been implicated in regulating BAT metabolism in separate studies, it is not yet known whether eCBs act specifically in the DMH to control BAT activity. Future research should consider the role that intra-DMH eCBs might play in regulating BAT in the periphery. It is possible that eCBs could dampen BAT activity by activating DMH neurons, an effect that could be accomplished with an eCB-mediated decrease in GABA release onto DMH neurons.

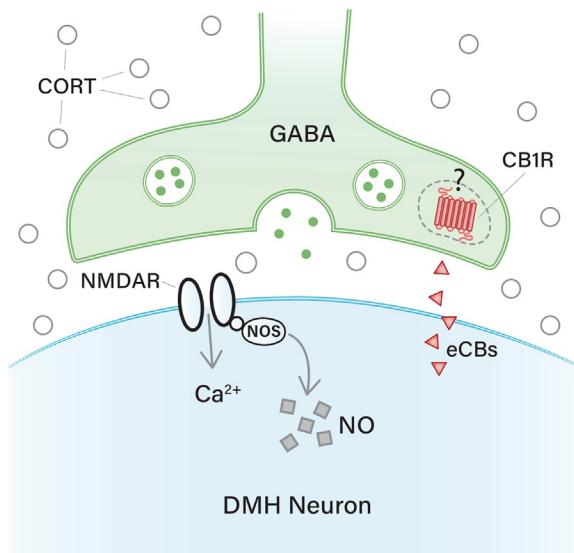
*Future Questions: What is the mechanism underlying the eCB-mediated changes in food intake in the DMH? Does intra-DMH administration of eCBs alter BAT activity?*

## Stress, anxiety, and panic

### Stress

The DMH is important in the physiological response to stress. DMH neurons send major projections to neuroendocrine cells in the PVN ([Thompson et al., 1996; Ulrich-Lai et al., 2011](#)), putting them in an ideal position to modulate the stress response. DMH neurons also project to brain stem nuclei including the RVLM to mediate the sympathetic response to stress ([Brasil et al., 2019; Fontes et al., 2011](#)). The DMH might also be a target of stress hormones following activation of the stress response, as data suggest that glucocorticoid receptors are expressed in the DMH ([Cintra et al., 1990](#)). Although much remains to be studied regarding the role of the DMH in the stress response, there is growing evidence of a link between stress and eCBs in the DMH ([Crosby & Bains, 2012](#)). At the synaptic level, exposure to stress in rats decreases eCB signaling at CB1Rs. This disruption in CB1R signaling is observed in animals experiencing modest, but prolonged, stress resulting from food deprivation ([Crosby et al., 2011](#)) and is prevented in food-deprived animals when glucocorticoid receptors are blocked. The evidence to support a disruption in CB1R signaling stems from observations that the CB1R agonist, WIN 55,212-2, which significantly decreases GABA release onto DMH neurons in naive animals, fails to do so in stressed animals. In addition, eCB-mediated long-term depression of GABA release following high-frequency stimulation of axons in naive animals is not observed in stressed animals, unless glucocorticoid receptors are blocked. These findings suggest that glucocorticoids, including corticosterone in rats, are somehow acting to decrease the function of CB1Rs, possibly through receptor internalization (see [Fig. 3](#)). A similar stress-induced downregulation in CB1Rs has been observed in other brain regions, including the PVN ([Wamsteeker et al., 2010](#)), hippocampus, and striatum ([Hill et al., 2008](#)). In the DMH, unpublished data also suggest that exposure to stress during the prenatal stress disrupts eCB signaling in the postnatal period, or in young rats, or somehow identify when those changes were observed. No changes were observed at glutamate synapses in males or at either type of synapse in females (Welsh, Hildebrand, Pond, & Crosby, unpublished observations). The mechanisms underlying these stress-induced disruptions in eCB signaling are not fully understood, but it is possible that the excess corticosterone during the prenatal period somehow disrupts CB1R signaling or expression.

In addition to stress affecting the eCB system, eCBs can also modulate the stress response. For example, in response to an acute stressor and increased corticosterone levels, eCBs are released in the PVN where they suppress glutamate release onto corticotropin-releasing hormone neurons ([Di et al., 2003](#)), mediating negative feedback inhibition of the HPA axis. Such effects have not yet been studied in the DMH, but future research should examine whether eCBs can modulate the



**FIG. 3** Prolonged stress triggers a decrease in CB1R function in the DMH, possibly via internalization of CB1Rs. *CB1R*, type I cannabinoid receptor; *CORT*, corticosterone; *eCB*, endocannabinoid; *NMDAR*, *N*-methyl-D-aspartate receptor; *NO*, nitric oxide; *NOS*, nitric oxide synthase.

stress response through actions in the DMH. Indeed, glucocorticoid receptors are expressed in the DMH and could lead to increases in eCB levels, and there are extensive projections from DMH neurons to PVN neuroendocrine cells that orchestrate the stress response (Thompson et al., 1996; Ulrich-Lai et al., 2011).

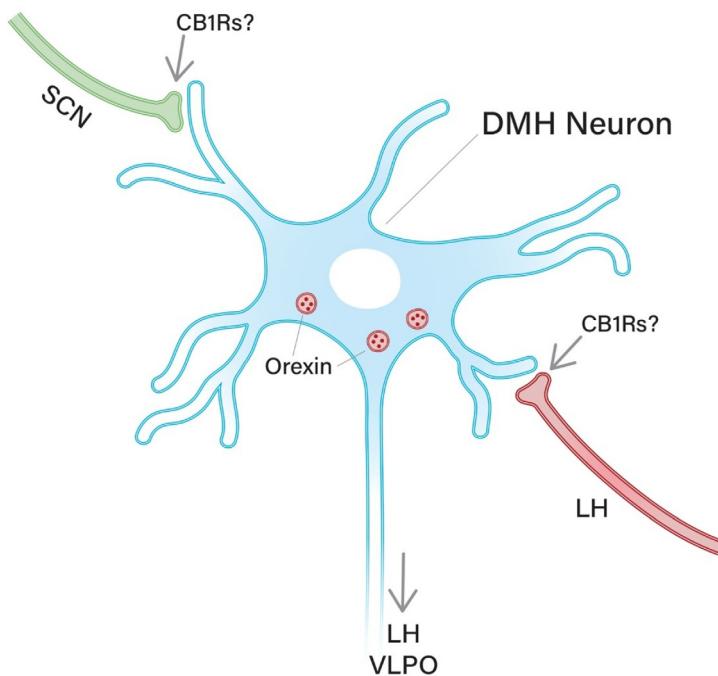
### Anxiety/panic

Endocannabinoid activity in the DMH has also been implicated in panic-like defensive behavior. Administration of anandamide into the mouse DMH triggers a significant panicolytic-like effect when mice are exposed to a predator (Dos Anjos-Garcia & Coimbra, 2019). This panicolytic response is prevented by pretreatment with a CB1R blocker, suggesting that anandamide-induced CB1R activation mediates this process. Endocannabinoid activity in brain regions upstream of the DMH also appears to be involved in preventing panic-like responses. In one study, an anxiety response was induced with microinjection of a GABA<sub>A</sub> receptor antagonist (bicuculline) into the DMH. Blockade of CB1Rs with AM251 specifically in the prelimbic division of the medial prefrontal cortex (mPFC) inhibited the panic-like behavior (de Freitas et al., 2013). Physiologic responses related to panic provoked by electrical stimulation of the DMH were similarly abolished by microinjection of a CB1R antagonist into the dorsal periaqueductal gray (PAG; Dean, 2011). Intra-DMH administration of NMDA is also capable of producing panic-like behavior in rats. In this case, local, but not systemic, injection of a 2-AG hydrolysis inhibitor prevents the effect (Viana et al., 2019). Both CB1Rs and CB2Rs appear to be implicated in this process, as agonists mimic the action of 2-AG hydrolysis inhibitor, whereas antagonists have the opposite effect. In addition to solidifying the role between eCBs and DMH-mediated panic-like behavior, this finding is also one of few to implicate CB2Rs in eCB-mediated responses within the DMH.

*Future Questions:* What is the mechanism and function of CB1R down-regulation? Does downregulation also occur in females? How are anandamide, 2-AG, CB1Rs, and CB2Rs all involved in panic-like behaviors in the DMH?

### Sleep/arousal

The DMH is part of a network of nuclei within the hypothalamus that regulate the sleep–wake cycle. Neurons in the DMH receive information from the suprachiasmatic nucleus (the circadian clock in the hypothalamus) and are involved in circadian rhythms of sleep and waking. DMH neurons are also extensively innervated by orexin neurons in the lateral hypothalamus, and orexin directly depolarizes DMH neurons (Li et al., 2021). In addition to receiving orexinergic input, some DMH neurons also express orexin (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). In turn, DMH neurons involved in sleep–wake cycles project to other important sleep- and wake-promoting nuclei such as the ventrolateral preoptic nucleus (VLPO) and lateral hypothalamus (LH), respectively (Chou et al., 2002; Yoshida et al., 2006). See Fig. 4 for a simple overview of the sleep-related projections involving DMH neurons.



**FIG. 4** A schematic illustration of a DMH neuron involved in sleep–wake cycles demonstrating the relevant projections to and from the DMH. DMH neurons can receive input from the suprachiasmatic nucleus (SCN) and lateral hypothalamus (LH) and can project to the LH and ventrolateral preoptic nucleus (VLPO). Some DMH neurons also express orexin, but it is unknown if the same populations of neurons receive and send these projections.

Cannabinoid modulation of these sleep–wake circuits has been reported to affect sleep patterns. Prolonged exposure to a CB1R agonist during adolescence in rats leads to significant sleep disturbances later in adulthood that are associated with increased neuronal activation of neurons in the DMH (Macías-Triana et al., 2020). It remains unknown, however, which CB1Rs are specifically being activated to alter neuronal activity in the DMH. DMH neurons receive orexin/hypocretin input from the LH, so it is possible that CB1Rs could be localized on these terminals, but this has not yet been investigated. Cannabinoids also play a role in arousal via their actions in the DMH. Intra-DMH administration of a CB1R antagonist promotes arousal following anesthesia (Zhong et al., 2017), suggesting that basal eCB signaling may trigger a decrease in arousal or promote sleep. Although eCBs have been implicated in arousal and sleep in the DMH, many questions remain about the role of eCBs in these processes.

*Future questions: How are eCBs involved in arousal and sleep? Are CB1Rs localized on SCN or LH inputs to the DMH (see Fig. 4)?*

## Cannabinoid interactions

This chapter thus far has examined the role of cannabinoids in several important homeostatic functions in the DMH. The role of eCBs in these processes largely involves their ability to alter the release of classical neurotransmitters, such as glutamate and GABA. In addition to altering the release of these neurotransmitters, eCBs have also been reported to interact with other neurotransmitters, including NO and opioids. Here we will outline some of these interactions and attempt to draw conclusions about how and when these interactions are involved in key DMH functions (see Table 2 for a summary).

Interactions between NO and eCBs have been documented in multiple brain regions and are implicated in a variety of functions (for a review, see Gambino et al., 2020). Within the DMH, both eCBs and NO are retrograde transmitters that are synthesized in the same DMH neurons in response to the same stimuli, yet they have opposing effects on GABA release from presynaptic terminals. eCBs decrease, whereas NO increases, GABA release in rats (Crosby et al., 2011). The same appears to be true at glutamate synapses (Poole et al., 2020), but these results have not fully been published (Sukkar, Bobbitt, From, & Crosby, unpublished observations), and it is not yet clear under what circumstances eCBs and NO specifically act on glutamatergic vs GABAergic synapses. In addition to having opposing actions on the same transmitters, eCBs and NO appear to interact with one another in various complex ways. For example, eCBs have been shown to disrupt NO signaling in the DMH. Following activation of CB1Rs with WIN 55,212-2, a NO donor no longer increases GABA release onto DMH neurons (Crosby et al., 2011). A similar phenomenon is observed at glutamate synapses. Application of

**TABLE 2** Interactions between the endocannabinoid system and other transmitters.

Transmitters	Interaction	Findings
eCBs and NO	eCBs disrupt NO signaling	CB1R activation prevents NO-induced increase in GABA signaling ( <a href="#">Crosby et al., 2011</a> )
		2-AG prevents NO precursor-induced increase in glutamate signaling ( <a href="#">Poole et al., 2020</a> )
	NO is required for eCB signaling	CB1R activation does not decrease GABA signaling when NO synthesis is blocked ( <a href="#">Crosby et al., 2011</a> )
eCBs and opioids	Cannabinoids increase opioid signaling	THC increases the expression of mu-opioid receptors ( <a href="#">Corchero et al., 2004</a> )
	Opioids are required for cannabinoid signaling	Opioid receptor blockade prevents THC-induced c-Fos expression ( <a href="#">Singh et al., 2004</a> )

WIN 55,212-2 or 2-AG to brain slices containing the DMH prevents NO from increasing glutamate release ([Poole et al., 2020](#)). In contrast, NO appears to be required for eCB signaling; inhibition of NO synthesis prevents WIN 55,212-2 from decreasing GABA release onto DMH neurons. The same holds true with eCB-mediated long-term depression at GABA synapses; this form of long-term plasticity is not observed when NO synthesis is blocked. While these interactions appear to be contradictory, they could be a mechanism to ensure that the actions of eCBs are dominant. eCBs suppress the actions of NO on neurotransmitter release, but NO can still aid eCB signaling. Behavioral studies have corroborated the synaptic data related to eCB-NO interactions in the DMH. As discussed earlier, eCBs and NO interact to influence food intake in rats: both 2-AG and NO are required to enhance regular chow intake, but 2-AG hinders the ability of NO to increase the consumption of high-fat food ([McGavin et al., 2019](#); [Poole et al., 2020](#)).

Interactions between eCBs (or exogenous cannabinoids) and opioids have also been documented at both the cellular and behavioral level. In rats, an increase in mu-opioid receptor density in the DMH is seen with repeated intraperitoneal administration of the partial CB1R agonist, delta-9-tetrahydrocannabinol (THC; [Corchero et al., 2004](#)). Conversely, the CB1R antagonist SR141716 has inhibitory effects on opioid-related activation of the DMH. While both intraperitoneal morphine and SR141716 increase c-Fos expression when delivered alone, pretreatment with SR141718 prevents the morphine-induced increase ([Singh et al., 2004](#)). The same authors demonstrated that SR141716 also attenuates the effects of morphine at a behavioral level, inhibiting hyperthermia and locomotor activity induced by morphine administration. Opioids also modulate cannabinoid activity in the DMH. For example, the administration of naloxone inhibits a THC-induced increase in c-Fos expression in the DMH. This is in contrast to other brain regions, where naloxone and THC have an additive effect on c-Fos expression ([Allen et al., 2003](#)). While these observations may reflect interactions between two common exogenous drugs, they also raise the question as to whether the endogenous cannabinoid and opioid systems can influence each other in the DMH and how such interactions might be involved in DMH functions. In other brain regions, well-documented interactions between eCBs and opioids have been implicated in a variety of functions including reward and pain management ([Desroches & Beaulieu, 2010](#); [Fattore et al., 2004](#)). Further research is needed in order to elucidate the behavioral changes that may be related to these cellular effects within the DMH.

Taken together, these results highlight the DMH as an important site of study for interactions with other signaling molecules.

## Applications

The endocannabinoid system is involved in a multitude of homeostatic functions in the DMH. In this chapter, we have provided information on the role of eCBs in the regulation of appetite, the response to stress or panic, and sleep-wake cycles. We have also discussed how eCBs can interact with other neurotransmitters to modulate synaptic activity, receptor expression, and behavior. Understanding how eCBs are involved in these processes in the DMH in non-human animals could potentially lead to a better understanding of the pathophysiology underlying diseases in humans where homeostasis is disrupted. Improved understanding of these processes may reveal new therapeutic targets for intervention in obesity or eating disorders, disorders of stress and anxiety, or disordered sleep. The major issue in translating this work from lab animals to humans, however, is that the majority of these studies have been conducted in male animals. Although there

has been a shift in recent years to include more females in research experiments, there is still a gaping hole in the literature on the effects of endocannabinoids in females. Thus, for these findings to be applied to humans, future studies need to consider the effect of endocannabinoids on synaptic function, expression of target proteins, and behavior in females.

## Summary points

- The dorsomedial hypothalamus is a brain region that is involved in maintaining homeostasis
- The endocannabinoid system is expressed in the dorsomedial hypothalamus
- Endocannabinoids alter synaptic transmission in the dorsomedial hypothalamus
- Endocannabinoids modulate various functions in the dorsomedial hypothalamus, including appetite regulation, the stress response, and sleep–wake cycles
- Endocannabinoids interact with other neurotransmitters in the dorsomedial hypothalamus

## Key facts

- The dorsomedial hypothalamus (DMH) is a brain region that is involved in the regulation of appetite, the response to stress, and sleep–wake cycles
- The DMH expresses all components of the endocannabinoid system
- Endocannabinoids bind to receptors in the DMH to control synaptic transmission between neurons
- Endocannabinoids appear to play an important role in the control of appetite, the response to stress, and sleep–wake cycles
- Interactions between endocannabinoids and other transmitters affect synaptic function and behavior in the dorsomedial hypothalamus

## Mini-dictionary of terms

- DMH: nucleus in the hypothalamus involved in the regulation of homeostatic functions including food and water intake, the response to stress, and sleep–wake cycles homeostasis
- Homeostasis: a state of internal stability in the face of external changes
- Retrograde neurotransmitter: chemical signal that travels backward across a synaptic cleft to modulate presynaptic activity
- Nitric oxide: A retrograde neurotransmitter that can be released from DMH neurons to modulate presynaptic activity; interacts with endocannabinoids
- GABA synapse: A connection between neurons in which the major inhibitory neurotransmitter is released
- Glutamate synapse: A connection between neurons in which the major excitatory neurotransmitter is released
- Brown adipose tissue: A type of adipose tissue that can be activated to generate heat

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## Chapter 35

# Key role of the endocannabinoid system with special emphasis in anandamide on the modulation of cardio-renal homeostasis

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## Abbreviations

2-AG	2-arachidonoylglycerol
AEA	anandamide
CB1	type 1 cannabinoid receptor
CB2	type 2 cannabinoid receptor
CBD	cannabidiol
CYP	cytochrome P450
DOCA	deoxycorticosterone acetate
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
MAGL	monoacylglycerol lipase
NAGLY	N-arachidonoyl glycine
PPAR- $\alpha$	peroxisome proliferator-activated receptor-alpha
SHR	spontaneously hypertensive rats
TRPV1	transient receptor potential cation channel subfamily V member 1

## Introduction

It has been suggested that the upregulation of endocannabinoid system (ECS) may play a protective role in the attenuation of cardio-renovascular disease development and progression. Therefore, the improvement of endocannabinoid tone by inhibiting both endocannabinoid degradation and its cellular uptake may be a useful therapeutic tool during this kind of pathologies (M. Toczek and Malinowska 2018). Of special interest, the little explored pharmacotherapeutic potential of the endocannabinoid anandamide (AEA) and ECS in the prevention and treatment of several cardio-renal diseases has already been explored in some previous studies (Martín Giménez et al. 2018).

Notably, not all effects of cannabinoids are consequence to their interaction with type 1 and 2 cannabinoid receptors (CB1 and CB2). In fact, other types of receptors as well as transcription factors, ion channels, enzymes, and transporters, among others, may be capable of mediating multiple effects of these active compounds (Lin, 2021).

Oxidative stress and inflammation have proven to be some of the most important triggering factors for the development of cardiovascular and renal diseases. In this regard, it has been shown that AEA, 2-AG, and some of their analogs may modulate multiple oxidative and inflammatory signaling pathways through their actions on CB1, CB2, transient receptor

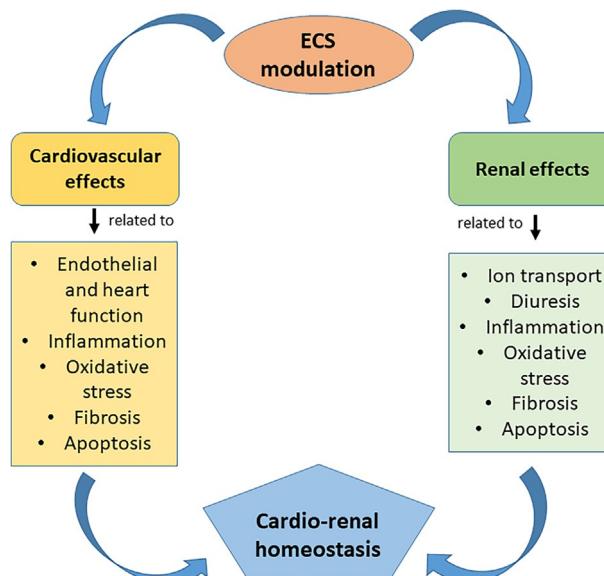
potential cation channel subfamily V member 1 (TRPV1), peroxisome proliferator-activated receptor-alpha (PPAR- $\alpha$ ), G-protein-coupled receptor 18 and 25 (GPR18 and GPR25) receptors, among others, thus preventing or attenuating the oxidative stress and the inflammatory process (Gallelli et al., 2018). Indeed, exacerbated oxidative stress and hyperinflammation are characteristic conditions in animal models of hypertension, including spontaneously hypertensive rats (SHRs) and hypertensive deoxycorticosterone acetate (DOCA)-salt rats. However, it was observed that although the increase in endocannabinoid plasma levels usually causes a reduction in oxidative stress and inflammation in hypertensive animals, it would be also able to provoke a rise in these parameters in normotensive animals. This is one of several examples where ECS modulation would be involved in the development of deleterious effects. Therefore, the therapeutic management of ECS should be interpreted carefully, since this may depend on multiple circumstantial factors (Biernacki, Ambrożewicz, et al., 2018; Biernacki, Malinowska, et al., 2018).

In this context, this chapter summarizes the most recent findings on endocannabinoid modulation through phyto, endo, and synthetic cannabinoids, which may mediate both harmful and beneficial effects at the cardiovascular and renal level.

## Cardiovascular effects derived from pharmacological manipulation of the endocannabinoid system

Pharmacological manipulation of ECS has demonstrated to play a key role in the modulation of cardiovascular homeostasis (Fig. 1). For instance, it has been suggested that cannabidiol (CBD) would be an attractive therapeutic alternative in the stroke prevention by stimulating the homeostatic effects of the ECS and preventing or attenuating the metabolic syndrome and atherosclerosis (Scharf, 2017). Likewise, the activation of CB1 receptor at the dorsal hippocampus level may prevent or attenuate the cardiovascular responses induced by stress in rats, such as tachycardia and mean blood pressure increase (Hartmann et al., 2019). In addition, CB2 receptor activation may attenuate inflammation by affecting immune cell recruitment and macrophage polarization, among other effects, which results in special interest considering that inflammation is one of the main underlying mechanisms for the development and progression of cardiovascular disease (Puhl, 2020).

In relation to ischemia/reperfusion injury, ECS has a crucial role in the preservation of tissue and function integrity during this condition, since it may modulate the oxidative stress and inflammation generated as a consequence of this physiopathological process. In fact, after induced bilateral common carotid artery occlusion followed by reperfusion, an increase in AEA plasma levels was detected, in an attempt to counteract the oxidative and inflammatory damage caused (Quarti et al., 2017). To highlight, a recent study revealed that AEA was one of the main endocannabinoids released by murine aorta in response to an induced inflammatory environment, suggesting that AEA release would act an antiinflammatory mechanisms of compensation at the cardiovascular level. Additionally, the same study showed that AEA



**FIG. 1** Key role of endocannabinoid system modulation in the regulation of cardio-renal homeostasis. This figure shows the specific cardiovascular and renal effects that may be regulated through endocannabinoid system (ECS) modulation, which directly influence on the cardio-renal homeostasis.

pretreatment of both murine aorta and human vascular smooth muscle cells under inflammatory conditions inhibited the expression of several proinflammatory genes, including CCL2 cytokine, through an epigenetic mechanism mediated by H3K27me3, H3K27ac, HDAC4, and NCoR1. These findings confirm the antiinflammatory cardiovascular effects of AEA (Pflüger-Müller et al., 2020). Moreover, it has been demonstrated that the cardioprotective mechanisms of propofol pretreatment before myocardial reperfusion would be related with its ability to increase endocannabinoid plasma levels (mainly AEA and 2-AG). These cardioprotective actions of propofol would be associated with antioxidative, antiinflammatory, and antiapoptotic effects mediated by CB2 receptor activation (Sun et al., 2017). AEA was also able to significantly increase the cell viability and to reduce the apoptosis and oxidative stress in cultured cardiomyoblasts under induced hypoxia/reoxygenation (Santa-Helena et al., 2017).

As adaptive response during heart disease, an increase in AEA levels and CB2 receptor expression was observed in mice with induced myocardial hypertrophy, in order to prevent or attenuate inflammation, fibrosis, and apoptosis of cardiomyocytes. Therefore, the ECS has a key role in myocardial adaptation in response to pressure overload (Duerr et al., 2019). Likewise, the activation of CB1 receptors may induce vasodilation and cardiac contractility, thus modulating blood pressure and enhancing heart function. CB1 receptor activation would also stimulate to the energy-sensing AMP-activated protein kinase to reduce inflammation and consequently attenuate the insulin resistance at cardiomyocyte level, showing beneficial effects not only on heart, but also on blood vessels. In addition, CB2 receptor activation would be able to attenuate the inflammation in atherosclerotic plaques, reducing their vulnerability to rupture and thrombosis risk (Chanda et al., 2019). Endocannabinoids not only have cardioprotective and antiinflammatory properties by itself, but also some enzymes such as cytochrome P450 (CYP) epoxygenase CYP2J2 (the most abundant CYP in the heart) are able to catalyze the metabolism of endocannabinoids to bioactive epoxides that are involved in the maintenance of multiple cardiovascular functions (Carnevale et al., 2018; Arnold et al., 2018).

Regarding pulmonary hypertension, ECS may also regulate vascular responses facing different vasoconstrictor stimuli. Indeed, pulmonary vasoconstriction evoked by angiotensin-II and U46619 (a thromboxane A2 analog) may induce endothelial endocannabinoid release, which leads to vasorelaxation. Therefore, ECS activation may mediate powerful vasorelaxant properties, which may be very useful in the treatment of pulmonary hypertensive pathology and other related diseases (Karpińska et al., 2017). In the same way, by assessing the effects of cannabidiol on rats with induced pulmonary hypertension, it was demonstrated that this phytocannabinoid was able to improve the function and efficiency of endothelium, normalizing hemostatic impairments, reducing leukocyte count on enhancing pulmonary levels of several endocannabinoids, including AEA. Therefore, this suggested that cannabidiol therapy may be useful in the treatment of human pulmonary hypertension (Sadowska et al., 2020).

Delving into the vasodilator effects caused by ECS stimulation, TRPV1 channels, one of the most important cannabinoid receptors, which integrate this system (highly expressed in human skeletal muscle feed arteries and other blood vessels), are able to mediate vasorelaxation induced by different endocannabinoids. TRPV1 acts counteracting the vasoconstrictor effects from  $\alpha$ -adrenergic agonists and potentiating the vasodilator effects from different compounds such as acetylcholine and sodium nitroprusside. Thus, TRPV1 may be an important therapeutic target during multiple cardiovascular diseases characterized by vasoconstriction (Ives et al., 2017). However, TRPV1 is not the only target involved in vasorelaxation induced by ECS activation. The vasodilator effect of cannabinoids even was observed in an ex vivo rat model of retinal capillaries precontracted by noradrenaline. Specifically, 2-AG, AEA, and the synthetic cannabinoid R-(+)-WIN55212-2 provoked, in a dose-dependent manner, vasorelaxant effects in these blood vessels through a mechanism mediated, at least in part, by CB1 receptors and the nitric oxide–cyclic guanosine monophosphate signaling pathway (Zong et al., 2017). Furthermore, the first phase of hypotension of the triphasic response of AEA (hypotension–hypertension–hypotension) observed in anesthetized animals would be mediated, at least in part, by sphingosine kinase-1/sphingosine-1-phosphate endothelial regulatory axis, which provokes peripheral vasodilation and reduction of blood pressure (Greig et al., 2019). Likewise, the treatment of small mesenteric arteries isolated from SHR rats with fatty acid amide hydrolase (FAAH—responsible for the metabolism of AEA) inhibitors such as URB597 caused an increase in AEA levels, showing vasodilator effects that may be beneficial in the treatment of hypertension (Baranowska-Kuczko et al., 2021). N-arachidonoyl glycine (NAGLY) is an endocannabinoid that activates the GPR18, which is also part of ECS. In this regard, NAGLY may also cause vasodilator and hypotensive effects. This vasorelaxation is mainly mediated by the activation of nitric oxide–cGMP pathway and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger and likely by the “endothelial AEA receptor,” whereas the hypotension observed does not appear to be associated with this receptor (Al Suleimani and Al Mahrui, 2017). Even, a potent vasodilator effect of AEA in cerebral arteries from baboons’ fetuses exposed to high concentrations of alcohol was observed, which would be mediated by CB2 receptors (Seleverstov et al., 2017).

It is well known that AEA presents several difficulties at the moment of being administered exogenously, since it is highly susceptible to degradation by different environmental factors and may provoke some adverse effects such as

mentioned triphasic response. For this reason, AEA delivery nanosystems were designed in order to overcome these limitations. AEA incorporated into polymeric nanocarriers showed to be effective not only in the treatment of hypertension, but also in the therapy of associated comorbidities such as cardiac remodeling. Indeed, AEA reduced left ventricular mass index and serum cytokines such as IL-1, IL-6, TNF $\alpha$ , extracellular Hsp70, and ultrasensitive C reactive protein, without causing reduction in ejection fraction. AEA incorporated into polymeric nanocarriers also showed antiinflammatory, antioxidative, and antiapoptotic properties both peripherally and at the central nervous system level, specifically within the cerebral cortex in rats. This nanoformulated endocannabinoid downregulated the AT1-WT-1-iNOS signaling pathway and increasing intracellular Hsp70 in cerebral cortical cells. Therefore, in addition to acting as an antihypertensive, it could modulate altered behaviors in SHR without producing central nervous adverse effects.

On the contrary, it has also been reported that CB1 receptor activation would be involved in the development of the cardiovascular dysfunction associated with an acute excessive alcohol intake (Paloczi et al., 2019), which would represent one of the several controversial effect of ECS stimulation. Similarly, AEA may promote the expression of cardiometabolic phenotype, as happens in diabetic patients with coronary artery disease under caloric restriction, in which a strong correlation between their AEA plasma levels and adipose tissue accumulation was observed (van Eyk et al., 2018). Indeed, some studies reported that, sometimes, CB1 receptors activation may also aggravate the inflammatory response and would be related to the development of obesity and diabetes, whereas its antagonism would cause the improvement of lipids and glucose plasma profile (Puhl, 2020). Likewise, obese mice have higher myocardial levels of endocannabinoids, including AEA and 2-AG and a greater expression of myocardial CB1 receptors than nonobese mice. Therefore, obesity may predispose to development and progression of cardiac dysfunction in obese individuals through ECS activation (Valenta et al., 2018). Additionally, a positive correlation between endocannabinoid and triglyceride plasma levels in patients who consumed beverages sweetened with high-fructose corn syrup during 2 weeks was also observed, which would suggest that, under some circumstances, high levels of AEA and 2-AG could be related to cardiovascular disease development (Price et al., 2018).

## **Endocannabinoid system management and its consequences on kidney functions**

ECS is present in several organs and systems, including the kidney. Usually, CB1 receptor activation may regulate renal vascular hemodynamics and modulate ions, water, and proteins transport in various nephron compartments in a beneficial way (Fig. 1). However, in some animal models of obesity and diabetes mellitus, endocannabinoids produced by renal cells may induce oxidative, inflammatory, and fibrotic effects at the kidney level through the activation of CB1 receptors, which may be prevented or attenuated by the use of CB1 receptor antagonists (Tam, 2016; Dao and François, 2021). In fact, it has been recently suggested that renal ECS would also participate in the pathogenesis of some renal illnesses including diabetic nephropathy, drug nephrotoxicity, and progressive chronic kidney disease. Furthermore, several cases of acute kidney injury associated with the use of synthetic cannabinoids have been reported (Barutta et al., 2018).

Conversely, renal CB2 receptors activation may reduce the harmful effects of these chronic pathologies, thus attenuating some of the deleterious tubular effects that occur as a consequence of acute kidney injury. In fact, the administration of SMM-295 (a new agonist of CB2 receptors) to anesthetized mice caused an increase in cortical renal perfusion and a vasodilator effect on afferent arterioles from these mice (Pressly et al., 2019). In addition to CB2 receptors, AEA and 2-AG also mediate multiple beneficial effects at the renal level. As example, AEA not only may function as a neuromodulator of the renal sympathetic nervous system, but also together with prostamides (its cyclooxygenase-2 metabolites) may represent a powerful antihypertensive system implicated in the control of blood pressure at the renal medulla level. Likewise, AEA and its metabolites may exert important therapeutic effects during chronic kidney disease associated with inflammation and cardiovascular pathologies, specially accompanied by hyperhomocysteinemia (Ritter et al., 2016). For its part, a rat model of renal ischemia/reperfusion injury pretreated with URB602 [a monoacylglycerol lipase (MAGL—responsible for the metabolism of 2-AG) inhibitor] showed significant antioxidant, antiinflammatory, and renoprotective effects through a mechanism mediated by 2-AG increased levels and CB2 receptor activation (Li et al., 2020).

Hence, FAAH or MAGL inhibition is another common way of pharmacological manipulation of ECS that may be therapeutically useful in the modulation of kidney homeostasis. In this regard, it was observed that FAAH inhibition by URB597 was able to reduce the activity of prooxidant enzymes, which resulted in a decrease of oxidative stress, added to a tendency to enhance the antioxidant defense in the kidneys from SHR and DOCA-salt hypertensive rats. Moreover, URB597 administration decreased inflammation, particularly in the kidneys from DOCA-salt hypertensive rats. The treatment with URB597 also caused an increase in the levels of renal endocannabinoids in both groups of hypertensive rats, added to an increased expression of the CB1 and CB2 receptors in SHR as well as TRPV-1 receptors in DOCA-salt rats. Conversely, URB597 administered to normotensive rats caused an increase in renal oxidative stress (resulting in

higher levels of both neuroprostanes in kidneys from Wistar Kyoto rats and reactive aldehydes in kidneys from Wistar rats) despite the levels of endocannabinoids and their receptors being also higher in both control groups of rats. Therefore, in hypertensive rats, FAAH inhibition may prevent or attenuate kidney disorders in a model-dependent manner, but may also provoke kidney alterations in normotensive rats, which requires more investigation (Biernacki, Ambrożewicz, et al., 2018; Biernacki, Malinowska, et al., 2018). Of interest, URB597 administration to DOCA-salt hypertensive rats during 2 weeks resulted in an age-dependent decrease in blood pressure and heart rate (in older but not in younger rats), as well as a reduction in cardiac and renal hypertrophy (in younger but not in older rats). This potentially would indicate that FAAH inhibitors not only may mediate model-specific effects, but also may cause age-specific effects on cardio-renal system (M. Toczek et al., 2016).

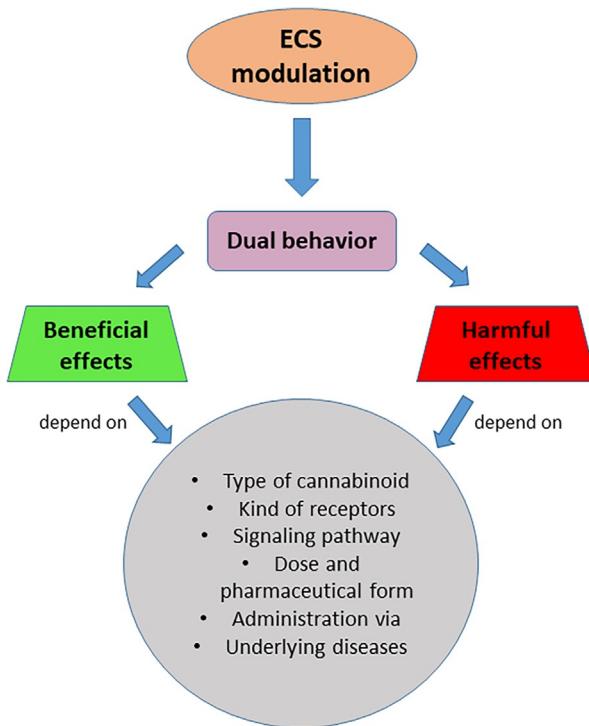
In relation to the transport of ions at the renal level, it was observed both *in vivo* and *in vitro* that the ECS and the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump expression and activity are downregulated after induced ischemia/reperfusion injury on kidney cells. These alterations were reversed by WIN55,212 (a CB1/CB2 receptor agonist), in a CB1-receptor-dependent manner. This would suggest that CB1 receptor agonists may be used as a potential strategy to mitigate the consequences of ischemia/reperfusion injury at the kidney level (Sampaio et al., 2018). However, CB1 receptor activation by different cannabinoids such as CP55940 (a CB1/CB2 agonist), AEA, or PF3845 (an FAAH inhibitor) may also cause sodium transport inhibition in thick ascending limb tubules of mice, at least in part, by Na<sup>+</sup>-K<sup>+</sup>-ATPase blockade. This would explain the natriuretic and diuretic effects of cannabinoids at the renal level, which directly influence the blood pressure modulation (Ritter et al., 2021). Similarly, the treatment of epithelial cells of the human proximal tubule with AEA encapsulated in polymeric nanoparticles showed a decrease in the Na<sup>+</sup>/K<sup>+</sup> ATPase activity in a dose-dependent manner, in a mechanism mediated by inducible nitric oxide enzyme and nitric oxide, whose levels were increased after the nanoencapsulated AEA administration (Martín Giménez et al., 2020). Of special interest, the infusion of isopropyl dodecyl fluorophosphate (an FAAH and MAGL enzymes inhibitor) into the renal medulla of mice raised both the urine formation rate and sodium excretion in a COX-2 and FAAH-dependent manner, as well as increased the renal medullary blood flow, without affecting glomerular filtration rate or renal blood flow. This confirms that AEA and 2-AG would be capable of modulating natriuresis and diuresis at the kidney level (Ahmad et al., 2017). Furthermore, the intramedullary infusion of PF-3845 (an FAAH inhibitor) was able to cause not only an increase in diuresis, but also a reduction of blood pressure. These effects would be mediated by both increased kidney tissue levels of AEA and enhanced plasma levels of 2-AG (Ahmad et al., 2018).

## Conclusion and prospects

Undoubtedly, ECS and especially AEA as one of its key components play an essential role in the modulation of cardio-renal homeostasis, since they may beneficially regulate multiple processes related to the adequate function of the heart, blood vessels, and kidneys, including inflammation, oxidative stress, fibrosis, apoptosis, endothelial function, transport of ions, diuresis, among others. However, ECS is also able to mediate harmful effects, which depends on multiple factors, such as the type of cannabinoid used, the kind of receptors that act as target, the signaling pathway involved, the dose and pharmaceutical form administered, the administration via used, the coexistence of underlying diseases (diabetes, obesity, etc.), among others (Fig. 2). In addition to this dual behavior of ECS, it is important to consider that endocannabinoid modulation in humans may sometimes be different from endocannabinoid modulation in animals. Accordingly, more studies in humans should be done in order to clarify this point (Hoyer-Allo et al., 2021).

## Application to other areas

In this chapter, we have reviewed the most recent findings on endocannabinoid modulation through phyto, endo, and synthetic cannabinoids, which may mediate both harmful and beneficial effects at the cardiovascular and renal level. Although the main objective of this chapter was to highlight the key role of pharmacological manipulation of the endocannabinoid system in the maintenance of cardio-renal homeostasis for the prevention and treatment of cardiovascular and kidney diseases, the versatility of this system would allow the application of the therapeutic potential of cannabinoids and their derived compounds to the management of other related and nonrelated diseases, which has inflammation, oxidative stress, fibrosis, and apoptosis as the common underlying mechanisms. Within this group of affections, we may find cancer, neurodegenerative pathologies, autoimmune and infectious diseases, among many others. Likewise, the analysis of this chapter allows us to know what signaling pathways may be deleterious in relation to pharmacological manipulation of endocannabinoid system and under what circumstances. This would allow to orientate the development of novel strategies for drug targeting, such as the use of nanostructures for carrying cannabinoids in order to localize their actions only on molecular targets in which their effects are beneficial, instead those in which may exert deleterious consequences. Therefore, another



**FIG. 2** Dual behavior of endocannabinoid system. This figure describes some of the most important factors that determine the duality of endocannabinoid system (ECS) modulation, which results in either beneficial or harmful effects.

application area that could be enriched from the knowledge described in this chapter is related to the field of nanomedicine, through which it is possible to obtain new pharmaceutical forms to administrate cannabinoids as therapeutic compounds maximizing their efficacy and minimizing their pharmacokinetic and pharmacodynamics limitations, as well as their adverse effects.

### Mini-dictionary of terms

- **Cardio-renal.** Referred to both cardiovascular and renal systems.
- **Nano(system/carrier/formulation/particle).** Referred to platforms with sizes from 1 to 1000 nm, which act as vehicles of different pharmaceutical drugs.
- **H3K27me3/H3K27ac.** Different types of epigenetic modifications.
- **HDAC4.** A kind of histone deacetylase.
- **NCoR1.** A kind of nuclear receptor corepressor.
- **IL-1/IL-6/TNF $\alpha$ /CCL2.** Different types of cytokines.
- **Hsp70.** A kind of heat shock protein.
- **WT1.** Wilms' tumor 1 protein.
- **AT1.** A kind of angiotensin-II receptor.
- **iNOS.** Inducible nitric oxide synthase.

### Key facts of cardio-renal diseases

- Cardiovascular disease is the main cause of death in the worldwide, even over cancer.
- Most of cardiovascular diseases such as hypertension are silent pathologies; therefore, the delay diagnosis may difficult their treatment.
- Kidneys are highly sensitive organs; therefore, any damage on renal cells may be irreversible and lead to dialysis therapy, which significantly affects the life quality and life expectancy of patients that suffer this kind of pathologies.

- Most of conventional pharmacological therapies for the treatment of cardio-renal diseases have multiple limitations and disadvantages, such as poor oral bioavailability, short plasma half-life, high dosage frequency, many side effects, low specificity, among others, which leads to low adherence to the treatment and a deficient efficacy of the therapies.
- As novelty, drug delivery nanosystems may overcome most of the limitations of conventional drugs used in the treatment of cardio-renal diseases.

## Summary points

- The imbalance of cardio-renal homeostasis may lead to the development of multiple cardiovascular and kidney diseases, which are highly prevalent in worldwide population and have elevated morbidity and mortality rates.
- Currently, the available therapeutic options are not enough to achieve an adequate control of cardio-renal diseases.
- The pharmacological manipulation of endocannabinoid system arises as an attractive way to improve the prevention and treatment of cardio-renal pathologies.
- Anandamide and other phyto, endo, and synthetic cannabinoids have vasodilator, antiinflammatory, antioxidant, anti-fibrotic, antiapoptotic, natriuretic, and diuretic properties.
- Because of dual behavior of endocannabinoid system, under certain circumstances, its pharmacological modulation may also cause opposite effects, which may be deleterious for cardio-renal homeostasis.

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## Chapter 36

# The endocannabinoid system: Signaling and social motivation

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## Abbreviations

<b>2-AG</b>	2-arachidonoyl glycerol
<b>AEA</b>	anandamide
<b>ASD</b>	autism spectrum disorder
<b>BLA</b>	basolateral amygdala
<b>BNST</b>	stria terminalis
<b>CB1</b>	cannabinoid receptor type 1
<b>CB2</b>	cannabinoid receptor type 2
<b>CPP</b>	conditioned place preference
<b>DAGL</b>	diacylglycerol lipase
<b>ecbs</b>	endocannabinoids
<b>ECS</b>	endocannabinoid system
<b>FAAH</b>	fatty acid amide hydrolase
<b>GPCR</b>	G-protein-coupled receptor
<b>HA</b>	anterior hypothalamus
<b>HIP</b>	hippocampus
<b>LPI</b>	lysophosphatidylinositol
<b>LS</b>	lateral septum
<b>MAGL</b>	monoacylglycerol lipase
<b>meAMG</b>	medial amygdala
<b>MGL-Tg</b>	transgenic mouse model
<b>NAc</b>	nucleus accumbens
<b>NADA</b>	N-arachidonoyl-dopamine
<b>NAPE-PLD</b>	N-acylphosphatidylethanolamine-specific phospholipase D
<b>OAE</b>	virodhamine
<b>OT</b>	oxytocin
<b>OTR</b>	oxytocin receptor
<b>PAG</b>	midbrain periaqueductal gray
<b>PFC</b>	prefrontal cortex
<b>POA</b>	preoptic area
<b>SBN</b>	Social Behavior Network
<b>sCPP</b>	CPP for social contact
<b>SDMN</b>	Social Decision-Making Network
<b>VMH</b>	ventromedial hypothalamus
<b>VP</b>	ventral pallidum
<b>VTA</b>	ventral tegmental area

## Introduction

In recent decades, the endogenous cannabinoid system has been studied to better understand the neurobiology underlying social motivation. This chapter will provide an overview of endocannabinoid role on social reward and motivation, describing the presence of cannabinoid receptors in key structures involved in social-emotional processing, biochemical events involving endocannabinoids and its interaction with other neurotransmitter system.

## The endocannabinoid system (ECS)

The endocannabinoid system (ECS) is composed of type 1 and 2 cannabinoid receptors, endogenous ligands, known as endocannabinoids (eCBs), and the enzymes involved in biosynthesis and degradation. The eCBs are signaling lipids that activate cannabinoid receptors. To date, there are six molecules recognized: N-arachidonoyl-ethanolamine (Anandamide, AEA), 2-arachidonoyl glycerol (2-AG), N-arachidonoyl-dopamine (NADA), 2-arachidonyl glyceryl ether (noladin ether), virodhamine (OAE), and lysophosphatidylinositol (LPI) (Ye et al., 2019) (Table 1). The enzymes involved in the synthesis of AEA and 2-AG are N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively, and the enzymes responsible for the inactivation are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Lu & Mackie, 2021).

In contrast with classic neurotransmitters that are synthesized, stored in vesicles, and released after action potential reach the presynaptic bouton, the eCBs are produced on-demand at postsynaptic neurons via increased intracellular calcium in response to sustained synaptic activity. Once released from postsynaptic neurons, endocannabinoids act as retrograde lipid messengers or local modulators on the CB1 presynaptic receptors to control presynaptic firing and the release of other neurotransmitters (e.g., dopamine, opioids, GABA, glutamate, and acetylcholine), enabling the ECS to regulate multiple physiological functions in the central nervous system (Kendall & Yudowski, 2017; Silver, 2019).

## Cannabinoid receptors

The two main cannabinoid receptors, termed CB1 and CB2, are members of G-protein-coupled receptor (GPCR) family coupled to Gi/o protein, the stimulation of which inhibits adenylyl cyclase activity leading to a decrease of cAMP levels. In the brain, CB1 receptors are abundantly expressed in neuronal terminals and considered the primary target for the action of cannabinoids. CB2 receptors, initially believed to be restricted on immune cells due to its low baseline expression, are widely distributed in the brain and implicated in neuromodulatory roles. Both receptors are found in brain social network, termed Social Decision-Making Network (SDMN), introduced by O'Connell and Hofmann (2011, 2012) (Table 2).

## Social decision-making network (SDMN)

The research in neuroscience field has been elucidating the network that specifically processes social information. The first social behavior network (SBN) was described by Sarah Newman (1999) based on studies of brain regions sensitive to sex steroids hormones that regulate multiple forms of expression of social behavior (sexual behavior, aggressive behavior, parental behavior, affiliation, and attachment). The brain's social behavior network comprises highly interconnected nodes: the lateral septum (LS), the preoptic area (POA), the ventromedial hypothalamus (VMH), anterior hypothalamus (HA), the midbrain periaqueductal gray (PAG), the extended medial amygdala (i.e., the medial amygdala (meAMG), and the bed nucleus of the stria terminalis (BNST). The original SBN lacked brain regions needed to detect, assess, and respond to social cues from multiple sensory modalities of other individuals in the group, through the evaluation of aspects potentially challenging or rewarding of social interactions, transforming a sensorimotor experience into a reciprocal behavioral

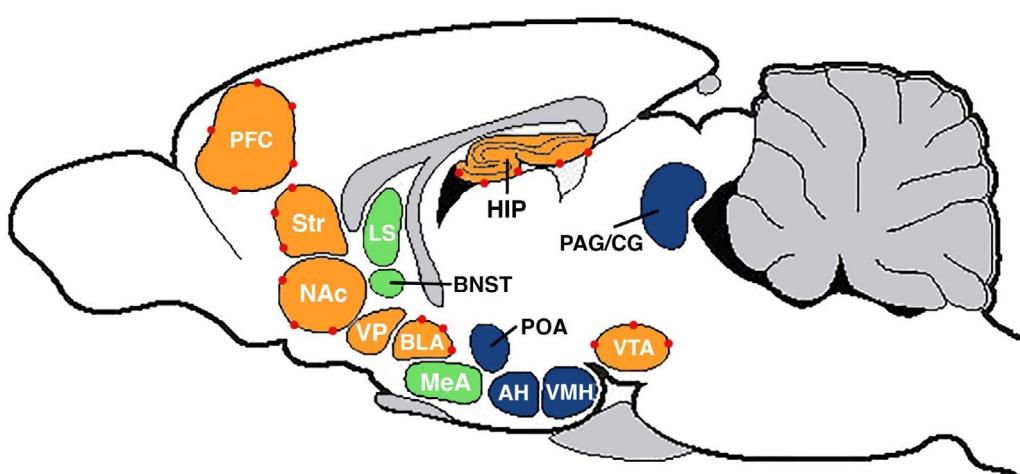
**TABLE 1** Main endocannabinoids and enzymes for synthesis and inactivation.

Endocannabinoid molecule	Enzyme for synthesis	Enzyme for inactivation
N-arachidonoyl-ethanolamine (Anandamide, AEA)	N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD)	Fatty acid amide hydrolase (FAAH)
2-Arachidonoyl glycerol (2-AG)	Diacylglycerol lipase (DAGL)	Monoacylglycerol lipase (MAGL)

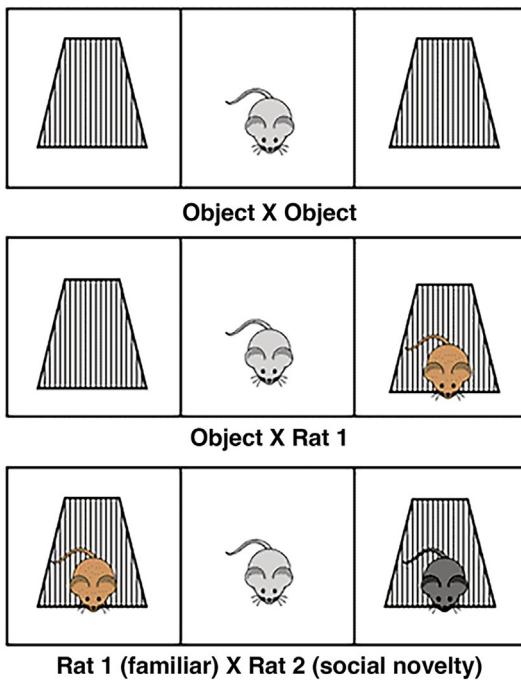
**TABLE 2** Distribution of CB1 and CB2 receptor in brain regions related to social decision-making network (SDMN).

Brain structures	CB1	CB2
Basolateral amygdala (BLA)	Marsicano et al. (2002), Katona et al. (2001)	Argue et al. (2017)
Prefrontal cortex (PFC)	Egerton et al. (2006), McLaughlin and Gobbi (2012), Heng et al. (2011)	Mackie (2005)
Hippocampus (HIP)	Herkenham et al. (1991), Fride (2005)	Li and Kim (2015), Komorowska-Müller et al. (2021), Visvanathan et al. (2022)
Dorsal striatum (DS)	Hohmann and Herkenham (2000)	Sagredo et al. (2009)
Ventral tegmental (VTA) area and Nucleus accumbens (NAc)	Fitzgerald et al. (2012), Winters et al. (2012)	Zhang et al. (2014), Zhang et al. (2017)

decision between two decision-making individuals (Chen & Hong, 2018; Rogers-Carter & Christianson, 2019). O'Connell and Hofmann (2011, 2012) observed that the mesocorticolimbic system, implicated in encoding the rewarding properties of "natural rewards," expresses many of the same receptors and genes that mediate social behaviors and thus, merged the SBN with the mesocorticolimbic dopamine system to coin the Social Decision-Making Network (SDMN), adding to this brain network, the nucleus accumbens (NAc), the Ventral pallidum (VP), the Basolateral Amygdala, (BLA), the Hippocampus (HIP), the ventral tegmental area (VTA), and the prefrontal cortex (PFC). How the sensory information is transmitted in the brain to shape a behavioral response is not well understood; however, the prefrontal cortex was associated with top-down control of social decision-making; the amygdala, with the modulation of emotional processing and with the processing of sensory stimuli, and in the case of rodents, the social odor; the hypothalamus, with the stress response and integration of internal and external stimuli; the hippocampus, considered the site of social memory, representing different dimensions of social space; and the nucleus accumbens, associated with social reward and motivation (Greenberg & Trainor, 2016; Ko, 2017; Montagrin et al., 2017) (see Fig. 1). Accumulated evidence has been showing that the insular cortex also plays a relevant role in this network, integrating socioemotional cues to SDMN to lead a behavioral response (Rogers-Carter & Christianson, 2019). The existence of a circuitry that specifically processes social information was also identified by neuroimaging and cognitive-neuroscience experiments (Insel & Fernald, 2004; Stanley & Adolphs, 2013) as well by molecular studies that evidenced a distinct brain activity associated with social interaction (Matthews et al., 2016).



**FIG. 1** CB1 and CB2 receptors' presence in structures of SBN and SDMN. Presence of CB1 and CB2 receptors (in red) in structures of Social Behavior Network (SBN) and Social Decision-Making Network (SDMN). Brain areas in blue (POA, AH, VMH, and PAG/CG) represent the SBN. Brain area in orange (NAc, Str, VP, BLA, HIP, VTA, and PFC) represent additional structures of SDMN. The LS, MeA, and BNST (green) are included in both SBN and SDMN. LS, lateral septum; BNST, bed nucleus of the stria terminalis; POA, preoptic area of the hypothalamus; MeA, medial amygdala; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; PAG/CG, periaqueductal gray/ central gray; NAc, nucleus accumbens; Str, striatum; VP, ventral pallidum; BLA, basolateral amygdala; HIP, hippocampus; VTA, ventral tegmental area; PFC, prefrontal cortex. Elaborated by this chapter authors, Roberta Cysneiros and Fernanda Ribeiro, based in Prounis, G. S., & Ophir, A. G. (2020). One cranium, two brains not yet introduced: Distinct but complementary views of the social brain. *Neuroscience & Biobehavioral Reviews*, 108, 231–245. <https://doi.org/10.1016/j.neubiorev.2019.11>.



**FIG. 2** Three-chambered social approach task. Rodents normally prefer to spend more time with another rodent (sociability) and will investigate a novel intruder more so than a familiar one (social novelty). Testing occurs in four sessions (10 min each) within a three-chambered box with openings between the chambers. Phase 1 is habituation in the center; in phase 2, the subject encounters two empty boxes; in phase 3, encounters a never-before-met intruder. In phase 4, subject then encounters the first intruder (familiar) as well as a second never-before-met intruder (social novelty). The time spent investigating, the time spent in each chamber, and the number of entries into each chamber are presented. This test is useful for quantifying deficits in social behavior as well as evaluating pharmacological effects on social behavior. Adapted from Stanford Behavioral Neuroscience Laboratory. Original site: <https://med.stanford.edu/>.

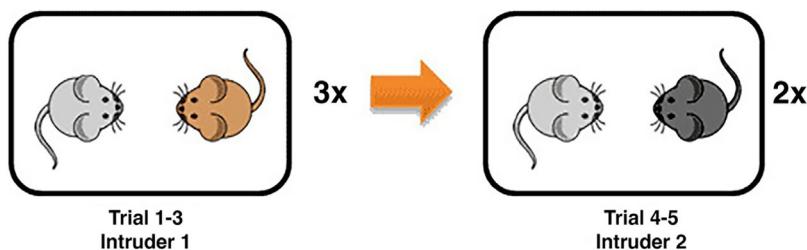
## Behavioral paradigms to assess sociability in rodents

The behavioral paradigms often used to assess the sociability in rodents are conditioned place preference (CPP) task, Three-Chambered Social Approach Task, Social interaction test, and Social Recognition Test. These paradigms can be also combined with pharmacologic and genetic approaches and in different context to gain a comprehensive understanding of sociability (Table 3).

**TABLE 3** Paradigms often used to assess sociability in rodents.

Paradigm	Description	References
Conditioned place preference (CPP) task	Assessment of positive reinforcement by assessing the amount of time an animal spends in an area that has been associated with a stimulus	Tanaka et al. (2011)
Three-chambered social approach task (Fig. 2)	Assessment of direct approach behaviors when an animal is presented with the choice of spending time with either a novel conspecific or a novel object	Yang et al. (2011)
Social interaction test	Assessment of time spent in active social investigation by pairs	File and Seth (2003)
Social recognition test (Fig. 3)	Assessment of time an animal spends investigating a conspecific introduced in successive expositions into the arena of test, considering that the time of investigating decrease with exposure to the same social stimulus and increase upon exposure to a novel conspecific	Thor et al. (1981)
Social play behavior	Assessment of a set of criteria associated with sociability, as: pouncing, boxing and pinning.	Vanderschuren and Trezza (2013)

**FIG. 3** Social Recognition Test. The subject is given three exposures to the same intruder. In the fourth trial, the subject encounters an entirely novel intruder. This test is useful in identifying social memory deficits or other social interaction deficits, as well as evaluating pharmacological effects in social memory. Adapted from Stanford Behavioral Neuroscience Laboratory. Original site: <https://med.stanford.edu/>.



## Endocannabinoids's role on social reward and motivation

Social motivation and rewards are fundamental processes to keep individuals engaged in social activities, for establishing and maintaining social bonds, being critical to survival, reproduction, and overall healthy (Kahrson et al., 2016). Social play behavior is a highly rewarding behavior in juvenile and adolescent mammals (Vanderschuren et al., 2016; Vanderschuren & Trezza, 2013), and it is modulated by molecules involved in motivation and rewards, such as AEA, 2-AG, opioid, and dopamine (Fattore et al., 2005; Maldonado et al., 2006; Oleson et al., 2021; Solinas & Goldberg, 2005; Spanagel, 2020). Our knowledge of EC system as modulator of natural rewards, as feeding and social contact, has been growing in recent years in large part due the utilization of genetic and pharmacologic approaches, such as specific enzyme inhibitors of endocannabinoids formation and degradation, selective agonists, and antagonists of cannabinoids receptors, as well as transgenic mouse model. Studies with systemic drug administration have shown that the effect of pharmacological manipulation of ECS system on socioemotional processes depends on strain, developmental ages, and experimental context, which may account for contradictory effects reported by different research groups (Manduca et al., 2014, 2015, 2016).

### Anandamide

In the brain, AEA is found in a proportion 10–100 times lower than 2-AG, but it is the most widely studied endocannabinoid (Biringer, 2021).

Social play with an unfamiliar rat increases AEA levels in the nucleus accumbens, amygdala, and dorsal striatum as compared with encounter with a familiar partner or nonsocial controls (Marco et al., 2011; Trezza et al., 2012). Corroborating these findings, the FAAH inhibition, the main AEA-metabolizing enzyme, by URB597 administration, increases social play behavior, such as pinning and pouncing, as well as anandamide levels in the nucleus accumbens and amygdala. Interestingly, FAAH inhibition by using URB597 did not affect CPP for social contact (sCPP) in rats (Achterberg et al., 2016). A possible explanation for the lack of effect was attributed to the test protocol, as the ceiling effect induced by social isolation prior to the behavioral task, so that the URB597 treatment did not further increase animals' performance, or alternatively, AEA is not relevant for memory consolidation of social play's pleasurable properties. In opposition to these findings, FAAH knockout mice exhibited high levels of sCPP, which was decreased by CB1 inverse agonist (AM251) in both FAAH knockout mice and wild-type mice (Wei et al., 2015). The same study reported that male wild-type and FAAH knockout mice socialized after 24-h isolation period exhibited increased AEA levels in the nucleus accumbens and in ventral hippocampus, brain regions implicated in reward learning, which was abolished by oxytocin receptor blockade. Conversely, oxytocin receptor agonist elicited nucleus accumbens AEA mobilization in the absence of social contact. In line with behavioral and neurochemical analysis, the immunohistochemical approach showed increased c-Fos immunostaining in nucleus accumbens following resocialization, which was abolished by oxytocin antagonist administration. The role of oxytocin in processes involving emotional attachments and in recompense related to social encounters is well established. The oxytocin receptor (OTR) is largely distributed in the central nervous system and specifically, OTRs are highly expressed in SDMN structures, such as the nucleus accumbens, the amygdala, the hippocampus, as well the ventral tegmental area (VTA) (Dölen et al., 2013; Gordon et al., 2011). Therefore, the evidence supports the assertion that social rewarding processing requires a coordinated activity of AEA and oxytocin, the dysregulation of which may be implicated in social impairment conditions, such as autism spectrum disorder (ASD) (Wei et al., 2015). In accordance with these findings, the animal model of early life seizures, which exhibits deficit in oxytocin signaling (Pacífico et al., 2020) and low social interaction and motivation (Castelhano et al., 2013; Leite et al., 2016; Pacífico et al., 2020), the pretreatment with

JZL195, a dual FAAH/MAGL inhibitor, did not enhance social interaction with unfamiliar partner assessed in three-chambered social discrimination task and social recognition task (Ribeiro et al., 2020). Conversely, control animals, young adults Wistar rats, treated with JZL195, displayed reduced time investigating the conspecifics, and impaired social discrimination. Ribeiro's study has two key features: (i) sociability was studied in young adult rats instead of juvenile or adolescent ones, whereas the initiative to seek for social stimuli, familiar, or novelty is less rewarding than social play behavior in juvenile and adolescence phase of life due the reciprocal interactions between peers (Achterberg et al., 2016); (ii) following pretreatment, animals were kept with cage mates instead of being kept socially isolated to increase social motivation during testing. Combining these two features, a likely explanation for the reduction in sociability of adult intact animals treated with JZL195 is that endocannabinoids enhancement in group-housed animals damped the social novelty-seeking behavior.

Influence of genetic, developmental ages, and environmental factors on endocannabinoid effect has been frequently shown. For instance, FAAH inhibition by using URB597 did not change the sociability of adolescent or adult Sprague–Dawley strain in any experimental conditions, while it enhanced social play behavior and vocalization in adolescent Wistar rats under all experimental conditions, but, in adults, only increased social interaction under unfamiliar/high light condition. Of note, Wistar rats exhibit lower baseline levels of social interaction as compared with Sprague–Dawley strain (Manduca et al., 2014). These results converge with the Naidu et al. (2007) findings when evaluating rats' anxiety in a plus-maze test: URB597 treatment did not produce anxiolytic effects when animals were tested under low light or when habituated to the testing room. In contrast, robust anxiolytic effects were noticed when rats were tested under high light without habituation or when habituated rats were subjected to sudden changes in illumination during testing. Thus, it seems that anandamide signaling induced by URB597 increases the motivational and rewarding properties of social play only under aversive environmental conditions.

## 2-Arachidonoyl glycerol (2-AG)

2-Arachidonoyl glycerol (2-AG), the most abundant endocannabinoid in the brain (Justinova et al., 2011; Karhson et al., 2016), also modulates social reward, but its role on socioemotional behavior has been less investigated. The dual FAAH/MAGL inhibitor JZL195, at a dose that enhances 2-AG but not anandamide, increases social play behavior, the effect of which was blocked by pretreatment with cannabinoid receptor antagonist/inverse agonist SR141716A (Manduca et al., 2015). Curiously, the enhancement of 2-AG increased specifically social play behavior, with no effect over social exploration, anxiety, or locomotor activity (Manduca et al., 2015). Corroborating with previous findings, transgenic mouse model (MGL-Tg), in which forebrain 2-AG levels were selectively reduced, exhibited CPP impairment for social contact (sCPP), but similar levels of social interaction and social interest when compared with wild-type mice. In the same study, social contacts following resocialization elicited 2-AG mobilization mainly in the nucleus accumbens, while the isolation decreased 2-AG in ventral hippocampus and in medial prefrontal cortex as compared with animals' group housed. Thus, forebrain 2-AG seems to be relevant to memory consolidation rather than to reward processing, while 2-AG in the nucleus accumbens was identified as relevant for social-reward processes (Wei et al., 2016). Moreover, there is evidence that 2-AG may interact with other neurotransmitter system to modulate reward learning and motivation. For instance, the MAGL inhibition, the enzyme that preferentially catabolizes 2-AG, by JZL184 administration, or the opioid receptor agonist (morphine) increased social play behavior in adolescent rats, the effects of which were blocked by either CB1 cannabinoid receptor or mu-opioid receptor antagonists. The morphine or JZL184 effect was also abolished by the CB1 antagonist and mu-opioid receptor antagonists applied into the nucleus accumbens core. In addition, CB1 antagonist blocks the effect of the mu-opioid receptor agonist, and conversely, mu-opioid receptor antagonist blocks the effect of cannabinoid agonist. Thus, data show that endocannabinoid and opioid interact to regulate rodent socioemotional behaviors (Manduca et al., 2015).

In summary, behavioral studies have shown that the endocannabinoids modulate social motivation and rewards. The endocannabinoids effects are strain and context-dependent and coordinate with the oxytocin, endogenous opioid, and dopaminergic systems, involving limbic brain areas, such as the nucleus accumbens and amygdala.

## Applications to other areas

*In this chapter, we have reviewed the endocannabinoid system (ECS) and its presence in social brain's structures. We presented behavioral results that show this system's role in social motivation. A better comprehension about ECS participation in social motivation can be crucial to understand and plan therapeutic targets to disorders that are social deficit symptoms, such as autism spectrum disorder (ASD).*

## Mini-dictionary of terms

- **Autism Spectrum Disorder (ASD).** *Developmental disorder characterized by difficulty with communication and social interaction and repetitive behaviors.*
- **Endocannabinoids.** *Endogenous lipidic messengers from endocannabinoid system. Most studied endocannabinoids are anandamide (AEA) and 2-AG.*
- **Cannabinoid receptors.** *G-protein-coupled receptors, which bind with endocannabinoids and exogenous cannabinoids. Abundant in the central nervous system (CSN), mainly in structures of SDMN (below), they can be type 1 or type 2.*
- **Social Decision-Making Network (SDMN).** *Integration of the mesolimbic reward system and social behavior pathways, which recruits simultaneously associational prefrontal cortical regions and subcortical structures that underpin human social-emotional functioning.*
- **Social motivation.** *Set of behavioral dispositions and biological mechanisms biasing the individual to seek and take pleasure in social interactions (social reward).*

## Key facts of social motivation

*Endocannabinoid system has a crucial role in social reward, although neurobiological circuitry underpinning it is not clear.*

*Its role is specifically related to social novelty-seeking behavior.*

*Social novelty-seeking behavior mediated by the endocannabinoid system is affected in a context-dependent way (such as aversive environmental conditions).*

*Endocannabinoid anandamide acts in a coordinated way with the oxytocin system during social reward processes.*

*Social motivation mediated by endocannabinoids is totally affected by social stress context (such as isolation) before social encounter assessing.*

## Summary points

*Unlike classic neurotransmitters, endocannabinoids are synthesized and released on demand and are transmitted by retrograde signaling (from postsynaptic to presynaptic neuron).*

*Cannabinoid receptors, specially type 1, are highly expressed in brain structures of Social Decision-Making Network (SDMN), such as the hippocampus, basolateral amygdala, dorsal striatum, ventral tegmental area, nucleus accumbens, and prefrontal cortex.*

*The behavioral paradigms often used to assess the sociability in rodents are conditioned place preference (CPP) task, Three-Chambered Social Approach Task, Social interaction test, and Social Recognition Test.*

*The combination of behavioral paradigms for assessing sociability with pharmacologic and genetic approaches in different contexts is being useful to gain a comprehensive understanding of sociability.*

*Studies with systemic drug administration have shown that the effect of pharmacological manipulation of ECS system on socioemotional processes depends on strain, developmental ages, and experimental context, which may account for contradictory effects reported by different research groups.*

*Social play with an unfamiliar rat increases anandamide (AEA) levels in the nucleus accumbens, amygdala, and dorsal striatum as compared with encounter with a familiar partner or nonsocial controls.*

*Enhancement of 2-AG increased specifically social play behavior, with no effect over social exploration, anxiety, or locomotor activity.*

*Behavioral studies have shown that the endocannabinoids modulate social motivation and rewards.*

*The endocannabinoids effects are strain and context-dependent and coordinate with the oxytocin, endogenous opioid, and dopaminergic systems, involving limbic brain areas, such as the nucleus accumbens and amygdala.*

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## Chapter 37

# Safety and toxicology of the dietary cannabinoid $\beta$ -caryophyllene

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## Abbreviations

ALT	alanine transaminase
AST	aspartate transaminase
CB <sub>2</sub>	cannabinoid receptor 2
GHS	Globally Harmonized System of Classification
i.p.	intraperitoneal
IL	interleukin
LD <sub>50</sub>	medium lethal dose
LDH	lactate dehydrogenase
OECD	Organization for Economic Co-operation and Development
SOD	superoxide dismutase

## Sesquiterpene: $\beta$ -Caryophyllene

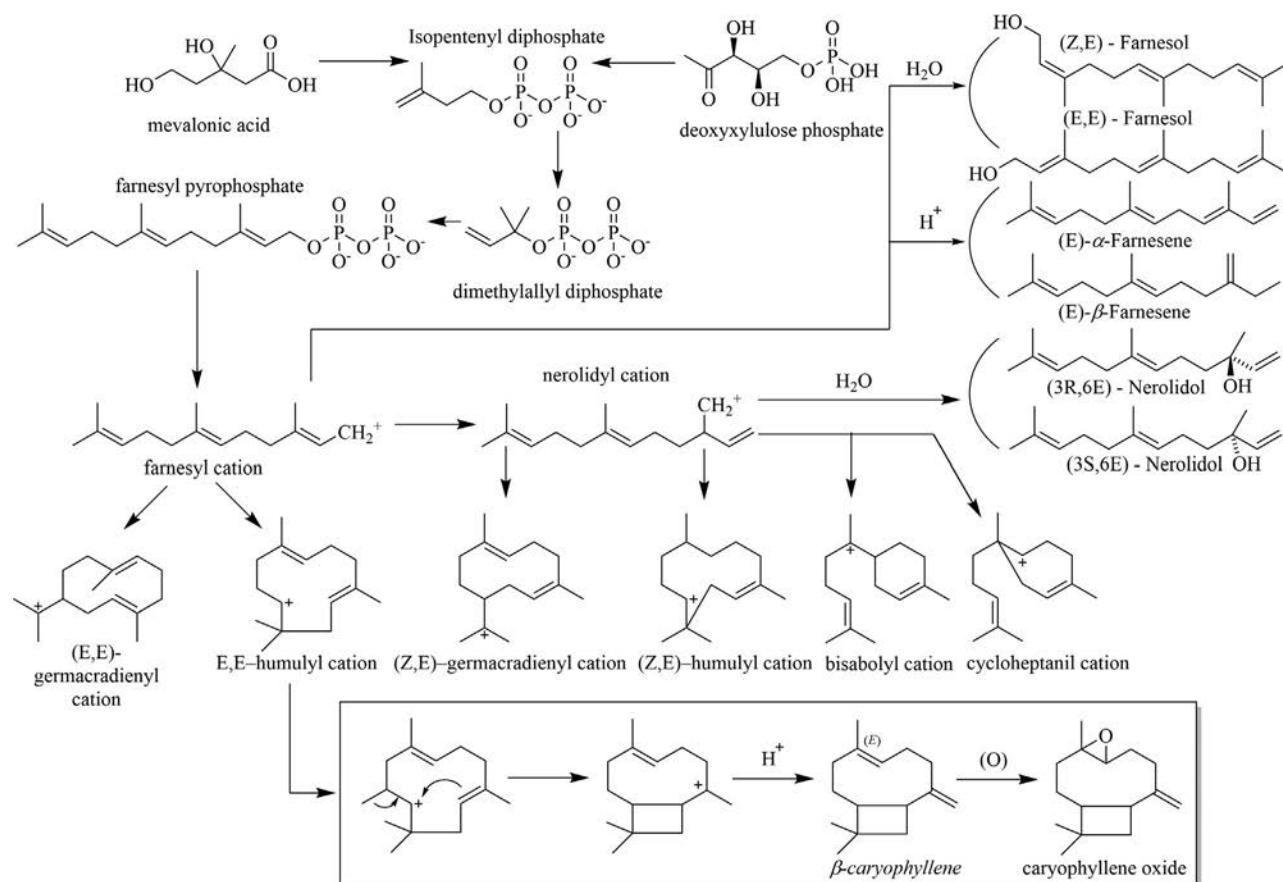
Being mainly hydrocarbons, terpenes ( $C_5H_8$ )<sub>n</sub> are widely found as constituents of essential oils and are classified according to the number of isoprene units into monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), triterpenes (6 units), and tetraterpenes (8 isoprene units). Specifically, sesquiterpenes are divided into groups when considering their chemical structure as acyclic or cyclic (Christianson, 2017; Liao et al., 2016).

The biosynthesis of sesquiterpenes can occur via either the mevalonic acid pathway or the deoxyxylulose phosphate (1-deoxy-D-xylulose-5-phosphate) pathway as highlighted in Fig. 1 (Chappell & Coates, 2010; Rohdich et al., 2003). The biochemically active compounds isopentenyl diphosphate and its isomer dimethylallyl diphosphate in both pathways undergo chemical combinations with the sesquiterpene precursor farnesyl pyrophosphate. Ionization at the second carbon of farnesyl pyrophosphate and stereochemical changes (E or Z configuration) of the double bond closest to the diphosphate result in the formation of farnesyl cations (E and Z) and the nerolidyl cation. Chemical modifications due to reduction, oxidation, and cyclization from the farnesyl and nerolidyl cations will form various acyclic and cyclic sesquiterpenes.

As highlighted earlier, sesquiterpenes comprise a subdivision of terpenes and can be found in linear, monocyclic, bicyclic, and tricyclic structure format (Table 1). The sesquiterpenes can be considered the most diversified group of terpenes and have broad-ranging pharmacological properties.

Among the sesquiterpenes that are formed according to Fig. 1 by cyclization of farnesyl pyrophosphate,  $\beta$ -caryophyllene can be highlighted, and it is among the main constituents found in plant foods such as oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum* spp.), pepper (*Piper nigrum* L.), and clove (*Eugenia caryophyllata*) (Buchbauer & Ilic, 2013; Gertsch et al., 2008). In addition,  $\beta$ -caryophyllene comprises one of the main active ingredients of essential oils from *Cannabis sativa*, *Ocimum gratissimum*, *Lantana camara*, *Cordia verbenaceae*, *Spiranthera odoratissima*, *Croton campestres*, *Vernonia cinerea* Less, *Pellia endiviifolia*, *Murraya paniculata* L., and several *Copaifera* L. species (Almeida et al., 2013; Galdino et al., 2012; Oliveira-Tintino et al., 2018; Selestino Neta et al., 2017).

This bicyclic sesquiterpene (synonyms: caryophyllene, *trans*-caryophyllene, (−)-*trans*-caryophyllene, (−)(E)-caryophyllene, (E)-(1R,9S)-(−)-Caryophyllene, and L-caryophyllene) is on the list of food additives and flavoring agents approved by the United States Food and Drug Administration (FDA, *Code of Federal Regulations*/no. 21CFR172.515), and

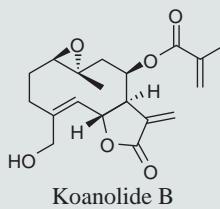
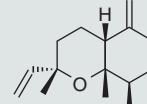
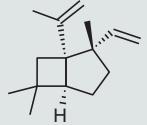
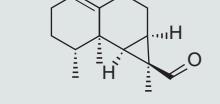


**FIG. 1** General pathway for sesquiterpene biosynthesis with formation of  $\beta$ -caryophyllene [(1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene].

**TABLE 1** Classification of sesquiterpenoids according to the functional groups/structure.

Type of sesquiterpene	Example/chemical structure	Reference
Acid		Otto et al. (2014)
Ketone		Bao et al. (2021)
Oxide		Novaković et al. (2019)

**TABLE 1** Classification of sesquiterpenoids according to the functional groups/structure—cont'd

Type of sesquiterpene	Example/chemical structure	Reference
Lactone	 Koanolide B	Castillo et al. (2022)
Ether	 Dactyloxene D	Ayyad et al. (1994)
Hydrocarbon	 Panaxene	Richter et al. (2005)
Aldehyde	 1(10)-Aristolen-13-AL	Rodríguez et al. (1995)

studies in animal models have demonstrated pharmacological activities that include the treatment of alcoholism (Al Mansouri et al., 2014), antiviral (Hassanin et al., 2020), antibacterial (Liu et al., 2021), antihyperglycemic (Basha & Sankaranarayanan, 2016), hypolipidemic effect (Baldissera et al., 2016), prevents nonalcoholic fatty liver disease (Kamikubo et al., 2016), neuroprotective (Assis et al., 2014), antidepressant, anxiolytic (Bahi et al., 2014), anticonvulsant (Liu et al., 2015), anticancer (Fidyt et al., 2016), analgesic (Quintans-Júnior et al., 2016), antinociceptive (Paula-Freire et al., 2014), antimelanogenic effect (Yang et al., 2015), antiinflammatory (Cho et al., 2015), and antioxidant activities (Ames-Sibin et al., 2018; Dahham et al., 2015) and acts as a selective agonist of the cannabinoid type 2 receptor (CB<sub>2</sub>) (Askari & Shafiee-Nick, 2019; Bento et al., 2011; Sharma et al., 2016). Due to its aromatic characteristics,  $\beta$ -caryophyllene can be used in cosmetics (Sköld et al., 2006).

Therefore, due to the pharmacological potential that has sparked increasing interest in the development of new research and few have addressed toxicological parameters, the aim of the present review study is to detail the *in silico*, *in vitro*, and *in vivo* toxicological activities of  $\beta$ -caryophyllene.

## Research strategy

This review was carried out through a comprehensive analysis of articles. The articles were searched in the following databases: ScienceDirect, MEDLINE/PubMed, Wiley Online Library Google Scholar, ACS Publications, and Web of Science. The descriptors used were “ $\beta$ -caryophyllene,” “ $\beta$ -caryophyllene and safety,” “ $\beta$ -caryophyllene and toxicology,” “ $\beta$ -caryophyllene and toxicology effect,” “ $\beta$ -caryophyllene and genotoxicity,” “ $\beta$ -caryophyllene and mutagenicity,” and all the references found during the conduction of this research were studied in detail; being considered valid the full-text articles of original research. We also manually searched the reference lists of the selected articles for additional studies. Articles published in English and Portuguese up to January 2022 were analyzed in this review. After the identification and selection of articles in all databases; they were imported into EndNote® X7 (Thomson Reuters; New York; USA) (Oliveira & de Freitas, 2015).

## Safety and toxicology

The present study organized the toxicological results of  $\beta$ -caryophyllene as follows:

### In silico toxicity studies

The result of the toxicity parameters predicted by the ProTox-II server ([https://tox-new.charite.de/protox\\_II/index.php?site=compound\\_input](https://tox-new.charite.de/protox_II/index.php?site=compound_input)) demonstrated that  $\beta$ -caryophyllene can be immunocytotoxic (the specific cell type is not specified in the model), and it is classified in category 5 ( $2000 < LD_{50} \leq 5000$ ) of the Globally Harmonized System of Classification (GHS). Differently, the toxicity result predicted by the AdmetSAR 2.0 server (<http://lmmecust.edu.cn/admetsar2>) resulted in category 3 (Table 2). It is highlighted that the in silico toxicity result of  $\beta$ -caryophyllene by the ProTox-II server conforms to the repeated dose toxicity studies of Oliveira et al. (2018), which can be classified in category 5 (toxicity greater than 2000 mg/kg body weight) of the Globally Harmonized System of Classification (GHS) of OECD guideline 423 (OECD, 2001). The in silico hepatotoxicity result is in accordance with an in vitro analysis developed by Kamikubo et al. (2016) that showed that treatment with  $\beta$ -caryophyllene (40  $\mu$ M) resulted in the absence of cytotoxicity against hepatocyte cell line (HepG2) over a 24 h period. This result was also observed in rodents as will be highlighted later in the single and repeated dose toxicity studies. In addition to the results described in Table 2 indicating no cytotoxicity,  $\beta$ -caryophyllene shows cytotoxic effect against B16F10 murine melanoma-derived cell lines ( $CI_{50} = 3.88 \mu\text{g/mL}$ ; 19  $\mu\text{moL/L}$ ) (Kubo et al., 1996).

**TABLE 2** In silico results of the toxicity of  $\beta$ -caryophyllene.

Parameters/target	In silico toxicity studies			
	ProTox-II		AdmetSAR 2.0	
Prediction	Probability	Prediction	Probability	
Hepatotoxicity	Inactive	0.80	Inactive	0.77
Carcinogenicity	Inactive	0.70	Inactive	0.75
Immunotoxicity	Active	0.54	N.C.	–
Mutagenicity	Inactive	0.95	N.C.	–
Ames mutagenesis	N.C.	–	Inactive	0.99
Cytotoxicity	Inactive	0.75	N.C.	–
Respiratory toxicity	N.C.	–	Inactive	0.51
Reproductive toxicity	N.C.	–	Inactive	0.78
Mitochondrial toxicity	N.C.	–	Inactive	0.51
Crustacea aquatic toxicity	N.C.	–	Active	0.73
Fish aquatic toxicity	N.C.	–	Active	0.99
Honey bee toxicity	N.C.	–	Inactive	0.89
Acute oral toxicity (c)	N.C.	–	3 <sup>b</sup>	0.82
Predicted LD <sub>50</sub> <sup>a</sup>	5300 mg/kg	–	N.C.	–
Predicted toxicity class	5	–	3	0.82

N.C. (Not Calculated).

<sup>a</sup>Prediction accuracy: 70.97%.

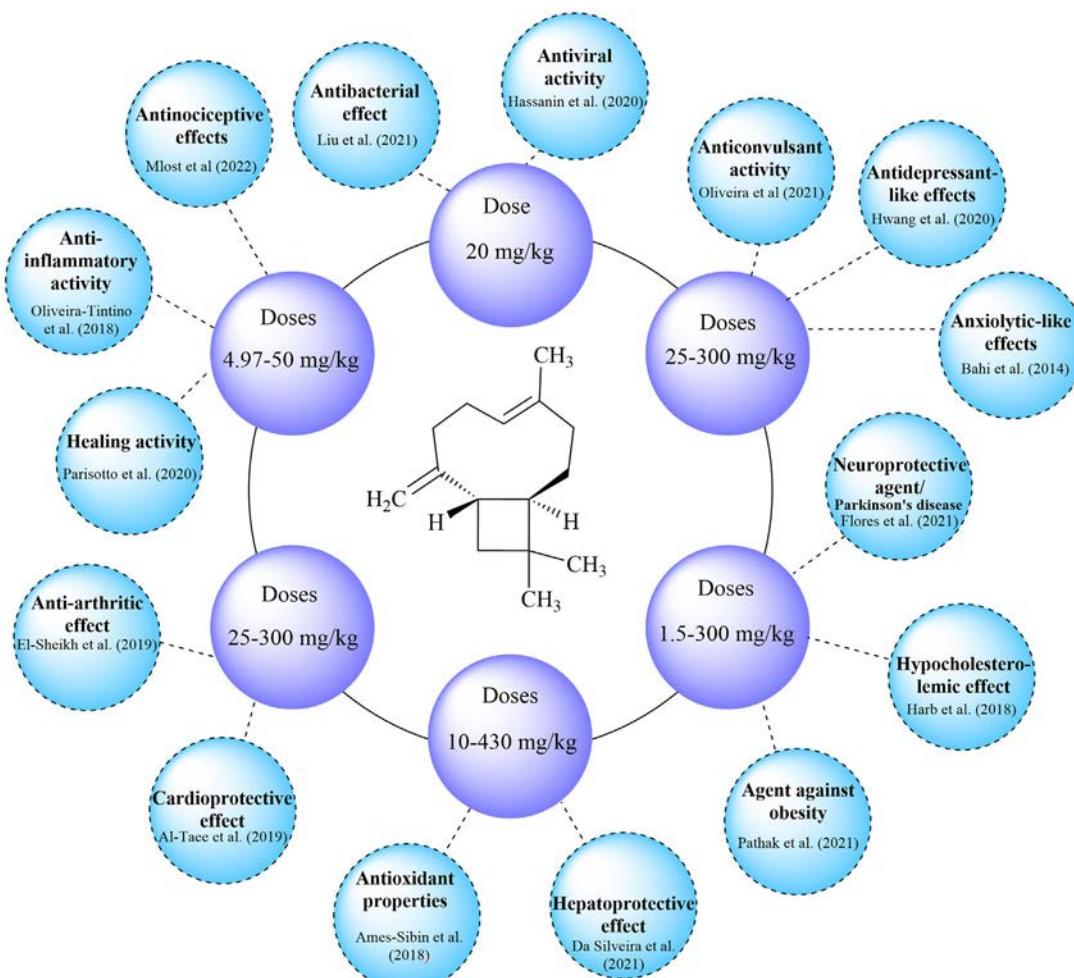
<sup>b</sup>AdmetSAR (Category III includes compounds with LD<sub>50</sub> values greater than 500 mg/kg but less than 5000 mg/kg).

## General toxicology evaluation

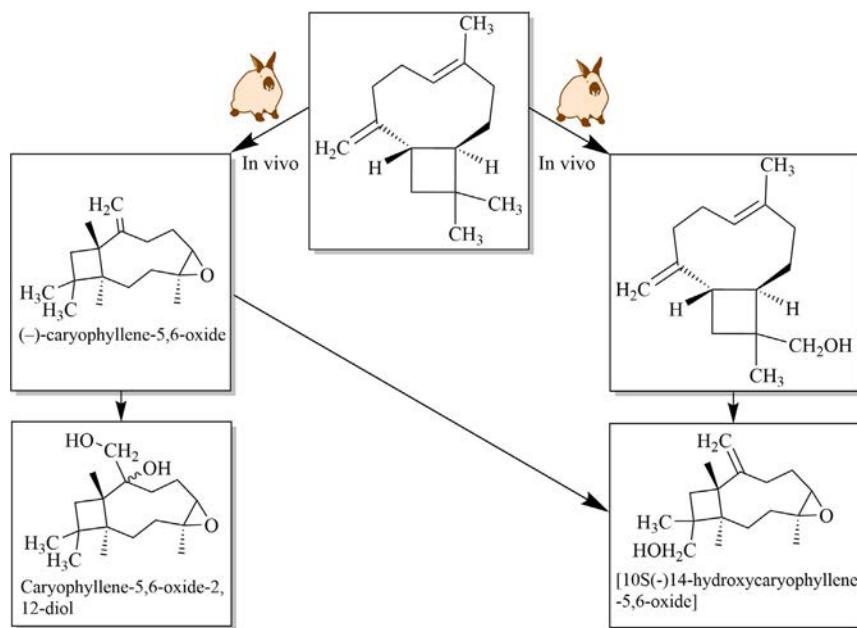
Human exposure to  $\beta$ -caryophyllene can be common by ingestion of different plant/spice foods or by inhalation because it is naturally present in the atmosphere (ca. 5–7 Tg year<sup>-1</sup>) (Maclean et al., 2021). There is still an expansion of pharmacological studies by various research groups and few have addressed toxicological parameters in animal models. As a compound with a wide range of therapeutic potential, treatment in animal models with  $\beta$ -caryophyllene spans a range of doses and time in which clinical toxicity effects are little discussed (Fig. 2). It is estimated that the daily human intake of  $\beta$ -caryophyllene can be between 10 and 200 mg, which influences the modulation of the various pharmacological responses described so far in in vitro and in vivo studies (Gertsch et al., 2008).

The in vivo metabolism or biotransformation of  $\beta$ -caryophyllene is still little discussed in the scientific literature. Of the few studies, Asakawa et al. (1986) demonstrated in rabbits that  $\beta$ -caryophyllene can be converted into an intermediate metabolite (−)-caryophyllene-5,6-oxide that undergoes metabolism into another compound named [10S-(−)-14-hydroxy-caryophyllene-5,6-oxide] (80%) or caryophyllene-5,6-oxide-2,12-diol (Fig. 3). It can be pointed out that the products resulting from metabolism or biotransformation are not considered toxic in animals as can be suggested by the toxicological studies conducted by Hart and Wong (1971), Oliveira et al. (2018), Molina-Jasso et al. (2009), and Bastaki et al. (2020).

The constant development of research to clarify the toxicological profile of  $\beta$ -caryophyllene is of great importance. For example, the acute (14-day) repeated-dose (28-day) toxicity study by Oliveira et al. (2018) reported that Hypocratic Screening for animal evaluation after administration of the 300 and 2000 mg/kg doses of  $\beta$ -caryophyllene orally did not induce any adverse effects such as ptosis, abdominal contortions, straightening reflex, irritability, piloerection, tremors,



**FIG. 2** Representation of the doses that are widely used in various pharmacological studies with  $\beta$ -caryophyllene.



**FIG. 3** Biotransformation of  $\beta$ -caryophyllene in rabbits (Asakawa et al., 1986).

and convulsions. In the same study, no death was recorded in the single dose and repeated dose toxicity study during the exposure period with  $\beta$ -caryophyllene.

Thus, it can be highlighted that the toxicity of  $\beta$ -caryophyllene can be classified in the category (5toxicity greater than 2000mg/kg body weight) of the Globally Harmonized System of Classification (GHS) of OECD guideline 423 (OECD, 2001). It is worth noting that the study by Oliveira et al. (2018) was only with female Swiss mice, which is generally considered sufficient for preliminary animal toxicity study as indicated in OECD guideline 423. Among the first toxicity studies in animal model, Hart and Wong (1971) pointed out that the acute toxicity of  $\beta$ -caryophyllene orally *in rats* resulted in the mean lethal dose ( $LD_{50}$ ) greater than 5000mg/kg as already evidenced by the study of Molina-Jasso et al. (2009) and *in silico* (Table 2).

Similarities between toxicological results from different authors are observed in the scientific literature. For example, the repeated dose toxicity results of Oliveira et al. (2018) are in agreement with the subchronic toxicological evaluation (90 days) performed by Schmitt et al. (2016) that reported the lack of symptoms indicative of toxicity and death in *Wistar* rats treated orally with  $\beta$ -caryophyllene at doses of 150, 450, and 700mg/kg/day. In the study determining  $LD_{50}$  in 6-week-old male mice (NIH), Molina-Jasso et al. (2009) highlighted the absence of signs of toxicity after oral administration of up to 5000mg/kg  $\beta$ -caryophyllene.

Another study worth mentioning was conducted by Bastaki et al. (2020), as it was shown that dietary administration of  $\beta$ -caryophyllene did not result in deaths or clinical manifestation indicative of toxicity in male (516.4, 1546.8, or 3568.9 mg/kg) and female (528.2, 1582, or 4438.5 mg/kg) of Sprague–Dawley strain rats. Additionally,  $\beta$ -caryophyllene does not induce body weight changes in rodents subjected to the single and repeated dose toxicity studies as observed in the works conducted by Oliveira et al. (2018) and Schmitt et al. (2016) at doses up to 2000mg/kg. Already high doses of  $\beta$ -caryophyllene such as 3568.9 and 4438.5mg/kg when administered in Sprague–Dawley rats can induce metabolic adverse effects resulting in significant body weight loss (Bastaki et al., 2020).

Despite this result, the doses of  $\beta$ -caryophyllene that are widely used in pharmacological studies as shown in Fig. 2 are well below the highest dose in the study by Bastaki et al. (2020). For example, the study by Al-Taei et al. (2019) demonstrated that the body weight loss of male *Wistar* albino rats treated with doxorubicin (12.5mg/kg, single dose, i.p.) was significantly reversed after 5-day treatment with  $\beta$ -caryophyllene (25 and 50mg/kg, i.p.). Cho et al. (2007) pointed out that oral administration of  $\beta$ -caryophyllene (300mg/kg) dissolved in olive oil once daily for 1 week reduced weight loss in Balb/c strain mice with DSS (Dextran Sulfate Sodium)-induced colitis.

One of the parameters that can be evaluated in rodents in acute and repeated dose toxicity studies is neurotoxicity, which involves one of the most complex areas of toxicology. Related to this topic, there are studies that highlight the absence of neurotoxicity of  $\beta$ -caryophyllene (300 and 2000mg/kg) in orally treated female *Swiss* mice by not compromising

spontaneous exploratory activity (open field test) in acute and repeated dose toxicity studies. Additionally, it does not induce muscle relaxation when compared with the control group for acute and repeated dose toxicity (Oliveira et al., 2018). Applying the open field test, the absence of neurotoxicity at lower doses than used by Oliveira et al. (2018) was verified in studies where  $\beta$ -caryophyllene ameliorated metabolic and psychological changes induced by the HFFD (high fat/fructose diet) [30mg/kg (Youssef et al., 2019)], showed anticonvulsant [100mg/kg (Liu et al., 2015; Oliveira et al., 2021)], antinociceptive [20, 40 and 80mg/kg (Paula-Freire et al., 2014)], antidepressant, and anxiolytic [50mg/kg (Bahi et al., 2014; Oliveira et al., 2021)] activity in rodents.

Considering the ability of  $\beta$ -caryophyllene to be distributed in the brain through the blood circulation when inhaled (Takemoto et al., 2021), the low risk of adverse effects of  $\beta$ -caryophyllene on the central nervous system is highlighted. In addition to not exhibiting neurotoxicity, it is relevant to highlight that  $\beta$ -caryophyllene administered intraperitoneally (50mg/kg BW once daily for 4 weeks) has the ability to inhibit rotenone-induced neurotoxicity in male Wistar rats (*Rattus norvegicus*) as highlighted in the results by reversing glutathione depletion and lipid peroxidation, restore the activities of antioxidant enzymes (SOD and catalase), and block the stimulation of release of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) and mediators of inflammation (NF- $\kappa$ B, COX-2, and iNOS). It also has the ability to inhibit the death of dopaminergic neurons of the substantia nigra pars compacta (SNpc) and dopaminergic fibers, reduced activation of astrocyte cells and microglia as demonstrated by decreased expression of GFAP and Iba-1, respectively (Javed et al., 2016). In this same study, the ability to inhibit neurotoxicity may be related to the antiinflammatory and antioxidant properties dependent on CB<sub>2</sub> receptor activation, as the observed pharmacological activities were blocked by pretreatment with AM630. Similarity in the results of inhibiting 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP)-induced neurotoxicity in male C57BL/6J mice was also demonstrated with repeated administration (10mg/kg BW for 5 days) of  $\beta$ -caryophyllene (Viveros-Paredes et al., 2017).

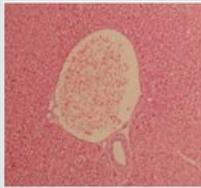
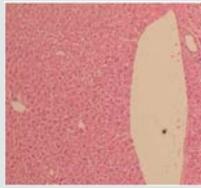
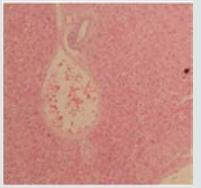
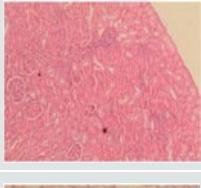
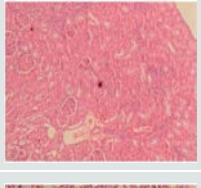
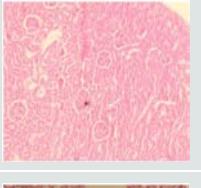
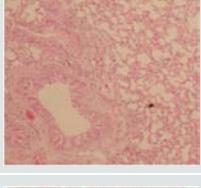
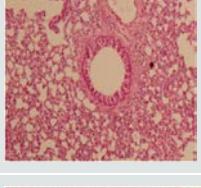
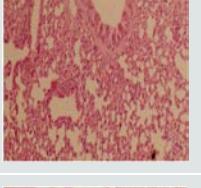
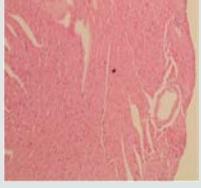
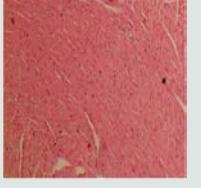
As shown in Fig. 2, the pharmacological activities of  $\beta$ -caryophyllene in animal models may be mainly related to organs such as the brain (Bahi et al., 2014; Lou et al., 2016; Tian et al., 2016), liver, and kidney (Baldissera et al., 2016; Calleja et al., 2013; Kamikubo et al., 2016). Thus, it is important that  $\beta$ -caryophyllene does not impair the proper metabolic functioning of these organs. Considering the liver and kidney, a study by Oliveira et al. (2018) showed that the variations in the values of serum biochemical indicators of liver function (ALT and AST) and kidney function (urea and creatinine) were not significant in female animals treated with  $\beta$ -caryophyllene in the acute toxicity study (14 days) and in repeated doses (28 days). Whereas, the sub-chronic toxicity result in *Wistar* rats of both sexes obtained by Schmitt et al. (2016) demonstrated significant reduction of biochemical parameters such as ALT, AST, and urea, but that was not considered an indication of hepatotoxicity and nephrotoxicity associated with the 90-day treatment.

In a complementary way, the histopathological analysis of the study of Oliveira et al. (2018) highlighted in Table 3 resulted in the absence of hepatotoxicity and nephrotoxicity for the dose of up to 2000mg/kg. Whereas in the study by Bastaki et al. (2020), it was reported that the changes in liver function indices and renal function are related to some renal/hepatic pathology in male (1546.8 or 3568.9 mg/kg) and female (1582 or 4438.5 mg/kg) Sprague–Dawley strain rats in the repeated dose (90 days) toxicity study. The increase in mean liver weight of male (3568.9 mg/kg) and female (1582, 4438.5 mg/kg) rats is another observation in the study by Bastaki et al. (2020) that may be related to the increase in hepatocyte area that probably stems from the swelling of these cells. In this same study, it was important to have performed the determination of oxidative biomarkers (catalase and superoxide dismutase, lipid peroxidation, nitrite, and reduced glutathione) in the livers of kidneys of the animals treated with the higher doses, since the administration of the established doses of  $\beta$ -caryophyllene in animals of both sexes may have resulted in oxidative injury in the liver and kidneys that induced hepatotoxicity and nephrotoxicity.

Additionally, hepatotoxicity in rodents is usually accompanied by disturbances in lipid and glucose metabolism, as the loss of balance of hepatic glucose and lipid metabolism leads to triglyceride accumulation in the liver and elevated carbohydrate concentration such as glucose in the blood, which is possible or suggestible as per the changes observed in the biochemical analyses by Bastaki et al. (2020). In the analysis of the study performed by Ames-Sibin et al. (2018), treatment with  $\beta$ -caryophyllene (gavage, 215 and 430mg/kg) was not related to liver pathology and still showed pharmacological activity by reducing systemic inflammation and oxidative stress in liver and plasma of rats with adjuvant-induced arthritis.

With similar in vitro result already discussed above, Kamikubo et al. (2016) highlighted the absence of hepatotoxicity and further reported that treatment with  $\beta$ -caryophyllene (5–10 $\mu$ M) by CB type 2 receptor activation significantly reversed the increase in lactate dehydrogenase (LDH) and AST activity that was induced by palmitate (0.5 mM) in HepG2 cells. Additionally,  $\beta$ -caryophyllene dissolved in olive oil (>99% pure/1, 3, and 10mg/kg, i.p) attenuated cisplatin-induced nephrotoxicity in male C57BL/6J mice by decreasing renal proinflammatory cytokine expression, apoptosis, tubular necrosis, oxidative stress, and preserving renal morphology via CB activation (Horváth et al., 2012). Also considering the various organs in rodents, in histopathological studies of animals treated with  $\beta$ -caryophyllene (300 and 2000mg/kg) after the

**TABLE 3** Histopathological analysis of organs treated with  $\beta$ -caryophyllene in the repeated-dose toxicity (H and E  $\times 100$ ).

Organs	Histopathology evaluation		
	CN	$\beta$ -CFN <sub>300</sub>	$\beta$ -CFN <sub>2000</sub>
Liver			
Kidney			
Lung			
Heart			

(CN) Control (vehicle), ( $\beta$ -CFN300) dose of 300 mg/kg, ( $\beta$ -CFN2000) dose of 2000 mg/kg. Scale bar = 50  $\mu$ m. Authorized by Oliveira et al. (2018).

repeated dose toxicity study, Oliveira et al. (2018) detected no pathological indications in the brain, heart, and lung of female Swiss mice when compared with the control groups (Table 3).  $\beta$ -Caryophyllene shows no toxicity to heart cells of male Wistar albino rats treated with the dose of 100 mg/kg (i.p.) for 5 days according to the histological observation of intact muscle fibers without degradation.

The induction of oxidative stress can be considered a possible pathway for toxicity triggered by compounds such as sesquiterpenes and is therefore suggested to be evaluated in a variety of toxicological studies in vitro and in vivo. In this context, the oxidative stress analysis performed by Oliveira et al. (2018) in the livers and kidneys of female Swiss mice subjected to the repeated dose toxicity study demonstrated no significant changes in oxidative biomarkers (catalase and superoxide dismutase, lipid peroxidation, nitrite, and reduced glutathione) related to the administration of the doses of  $\beta$ -caryophyllene (300 and 2000 mg/kg). Besides not compromising the antioxidant system in the liver and kidney of female Swiss mice,  $\beta$ -caryophyllene (25 and 50 mg/kg, i.p.) reduces oxidative stress in the heart of male Wistar albino rats subjected to cardiotoxicity with doxorubicin (12.5 mg/kg, single dose, i.p.) through restoration of catalase and superoxide dismutase enzymes, increase in reduced glutathione, and inhibition of lipid peroxidation (Al-Taee et al., 2019; Meeran et al., 2019). Possibly, the cardioprotective effect  $\beta$ -caryophyllene shows association with CB<sub>2</sub> receptor activation, since the observed pharmacological activities were blocked by pretreatment with AM630 (1 mg/kg) (Meeran et al., 2019).

The study by Meeran et al. (2021) also highlights the importance of CB<sub>2</sub> receptor activation in the cardioprotective effect of  $\beta$ -caryophyllene by reducing oxidative stress via modulation of PI3K/Akt/Nrf2 signaling was reversed by pre-treatment with AM630. The ability of  $\beta$ -caryophyllene to restore the antioxidant defense system has been demonstrated

in other organs such as the brain [(10 mg/kg/orally, Flores-Soto et al., 2021)], liver [(100 mg/kg, i.p., Da Silveira et al., 2021)], and pancreas [(200 mg/kg/intragastric, Basha & Sankaranarayanan, 2016)].

## Mutagenicity/genotoxicity studies

The genotoxicity/mutagenicity study of a given substance is a mandatory procedure due to the relevance for determining the risk related to human health. Whereas genotoxicity is related to the ability of a given compound to damage genetic material that may result in mutations, treatment with  $\beta$ -caryophyllene is not known to cause structural damage to DNA that might result in interference with the machinery of cell division, abnormal chromosome segregation, and numerical chromosomal changes.

The absence of cytotoxicity and in vitro genotoxic effects of  $\beta$ -caryophyllene (100  $\mu$ g/mL/purity  $\geq$ 98.5%) by determination of nuclear division index (NDI) and micronucleus frequency, respectively, was reported by Di Sotto et al. (2010) in human peripheral lymphocytes. However, considering that NDI provides a parameter of the proliferative state of viable cells and can be used as an indicator of cytotoxicity, Di Sotto et al. (2010) also demonstrated that  $\beta$ -caryophyllene at 200  $\mu$ g/mL can result in cytotoxicity, since nucleation index was significantly reduced. Another study by the same author highlighted that  $\beta$ -caryophyllene (100  $\mu$ g/mL/purity  $\geq$ 98.5%) does not induce positive mutagenic response in *Salmonella typhimurium* TA 98 and TA 100 (Di Sotto et al., 2008), as demonstrated by means of *in silico* study conducted by AdmetSAR 2.0 (Table 2). It is worth noting that the study by Di Sotto et al. (2008) is not fully in accordance with current guidelines as it did not include a bacterial test strain to detect mutagens that act specifically on AT base pairs.

Additionally, the study by Alvarez-González et al. (2014) verified antioxidant and antigenotoxic activity of  $\beta$ -caryophyllene on the damage induced by the environmental contaminant benzo(*a*)pyrene (200 mg/kg, i.p.) in male Swiss Webster mice. The study was developed with doses of 20, 200, and 2000 mg/kg (i.p.) and demonstrated absence of cytotoxicity by not compromising the mitotic index, absence of genotoxicity by not resulting in significant chromosomal aberrations and also showed an inhibitory action of more than 50% on the frequency of sister chromatid exchanges (SCE) and chromosomal aberrations induced by benzo(*a*)pyrene. In the study performed by Molina-Jasso et al. (2009),  $\beta$ -caryophyllene (97% pure) within 96 h after oral treatment at doses of 20, 200, and 2000 mg/kg in mice NIH (National Institutes of Health) resulted in no effect on clastogenic activity indices in the micronucleus test.

## Conclusion

An increase in the number of experimental studies with  $\beta$ -caryophyllene has been observed, mainly because it acts as a selective agonist of the cannabinoid receptor type 2. However, it is pointed out that the toxicological profile of  $\beta$ -caryophyllene is not fully known, and therefore, further studies are needed, including those related to its genotoxic potential. The toxicity studies by various routes of administration of  $\beta$ -caryophyllene in animals are mainly in rodents, and the currently available toxicological information shows no evidence that it is neurotoxic, hepatotoxic, cardiotoxic, nephrotoxic, and genotoxic, mutagenic or shows evidence of toxicity such as animal death at the doses that are evaluated (30–5000 mg/kg). Considering that the doses widely used in pharmacological studies hardly exceed 300 mg/kg, which is well below the doses that are used in toxicological studies,  $\beta$ -caryophyllene can be considered safe when considering rodent, *in vitro* and *in silico* studies.

## Key facts of $\beta$ -caryophyllene

- $\beta$ -Caryophyllene is a food additive
- $\beta$ -Caryophyllene is a bicyclic sesquiterpene
- $\beta$ -Caryophyllene has low solubility in water
- Acts as a selective agonist of the cannabinoid type 2 receptor (CB<sub>2</sub>)
- Has broad-ranging pharmacological potential

## Mini-dictionary of terms

- AdmetSAR 2.0—web service for prediction and optimization of chemical ADMET properties
- Oxidative stress—excess production of reactive oxygen species (ROS) relative to antioxidant defense
- ProTox-II—a virtual lab for the prediction of toxicities of small molecules
- Sesquiterpene—are C15-terpenoids built from three isoprene units.

## Summary points

- This chapter focused on the in silico, in vitro, and in vivo toxicological activities of  $\beta$ -caryophyllene
- The in silico study demonstrated that  $\beta$ -caryophyllene can be immunocytotoxic.
- $\beta$ -Caryophyllene can be considered safe when considering rodent, in vitro, and in silico studies.

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## Chapter 38

# Synthetic cannabinoid receptor agonists: An overview

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## Abbreviations

CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBD	cannabidiol
CNS	central nervous system
<b>μM</b>	micromolar
<b>nM</b>	nanomolar
PET	positron emission tomography
SCRA(s)	synthetic cannabinoid receptor agonist(s)
THC	( <i>–</i> )- <i>trans</i> -Δ <sup>9</sup> -tetrahydrocannabinol

## Introduction

The cannabinoid system has been of medical interest even before the identification of its two receptors in humans, simply named cannabinoid receptor 1 (CB1) and 2 (CB2). The cannabinoid system is involved in a number of physiological processes including memory, appetite, pain, and psychological conditions (Zou & Kumar, 2018) and has been implicated in various disease states (Lu & Anderson, 2017). Compounds that target the cannabinoid receptors have been created with the intention of treating nausea and pain, as well as alleviating symptoms of epilepsy, Parkinson's disease, and Alzheimer's disease (Kendall & Yudowski, 2017; Maccarrone et al., 2011). However, the recreational use of marijuana, the main constituent of which is (*–*)-*trans*-Δ<sup>9</sup>-tetrahydrocannabinol (Δ<sup>9</sup>-THC, **1**) and the compound associated with "getting high," has also driven interest in generating compounds that target the cannabinoid system. As a result, a vast number of chemically diverse compounds, called synthetic cannabinoid receptor agonists (SCRAs), have been generated that show varied behavior at the CB1 and CB2 receptor as well as other off-site receptors.

SCRAs are synthetically derived molecules that act as agonists at the CB1 and/or CB2 receptors. Historically, SCRAs have gone by many names, including synthetic cannabinoid, cannabimimetic, and synthetic marijuana and sold under brand names such as Spice or K2. However, scientifically, the SCRA terminology is best used to describe these compounds to distinguish them from their natural counterparts and the significantly different pharmacology that they exhibit (Darke et al., 2021). Given the definition of SCRAs, this chapter will focus on compounds that do not have their origins in natural systems, whether endocannabinoids or phytocannabinoids from the cannabis plant. Δ<sup>9</sup>-THC (**1**), Δ<sup>8</sup>-THC (**2**), and cannabidiol (CBD, **3**) have multiple reported procedures for their synthetic production (for a recent review see (Reekie et al., 2017)) and show some agonist activity at CB1 or CB2, but since their structure originated from *Cannabis sativa* and their main source of supply is still from plant material, they are not considered SCRAs. When produced in the laboratory, compounds such as THC or CBD are better classified as synthetic phytocannabinoids, rather than simply phytocannabinoids when extracted, or used directly from plant material and differ from SCRAs that can only come from human synthesis.

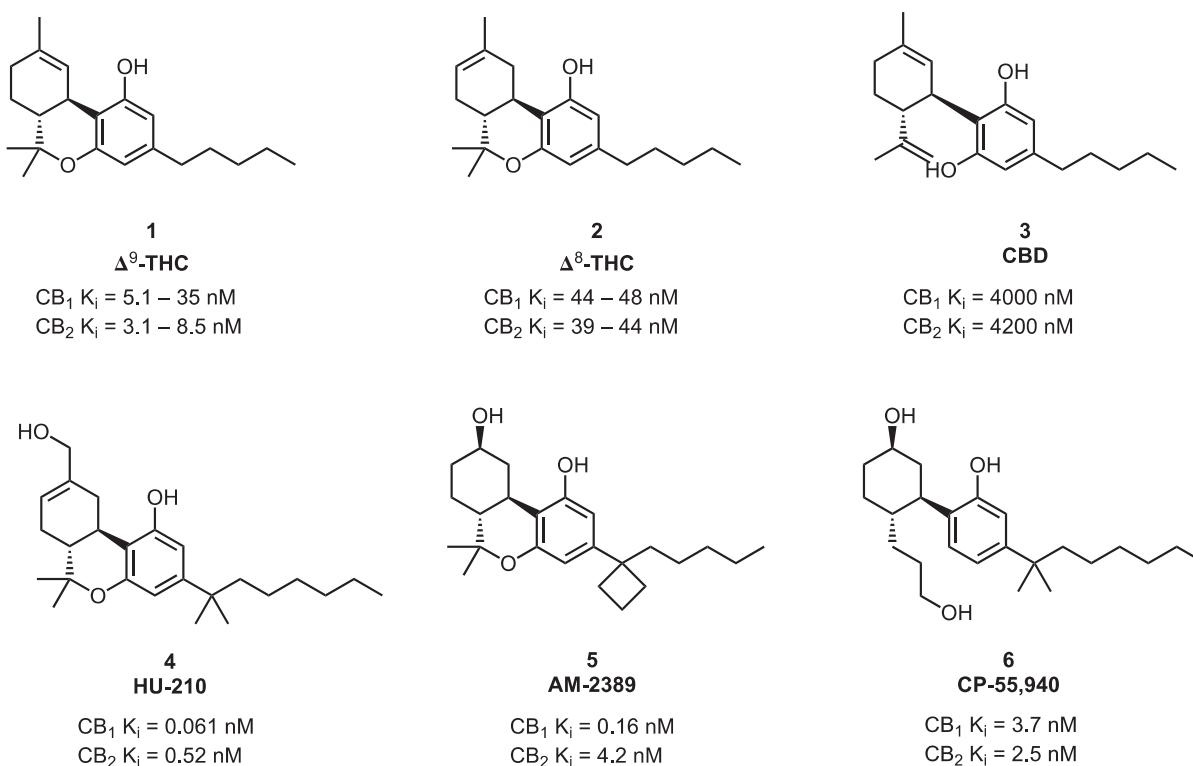
Originally produced through academic research, SCRAs gained prominence through their synthesis in clandestine labs and being sold as a legal high, not being the subject of regulation like marijuana was. These alternatives to marijuana came

in 2004, where an herbal mixture was showing cannabis-like effects, though this was later identified as innocuous plant material treated with one or multiple SCRAs. As regulations of particular SCRAs were implemented and increased, new structures reached the market as a way of staying ahead of law enforcement. While structures differed, the general principle was the same. Herbal material was used as a carrier that matched the appearance of marijuana, but was treated with one or more SCRAs to give the desired psychoactive effect. While most commonly smoked, other routes of administration often used for marijuana could also be used. In the years 2008–20, over 200 different SCRAs have been notified to the European Union Early Warning System from the illicit/non-pharmaceutical drug market ([European Monitoring Centre for Drugs and Drug Addiction, 2021](#)), with additional structures being reported in the academic literature. This chapter will explore the key structural features of SCRAs and examine their pharmacological profile at the CB1 and CB2 receptors.

## Phytocannabinoid-like structures

Given that the structure of  $\Delta^9$ -THC (**1**), the double-bond isomer  $\Delta^8$ -THC (**2**) and related phytocannabinoid CBD (**3**) had already been elucidated, it is of no surprise that some of the first SCRAs produced incorporated some of the key functionality seen with phytocannabinoids. Selected examples are shown in [Fig. 1](#). The phytocannabinoids **1–3** are considered partial agonists of the CB1 and CB2 receptors with varied activities at these receptors. For example,  $\Delta^9$ -THC (**1**), the main compound responsible for marijuana's high, is much more active at the cannabinoid receptors than CBD (**3**), a compound often touted as being responsible for a number of the health benefits of the plant ([Rapin et al., 2021](#)). In comparison, the synthetically derived analogs are often much better at interacting with the cannabinoid receptors. The HU series (developed by the Raphael Mechoulam group at the Hebrew University, representative example **HU-210** (**4**)), the AM series (developed by the Alexandros Makriyannis group at Northeastern University, representative example **AM-2389** (**5**)), and the CP series (developed by Pfizer, representative example **CP-55,940** (**6**)) all have structures that mimic the naturally occurring phytocannabinoids ([Fig. 1](#)).

**HU-210** (**4**) has a number of similarities with  $\Delta^9$ -THC (**1**) differing only with hydroxylation of the top carbon atom and changes to the aliphatic tail by extending the chain and including two methyl groups adjacent to the aromatic ([Mechoulam et al., 1990](#)). Interestingly, the C9 hydroxylation mimics the *in vivo* metabolism profile of **1** ([Aizpurua-Olaizola et al., 2017](#)).



**FIG. 1** Key phytocannabinoids and synthetic cannabinoid receptor agonists based on the phytocannabinoid structure.

**HU-210 (4)** shows significant binding at CB1 ( $K_i = 0.061\text{ nM}$ ), which is significantly better than  $\Delta^9\text{-THC}$  (1), as is the binding for CB2 ( $K_i = 0.52\text{ nM}$ ) (Devane et al., 1992; Stern & Lambert, 2007).

**AM-2389 (5)** has structural changes further differentiating it from phytocannabinoids. These include incorporation of a cyclobutyl group adjacent to the aromatic (cf. dimethyl) (Papahatjis et al., 2007) and inclusion of a hydroxyl group rather than methyl at the C9 position (Nikas et al., 2010). This necessitates removal of the double bond and therefore the addition of another stereocenter. **AM-2389 (5)** shows good binding at both CB1 and CB2 receptors, 0.16nM and 4.2nM, respectively (Järbe et al., 2012; Nikas et al., 2010).

**CP-55,940 (6)** has features of both 4 and 5 including the dimethyl adjacent to the aromatic and hydroxyl incorporation at C9 and double bond removal. However, it has further modifications to the southern portion of the molecule where the third ring has been opened to result in a hydroxybutyl chain (Devane et al., 1988). **CP-55,940 (6)** has good binding at both CB1 and CB2 receptors, 3.7nM and 2.5nM, respectively. As a result of this good and almost equal binding, it is often used as a comparative compound in functional assays for both receptors (Longworth et al., 2017).

While this section has only highlighted a few select phytocannabinoid-based synthetic compounds, there are a number of related compounds reported, these are covered by a broad review on the topic (Stern & Lambert, 2007). However, their more complex structures including multiple sites of chirality means they have been usurped in a lot of ways by structurally simpler SCAs detailed in the following sections.

## Indole-based structures—Historic development

Moving away from complex SCAs, the major chemotype in this area is a substituted indole core, the general form is represented by structure 7 (Fig. 2). The nitrogen atom of the indole is normally alkylated (shown in blue), with a 5-carbon chain being the most common, mimicking the tail seen in  $\Delta^9\text{-THC}$  (1) and other phytocannabinoids. Though cyclic and halogen substituted chains can also be incorporated while maintaining cannabinoid receptor activity. The 3-position of the indole is then substituted through a linking group (shown in green), which mostly incorporates a carbonyl functionality, though the exact form, ketone, amide or ester, does not seem to matter for cannabinoid receptor activity. The 3-position is linked to a hydrophobic group (shown in red) with huge variation in what can actually be incorporated there while still maintaining cannabinoid activity. A select number of SCAs that are based on the indole moiety will now be discussed to give a broad representation of this family of compounds.

Identification of the indole core as an active moiety at the cannabinoid receptors came about from investigation of the analgesic pravadolone (8) (Haubrich et al., 1990). Eventually, the in vivo response from pravaoldine was shown to be acting in part through activation of cannabinoid receptors (Bell et al., 1991). Further investigation of this structure led to **WIN 55,212-2 (9)** the first purposely designed synthetic cannabinoid structurally distinct from phytocannabinoids (Compton et al., 1992). However, this compound was still relatively structurally complex, containing multiple rings and a stereogenic center. The structure-activity relationship studies performed by Hoffman and coworkers simplified the pharmacophore required for cannabinoid activity leading to a number of active compounds of which **JWH-018 (10)** is the most significant (Wiley et al., 1998). With its structural simplicity and good binding at both cannabinoid receptors (CB1  $K_i = 9.0\text{nM}$ , CB2  $K_i = 2.9\text{nM}$ ) (Aung et al., 2000), **JWH-018 (10)** is often considered the compound that really initiated this area of research. Concurrent studies showed that variation in alkyl chain length maintained similar activity, by shortening by one methylene group (11, CB1  $K_i = 8.9\text{nM}$ , CB2  $K_i = 38.0\text{nM}$ ) or by lengthening by one methylene group (12, CB1  $K_i = 9.8\text{nM}$ , CB2  $K_i = 5.55\text{nM}$ ) (Aung et al., 2000). While a significant research breakthrough, **JWH-018 (10)** soon gained notoriety when it was detected in samples of Spice, sold as synthetic marijuana (Lindigkeit et al., 2009). Following from this, authorities listed **JWH-018** as a controlled substance, and within a month of this ban, it was quickly replaced by compound 11 as the active ingredient in Spice (Lindigkeit et al., 2009). These events led to a surge in new compounds that activated the cannabinoid receptors as clandestine labs generated new compounds to circumvent new regulations. Authorities were also concurrently identifying and testing the new active compounds to control their use while academic and industrial researchers pursued SCAs as potential pharmaceuticals to treat varied disease states.

## Alkyl chain variation

As discussed, variation on alkyl length maintains cannabinoid receptor activity (compounds 10–12) (Aung et al., 2000; Wiley et al., 1998), but to further probe the structure activity relationship of this site and avoid detection from authorities, a number of modifications have been made. One common alteration is the isosteric replacement of a hydrogen atom with a fluorine atom, for example, compound 13, which showed enhanced binding at the CB1 receptor ( $K_i = 1.0\text{nM}$ ) (Makriyannis & Deng, 2001; Wilkinson et al., 2015). The fluorine handle also has potential applications for positron

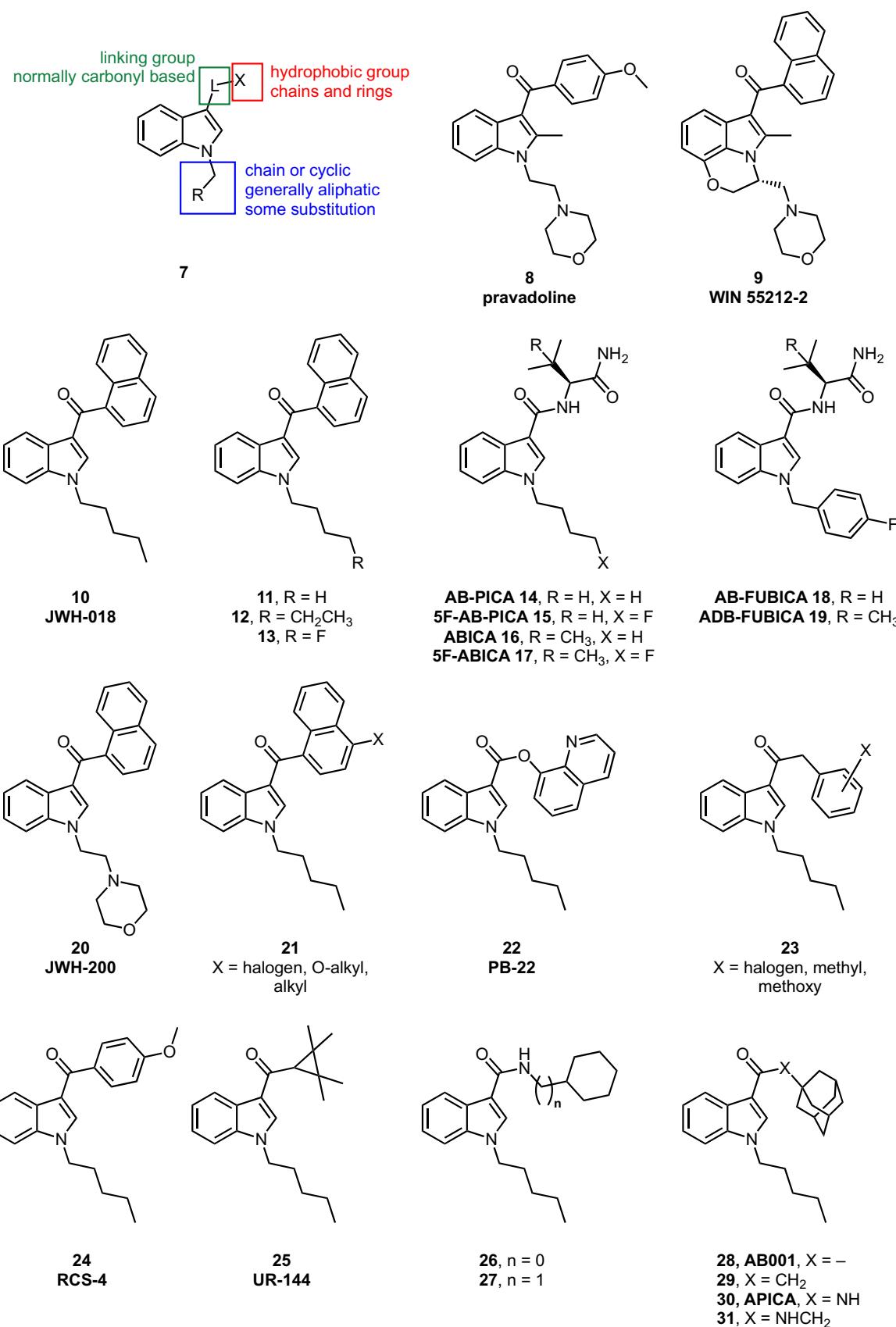


FIG. 2 Indole-based synthetic cannabinoid receptor agonists.

emission tomography (PET) to image the cannabinoid receptors. The 5-fluoropentyl chain has been incorporated in a number of other analogs as the linking and hydrophobic groups have been altered such as **AB-PICA** (**14**) to **5F-ABPICA** (**15**) and **ABICA** (**16**) to **5F-ABICA** (**17**) among others (Wilkinson et al., 2015).

Another common tail group modification is incorporating a phenyl group, also with the potential for additional fluorination. This is best represented by **AB-FUBICA** (**18**) and **ADB-FUBICA** (**19**) (Banister, Moir, Stuart, et al., 2015). Bringing back the original lead **WIN 55,212-2** (**9**), simplified versions that still incorporate the morpholine in the alkyl chain, such as **JWH-200** (**20**) moiety have also shown activity (Dutta et al., 1997). While many alterations to the alkyl substituent have been introduced, greater variation is seen with the hydrophobic group at the C3 position, which will be discussed in the next section.

## Hydrophobic group modification

Given that the naphthyl moiety was present in **WIN 55,212-2** (**9**), it has continued its presence in a number of SCRAs. Additional substitution on the naphthyl group is tolerated with halogens, ethers, and alkyl groups all commonly incorporated (Huffman, 2009). While sites of substitution are varied, the 4-position on the naphthyl ring, as represented by compound **21**, has been the most explored and generally leads to retained cannabinoid receptor activity (Aung et al., 2000). Converting the naphthyl to 8-quinoline gives **PB-22** (**22**) (Uchiyama et al., 2013) resulting in reduced hydrophobicity, but with activity at both CB1 and CB2 receptors, though there is a sevenfold preference for the former ( $EC_{50}$  CB1 = 5.1 nM, CB2 = 37 nM) (Banister, Stuart, Kevin, et al., 2015).

Given the success of the naphthyl group, research quickly turned to compounds with other aromatic rings. Phenylacetyl with various substitutions also appended to the 3 position of alkyl indoles (**23**) showed broad activity at the CB1 receptor with  $K_i$  values generally below 100 nM (Huffman et al., 2005). Binding was generally lower at the CB2 receptor though general tolerance for this modification and broad scope was identified. Directly bonding the aromatic to the carbonyl is also tolerated with **RCS-4** (**24**) being a representative example of this phenotype.  $EC_{50}$  values reported for **RCS-4** (**24**) were 146 nM for the CB1 receptor and 46 nM for the CB2 receptor (Banister, Stuart, Conroy, et al., 2015).

Substituting aromatics with aliphatic rings, particularly large and/or bulky rings, maintains the cannabinoid receptor activity. Taking **UR-144** (**25**), for example, the small cyclopropyl ring is enhanced by the tetramethyl substitution to give a bulky hydrophobic substituent. **UR-144** (**25**) has lower affinity for CB1 than CB2 (150 nM vs 1.8 nM, respectively) (Frost et al., 2010), which also translates to functional activity ( $EC_{50}$  CB1 = 421 nM, CB2 = 72 nM) (Banister, Stuart, Kevin, et al., 2015). The cyclohexyl group linked via an amide, either directly (compound **26**) or with a methylene group (compound **27**), affords compounds with greater selectivity for the CB1 receptor ( $EC_{50}$  **26** CB1 = 37 nM, CB2 = 102 nM, **27** CB1 = 16 nM, CB2 = 216 nM) (Banister et al., 2013). As it became clear that bulky aliphatic groups could be incorporated into this position, compounds with adamantine linked via a ketone at this position (compound **28** and **29**) were soon identified in herbal blends (Grigoryev et al., 2012; Jankovics et al., 2012). Academic investigation into these compounds showed they had activity at both cannabinoid receptors ( $EC_{50}$  **28** CB1 = 35 nM, CB2 = 48 nM, **29** CB1 = 37 nM, CB2 = 89 nM) (Banister et al., 2013). These compounds then progressed to the amide-linked analogs **30** (APICA) (Uchiyama et al., 2012) and **31**, again both possessing activity at the cannabinoid receptors ( $EC_{50}$  **29** CB1 = 34 nM, CB2 = 29 nM, **30** CB1 = 43 nM, CB2 = 57 nM) (Aizpurua-Olaizola et al., 2017).

As already alluded to with compounds **14–19**, bulky amino acid-based substituents are also tolerated at this position. Predominately valine or *tert*-leucine derived, these side chains add the sufficient bulk needed to impart cannabinoid receptor activity. Compounds **14–19** depict this amino acid capped with a terminal primary amine, but methyl esters and free carboxylic acids have also been reported (Longworth et al., 2017). While these structures might appear to add additional complexity with the chiral center, given that these amino acids are readily available as single enantiomers, this does not add a significant challenge to the synthesis of these molecules and explain why they have appeared in herbal blends.

## Linker variation

Linking the hydrophobic group to the 3-position of the indole has occurred through ketone, amide, and ester functionality (Fig. 2). Linker variation tends to have less to do with cannabinoid receptor activity and more relevant to the synthetic route to obtain the SCRA. For example, when a carboxylic acid functionalized hydrophobic group is available (for example, 1-naphthoic acid), then this can be easily installed through a Friedel-Crafts acylation reaction. Compare this with compounds **14–19** where the amino acid is readily available, and so an amide linkage is employed for those substrates. Likewise, with the availability of cumylamine, this bulky hydrophobic group is appended to the 3-position with an amide linkage (Arikan

Ölmez et al., 2018). The ester linkage is observed in some SCAs, for example, **22**, but its greater susceptibility to cleavage is probably why it is not more commonly employed (Uchiyama et al., 2013).

As illustrated by general structure **7**, functionalizing indole through N-alkylation and C3-carbonyl linkage to hydrophobic groups generates compounds activating the CB1 and CB2 receptors. While that activity is varied and can be fine-tuned by tweaking each of the functionalities, findings from various research groups, clandestine labs and by authorities show the broad scope of indole-based SCAs.

## Indazole-based structures

With the structural similarity between indole and indazole moieties, it was not long until this swap was made to investigate the biological activity of these related analogs. Many of the changes to the *N*-alkyl chain, linking group and hydrophobic moiety were all transferable to the indazole group (general structure **32**, new nitrogen atom highlighted in purple (Fig. 3)). While some variation in activity was observed, this did allow a brand-new class of compounds to investigate and avoid detection of authorities. For example, the adamantly-containing compounds **33** and **34**, which provide a direct comparison to compounds **30** and **31**, with the only difference being an indazole core rather than indole. Rather than just using the *N*-pentyl chain, many variations of the alkyl chain and linking group are also reported for the adamantly substituted indazole (Asada et al., 2017; Hess et al., 2016; Longworth et al., 2019). The indazole analogs of compounds **14–19** have also been reported, for example, AB-PINACA (**35**) and ADB-PINACA (**36**) (Banister, Moir, Stuart, et al., 2015). A comparison of indole analog AB-PICA (**14**) ( $EC_{50}$  CB1 = 12 nM, CB2 = 12 nM) (Banister, Moir, Stuart, et al., 2015) and indazole analog AB-PINACA (**35**) ( $EC_{50}$  CB1 = 1.2 nM, CB2 = 25 nM) shows increased potency for the latter (Aizpurua-Olaizola et al., 2017; Wiley et al., 2015).

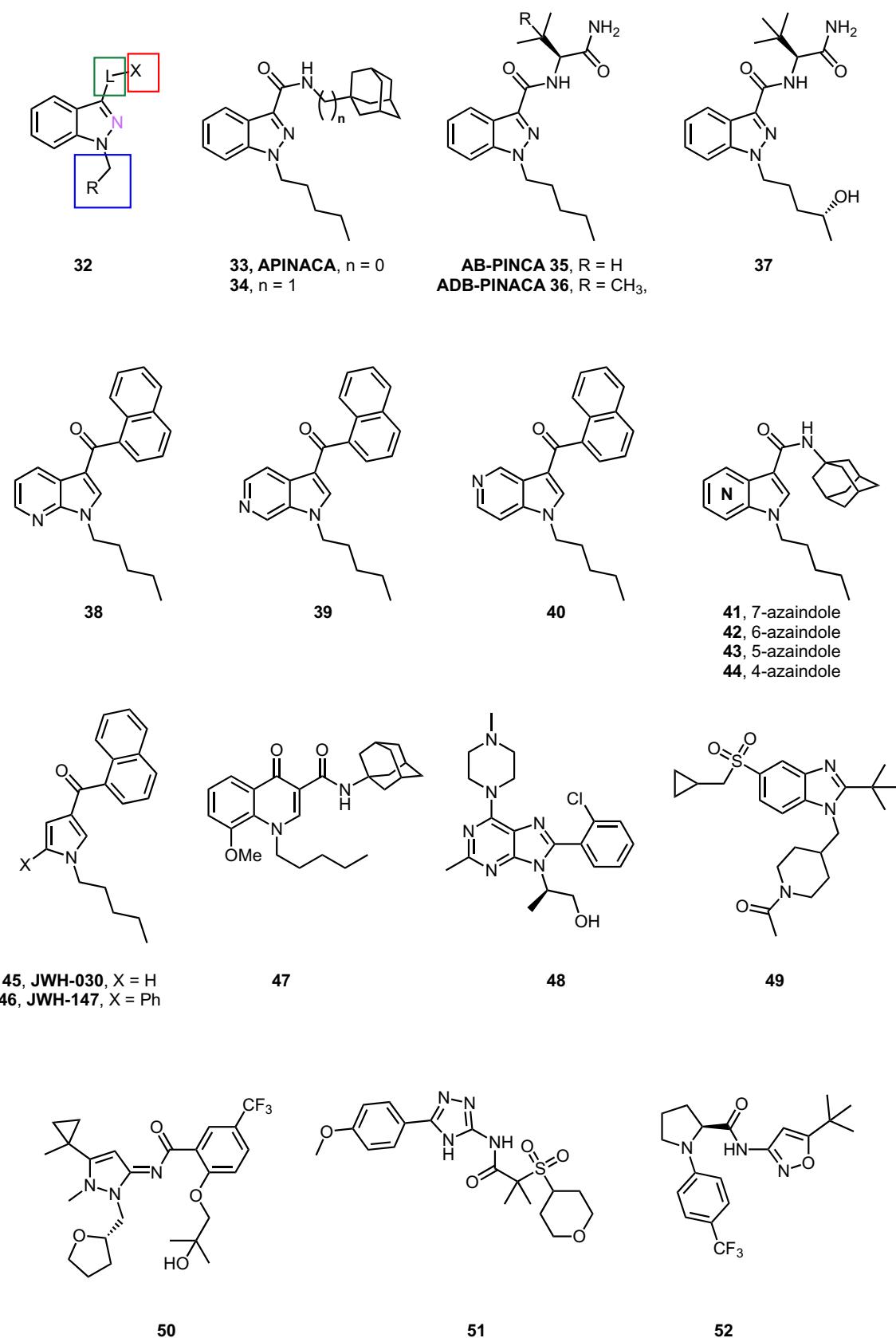
By working with the indazole core, the additional nitrogen atom allows for the generation of regioisomers by alkylating at the N2-positon. Generally this change significantly lowers activity at both cannabinoid receptors, but these regioisomers have been identified as minor contaminants in samples of N1-analogs (Longworth et al., 2016).

The in vivo metabolism of phytocannabinoids, often involving oxidative processes, generates compounds not active, or with significantly reduced activity, at the cannabinoid receptors (Aizpurua-Olaizola et al., 2017). SCAs also undergo oxidative metabolism; however, a number of these metabolites are still active at the cannabinoid receptors (Longworth et al., 2017). One site commonly recognized for metabolism is the tail group. For example, compound **34** undergoes tail group oxidation to **35** (among others), and while activity at both receptors drops (CB1  $EC_{50}$  from 1.3 nM to 17 nM and CB2  $EC_{50}$  from 2.6 nM to 13 nM), this is still similar to what is seen with  $\Delta^9$ -THC (**1**) (Longworth et al., 2017). This means longer-term activation of the receptors by SCAs over what is seen with phytocannabinoids and is likely part of the reason behind the negative health effects seen when taking these compounds.

## Azaindoles-based structures

Continuing the trend of additional nitrogen atoms in the core, azaindole has also been explored as a viable core for cannabinoid receptor agonism (Blaazer et al., 2011). Also identified from herbal blends (Bovens et al., 2017; Liu et al., 2017; Qian et al., 2017), the structural similarities between the azaindoles and the previously identified indoles became apparent. The *N*-alkylation and C-3 carbonyl linkage to a hydrophobic group are present across a range of azaindoles (Fig. 3) (Blaazer et al., 2011; Longworth et al., 2019). However, with an azaindole present, the location of the second nitrogen atom leads to further analogs. The herbal blends initially used 7-azaindole as the core (Bovens et al., 2017; Liu et al., 2017; Qian et al., 2017), but this is no means a requirement for cannabinoid receptor activity. Three of the azaindole analogs of JWH-018 (**10**) have been made and screened (Blaazer et al., 2011). The 7-azaindole compound **38** possesses an  $EC_{50}$  of 32 nM and 158 nM at the CB1 and CB2 receptors, respectively. Compared with the isomeric 6-azaindole analog **39**, a loss in activity is seen at both receptors ( $EC_{50}$  CB1 = 100 nM, CB2 = 501 nM) suggesting some detrimental effect of shifting the nitrogen atom. That activity is mostly restored with the 5-azaindole analog **40** with a CB1  $EC_{50}$  of 50 nM (CB2 activity not reported for comparison). Interestingly the same trends of nitrogen atom positioning are not observed across SCAs.

The azaindole analogs of indole APICA (**30**) and indazole APINACA (**33**) have also been synthesized and screened (**41–44**) (Longworth et al., 2019). The 7-azaindole analog **41** has an  $EC_{50} > 10 \mu\text{M}$  but retains pretty significant CB2 activity ( $EC_{50} = 20 \text{ nM}$ ). The 6-azaindole analog gains some CB1 activity ( $EC_{50} = 1022 \text{ nM}$ ) but also with a loss of CB2 activity ( $EC_{50} = 158 \text{ nM}$ ). The two other N-isomers **43** and **44** show no activity at either receptor with  $EC_{50}$  values  $> 10 \mu\text{M}$ . These results show that the structure-activity relationship between azaindole cores and activity at the cannabinoid receptors is not straight forward and could be explored further, though they tend to be less active than their indole or indazole analogs.



**FIG. 3** The broad structural class of compounds that can act as cannabinoid receptor agonists, particularly targeting the CB<sub>2</sub> receptor.

## Miscellaneous structures

The particular focus on indole and indole-like SCAs has in most part been driven by clandestine labs synthesizing and supplying these compounds for recreational use. However, there have also been other chemical cores that have been identified as being appropriate for cannabinoid activity. Generally, they consist of hydrophobic substituted heterocycles. Pyrroles were investigated at a similar time to indoles but a combination of lower activity and inherent instability meant they were not as heavily explored (Lainton et al., 1995; Tarzia et al., 2003). **JWH-30 (45)** is the direct pyrrole analog of **JWH-018 (10)** and sees a reduction in CB1 binding from 9.0 nM to 87 nM (Fig. 3) (Lainton et al., 1995). The phenyl substituted analog **JWH-147 (46)**, which can also be considered as resulting from indole ring opening, has improved affinity again ( $K_i$  CB1 = 11.0 nM) with some minor selectivity for CB2 ( $K_i$  = 7.1 nM) (Huffman et al., 2006).

With clandestine labs driving the development of SCAs, academic research has generally focused on developing compounds that are selective for the CB2 receptor due to its implications in a wide range of diseases and disorders (Aghazadeh Tabrizi et al., 2016). This has resulted in a number of structural classes some of which have been identified here (Fig. 3). While only single compounds have been drawn here, structurally similar analogs also show activity.

Quinolone-based compound **47** showed high activity at CB2 ( $EC_{50}$  = 0.6 nM) and was functionally selective over CB1 ( $EC_{50} \geq 10 \mu M$ ) (Pasquini et al., 2011). Interestingly, this compound shows how the functionality identified as being important for activity in the indole series has translated to this quinolone form. The pentyl chain and adamantyl linked amide both feature in these compounds.

The purine moiety has been developed as a suitable core from which to build CB2 selective molecules. Compound **48** showed selective agonism at the CB2 receptor ( $EC_{50}$  CB2 = 22.4 nM) over the CB1 receptor CB1 ( $>10 \mu M$ ) (Guidetti et al., 2014). The benzimidazole moiety has some structural similarity to indoles and indazoles, but as a result of the shift in position of the nitrogen atom, 3-substitution is not possible. Including a bulky hydrophobic *tert*-butyl group at the 2-position instead, such as in compound **49**, appears a viable alternative with excellent CB2 activity and selectivity ( $EC_{50}$  CB2 = 8.2 nM, CB1  $> 10 \mu M$ ) (Gijsen et al., 2012). Related analogs that incorporate  $^{18}F$  labels have also been investigated for PET tracer development (Kallinen et al., 2019).

A number of 5-membered ring heterocycles have also shown utility in compounds that target the cannabinoid receptors. With a pyrazole greater stability is achieved as well as more options for *N*-alkylation. This is represented by compound **50** with exceptional CB2 activity ( $EC_{50}$  = 0.47 nM) and no CB1 activity ( $>10 \mu M$ ) (Carroll et al., 2010). Triazole cores have also shown promise for SCAs. The representative example of compound **51** is one example where *N*-alkylation as not been introduced. The lack of *N*-alkylation does not seem detrimental to activity with a selective preference for the CB2 receptor ( $EC_{50}$  CB1  $\geq 10 \mu M$ , CB2 = 0.66 nM) (Berry et al., 2009).

Dearomatizing the heterocycle core affords the proline-containing compound **52**, which is over 1000 times selective for the CB2 receptor ( $EC_{50}$  = 0.23 nM) over the CB1 receptor ( $EC_{50}$  = 307 nM) (Hickey et al., 2015). The chirality introduced as a result of this dearomatization is easily incorporated through the use of amino acid L-proline.

With this broad and diverse pool of compounds targeting the CB2 receptor, attempts have also been made to take key structural components from SCAs to understand key pharmacophore requirements and develop novel structures (Moir et al., 2021; Moir, Boyd, et al., 2019; Moir, Lane, et al., 2019). This section has illustrated that the space in which to explore cannabinoid receptor activity is broad with wide-ranging functional diversity.

## Conclusions

Synthetic cannabinoid receptor agonists (SCAs) come in many different forms. From the structures built on naturally occurring phytocannabinoids to entirely novel arrangements, the number of compounds known to activate cannabinoid receptors are significant. This in part has been driven by clandestine labs continually generating new SCAs to avoid detection and regulation. This chapter has provided an overview of SCAs and their varied cannabinoid receptor activity and has correlated this to diverse structural functionality.

## Applications to other areas

In this chapter, we have reviewed the key synthetic cannabinoid receptor agonists (SCAs), with a particular focus on their use as designer drugs. Despite their history as drugs of abuse, and their resulting legal regulation, compounds that target the cannabinoid receptors, particularly receptor 2, have the potential to find applicability as prescribed

pharmaceuticals. Potential clinical applications of SCRAs include treating inflammatory disease states, which also incorporates neuroinflammation, other diseases of the central nervous system such as Parkinson's disease and other neurodegenerative illnesses. This is just the beginning of the potential application of cannabinoid receptor agonists, and further research and understanding are required to further release their potential.

Compounds discussed in this chapter also have the potential to act as development substrates for tool molecules, such as for positron emission tomography (PET) imaging. Development of molecules for PET imaging would allow us to investigate the cannabinoid receptors *in vivo* in a noninvasive manner. Such research would allow us to explore how this receptor is involved in different disease states and its role in normal human function. This in turn would allow for further molecule development and pharmaceutical design.

Investigating SCRAs also has an important public health consideration as they continue to be used as drugs of abuse with little understanding as to their health impacts. Further investigation into their pharmacological profiles, *in vivo* metabolism, and toxicology would allow informed public health debate and appropriate policy generation and legal protections.

## Mini-dictionary of terms

- **Agonist:** A compound that activates a receptor.
- **EC<sub>50</sub>:** The concentration at which a compound produces a response 50% of the maximum.
- **K<sub>i</sub>:** The concentration at which a compound binds to the active site of a receptor.
- **Phytocannabinoid:** Compounds that have originally been produced by plants and target the cannabinoid receptors.
- **Positron emission tomography (PET):** An isotope-labeled compound is used to visualize processes in the body in a non-invasive manner.
- **Synthetic cannabinoid receptor agonist (SCRA):** Compounds that are entirely synthetic in nature that target either, or both, cannabinoid receptors.
- **Synthetic marijuana:** Also known by commercial names Spice or K2, it is an innocuous herbal plant mixture treated with a synthetic cannabinoid receptor agonist.

## Key facts on legal regulation of synthetic cannabinoid receptor agonists

- When first identified in herbal blends SCRAs were not legally regulated
- Most countries responded by introducing laws banning these compounds
- Laws identified specific compounds, for example, **JWH-018 (10)**, which led to its control
- New analogs that were not explicitly controlled quickly emerged to replace those that could lead to prosecution
- These too were then controlled but a lag between emergence and law changes led to challenges for prosecution
- Countries then moved to control broad structural classes, such as general structure 7, even if they had yet to be made or actually show cannabinoid receptor activity
- Even so, there continues to be new compounds generated that avoid the general structures, which requires further intervention
- A summary of the German specific response can be found in the European Union review ([European Monitoring Centre for Drugs and Drug Addiction, 2021](#)).

## Summary points

- Synthetic compounds can be used to agonize the cannabinoid receptors.
- Generation of new cannabinoid receptor agonists has been driven by clandestine labs to generate compounds that avoid detection.
- A number of chemotypes have been developed that target the cannabinoid receptors.
- Core functionality with variable appendages can be used to tune cannabinoid receptor activity.
- The indole moiety is the most commonly employed core for compounds that target the cannabinoid receptors.
- Cannabinoid agonists that target the cannabinoid receptor 2 have been developed with for potential medicinal applications.

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## Chapter 39

# Nonneurological aspects of the endocannabinoid system: Nonalcoholic fatty liver disease

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## Abbreviations

<b>2AG</b>	2-archidonylglycerol
<b>ALD</b>	alcoholic liver disease
<b>ACC</b>	acetyl Co-A carboxylase
<b>ACO</b>	acyl-CoA oxidase
<b>BMI</b>	body mass index
<b>CPT-1</b>	carnitine palmitoyl transferase-1
<b>CBD</b>	cannabidiol
<b>CB1</b>	cannabinoid receptor 1
<b>CB2</b>	cannabinoid receptor 2
<b>CCl4</b>	carbon tetrachloride
<b>DIO</b>	diet-induced obesity
<b>ER</b>	endoplasmic reticulum
<b>FAAH</b>	fatty acid amid hydrolase
<b>FAAs</b>	free fatty acids
<b>FAS</b>	fatty acid synthase
<b>FEP</b>	first episode
<b>FIB-4</b>	fibrosis-4
<b>FLI</b>	fatty liver index
<b>FXR</b>	farnesoid X receptor
<b>GWASs</b>	genome-wide association studies
<b>HCC</b>	hepatocellular carcinoma
<b>HDL</b>	high density lipoprotein
<b>MGL</b>	monoacylglycerol lipase
<b>NAFLD</b>	nonalcoholic fatty liver disease
<b>NAFL</b>	nonalcoholic fatty liver
<b>NASH</b>	nonalcoholic steatohepatitis
<b>PPAR</b>	peroxisome proliferator-activated receptor
<b>THC</b>	tetrahydrocannabinol
<b>THCV</b>	tetrahydrocannabivarin
<b>Δ<sup>9</sup>-THCA</b>	delta-9 tetrahydrocannabinolic acid
<b>TNC</b>	tenascin C
<b>TNF-α</b>	tumor necrosis factor-alpha
<b>SCD1</b>	stearoyl-CoA desaturase
<b>SREBP-1c</b>	sterol regulatory element binding protein
<b>VLDL</b>	very low density lipoprotein

## Introduction

Nonalcoholic fatty liver disease (NAFL) is one of the most prevalent hepatic diseases globally, with incidence increasing to affect one-fourth of the population (Castera et al., 2019; Samuel & Shulman, 2018). The occurrence of NAFLD increases with age, affecting most males before the age of 50, but females tend to be more affected by this disease after the age of 50 (Lonardo et al., 2015). The pathogenesis of the disease begins with simple steatosis, which is characterized by the occurrence and accumulation of lipid droplets, mainly as triglycerides in hepatocytes, and the presence of this alone is called nonalcoholic fatty liver (NAFL; Brunt et al., 2015; Friedman et al., 2018). In around 40% of patients, simple steatosis can then develop into lobular inflammation accompanied by the presence of hepatocellular injury and ballooning; this stage is known as nonalcoholic steatohepatitis (NASH). Fibrosis and cirrhosis can later progress in a low percentage of patients, and it may then lead to hepatocellular carcinoma (HCC; Carr et al., 2016). HCC incidence has increased dramatically, and the number of cases associated with NAFLD accounts for around 25% of the total cases in Western countries (Samuel & Shulman, 2018).

Several studies have hypothesized that the endocannabinoid system is highly implicated in the pathogenesis of NAFLD (Berk et al., 2021). However, further study is needed to determine whether endocannabinoids are correlated to the development of NAFLD. In recent years, there has also been considerable interest in finding a suitable therapy for NAFLD due to the increased prevalence worldwide and the lack of a beneficial treatment. Therefore, the aim of this chapter is to address the role of the endocannabinoid system in the development of NAFLD and to evaluate the efficacy of cannabinoids in treating this chronic disease.

## The mechanism of the pathogenesis of NAFLD

The mechanisms behind the onset of steatosis are highly attributed to many metabolic factors, such as the elevated de novo synthesis of fatty acids, high flux of fatty acids from the organs' peripheral tissue to the liver, reduction of fatty acid oxidation, and impairment of the transport of fatty acids in the form of triglycerides from the liver to circulation and thereafter to the peripheral tissues (Purohit et al., 2010).

NAFLD is strongly correlated with obesity, as well as some metabolic syndromes, including hyperlipidemia, hypertension, type 2 diabetes mellitus (T2DM), and insulin resistance (Carr et al., 2016; Younossi et al., 2016). According to the results of many studies conducted on humans and animals, NASH results from an overload and high consumption of dietary fat and carbohydrates. This results in increased levels of some principal metabolic substrates, such as fatty acids, fructose, and glucose. The accumulation of free fatty acids (FFAs) in the liver promotes the activation of some metabolic pathways, including lipogenesis, leading to hepatocellular injury (Ertunc & Hotamisligil, 2016; Ghazali et al., 2020; Neuschwander-Tetri, 2010).

It is well known that obesity caused by high caloric intake leads to the occurrence of insulin resistance, which is believed to increase lipolysis in the adipose tissue and, consequently, to elevate the level of plasma FFAs; this is known as the “first-hit theory.” FFAs, in turn, flux to the liver and, if not oxidized, they accumulate and lead to the onset of hepatic steatosis. This in turn makes the liver more susceptible to mitochondrial dysfunction, adipokines, cytokines, bacterial endotoxins, and endoplasmic reticulum (ER) stress, and this is called the “second-hit” hypothesis. However, this original “two-hits” theory has been altered into a “multiple parallel hits” theory. Tilg and Moschen (2010) proposed that NAFLD is a multifactorial disease in which many risk factors, including insulin resistance, nutritional factors, adipocyte dysfunction, gut microbiota, and epigenetic and genetic factors, instantaneously act on the extra and intrahepatic pathways, resulting in the onset of steatosis, which may develop further into NASH. This theory suggests that in NAFLD, insulin resistance results in increasing lipogenesis and the uptake of FFAs to the liver, causing lipotoxicity. Thus, sensitizing liver injuries by various hits, such as hepatic stellate cell activation, oxidative stress, fibrogenic pathway activation, and adipokine expression alteration, consequently leads to the prognosis of NASH (Nassir & Ibdah, 2014).

## Endocannabinoid system

The endocannabinoid system includes endocannabinoid receptors, ligands, transports, and degradative enzymes (Goyal et al., 2017). There are three species of cannabis plant: *cannabis indica*, *cannabis sativa*, and *cannabis ruderalis*. The cannabis plant has 60 hydrocarbon aromatic compounds, referred to as cannabinoids. Cannabinoids involve many agents, including delta-9 tetrahydrocannabinol (THC), which has a psychotropic effect, and cannabidiol (CBD), which has an impact on inflammation, analgesia, and motility. There is also another element known as cannabigerol, but its effect is yet to be elucidated (Cohen & Neuman, 2020).

These agents act by binding to two cannabinoid receptors (CB1 and CB2). The CB1 receptors are located in the peripheral and central neurons, while the CB2 receptors are situated in the immune system and in the nerve terminals (Hornby & Prouty, 2004; Pertwee, 2001). In the liver, CB1 mainly occurs in the stellate cells, hepatocytes, and hepatic sinusoidal cells, whereas CB2 presents in the Kupffer and stellate cells, where they show a high expression in pathology, particularly with inflammation, like in NASH, but seem weak in healthy people (Tam et al., 2011; Wu et al., 2019). In recent years, there has been considerable interest in the CB1 receptor b isoform due to its predominant presence in pancreatic B cells and hepatocytes only and not in the brain (González-Mariscal et al., 2016). THC normally binds to CB1 receptors and produces a euphoric effect. On the other hand, CBD weakly binds to CB1 and may affect the binding of THC to CB1 and subsequently reduce the euphoria. It has also been found that CBD acts as an agonist and binds to CB2 (Cohen & Neuman, 2020). According to the positions of CB2 receptors, the binding of endogenous or exogenous cannabinoids, such as 2-archidonylglycerol (2AG) and CBD, respectively, may modify the inflammatory expression with T and B cell subtypes, neutrophils, and macrophages (Cohen & Neuman, 2020; Hornby & Prouty, 2004).

During the metabolic influence of cannabinoids, various presumed cannabinoid receptors are involved. These receptors are known as nonCB1 and nonCB2 receptors and G-protein-coupled receptors (GPCRs). There are three cannabinoid-related GPCRs: GPCR18, GPCR55, and GPCR119 (Berk et al., 2021). GPCR55 has been investigated broadly in the adipocytes, but its role in the signaling pathways in the hepatocytes has not been elucidated. Mice studies reported that GPCR55 plays a role in either sustaining or reenforcing the action of insulin in the liver (Lipina et al., 2019). In immune cells, GPCR55 also showed potential cooperation with CB2 (Balenga et al., 2011). This is supported by other studies in which there was a supposed relation between CB2 and GPCR55 when they both coexpressed in the same tissue such as in the liver, and this may then affect the liver glucose metabolism (Simcocks et al., 2014). Meanwhile, it is believed that the GPCR119 receptor is involved in inhibiting lipid accumulation by stimulating adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, which in turn decreases the expressions of some enzymes and sterol regulatory element binding protein (SREBP)-1, the transcriptional factors implicated in lipogenesis (Yang et al., 2016). Contrary to GPCR55 and GPCR119, GPCR18 was reported to be involved in inflammation associated with obesity; hence, evidence of the effect of these receptors on hepatocytes is poorly documented (Rajaraman et al., 2016). It has been stated that the use of the GPCR18, GPCR55, and GPCR119 receptors in treating and reducing the risk of such metabolic disorders as NAFLD is promising (Berk et al., 2021).

## **Endocannabinoid system and NAFLD**

It has been stated that various hepatic disorders, such as liver steatosis, fibrosis, and alcoholic liver disease (ALD), are correlated to cannabinoids. The effect of the endocannabinoid system in the peripheral tissues involves lipogenesis induction and lipolysis inhibition, along with appetite elevation caused by the endocannabinoids anandamides and 2-AG and, consequently, the promotion of fat accumulation, which are considered the main characteristics of the onset of NAFLD (Berk et al., 2021). Furthermore, the activation of CB1 receptors may induce steatosis by stimulating lipogenesis and decreasing the rate of fatty acid oxidation, whereas the antagonist suppression of CB1 reduces the risk of hepatic steatosis (Goyal et al., 2017).

### *The role of a high-fat diet in CB1 activity*

As mentioned previously, a high-fat diet is one of the causative components of NAFLD, as this disease is significantly associated with obesity. Normally, the expressions of the CB1 and CB2 receptors in the liver are faint, and the latter can also be absent. However, the expression of CB1 has been found upregulated in liver steatosis, increased food intake, and lipogenesis, while CB2 is overexpressed in inflammation and fibrogenesis (Alswat, 2013).

It has been demonstrated by some experiments conducted on laboratory animals that the administration of THC and endocannabinoids (anandamides and 2-AG) increases food intake, and this effect was antagonized by a CB1 receptor blockade (Kirkham et al., 2002; Ravinet Trillou et al., 2004). From these data, it can be suggested that cannabinoids may increase appetite and upregulate the expression of CB1, which can be antagonized by a CB1 antagonist.

This is supported by another study, which found that mice with CB1 receptor knockout (CB1[−/−]) were lean and resistant to weight gain after they were introduced to diet-induced obesity (DIO), whereas wild mice with the CB1 receptors (CB1[+/+]) showed an increased weight and developed obesity (Ravinet Trillou et al., 2004). It has been also found that DIO mice being treated by Rimonabant (SR141716), a CB1 antagonist, for 5 weeks exhibited a reduced diet intake in the first week, along with a significant reduction in adiposity and body weight (Ravinet Trillou et al., 2003). Furthermore, Osei-Hyiaman et al. (2005) stated that the level of anandamides, the expression of CB1 receptors in the liver, and the rate of fatty

acid synthesis were all increased in response to a high-fat diet in animals. They also noticed that the rate of fatty acid synthesis decreased following the use of a CB1 blockade treatment. In contrast, isolated hepatocytes and the liver, where CB1 receptors are predominant, showed an elevation in the rate of the synthesis of fatty acids when treated with a CB1 agonist (Brown & Goldstein, 1998). In another study focused on the impact of a high-fat diet and the role of CB1 in the development of NAFLD, Gary-Bobo et al. (2007) found that a CB1 antagonist (Rimonabant) reduced liver steatosis in obese rats, where obesity in these rats was induced genetically with a defect in leptin receptors, leading to severe steatosis. They also found that Rimonabant reduced the level of liver enzymes and tumor necrosis factor alpha (TNF- $\alpha$ ) and increased the level of adiponectin, an anti-inflammatory hormone, and all these changes are considered features of metabolic syndrome. On the other hand, their data demonstrated that pair-fed obese rats, which were consuming the same amount of food as treated rats, showed a slight or no significant effect on hepatic steatosis and liver enlargement. In addition, no changes were observed in all the above parameters, proposing that improvement of steatosis can be achieved using Rimonabant only, and there is no need to reduce caloric intake.

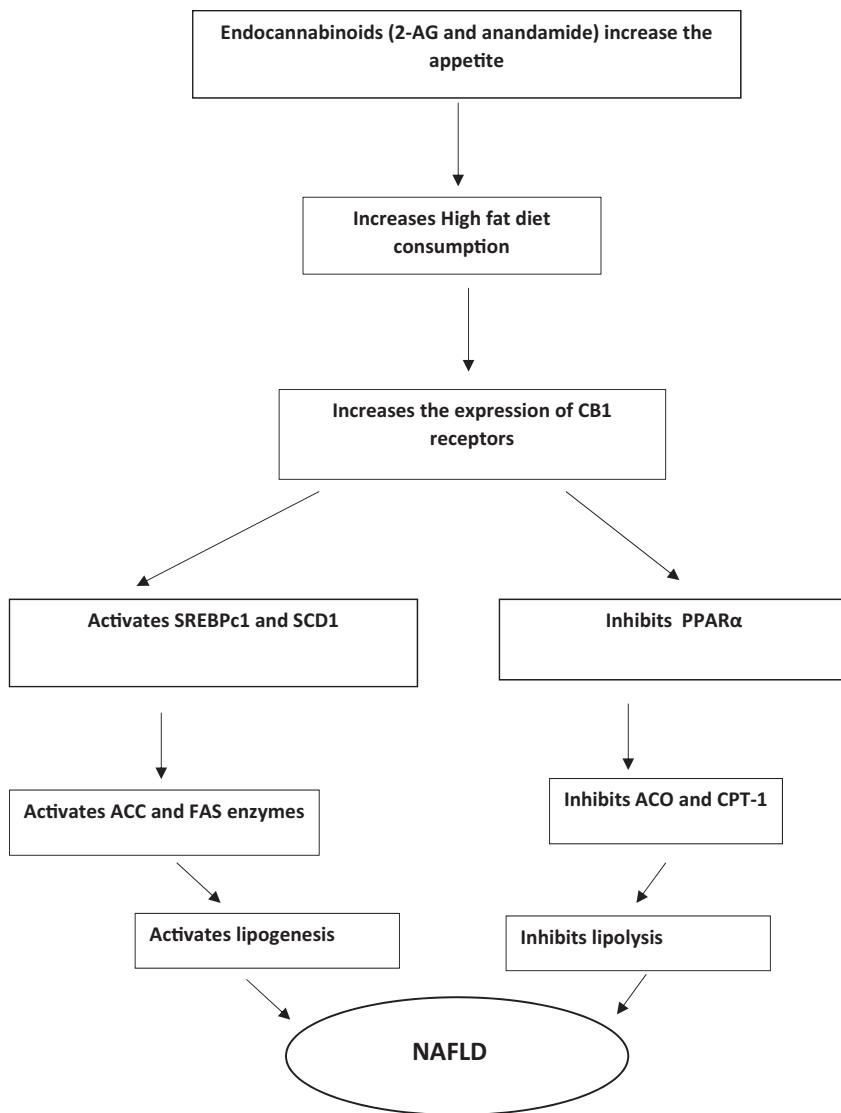
The results of Auguet et al. (2014) also clarified the role of CB1 in the prognosis of NAFLD in 72 morbidly obese females and found that the expression of CB1 was high in NASH accompanied by a reduction in the expression of peroxisome proliferator-activated receptor (PPAR $\alpha$ ). Another study conducted in 2020 by Ghazali et al. on the human hepatoma cell line VL-17A derived from the HepG2 cell line supports Auguet et al.'s (2014) findings. In this study, VL-17A cells were treated with various increasing omega 6/omega 3 ratios, and the expressions of some lipolytic, lipogenic, and endocannabinoid system mediators were evaluated (Ghazali et al., 2020). The findings of this study showed an increase in the expressions of CB1 and stearoyl-CoA desaturase (SCD), which are involved in lipogenesis, while the PPAR $\alpha$  expression was downregulated with a high omega6/ omega3 ratio.

Thus, it can be summarized that diet induces obesity, and it may lead to an upregulation in the expression of CB1 receptors. Furthermore, the overexpression of these receptors was observed in steatosis, as mentioned previously, and steatosis is considered a hallmark of NAFLD. The role of CB1 receptors in obesity and food intake can be attributed to their impact on the SREBP-1c and SDC1 transcriptional factors, which are involved in lipogeneses via the activation of some lipogenic enzymes, including acetyl Co-A carboxylase (ACC) and fatty acid synthase (FAS), and consequently stimulating the de novo synthesis of fatty acids (Berk et al., 2021; Brown & Goldstein, 1998). Meanwhile, the impact of CB1 on lipolysis can be due to their effect on PPAR $\alpha$ , which in turn inhibits some lipolytic enzymes such as acyl-CoA oxidase (ACO) and carnitine palmitoyl transferase-1 (CPT-1) involved in fatty acid oxidation (Levy et al., 2004) (Fig.1).

### **Endocannabinoid system and obesity (human trial)**

Several clinical studies clarified the correlation between endocannabinoids and obesity and that obesity is strongly associated with endocannabinoid system dysregulation. For instance, many studies found that type 2 diabetic and obese patients with low levels of high-density lipoprotein (HDL) cholesterol, insulin resistance, and low triglycerides express elevated levels of endocannabinoids (Bennetzen et al., 2011; Bluher et al., 2006; Cote et al., 2007; Di Marzo et al., 2009; Matias et al., 2006). In addition, physical exercise intervention in 49 obese males showed a significant reduction in the levels of both 2-AG and anandamide. This was also accompanied by a marked decrease in body weight, vesicular adipose tissue, and waist circumference (Di Marzo et al., 2009).

Thirty obese or overweight middle-aged females underwent one of three 20-week weight loss programs: caloric restriction only, moderate exercise with caloric restriction, or intensive aerobic exercise with caloric restriction, to investigate the expression of CB1 receptors and fatty acid amid hydrolase (FAAH), an enzyme responsible for the degradation of anandamides (Berk et al., 2021; You et al., 2011). They noticed that caloric restriction reduced the expression of CB1 and FAAH in the gluteal but not abdominal adipose tissue. Caloric restriction either with moderate or intensive exercise alone did not show any change, whereas caloric restriction with both moderate and intensive exercise expressed a decrease in FAAH expression only. This study is corroborated by Bennetzen et al. (2011), who found that the 2-AG level in the subcutaneous adipose tissue in 21 obese individuals was low. However, this level increased after they underwent a diet regimen for 12 weeks. Indeed, the low expression of CB1 in the abdominal adipose tissue was normalized postweight loss. Regardless, the gluteal adipose tissue expressed further reductions in the expression of CB1 after weight reduction. Evaluating the relation between the expressions of the endocannabinoid system catabolic enzymes FAAH and monoacylglycerol lipase (MGL), a hydrolytic enzyme involved in the catabolism of 2-AG, and body mass index (BMI) in 28 healthy obese individuals indicated a positive correlation between high levels of the FAAH enzyme in mature subcutaneous adipocytes and elevated BMI (Berk et al., 2021; Cable et al., 2011). However, MGL activity did not show any association with BMI or other metabolic markers, such as serum glucose, insulin, or adiponectin.



**FIG. 1** The role of endocannabinoid system in the development of NAFLD. This figure shows the implication of the endocannabinoid system in the pathogenesis of NAFLD. Endocannabinoids can increase the appetite, which consequently increases the consumption of high-fat diet. This in turn leads to increase the expression of CB1 receptors and subsequently stimulates lipogenesis by activating SREBPc1 and SCD1 transcriptional factors and inhibiting lipolysis by down-regulating the activity of PPAR $\alpha$ . The activation of SREBPc1 and SCD1 increases the activity of lipogenic enzymes; ACC and FAS while the downregulation of PPAR $\alpha$  inhibits ACO and CPT-1 involved in fatty acid oxidation. (SCD1) Stearoyl-CoA desaturase; (SREBP-1c) Sterol regulatory element binding protein; (PPAR) Peroxisome proliferator-activated receptor; (ACC) Acetyl Co-A carboxylase; (FAS) Fatty acid synthase; (ACO) Acyl-CoA oxidase; (CPT-1) Carnitine palmitoyl transferase-1; (NAFLD) nonalcoholic fatty liver disease.

From the data above on obesity, it seems the level of 2-AG decreases when accompanied by an increase in the expressions of CB1 receptors and the FAAH enzyme level. Therefore, it can be concluded that the endocannabinoid system is highly associated with obesity and hence with NAFLD, as obesity is considered as a component of the metabolic syndrome (Fig.1).

## NAFLD and CB2 receptors

CB2 receptors have been implicated as modulators in bone loss, pain, inflammation, and hepatic pathophysiology, particularly hepatic inflammation and fibrogenesis, which are correlated with chronic disease and liver encephalopathy related to acute liver disease. However, the potential role of CB2 receptors in the pathogenesis of NAFLD is still to be elucidated (Alswat, 2013).

Previously in this chapter, it was mentioned that the expression of CB2 in a normal liver is faint, but it can be overexpressed in cases of inflammation and fibrogenesis. The findings of a study conducted on 72 morbidly obese females with various stages of NAFLD found that the expression of the CB2 gene is positively correlated with antiinflammatory adiponectin hormone, as well as PPAR $\gamma$  and Acetyl-CoA carboxylase 1 (ACC1). However, this correlation was accompanied by an increase in the levels of some inflammatory cytokines, such as IL6, TNF $\alpha$ , and resistin hormone, which suggests that

CB2 may have a dual effect (August et al., 2014). Another study on rats with cirrhosis showed that the activation of CB2 receptors in the liver decreased the content of hepatic collagen, in addition to enhancing the response of regeneration in the case of acute hepatic injury (Muñoz-Luque et al., 2008). Liver injury was induced in CB2 ( $-/-$ ) mice by the administration of carbon tetrachloride (CCl<sub>4</sub>). However, treating CB2 ( $-/-$ ) mice with a CB2 agonist (JWH-133) reduced liver injury and augmented hepatic regeneration (Teixeira-Clerc et al., 2010). Some other reports also indicated that CB2 receptors are protective during hepatic injury. This can be attributed to the antifibrogenic and antiinflammatory impacts of CB2 receptors due to their expression in liver immune cells and liver myofibroblasts (Louvet et al., 2011; Mallat et al., 2013). It has been also noted that CB2 receptor induction, by either endogenous or exogenous causes, exerted a protective effect in many liver injury models (Agudo et al., 2010; Lotersztajn et al., 2008; Mendez-Sanchez et al., 2007; Pacher & Mechoulam, 2011). Several studies on humans reported that CB2 receptors were expressed in the liver of patients with steatosis or steatohepatitis. These receptors were specifically located in the hepatocytes, liver stellate cells, and cholangiocytes. Meanwhile, a normal liver biopsy did not show any CB2 expression in parenchymal or nonparenchymal cells (Mendez-Sanchez et al., 2007). On the other hand, after 6 weeks of being fed a high-fat diet, wildtype mice showed high expressions of CB2 receptors after CB2 agonist (JWH-133) administration. This effect was associated with hepatic triglyceride accumulation, insulin resistance, and fat accumulation (Deveaux et al., 2009). Conversely, following the inactivation of CB2, a reduction in steatosis, as well as a decrease in the content of triglycerides, was observed (Deveaux et al., 2009). In addition, the inactivation of CB2 receptors by genetic or pharmacological factors reduced the infiltration of adipose tissue macrophages and played a potential role in protecting mice from insulin resistance, which can be diet- or age-related (Agudo et al., 2010). Moreover, the expression of CB2 receptors was upregulated in patients with different stages of liver cirrhosis, which was apparent in the liver fibrogenic cells, while normal livers did not express any CB2 receptors. Cultured myofibroblasts, as well as stimulated stellate cells, also showed upregulated CB2 receptors (Julien et al., 2005). According to a study performed by De Gottardi et al. (2010) on HepG2 cells, lipid metabolism can be modulated by CB2 receptors, resulting in inducing some genes involved in lipid synthesis. This was also accompanied by an increase in the expressions of CB1 receptors. These findings indicated that the activation of CB2 receptors contributed to lipotoxicity and may play a role in the prognosis of NAFLD.

Altogether, the role of CB2 receptors in the pathogenesis of NAFLD is still unclear. However, the high expressions of these receptors in human and animal studies can be ascribed to their antiinflammatory or antifibrogenic effects. In addition, the high expression of CB2 in the abovementioned studies may be highly related to the occurrence of inflammation, which results from high fat consumption, and the overexpression of CB2 receptors can be explained by the theory that CB2 may act as an antiinflammatory mediator, which in turn inhibits the activity of some inflammatory transcriptional genes.

## NAFLD treatment

It is believed that the early stages of NAFLD can be reversed (Maciejewska-Markiewicz et al., 2021). To date, there is no approved treatment for NAFLD. However, currently, the most effective strategy in reversing this disease is by improving patients' lifestyles. This involves diet management and regular physical exercise (Brunt et al., 2015). Diet management can be achieved either by reducing the delivery of metabolic substrates, such as glucose, fatty acids, or fructose, to the liver, or by facilitating their disposal. This strategy can help in improving the features of NASH, including fibrosis (Vilar-Gomez et al., 2015). Furthermore, bariatric surgery has to some extent played a crucial role in reversing the initial phases of NAFLD (Brunt et al., 2015).

Various pharmaceutical therapies have been used to help treat this disease. PPAR is a member of the superfamily of nuclear hormone receptors (Yi et al., 2017), and it has three isoforms: PPAR $\beta/\delta$ , PPAR $\alpha$ , and PPAR $\gamma$  (Berger & Moller, 2002). It is known that PPAR $\alpha$  plays a crucial role in increasing the oxidation of fatty acids, while PPAR $\gamma$  acts as an anti-inflammatory component (Friedman et al., 2018). Elucidating the roles of these receptors can help in understanding the mechanisms of some PPAR $\gamma$  ligands and PPAR $\alpha/\gamma$  agonists. The latter have shown a good impact by improving insulin sensitivity, which is known—as previously mentioned—as one of the metabolic syndromes highly implicated in NAFLD (Ratziu et al., 2016). Therefore, PPAR $\alpha/\gamma$  agonists may help in accelerating the disposal of metabolic substrates safely, but these agents are still under study. On the other hand, PPAR $\gamma$  ligands have also shown promising results in improving steatosis, hepatocellular swelling, inflammation, and fibrosis, despite its side effects, including weight gain, osteopenia and fluid retention, and elevated bone fractures. Particularly in females.

The involvement of the Farnesoid X receptor (FXR) in regulating the metabolism of lipids and glucose is well documented. Therefore, synthetic FXR ligands, which play a role in activating other nuclear receptors, have also been designated effective in mouse studies with NASH and human tissues by improving insulin resistance via their antifibrotic and antiinflammatory effects (Kong et al., 2009; Mudaliar et al., 2013).

It is also believed that cannabis may play a role in reducing hepatic inflammation, which is considered one of the major features of NASH (Cohen & Neuman, 2020). This role will be discussed further in this chapter.

### *The role of endocannabinoids in NAFLD treatment*

Reviewing various studies and understanding the role of the endocannabinoid system in the development of NAFLD in this chapter have led to significant interest in studying cannabis use in the treatment of NAFLD. It is believed that NAFLD is one of the most common liver diseases and one of the leading causes of hepatocellular carcinoma worldwide (Berk et al., 2021). This chapter previously discussed some of the therapeutic targets that can help in curing NAFLD, and the only reliable strategy mentioned is changing the lifestyle of the patient by altering eating habits and increasing physical exercise. Although the consumption of cannabis is considered an unhealthy and addictive practice, currently the usage of cannabis is common for preventing the development of many metabolic disorders that lead to the occurrence and progression of hepatic steatosis. However, endocannabinoid system dysregulation, which has been discussed previously, is one of the major implicated factors in the pathogenesis of NAFLD.

### *The role of cannabis in NAFLD*

It has been predicted that the use of cannabis will be promising in treating many diseases (Berk et al., 2021). In a study conducted by Vázquez-Bourgon et al. (2019), the efficacy of cannabis consumption was evaluated in 390 first-episode (FEP) nonaffective psychosis patients with NAFLD. They were assessed at the starting point and 3 years from the first use of cannabis. Only 6.7% reported the use of cannabis at the start of their participation in the study. Hepatic steatosis along with fibrosis was assessed using validated clinical scores: fatty liver index (FLI) to indicate the level of inflammation, Fibrosis-4 (FIB-4), and NAFLD. Their data showed a significant reduction in the inflammation score at 3 years compared with nonusers ( $P < 0.001$ ). After 3 years, patients who continued consuming cannabis presented a further improvement in the inflammation score compared with patients who discontinued using cannabis ( $P = 0.022$ ) and compared with individuals who had never consumed cannabis ( $P = 0.016$ ). However, no change was noticed in the fibrosis score in relation to cannabis consumption.

It is also well known that chronic cannabis use is associated with metabolic disorders that are highly correlated with the development of NAFLD. This is due to the impact of cannabis on increasing appetite and inducing high caloric consumption, mainly fat and simple sugar (Foltin et al., 1988; Kirkham, 2009; Rajavashisth et al., 2012; Rodondi et al., 2006; Smit & Crespo, 2001).

On the other hand, several studies have revealed the benefits of prolonged cannabis use, believing that cannabis can hinder the development of NAFLD via reducing the frequency of insulin resistance, hyperlipidemia, T2DM, and other metabolic syndromes (Carrieri et al., 2015; Le Strat & Le Foll, 2011; Penner et al., 2013; Rajavashisth et al., 2012; Vidot et al., 2016). The major component of marijuana is phytocannabinoids (natural cannabinoids), and several studies have indicated a reduction in the incidence of obesity in individuals using marijuana (Berk et al., 2021). This is supported by Adejumo et al. (2017), who evaluated the impact of using cannabis in patients with NAFLD aged  $\geq 18$ , revealing that the pathogenesis of the disease is reversed or suppressed with cannabis consumption. It has also been found that cannabis use is correlated with a reduction in BMI (Pasman et al., 2018).

The reason behind the positive effect of cannabis in reducing the development of NAFLD might be due to the antagonistic impact of cannabinoids as CBD and tetrahydrocannabivarin (THCV) on CB1 receptors (Thomas et al., 2005, 2007). For instance, investigating the role of CBD in modulating CB1 in HEK 293A and STHdhQ7/Q7 cells, where CB1 receptors are highly expressed, showed downregulation in the activity of CB1. The data of various studies confirmed this positive correlation between the antagonism of CB1 and the development of NAFLD, as mentioned previously in this chapter. In addition, insulin sensitivity was improved after antagonizing CB1 in the hepatocytes, and this can result in improving hepatomegaly, hepatic steatosis, and metabolic syndromes by decreasing the accumulation of fat in the liver and normalizing the metabolism of glucose in the liver. Counteracting CB1 in the cells also showed a reduction in the secretion of very low density lipoprotein (VLDL) and a decline in triglyceride synthesis (Mu et al., 2019; Osei-Hyiaman et al., 2005, 2008; Smit & Crespo, 2001; Tam et al., 2011). Therefore, the significant benefits of using cannabis as a drug to treat NAFLD can be summarized as follows: cannabis may antagonize CB1 receptors, and this consequently downregulates some transcriptional factors that are involved in lipogenesis, such as SREBP-1c and SDC1, thus decreasing the activity of ACC and FAS as key enzymes in the de novo synthesis of fatty acids. In addition, blocking CB1 via cannabis use may activate PPAR $\alpha$  involved in lipolysis and activate the oxidation of fatty acids.

However, in a study conducted by Wang et al. (2020) using meta-analysis data from three large genome-wide association studies (GWASs) on lifetime cannabis users, cannabis use dependence, and cannabis use disorder, they found no relation between the use of cannabis and its presumed effect of protecting against NAFLD.

Clinically, cannabis is primarily consumed as a therapeutic agent to treat types of pain, including that related to cancer, spasticity, nerve damage, and inflammation (Wang et al., 2020). The latter is a key stage in the pathogenesis of NASH.

*Cannabis sativa* excretes a valuable effect on rats whose high fat diet-induced fatty liver disease is indicated by hypercholesterolemia, inflammation, and oxidative stress, which is considered one of the pathological characteristics of NAFLD. Cannabis sativa ameliorated oxidative stress and inhibited inflammatory mediators, such as hPGDS, mPGES, Cox-2, IL-4, sEH, and TNF- $\alpha$ . However, the therapeutic effect of *cannabis sativa* in this study was due to the polyunsaturated fatty acid content, where omega 3 (alpha linolenic acid) is higher than omega 6 in their optimal proportions (Kaushal et al., 2020). This is consistent with a study that identified the effect of a high omega 6/omega 3 ratio on the expression of CB1, where the CB1 receptor expression was high in VL-17A cells (Ghazali et al., 2020). In terms of the cannabis content of cannabinoids, a 3-week administration of a high level of CBD exerted a proinflammatory effect in the liver of mice fed a diet rich in fat and cholesterol for 6 weeks. In the same study, CBD also increased the fasting glucose level, whereas elevated THC improved the fasting glucose level and reduced the activity of liver enzymes, as well as the expression of two inflammatory genes: TNF $\alpha$  and inducible nitric oxide synthase (iNOS). Despite the positive impact of THC, it caused a moderate increase in body weight. No marked effect was noticed on the expressions of CB1 and CB2 receptors after the administration of CBD and THC, despite the information stated in this section regarding the antagonizing of CB1 and the development of NAFLD (Assa-Glazer et al., 2020). To ascertain the effect of THC, the THC precursor nonphysico  $\Delta^9$ -tetrahydrocannabinolic acid ( $\Delta^9$ -THCA) was used to investigate its effect on liver fibrosis and inflammation in vitro using LX-2 and NIH-3 T3-Col1A2-luc cell lines and in vivo by inducing nonalcoholic fibrosis in mice by either treating them with CCl<sub>4</sub> or feeding them a high-fat diet for 23 weeks. The findings revealed that the expressions of Col3A1 and Tenascin C (TNC), which were stimulated by TGF $\beta$ , were inhibited in LX-2 cells. In fibroblasts, the activity of the transcriptional factor Col1A2 promoter was also inhibited by the administration of  $\Delta^9$ -THCA.  $\Delta^9$ -THCA also ameliorated liver fibrosis and inflammation, which are induced by CCl<sub>4</sub>, and reduced macrophage infiltration and T cells in mice. Furthermore, mice with fatty liver, along with fibrosis and infiltration in the immune cells induced by a high-fat diet, showed an improvement in and attenuation of all these symptoms (Carmona-Hidalgo et al., 2021). In contrast, it has been stated that THC agonists, the expressions of both CB1 and CB2 increased the levels of anandamide and 2-AG in the hepatocytes. The increased level of endocannabinoids is due to the competition between THC and endocannabinoids to bind to fatty acid binding protein-1, which causes a reduction in the availability of the endogenous ligands needed for the enzymatic degradation of endocannabinoids (McIntosh et al., 2018).

These findings could provide new insights to support the concept of using cannabis as a therapeutic agent in NAFLD. However, the psychotropic impact has been observed after THC use in many studies (Cohen & Neuman, 2020). The side effects of the acute consumption of cannabis involve panic, anxiety, tachycardia, increased appetite, dry mouth, and psychosis. Therefore, further investigation is required to understand precisely the impact of cannabis use in the treatment of NAFLD while avoiding the occurrence of these adverse effects.

## Conclusion

NAFLD is a chronic disease with a high incidence worldwide, and it is highly associated with metabolic syndromes, including obesity and insulin resistance. It begins with simple steatosis, which is characterized by the accumulation of fat in the liver. This then develops into steatohepatitis, accompanied by ballooning, fibrosis, and inflammation, which in some cases advances into cirrhosis and may end lead to HCC. Many factors are implicated in the development of this disease, with high dietary fat and simple sugar consumption considered the prime causes of NAFLD. The endocannabinoid system has been correlated to the development of NAFLD, and this can be through the activation of CB1 receptors by the endocannabinoids anandamides and 2-AG, which consequently activate some lipogenic enzymes involved in the synthesis of fatty acids. CB2 receptors are found to be highly expressed in NASH and during inflammation. The role of CB2 receptors is still unclear, but the high expression can be due to their antiinflammatory protective effect via their action to reduce the expression of some inflammatory transcriptional factors, including IL6 and TNF $\alpha$ . Currently, the only reliable strategy in treating NAFLD is by reducing body weight, which can be achieved by increasing physical exercise and reducing the consumption of simple sugar and fats. Indeed, the role of cannabis in treating NAFLD seems promising and could provide new insights in the search for a new therapy for this chronic disease. This can be due to the effect of cannabis in ameliorating some metabolic syndromes related to NAFLD, in addition to its role in decreasing the activity of CB1 receptors and consequently the de novo synthesis of fatty acids. Despite several reviews in the literature that addressed the effectiveness of cannabis, very few recently published articles have comprehensively discussed the important role of cannabis use on CB2 activity. However, whether cannabis use is beneficial in treating NAFLD requires further investigation.

## Applications to other areas

In this chapter, we have reviewed the relation between the endocannabinoid system and NAFLD. An overwhelming number of studies have shown a high correlation between obesity and insulin resistance, which are considered some of the most common metabolic syndromes related to the development of NAFLD and increased levels of endocannabinoids, including 2-AG and anandamide, hence the overexpression of the CB1 and CB2 receptors in the endocannabinoid system. This in turn will stimulate fatty acid oxidation and downregulate the activity of fatty acid synthesis. Cannabis use in treating NAFLD is a promising approach, especially the use of CBD and THC, as these have been confirmed by some researchers to downregulate the expression of CB1 and consequently ameliorate NAFLD-related metabolic syndromes, including insulin resistance, hyperlipidemia, and T2DM.

Cannabis use, like THC, in treating other diseases, such as anorexia nervosa and AIDS, due to its orexigenic and anti-vomiting impact has been reported. It has also been found useful in treating chronic and neuropathic pain (Borgelt et al., 2013; Kirkham, 2009; Koppel et al., 2014; Maroon & Bost, 2018). We thus speculate that cannabis may not only be effective in treating NAFLD, but it could also be helpful in curing other diseases.

## Mini-dictionary of terms

- **Endoplasmic reticulum stress (ER stress):** defined as a chronic disorder that affects ER homeostasis and is characterized by lost protein accumulation and consequently disturbs the capacity of the ER to fold these proteins properly.
- **5' adenosine monophosphate-activated protein kinase (AMPK):** an enzyme involved in cellular energy homeostasis to stimulate oxidation and uptake glucose and fatty acids when cellular energy is reduced.
- **Oxidative stress:** defined as the imbalance between the antioxidant defense and the production of reactive oxygen species (ROS).
- **Fatty liver index (FLI):** a procedure used for fatty liver prediction, based on measuring gamma-glutamyl transferase (GTT), triglycerides, BMI, and waist circumference.
- **Adiponectin:** a hormone secreted by adipocytes that plays a key role in the modulation of lipid and glucose metabolism in the human tissues, which are known to be insulin-sensitive.

## Key facts of NAFLD

- Nonalcoholic liver disease (NAFLD) plays a crucial role in increasing morbidity and mortality rates worldwide.
- The annual projected burden of NAFLD on the economy is estimated around \$103 billion in the United States and €35 billion in Italy, the United Kingdom, Germany, and France combined.
- The most common causes of morbidity related to the liver are cirrhosis and hepatocellular carcinoma.
- Liver biopsy is the gold standard diagnostic tool for NAFLD.
- NADFLD cirrhosis is a principal indicator for liver transplantation.

## Summary points

- NAFLD is one of the commonest liver diseases in the world, and it contributes significantly to the increased incidence of HCC.
- NAFLD is a multifactorial disease that is highly associated with several metabolic syndromes, including hyperlipidemia, hypertension, T2DM, insulin resistance, and obesity.
- The pathogenesis of NAFLD ranges from simple steatosis to steatohepatitis, and it may end with the incidence of HCC.
- The reason for the occurrence of simple steatosis is an imbalance between the storage of lipid and liver clearance of fat.
- The role of CB1 in the pathogenesis of NAFLD is due to the high influx of triglycerides into the liver, which activates the expressions of CB1 receptors and stimulates some lipogenic transcriptional factors, including SREBP-1c and SDC1, and downregulating the expression of PPAR $\alpha$ .
- SREBP-1c and SDC1 play key roles in lipogenesis due to the activation of some lipogenic enzymes, including ACC and FAS, which in turn stimulate the de novo synthesis of fatty acids while playing a role in lipolysis.
- The role of CB2 receptors in NAFLD could be attributed to their protective effect during the presence of inflammation in NASH.
- Cannabis, including CBD and THC, can be promising in treating NAFLD, although their adverse effects should be considered.

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## Chapter 40

# Components of the endocannabinoid system: Hepatic expression levels of the cannabinoid receptors and microRNAs

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## Abbreviations

<b>2-AG</b>	2-arachidonyl glycerol
<b>ABHD</b>	alpha, beta-hydrolase
<b>ACC</b>	acetyl-coenzyme A carboxylase
<b>AEA</b>	N-arachidonoyl-ethanolamine, anandamide
<b>ALD</b>	alcohol-induced liver disease
<b>BMM</b>	bone marrow-derived monocytes/macrophages
<b>BTG2</b>	B-cell translocation gene 2
<b>CB1R</b>	cannabinoid receptor 1
<b>CBD</b>	cannabidiol (CBD)
<b>CBRs</b>	cannabinoid receptors
<b>CCI4</b>	carbon tetrachloride
<b>CNR</b>	cannabinoid receptor gene
<b>CNS</b>	central nervous system
<b>Con A</b>	Concanavalin A
<b>DAGL</b>	diacylglycerol lipases
<b>ECB</b>	endogenous cannabinoid, endocannabinoid
<b>ECS</b>	endocannabinoid system
<b>ER</b>	endoplasmic reticulum
<b>FAAH</b>	fatty acid amide hydrolase
<b>GalN</b>	D-galactosamine
<b>GEM</b>	gemcitabine
<b>GPCR</b>	G-protein-coupled receptor
<b>GPR55</b>	G-protein-coupled receptor 55
<b>HuR</b>	human antigen R
<b>LPI</b>	L- $\alpha$ -lysophosphatidylinositol
<b>LPS</b>	lipopolysaccharide
<b>MAGL</b>	monoacylglycerol lipase
<b>MAPK</b>	mitogen-activated protein kinase
<b>miRNA (miR)</b>	microRNA
<b>MMP-2</b>	matrix metalloproteinase-2
<b>mt</b>	mitochondria
<b>NAFLD</b>	nonalcoholic fatty liver disease
<b>NAPE-PLD</b>	<i>N</i> -acyl-phosphatidyl-ethanolamine-selective phospholipase D
<b>NF-<math>\kappa</math>B</b>	nuclear factor kappa B

<b>NLR</b>	nod-like receptor
<b>NLRP3</b>	nod-like receptor family pyrin domain containing 3
<b>NSCLC</b>	nonsmall-cell lung cancer
<b>PDAC</b>	pancreatic ductal adenocarcinoma
<b>PIP2</b>	phosphatidylinositol 4,5-bisphosphate
<b>PPARs</b>	peroxisome proliferator-activated receptors
<b>SIRT1</b>	sirtuin 1
<b>TGF-<math>\beta</math></b>	transforming growth factor-beta
<b>THC</b>	tetrahydrocannabinol
<b>TLR</b>	toll-like receptor
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor-alpha
<b>TRP</b>	transient receptor potential
<b>TRPA1</b>	transient receptor potential ankyrin-1
<b>TRPV</b>	transient receptor potential vanilloid
<b>T<math>\beta</math>R1</b>	transforming growth factor beta receptor 1
<b>WIN</b>	WIN55, 212-2
<b>YY1</b>	Yin Ying 1

## Introduction

This chapter covers the components of the endocannabinoid system (ECS) and progresses in elucidating the relationship between the hepatic expression of cannabinoid receptors, which are a component of ECS, and the expression of microRNAs (miRNAs). The ECS, also called the endogenous cannabinoid system, is a cell signaling system that begins in postsynaptic neurons and acts on presynaptic terminals. The ECS is a widespread neuromodulator system with important roles in central nervous system (CNS) development and response to endogenous and environmental attacks, as well as a widely distributed system in various tissues containing endogenous ligands and receptors (Crocq, 2020; Lu & Mackie, 2021). The ECS is very important for the organism as it plays a critical role in maintaining the homeostasis of the human body, which includes the brain, endocrine, and immune system (Borowska et al., 2018; Fitzgibbon et al., 2019). Moreover, studies in the last 10 years indicate that ECS may be a potential new target for the treatment of various cancer subtypes due to its role in both tumor formation and growth and tumor spread (Moreno et al., 2019; J. Yang et al., 2019).

When we look at the history of ECS, it is seen that ECS research gained momentum with the discovery of the structure of tetrahydrocannabinol (THC), the main psychoactive phytocannabinoid, by Mechoulam and Gaoni in 1964 (Crocq, 2020; Mechoulam & Gaoni, 1965). The identification and cloning of cannabinoid receptor 1 (CB1R) in 1992 led to the discovery of its first endogenous agonist, N-arachidonoyl-ethanolamine (AEA, anandamide) (Devane et al., 1992). ECS consists of three components. These are cannabinoid receptors (CBRs), endogenous cannabinoid (ECB) ligands, and enzymes responsible for the biosynthesis, transport, and breaking down of ECBs (Lu & Mackie, 2016).

## What are the components of the endocannabinoid system?

Although ECS is known to contain several accepted ECBs such as AEA and 2-arachidonoyl glycerol (2-AG) and two major CBRs (CB1R and CB2R), GPR55 has recently emerged as a putative “type 3” CBR (Crocq, 2020; H. Yang et al., 2016). The components of the ECS system are listed in the Table 1. The first discovered components of ECS were CBRs, which are THC target sites in the brain. The first cloned receptor, CB1R, was observed to cause psychotropic effects in the CNS, while the second identified ECS receptor, CB2R, was found in non-CNS regions, mostly in cells of the immune system (Munro et al., 1993). Chromosomal localization of cannabinoid receptor gene 1 (*CNR1*) is known in humans as chromosome 6 (6q14-q15). Cannabinoid receptor gene 2 (*CNR2*) is located on chromosome 1 (1p35-p36.1). *CNR1* encodes three isoforms of CB1R protein (60 kDa). *CNR2* encodes multiple isoforms of CB2R protein (40 kDa) (Lutz, 2002; Moreno et al., 2019). These two receptors belong to a family of A-class rhodopsin-like G-protein-coupled receptors (GPCR), the most extensive human membrane protein family with exceeding 800 members in the human proteome (Munro et al., 1993; Shimada et al., 2019).

In 2007, Ryberg et al. found that orphan GPR55 showed binding with some cannabinoids and non-cannabinoid ligands. Thus, it was suggested that GPR55 could be a candidate to be considered as the “third” CBR (Ryberg et al., 2007; H. Yang et al., 2016). Cannabinoids bind not only to classical CBRs but also to certain orphan receptors (GPR18 and GPR119), transient receptor potential (TRP) ion channels, and peroxisome proliferator-activated receptors (PPARs) (Luschnig & Schicho, 2019).

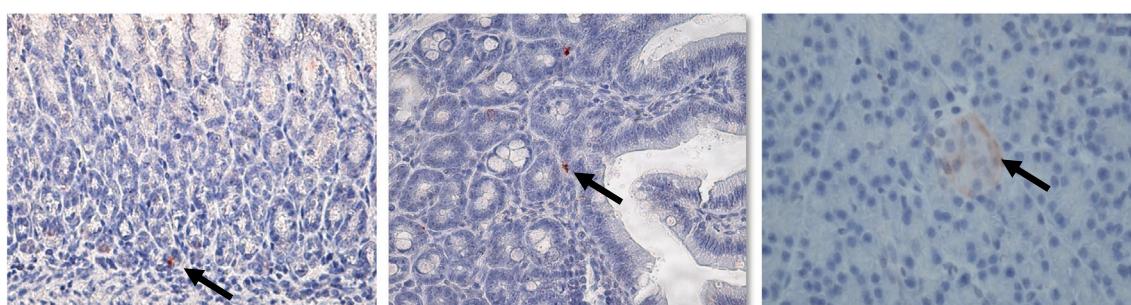
**TABLE 1** Components of endocannabinoid system.

Endocannabinoid system		
Cannabinoid receptors	Endocannabinoids (Endogenous cannabinoids)	Enzymes
<b>Main receptors</b>		
CB1R	AEA	NAPE-PLD
CB2R	2-AG	DAGL $\alpha$
GPR55 (a putative “type 3” CBR)		DAGL $\beta$
<b>Other receptors</b>		
GPR18		FAAH
GPR119		MAGL
Ion channels such as TRPV1, TRPV2, and TRPA1		ABHD6
PPARs		ABHD12

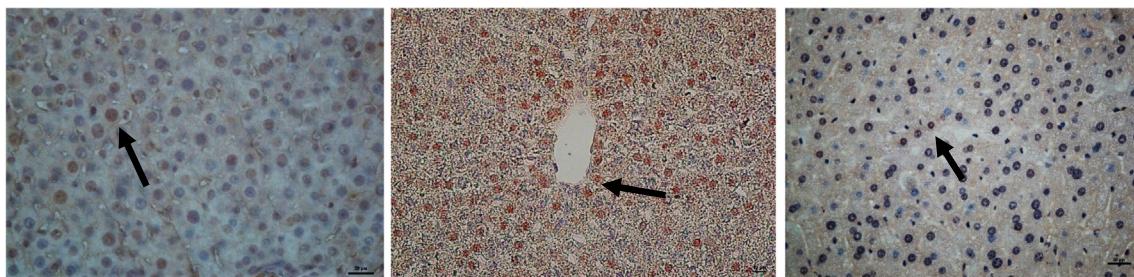
The first identified ECB, AEA, was found in 1992, and the other ECB, 2-AG, was found in 1995 (Devane et al., 1992; Mechoulam et al., 1995). ECBs are signaling lipids that activate CBRs. Although both AEA and AG-2 contain arachidonic acid, their synthesis and degradation pathways differ. Arachidonic group hydrolysis of 2-AG or AEA causes termination of the ECB signal. Most AEA appears to be produced from N-arachidonoyl phosphatidyl ethanol (NAPE), whereas fatty acid amino hydrolase (FAAH) primarily terminates the action of AEA. 2-AG is produced from primarily arachidonoyl-containing phosphatidylinositol 4,5-bisphosphate (PIP2), and then these are metabolized by the diacylglycerol lipases (DAGL  $\alpha$  and DAGL  $\beta$ ), but its hydrolysis is mainly carried out by monoacylglycerol lipase (MAGL) at the highest levels presynaptically or by alpha/beta-hydrolase (ABDH6) found mostly in dendrites (Blankman et al., 2007; Cravatt et al., 2001; Marrs et al., 2010; Murataeva et al., 2014).

## Cannabinoid receptors and microRNAs in all organs

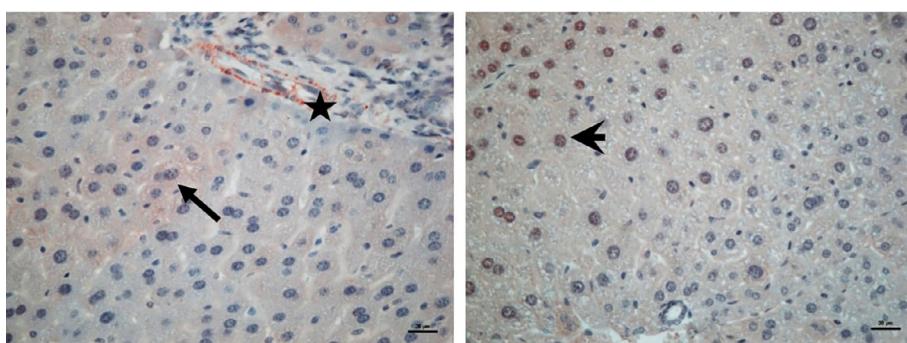
As mentioned above, the effects of endocannabinoids are primarily mediated by CB1R and CB2R. Both CB1R and CB2R are greatly expressed in the immune cells, glands, tissues, and organs, as well as the CNS (Joshi & Onaivi, 2019). Although the CB1R is mostly expressed in CNS, especially in the cerebellum, hippocampus, cortex, and basal ganglia, it is also found in the periphery (Figs. 1 and 2). Unlike CB1R, CB2R is found in low amounts in the CNS. The CB2R is predominantly present in peripheral tissues of the immune system (spleen, thymus, etc.) (Galiègue et al., 1995). It has been shown that CBRs are not only localized on plasma membranes, but also in intracellular compartments in the endoplasmic reticulum (ER), endosomes, lysosomes, mitochondria (mt), and nuclei (Hebert-Chatelain et al., 2017) (Fig. 3). CB1R and CB2R both inhibit adenylyl cyclase and certain voltage-dependent calcium channels and activate several mitogen-activated protein



**FIG. 1** CB1R immunopositive cells in different tissues. CB1R immunopositive cells (arrow) are shown in the stomach, duodenum, and pancreas of control rats from left to right, respectively. Streptavidin–biotin–peroxidase technique. Counterstain, hematoxylin (The total Magnifications X400).



**FIG. 2** CB1R immunopositive cells in liver. CB1R immunopositive signals (arrow) are seen in healthy adult rat and neonatal rat and mouse liver, particularly in the nuclear region, from left to right, respectively. Streptavidin–biotin–peroxidase technique. Counterstain, hematoxylin.



**FIG. 3** CB2R immunopositive cells in liver. Cytoplasmic (arrow) and nuclear CB2R immunopositive cells are localized (arrowhead) in healthy mice liver. CB2R immunopositive cells are also seen in portal triad (asterisk). Streptavidin–biotin–peroxidase technique. Counterstain, hematoxylin.

kinases (MAPK) and various types of potassium channels depending on the particular type of cell. For this reason, the activation of these receptors leads to various consequences on cellular physiology, consisting of gene transcription, cell motility, etc. (Howlett et al., 2002).

miRNAs, short RNA molecules 19–25 nucleotides in size, are a class of small, endogenous, evolutionarily conserved RNAs that regulate posttranscriptional gene expression (Lu & Rothenberg, 2018). miRNAs were first discovered in 1993 by two different research groups simultaneously as a posttranscriptional regulator of gene expression in the nematode *Cae-norhabditis elegans* (*C. elegans*). One of these groups, Lee et al. (1993) found that the LIN-14 protein, which is essential for the progression of postembryonic developmental events, is downregulated in these organisms. They observed that the transcription of a second gene called *lin-4* reduces the expression of the target gene. Another of these groups showed that multiple sites in the 3' UTR of *lin-14* mRNA bind to the *lin-4* RNAs in a cooperative manner to decrease the LIN-14 protein expression (Lee et al., 1993; Wightman et al., 1993). Later, *let-7* RNAs were discovered in adult in *C. elegans*. *Let-7* homologs were subsequently recognized in many other organisms, including mammals (Pasquinelli et al., 2000).

Only 1%–2% of human genes are the most regulated by miRNAs. However, over 3000 encoded mature miRNAs are found in the human genome. Hundreds of mRNAs can bind to a single miRNA, and so miRNA can affect the expression of many genes included in a functional interactive pathway (Lu & Rothenberg, 2018; miRTarBase, 2021). It was shown that miRNAs play a significant role in physiological processes and pathologies, including cancer, cardiovascular and metabolic diseases. On the other hand, some miRNAs have been reported to have the potential to be developed into new therapeutic targets by key pathogenic pathways involved in allergic inflammation (Correia de Sousa et al., 2019; Lu & Rothenberg, 2018). Regulation of mRNA expression by miRNAs is mostly cell or organ-specific but may vary according to the stress and metabolic state of the organism (Correia de Sousa et al., 2019).

The studies showed that there is an interaction between GPCRs and miRNAs, and also miRNA regulates the expression levels of some GPCR (Mandal et al., 2021). Furthermore, evidences suggested that miRNAs also play an important role in GPCR signaling (Nohata et al., 2017). Interestingly, in contrast, researchers reported that THC, a cannabinoid receptor agonist, causes downregulation of miR-185, which is associated with an increase in proapoptotic gene targets (Mohammed et al., 2020). Thus, miRNAs can be considered to play an important role in the expression of CBRs. X. He et al. (2020) revealed that AM1241, a CB2R agonist, attenuates Parkinson's disease through upregulation of the Xist/miR-133b-3p/Pitx3 axis. According to Notaro et al. (2019), a synthetic agonist of cannabinoid receptors, WIN55,212-2 (WIN), affects cell migration, dependent on miR-29b1, and so it could be a potential therapeutic agent

in the treatment of osteosarcoma. Although recent studies have predicted that the relationship between CBRs and miRNA can guide the treatment of various diseases, more work is needed in this area.

The other CBR, GPR55, is expressed in various tissues involved in regulating energy expenditure and intake, such as the pancreas, gastrointestinal tract (colon, stomach, intestine, etc.), hypothalamus, and adipose tissues (Lin et al., 2011; Romero-Zerbo et al., 2011; Ryberg et al., 2007). In addition, the mRNA and protein expressions of this receptor have been demonstrated in the livers of both rodents and humans (Lipina et al., 2019; Moreno-Navarrete et al., 2012). The localization of GPR55 in these tissues indicates that GPR55 plays a role in maintaining energy homeostasis. Furthermore, GPR55 and its endogenous ligand L- $\alpha$ -lysophosphatidylinositol (LPI) have been reported to play a role in various cancers (Emmanouilidi et al., 2020; Ferro et al., 2018; D. He et al., 2015).

## Expression states and relationships of cannabinoid receptors and microRNAs in the liver

It is emphasized that CBRs can be an important target in liver diseases as in many other diseases (Mallat & Lotersztajn, 2008). Unfortunately, it is still controversial whether CBRs antagonists or agonists are effective in treatment. A study by Kojima et al. (2009) showed that SR141716A, a CB1R antagonist, is hepatoprotective with respect to a Concanavalin A (Con A)-induced mouse liver injury. On the other hand, targeting CB1R and CB2R using exogenous and endogenous cannabinoids has been found to modulate experimental autoimmune hepatitis (Hegde et al., 2008). A study using hepatocyte-specific CB1R-null mice (hCNR1 $^{−/−}$ ) suggested that CB1R antagonists may be hepatoprotective against toxin-induced liver damage (Kim et al., 2020).

Studies of the last decade provide clear evidence that miRNAs are abundant in the liver and modulate various liver functions. It has been suggested that dysregulation of miRNA expression may be an important pathogenetic factor in many liver diseases, including hepatocellular cancer, polycystic liver diseases, and viral hepatitis (Chen, 2009). After mir-122, which was the first miRNA identified in the liver, many other miRNAs such as miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, and let-7 family were also identified. It was determined that they are abundantly expressed in adult liver tissue. However, it has been reported that the liver shows a different miRNA expression profile during its development (J. Chang et al., 2004; Girard et al., 2008).

According to L. Yang et al. (2020), CB1R expression showed an increase in the liver injury model, while CB1R blockade by AM281, a CB1R antagonist, reduced activation of Nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome and liver inflammation. In addition, the level of hepatic miR-30b-5p increased by CB1R blockade. Depending on these findings, researchers suggested that miR-30b-5p may alleviate liver inflammation by targeting NLRP3. The study by Azar et al. (2020) showed that CB1R blockade may contribute to the amelioration of hepatic steatosis, caused by diet-induced obesity, depending on PPAR $\alpha$  and sirtuin 1 (SIRT1). Moreover, modulation of the hepatic expressions and activities of PPAR $\alpha$  and SIRT1 by the CB1R has been suggested to be controlled by a p53/miR-22 signaling pathway. In another study, it was reported that the level of CB1R mRNA expression in diabetic rats may be inversely proportional to the level of miR-33a expression, but its relationship with miR-122 was not reported (Coskun et al., 2019). It is thought that melatonin may ameliorate alcohol-mediated hepatic bile acid synthesis via the CB1R-B-cell translocation gene 2 (BTG2)-Yin Ying 1 (YY1) signaling pathway by increasing miR-497 expression (Kim et al., 2017). In a chronic liver injury study, it is reported that a CB1R agonist, ACEA, can induce bone marrow-derived monocytes/macrophages (BMM) migration. The human antigen R (HuR) and miR-29 family have also been observed to competitively regulate CB1 expression and increase ACEA-induced BMM migration (N. Chang et al., 2020).

When we look at the interaction studies of CB2R and miRNAs, they are less in number than CB1R. A miRNA microarray analysis study revealed that JWH-133, a CB2R agonist, treatment changed a few miRNAs in the liver mononuclear cells. Among these miRNAs, miR-145, in particular, was observed to be most markedly decreased. Thus, researchers suggested that JWH-133 may protect mice from D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced acute liver failure by regulating miRNA expression, which acts as a regulator of Toll-like receptor (TLR) signaling (Tomar et al., 2015). Similarly, Wu et al. (2019) showed that activation of CB2R by JWH-133 inhibits transforming growth factor beta (TGF- $\beta$ ) receptor 1 (T $\beta$ R1) through miR-27b and thus contributes to the prevention of TGF- $\beta$ -mediated hepatocyte damage. Consistently, Ali et al. (2021) reported that AM1241, a synthetic analog of CB2R, has strong hepatoprotective potential in thioacetamide-induced liver fibrosis. The researchers emphasized that this effect may be due to a reduction in the hepatic TLR4/miR-155 signaling pathway, which controls NF $\kappa$ B p65/tumor necrosis factor-alpha (TNF- $\alpha$ ) signaling. As a result, there is certainly a relationship between miRNAs and CBRs in the liver. However, it is seen that few studies have not been able to fully reveal this relationship.

When we look at the relationship of GPR55 with miRNAs, we found only a few studies on cancer. According to a study by Ferro et al. (2018), GPR55 inhibition decreased pancreatic cancer cell growth. The researchers administered cannabidiol

(CBD), a GPR55 antagonist, in addition to gemcitabine (GEM), one of the most widely used drugs to treat pancreatic ductal adenocarcinoma (PDAC), into a mouse model of PDAC and allowed the mice to survive three times longer. They also suggested that the tumor suppressor p53 regulates GPR55 protein expression through modulation of the miR34b-3p. Another study was with non-small-cell lung cancer (NSCLC) cells. In the study, cell growth and proliferation, colony formation, migration, and invasion were promoted with downregulation of miR-675-5p. Moreover, it was suggested that overexpression of miR-675-5p may cause the downregulation of GPR55 and its signaling pathway. Researchers reported that GPR55 can be a target gene of miR-675-5p (D. He et al., 2015).

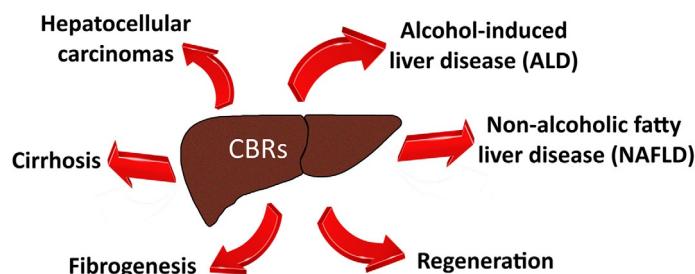
## CBRs in liver diseases

Both CB1R and CB2R expressions are low in the healthy liver, these receptors play a significant role in the pathophysiology of liver diseases including alcohol-induced liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), and cardiovascular alterations associated with cirrhosis, etc. (Caraceni et al., 2008) (Fig. 4). Patsenker et al. (2011) reported that SR141716, a CB1R antagonist, protects against alcoholic-induced fibrosis by inhibiting CB1R activation. On the contrary, CB2R activation inhibits alcohol-induced inflammation and steatosis by regulating Kupffer cell activation that are resident macrophages of the liver and protects against ALD. Also, CB2R activation in macrophages prevents alcohol-induced steatosis by reducing hepatic inflammation via an autophagy-dependent pathway (Denaës et al., 2016).

NAFLD is a disease spectrum ranging from simple steatosis to inflammatory steatohepatitis, a condition that can progress to cirrhosis. An in vitro study showed low levels of CB1R expression in the human hepatocyte carcinoma cell line (HepG2), whereas it was high in fatty liver cells. Researchers suggested that CB1R inhibition improves lipogenesis in an in vitro NAFLD model (Shi et al., 2014). Consistently, the study by Jorgačević et al. (2019) demonstrated that a CB1R antagonist, rimonabant, modulated plasma insulin and glucose concentrations as well as hepatic interleukin (IL-6) and interferon gamma (IFN- $\gamma$ ). It is suggested that CB1R blockade may be useful for the treatment of NAFLD in mice as it can regulate the adipokine profile and proinflammatory cytokines in adipose and liver tissues as well as glucose metabolism. In contrast to CB1R, the role of CB2R in the development of fatty liver has not been adequately studied. In the study on human liver biopsies with a diagnosis of NAFLD, Mendez-Sanchez et al. (2007) reported that CB2R is found in hepatocytes in NAFLD but not healthy liver. In a recent study, it has been reported that the GPR55-LPI system has a role in the development of NAFLD. They suggested that GPR55 expression is elevated in liver damage and LPI increased lipid levels in hepatocytes by inducing GPR55 and activation of acetyl-coenzyme A carboxylase (ACC) (Fondevila et al., 2021).

A chronic liver injury study showed that CB1R blockade modulated neutrophil infiltration and liver inflammation via Goxi/o/ROS/p38 MAPK signaling pathway in carbon tetrachloride (CCl4)-induced mice (Zhou et al., 2020). On the other hand, JWH-133 treatment accelerated liver regeneration and decreased liver injury by increasing TNF- $\alpha$  and IL-6 and reducing matrix metalloproteinase-2 (MMP-2). Thus, CB2R agonists have hepatoprotective properties (Teixeira-Clerc et al., 2010). CB1R was found to be increased in samples from patients with liver fibrosis and mouse models. However, CB1R blockade with JD5037 has been reported to reduce activation of CB1R-regulated hepatic stellate cells and liver fibrosis by suppressing  $\beta$ -arrest1/Akt signaling (Tan et al., 2020). Julien et al. (2005) suggested that CB2R activation displays antifibrogenic properties via inducing growth inhibition and apoptosis. Moreover, Suk et al. (2016) drew attention to the opposing effects of CB1R and CB2R in hepatocarcinogenesis. Researchers have stated in their studies that CB1R antagonists or CB2R agonists can prevent the development of hepatocellular carcinomas. Accumulating evidence indicates that CB1R and GPR55 antagonism and CB2R agonism may be involved in liver disease and related complications.

In summary, it is known that the endocannabinoid system has a role in hepatic physiology and pathological conditions. Studies have shown that CBRs are found in many tissues, including the liver. It has been suggested that various miRNAs



**FIG. 4** Several instances associated with cannabinoid receptors (CBRs).

found in the liver and also they may be promising targets for the development of strategies to identify, prevent, or treat liver diseases. Therefore, consideration of CBRs and associated miRNAs may be recommended because of their effects on the development and progression of liver diseases pathogenesis. In the future, CBR agonists/antagonists may be preferred for their protective benefits in liver diseases.

## Mini-dictionary of terms

- **Agonist.** a substance or drug that binds to the receptor on the cell surface or inside the cell and induces a similar response as the substance that normally binds to the receptor.
- **Antagonist.** a substance or drug that binds to the receptor on the cell surface or inside the cell and induces an adverse reaction with the substance that normally binds to the receptor.
- **Central nervous system.** The central nervous system, the body's processing center, consists of two parts: the brain and the spinal cord. This system is responsible for unifying and coordinating all bodily functions, processing all incoming nerve messages, and sending commands to different parts of the body.
- **Endogenous ligands.** A ligand is any molecule (e.g., neurotransmitter, hormone, drug) that binds to a site on a target protein. Ligands originating from an organism, tissue, or cell are called endogenous ligands.
- **G-protein-coupled receptor.** These receptors, known as seven-(pass)-transmembrane domain receptors, are integral membrane proteins and are found only in eukaryotes. They are used by cells to convert extracellular signals into intracellular responses, such as responses to hormones, neurotransmitters, vision, olfaction, and taste signals.
- **G-protein-coupled receptor 55 (GPR55).** The receptor, which is widely expressed in both brain and peripheral tissues, was firstly identified as a putative cannabinoid receptor because of its affinity for cannabinoid ligands.
- **Liver.** An organ of the digestive system, the liver, is found only in vertebrates. This organ detoxifies various metabolites, synthesizes proteins, and produces biochemicals necessary for digestion and growth.
- **MicroRNA (miRNA, miR).** miRNAs are short non-coding RNAs 19–25 nucleotides in size that play important gene regulatory roles in eukaryotes by pairing with the mRNAs of protein-coding genes to direct their post-transcriptional repression.

## Key facts of cannabinoid receptors

- Cannabinoid receptors are members of the G-protein-coupled receptor superfamily.
- There are two main cannabinoid receptors as CB1R and CB2R in the endocannabinoid system.
- The CB1 receptors are one of the most abundant G-protein-coupled receptors in the CNS.
- The CB2 receptors are found predominantly in cells and tissues of the immune system
- Recently, the GPR55 appeared as a putative “type 3”.

## Key facts of microRNA

- microRNA is one of the classes of small endogenous RNA molecules.
- microRNAs are functional in translational silencing and posttranscriptional regulation of gene expression
- miRNAs are found in many mammalian cell types as well as in extracellular circulating miRNAs.
- Circulating miRNAs are released into biofluids, including serum, saliva, and cerebrospinal fluid.
- Circulating miRNAs have the potential to be used as biomarkers in a number of diseases.

## Key facts of liver disease

- Consisting of four lobes, the liver is the largest internal organ in the human body.
- Apart from synthesizing and storing certain macromolecules, the functions of the liver include filtering blood from the digestive tract, metabolizing drugs, and detoxifying chemicals, as well as regulating blood sugar levels.
- There are many types of liver diseases caused by viruses, drugs, poison, or too much alcohol.
- Some miRNAs may have a curative effect on liver diseases by targeting various signaling molecules.
- Cannabinoid receptor agonist/antagonist can be used to treat some liver diseases.

## Summary points

- Cannabinoid receptors, members of the G-protein-coupled receptors family, are found in the liver.
- MicroRNAs, which are involved in the regulation of gene expression, are also associated with cannabinoid receptors.
- It has been stated that dysregulation of miRNAs, which are abundant in the liver, is important in terms of liver diseases.
- The expressions of CB1R and CB2R are associated with liver disease, such as liver steatosis, nonalcoholic fatty liver disease, and chronic liver injury.
- It has been suggested that some miRNAs control cannabinoid signaling by regulating the expression of CBRs.

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## Chapter 41

# Tumor growth and the endocannabinoid system: Investigating CB<sub>2</sub> agonists

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## Abbreviations

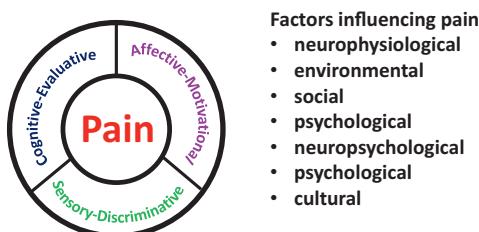
<b>AEA</b>	arachidonoyl ethanolamine
<b>AKT</b>	protein kinase B
<b>Ang</b>	angiogenesis
<b>Apo</b>	apoptosis
<b>CBD</b>	cannabidiol
<b>CDP</b>	cannabis-derived product
<b>CB1</b>	cannabinoid receptor 1
<b>CB2</b>	cannabinoid receptor 2
<b>CIPN</b>	chemotherapy-induced peripheral neuropathy
<b>COX-2</b>	cyclooxygenase 2
<b>CXCR4</b>	chemokine receptor 4
<b>Δ<sup>9</sup>-THC</b>	delta-9 tetrahydrocannabinol
<b>ERK</b>	extracellular signal-regulated kinase
<b>EGF</b>	epidermal growth factor
<b>EGFR</b>	epidermal growth factor receptor
<b>GABA</b>	gamma-aminobutyric acid
<b>GIRK</b>	G-protein-regulated inward-rectifying potassium channels
<b>ID-1</b>	inhibitor of differentiation 1
<b>IGF-IR</b>	insulin-like growth factor type 1 receptor
<b>IP</b>	intraperitoneal
<b>IV</b>	intravenously
<b>JWH-133</b>	CB2 agonist
<b>JWH-015</b>	CB2 agnoist
<b>MMP2</b>	matrix metalloproteinase 2
<b>NMDA</b>	<i>N</i> -methyl-D-aspartate
<b>NSAIDs</b>	nonsteroidal anti-inflammatory drugs
<b>PAG</b>	periaqueductal gray
<b>PEG2</b>	prostaglandin E2
<b>PKC</b>	protein kinase C
<b>PSNL</b>	partial sciatic nerve ligation
<b>PT</b>	peritumoral
<b>SNRI</b>	serotonin norepinephrine reuptake inhibitor
<b>SR1</b>	SR141716A
<b>SR2</b>	SR144528
<b>SSRI</b>	selective serotonin reuptake inhibitor
<b>2-AG</b>	2-arachidonoyl glycerol
<b>US</b>	United States
<b>WIN-2</b>	WIN55,212-2 (mixed CB1/CB2 agonist)

## Introduction

Economic and societal impact of cancer is tremendous, accounting for nearly 10 million deaths worldwide in 2022 (Siegel et al., 2022). The prediction for 2022 in the United States estimates that 1,918,030 new cancer cases and 609,360 cancer deaths are projected to occur (Siegel et al., 2022). Incidence during 2014 through 2018 continued with a slow increase for female breast cancer (by 0.5% annually), but remained stable for prostate cancer (Siegel et al., 2022). Effective treatment and management of cancer are critical for cancer patients. Development of safe and effective treatments that improve cancer therapy and alleviate chemotherapy-induced peripheral neuropathy (CIPN) unwanted side effects of treatment with chemotherapeutic agents remains an important clinical need.

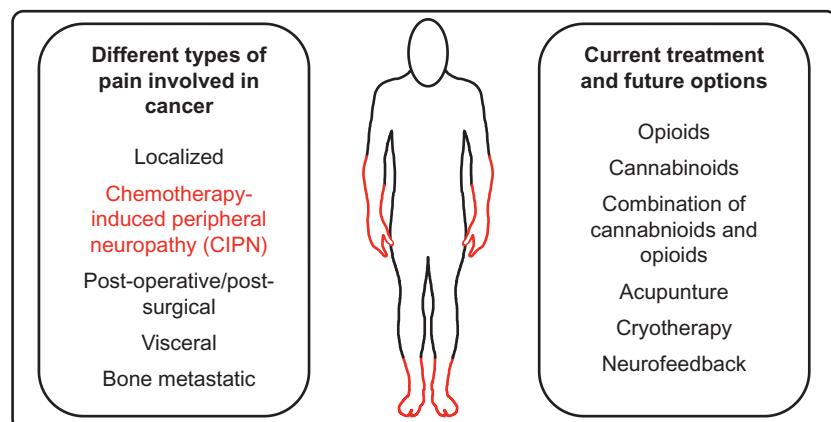
Pain theory has evolved greatly throughout the years (Guindon & Hohmann, 2009). The most recent definition of pain by the international association for the study of pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with, or resembling that associated with actual or potential tissue damage” (Raja et al., 2020). Currently, pain is associated with three components: sensory-discriminative, affective-motivational, and evaluative-cognitive (Fig. 1) (Guindon & Hohmann, 2009). The sensory component is associated with the intensity and sensory aspect of pain, the affective component referred to the affective part that captures how bad or unpleasant it feels and is related to the emotions associated with this pain, and the evaluative component corresponds to the evaluation of the pain and involves the cognitive functions to address the situation (Fig. 1) (Guindon & Hohmann, 2009). However, it is important to emphasize that pain remains a personal, complex, and multidimensional experience that is influenced by different factors (social, cultural, environmental, neurophysiological, neuropsychological, psychological, and/or psychophysical) (Fig. 1).

Indeed, different types of cancer will generate difference in pain experiences (McHann et al., 2021). These differences in pain experiences are associated with the types of cancer (chemotherapeutic agents, radiation, surgical removal, and hormonal therapy) treatments received, localization of the tumors, compression or not of nerves and/or internal organs (Fig. 2) (McHann et al., 2021). The different stages of cancer progression (such as metastasis to bone, spinal cord, or bowel obstruction) can also influence the experience of pain (McHann et al., 2021). Localized pain referred to pain felt in one part or specific area of the body (Fig. 2). Postoperative/postsurgical pain will be felt, for example, after breast cancer surgical removal such as pain at the incision site (Guindon & Hohmann, 2009) (Fig. 2). Visceral pain is defined as pain emanating from the internal thoracic, pelvic, or abdominal organs (Guindon & Hohmann, 2009). Chemotherapy-induced peripheral neuropathy (CIPN) is occurring in cancer patients after treatment with chemotherapeutic agents (Fig. 2), which



**FIG. 1** Dimensions of pain and influencing factors. Pain comprises three components: sensory-discriminative, affective-motivational and evaluative-cognitive.

**FIG. 2** Different types of pain and treatment options for cancer related pain. Schematic representation of different types of pain experienced in cancer conditions is illustrated as well as current treatment and future options.



are characterized by three classes (platinums, taxanes, and vinca alkaloids) (Blanton et al., 2019). Moreover, pain from bone metastasis is felt from metastasis in the bone and is described by patients, as slowly increasing over a period until it becomes unbearable (McHann et al., 2021). Therefore, there is a critical and important clinical need to develop new safe and effective therapies that reduce cancer treatments and chemotherapy-induced peripheral neuropathy (CIPN) side effects from chemotherapeutic agents.

Cannabis has been used for more than 12,000 years for different purposes. It is one of the oldest documented medicines in history. One of the earliest known references to the use of cannabis for medicinal purposes dates to its appearance in a Chinese pharmacopoeia in 2800 BCE (Blanton et al., 2019). The ever-increasing number of states with legalized medical (37) and adult-recreational (19) cannabis use have likely contributed to these increases in reported use (Al-Shammari et al., 2017; NORML, 2022a; NORML, 2022b; Sandler et al., 2019). Cannabinoid-based therapies are increasingly being used by cancer patients to treat chemotherapy-induced nausea and vomiting (Blanton et al., 2019; Sexton et al., 2021). In recent years, cannabis and associated products have increased in availability and decreased in cost, and a softening of prior social stigma around the use of cannabis has taken place. Within the states having medical cannabis programs, chronic pain is the most common qualifying medical condition reported by patients (Blanton et al., 2019).

Cannabinoids are implicated in a variety of physiological and pathological conditions including inflammation, immunomodulation, analgesia, cancer, and others (Blanton et al., 2021; Blanton et al., 2022). Cannabis main active ingredient, delta-9 tetrahydrocannabinol ( $\Delta^9$ -THC), produces its effects through activation of G-protein-coupled CB1 and CB2 receptors (Blanton et al., 2021; DiMarzo, 2006). Endocannabinoids are endogenous lipid-signaling molecules that are generated in the cell membrane from phospholipid precursors (DiMarzo, 2006). They bind and activate one or more cannabinoid receptor subtypes, thus producing cannabimimetic properties (Blanton et al., 2021; DiMarzo, 2006). Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the two best studied endocannabinoids (DiMarzo, 2006).

Cannabinoids have recently been shown to produce antitumor actions (cell proliferation and cell survival) in different models of cancer (Table 1). Animal models have been developed to assess experimentally pathophysiological mechanisms implicated in the clinical aspect of cancer. Animal models provide insight into pathophysiological processes of cancer (disease state) and help elucidate mechanisms of action that could be targeted by new drug discovery efforts aimed to identify new therapeutic targets (Guindon & Hohmann, 2009). Preclinical models are necessary to evaluate and validate therapeutic efficacy of new pharmacotherapies. The mechanisms by which cannabinoid agonists and cannabinoid (CB) receptors type 1 (CB1) and type 2 (CB2) impact proliferation, migration, and apoptosis in preclinical cancer models are discussed in the section below.

## Cannabinoids breast cancer *in vivo* preclinical studies

In recent *in vivo* studies, the focus has shifted to mostly CB<sub>2</sub> selective agonists as they have shown the most promise in reducing tumor size, metastasis, proliferation, and angiogenesis, using different cannabinoids, cell lines, and mice (Table 1) (Caffarel et al., 2010; Nasser et al., 2011; Qamri et al., 2009). CB<sub>2</sub> agonists also do not have the unwanted psychotropic effects that cannabinoids such as  $\Delta^9$ - THC can produce (Guindon & Hohmann, 2008). For example, JWH-133 decreases tumor growth and metastasis in CB-17 immune-compromised mice with three different cell lines (Qamri et al., 2009) (Table 1). The CB<sub>2</sub> action on tumor growth was confirmed in this study in two ways: in the first part they use JWH-133 as a CB<sub>2</sub> selective agonist and then block the CB<sub>2</sub> receptor showing tumor growth increase (Qamri et al., 2009). JWH-133 was also used in MMTV-neu mice in an ErbB2 breast cancer model and was also able to decrease tumor size, metastasis, angiogenesis, and proliferation (Table 1) (Caffarel et al., 2010). Another group used a different CB<sub>2</sub> agonist called JWH-015 in FVB mice with NT2.5 cells (Nasser et al., 2011). They found that the primary tumor expressed CB<sub>2</sub> receptors and that JWH-015 was successful at decreasing the primary tumor size and proliferation (Nasser et al., 2011). In a study using nu/nu mice with MCF-7 cells and SUM159 cells, it was found that JWH-015 was successful at treating the primary tumors and decreasing proliferation (Table 1) (Elbaz et al., 2017). JWH-133 and JWH-015 are not currently being used recreationally or clinically.

However, CBD is a clinically and recreationally available cannabinoid used in preclinical (Table 1) and clinical studies. Studies have shown that CBD can reduce tumor size, metastasis, proliferation, and angiogenesis in several different mouse models. In BalB/c mice using MDA-MB-231 cells, 4 T1 cells, 4 T1.2 cells, or MVT-1 cells, CBD was found to decrease the primary tumor size, metastasis, angiogenesis, and proliferation (Table 1) (Elbaz et al., 2015; Ligresti et al., 2006; McAllister et al., 2011). Moreover, O-1663 was used for its selectivity for CB<sub>2</sub> receptors, and when compared with CBD, its antitumor action is significantly more potent (Murase et al., 2014).

**TABLE 1** Cannabinoids breast cancer *in vivo* preclinical studies.

Compound	Dose and Route	Mouse Strain	Tumor Induction (cell line)	Tumor		Metastasis				Mediated by		References
				Size	Number	Size	Number	Proliferation (pro)/ Apoptosis (apo)/ Angiogenesis (ang)	Mechanism	CB <sub>1</sub>	CB <sub>2</sub>	
JWH-133	5 mg/kg p.t.	CB-17 immuno	MDA-MB-231 flank	↓ by JWH-133	–	–	–	JWH-133 ↓ pro, ↓ ang	↓ COX2 expression, ↓ PGE2 levels	–	SR2	Qamri et al. (2009)
JWH-133	5 mg/kg i.p.	CB-17 immuno	MDA-MB231-luc-D3H2LN lateral vein	–	–	↓ by JWH-133	↓ by JWH-133	–	↓ COX2 expression, ↓ PGE2 levels	–	–	Qamri et al. (2009)
JWH-133	5 mg/kg i.p.	PyMT	NA	↓ by JWH-133	–	–	–	JWH-133 ↓ pro, ↓ ang	↓ COX2 expression, ↓ PGE2 levels	–	–	Qamri et al. (2009)
JWH-133	0.05 mg p.t.	MMTV-neu	NA	↓ by JWH-133	↓ by JWH-133	↓ by JWH-133	–	JWH-133 ↓ pro, ↑ apo, ↓ ang	↓ Akt, ↓ MMP2	Not SR1 pro	SR2 pro	Caffarel et al. (2010)
JWH-015	5 mg/kg p.t.	FVB	NT 2.5 Cells Fourth Mammary Pad	↓ by JWH-015	–	–	–	JWH-015 ↓ pro				
	↓ CXCR4 Phosphorylation, ↓ Erk Phosphorylation	–	JWH-015	Nasser et al. (2011)								
JWH-015	10 mg/kg p.t.	Nu/Nu	SUM159 Fourth Mammary Pad	↓ by JWH-015	–	–	–	JWH-015 ↓ ang	↓ EGF/EGFR, ↓ IGF-IR, ↓ ERK, ↓ Akt	–	JWH-015	Elbaz et al. (2017)
JWH-015	10 mg/kg p.t.	Nu/Nu	SUM159 Fourth Mammary Pad	↓ by JWH-015	–	–	–	JWH-015 ↓ ang	↓ EGF/EGFR, ↓ IGF-IR, ↓ ERK, ↓ Akt	–	JWH-015	Elbaz et al. (2017)
Δ <sup>9</sup> -THC	0.5 mg p.t.	MMTV-neu	NA	↓ by Δ <sup>9</sup> -THC	↓ by Δ <sup>9</sup> -THC	–	↓ by Δ <sup>9</sup> -THC	Δ <sup>9</sup> -THC ↓ pro, ↑ apo, ↓ ang	↓ Akt, ↓ MMP2	Not SR1 pro	SR2 pro	Caffarel et al. (2010)
Δ <sup>9</sup> -THC	45 mg/kg i.p.	Nu/Nu	T47D, BT4T4, MDA-MB-231 Subcutaneous	Δ <sup>9</sup> -THC no Δ	–	–	–	–	–	–	–	Blasco-Benito et al. (2018)

$\Delta^9$ -THC	45 mg/kg i.p.	Nu/Nu	T47D, BT4T4, MDA-MB-231 Subcutaneous	$\Delta^9$ -THC no $\Delta$	–	–	–	–	–	–	–	–	Blasco-Benito et al. (2018)
WIN-2	5 mg/kg p.t.	CB-17 immuno	MDA-MB-231 flank	↓ by WIN-2	–	–	–	WIN-2 ↓ pro, ↓ ang					
	↓ COX2 expression, ↓ PGE2 levels	AM251	SR2	Qamri et al. (2009)									
JWH-133 + WIN-2	5 mg/kg i.p.	CB-17 immuno	MDA-MB231-luc-D3H2LN lateral vein	–	–	↓ by WIN-2	↓ by WIN-2	–	↓ COX2 expression, ↓ PGE2 levels	AM251	SR2	Qamri et al. (2009)	
CBD	5-6.5 mg/kg s.c.	Athymic	MDA-MB-231 dorsal side	↓ by CBD	–	–	–	–	–	–	–	–	Ligresti et al. (2006)
CBD	5-6.5 mg/kg s.c.	BalB/C	MDA-MB-231 paw	–	–	–	↓ by CBD	–	–	–	–	–	Ligresti et al. (2006)
CBD	1-5 mg/kg i.p.	BalB/C	4 T1 orthotopically	↓ by CBD	–	↓ by CBD	↓ by CBD	CBD ↓ pro	↓ Id-1 expression, ↓ S phase, ↑ G0/G1	–	CBD	McAllister et al. (2011)	
CBD	1 mg/kg i.p.	BalB/C	4 T1 tail vein (i.v.)	–	–	↓ by CBD	↓ by CBD	CBD ↓ pro	Partial ↓ Id-1 expression	–	CBD	Murase et al. (2014)	
CBD	1 mg/kg i.p.	BalB/C	4 T1 orthotopically	CBD no $\Delta$	–	↓ by CBD	↓ by CBD	CBD ↓ pro	Partial ↓ Id-1 expression	–	CBD	Murase et al. (2014)	
CBD	1 mg/kg i.p.	Athymic nu/nu	MDA-MB231 tail vein (i.v.)	–	–	↓ by CBD	↓ by CBD	CBD ↓ pro	↓ Id-1 expression	–	CBD	Murase et al. (2014)	
CBD	10 mg/kg i.p.	BalB/C	4 T1.12 + MTU1 fourth mammary gland	↓ by CBD	–	↓ by CBD	↓ by CBD	CBD ↓ pro, ↓ ang	Induces EGF, Activates EGFR and ERK, Decreases MΦ Recruitment	–	CBD	Elbaz et al. (2017)	
CDP	45 mg/kg i.p.	Nu/Nu	T47D, BT4T4, MDA-MB-231 Subcutaneous	↓ by CDP	–	–	–	CDP ↓ pro	↑ Reactive Oxygen Species				
	–	CDP	Blasco-Benito et al. (2018)										
O-1663	1 mg/kg i.p.	BalB/C	4 T1 tail vein (i.v.)	–	–	↓ by O-1663	↓ by O-1663	O-1663 ↓ pro	Complete ↓ Id-1 expression	–	O-1663	Murase et al. (2014)	

↑, Increase; ↓, decrease; no  $\Delta$ , no change; –, not tested; NA, not applicable; *ang*, angiogenesis; *apo*, apoptosis; *pro*, proliferation; *i.p.*, intraperitoneal; *i.v.*, intravenously; *p.t.*, peritumoral; *s.c.*, subcutaneous; *BalB/C mice*, albino mice that are immunodeficient; *CB1*, cannabinoid receptor 1; *CB2*, cannabinoid receptor 2; *CBD*, cannabidiol; *CDP*, cannabis-derived product; delta-9-tetrahydrocannabinol; *FVB mice*, genetically engineered immunocompetent mice prone to blindness and susceptible to friend leukemia virus B; *immuno*, immunodeficient; *PyMT mice*, transgenic mice developing mammary gland tumors; *MMTV-neu mice*, genetically engineered mice of ErbB2 (tyrosine kinase receptor)-driven metastatic breast cancer; *MΦ*, macrophage; *Nu/Nu mice*, immunodeficient nude mice; *SR1*, SR141716A; *SR2*, SR144528;  $\Delta^9$ -THC, WIN-2, WIN55,212-2.

Since CB<sub>2</sub> agonist influences breast cancer tumor growth, it is important to investigate dual agonists (WIN55,212-2) and partial agonists ( $\Delta^9$ - THC). When WIN55,212-2 was administered to MDA-MB-231 cells in CB-17 immune-compromised mice, it was effective in the similarly as JWH-133; however, it was less potent (Table 1) (Qamri et al., 2009).  $\Delta^9$ - THC, however, was used in MMTV-neu mice in the same ErbB2 breast cancer model as described previously (Caffarel et al., 2010). In this model,  $\Delta^9$ -THC was effective in minimizing primary tumor size, angiogenesis, and metastasis to the lungs (Caffarel et al., 2010). On the contrary in a study comparing  $\Delta^9$ -THC and CDP (cannabis-derived product), on several different cell lines (Table 1), it was found that  $\Delta^9$ -THC had no effect on the tumor, proliferation, angiogenesis, or metastasis (Blasco-Benito et al., 2018). Whereas they did find significant effect with CDP showing a decrease in tumor growth with each cell line (Blasco-Benito et al., 2018). Overall, the current preclinical in vivo studies seem to show that the role of CB<sub>2</sub> has a major role in tumor size, angiogenesis, proliferation, and metastasis when compared with CB<sub>1</sub>. Although most of the studies mentioned above suggest that CB2 (JWH-133, JWH-015, and O-1663) or mixed (WIN55,212-2,  $\Delta^9$ -THC) agonists decrease tumor size, angiogenesis, and metastasis (Table 1), further studies are needed to assess the role of cannabinoid agonists in affecting tumor growth, angiogenesis, and metastasis. Tumor size, hormones, stressors, and environmental factors could influence negatively or positively tumor growth.

Nonetheless, other types of cancer such as head and neck mouse model (cell line) have shown that CB<sub>2</sub> agonists increase tumor growth (Liu et al., 2020). Furthermore, ovarian cancer studies in SCID mice have also shown that JWH-133 (CB<sub>2</sub> agonist), administered chronically for 30 days in an established cancer (30 days of tumor growth prior to cannabinoid administration), significantly increases tumor growth (Blanton et al., 2022).

## Cannabinoids in clinical cancer studies

Cannabinoids have been clinically researched for their potential in managing emesis, cancer pain, and cachexia in cancer patients (Fig. 2). Recent studies have found patient-reported benefits in sleep disorders, fatigue, anxiety and depression, and nausea/vomiting (Schleider et al., 2018; Sexton et al., 2021). While not currently a first-line treatment for chemotherapy-induced nausea and vomiting (CINV), the synthetic tetrahydrocannabinol dronabinol has been found to perform similarly to conventional treatments, due to the greater incidence of adverse effects (Fig. 2). However, it is currently only recommended for refractory cases of CINV (Badowski, 2017; Rock & Parker, 2016; Smith et al., 2015). With regard to cancer pain, the research has been more divided as to the efficacy of cannabinoids.

A randomized controlled clinical trial (RCT) by the Portenoy group found that Nabiximols (Sativex), an oral spray consisting of  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) in a 1:1 ratio, provided a small but statistically significant benefit to cancer pain (Portenoy et al., 2012). This effect was supported by reviews and meta-analyses, which also found a similar effect for cannabis and synthetic forms of THC in managing cancer pain (Darkovska-Serafimovska et al., 2018; Wang et al., 2021). However, a second set of RCTs by the Fallon group failed to find any significant difference from placebo in the management of cancer pain by Nabiximols. Moreover, other meta-analyses similarly found either no statistically significant benefit of Nabiximols, cannabis, and synthetic forms of THC or raised concerns regarding bias and the quality of evidence in the trials that they examined (Blake et al., 2017; Fallon et al., 2017; Fisher et al., 2021). Similarly, despite long-held associations of cannabinoids with increased appetite, repeated meta-analyses and RCTs have found no benefit of cannabis, THC, Nabiximol, Dronabinol, or Nabilone, another synthetic THC, in managing cancer-related cachexia (Strasser et al., 2006; Kirkham, 2009; Mücke et al., 2018; Simon et al., 2022).

Inspired by promising preclinical research involving the therapeutic potential of cannabinoids for solid tumors, including breast cancer, a recent clinical study examined the effect of CBD on tumor size/presence, disease progression, and median survival on 119 patients (Kenyon et al., 2018). In this study, 92% of the 39 patients with breast cancer showed a clinical response to CBD. Cannabinoids have also begun to be evaluated for the management of other cancers, including various brain cancers and leukemias. A pilot study found that intratumor injections of THC caused regression of glioblastoma multiforme (GBM) tumors in two of nine patients (Guzmán et al., 2006). Later clinical trials involving Nabiximols as adjuvant to Temozolamide chemotherapy found similarly promising results in the management of GBM; further clinical trials are underway for the use of cannabinoids as both adjuvant and monotherapy for GBM (Dumitru et al., 2018). Additionally, a case report found that two pediatric patients had regression of their pilocytic astrocytoma tumors following management of inhaled cannabis (Foroughi et al., 2011). With regard to leukemia, a case report of a 14-year-old patient showed dose-dependent management of their acute lymphoblastic leukemia with oral cannabinoid extracts (Singh & Bali, 2013). The results of these studies and the lack of RCTs involving cannabinoids and cancer highlight the need for further research into the mechanisms of action for the antitumor effects of cannabinoid compounds.

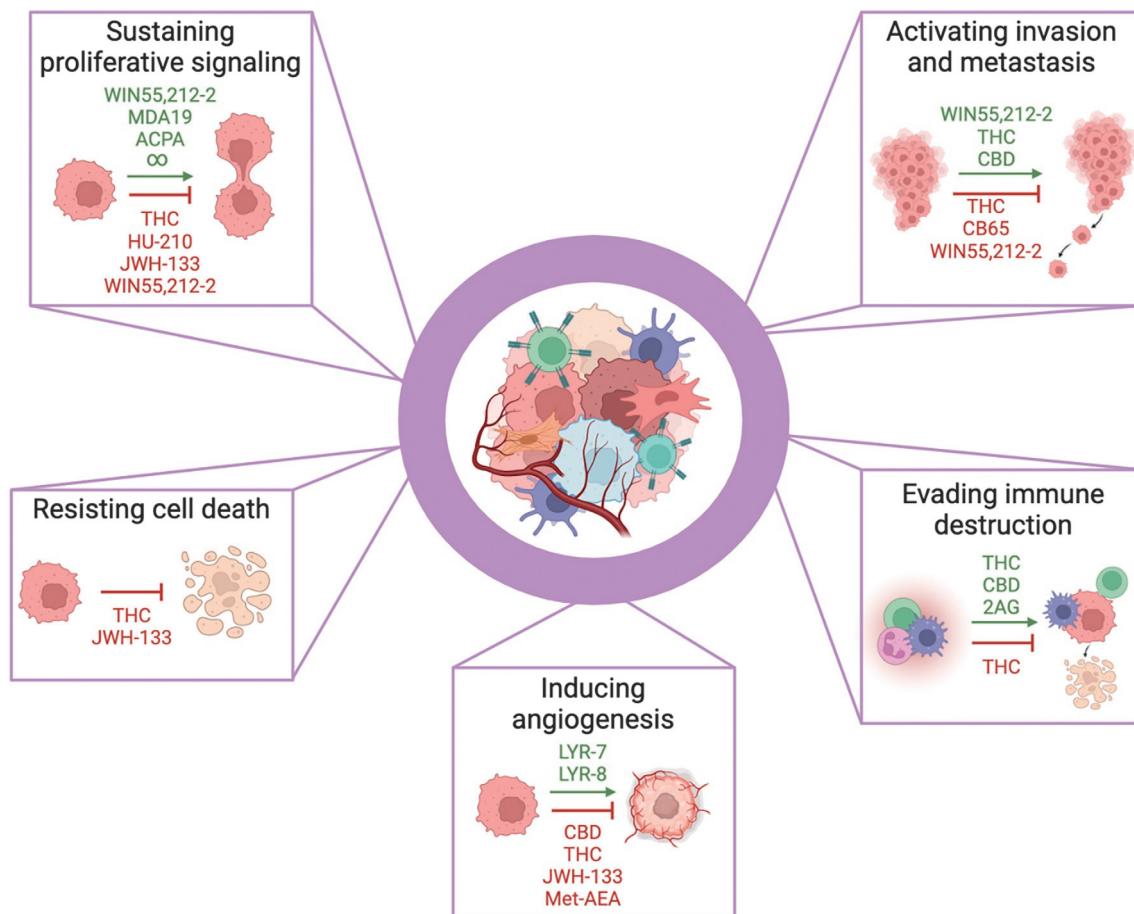
## Cannabinoids mechanisms of action in cancer

In order to understand the transition of a human cell to a neoplastic state, it is noted that these cells will acquire new functional capabilities, which allow them to form malignant tumors. These new capabilities are known as the hallmarks of cancer (Hanahan & Weinberg, 2000), as the knowledge of cancer mechanisms has grown and new facets have emerged as potential hallmarks. In 2000, six hallmarks were proposed, which include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Fig. 3) (Hanahan & Weinberg, 2000). In 2011, these hallmarks were expanded to include: altering energy metabolism, evading immune destruction, tumor-promoting inflammation, and genome instability and mutation (Hanahan & Weinberg, 2011). In 2022, new additional emerging hallmarks were incorporated: unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells (Hanahan, 2022).

A considerable number of reports are suggesting a role of cannabinoids in tumor hallmarks (Hinz & Ramer, 2022; Pagano et al., 2021). One of the first reports dated in the mid-1970s showing that THC,  $\Delta^8$ -THC, and cannabidiol reduced lung adenocarcinoma *in vivo* (Munson et al., 1975).

One of the most characteristic features of cancer cells involves their ability to maintain chronic proliferation known as “sustaining proliferative signaling.” Noncancer cells perform an exquisite control of the cell cycle and growth-promoting pathways to ensure the tissue homeostasis and function. Cancer cells can deregulate signaling pathways to assure progression through the cell cycle as well as cell growth independently of external signals such as growth factors relaying in proliferative pathways such as: AKT, MAPK/ERK, and/or MTOR (Hanahan & Weinberg, 2000).

Cannabinoids through CB<sub>2</sub> receptor and/or CB<sub>1</sub> receptor mediate the inhibition of AKT and inhibit tumor growth in syngenic tumor models as ErbB2-positive breast cancer exposed to  $\Delta^9$ -THC (Caffarel et al., 2010), human gastric cancer



**FIG. 3** Cannabinoids and the hallmarks of cancer. Schematic illustration of the multiple roles that cannabinoids play in the pathogenesis of cancer and modulate several key hallmarks of cancer: sustaining of the proliferative signaling, resisting cell death, inducing angiogenesis, activation of invasion and metastasis, and evasion of the immune response.

exposed to WIN 55,212-2 (Xian et al., 2010), hepatocellular carcinoma exposed to MDA19, a CB<sub>2</sub> agonist (Rao et al., 2019), and small cell lung cancer exposed to ACPA (Arachidonoylcyclopropylamide), a synthetic CB1R-specific ligand (Fig. 3) (Boyacioglu et al., 2021).

CB<sub>2</sub> antiproliferative action has been associated also to affect cell arrest by blocking the transition of the G1/S in human breast cancer by THC (Caffarel et al., 2006) and prostate cancer by WIN 55,212-2 (Roberto et al., 2019). Δ<sup>9</sup>-THC can also reduce cell cycle progression in hormone-sensitive (HS) and hormone-resistant (HR) breast cancer cell lines by reduction of Cdc2 leading to a stop of the cell cycle at the G2-M phase (Fig. 3) (Kiskova et al., 2019).

Conversely, CB<sub>2</sub> was also linked to an increase of tumor progression in human papillomavirus-positive squamous cell carcinomas of the head and neck in vivo (Liu et al., 2020) and in lung, gastric, ovarian, and colon cancer cell lines in vitro (Fig. 3) (Blanton et al., 2022; Hart et al., 2004; Martinez-Martinez et al., 2016; Miyato et al., 2009).

Cancer cells evade apoptosis altering mechanisms that detect cell damage or irregularities, and preventing cell death, this hallmark is known as “resisting cell death” (Hanahan & Weinberg, 2000). Interestingly, CB<sub>2</sub> has been associated with induction of apoptosis through de novo ceramide synthesis in glioma cells (del Pulgar et al., 2002; Sanchez et al., 2001) and activating endoplasmic reticulum stress-related genes such as p8 and its downstream endoplasmic reticulum stress-related targets activating transcription factor 4 (ATF-4) and TRB3 in pancreatic cancer cells (Fig. 3) (Carracedo et al., 2006). In breast cancer cell lines, both receptor-positive and receptor-negative, CBD induced apoptosis in a concentration-dependent manner via activation of the intrinsic apoptotic pathway and increasing the generation of reactive oxygen species (ROS) (Shrivastava et al., 2011).

Cancer cells will stimulate the formation of new blood vessels from preexisting blood vessels in order to supply nutrients to support tumor growth, known as angiogenesis. Several studies suggest an antiangiogenic effect of CB<sub>2</sub>, for example, CB<sub>2</sub> agonist (JWH-133) regulates hypoxia-angiogenesis markers associated with vascular endothelial growth factor (VEGF) pathway in gliomas (Fig. 3) (Blazquez et al., 2004). Interestingly, CBD inhibits angiogenesis thought inhibition of MMP-2 expression in human endothelial cells (HUVEC) (Solinas et al., 2012). Similarly, the exposure of HUVEC to conditioned media of lung cancer cells (A549) treated with CBC, Δ<sup>9</sup>-THC, Met-AEA, and JWH-133 conferred them antiangiogenic properties by the induction of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) leading to a reduction of migration, tube formation as well as sprout formation (Fig. 3) (Ramer et al., 2014).

Intriguingly, hexahydrocannabinol analogs, LYR-7 and LYR-8, inhibit endothelial (HUVEC) and breast cancer cell growth (MCF7 and tamoxifen-resistant MCF7 cell lines), as well VEGF-induced angiogenesis, but neither CB<sub>2</sub> antagonist AM630 nor CB<sub>2</sub> antagonist AM281 rescued the phenotype suggesting that the antiangiogenic activity of both LYR analogs may be induced by an cannabinoid receptor-independent mechanism (Thapa et al., 2011).

Cancer cells invade adjacent local tissue and/or disseminate to distant sites by two distinct mechanisms known as invasion and metastasis, respectively. Literature indicates an inhibitory effect of CB<sub>2</sub> on tumor invasion and metastasis via modulation of the extracellular matrix proteolysis by Δ<sup>9</sup>-THC in glioma cells (Blazquez et al., 2008), mixed agonist (WIN 55,212-2) in osteosarcomas (Notaro et al., 2019), or CB<sub>2</sub> agonists (CB65) in hepatocarcinoma (Fig. 3) (Pourkhalili et al., 2013). CB<sub>2</sub>-mediated antiinvasive effect has been published using CB<sub>2</sub> agonists in osteosarcomas (Notaro et al., 2019) and breast cancers (Murase et al., 2014) or inhibitor studies (Ramer et al., 2012; Ramer & Hinz, 2008).

Cancer cells’ adaptation mechanism to evade the host’s immune system by bypassing normal mechanisms of immune check point control, this hallmark is known as “evading immune destruction.” New-generation sequencing studies, specifically single-cell sequencing, revealed that Δ<sup>9</sup>-THC significantly affects transcriptomic profile in immune cell sub-clusters altering genes involved in immune response, cytokine production, cell proliferation, and apoptosis (Fig. 3) (Hu et al., 2020). In pancreatic cancer cells, cannabinoids (CBD and Δ<sup>9</sup>-THC) reduce the expression of a key target of immune checkpoint blockage (Programmed Death Ligand 1, PD-L1). Furthermore, in a pancreatic orthotopic mouse model, 2AG induced maturation of dendritic cells, proliferation myeloid-derived suppressor cells with no effect on the population of CD8(+) T cells and CD4(+) T cells (Qiu et al., 2019).

In contrast, other studies using murine mammary cell carcinomas 4 T1 and EMT6 reported that Δ<sup>9</sup>-THC increases breast cancer tumor growth and promotes tumor cell spread by inhibiting the antitumor immune response (McKallip et al., 2005).

The mechanisms through which cannabinoids/cannabinoid receptors impact proliferation, migration, and apoptosis of cancer cells are quite complex, and our understanding of these processes remains incomplete. Moreover, these mechanisms differ according to the types of cancer, and both pro- and anti-apoptotic effects of cannabinoids have been reported. However, the basic research findings are far from being completely understood, and further research is warranted to better understand the complexity of dynamic changes in the endocannabinoid system in cancer. One of the reasons for this complexity is likely attributable to the highly interactive nature of lipid signaling pathways, which recruit different mechanisms of action. Several mechanisms are likely to underline the pro- and anti-apoptotic effects of cannabinoids and explain their role in cancer.

## Conclusion and limitations

The need for additional clinical trials of cannabinoid therapeutic efficacy in cancer patients appears beyond doubt. Indeed, only few studies have evaluated the effect of cannabinoids on tumor growth or in alleviating cancer, in contrast to the extensive literature supporting efficacy of cannabinoids as antiemetics. Furthermore, future research studies need to explore the therapeutic potential of multimodal analgesic strategies that combine cannabinoids with other commonly used medications (opioids) (Fig. 2). The use of different pharmacotherapies in combination may increase the likelihood of synergistic interactions between compounds with multiple distinct mechanisms of action. Literature reports show a role of CB<sub>2</sub> in the transition to a neoplastic state, but contradictory data also indicate anticancer action. Future studies must be aimed to elucidate the mechanisms of action of CB<sub>1</sub>, CB<sub>2</sub>, and mixed (CB<sub>1</sub>/CB<sub>2</sub>) agonists in cancer. Further basic pre-clinical studies on cannabinoids anti- and/or pro-apoptotic effects and their role on tumor growth are critically needed. Moreover, clinical trials evaluating cannabinoid efficacy and their effect on tumor growth are required before cannabinoid use can be established and accepted as effective and safe adjunct to cancer therapy.

## Applications to other areas

Cannabis has been used for more than 12,000 years for different purposes. Medical cannabis is legalized in 37 states and is increasingly used by cancer patients. This increase is due to the softening of social stigma and beneficial effects of cannabinoids in cancer patients by the media. Societal and economic impact of cancer is tremendous, accounting, in 2022, for nearly 10 million deaths worldwide. This translational review of the endocannabinoid system and its role in cancer therapy highlight the need to develop new safe and effective treatments that improve cancer therapy and alleviate chemotherapy-induced peripheral neuropathy (CIPN) side effects. It is a critical and important medical need and to investigate cannabinoid compounds beyond their known antinausea/vomiting effects in cancer patients. Indeed, few clinical studies have shown their ability to alleviate CIPN in cancer patients. Similarly, only few preclinical and clinical studies have investigated the impact of cannabinoid compounds on tumor growth. With the limited number of preclinical and clinical studies on cannabinoids and their effects (positive or negative) on tumor growth, it is not currently possible to draw any conclusions. Indeed, the basic research findings are far from being completely understood, and further research is warranted to better understand the complexity of dynamic changes in the endocannabinoid system in cancer. One of the reasons for this complexity is likely attributable to the highly interactive nature of lipid signaling pathways, which recruit different mechanisms of action. Several mechanisms are likely to underline the pro- and anti-apoptotic effects of cannabinoids and explain their role in cancer.

## Mini-dictionary of terms (5–15 terms) (A–Z order)

- **Analgesia.** This is a removal of sensitivity to pain; when one takes an analgesic medication they are reducing the sensation of pain.
- **Angiogenesis.** Tumor angiogenesis is the growth of new blood vessels that tumors need to grow.
- **Apoptosis.** Type of cell death in which a series of molecular steps in a cell lead to its death. It is a method the body uses to get rid of abnormal cells. This process may be blocked in cancer cells.
- **Cancer.** Disease marked by uncontrolled cell division and cell death emerging from cumulative damage of important regulatory genes. Multiple genes likely need to be damaged in order for a cancer to grow and develop the ability to spread (i.e., metastasize).
- **Cannabis.** main active ingredient, delta-9 tetrahydrocannabinol ( $\Delta^9$ -THC), produces its effects through activation of G-protein-coupled CB<sub>1</sub> and CB<sub>2</sub> receptors.
- **Analgesia.** This is a removal of sensitivity to pain; when one takes an analgesic medication, it reduces the sensation of pain.
- **Chronic Pain.** This form of pain is long-term pain that persists after tissue injury should have healed and does not serve an adaptive function to the organism. This pain isn't a symptom of an injury or disease but rather its own medical condition requiring unique approaches to treatment from acute pain.
- **Neuropathy.** A state in which damage to peripheral nerves can create motor weakness, numbness, and painful sensations of burning, tingling, or throbbing, especially in the hands and feet.
- **Proliferation.** Increase in the number of cells resulting from cell growth and cell division.
- **Tumor.** Abnormal mass of tissue forming when cells grow and divide more than they should or don't die as they are supposed. Tumors can be benign (not cancer) or malignant (cancer).

## Key facts of cannabinoids and tumor growth

- Cancer pain affects millions of people worldwide
- Medical cannabis is legalized in 37 states
- Cannabinoid-based therapies are used increasingly in cancer patients for nausea/vomiting
- Cannabinoid compounds are used to alleviate chemotherapy-induced peripheral neuropathy (CIPN)
- Preclinical studies have shown that cannabinoids can reduce or promote cancer
- Few clinical studies have been conducted and demonstrated beneficial effects of cannabinoids on cancer
- Only few preclinical and clinical studies have investigated the impact of cannabinoids on tumor growth
- Cannabinoids show promise in cancer therapies, but no consensus in their anti or protumor effect due to limited number of studies
- Further research needs to investigate the mechanisms of action of cannabinoids in cancer

## Summary points

- This chapter focuses on the effects of cannabinoids on cancer pain
- Cannabinoid-based therapies are effective and used in cancer patients for nausea/vomiting
- Recent studies show the use of cannabinoid compounds to alleviate chemotherapy-induced peripheral neuropathy (CIPN)
- Preclinical studies are limited and have shown that cannabinoids can reduce or promote cancer
- Only few clinical studies have been conducted and demonstrated beneficial effects of cannabinoids on cancer
- There is a lack of preclinical and clinical studies investigating the impact of cannabinoids on tumor growth
- Cannabinoid compounds are promising in cancer therapies with no consensus for their anti- or pro-tumor effects due to limited number of studies
- Critical need for further research to investigate the mechanisms of action of cannabinoids in cancer

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## Chapter 42

# Linking endocannabinoid system, palmitoylethanolamide, and sarcopenia in view of therapeutic implications

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## Abbreviations

<b>2-AG</b>	2-arachidonoylglycerol
<b>AEA</b>	anandamide
<b>ATP</b>	adenosine triphosphate
<b>BMP</b>	bone morphogenetic proteins
<b>CB1</b>	cannabinoid receptor 1
<b>CB2</b>	cannabinoid receptor 2
<b>COX-2</b>	cyclooxygenase 2
<b>DAPI</b>	4',6-diamidino-2-phenylindol
<b>eCBs</b>	endocannabinoids
<b>ECS</b>	endocannabinoid system
<b>EWGSOP</b>	European Working Group on Sarcopenia in Older People
<b>FAAH</b>	fatty acid hydrolase
<b>FAEs</b>	fatty acid ethanolamides
<b>FAPs</b>	fibro/adipogenic progenitors
<b>GPRs</b>	G-protein-coupled receptors
<b>GRAS</b>	generally recognized as safe
<b>IGF1</b>	insulin-like growth factor 1
<b>IL-1h</b>	interleukin-1h
<b>INOS</b>	inducible nitric oxide synthase
<b>LMM</b>	low muscle mass
<b>LMS</b>	low muscle strength
<b>LPP</b>	low physical performance
<b>mTORC1</b>	mammalian target of rapamycin complex 1
<b>MyHC</b>	myosin heavy chain
<b>NAAA</b>	N-acylethanolamine-hydrolyzing acid amidase
<b>NAPE</b>	N-acyl-phosphatidylethanolamine
<b>NAPE-PLD</b>	N-acyl-phosphatidyl-ethanolamine-selective phospholipase D
<b>NMJ</b>	neuromuscular junction
<b>NPPE</b>	N-palmitoyl-phosphatidyl-ethanolamide
<b>OEA</b>	N-oleoylethanolamide
<b>PEA</b>	N-palmitoyl ethanolamine
<b>PGC1a</b>	PPAR gamma coactivator-1a
<b>PGE2</b>	prostaglandin E2
<b>PLD</b>	phospholipase D
<b>PLGA</b>	poly(lactic-co-glycolic acid)
<b>PPAR<math>\alpha</math></b>	peroxisome proliferator-activated receptors alpha
<b>PPRE</b>	peroxisome proliferator response element
<b>ROS</b>	reactive oxygen species

<b>RXR</b>	retinoid X receptor
<b>SLNs</b>	solid lipid nanoparticles
<b>TGF<math>\beta</math></b>	transforming growth factor beta
<b>TNF<math>\alpha</math></b>	tumor necrosis factor-alpha
<b>TRPV1</b>	transient receptor potential vanilloid 1
<b>UPS</b>	ubiquitin-proteasome pathway

## Introduction

The relationship between the Endocannabinoid System (ECS) and sarcopenia passes through the regulation of palmitoyl-ethanolamide (PEA), an endocannabinoid-like molecule, whose role in the disease therapy is extremely promising. To understand all the steps of this rational, the function and structure of skeletal muscle represent a necessary preamble.

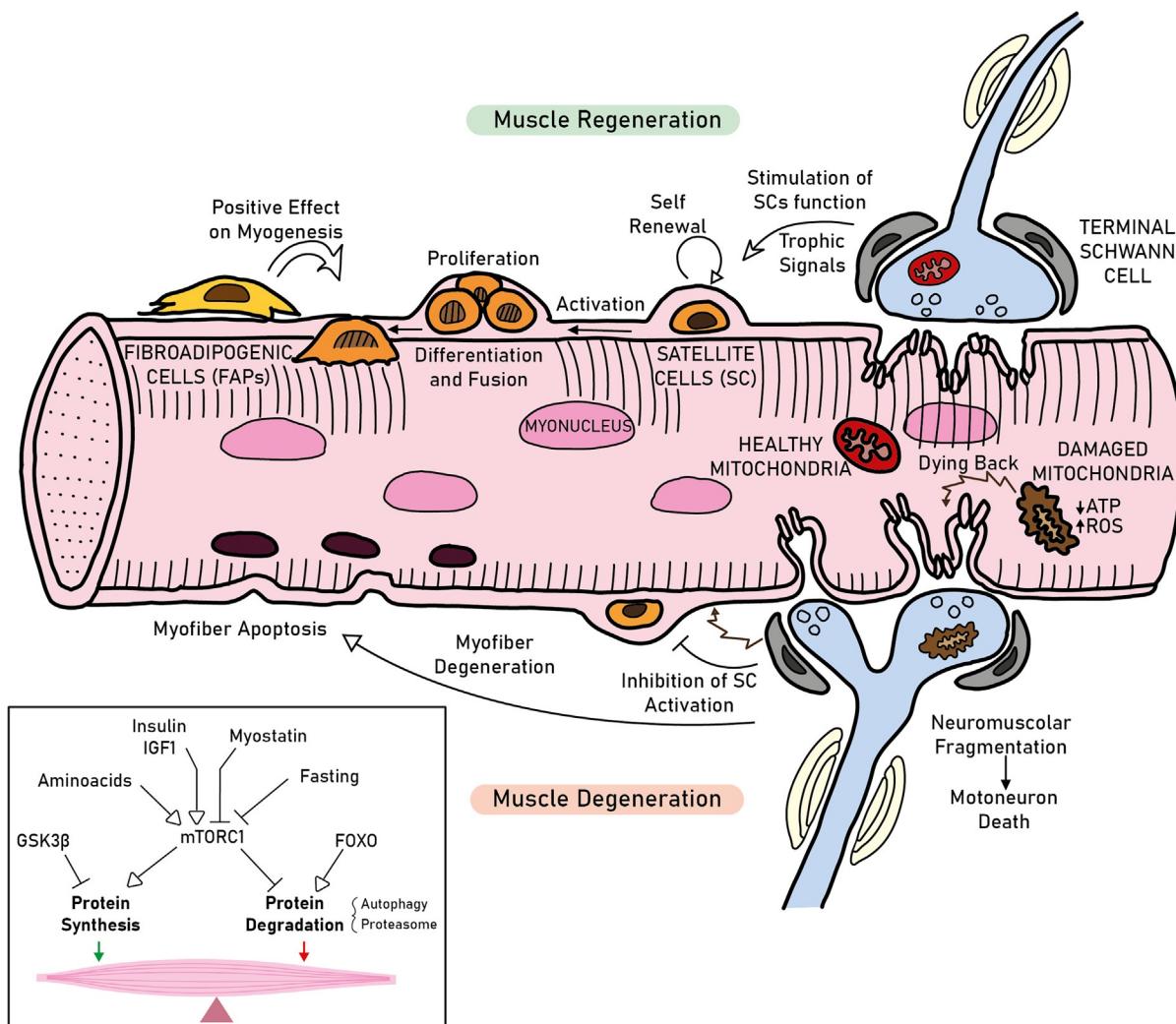
Skeletal muscle tissue plays key roles as the motor for voluntary movements and in the maintenance of posture; in addition, it regulates metabolic homeostasis, being the major site of glucose and fatty acids utilization, given that it accounts for about 30%–40% of body mass in healthy individuals. Skeletal muscles are composed of cylindrical multinucleated cells called myofibers, which, in vertebrates, are highly heterogeneous. It is possible to distinguish type I slow twitch, type II fast twitch (IIA, IIX, and IIB in rodents) myofibers, classified according to the expressed myosin heavy chain (MyHC) isoenzyme ([Schiaffino & Reggiani, 2011](#)). Skeletal myofibers differ for their contractile kinetics and metabolic properties: type I and IIA are mainly oxidative, type IIX and IIB glycolytic. The different types of myofibers are established during development, independently from external hints. Notably, adult muscle is a highly plastic tissue that can be remodeled both in term of mass and fiber type by changing work demands. Maintenance of skeletal muscle mass and function (homeostasis) not only ensures a good quality of life and preserves physical independence, but also protects toward metabolic diseases and is considered to work as a disease modifier ([Sartori et al., 2021](#)).

## Skeletal muscle homeostasis

Muscle homeostasis is the result of a fine balance between anabolic and catabolic processes that are controlled by several signaling pathways. A prominent role is played by the insulin/insulin-like growth factor 1 (IGF1) or transforming growth factor beta (TGF $\beta$ )/Activin/bone morphogenetic proteins (BMP) cascades. Muscle hypertrophy is stimulated by exercise, amino acids, insulin, and hormones released during muscle contraction that induces increased protein synthesis, which is controlled by the mammalian target of rapamycin complex 1 (mTORC1) pathway. Muscle protein catabolism is mediated by two main degradation systems, the ATP-dependent ubiquitin-proteasome pathway (UPS) (responsible for degrading 80%–90% of the proteins) and the (macro)autophagy pathway that is mainly implicated in the clearance of dysfunctional organelles ([Lilienbaum, 2013](#)). Homeostasis of muscle cells is ensured by myocyte apoptosis, the process of programmed cell death and by the activity of a small population of resident muscle stem cells, known as satellite cells ([Mauro, 1961](#)). Satellite cells are quiescent in resting muscle, but they are primed for activation upon an injury or in response to exercise, giving rise to proliferative muscle progenitors (myoblasts) that repair injured muscle or contribute to muscle growth. Satellite cells also maintain their own population by the process of self-renewal. The activity of satellite cells is supported by a population of muscle-resident mesenchymal stromal cells, named fibro-adipogenic progenitors (FAPs), which provide an optimal environment for muscle regeneration and growth. The mechanisms underlying skeletal muscle homeostasis are represented in [Fig. 1](#).

## Sarcopenia

The word sarcopenia derives from the Greek and literally means “poverty of flesh.” It is defined as poor muscle function (strength or performance) together with poor muscle mass; it is accompanied by increased risk of falls, loss of independence, and mortality ([Cruz-Jentoft et al., 2019](#)). Muscle loss consists of a reduction of the number and the size of myofibers per muscle ([Silventoinen et al., 2008](#)). Sarcopenia is associated especially to aging (primary sarcopenia) but also to other pathologies (secondary sarcopenia) such as rheumatologic disturbs, common in elderly, or rheumatoid arthritis in women ([Giles et al., 2008](#)). Sarcopenia coupled with obesity is an important and not rare subtype. In fact, the infiltration of adipose tissue in skeletal muscle is one of the clinical signs of this pathology and plays an important role in sarcopenia ([Zamboni et al., 2019](#)). Other disease states that lead to secondary sarcopenia are hypogonadism and diabetes mellitus. Sarcopenia is



**FIG. 1** Mechanisms underlying homeostasis of skeletal muscle. In the figure, a skeletal myofiber and mechanisms of muscle cells homeostasis are represented. Resident stem cells (satellite cells, SC) are mainly quiescent; when stimulated (muscle damage, exercise), they activate and become proliferating myoblasts, and subsequently, they differentiate and fuse to existing myofibers. Fibroadipogenic progenitors (FAPs) stimulate the myogenic fate of SC. Myogenesis and myofiber trophism are also promoted by healthy neuromuscular junctions (NMJs). This positive effect is reciprocal, and healthy muscle has a positive effect on the NMJ. Mitochondrial dysfunction in the myofiber results in unfavorable bioenergetics for the fiber and increase of reactive oxygen species. Dysfunctional mitochondria send a signal to the NMJ (a mechanism called dying back); consequently, the NMJs start to fragment, and the motoneuron dies stopping the trophic signal to the fiber, consequently, SC function is impaired, and myofiber eventually dies for apoptosis. The scheme reported below on the left summarizes the anabolic and catabolic pathways implicated in muscle proteostasis and the main stimuli that activate them. Protein synthesis is stimulated by the mTORC1 pathway, which is activated by insulin, insulin-like growth factor, and amino acids. mTORC1 function is repressed by glycogen synthase kinase 3 beta (GSK3beta) and by members of the class O of forkhead transcription factors (FOXO). FOXO proteins are activated by fasting, myostatin and stimulate protein catabolism through the ubiquitin proteasome system and the autophagy cascade.

very debilitating and is the most important cause of physical frailty, falls, and mortality in older people (Balogun et al., 2019).

This pathology was not much investigated in the past. The reason lies in the lack of standardized criteria to determine exactly the loss of muscle mass and the reduction of their strength and, consequently, to identify accurately sarcopenia disease. In the last years, the European Working Group on Sarcopenia in older People (EWGSOP) has developed an algorithm based on gait speed measurement for the screening of this pathology and has proposed the following criteria for the identification of sarcopenia:

- Low muscle strength (LMS) assessed by handgrip strength  $< 27 \text{ kg}$  (men) and  $< 16 \text{ kg}$  (women);
- Low Muscle Mass (LMM) assessed by skeletal muscle mass index  $\leq 7.0 \text{ kg/m}^2$  (men) and  $\leq 5.5 \text{ kg/m}^2$  (women);

- Low physical performance (LPP) assessed by gait speed  $\leq 0.8$  m/s.

The methods to determine LMS, LMM, and LPP are well described in the literature (Cruz-Jentoft et al., 2019).

### Sarcopenia triggers

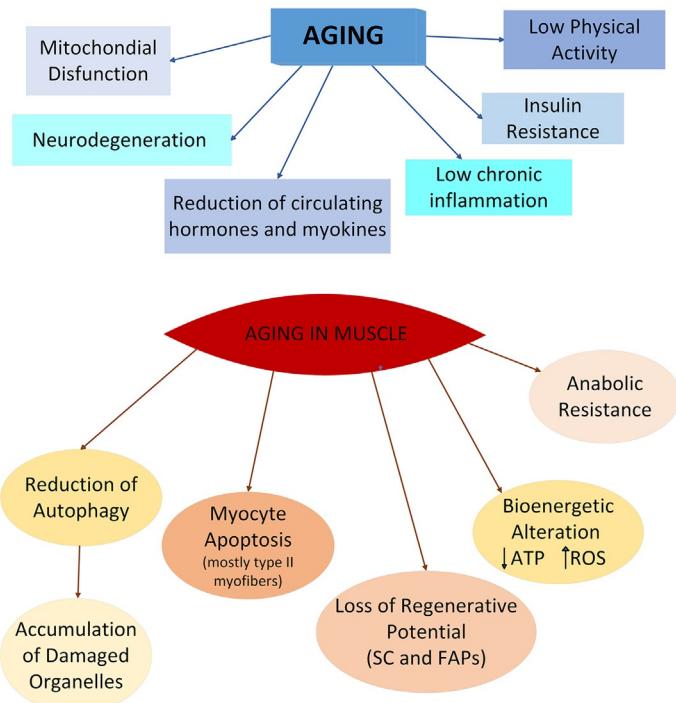
Aging is of the greatest importance in the onset and progression of sarcopenia, as it leads to poor physical activity (disuse), systemic inflammation, age-related decline in the levels of systemic hormones, such as insulin, testosterone, and estrogen, disturbed production of muscle growth regulators, including IGF1, myostatin, and insulin resistance.

Muscle disuse causes a reduced release of myokines that are cytokines synthesized and released by myocytes during muscular contractions, which regulate muscle mass and function. Insulin resistance is another aspect that is often observed in aged people. It synergizes with pro-inflammatory cytokines in impairing muscle protein synthesis due to the lower ability of the mTORC1 pathway to be stimulated by physiologic stimuli, a condition that is called “anabolic resistance” (Cleasby et al., 2016; Drummond et al., 2012). In older people, the number of satellite cells is lower, and their function is compromised by alterations of their niche and of the level of systemic factors that regulate their activity (Walston, 2012) leading to an impaired ability of the old muscle to regenerate itself after immobilization atrophy (Carlson et al., 2009).

An increasing number of studies indicate that mitochondrial dysfunction plays a central role in the pathogenesis of sarcopenia (Romanello & Sandri, 2015). Parameters such as mitochondrial DNA copy number and ATP levels decline with age. The trigger can be the reduction of skeletal muscle autophagy in the elderly with a consequent accumulation of damaged mitochondria (Jiao & Demontis, 2017). The number of type IIA and type IIX myofibers declines by 14% and 10% respectively per decade (Buch et al., 2016); this decline leads to a diminished reliance on glycolysis and glucose utilization in the cytosol of the cells and a high reliance on oxidative phosphorylation, contributing to increasing fat utilization in the mitochondria for energy metabolism (Dao et al., 2020).

Malfunction of mitochondrial metabolism and oxidative phosphorylation results in impaired bioenergetics with the result of augmented level of reactive oxygen species (ROS), which promote apoptosis and contribute to chronic inflammation, which in turn represses protein anabolism. A less efficient proteostasis in turn results in a decrease in mitochondrial biogenesis, creating a vicious circle that exacerbates mitochondrial malfunction (Fig. 2). Therefore, any intervention aimed to improve mitochondrial function and/or reduce chronic inflammation state is a putative therapeutic approach to counteract sarcopenia.

**FIG. 2** Alterations caused by aging. In the figure, the main alterations occurring during aging in the whole organism and in muscle are summarized. Most of them influence each other. ATP (Adenosine triphosphate); FAPs (Fibro/Adipogenic Progenitors); ROS (Reactive oxygen species); SC (satellite cells).



## Sarcopenia therapeutic approaches

Until now, sarcopenia affects > 50 million people, and in the next 40 years it will affect more than 200 million people (Dhillon & Hasni, 2017). Because of the important role that sarcopenia plays in terms of morbidity, disability, health care, and mortality, the interest in this disease has changed and improved in the last few years due to the worldwide aging of the population, which derives from the increase in average life expectancy and the decrease in birth rate. Nevertheless, until now there are no medical guidelines and no definitive therapy. Current treatment for sarcopenia combines drug and non-drug therapies. Exercise has proven to be very important against muscle atrophy; however, immobile patients and inter-individual differences in physical abilities limit its potential. Resistance exercise is often combined with nutritional therapy with vitamin D, polyunsaturated fatty acids, amino acids, and proteins. The most important amino acid is leucine, which is a reasonable supplement in case of low protein intake (Cederholm & Morley, 2016). Thus, the Prot-Age study group recommends 1–1.2 g/kg body weight per day and higher intake of leucine in case of pathologies or chronic disease (Bauer et al., 2013). Among the pharmacological therapies, there are the use of testosterone and anabolic steroids supplements in hyper-gonadal men in order to reduce the muscle mass loss (Saad et al., 2017). Also β-agonist, myostatin antibodies, and activin receptor antibodies can be administered to preserve muscle functionality (Lynch & Ryall, 2008; Morley, 2018). These drugs are used only in severe cases and cause many side effects.

Therefore, effective therapy without side effects is required. For this purpose, it is of great importance to understand the influence that complex signaling systems, such as the ECS, have in this disease. Indeed, skeletal muscle tissue expresses all endocannabinoid elements; thus, it is fundamental to understand the role that this system has in the pathophysiology of the muscle.

## The endocannabinoid system (ECS) in skeletal muscle tissue

The ECS is a widespread signaling system that plays important roles in regulating many physiological processes including inflammation.

The ECS includes cannabinoid (CB) receptors, endogenous cannabinoids (endocannabinoids, eCBs), and the enzymes responsible for the synthesis and degradation of the eCBs. Currently, two cannabinoid receptors are well known, type 1 (CB1) and 2 (CB2) receptors. CB1 receptor is widely expressed in the central nervous system and peripheral tissues, including white and brown adipose tissues, liver, myocardium, and skeletal muscle. CB2 receptor, on the other hand, shows a more limited expression pattern and can be found mainly in immune cells and to a lesser extent in the cerebral cortex, cerebellum, and gastrointestinal tract (Maccarrone, 2020). The existence of alternative cannabinoid receptors is still a topic of debate; however, the G-protein-coupled receptors GPR18, GPR55, and GPR119 respond to a variety of both endogenous and exogenous cannabinoid ligands such as phytocannabinoids and are therefore considered novel cannabinoid receptors or at least cannabinoid-related receptors (Schönke et al., 2020). Other receptors with central role in ECS are peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor mainly expressed in energy-demanding tissues, and transient receptor potential vanilloid 1 (TRPV1), a non-selective ion channel involved in the transmission and modulation of pain. The circulating eCBs, anandamide (AEA), and 2-arachidonoylglycerol (2-AG) bind to G-protein-coupled CB1 and CB2 receptors. Other endogenous ligands are called endocannabinoid-like compounds, because they share this enzymatic system, but are unable to directly activate CB receptors. Among these compounds are fatty acid ethanolamides (FAEs), such as N-oleoylethanolamide (OEA) and N-palmitoylethanolamine (PEA), which modulate lipid metabolism and exhibit antiinflammatory properties, respectively.

The ECS is a key regulator of skeletal muscle formation (myogenesis) and metabolism, even if its functions are very different depending on the ligand-receptor considered. The endocannabinoid receptors in skeletal muscle tissue include CB1 and CB2 receptors, PPARα and TRPV1 as well as the fatty acid hydrolase (FAAH) enzyme, which modulate eCBs levels (Cavuoto et al., 2007). PPARα is enriched in skeletal muscle tissue where, together with PPAR gamma coactivator-1a (PGC1a), it regulates muscle mitochondrial biogenesis. OEA and PEA enhance mitochondrial biogenesis and lipid oxidation via stimulation of PPARα. In addition, TRPV1 activation is related to increased glucose metabolism and mitochondrial biogenesis (Guzmán et al., 2004; Luo et al., 2012; Page et al., 2019) as well as correlates with an enhancement of muscle differentiation and regeneration (Kurosaka et al., 2016).

Opposing effects of endocannabinoid ligands and receptors are also observed in the myogenesis process. The activation of CB1 receptor represses insulin sensitivity, glucose uptake, and mitochondrial function in skeletal muscle cells (Lindborg et al., 2010; Mendizabal-Zubiaga et al., 2016). Moreover, the action of natural or synthetic agonist of CB1 inhibits cultured myoblasts terminal differentiation and promotes their proliferation and CB1 transcripts are elevated in skeletal muscle of

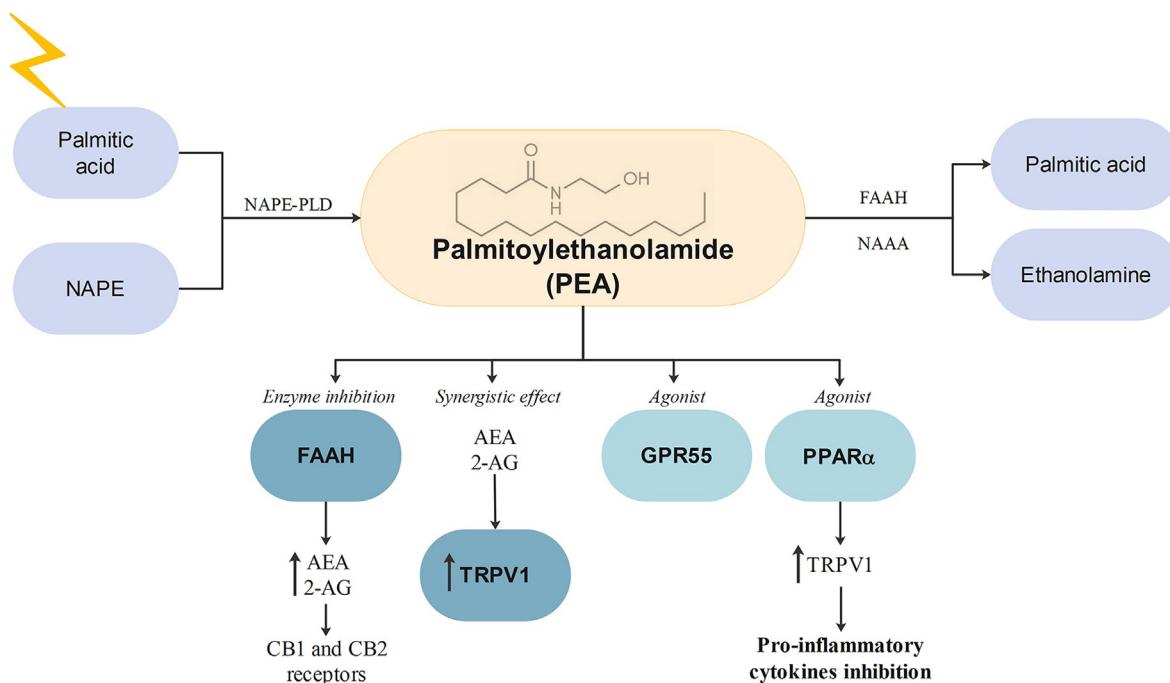
mdx mice, a mouse model of Duchenne Muscular Dystrophy. Importantly, CB1 antagonism in mdx mice may counteract the loss of muscle strength associated to muscle dystrophy, by triggering muscle regeneration (Iannotti et al., 2018).

The endocannabinoid-like molecule PEA plays a key role in the regulation of the ECS in the muscle because it is a direct agonist of PPAR $\alpha$  and the orphan receptor GPR55 even if exhibits only weak binding efficacy at the classical cannabinoid receptors CB1 and CB2. PEA is produced as a pro-homeostatic protective response to cellular injury and is usually upregulated in inflammation states.

## Palmitoylethanolamide (PEA)

PEA is structurally the amide of palmitic acid and ethanolamine. Like the rest of FAEs, PEA is biosynthesized on demand in membranes of various cell types starting from palmitic acid (C16:0), the most common fatty acid in animals. The most investigated pathway involved the N-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD) to form N-palmitoyl-phosphatidyl-ethanolamide (NPPE). The subsequent step provides for the release of N-palmitoyl ethanolamine from NPPE by a phosphodiesterase of the phospholipase D (PLD) type. This hydrolase recognizes different types of N-acyl phosphatidyl ethanolamine (NAPE), so it can generate PEA and other species of FAEs (Okamoto et al., 2004).

Over the years, the interest about PEA has greatly increased, and several studies have been carried out to understand the multiple mechanisms of action through which PEA exerts its pharmacological effects. The first mechanism of action was proposed by Nobel Prize-awarded Rita Levi-Montalcini's research group, who found out that PEA is effective in reducing mast cell migration and degranulation (Aloe et al., 1993). Later studies have proved that the antiinflammatory action of PEA is directly connected to the activation of at least two different receptors: the PPAR $\alpha$  (Lo Verme et al., 2005) and the orphan GPR55 (Ryberg et al., 2007). Other important targets of PEA are TRPV1 and CB receptors. These last are identified as "indirect" targets, according to the fact that PEA causes the inhibition of AEA hydrolysis and potentiates its activity to bind these receptors (Lo Verme et al., 2005). Different studies suggested a short-lived action of PEA, probably linked to its degradation by two different enzymes. PEA is degraded to palmitic acid and ethanolamine by the action of two hydrolases: FAAH (Cravatt et al., 1996) and N-acylethanolamine-hydrolyzing acid amidase (NAAA) (Ueda et al., 1995) (Fig. 3).



**FIG. 3** PEA synthesis, degradation, and mechanism of action. This diagram represents the anabolism and catabolism of PEA and the enzymes involved. At bottom, the action of PEA inside endocannabinoid system is represented. 2-AG (2-arachidonoylglycerol); AEA (Anandamide); CB1 and CB2 (cannabinoid receptor 1 and 2); FAAH (Fatty acid hydrolase); GPR55 (G-protein-coupled receptor 55); NAAA (N-acylethanolamine-hydrolyzing acid amidase); NAPE (N-acyl-phosphatidyl ethanolamine); NAPE-PLD (N-acyl-phosphatidyl-ethanolamine-selective phospholipase D); PPAR $\alpha$  (Peroxisome proliferator activated receptors alpha); TRPV1 (Transient Receptor Potential Vanilloid 1).

Bachur's group (1965) quantified the content of PEA in different tissues of rats, demonstrating that the amount of PEA in the liver is variable, while in the brain and muscles it is present at high and stable concentration. Some studies suggested that the intake of dietary fatty acids could hardly influence the tissue levels of PEA, except in the small intestine, where it can lead to a decrease of levels of this cannabinoid. Furthermore, during inflammation, the free levels of PEA increase (Balvers et al., 2013). For all these reasons, it is already known that there is a perfect balance between synthesis and breakdown that regulates tissue levels of PEA, which is synthesized when necessary to act locally.

## Direct and indirect targets of PEA

The effects of PEA can be exerted by both the direct and the indirect interaction with molecular targets, as mentioned above and reported in Fig. 3. Within the direct interactions, the most important ones involve the PPAR $\alpha$  and the GPR55 receptors. The nuclear PPAR $\alpha$  is the most important "direct" target of PEA, and it is responsible for the antiinflammatory action (Petrosino & Di Marzo, 2017). PPAR $\alpha$  is activated by endogenous and synthetic drugs. Endogenous ligands are fatty acids and/or their derivatives, such as PEA. Lo Verme et al. (2005) reported, for the first time, that also PEA is an agonist of this nuclear receptor. It is a potent regulator of systemic and cellular metabolism as well as energy homeostasis and is mainly expressed in energy-demanding tissues such as the liver, kidney, heart, and muscle (Daynes & Jones, 2002). The interaction between PEA and this target induces a hetero-dimerization with retinoic acid receptor (RXR), leading to the activated receptor complex. This one, once in the nucleus, binds a peroxisome proliferator response element (PPRE) and consequently reduces the pro-inflammatory genes transcription (Petrosino et al., 2010). Among other roles, it has an important function in fatty acid catabolism. After being activated, PPAR $\alpha$  can activate or repress specific genes, as well as regulate lipid and glucose metabolism protecting cells from excessive mitochondrial ROS generation.

The GPR55 receptor belongs to the G-protein-coupled receptors and is expressed mostly in different areas of the brain and in the gastrointestinal tract (Petrosino & Di Marzo, 2017). It can be activated by the endocannabinoid AEA and also by low concentrations of PEA (Ryberg et al., 2007).

Among the indirect targets, CB1 and CB2 receptors are regarded as such because PEA does not interact with them, but it could cause an increasing activation of them. Indeed, as described above, PEA may rise AEA tissue levels, competing with this compound for FAAH (Cravatt & Lichtman, 2004) and consequently leads an improved activation of especially CB2 receptor, which instead is a direct target of AEA.

The TRPV1 channel is another indirect target of PEA, also named *Capsaicin receptor*, and it is a non-selective ion channel. Several stimuli could activate this receptor and among these also eCBs and the activation of PPAR $\alpha$  (Di Marzo & De Petrocellis, 2010).

## PEA pharmacological uses

The most important activity of this compound is the antiinflammatory effect, and in several diseases the action of PEA has been evaluated. Its role in the inflammatory cascades has been demonstrated with the decrease of pro-inflammatory enzymes such as cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (INOS) activity. Furthermore, PEA acts by reducing inflammatory cytokines, such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and prostaglandin E2 (PGE2) (Costa et al., 2002).

PEA may be also used to treat influenza virus, common cold, and some types of infections. This is broadly expounded in several studies, where it has also shown that a prolonged induction of inflammatory cytokines (even named Hypercytokinemia) is linked to virulent virus and especially to the death of patients infected, improving significantly the risk of mortality (Meunier et al., 2012).

It was against this background that several clinical trials in recent years investigating the use of oral PEA for Covid-19 to reduce inflammation and oxidative stress induced by virus (Albanese et al., 2022).

Another potential application of this compound concerns inflammatory bowel diseases and ulcerative colitis. In both those studies, the results suggested the possibility of PEA to improve the action of eCBs, such as AEA, on CB1 and CB2 receptors, restoring the pre-inflammatory condition (Capasso et al., 2014; Sarnelli et al., 2016).

Moreover, it was demonstrated that the oral administration or eye-local application of PEA can improve ocular pathologies such as glaucoma and ocular hypertension. Particularly, clinical trials have shown that oral administration of PEA reduced pro-inflammatory cytokines and intra-ocular pressure similarly to the current drugs. Also patient's compliance of the medication when PEA is associated with antiglaucoma drugs was increased (Di Zazzo et al., 2017; Rossi et al., 2020).

Considering several PEA-based nutraceutical products on the market, case studies are reported in the literature in which their use is linked to muscle pathologies (Table 1).

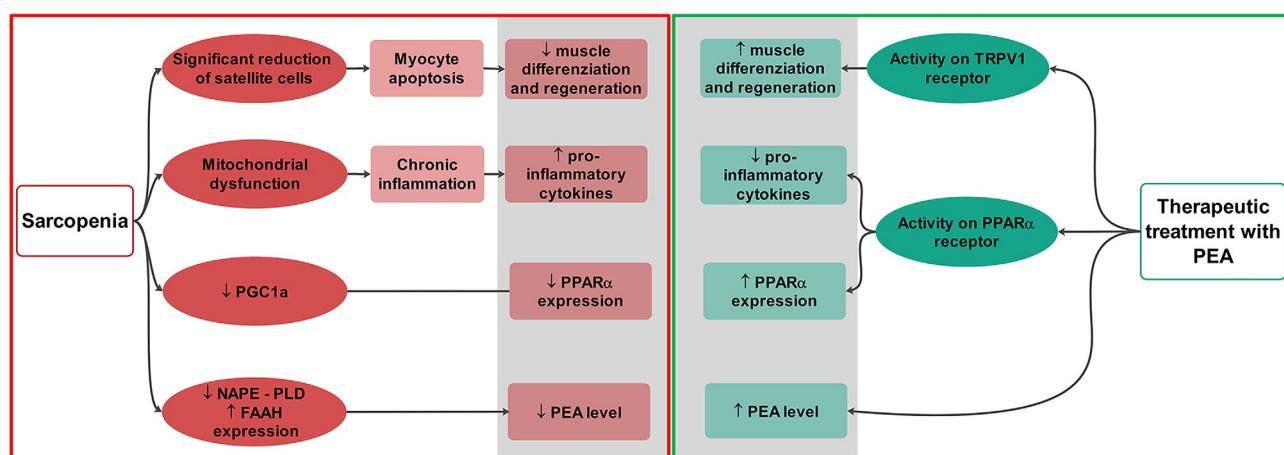
**TABLE 1** Case study reports of PEA treatment in muscular diseases. For each study reported in the reference column, muscle disease, administered therapy, type of study, and results were considered (Unpublished table).

Muscle disease	Administered therapy	Type of study	Results	References
Nocturnal muscle cramps	PeaPure 400mg capsules	Three case patients	Agonistic action on TRPV1	Keppel Hesselink and Kopsky (2016)
Amyotrophic lateral sclerosis	μm-PEA 600mg twice a day	Single case patient	Reduction of microglia and mast-cell activity	Clemente (2012)
Myasthenia gravis	μm-PEA 600mg twice a day	Pilot study on 22 patients	Reduction of disability level and improve muscular response to fatigue	Onesti et al. (2019)
Fibromyalgia syndrome	Normast® + personal current therapy	Retrospective observational study (407 patients)	Reduction of pain and improvement of the quality of life	Schweiger et al. (2019)

### PEA and its potential use in sarcopenia

Recent study on aged rats in comparison with adult ones was conducted. Le Bacquer et al. (2022) demonstrated a reduction of OEA and PEA levels in aged rats. In particular, it was observed a decrease of enzymes involved in biosynthesis of these molecules, while level of FAAH, involved in catabolism, was three times higher than those in adult animals. Moreover, the low plasma levels of PEA and OEA were related to the loss of muscle mass and reduction of muscle function, as contractility. These data suggest that PEA could be considered a therapeutic candidate to counteract age-related muscle loss.

In sarcopenic muscle, the expression level of both PPAR $\alpha$  and its coactivator PGC1a decreases with age, suggesting a causative relation between the lower PPAR $\alpha$  function and sarcopenia. Promoting PPAR $\alpha$  activity in skeletal muscle could be beneficial to counteract muscle wasting also for its ability to inhibit inflammatory signaling (Grabacka et al., 2021). Therefore, increasing level of PPAR $\alpha$  agonists such as OEA and PEA can represent a response to control the inflammation and progression of sarcopenia (Fig. 4), since the activation of PPAR $\alpha$  promotes a cascade that inhibits the release of pro-inflammatory cytokines, a central point to treat the strong inflammation characteristic of sarcopenia (Cifelli et al., 2022). In this regard, recently, to better achieve muscle cells, PEA was delivered by nanoparticles (Maretti et al., 2022).



**FIG. 4** Effects on sarcopenia of a therapy with PEA. The scheme represents potential effects of administered (exogenous) PEA on altered metabolic pathways in sarcopenia. FAAH (Fatty acid hydrolase); NAPE-PLD (N-acyl-phosphatidyl-ethanolamine-selective phospholipase D); PPAR $\alpha$  (Peroxisome proliferator-activated receptors alpha); TRPV1 (Transient Receptor Vanilloid 1).

## Nanoparticles for PEA delivery and for muscle cell targeting

PEA is on the market in its native or ultramicronized form as a nutraceutical or as a medical device but not as a drug. Indeed, over the important therapeutic effects of PEA in the control of inflammation, this compound presents two important disadvantages that make difficult its clinic uses: poor bioavailability and poor solubility in any solvent. For these reasons, much attention has recently been paid to nanotechnology-based formulations (nanoparticles) in order to achieve the clinical success of PEA.

These nano-formulations could be embedded with drugs through encapsulation or covalent bond, with the aim of increasing their solubility and bioavailability and also to prevent their degradation. Another critical profit obtained with nanoparticles is that these systems are employed to reach a development of drugs with a more specific target.

The employment of PEA-loaded nanoparticles has been considered in order to overcome the aforementioned PEA drawbacks. [Tronino et al. \(2016\)](#) presented nanoparticulate systems containing PEA to resolve its pharmacokinetics problems and increase its percutaneous diffusion, by raising the possibility of using PEA in dermal and transdermal formulations. Other studies have been performed to investigate the effect of nanoparticles in ocular or intestine delivery of PEA ([Puglia et al., 2020](#)).

At the muscle level, the use of nanoparticles could potentially be useful in order to promote drug delivery to this tissue. Indeed, there are some experiments in which C2C12 muscle cell line was used to explore the uptake and the consequential effect of labeled nanoparticles on muscles ([Costanzo et al., 2019](#)).

It has been shown that the myoblasts treated with the nanoparticles are able to differentiate into myotubes. [Poussard et al. \(2015\)](#) have reported that, after several days of differentiation, nanoparticles are still present into the nucleus of myotubes and their formation is possible also in the presence of nanoparticles.

In another study, C2C12 skeletal myoblasts have been treated with polymeric PLGA nanoparticles with the purpose of examining their biocompatibility and bioactivity. After several days, the cellular behavior was evaluated, and the results suggest an increasing proliferation of cells during the culture period. This proves that the treatment with polymeric nanoparticles doesn't negatively affect the growth of cells. This is probably associated to the hydrophilicity of the polymeric matrix, which represents a supported microenvironment for the proliferation of muscle cells ([Shin et al., 2015](#)).

Also, Solid Lipid Nanoparticles (SLNs) were tested on C2C12 cell line. SLNs labeled with Myricitrin have been used to evaluate the antidiabetic and antioxidant effects. Furthermore, the vitro assessment showed that this nanoparticulate system provided a cellular survival and a differentiation ([Ahangarpour et al., 2018](#)).

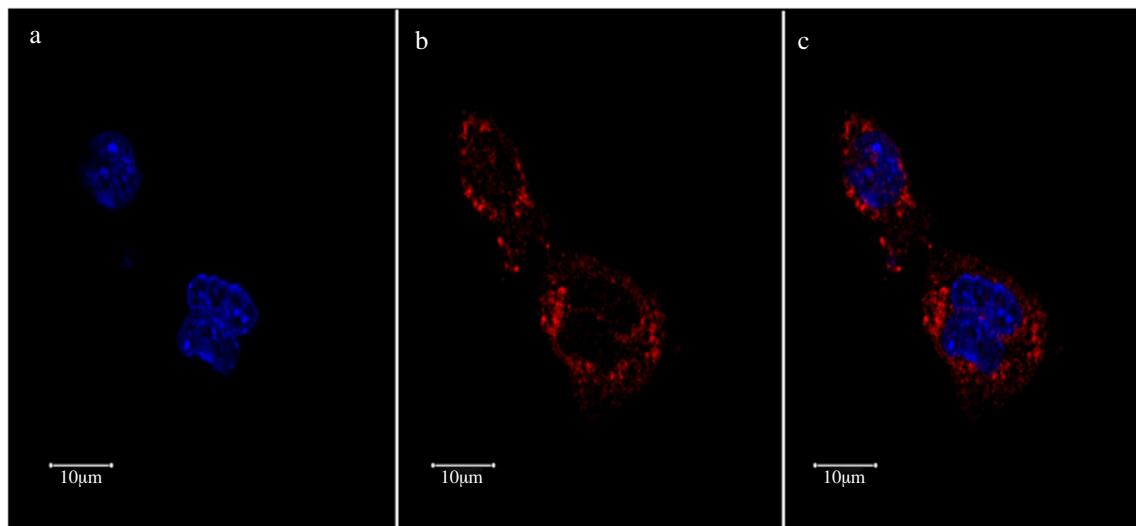
## PEA-loaded SLNs as potential treatment of sarcopenia

SLNs are colloidal carriers with a mean diameter ranging from 50 to 1000 nm, constituted by a solid lipid matrix made of generally recognized as safe (GRAS) lipids and surfactants. SLNs are widely used to optimize drug release and targeting, especially because of their prolonged-release effect and their ability to protect chemically labile ingredients.

PEA-loaded SLNs were designed for a parenteral administration in order to achieve an antiinflammatory treatment of sarcopenia ([Maretti et al., 2022](#)). The size of the SLNs was approximately 250 nanometers, and the encapsulation efficiency of drug reached 90%. PEA-SLNs labeled with Nile Red dye showed a high capacity to be internalized into myoblasts (flow cytometric values between 85% and 94% after 14h of incubation), without showing any sign of cytotoxicity. Confocal analysis confirmed the presence of SLNs in the cytoplasm of myoblasts suggesting that PEA-SLNs can be considered a potential strategy for the treatment of sarcopenia ([Fig. 5](#)).

## Applications to other areas

This chapter describes the translational aspects related to the role of PEA in the treatment of sarcopenia, as an endogenous agonist of PPAR $\alpha$ . As described, in addition to its affinity for PPAR $\alpha$ , PEA has a high affinity for the GPR55 receptor and a number of other targets, including the TRPV1 channel. This may be important, since some TRPV1 channel activators have recently been proposed for the treatment of muscle cramps ([Bean et al., 2013](#)). Indeed, the physiological mechanism of muscle cramping is still unknown, even if there are many speculations ([Keppel Hesselink & Kopsky, 2016](#)). For example, muscle cramps may be a symptom of peripheral vascular disease, radiculopathies and lumbar canal stenosis, diabetes mellitus, liver disorders and cirrhosis, metabolic myopathies, and neuropathies. Here it has been extensively discussed that PEA is produced in the body on demand and accumulates locally during inflammatory disorders. PEA can be synthesized in muscle tissue, and this synthesis appears to be disturbed in the case of fibromyalgia. Therefore, it is possible to speculate that PEA could play a role in stabilizing overexcited muscles that cause nocturnal leg cramps, and one of the mechanisms of



**FIG. 5** Representative confocal microscopy images of C2C12 cells exposed to PEA-SLNs-NR during 14 h of incubation. The confocal image represents nuclei (blue channel) (A) and SLNs (red channel), (B) stained with DAPI and Nile Red (NR), respectively; image (C) was obtained merging the two channels. Data are representative of three independent experiments.

action of the anticonvulsant activity of PEA could be its agonistic action on the TRPV1 receptor that induces a decrease of  $\alpha$ -motor neuron hyperexcitability.

### Mini-dictionary of terms

- **Mitochondria** are cellular organelles with the main role of converting nutrients into energy and controlling the metabolism
- **Muscle cells** or myocytes are contractile cells that constitute the muscular fibers. There are three types of muscle cells in our body: cardiac, skeletal, and smooth
- **Nanoparticles** in drug delivery are structures of different nature with the task of protecting, transporting, or controlling the release of drugs with particular problems such as stability or bioavailability
- **Oxidative metabolism** is a chemical process that involves oxygen in order to create energy from carbohydrates. It's also defined as aerobic respiration
- **Sarcopenia** is a muscle disease common in elderly leading to the loss of muscle mass, function, and strength
- **Skeletal muscle cells** are multi-nucleated striated cells that create connections with the skeleton. They contain many mitochondria to generate energy necessary to maintain tone and movement

### Key facts of PPAR $\alpha$

- PPAR $\alpha$  is one of the three isoforms of the peroxisome proliferator-activated receptor located in the nuclear region.
- It is one of the principal elements of endocannabinoid system with a role of nuclear transcription factor.
- It is a ligand-activated transcriptional factor, which regulates the activation of different genes and represents the major regulator of lipid metabolism in the liver.
- During inflammation, PPAR $\alpha$  is involved in the inactivation of NF- $\kappa$ B, a protein complex that promotes cytokine production.
- PPAR $\alpha$  pathway stimulates the expression of antioxidant enzymes reducing ROS concentration.

### Summary points

- Sarcopenia is a debilitating skeletal muscle disease closely connected with elderly.
- Understanding the role that the endocannabinoid system (ECS) plays in muscle tissue is necessary in order to find new therapies

- The skeletal muscle expresses all the ECS elements, and their regulation can influence the onset and progression of the disease
- A central role is played by the nuclear receptor PPAR $\alpha$  and its main endogenous ligand, palmitoylethanolamide (PEA)
- The administration of PEA to the muscle can be a promising approach to counteract sarcopenia
- In muscle, the expression level of PPAR $\alpha$  decreases, suggesting a causative relation between the lower PPAR $\alpha$  function and sarcopenia.
- To promote the PEA muscle targeting, innovative drug delivery systems such as solid lipid nanoparticles can be considered very promising

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## Chapter 43

# Recommended resources for studying and investigating the neurobiology and physiology of the endocannabinoid system

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## Introduction

Δ9-Tetrahydrocannabinol and cannabidiol are important components of cannabis (Chandra et al., 2019). These molecules stimulate the endogenous cannabinoid system (Russo, 2016). The constituents of this pathway include endocannabinoids (i.e., endogenous cannabinoids), receptors for these cannabinoids, and proteins that synthesize, transport, and break down endocannabinoids (Lu & Mackie, 2021).

Most of the constituents of the endocannabinoid system have multiple functions (Lu & Mackie, 2021). The endocannabinoid pathway interacts with and influences many other signalling pathways (Lu & Mackie, 2021). Similarly, many other signaling pathways influence the endocannabinoid system (Lu & Mackie, 2021). This point is important when assessing the actions of drugs that target the endocannabinoid pathways (Lu & Mackie, 2021).

The role of the endocannabinoid pathway is central in the developing nervous system (Lu & Mackie, 2021). In the mature nervous system, endogenous endocannabinoids modulate neuronal activity and networking (Lu & Mackie, 2021). The current paradigm of the function of the endocannabinoid system in adults is based on the concept that only endocannabinoids are produced on demand (Di Marzo et al., 1999).

Most classic neurotransmitters are produced and stored in vesicles awaiting signals for their release. In contrast, endocannabinoids are released at precise points at specific times (Lu & Mackie, 2021). However, the receptor engagement of administered exogenous cannabinoid ligands is indiscriminate and sustained (Lu & Mackie, 2021). Thus, the systemically administered cannabinoids' effects are likely to differ significantly from the physiologically effects of endocannabinoids (Lu & Mackie, 2021).

The endocannabinoid system is actively being investigated (Kroon et al., 2021), and thus, in recent years, information in this area has increased greatly. To assist those embarking on research into the endocannabinoid system, we have compiled a list of resources that were recommended by active researchers and practitioners. The list of acknowledgements below includes all of the experts who provided input into the ensuing tables.

## Resources

Tables 1–5 list the most up-to-date information on the regulatory bodies (Table 1), professional societies (Table 2), books (Table 3), emerging technologies, and platforms (Table 4), and other resources of interest (Table 5) that are relevant to an evidence-based approach to the neurobiology and physiology of the endocannabinoid system.

**TABLE 1** Websites, regulatory bodies, or organizations dealing with the neurobiology and physiology of the endocannabinoid system or related fields and areas.

Regulatory body or organization	Web address
Brazilian National Health Surveillance Agency (Anvisa)	<a href="https://www.gov.br/anvisa/pt-br">https://www.gov.br/anvisa/pt-br</a>
Brazilian Platform for Drugs Policies	<a href="https://pbpd.org.br/">https://pbpd.org.br/</a>
Canadian Centre on Substance Use and Addiction (CCSA)	<a href="https://www.ccsa.ca/">https://www.ccsa.ca/</a>
Cannabinoid Medicine in Brazil	<a href="https://www.cannabisesaude.com.br/">https://www.cannabisesaude.com.br/</a>
Cannabinoid Research Initiative of Saskatchewan	<a href="https://www.crisk.ca/">https://www.crisk.ca/</a>
Cannabis Education for Health Care Providers Toolkit: University of British Columbia	<a href="https://ubccpd.ca/learn/resources-recordings/toolkits/cannabis-education-toolkit">https://ubccpd.ca/learn/resources-recordings/toolkits/cannabis-education-toolkit</a>
Cannabis Resources for Family Physicians: The College of Family Physicians of Canada	<a href="https://www.cfpc.ca/en/education-professional-development/practice-tools-guidelines/cannabis-resources-for-family-physicians">https://www.cfpc.ca/en/education-professional-development/practice-tools-guidelines/cannabis-resources-for-family-physicians</a>
Cannabis-Based Medicines: Ministry of Health, Italy	<a href="https://www.salute.gov.it/portale/medicinaliStupefacenti/menuContenutoMedicinaliStupefacenti.jsp?lingua=italiano&amp;area=sostanzeStupefacenti&amp;menu=organismo&amp;idMat=null">https://www.salute.gov.it/portale/medicinaliStupefacenti/menuContenutoMedicinaliStupefacenti.jsp?lingua=italiano&amp;area=sostanzeStupefacenti&amp;menu=organismo&amp;idMat=null</a>
Cannabis: State of Colorado	<a href="https://cannabis.colorado.gov/">https://cannabis.colorado.gov/</a>
Cannabis: Health Canada	<a href="https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis.html">https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis.html</a>
Cannabis: Ontario College of Family Physicians	<a href="https://www.ontariofamilyphysicians.ca/tools-resources/timely-trending/cannabis">https://www.ontariofamilyphysicians.ca/tools-resources/timely-trending/cannabis</a>
Cannify	<a href="https://www.cannify.us/">https://www.cannify.us/</a>
Center for Cannabis Research: University of Washington	<a href="https://cannabis.uw.edu">https://cannabis.uw.edu</a>
Centers for Disease Control and Prevention (CDC)	<a href="https://www.cdc.gov/">https://www.cdc.gov/</a>
College on Problems of Drug Dependence	<a href="https://cpdd.org/">https://cpdd.org/</a>
FDA Regulation of Cannabis and Cannabis-Derived Products, Including Cannabidiol (CBD): U.S. Food and Drug Administration (FDA)	<a href="https://www.fda.gov/news-events/public-health-focus/fda-regulation-cannabis-and-cannabis-derived-products-including-cannabidiol-cbd">https://www.fda.gov/news-events/public-health-focus/fda-regulation-cannabis-and-cannabis-derived-products-including-cannabidiol-cbd</a>
Fundación CANNA: Scientific Research and Cannabis Testing	<a href="https://www.fundacion-canna.es/en">https://www.fundacion-canna.es/en</a>
Maryland Cannabis Policy Coalition	<a href="https://www.marylandcannabispolicy.org/">https://www.marylandcannabispolicy.org/</a>
Medical Cannabis Canada	<a href="https://patientaccess.ca/">https://patientaccess.ca/</a>
Medical Cannabis: Arthritis Society	<a href="https://arthritis.ca/treatment/medication/medical-cannabis">https://arthritis.ca/treatment/medication/medical-cannabis</a>
Medical Cannabis: Canadian Pharmacists Association	<a href="https://www.pharmacists.ca/advocacy/issues/medical-cannabis/">https://www.pharmacists.ca/advocacy/issues/medical-cannabis/</a>
National Institute on Alcohol Abuse and Alcoholism	<a href="https://www.niaaa.nih.gov/">https://www.niaaa.nih.gov/</a>
National Institute on Drug Abuse (NIDA)	<a href="https://nida.nih.gov/">https://nida.nih.gov/</a>
National Institutes of Health (NIH)	<a href="https://www.nih.gov/">https://www.nih.gov/</a>
Spanish Agency for Medicines and Health Products (AEMPS)	<a href="https://www.aemps.gob.es/">https://www.aemps.gob.es/</a>
Systematically Testing the Evidence on Marijuana	<a href="https://www.cannabisevidence.org/">https://www.cannabisevidence.org/</a>
U.S. Food and Drug Administration (FDA)	<a href="https://www.fda.gov/">https://www.fda.gov/</a>

This table lists the regulatory bodies and organizations involved with the neurobiology and physiology of the endocannabinoid system. Some of the links have indirect references to this topic. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the "search" tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to the neurobiology and physiology of the endocannabinoid system within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations; see also Table 2.

**TABLE 2** Professional societies relevant to the neurobiology and physiology of the endocannabinoid system or related fields and areas.

Society name	Web address
American Association for Accreditation of Laboratory Animal Care	<a href="https://www.aaalac.org/">https://www.aaalac.org/</a>
American Association for the Advancement of Science	<a href="https://www.aaas.org/">https://www.aaas.org/</a>
American Chemical Society	<a href="https://www.acs.org/content/acs/en.html">https://www.acs.org/content/acs/en.html</a>
American Medical Marijuana Association (AMMA)	<a href="https://americanmarijuana.org/">https://americanmarijuana.org/</a>
American Physiological Society	<a href="https://www.physiology.org/?SSO=Y">https://www.physiology.org/?SSO=Y</a>
American Society for Pharmacology and Experimental Therapeutics	<a href="https://www.aspet.org/">https://www.aspet.org/</a>
Association for Research and Development of Medicinal Cannabis in Brazil (CANNAB)	<a href="https://cannab.com.br/">https://cannab.com.br/</a>
Brazilian Association of Medical Cannabis Patients	<a href="https://amame.org.br/">https://amame.org.br/</a>
Brazilian Association of Support in Cannabis (Abrace)	<a href="https://abraceesperanca.org.br/">https://abraceesperanca.org.br/</a>
Brazilian Society For Cannabis Sativa Studies—Sociedade Brasileira de Estudos da <i>Cannabis sativa</i> (SBEC)	<a href="https://sbec.med.br/">https://sbec.med.br/</a>
Canadian Consortium for the Investigation of Cannabinoids	<a href="https://ccic.net/">https://ccic.net/</a>
Cannabis Regulator Associations (CANNRA)	<a href="https://www.cann-ra.org/">https://www.cann-ra.org/</a>
International Association for Cannabinoid Medicines	<a href="https://www.cannabis-med.org/">https://www.cannabis-med.org/</a>
International Association for the Study of Pain (IASP)	<a href="https://www.iasp-pain.org/">https://www.iasp-pain.org/</a>
International Cannabinoid Research Society (ICRS)	<a href="http://icrs.co">www.icrs.co</a>
Maryland Cannabis Industry Association (MDCIA)	<a href="http://mdcia.org/">http://mdcia.org/</a>
Medical Cannabis Clinicians Society	<a href="https://www.ukmccs.org/">https://www.ukmccs.org/</a>
Minority Cannabis Business Association (MCBA)	<a href="https://minoritycannabis.org/">https://minoritycannabis.org/</a>
National Association of Cannabis Businesses (NACB)	<a href="https://nacb.com/">https://nacb.com/</a>
National Cannabis Industry Association (NCIA)	<a href="https://thecannabisindustry.org/">https://thecannabisindustry.org/</a>
Society for Neuroscience	<a href="https://www.sfn.org/">https://www.sfn.org/</a>
Spanish Society for Cannabinoid Research (SEIC)	<a href="https://www.seic.es/">https://www.seic.es/</a>
Toronto Cannabis and Cannabinoid Research Consortium (TC3)	<a href="https://www.tc3.utoronto.ca/">https://www.tc3.utoronto.ca/</a>

This table lists the professional societies involved with the neurobiology and physiology of the endocannabinoid system. Some of the links have indirect references to this topic. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the “Search” tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to the neurobiology and physiology of the endocannabinoid system within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations; See also Table 1.

**TABLE 3** Books on the neurobiology and physiology of the endocannabinoid system or related fields and areas.

Book title	Authors or editors	Publisher	Year of publication
Addictions: A comprehensive guidebook	McCrady BS, Epstein EE	Oxford	2013
Anandamide, an endogenous cannabinoid	Litwack G	Academic Press	2013

Continued

**TABLE 3** Books on the neurobiology and physiology of the endocannabinoid system or related fields and areas—cont'd

Book title	Authors or editors	Publisher	Year of publication
ASAM principles of addiction medicine	Miller SC, Feillin DA, Rosenthal RN, Saitz R	Wolters Kluwer	2019
Behavioral neurobiology of the endocannabinoid system	Kendall D, Alexander S	Springer	2009
Cannabinoid modulation of emotion, memory, and motivation	Camplongo P, Fattore L	Springer	2015
Cannabinoids pharmacology	Kendall D, Alexander S	Academic Press	2017
Cannabinoid receptors	Reggio PH	Human Press	2009
Cannabinoids and sleep: Molecular, functional and clinical aspects	Murillo-Rodríguez E, Pandi-Perumal SR, Monti JM	Springer	2021
Cannabinoids	Di Marzo V	Wiley Blackwell	2014
Cannabinoids	Pertwee RG	Springer	2005
Cannabinoids and neuropsychiatric disorders	Murillo-Rodríguez E, Pandi-Perumal SR, Monti JM	Springer	2021
Cannabinoids and the brain	Parker LA	MIT Press	2018
Cannabinoids and the brain	Köfalvi A	Springer	2010
Cannabinoids and their receptors	Reggio PH	Elsevier Science	2017
Cannabinoids as therapeutics	Mechoulam R	Springer	2005
Cannabinoids in health and disease	Meccariello R, Chianese R	Intechopen	2016
Cannabinoids in neurologic and mental diseases	Fattore L	Elsevier Science	2015
Cannabis medicinal no Brasil	Callado TM, Lo Prete, AC, Kishi MA,	Cia Farmacêutica	2021
Cannabis: A history	Booth M	Picador	2005
CBD? What does science say?	Rock EM, Parker L, Mechoulam R	MIT Press	2022
Diagnosis and treatment of traumatic brain injury	Preedy V	Elsevier	2021
Endocannabinoid signaling: Methods and protocols	Maccarrone M	Springer New York	2016
Endocannabinoid system in local and systemic inflammation	Kelly MEM, Lehmann C, Zhou J	Morgan and Claypool	2017
Endocannabinoid system: Genetics, biochemistry, brain disorders, and therapy	Murillo-Rodriguez E	Elsevier Science	2017
Endocannabinoidome: The World of endocannabinoids and related mediators	Di Marzo V, Wang J	Elsevier Science	2014
Endocannabinoids and lipid mediators in brain functions	Melis M	Springer	2017
Endocannabinoids	Pertwee RG	Springer	2015
Endocanna's binoids	Di Marzo V, Onaivi ES, Sugiura T	Taylor and Francis Group	2019
Endocannabinoids	Onaivi ES, Sugiura T, Di Marzo V	Taylor and Francis	2006

**TABLE 3** Books on the neurobiology and physiology of the endocannabinoid system or related fields and areas—cont'd

Book title	Authors or editors	Publisher	Year of publication
Endocannabinoids: Molecular, pharmacological, behavioral and clinical features	Murillo-Rodriguez E	Bentham Books	2013
Handbook of cannabis	Pertwee RG	Oxford University Press	2014
Handbook of cannabis and related pathologies: Biology, pharmacology, diagnosis, and treatment	Preedy VR	Elsevier	2017
Health effects of cannabis and cannabinoids: The current state of evidence and recommendations for research	National Academies of Sciences, Engineering, and Medicine, Health and Medicine Division, National Academies of Sciences, Engineering, and Medicine (U.S.), Committee on the Health Effects of Marijuana: an Evidence Review and Research Agenda, Board on Population Health and Public Health Practice	National Academies Press	2017
Marijuana and cannabinoid research: Methods and protocols	Onaivi ES	Humana Press	2006
Marijuana and the cannabinoids	ElSohly MA	Human Press	2007
New tools to interrogate endocannabinoid signaling: From natural compounds to synthetic drugs	Maccarrone M	Royal Society of Chemistry	2021
PET and SPECT of neurobiological systems	Dierckx RAJO, Otte A, de Vries EFJ, Waarde A, Luiten PGM	Springer	2021
Phytocannabinoids	Kinghorn AD, Falk H, Gibbons S, Kobayashi J	Springer	2017
Phytocannabinoids: Unraveling the complex chemistry and pharmacology of Cannabis sativa	Kinghorn AD, Falk H, Gibbons S, Kobayashi J	Springer	2017
Science of marijuana	Iversen LL	Oxford University Press	2007
TRP channels in health and diseases: Implication for diagnosis and therapy	Szallasi A	Nova science	2011
Vanilloid receptor TRPV1 in drug discovery: Targeting pain and pathological disorders	Gomtsyan A, Faltynek CR	Wiley	2010

This table lists books relevant to the neurobiology and physiology of the endocannabinoid system.

**TABLE 4** Emerging techniques, instruments, and analytical platforms or devices for investigating the neurobiology and physiology of the endocannabinoid system or related fields and areas.

Organization or company name	Web address
Cannabis testing solutions: SC Labs	<a href="https://www.sclabs.com/cannabis/">https://www.sclabs.com/cannabis/</a>
CISBIO: Perkin Elmer	<a href="https://www.cisbio.eu/dd/by-category/gpcrs">https://www.cisbio.eu/dd/by-category/gpcrs</a>
DR. CANNABIS (platform of contact between doctors and patients)	<a href="https://www.doutorc.com.br/">https://www.doutorc.com.br/</a>
Phytoplant Research	<a href="https://www.phytoplant.es/es">https://www.phytoplant.es/es</a>
Proximity Ligation Assay (PLA) from Duolink, Sigma-Aldrich	<a href="https://www.sigmaaldrich.com/ES/es/technical-documents/technical-article/protein-biology/protein-and-nucleic-acid-interactions/how-pla-works">https://www.sigmaaldrich.com/ES/es/technical-documents/technical-article/protein-biology/protein-and-nucleic-acid-interactions/how-pla-works</a>

This table lists technologies or platforms relevant to the neurobiology and physiology of the endocannabinoid system. Please note, occasionally the location of the websites or web address changes.

**TABLE 5** Other resources of interest or relevance for health care professionals or patients related to cannabis, cannabinoids, and endocannabinoids or related fields and areas.

Name of resource or organization	Web address
Alliance for Cannabis Therapeutics (ACT)	<a href="https://www.maximumyield.com/definition/4852/alliance-for-cannabis-therapeutics">https://www.maximumyield.com/definition/4852/alliance-for-cannabis-therapeutics</a>
American Academy of Addiction Psychiatry	<a href="https://www.aaap.org/">https://www.aaap.org/</a>
American College of Neuropsychopharmacology	<a href="https://acnp.org/">https://acnp.org/</a>
Analgesic, Anesthetic, and Addiction Clinical Trial Translations, Innovations, Opportunities, and Networks (ACTTION)	<a href="https://www.acttion.org/">https://www.acttion.org/</a>
Cannabis (Marijuana) and Cannabinoids: National Center for Complementary and Integrative Health (NCCIH)	<a href="https://www.nccih.nih.gov/health/cannabis-marijuana-and-cannabinoids-what-you-need-to-know">https://www.nccih.nih.gov/health/cannabis-marijuana-and-cannabinoids-what-you-need-to-know</a>
Cannabis and Cannabinoids: Health Canada	<a href="https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/information-medical-practitioners/information-health-care-professionals-cannabis-cannabinoids.html">https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/information-medical-practitioners/information-health-care-professionals-cannabis-cannabinoids.html</a>
Cannabis and Cannabinoids: National Cancer Institute - National Institutes of Health (NIH)	<a href="https://www.cancer.gov/about-cancer/treatment/cam/patient/cannabis-pdq">https://www.cancer.gov/about-cancer/treatment/cam/patient/cannabis-pdq</a>
Cannabis Education and Research Program (CERP): University of Washington	<a href="https://adai.uw.edu/research/cannabis-research-education/">https://adai.uw.edu/research/cannabis-research-education/</a>
Gordon Research Conference (GRC) on Cannabinoid Function in the CNS; occurs every other year	<a href="https://www.grc.org/cannabinoid-function-in-the-cns-conference/2021/">https://www.grc.org/cannabinoid-function-in-the-cns-conference/2021/</a>
International Cannabis Society Links: Medical Cannabis Clinicians Society	<a href="https://www.ukmccs.org/about-us/international-cannabis-societies/">https://www.ukmccs.org/about-us/international-cannabis-societies/</a>
Military Pharmaceutical Chemical Plant Florence: Ministry of Defence, Italy	<a href="https://www.agenziaindustriedifesa.it/unita-produttive/stabilimento-chimico-farmaceutico-militare-firenze/">https://www.agenziaindustriedifesa.it/unita-produttive/stabilimento-chimico-farmaceutico-militare-firenze/</a>
Overdose Lifeline	<a href="https://www.overdoselifeline.org/">https://www.overdoselifeline.org/</a>
Presidential Task Force on Cannabis and Cannabinoid Analgesia: International Association for the Study of Pain (IASP)	<a href="https://www.iasp-pain.org/group/iasp-presidential-task-force-on-cannabis-and-cannabinoid-analgesia/">https://www.iasp-pain.org/group/iasp-presidential-task-force-on-cannabis-and-cannabinoid-analgesia/</a>
Research on Cannabis and Cannabinoids: National Institute on Drug Abuse (NIDA)	<a href="https://nida.nih.gov/drug-topics/marijuana/nih-research-marijuana-cannabinoids">https://nida.nih.gov/drug-topics/marijuana/nih-research-marijuana-cannabinoids</a>
US Centers for Disease Control and Prevention (CDC): Synthetic Cannabinoids	<a href="https://www.cdc.gov/nceh/hsb/chemicals/sc/About.html/#symptoms">https://www.cdc.gov/nceh/hsb/chemicals/sc/About.html/#symptoms</a>

This table lists other resources of interest or relevance for health care professionals or patients relevant to the neurobiology and physiology of the endocannabinoid system. Please note, occasionally the location of the websites or web address changes.

## Other resources

The Wellcome Collection (<https://wellcomecollection.org/collections>) and The British Library (<https://www.bl.uk/>) also hold material on topics related to the neurobiology and physiology of the endocannabinoid system. See also [Rajendram et al. \(2023a, 2023b\)](#).

Chapters on resources (recommended by authors and practitioners) could be pertinent to the neurobiology and physiology of the endocannabinoid system ([Rajendram et al., 2017](#); [Rajendram et al., 2022](#); [Rajendram et al., 2023c, 2023d, 2023e, 2023f](#); [Rajendram & Preedy, 2019](#)).

Disclaimer: This list of material in these tables is included to provide general information only. It does not constitute any recommendation or endorsement of the activities of these sites, facilities, or other resources listed in this chapter, by the authors or editors of this book.

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