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"The rat ventromedial prefrontal cortex in the neural circuitries of depression and sleep"

presented by

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candidate for the degree of Doctor of Philosophy
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The rat ventromedial prefrontal cortex in the neural circuitries of depression and sleep

A dissertation presented

by

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The rat ventromedial prefrontal cortex in the neural circuitries of depression and sleep

ABSTRACT

Major depressive disorder (MDD) is a debilitating disorder affecting hundreds of millions of people worldwide. The etiology of the disease is unknown, and how antidepressant medications reverse depression is unclear. However, imaging and postmortem studies of MDD patients show abnormalities in several limbic areas of the brain, including the prefrontal cortex. The involvement of the ventromedial prefrontal cortex (vmPFC) in depression has been particularly intriguing, for this region demonstrates reduced metabolic activity in remission, and this reduction is unique to treatment responders. In addition, deep brain stimulation targeting the subgenual cingulate cortex in the vmPFC has been shown to be effective in treating 'treatment-resistant' patients. Furthermore, neuroanatomical studies have shown that this region projects to many downstream limbic areas implicated to play roles in MDD. I therefore hypothesized that 1) the vmPFC may be an important target of antidepressant drugs, and that 2) this region may play a role in the generation of depression-associated behaviors. To test the first hypothesis, I administered desipramine (DMI), a tricyclic antidepressant, to rats. I found that the rat vmPFC was significantly activated by DMI, whereas the dorsomedial PFC (dmPFC) was not. I also found that the drug increases neuronal activity in the nucleus accumbens, but this activation was dependent on the integrity of the vmPFC. To test the second hypothesis, I induced neuronal lesions in the rat dmPFC or vmPFC and subjected the animals to behavioral tests. I found that

while lesions in both areas led to increased REM sleep, only vmPFC-lesioned animals had reduced REM latency, increased sleep fragmentation and increased forced swim test immobility. Together, these results demonstrate that the vmPFC may be an important region for both antidepressant action and the generation of depression-like behaviors.

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Dedication

I dedicate this dissertation to my parents, C.S. and Inja, and to my husband, Phil.

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Above all, I give thanks to God for everything, pass on the glory to Him, and hope He is blessed by this work and journey.

Chapter 1

Introduction to sleep, depression and their neural circuitries

Sleep deprivation is a growing concern for health, safety and productivity. In the US, a recent survey reported that adults sleep forty-five minutes less per night than they claim they need to be at their best (1). Long-term sleep loss has been associated with medical issues such as cardiovascular disease, diabetes, and certain cancers. In addition, psychiatric disorders such as depression are strongly associated with sleep changes and disturbances. Patients with depression complain of insomnia (and to a lesser degree, hypersomnia), and demonstrate characteristic alterations in rapid eye movement sleep: increased amounts per night, shortened latency to onset, and increased density of rapid eye movements (2; 3). In addition, most traditional antidepressant medications have been found to suppress rapid eye movement sleep. Despite the long-standing evidence of a relationship between sleep and depression, the neural circuitry underlying this relationship is unknown. A motivating factor for this dissertation was to unearth clues about the neurobiology of this relationship.

Sleep and Public Health

Although chronic sleep deprivation is not center stage among the nation's public health issues, societal awareness of its negative consequences appears to be increasing. U.S. regulatory bodies such as the Federal Aviation Association and National Transportation Safety Board (NTSB) have promptly responded to incidents related to fatigued air traffic controllers and sleepy drivers, and the medical industry frequently debates the issue of extended work hours for residents and interns. However, there are few broad regulations that attempt to prevent fatigue-related accidents or punish the harmful consequences of them. It can be argued that the passage of sweeping laws that address fatigue – as in the case of drunk driving a few decades ago – will be an indicator that the public has accepted the notion that fatigue is dangerous.

An obstacle to achieving this milestone is that there are many aspects of sleep we do not understand, including why we (and most organisms) have evolved to sleep. Although it has been found to improve learning and memory (4), be associated with immune function (6) and improve performance of basic tasks (5), the neurobiological functions that necessitate spending several hours of every day sleeping are unknown. Furthermore, the purported variability in sleep need (7) makes it difficult to distinguish people who are sleep-deprived from individuals who simply may require less sleep.

Nevertheless, the consequences of sleep deprivation have provided fodder for the argument that sleep is necessary for one's well-being. Randy Gardner, a teenager who volunteered to fend off sleep long enough to set a Guinness Record, suffered from short-term memory loss, moodiness, paranoia and hallucinations by the end of 264 hours (11 days; 10). Interestingly, even much more mundane instances of sleep deprivation have been connected to negative health consequences: the onset of daylight savings time, which much of the world

endures in the early spring, is associated with an increased incidence of heart attacks compared to when daylight savings time ends (12).

Prospective and retrospective epidemiological studies have demonstrated that sleep deprivation and rotating shiftwork schedules, which presumably disrupt circadian rhythmicity and result in sleep deprivation, can have long-term negative health consequences. For instance, sleep deprivation has been associated with increased blood pressure (8), increased inflammatory markers (9) and decreased levels of leptin, an appetite-suppressing hormone (16). In addition, the Nurses' Health Study at Harvard has demonstrated that chronically sleep-deprived women have a significantly increased risk of coronary heart disease (11) and diabetes (13; 14), and 15-20 years of regular rotating shiftwork is associated with increased risk of developing breast cancer (15) and colorectal cancer (17). Although these studies are extremely important and informative, the biological bases for these associations are yet to be understood.

In addition to mental and physical health issues related to sleep deprivation, fatigue can lead to serious accidents at work and on the road. A Swedish cross-sectional survey found that a worker was 1.89 times more likely to die in a work-related accident if he or she had continuous sleep problems during the prior two weeks (23). Medical interns made 35.9% more serious errors on a traditional schedule, which required work shifts of 24 hours or more every other shift, compared to when on an 'intervention' schedule that eliminated long shifts and reduced total number of hours worked per week (25). Long work hours, as well as shift work, changes in family life, illness, and difficult economic circumstances can contribute to sleepy people getting behind the wheel. As a result, sleep-deprived drivers cause an estimated 100,000 car accidents in the U.S. each year, and lead to over 1500 deaths (27). In one study, 17 hours of sustained

wakefulness was found to be comparable to a blood alcohol level of 0.05% in tests for cognitive psychomotor performance (29).

Sleep deprivation is also believed to have a significant economic impact. It has been estimated that $20.5 billion is needed to test and treat every sleep apneic patient in the U.S. (31). The cost due to shift workers' difficulty adjusting to their schedules is estimated to be $60 billion (33), and lost productivity due to absenteeism, sleep-related accidents, poor decision-making and other sources lost productivity are believed to cost businesses $150 billion annually (35). Furthermore, many major disasters have at least partially been attributed to fatigue, which may have been prevented by adequate sleep. In 1989, an *Exxon Valdez* crewmember was exhausted when the tanker ran onto rocks in Alaska, leading to 11 million gallons of spilled oil (37). The NTSB report on the 1997 Korean Airline crash in Guam noted that the pilot was fatigued, which may have led to his making poor decisions (39). More recently, the Bronx bus tragedy (2011) that killed 15 and seriously injured seven – including by maiming and decapitation – was determined by the NTSB to be a consequence of fatigue and sleep loss (41). An investigation determined that the driver had less than 4 hours sleep opportunity in the day leading up to the accident.

Despite the immense impact that lack of sleep has been found to have on public health and society, there are substantial gaps in our knowledge of its biology and in particular how it can lead to mental and physical illness. Therefore deeper pursuit of understanding the neural circuitry of sleep should be closely accompanied by research into how sleep dysfunction affects the brain and body.

The Basics of Sleep

Architecture of human sleep

Sleep can be divided into two parts: rapid eye movement (REM) and non-rapid eye movement (NREM). During REM sleep, the eyes display rapid movements back and forth underneath the eyelids. Most of the body is atonic (18), and blood pressure, heart rate, and respiration rate increase slightly compared to NREM sleep (44; 46; 48; 50). It is considered to be the 'active' stage of sleep (or 'paradoxical sleep') because neural activity is at a level similar to wakefulness as displayed on an electroencephalogram (EEG; 27). The REM sleep EEG waveform is dominated by theta waves, which are in the 4-7 Hz range (Fig. 1-1). Although REM sleep is most often associated with dreaming (53), particularly dreams that are recalled upon awakening, dreaming can occur during NREM sleep as well (55).

NREM sleep is characterized by synchronized EEG waves that have high amplitude and are of low frequency. There are three stages of NREM sleep, ranging from light (I) to deep (III) (recently changed from four stages; 31). The deepest sleep stage, also known as slow wave sleep, is typically dominated by delta waves in the 0.5-4 Hz range (59). During NREM sleep metabolic rate and body temperature are lowered (61), and the arousal threshold is elevated (19). However, muscle tone is not absent during this state, and parasomnias such as sleepwalking, night terrors and bed-wetting occur during NREM sleep (63; 65; 67; 69). A NREM sleep bout typically starts in stage I and gradually progresses to the deeper stages. After the first bout of slow wave sleep, the individual characteristically goes back through stage II and then I of NREM sleep before first entering into REM sleep; this progression then repeats at roughly 90-minute cycle intervals (59). The ratio of NREM to REM sleep in each cycle starts relatively high and gradually decreases

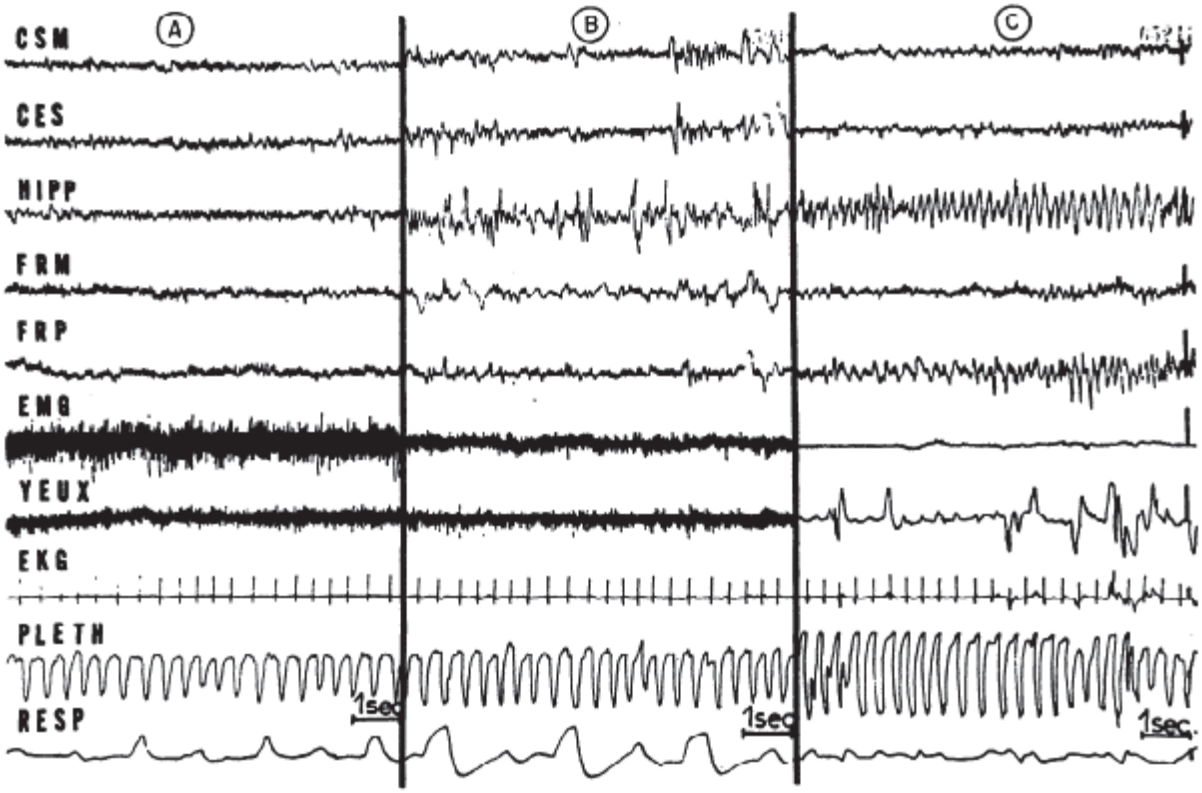

Figure 1-1. Representative human EEG waves in the different vigilance states: wake (A), NREM sleep (B), and REM sleep (C). Abbreviations indicate measurements from: CSM = sensorimotor cortex; CES = ectosylvian cortex; HIPP = hippocampus; FRM = mesencephalic reticular formation; FRP = pontine reticular formation; EMG = electromyogram; YEUX = eye movements; PLETH = plethysmographic index; RESP = respiratory activity. From (71); permission not required for use.

through the night and into the morning, such that most REM sleep occurs in the usually morning, and one usually wakes up from REM sleep.

Human sleep changes drastically from birth to adulthood. Newborns sleep for 16-20 hours a day, which is comprised of several sleep episodes ('fragmented' sleep). They enter sleep via REM sleep, which cyclically alternates with bouts of NREM sleep (20). Infants typically spend a much larger proportion of their time in the deepest stages of NREM sleep compared to adults. As infants progress into childhood, their total sleep amount gradually decreases and plateaus at about 10 hours per day at ages 6-7 (20). Sleep also becomes more consolidated, and the cycles change so that they start with NREM sleep. By puberty, although sleep characteristics change little aside from decreased slow wave sleep (21; 22), the homeostatic regulation of sleep appears to shift such that they need more sleep (22; 24). For instance, compared to adult males, adolescent males demonstrated greater sleep need across many different lengths of time spent awake (24). However, adolescence is also associated with a dramatic decrease in sleep amounts due to social influences and earlier school start times (26), suggesting that this segment of the population is continuously sleep-deprived (28). By adulthood, the recommended daily sleep amount is 7-8 hours (79; 81), although the most recent survey by the National Sleep Foundation reported that the average American sleeps less (45).

Neural circuitry of human sleep: Sleep-wake control regions

For many years, conscious wakefulness via cortical activation was believed to be driven by inputs from the thalamus and basal forebrain (30; 32). The thalamus has extensive reciprocal connections with the cortex, and is also a major relay structure for sensory inputs to the cortex (34). In addition, its firing patterns change between wake and sleep states, similar to cortical

EEG activity (88), suggesting that it is important for modulating cortical EEG. Retrograde tracing studies showed that the cholinergic lateral dorsal and pedunculopontine tegmental nuclei (LDT-PPT) innervate the reticular nucleus of the thalamus and surrounding nuclei (90; 92), and unit recordings *in vivo* revealed that the vast majority of cells in this region were wake- and/or REM-active (although it has never been confirmed that the cells that were recorded were cholinergic; 53). Therefore, it was proposed that the LDT-PPT—thalamus—cortex pathway was important for cortical EEG generation and arousal. However, a few studies have challenged the importance of the thalamus for driving cortical wakefulness and EEG activity. For instance, stimulation of the vast majority of the thalamus did not induce cortical activation (96), nor did thalamic lesions prevent cortical activation (98). Recently the role of the thalamus in arousal and sleep-wake circuitry was re-examined (36). Nearly complete lesions of the thalamus caused no significant change in any sleep-wake parameters, including total sleep or wake amounts and EEG activity (although long-term lesion effects were not studied). In addition, animals did not show any signs of increased sleepiness and had increased cortical cfos (an immediate early gene and marker of neuronal activation) expression following a prolonged bout of wakefulness, comparable to non-lesioned controls. Therefore, despite its neuroanatomical position as an important relay structure, the thalamus may not have a role in promoting wakefulness.

On the other hand, evidence of the importance of the basal forebrain in arousal has been strengthened. Large lesions in the basal forebrain produce a coma-like state (36; 38), including failure a continuous low-frequency EEG; there is also no cortical cfos expression despite continuous gentle handling. In addition, injections of glutamate agonists into the basal forebrain increase wakefulness (40; 42; 43). Monoaminergic nuclei in the brainstem and the histaminergic tuberomammillary nucleus in the hypothalamus send projections to the lateral hypothalamus

(LH), which in turn projects to the basal forebrain and cortex (Fig. 1-2). The LH includes the sole population of orexin neurons in the brain, although there are orexin receptors distributed throughout the brain (45; 47). The orexinergic neurons are wake-active, and appear to modulate the sleep-wake switch as well as the REM-on/off switch (see below). For instance, orexin knockout mice exhibit narcolepsy-like behavior with cataplexy and frequent vigilance state transitions, albeit normal total sleep and wake amounts (107). Optogenetic stimulation of the orexin neurons increases wakefulness (49), as do microdialysis applications of orexin into the basal forebrain (110; 112). Together, these results suggest that the LH orexinergic projections into the basal forebrain act to promote arousal.

The noradrenergic locus coeruleus (LC), serotonergic dorsal raphe (DR), dopaminergic ventral periaquductral gray (vPAG) and histaminergic tuberomammillary nucleus (TMN) have also been found to play roles in arousal. Neurons recorded in the LC were found to be most active during wake and least active during REM sleep (114). Infusion of noradrenergic alpha2 receptor agonists into the LC resulted in sleep (116), whereas ventricular administration of noradrenaline caused wakefulness (118). Similarly, in the DR, about half of recorded cells were wake-active and 18% were wake and REM-active (120), and extracellular levels of serotonin were highest during wake (51; 52). A recent study has addressed the presence of dopaminergic wake-promoting cells by combining cfos staining with immunohistochemistry against tyrosine hydroxylase (TH), which is found in dopamine-containing neurons in the midbrain (54). About half the TH-stained cells in the ventral periaqueductal gray matter (vPAG) were wake active, and lesions that selectively depleted the dopaminergic vPAG cells significantly increased sleep amounts. Lastly, bilateral lesions in the posterior hypothalamus, including the TMN,

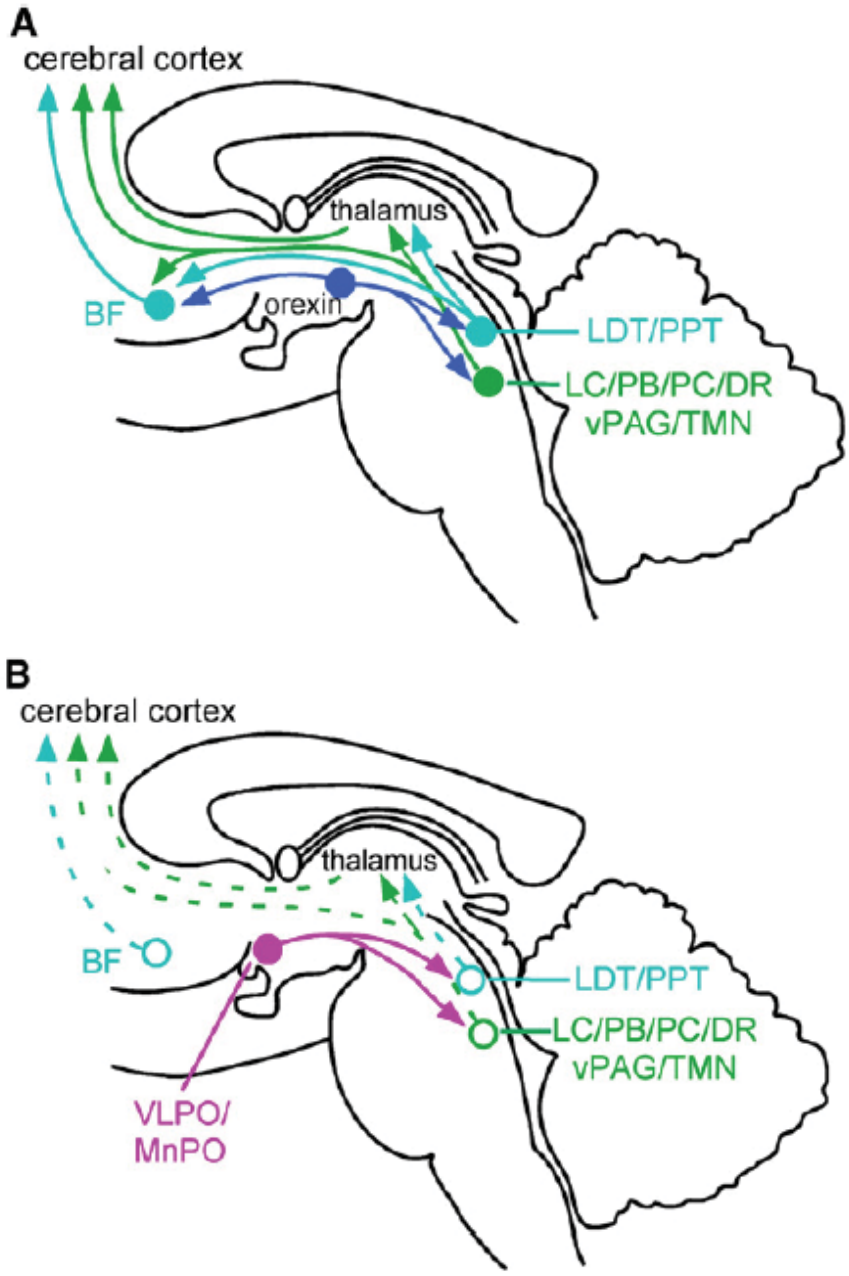

Figure 1-2. Diagram of sleep-wake circuitry. (A) Brainstem nuclei that promote arousal via the basal forebrain. (B) The VLPO and MnPO (magenta) promote sleep by inhibiting the arousal centers. Aqua: cholinergic projections. Green: monoaminergic and glutamatergic projections. Blue: orexinergic projections from the lateral hypothalamus. BF, basal forebrain; LDT, laterodorsal tegmentum; PPT, pedunculopontine tegmentum; LC, locus coeruleus; PB, parabrachial nucleus; PC, precoeruleus nucleus; DR, dorsal raphe nucleus; vPAG, ventral periaqueductal gray; TMN, tuberomammillary nucleus; VLPO, ventrolateral preoptic nucleus; MnPO, median preoptic nucleus. From (56); used with permission.

significantly increased sleep amounts (126) and extracellular unit recordings showed that histaminergic TMN neurons were exclusively wake-active (58). Projections from the LC, DR, vlPAG, and TMN to the LH have been confirmed by tracing studies (54; 60).

Strikingly, it was recently found that combined lesions of the parabrachial and precoeruleus nuclei (PB/PC) in the brainstem lead to a coma-like state, producing results very similar to complete lesions in the basal forebrain (36). As many cells in the PB/PC project to the basal forebrain. these results suggest that an essential wake-promoting pathway originates in the PB/PC in the brainstem. In contrast to the interpretation by Moruzzi and Magoun (130) that the ascending arousal influence originated in the reticular formation, these studies suggest that the electrolytic lesions or electrical stimulation in the paramedian midbrain reticular formation probably instead involved the ascending projections from the monoaminergic, cholinergic, and PB/PC glutamatergic pathways, which run through this region to the hypothalamus, basal forebrain, and cerebral cortex.

The arousal-promoting system in the brain is complicated and involves many different structures. On the contrary, just two regions have been established to promote sleep: the ventrolateral preoptic nucleus (VLPO) and the recently reported parafacial zone (PZ). A cfos study showed that neurons in the VLPO were highly activated following sleep bouts (62), and bilateral lesions led to significant decreases in NREM sleep amounts and delta power (64). Lesions encompassing the ‘core cluster’ of the VLPO led to the most sleep changes (64), and neuronal tracers showed that this region densely innervates the wake-promoting TMN (62). The more scattered cells surrounding the core also innervate the LC and raphe nuclei, although to a more moderate degree (134). It has been proposed that most of the VLPO cell population contains GABA, and thus has a mutually inhibitory relationship with the arousal regions (66).

The PZ was discovered following injection of a retrograde tracer cholera toxin B (CTB) from the wake-promoting medial parabrachial nucleus in rats (68). Animals were sacrificed under waking or sleeping conditions, and cells in the PZ that were stained for CTB only showed cfos expression if they had been asleep. Neuronal lesions of the PZ resulted in significant increases in wakefulness, and an experiment using mice that express GFP in vesicular GABA transporter (VGAT)-containing cells showed that these sleep-active neurons were primarily GABAergic. Lastly, selective knockout of the VGAT cells in the PZ using transgenic mice led to increased wake amounts and bout durations, confirming that inhibitory cells in the PZ were the key subpopulation that promotes sleep.

Neural circuitry of REM sleep

The REM sleep circuitry has been an area of intense investigation in recent years. A widely accepted circuit model proposed by Hobson and McCarley (138) suggested that the brainstem cholinergic neurons in the PPT-LDT promote REM sleep, and monoaminergic neurons in the DR and LC prevent it. However, this model was challenged by a number of findings. For instance, the target monoaminergic site that suppressed REM sleep was unclear, particularly serotonin (140). In addition, lesions of the monoaminergic brainstem nuclei had limited effects on REM sleep and its characteristic atonia (70; 72; 73). On the other hand, lesions in the SLD had been found to prevent REM atonia, causing cats to “act out” their dreams (74; 75). Furthermore, local administration of GABA antagonists bicuculline and gabazine into the SLD were found to induce REM sleep with very short latency, as did glutamate agonist kainic acid (76). These results suggested that the SLD was important in REM sleep generation, and GABA played a role in delaying REM onset while glutamate promoted it. The ventrolateral periaqueductal gray-

lateral pontine tegmentum (vlPAG-LPT) was a candidate ‘REM-off’ control region to the SLD because injections of GABA agonist muscimol in this region of the cat led to large increases in REM sleep (146). Subsequent studies showed that lesions in the ventrolateral periaqueductal gray-lateral pontine tegmentum (vlPAG-LPT) of rats led to an increase in REM sleep, and lesions in the sublaterodorsal nucleus-precoeruleus (SLD-PC) significantly decreased REM sleep (147). These results indicated that the vlPAG-LPT and SLD-PC were the REM-off and REM-on control areas, respectively. Tracing studies showed that these areas project to each other, and that many of the inputs from both were GABAergic (147; 148). Therefore, the authors proposed that these REM control areas mutually inhibit each other to form a ‘flip-flop switch’.

These REM control areas are modulated by many nuclei on both sides of the switch. As mentioned above, orexinergic neurons are found in the LH, and orexin knockout mice display narcolepsy-like behaviors (107). It is believed that the cataplexy that occurs in these animals is an incomplete display of REM sleep with atonia, so orexin may be important for controlling entry into REM. In addition, LH orexin neurons are relatively quiet during REM sleep (77; 78), so their projection to the vlPAG-LPT suggests that they may be assisting in the suppression of REM sleep by activating the REM-off region during wake.

Cells in the extended VLPO (eVLPO) were found to contain GABA and galanin, both inhibitory neurotransmitters, and be REM-active (80). This region also sends projections to the vlPAG-LPT (82), and VLPO lesions that include this region cause a decrease in REM sleep (64). Therefore, the eVLPO appears to block the vlPAG-LPT to allow REM sleep to occur.

The LDT-PPT, LC and DR that are important in arousal also appear to play roles in REM control. In the LDT-PPT, 97% of cells recorded in this region were found to be REM-active or REM- and wake-active (149). However, none of these studies identified the chemical phenotype

of the neurons being recorded, which will be very important in understanding their role in sleep. Although lesions of these regions do not change REM sleep amounts (147), injecting acetylcholine into the brainstem induces a REM-like state (150; 151). Although the circuitry by which the LDT-PPT modulates the REM switch is unclear, Lu and colleagues have proposed that the cholinergic and monoaminergic neurons modulate the activity in the vlPAG/LPT-SLD flip-flop switch.

Neurons in the LC are REM-off active (114). Again, although lesions here did not change the amount of REM sleep, they did cause a reduction in ponto-geniculo-occipital (PGO) spikes (70), which are most prominent immediately before REM sleep onset in cats. Compared to wake and NREM sleep, extracellular GABA concentrations were found to be higher in this region during REM sleep (84). Together, these data suggest that LC neurons assist the suppression of REM sleep, and are inhibited by GABA during REM sleep.

Serotoninergic neurons of the DR also may be REM suppressant. Microinjections of muscimol, a GABA agonist, into the DR lead to 67% increase in REM sleep, whereas injection of picrotoxin (a GABAA receptor antagonist) abolishes REM sleep (85).

REM sleep is characterized by muscle atonia and hippocampal theta waves in rodents. Lesions of the ventral SLD caused REM sleep to occur without atonia, suggesting that the mechanism for atonia depends on this nucleus (147). Initially it was believed that atonia was induced by inhibitory projections from the ventromedial medulla, as tracing studies in cats under identified glycinergic neurons that expressed cfos during carbachol-induced REM sleep (152). However, lesions in this area of the rat did not affect REM atonia (147). It was subsequently shown that the SLD may directly project to cells in the spinal cord, as a retrograde tracer injection into the ventral horn of the spinal cord labeled cells in the SLD (147). Furthermore,

nearly all the labeled cells contained *VGLUT2* mRNA, which is the transcript for a glutamate transporter protein. Anterograde tracer injection into the SLD demonstrated that most spinal cord neurons that the SLD neurons appose are inhibitory interneurons. Together, these results suggest that SLD neurons may inhibit muscle tone during REM sleep by excitation of interneurons that in turn suppress motor neurons.

It has been suggested that hippocampal theta rhythms are driven by GABAergic neurons in the medial septum (MS) (153). A retrograde tracer injection into the MS demonstrated labeled cells in the PC, and most of these cells are glutamatergic (147). Furthermore, ibotenic acid lesions in the PC abolished theta rhythms during sleep. Therefore, these results suggest that the PC may drive the signature theta rhythms of REM sleep.

Major Depressive Disorder and Sleep

There is a plethora of evidence that sleep is linked to psychiatric disorders. For instance, patients suffering from anxiety disorder report an increased latency to sleep, decreased sleep efficiency and decreased total sleep (154). Schizophrenic patients report difficulty initiating and maintaining sleep, daytime fatigue, and reduced REM latency (155). Major depressive disorder, one of the most common mental disorders in the U.S. (156), also has strong associations with sleep changes. The majority of those suffering from depression complain of insomnia (86; 87), and they also demonstrate reduced slow wave sleep (89) and shortened REM latency (91). However, the biology underlying the relationship between affect and sleep changes is unclear. I was interested in investigating this relationship, which I report on in this dissertation.

Major depressive disorder (MDD) is a debilitating disease characterized by a loss of interest in pleasurable things and a persistently depressed mood. MDD is primarily characterized as a disease of first-world countries: by one account, of the estimated 121 million that suffer from the disease worldwide, 14.8 million are in the U.S. (157). In addition, the lifetime prevalence of depression in the U.S. is 17% (93; 157). A patient is diagnosed to have MDD when he or she experiences depressed mood, loss of interest, and has at least three of the following additional symptoms for a minimum of two weeks: appetite/weight disturbance, sleep disturbance, psychomotor change, loss of energy, feelings of worthlessness/guilt, concentration difficulties/indecisiveness, or thoughts of death/suicide (158; 159). This symptom-based definition is a reflection of the difficulty scientists have faced in identifying a specific cause of depression. Furthermore, the biological basis of each of these symptoms is not well understood. Although epidemiologic studies show that 40-50% of the risk of depression is genetic (93; 95), non-genetic factors such as stress, emotional trauma, and nervous system abnormalities also

likely underlie the pathophysiology of MDD (160).

Sleep alterations in depression

As mentioned, patients with MDD show substantial alterations in their sleep architecture. Approximately 80% of depressed patients complain of insomnia, while 15-35% demonstrate hypersomnia (86; 161). Although abnormalities in both NREM and REM sleep have been reported, the latter are more pronounced and consistent. Typically patients experience increased REM sleep, shortened REM sleep latency (the interval of time between sleep onset and REM onset), and increased REM density (2; 3; 97; 99). Notably, these changes are observed in both insomniac and hypersomniac patients (100), so the REM sleep changes are unlikely to be related to increased sleep pressure following sleep loss.

REM sleep changes are also observed in animals exposed tochronic stress that are believed to exhibit behaviors related to depression. For example, two weeks of chronic mild stress led to increased REM sleep (101), and 15 days of inescapable footshocks resulted in reduced REM sleep latency as well as increased REM sleep (102). Flinders Sensitive Line rats, a line bred to be hypersensitive to cholinergic agonists (which is a trait observed in depressed humans), and prenatally stressed rats also show increased REM sleep (132; 162). On the other hand, most traditional antidepressant drugs have been found to suppress REM sleep, in humans as well as in animals (2; 103–106), although the mechanism of this suppression is unknown. Altogether, these data suggest that there is an interrelationship between REM sleep and depression-like behaviors.

Cortical involvement in depression: Human literature

Imaging studies have shown that depressed patients exhibit abnormal activity in certain brain regions compared to healthy subjects, including the amygdala, medial thalamus, and regions of the prefrontal cortex. Even in healthy subjects, clinical studies suggest that limbic structures, including subregions of the prefrontal cortex, play a role in affect regulation (158). When volunteers were asked to think of a sad memory during fMRI imaging, blood flow increased to the lateral orbital and ventrolateral PFC (vlPFC; 122). Another study found that happy memories caused a decrease in blood flow to the ventromedial PFC, while sad memories caused an increase (surprisingly, MDD patients demonstrated the opposite pattern; 123). Emotion-evoking photographs, either positive or negative, caused an increase in activity in the ventral and dorsal medial PFC and decrease in the lateral PFC compared to neutral photographs (164). The vlPFC was also found to be involved in active judgment of information that is held in cortical association regions, suggesting that the decision-making process of how to react to emotional thoughts may partially be made here (165).

The ventromedial PFC has particularly been receiving increased attention in human studies of depression in recent years. This region includes the rostral anterior cingulate (rACC, BA24), prelimbic (BA32) and subgenual cingulate cortices (SGC, BA25). A study showed that the rACC was hypometabolic in depressed patients overall, but those that responded to antidepressant treatment were actually distinctly *hyper*metabolic at baseline (109). On the other hand, the SGC of depressed patients displayed increased activity (111; 113) and decreased gray matter volume (111; 115; 117; 119). Six weeks of antidepressant treatment reduced blood flow in this region, and further analysis demonstrated that this reduction was specific to those that responded to treatment (121). These findings suggest that receptiveness to depression alleviation

via antidepressants may have a neurobiological basis (via the rACC), that the SGC may be a state marker of depression, and that generally the ventromedial PFC may be involved in the antidepressant response.

Deep brain stimulation (DBS) originally developed to treat Parkinson's Disease (166–168), was recently attempted in depressed patients for the first time (122). The electrode was placed just outside the SGC. Although the six patients had been labeled 'treatment-resistant', failing to respond to multiple modes of antidepressant therapy, three of them achieved remission or near-remission and one additional patient continued to experience an antidepressant response at the conclusion of the six-month study. A second study had similar results after targeting the same area in twenty patients (123). These patients also displayed decreased blood flow in the SGC after six months (122; 123), so it was suspected that DBS had local inhibitory properties. However, activity changes were observed in many brain areas that are downstream of the DBS target region in both MDD (122–124) and PD patients (125; 127; 128), suggesting that changes in neural network dynamics may be essential.

Indeed, tracing studies in *Macaca fuscata* monkeys found that the ventromedial prefrontal cortex in primates projects to many important limbic regions believed to participate in the depression circuitry, including the nucleus accumbens core and shell regions, amygdala, insular cortex, and septal nuclei (169). This region of the brain therefore appears positioned to influence activity in the limbic circuitry and play an important role in mood regulation.

The rodent as an animal model of depression

Although studying major depressive disorder exclusively in humans would be ideal, intrinsic variability among subjects and limitations in methods would make it difficult to significantly

advance our knowledge. For these reasons, animal models of depression are useful and necessary. Rodents, particularly rats, are good animal models because of their limited variability and uniform genetic background. We also have extensive knowledge of their neuroanatomy and neurocircuitry, including on regions of the brain believed to control emotion and reward. Based on neuroanatomical and functional studies, it has been accepted that rats possess a prefrontal cortex that shares many similarities with primate prefrontal cortex (170; 171). Although there has been some discrepancy about which specific human PFC subregion is homologous to the rat medial prefrontal cortex, a careful tracing study of the PFC of non-human primates revealed that BA25 and 32 in primates have very similar efferent projections to the rat vmPFC (169; 172). Therefore, the rat vmPFC is an excellent area to investigate neuroanatomical and behavioral questions regarding MDD, given the believed importance of the vmPFC in human depression (111; 121; 113; 122).

Although it is challenging to observe and measure depression in animals, several tests have been established in rodent depression research. In rats and mice perhaps the most common is the forced swim test (FST), also known as the Porsolt swim test (129). In this two-day test, a rat is placed in a cylinder of water of standard size and temperature for fifteen minutes the first day and five minutes the second day. The outcome measure is the amount of time an animal spends immobile during the second test session. A similar version is performed with mice, but with only one five-minute session (131). The FST was originally established as a model to detect antidepressant efficacy, and its predictive validity has been demonstrated from the wide range of antidepressants it detects as well as the non-antidepressants (such as anxiolytics) it does not (173). However, it is also used to demonstrate depression-like behaviors in animals that have not received antidepressant drugs (132; 133; 135–137). Although in this capacity it is acknowledged

that it has limited face validity, it is generally accepted that immobility is a quantifiable behavior that may be associated with depression (174). First, decreased immobility during the FST is observed following animal models that demonstrate aspects of depression, such as chronic mild stress (animals are exposed to a variety of stressors such as light during the night, food and/or water deprivation, cage tilt, etc. daily for weeks) (175; 176) and social defeat (animal is subjected to weeks of daily stress due to presence of a dominant animal) (139; 141). Second, the first swim session appears to change something in the animal, as it has been shown that animals that experience inescapable (but not escapable) water immersion are unable to learn a shock avoidance task compared to animals that could escape (177), suggesting that they are more vulnerable to feeling helplessness. Lastly, there is evidence that FST immobility is associated with a specific gene cluster. A group of researchers crossed two lines of mice that demonstrated the greatest and least amounts of immobility in the FST and tail suspension test (TST), and searched for genes that may inform the results in each (178). A whole genome quantitative trait locus analysis determined that a locus on mouse chromosome 11 was significantly associated with both FST and TST results, and candidate gene analysis pointed to a gene cluster that encodes GABAA receptor subunits alpha-1 and alpha-6. They found that between the two progenitor lines, the GABAA alpha-1 gene was expressed significantly more in one line, and in the GABAA alpha-6 subunit there was a single amino acid difference. A clinical study showed that polymorphisms on the human syntenic region of these genes was associated with mood disorders in female patients, compared to age-matched controls (179). In summary, although the FST is not an ideal rodent test in depression studies, there is evidence that it is a useful tool for studying behaviors that may be associated with depression.

Cortical involvement in depression: Rodent literature

There is mounting evidence that the rat mPFC may be involved in the regulation of stress and emotion. Chronic (daily for three weeks) and even one week of mild stress led to changes in dendritic morphology in the mPFC (142–144). Lesions of the rat ACC led to increased time spent immobile during the FST (180), and glial cell loss by astrocyte-specific toxin L-alpha-aminoapidic acid has been shown to produce increased immobility in the FST and decreased sucrose preference in rats (136). Since treatment-resistant depressed patients responded to deep brain stimulation in the SGC (122), the rodent vmPFC has been targeted for deep brain (135) and optogenetic stimulation (145) in recent studies. Deep brain stimulation in the rat during a FST protocol significantly decreased immobility compared to non-stimulated controls, suggesting an antidepressant effect. Optogenetic stimulation in the vmPFC of mice that displayed susceptibility to social defeat stress improved performance in a social interaction test and the sucrose preference test. In addition, the defeated mice had reduced expression of immediate early genes zif268, cfos and arc (145). Altogether, these studies suggest that reduced activity in the rodent vmPFC seems to be linked to depression-associated behaviors, whereas increased activity may be antidepressant.

Specific Aims

Studies of depressed patients demonstrate that the ventromedial prefrontal cortex displays abnormal metabolic activity and blood flow, which normalize upon successful treatment and/or remission. In addition, treatment responders display unique changes in imaging studies compared to nonresponders in this area. Lastly, recent evidence that treatment-resistant patients can successfully be treated with deep brain stimulation targeting the subgenual cingulate cortex suggests that this area may also be an important target for antidepressant therapies. For my dissertation work, I therefore hypothesized that the ventromedial prefrontal cortex may be a key target in antidepressant treatment as well as play a role in the generation of depressive behaviors.

Aim 1: Do antidepressant drugs target the ventromedial prefrontal cortex?

To study whether antidepressant drugs may be acting on or through the ventromedial prefrontal cortex, we administered desipramine (tricyclic antidepressant), fluoxetine (elective serotonin reuptake inhibitor), and ketamine (NMDA antagonist) to rats. We then examined the whole brain for regions that were selectively activated by each of these three drugs. Using tracing data in the literature as well as our own studies, we further asked what pathways appear to be important for the action of desipramine.

Aim 2: Does the ventromedial prefrontal cortex play a role in generating depressive behaviors, including sleep modulation?

Next, to understand the role the ventromedial PFC may have in giving rise to depression, we used ibotenic acid to induce neuronal lesions in the subregions of the rat medial prefrontal cortex: the dorsal mPFC (anterior cingulate) and ventral mPFC (prelimbic and infralimbic

cortices). We then recorded the animals' sleep-wake behavior and administered forced swim test, which are both measures of depressed state. Upon finding that lesions in the medial prefrontal cortex lead to REM sleep changes, we used anterograde and retrograde tracers to investigate possible neural pathways of sleep modulation.

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Chapter 2

Antidepressant drugs selectively activate the rat ventromedial prefrontal cortex

ABSTRACT

Research on traditional antidepressant drugs led to the monoamine hypothesis, which postulated that major depressive disorder (MDD) is a consequence of a chemical imbalance in the brain. However, this hypothesis is now considered unlikely and the pursuit of understanding the mood-elevating effects of antidepressants continues. The prefrontal cortex (PFC) of depressed patients displays abnormal activity, such as increased blood flow in the subgenual and ventrolateral PFC, which normalizes in patients who are asymptomatic or in remission. Furthermore, the subgenual and orbitofrontal PFC display decreased volume sizes that may be due to reductions in cell numbers, suggesting that dysfunction in these regions may be important for MDD etiology. We therefore hypothesized that antidepressants may target the PFC to reverse depression. We used the rat as an animal model to study the neural targets of desipramine, a tricyclic antidepressant. We found that cfos expression selectively increased in the ventromedial PFC following drug treatment, and increased expression only occurred in layers V-VI. This pattern was replicated following administration of fluoxetine, a selective serotonin reuptake inhibitor, and ketamine, an NMDA-antagonist recently found to have antidepressant properties. In addition, of the downstream limbic targets of the vmPFC only the NAc was activated by desipramine, and this activation was lost upon vmPFC neuronal lesions. These results suggest that the vmPFC may be

an essential target of antidepressant drugs, and vmPFC projections to the NAc may be a key circuit regulating antidepressant action.

Introduction

Monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and other classes of drugs have been prescribed to treat Major Depressive Disorder (MDD) over the past several decades. Limited knowledge of how these drugs worked gave rise to the monoamine hypothesis of depression, which postulated that a neurochemical imbalance in the brain contributed to the etiology of MDD (1). While this theory is currently considered overly simplistic, we still lack a complete understanding of the mood-elevating mechanism of these drugs. Greater insight into how these medications work may shed light on the etiology of MDD.

Antidepressant medications are taken systemically yet they have different effects on different regions of the brain (2). The changes observed before and after treatment in the prefrontal cortex (PFC) of MDD are particularly intriguing. For instance, depressed patients demonstrate increased activity in the subgenual and ventrolateral PFC (3; 4) and increased activity in the amygdala (4). The PFC activity changes are reversed in patients in remission (4; 5), and these changes may be essential for a positive response to antidepressant drugs. Responders to the SSRI fluoxetine after six weeks of treatment demonstrated changes in cerebral blood flow compared to non-responders, including decreases in the subgenual cingulate and increases in the dorsal PFC (5). In addition, volume decreases are observed in certain brain regions of depressed patients, and are most consistently reported in the orbitofrontal and subgenual PFC (4; 6–8). Post-mortem analyses of the brains of depressed patients demonstrated that both these regions have significantly reduced number of glial cells but do not show changes in neuronal cell numbers compared to healthy controls (8; 9), providing a possible explanation for the volume reduction. This notion is supported by animal work that has shown that chronic

restraint stress leads to dendritic atrophy, including reduced length and total branch numbers of apical dendrites in pyramidal cells of the rat mPFC (10–12).

Furthermore, there is mounting evidence that antidepressant treatment is associated with increased neuroplasticity and changes in cell morphology (13–17). For instance, chronic imipramine (a TCA) treatment resulted in increased levels of three plasticity-associated proteins in the rat PFC (15), and even a single dose of the MAOI iproniazid significantly increased the area of dendritic profiles (18). Therefore, the PFC may be an important region for both the underlying pathophysiology of MDD as well as the mood-alleviating effect of antidepressants.

Ketamine, an NMDA-antagonist typically used as an anesthetic agent, has recently been found to rapidly alleviate depression in humans and demonstrate antidepressant-like properties in rodents (19–22). Acute doses injected in rats have been found to increase synaptic spine density in the mPFC (22). In addition, its antidepressant-like effects may be dependent on the PFC, as infusion of rapamycin – which blocks an important signaling pathway involved in cell growth and survival – into the rat mPFC via cannulae prevents synaptogenesis and the antidepressant-like response of ketamine (22).

Despite the evidence for prefrontal cortical involvement in depression and its role in responding to antidepressants and ketamine, few studies have been performed that investigate the effect these drugs have on the prefrontal cortical-limbic neural circuitry. The rat mPFC is believed to have similar basic neuroanatomical traits as the human PFC (23–25), and therefore serves as an appropriate animal model. In particular, the rat vmPFC projects to many limbic areas implicated in control of emotion, such as the lateral septum, basolateral amygdala, insular cortex, and nucleus accumbens. These studies suggest that the mPFC may play a key role in both depression and in the therapeutic effects of antidepressant medications. To test this hypothesis, I

acutely administered desipramine, a TCA, fluoxetine, an SSRI or ketamine into healthy rats and examined patterns of neuronal activity via the immediate early gene transcript, cfos, in the mPFC and its downstream limbic targets.

Methods

Animals

All animals used were pathogen-free adult male Sprague-Dawley rats (350-400g) purchased from Taconic (Hudson, NY). They were housed in individual cages in rat-specific holding rooms controlled for temperature (22±1°C) and humidity. Food and water were available ad libitum, and lights were automatically switched on and off according to a 12:12 L:D cycle (lights on 8:00am – 8:00pm). The animals were cared for in accordance with National Institutes of Health standards, and all procedures were pre-approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Animal lesion surgery

Prior to surgery, animals were anesthetized with ketamine-xylazine (i.p., 800 mg/kg ketamine, 80 mg/kg xylazine, Med-Vet, Mettawa, IL) and then placed on a stereotaxic frame so that their head was fixed. Injections of ibotenic acid (IBO, Tocris, Ellisville, MO), 0.9% saline (Med-Vet, Mettawa, IL) or CTB (List Biological, Campbell, CA) were administered directly into the brain using a fine glass pipette (1 mm glass stock, tapering slowly to a 10-20um tip) connected to an air compression system. A series of 20-40psi puffs of air were used to deliver the compounds into and with the following coordinates (Paxinos and Watson, 2005) and volumes: vmPFC: AP+3.0mm DV-3.4mm RL+/-0.6mm, 66-99nL 5% IBO, 16.5nL 1.0% CTB; NAc: AP+2.0mm DV-6.8mm RL+/-1.0mm, 23.1nL 1% CTB. Incisions were closed with wound clips. Upon completion of the procedure, the animal was given a subcutaneous injection of analgesic meloxicam (1.0 mg/kg, Med-Vet, Mettawa, IL) and allowed to recover on a warm plate until awakened from anesthesia.

Forced swim test

The forced swim test procedure was conducted as described in Detke et al. (26) and Castagné et al. (27). The test was conducted over two days using acrylic cylinders (20cm x 40cm; Northeast Plastics, Philadelphia, PA) that were filled 30 cm with 25°C water. On the first day, animals were placed in the cylinder for 15 min while being video recorded using a computer running Ethovision (Noldus, Leesburg, VA). The animals were subsequently removed and gently handled and dried before being returned to their cages. On the second day, the swim test was repeated for 5 min. All animals were habituated to the room at 12:00 and the swim tests were completed between 13:00 and 15:00 to limit any circadian influences. Injections of drug or saline were administered three times: immediately following the first swim session, 4 hours prior to the second session, and 1 hour prior to the second session. Total amounts of immobility during the 5-min test session were scored using Ethovision and parameters validated for rats (28) (sampling rate 5 Hz; immobility threshold 11.5%). It has been shown that lesions in the rat mPFC do not impair locomotor activity (29), and we confirmed during the day 1 swim session that neither experimental group displayed movement deficits. FST immobility between lesion groups receiving saline or DMI were analyzed using unpaired t-test, using a significance threshold $p<0.05$.

Antidepressant Drug Treatments

To study neuronal activation patterns upon acute drug treatment, animals were placed in isolated chambers for at least two days. We chose these conditions (including performing injections in the morning) to maximize the likelihood that the animals would fall sleep following the

injection. At 10am after habituation, animals were gently handled and weighed. At 10am the following day, animals were injected i.p. with desipramine hydrochloride (10 mg/kg in saline, Sigma, St. Louis, MO), fluoxetine hydrochloride (Sigma, St. Louis, MO; 20 mg/kg in saline) ketamine (10 mg/kg in saline, Med-Vet, Mettawa, IL) or sterile saline (0.9%, Fisher Scientific, Pittsburgh, PA). The animals were then placed back in their cages and in their chambers for two hours, after which they were sacrificed via perfusion and fixation (see below) and their brains stained for cfos immunohistochemistry.

Perfusion and fixation

Animals were anesthetized with 7% chloral hydrate (i.p. 500 mg/kg, Sigma, St. Louis, MO). The body cavity was opened using surgical scissors and a 16G needle was inserted into the left ventricle of the heart. The top of the right atrium was cut to allow blood to be drained. About 100 mL of saline was flushed through the vascular system using an intravenous line, followed by 500mL of 10% buffered formalin (Fisher Scientific, Pittsburgh, PA). Upon fixation of the tissue the brain was removed from the skull and stored in 10% formalin for 4-5 hours. The brains were then moved to 20% sucrose and 0.02% azide solution overnight.

Histology and Immunohistochemistry

Brains were sliced into four series of 40um sections using a freezing microtome. The sections were stored in PBS-0.02% azide in 20°C.

Immunohistochemical staining was completed as follows: tissue sections were rinsed in PBS three times, 3-5 min each. They were then incubated for 30 min in 0.3% H_2O_2 (Sigma, St. Louis, MO) in PBT (phosphate buffer with Triton X-100; Sigma, St. Louis, MO) to oxidize any remaining blood. The sections were again rinsed in PBS and then incubated in primary antibody diluted in PBT-Azide for 1-2 nights, depending on the antibody (cfos Ab-5, PC38, rabbit polyclonal, 1:30,000, Calbiochem, Billerica, MA; Chemicon, Billerica, MA; CTB, 127H4810, goat polyclonal, 1:50,000, Sigma, St. Louis, MO). Tissue were then rinsed in PBS three times, and incubated in secondary antibody (1:1000, biotin SP-conjugated against appropriate species IgG, Jackson ImmunoResearch Laboratories, West Grove, PA) for 60-90 min. Sections were again rinsed in PBS and placed in ABC solution (1:1000 each Vectastain solutions A and B, Vector Laboratories, Burlingame, CA) for 60-90 min. Sections were rinsed in PBS and stained for 5 min in a solution consisting of: 1% DAB, 0.3% H_2O_2 (and 0.01% Ni, 0.005% $CoCl_2$ if desired a black stain). Tissue were then rinsed and mounted on microscope slides in gelatin.

Sections were Nissl stained by placing microscope slides of mounted tissue in ddH_2O for 5 min, followed by 10-30 sec in 0.1% thionin staining solution (t Sigma, St. Louis, MO). The slides were dehydrated step-wise by incubating in 50% EtOH, 70% EtOH, 95% EtOH, and 100% EtOH for 2 min each. Slides were then placed in xylene for several hours before covering with glass coverslips.

Cell Counting

All cfos-stained cells were counted at 25x magnification. For the ACC and PLC, three sections were counted and summed (rostral: Bregma +4.2mm, middle: Bregma +3.2mm, caudal: Bregma +2.5mm) and for the ILC two sections were counted (middle and caudal). Counting boxes used

were 400μm x 400μm. For the ACC, two boxes were placed corner to corner at the dorsomedial edge of the ACC, and for the ventral regions one box each was placed in the deep and superficial layers. Three sections were counted of the rostral NAc (Bregma +2.0 to +2.5mm): 400μm x 400μm counting boxes were placed immediately ventral to the anterior commissure (core) and lateral to the core along the medial edge of the NAc (shell). Three sections in the basolateral amygdala (Bregma -3.0mm to -3.3mm) were counted; two sections of the lateral septum (Bregma +1.7mm to +1.5mm) with counting box aligned to lateral ventricle; and two sections of the insular cortex (Bregma +3.0mm and +2.5mm) with counting box placed along the cortical edge. Counting box used for these structures was also 400μm x 400μm. The anterior-posterior levels were chosen based on projection patterns of the vmPFC (30). All cell counting data were corrected using Abercrombie's correction (31).

Statistical Analyses

All of the cell counting data were analyzed using the unpaired t-test, except in the case of the nucleus accumbens cfos comparisons in animals with and without vmPFC lesions when a 2-way ANOVA was used. In the comparison between fluoxetine, ketamine and saline injections, *p*-values were adjusted with Bonferroni's adjustment. $p<0.05$ was used as the threshold for significance.

Results

Desipramine activates deep layers of the rat vmPFC

To investigate whether antidepressant drugs may be targeting the prefrontal cortex, we administered desipramine (DMI) to adult male Sprague-Dawley rats (Fig. 2-1). The dose used (10 mg/kg) has been shown to be effective in the forced swim test, a rodent model of depression originally validated to demonstrate antidepressant efficacy (26; 32; 33). Saline was injected into animals under identical conditions as a control. Both groups showed sleeping postures in the interval between injection and perfusion, which was verified by EEG/EMG recording in two rats that received DMI (Fig. 2-2). REM sleep was completely suppressed by the drug for several hours, but NREM sleep and wakefulness parameters were not affected). In addition, animals of both groups showed a cfos activation pattern typical of sleeping animals, including high cfos expression in the sleep-active neurons of the ventrolateral preoptic nucleus, and low expression in the arousal regions such as the tuberomammillary nuclus (Fig. 2-3). Compared to controls, animals given DMI exhibited a significant increase in cfos expression in the ventral mPFC (i.e., the prelimbic and infralimbic cortices; average cell counts, saline v. DMI: 54.1±13.5 v. 120.1±22.4; $t(8)=2.52$ $p=0.036$), whereas this was not seen in the dorsal mPFC (anterior cingulate cortex; saline v. DMI: 4.4±1.8 v. 10.5±3.5; $t(8)=1.54$, $p>0.05$; Fig. 2-4A). The delineation of the mPFC was based on Paxinos and Watson's Rat Atlas (34) and cytoarchitectural characteristics as described in Hurley et al. (35).

As the mPFC consists of five cortical cell layers (I,II,III,V,VI), we divided the vmPFC into superficial (I-III) and deep layers (V-VI) and counted them separately to observe any

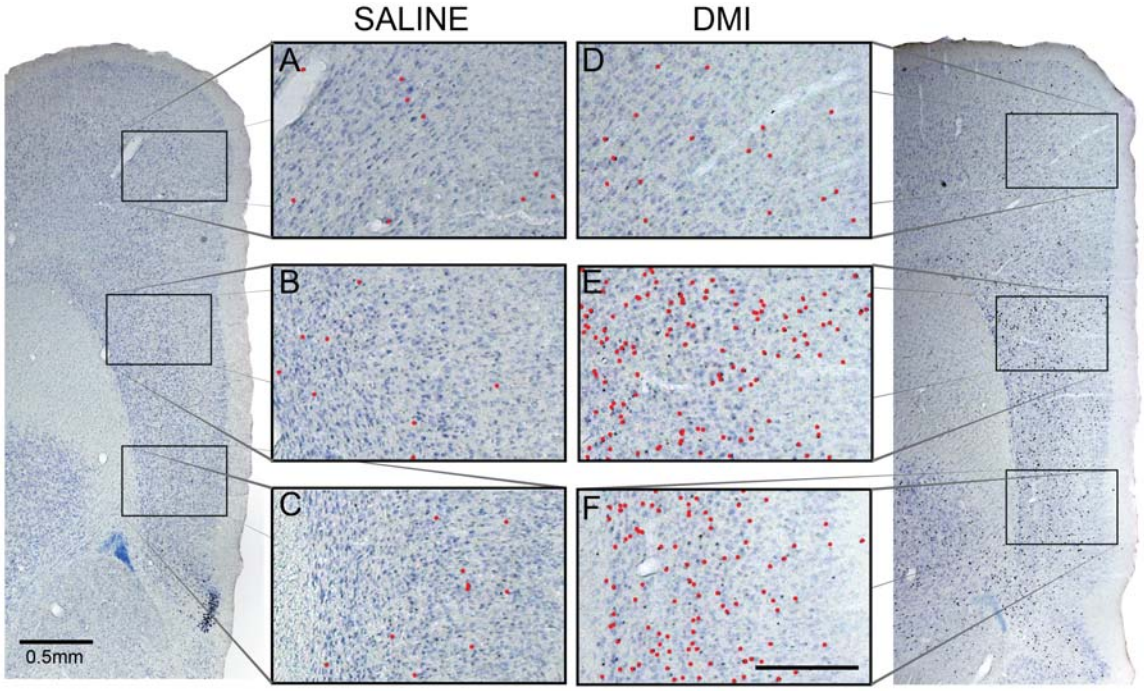

Figure 2-1. Animals were injected with saline (**A-C**) or DMI (**D-F**) (i.p., 10 mg/kg) at 10:00am and sacrificed two hours later. Cells in the mPFC stained with cfos are indicated by red dots in the anterior cingulate (**A** and **D**), prelimbic (**B** and **E**), and infralimbic (**C** and **F**) cortices. The prelimbic and infralimbic cortices, collectively called the vmPFC below, show a dramatic increase in cfos expression following drug administration whereas the anterior cingulate (dmPFC) is relatively quiet. Scale bar in **F** indicates 0.25mm.

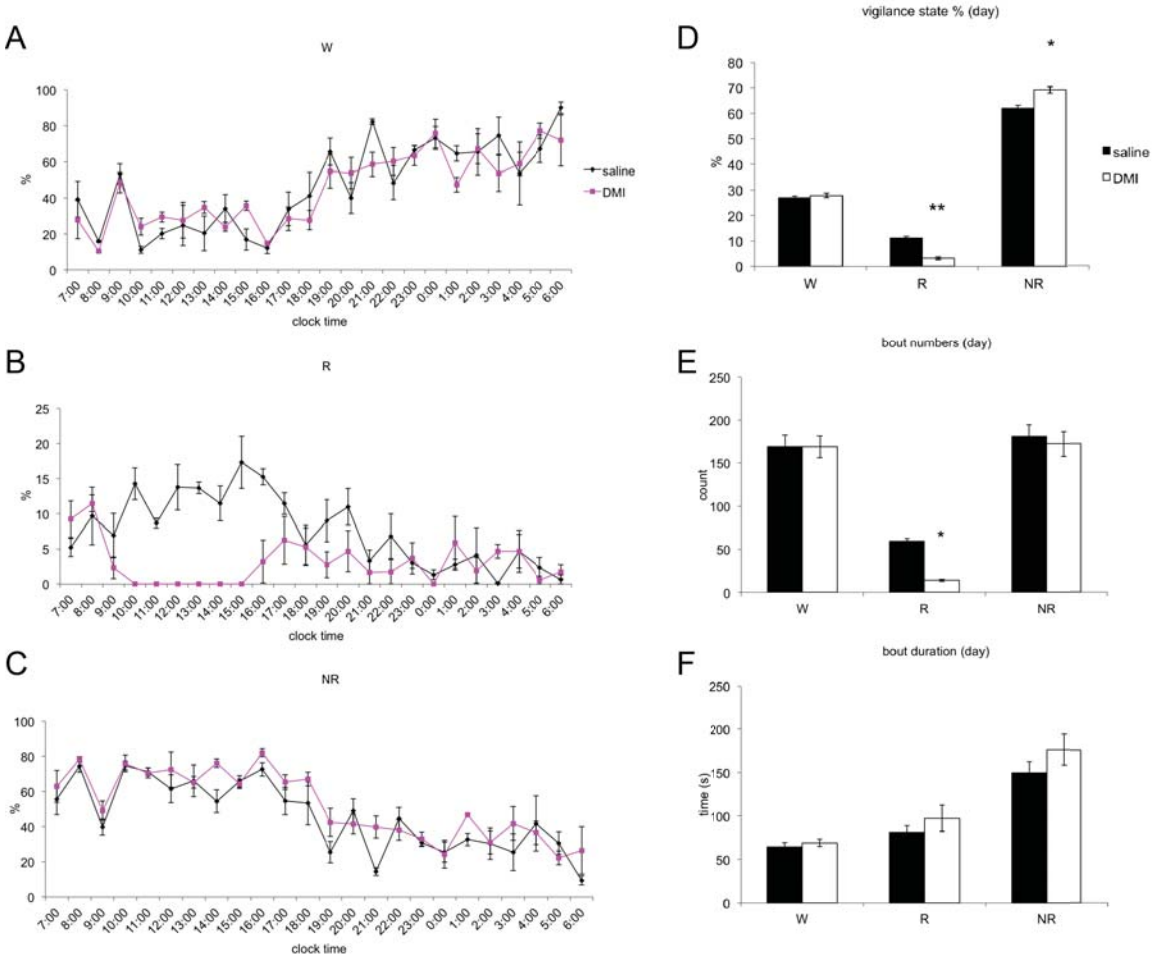

Figure 2-2. Sleep-wake analysis of animals administered saline or desipramine on consecutive days (n=4). **A**, **B**, and **C** show hourly wake, REM sleep, and NREM sleep percentages over 24h. **D**, **E**, and **F** show vigilance state percentages, bout numbers, and average bout durations during the 12h light period. Injections were given at approximately 9:00am.

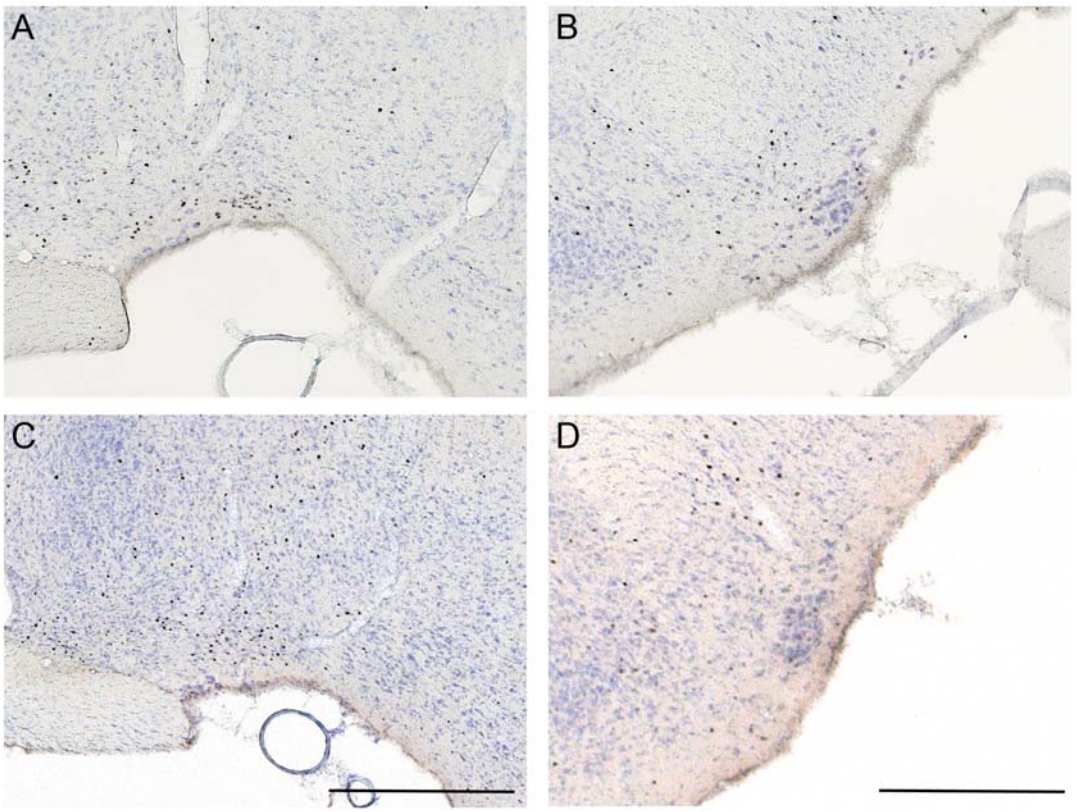

Figure 2-3. cfos expression in the ventrolateral preoptic nucleus (**A** and **C**) and tuberomammillary nucleus (**B** and **D**) following saline (**A** and **B**) or desipramine (**C** and **D**) injections indicate that animals were sleeping during the interval between injection and sacrifice. Scale bars indicate 0.5mm.

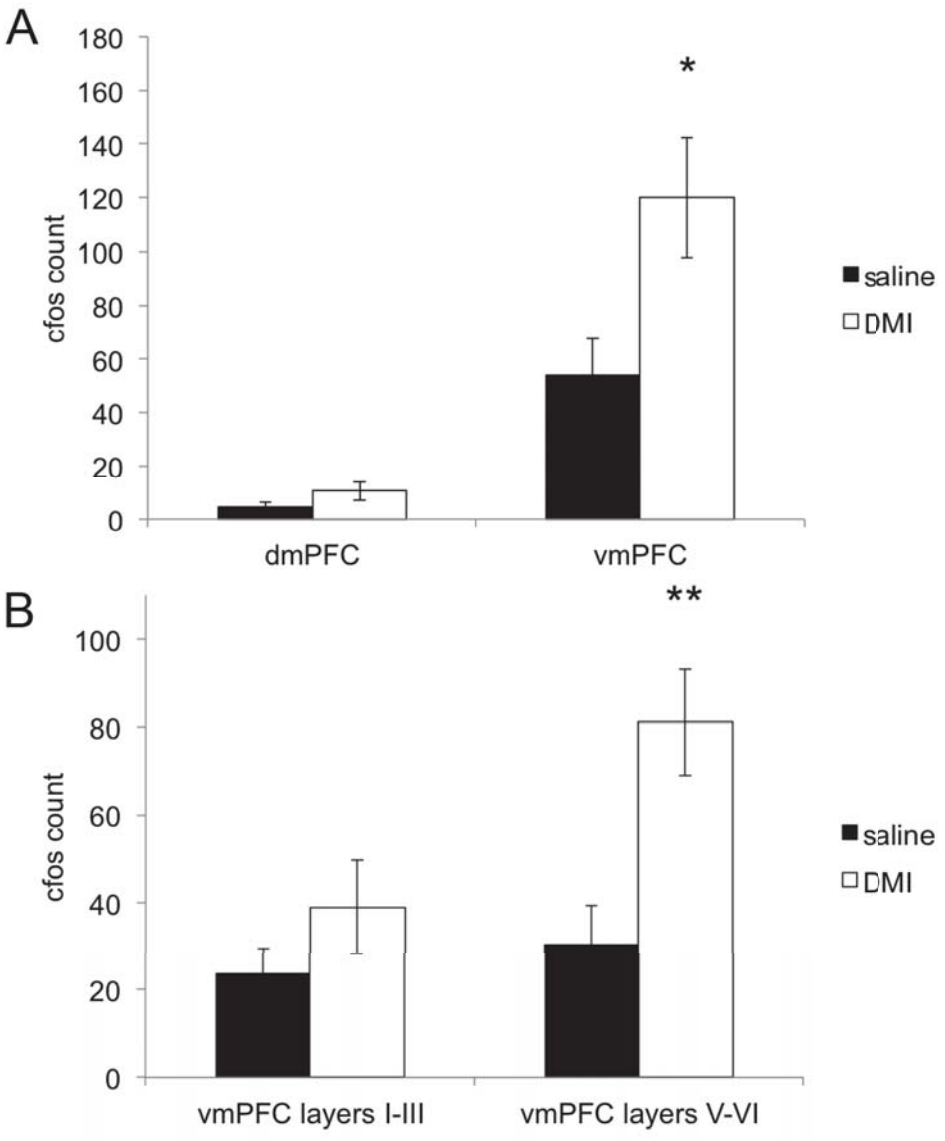

Figure 2-4. A The vmPFC, but not the dmPFC, was significantly activated upon DMI injection (i.p. 10 mg/kg, n=5) compared to saline-injected animals (n=5). **B** Within the vmPFC, there was a clear distinction in the number of cfos-stained cells between the deep layers (V-VI) and superficial layers (I-III).

differentiation in cfos expression. We found that the increase in cfos expression in the vmPFC due to DMI was entirely due to the deeper layers (Fig. 2-4B; saline v. DMI, layers V-VI: 30.5±8.9 v. 81.1±12.1, $t(8)=3.37$, $p=0.0099$), whereas the superficial layers were not statistically different between DMI and saline (saline v. DMI, layers I-III: 23.6±5.9 v. 38.9±10.8, $t(8)=1.25$, $p>0.05$).

Fluoxetine and ketamine also selectively activate vmPFC

To investigate whether pharmacological agents of classes other than TCAs produce similar patterns of neuronal activation in the mPFC, we treated animals under the same protocol with fluoxetine (FLX, 20 mg/kg) and ketamine (KET, 10 mg/kg; Fig. 2-5). The doses were chosen based on their effectiveness in decreasing immobility in the forced swim test (22; 26). We found that, like DMI, FLX induced an increase in cfos expression in the vmPFC compared to animals that received saline (saline v. FLX: 49.1±13.6 v. 150.3±29.0, $t(10)=2.47$, adj. $p=0.033$), while not significantly affecting the dmPFC (saline v. FLX: 3.2±1.8 v. 16.3±6.1, $t(10)=1.75$, adj. $p>0.05$; Fig. 2-6A). However, KET increased cfos expression both the dmPFC and vmPFC (dmPFC: saline v. KET: 3.2±1.8 v. 23.7±5.4, $t(8)=3.26$, adj. $p=0.0098$; vmPFC: saline v. KET: 49.1±13.6 v. 136.8±24.9, $t(8)=2.75$, adj. $p=0.023$). Furthermore, the increased vmPFC expression in both drug groups was exclusive to the deep cortical layers (layers V-VI: saline v. FLX: 31.0±9.8 v. 104.8±19.8, $t(10)=2.68$, adj. $p=0.023$; saline v. KET: 31.0±9.8 v. 96.0±14.9, $t(8)=3.33$, adj. $p=0.0088$; layers I-III: saline v. FLX: 18.1±5.3 v. 45.5±9.8, $t(10)=1.79$, adj. $p>0.05$; saline v. KET: 18.1±5.3 v. 40.8±10.5, $t(8)=1.43$, adj. $p>0.05$; Fig. 2-6B). As above, animals that received both FLX and KET showed sleeping postures and similar cfos expression

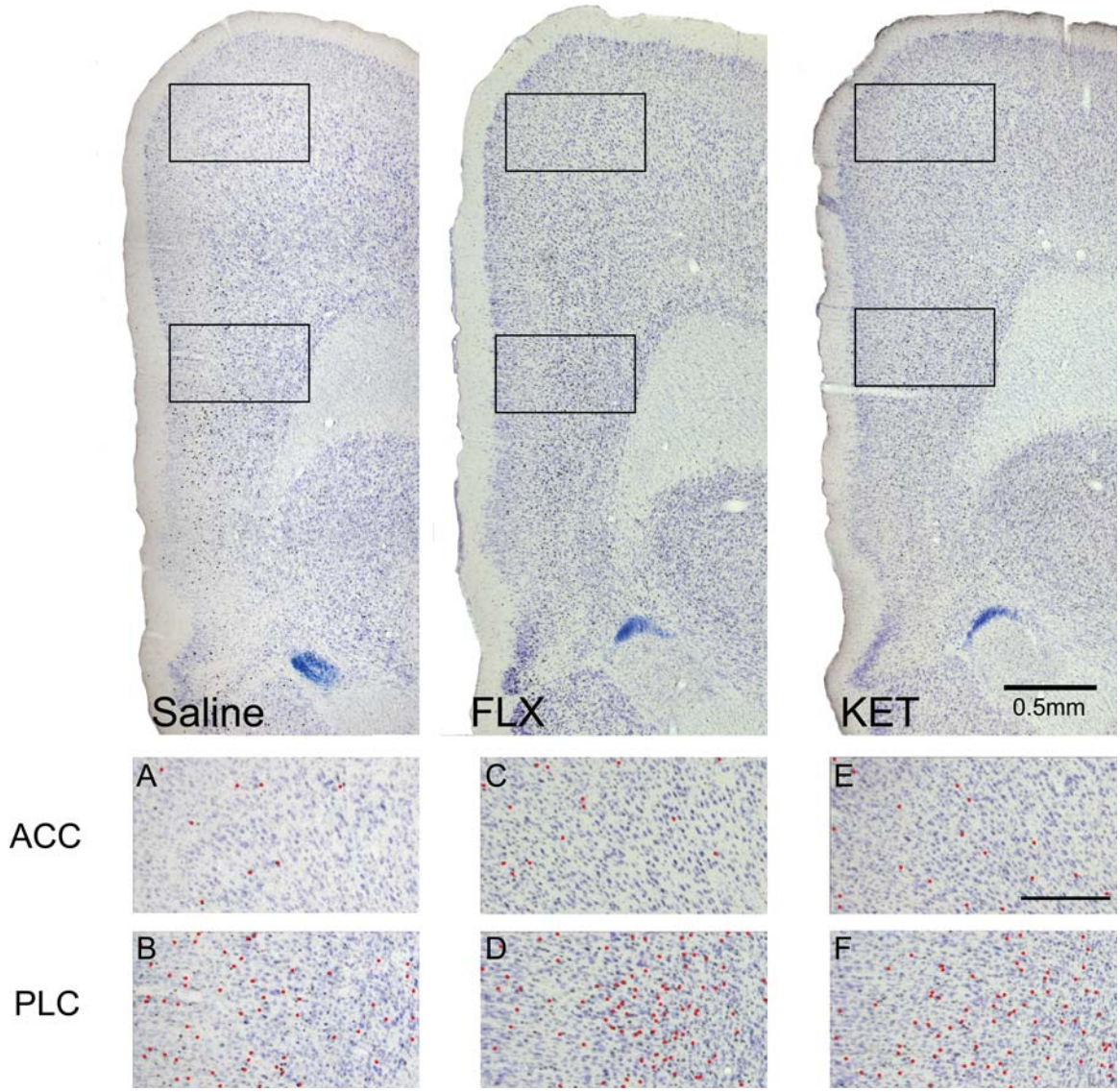

Figure 2-5. Animals were injected i.p. with saline, FLX (20 mg/kg), or KET (10 mg/kg) at 10:00am and sacrificed two hours later. The prelimbic cortex (**D** and **F**) demonstrated increased cfos expression following administration of both drugs compared to saline (**B**), whereas the anterior cingulate cortex did not (**A**, **C** and **E**). Scale bar in panel **E** indicates 0.25mm.

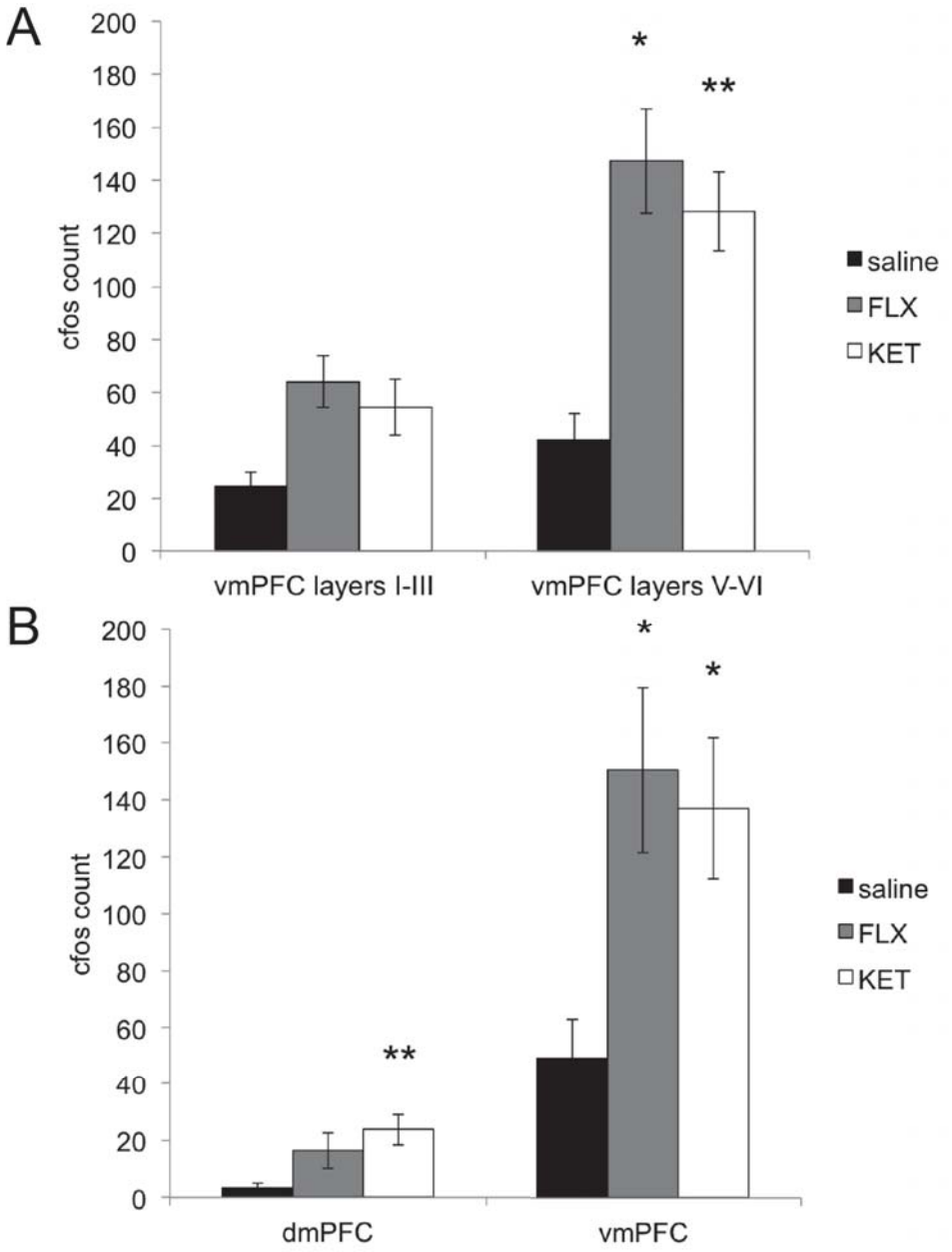

Figure 2-6. Animals were administered saline (n=6), FLX (20 mg/kg, n=5) or KET (10 mg/kg, n=5) at 10:00am and sacrificed two hours later. FLX and KET selectively activate the vmPFC (**A**), and the deep layers of this region (**B**), similar to DMI. '*', adj. $p<0.05$; '**', adj. $p<0.01$.

in the regions involved in sleep and arousal compared to control animals, indicating that these doses of antidepressant drugs do not significantly influence sleep-wake behavior.

Desipramine activates the nucleus accumbens via the vmPFC

We next asked whether the limbic structures that receive projections from the vmPFC are also activated by DMI (30; 34). We found that drug administration did not significantly activate the lateral septum (saline v. DMI: 15.2±2.3 v. 25.3±8.5, $t(8)=1.12$, $p>0.05$), insular cortex (saline v. DMI: 4.6±2.0 v. 2.3±0.7, $t(8)=1.08$, $p>0.05$), nor basolateral amygdala (saline v. DMI: 6.7±1.7 v. 8.7±3.3, $t(8)=0.5$, $p>0.05$) compared to saline injections (Fig. 2-7). On the other hand, the NAc exhibited significantly increased cfos expression (saline v. DMI: 10.6±3.2 v. 36.8±9.1, $t(8)=2.72$, $p=0.026$; Fig. 2-8). As the NAc consists of the core and shell subregions, which are believed to subserve separate behavioral functions (36), we analyzed the anatomic specificity of the vmPFC-dependent activation of the NAc by DMI (Fig. 2-9). We found that DMI significantly increased the number of cfos-stained neurons in the NAc core compared to that of controls (saline v. DMI: 3.9±0.6 v. 16.8±4.7, $t(8)=2.73$, $p=0.026$). The NAc shell had about 190% more cfos positive neurons after DMI, but this did not reach statistical significance, likely due to the size of the standard error (saline v. DMI: 6.7±2.7 v. 20.0±5.2, $t(8)=2.27$, $p=0.053$). However, lesions in the vmPFC led to decreases in cfos-stained neurons in both structures (DMI, intact v. lesion, NAc core: 16.8±4.7 v. 7.7±2.7; NAc shell: 20.0±5.2 v. 9.1±2.4), whereas vmPFC lesions did not affect NAc expression when saline was administered (intact v. lesion: NAc core: 3.9±0.6 v. 9.1±1.1; NAc shell: 6.7±2.7 v. 11.2±4.0). A 2x2 ANOVA confirmed that drug injection alone or lesion alone did not cause a significant difference in the group means. Note that although some lesions included dorsal peduncular cortex (DPC, as in Fig. 2-8), a tracing study (37), which I

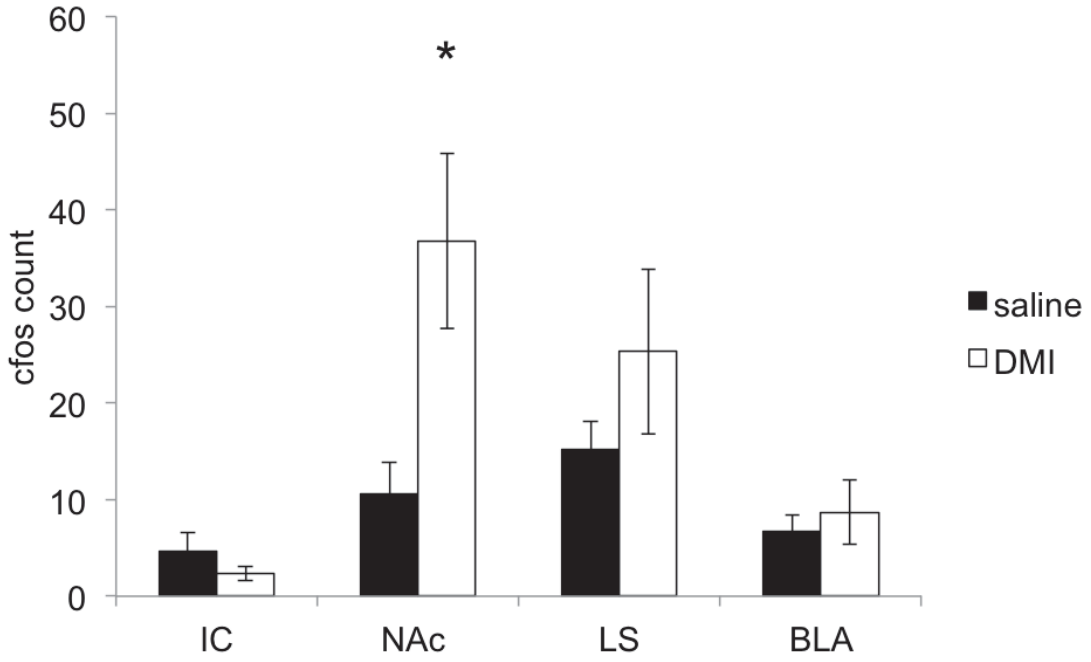

Figure 2-7. cfos-labeled cells were counted in the insular cortex (IC), nucleus accumbens (NAc), lateral septum (LS), and basolateral amygdala (BLA) in animals given i.p. saline (n=5) or DMI (10 mg/kg, n=5). Among these structures, only the nucleus accumbens was selectively activated by the drug. '*', $p<0.05$.

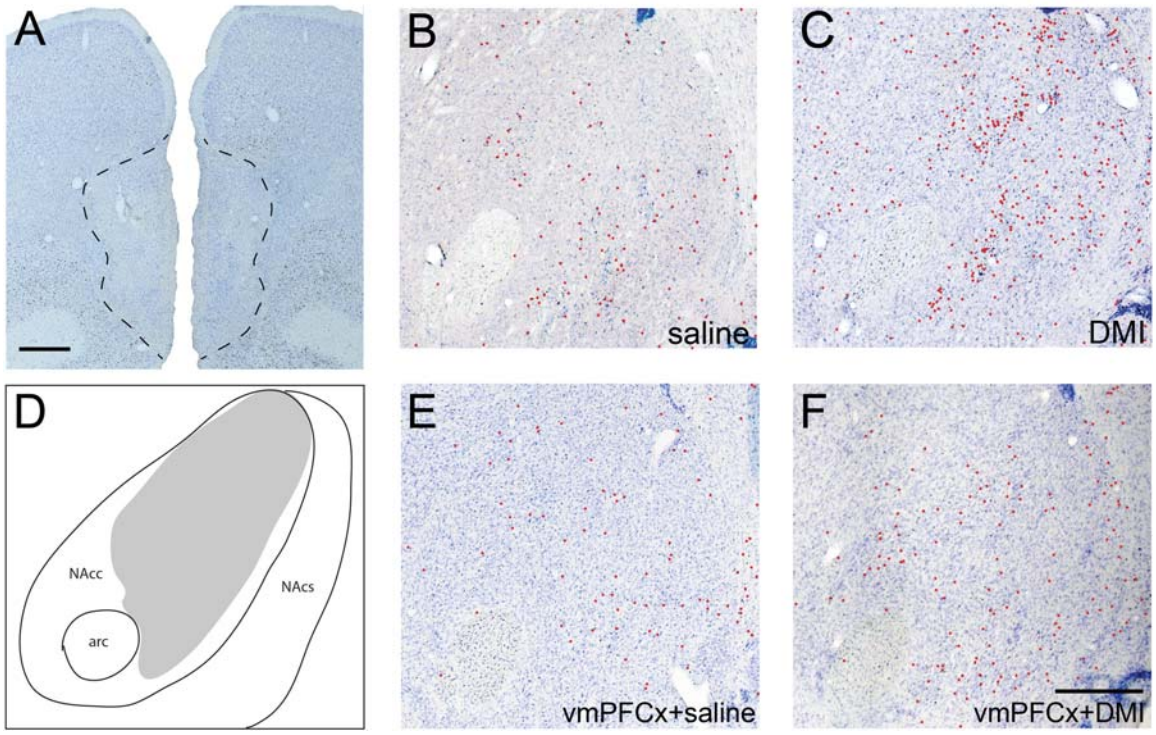

Figure 2-8. Cells in the nucleus accumbens are also activated by DMI (10 mg/kg) (**C**) compared to animals that received saline (**B**). However, cfos staining was reduced when DMI was injected into animals with neuronal lesions in the vmPFC (**A**, **E**). mPFC: Bregma +3.5mm; NAc: Bregma +2.0mm. Scale bars in **A** and **E** are 0.5mm.

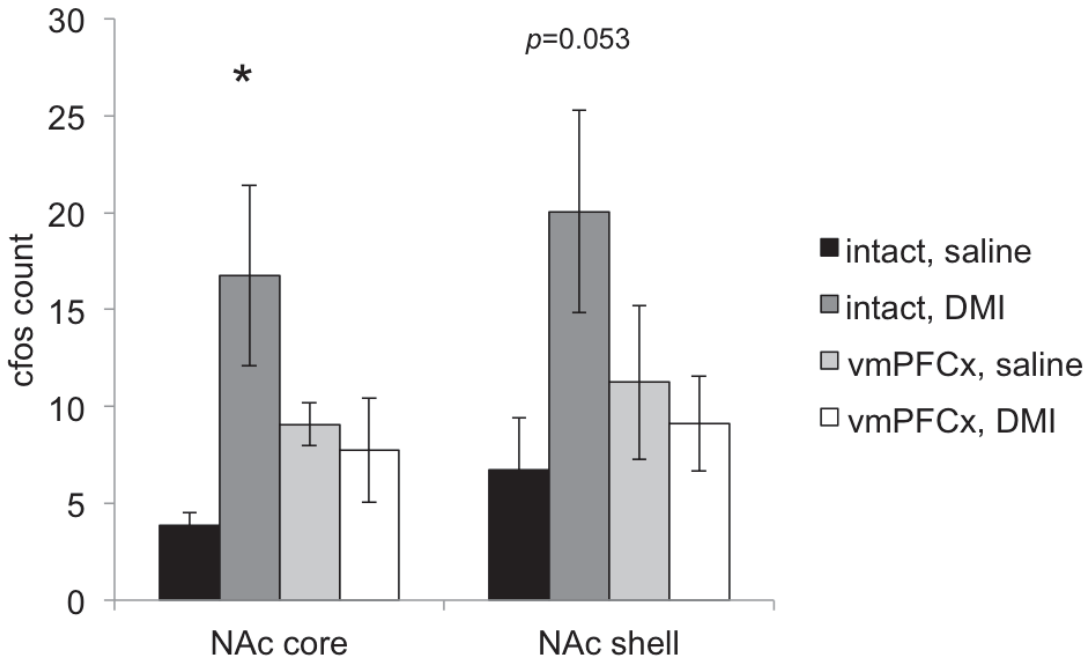

Figure 2-9. Sham- and vmPFC-lesioned animals were administered saline or DMI (10 mg/kg) i.p. (10:00am) and sacrificed two hours later. In intact animals, the number of cells that were stained by cfos in the NAc was significantly greater in animals that received DMI (n=5) compared to those that received saline (n=5). However, the levels of cfos expression in the core and shell were reduced by vmPFC lesions (n=6). n=6 for lesion group that received saline injection. '*', $p<0.05$.

confirmed, showed that only a few cells in this structure project to the NAc ipsilaterally, whereas the vmPFC heavily projects to NAc from both sides. Therefore inclusion of the DPC in lesions is unlikely to play a role in cfos counts in the NAc.

To examine whether the activation of the NAc by DMI via the vmPFC could be mediated by direct projections from the vmPFC to the NAc, we injected the retrograde tracer cholera toxin subunit B (CTB) unilaterally into the NAc of the animals (n=4; Fig. 2-10). After surgical recovery, we administered DMI as before and stained brain tissue for both CTB and cfos. We specifically looked for cells in the vmPFC that had cytoplasmic staining for CTB (brown, DAB) and nuclear cfos stain (black, DAB with Ni and Co), which would indicate cells in the vmPFC that were activated by DMI and project to the NAc. As before, DMI injection resulted in increased cfos staining in the NAc (Fig. 2-11A) and vmPFC (Fig. 2-11C). Of the cell bodies in the vmPFC stained with CTB, 12.6±2.1% had cfos-labeled nuclei on the ipsilateral side (Fig. 2-11D). These results suggest that these neurons are both activated by DMI and project to the NAc.

vmPFC lesions partially block the DMI effect on forced swim test immobility

Given that the vmPFC appears to be an important structure activated by DMI, I asked whether this region was the main avenue by which DMI induces its antidepressant effects. I therefore asked whether vmPFC lesions would block the antidepressant effects of DMI in the forced swim test (FST). In this procedure, an animal is placed in an escapable container of water for 15 minutes on day 1. On the second day, the animal is returned to the cylinder for 5 minutes and the total time of time spent immobile is measured, for increased immobility is considered to be associated with depression. We induced lesions in the vmPFC with ibotenic acid, and administered the FST to animals while administering desipramine or saline.

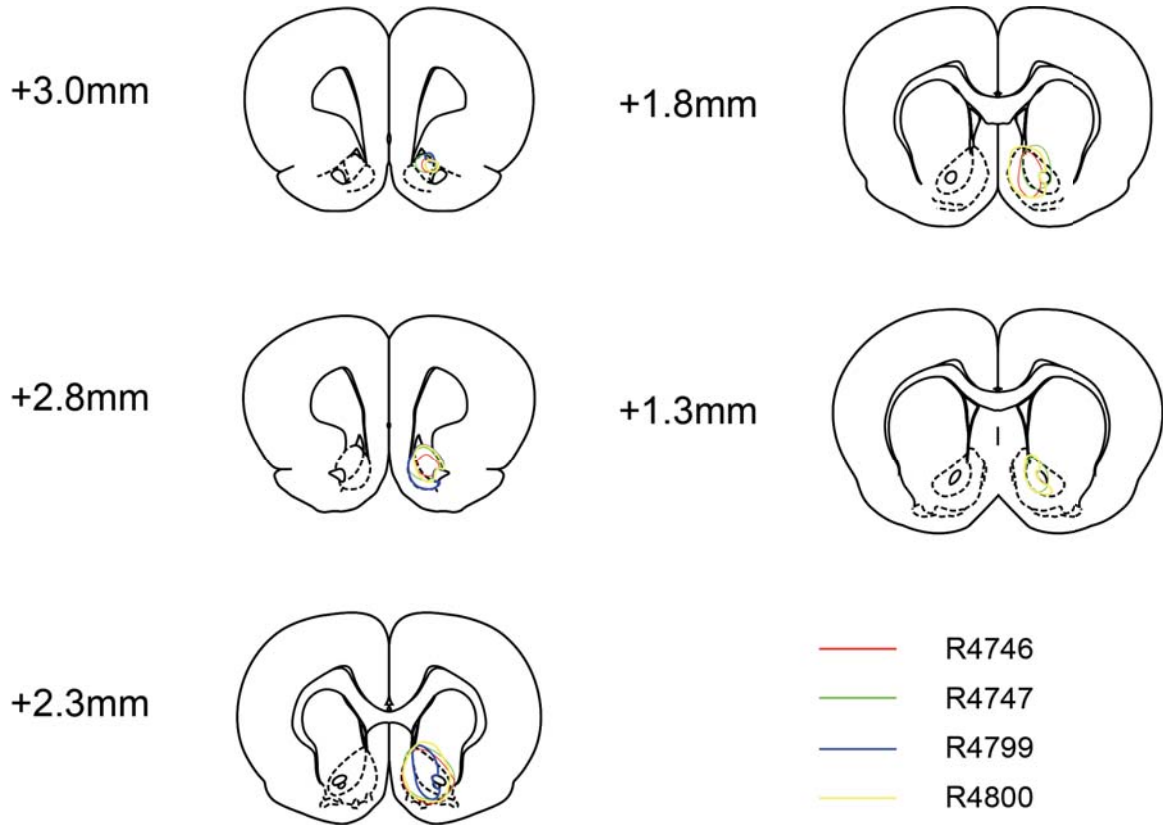

Figure 2-10. Retrograde tracer (CTB) injections into the nucleus accumbens of four animals. Labels indicate distance from Bregma.

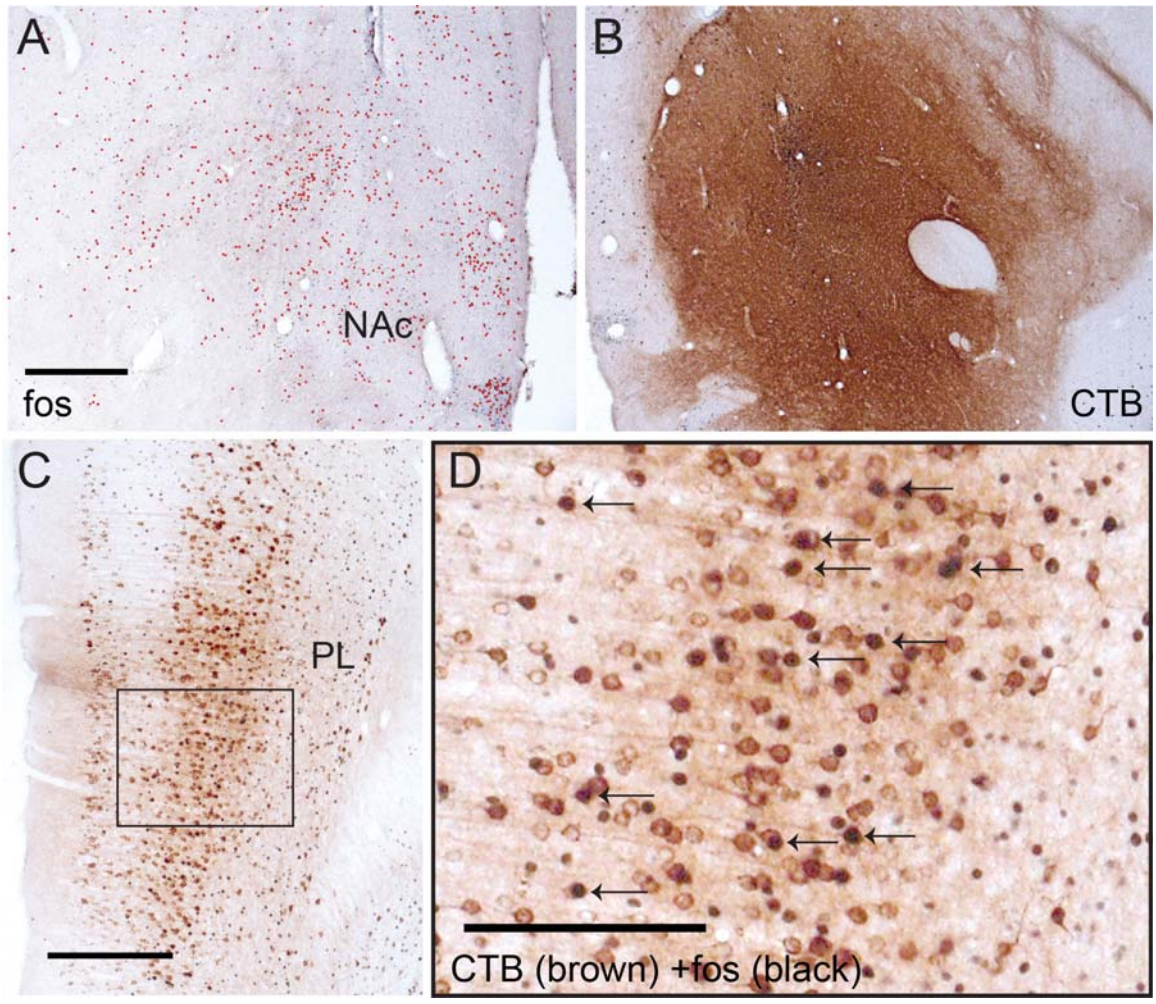

Figure 2-11. B Retrograde tracer CTB was unilaterally injected into the NAc (Bregma + 2.0mm), and upon postsurgical recovery the animal was injected with DMI (10:00am, 10 mg/kg) and sacrificed (12:00pm). **A** indicates opposite side that did not receive injection. **C** Many cell bodies were stained brown in the ventromedial prefrontal cortex, particularly layer V (Bregma +3.2mm). The black arrows in **D** point to cell bodies stained brown for CTB that had cfos staining in the nuclei, indicating possible activation by DMI. Scale bars in **A, C** and **D** indicate 0.5mm, 0.5mm and 0.25mm.

In intact animals, in comparison to saline-injections (n=5), DMI injections (n=4) reduced FST immobility time (saline v. DMI: 53.9±10.7s v. 13.9±2.4s, $t(7)=3.25$, $p=0.014$; Fig. 2-12). DMI injections into vmPFC-lesioned animals (n=6) also decreased FST immobility time, although not to the same levels as in intact animals (n=6; DMI in lesion v. intact: 36.07±7.57s v. 13.9±2.37s, $t(10)=4.28$, $p=0.0016$). The immobility time of the DMI-treated lesioned animals was greater than that in the intact animals DMI-injected animals, but this did not quite reach statistical significance ($t(8)=2.30$, $p=0.0507$). Furthermore, DMI reduced immobility in the lesioned animals, albeit not to the level of intact animals. These results suggest that vmPFC lesions may partially block the immobility-reducing effects of DMI in the FST.

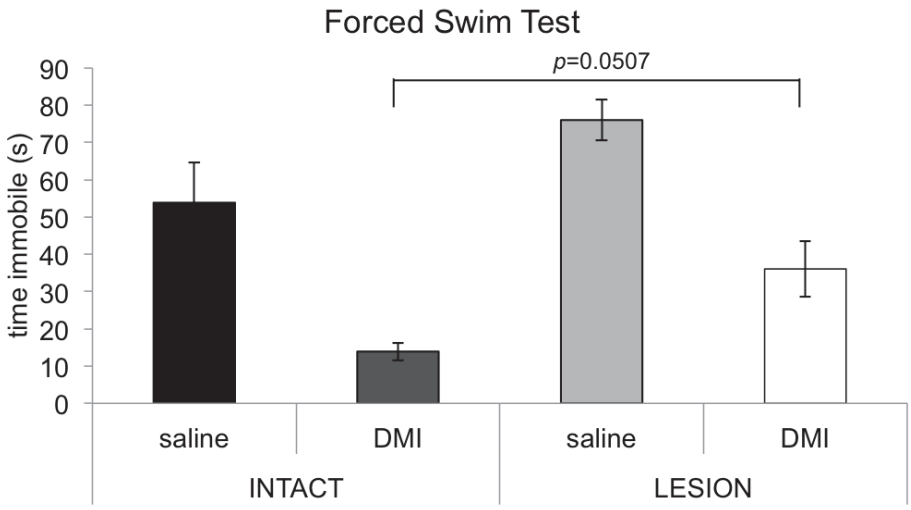

Figure 2-12. The forced swim test was performed on animals with vmPFC or sham lesions, that were administered saline (n=6 for each) or DMI (sham: n=4, lesion: n=5). Lesions increased immobility time in saline- and particularly DMI-treated animals, but did not reach statistical significance (saline: 0.0836, DMI: p=0.0507).

Discussion

Although antidepressant medications have been used for decades, how and where they work in the brain is unclear. In this study, we found that acute administration of the tricyclic antidepressant desipramine, selective serotonin reuptake inhibitor fluoxetine, and NMDA-antagonist ketamine selectively activate a common set of structures in the rat brain, including the ventromedial prefrontal cortex. All three drugs also selectively target the deep layers of the vmPFC, which contain neurons that project to the striatum, hypothalamus, and brainstem, suggesting that specific neurons within the vmPFC may be important in mediating the drug effect. In addition, we found that the nucleus accumbens (NAc), a structure implicated in MDD, was also activated by DMI, and that this activation was dependent on the vmPFC. Finally, vmPFC lesions increased immobility in the FST, a behavior that has been associated with depression. DMI was still partially effective in reducing the immobility time even in the vmPFC lesioned rats, but although the immmobility time was still three times that of intact, DMI-treated rats, this difference did not quite reach statistical significance. It should be noted that in the subsequent chapter, I found a significant increase in FST immobility in vmPFC-lesioned animals compared to sham-lesioned animals, whereas here statistical comparison of saline-injected sham-lesioned animals and vmPFC-lesioned animals did not quite reach significance (p=0.08). This may be because the three i.p. injections, despite being of saline, differentially affects sham-lesioned and vmPFC-lesioned animals, particularly stressing the sham-lesioned animals and thus making the difference between the groups less pronounced. This possibility is supported by the finding that immobility times in the vmPFC-lesioned groups with and without i.p. saline were similar (76.0±5.5s v. 66.0±6.8s, $t(26)=0.74$, $p=0.46$), whereas for the sham-lesioned groups with and without i.p. saline were dissimilar (53.9±10.7s v. 24.9±4.0s, $t(9)=2.92$, $p=0.017$). In addition,

i.p. injections introduced greater variability in the control group which likely contributed to the lack of statistical significance.

Technical considerations

One caveat to our findings is that we used healthy animals in our experiments, and it is conceivable that depression in humans may depend upon different circuitry. However, because we started with circuitry that is activated by antidepressants, our results are likely to apply to the antidepressant effects of DMI and other antidepressant drugs in humans. Another technical issue is that the dosages of antidepressant drugs that we used were derived from earlier literature about drug effects on tests in rats that have been thought to improve depression-like behaviors. However, the dosage at which the drugs may work in actual depression in humans may be different. Nevertheless, the results from functional imaging studies support that the vmFPC is likely to play a role in human depression, and that antidepressant drugs alter that activity.

Why do antidepressants selectively activate the deep layers of the vmPFC?

The vmPFC, as opposed to the dmPFC, has many connections to limbic areas of the brain that are involved in the control of emotion and mood (38). DMI is a reuptake inhibitor of norepinephrine and serotonin, and FLX is a serotonin reuptake inhibitor, so the increased extracellular concentration of these neurotransmitters may result in the activation of cells in the deep layers. For instance, there is evidence that alpha-2 receptors (alpha-2R) mediate NE action in depression, as alpha-2R antagonists blocked the effect of DMI in reducing immobility in the FST (39). However, the cellular elements where the alpha-2Rs are located are not fully

understod. There is evidence that presynaptic alpha-2R's moderate norepinephrine release (40), but that they also are on that they are on cortical GABAergic interneurons (41). The latter report provides evidence that alpha-2Rs cause cellular hyperpolarization, so they propose that norepinephrine reuptake inhibitors may be causing excitation in the vmPFC by disinhibiting pyramidal cells via inhibitory interneurons.

As for serotonin, it is estimated that 80% of PFC pyramidal cells contain both 5-HT1A and 5-HT2C receptors (42). However, the inhibitory effect of 5-HT1A receptors is believed to dominate the excitatory 5-HT2C receptors due to their localization around the soma and axon initial segment. In addition, a recent study showed that most of the fast-spiking interneurons in the rat mPFC are inhibited via the 5-HT1A receptors (43). Therefore, the net excitaory effect on the pyramidal cells in the vmPFC may also result from inhibition of the interneurons.

Pyramidal cell activation in the vmPFC by ketamine is also likely through interneurons, as KET blocks excitation by glutamate receptors. Low doses of KET in the mPFC may reveal its affinity for interneurons, and result in blockage of the inhibitory effects on pyramidal neurons (44). We therefore hypothesize that the vmPFC interneurons that inhibit deep-layer pyramidal cells are more sensitive to glutamate and the monoamines than the pyramidal neurons.

However, it is unclear why layers V-VI pyramidal neurons in this region are selectively activated following administration of the drugs, while the pyramidal cells in layers II-III are not. The localization of receptor subtypes may provide a clue; for instance, 5-HT2C receptor mRNA expression is light in the superficial layers of the mPFC (45), and there appears to be increased expression of 5-HT1A receptor mRNA in the vmPFC layer VI (46). However, in the mPFC receptors are not necessarily localized to the cell layer that they influence, and thus an explanation for the cfos expression pattern in the mPFC remains to be elucidated.

Is neuronal plasticity a common endpoint of all three drugs?

One of the key questions in comparing various classes of antidepressant drugs is whether their effects ultimately converge on the same molecular and physiological endpoints to produce their mood-elevating effects. The deep layers of the mPFC project to subcortical regions (47), so specific activation of the deep-layer cells may increase activation of the downstream limbic structures (see below). On a cellular level, the prevailing hypothesis is that enhancing neuroplasticity is key to combating depression, given that stress leads to dendritic atrophy (48) and depressed patients have decreased gray matter volumes (8). There is mounting evidence that ketamine in particular encourages synaptic growth, including increased spine density and new spine formation (22). Furthermore, as mentioned above, blocking a pathway important for cell growth and survival prevents synaptogenesis and blocks the behavioral antidepressant response (22), pointing to a potential link between neuroplasticity and the antidepressant effect.

Evidence that the rodent vmPFC plays a role in depression

The rodent vmPFC is a growing focus of depression research because some believe it – particularly the infralimbic region – is most homologous to the human subgenual prefrontal cortex (BA25; 30; 31), which has been reported to show changes in activity in depressed patients (see above). The projection patterns of the rat vmPFC and non-human primate BA25 are very similar (24; 30). There is preclinical evidence that the rodent vmPFC may be linked to depression-like outcomes. For instance, chronic social defeat stress resulted in decreased levels of transcripts for *Zif268*, an immediate early gene, in the rat infralimbic cortex (51), suggesting that the vmPFC in stressed animals may have impaired function. More recently, deep brain

stimulation (DBS) was performed in rats (49) and optogenetic stimulation in mice (52) targeting the vmPFC. DBS decreased immobility in the FST, appeared to decrease anxiety and reduce the effects of footshock stress. Optogenetic stimulation increased *cfos* and *Zif268* mRNA expression (where?) that was normally reduced following chronic social defeat stress, and reversed depression- and anxiety-like behaviors in a battery of tests. Furthermore, our work in the current study and that in Chapter 3 of this dissertation has shown that vmPFC lesioned animals demonstrate depression-like behaviors: increased rapid eye movement (REM) sleep, shortened REM latency, and increased immobility in the forced swim test. In contrast, dmPFC-lesioned animals only showed increased REM sleep. Altogether, these findings suggest that the vmPFC may be important in both the expression of depression-like behaviors and mechanism of antidepressant therapies.

NAc activation by DMI

Along with the vmPFC, the NAc was the only limbic region I found that showed significant activation with DMI compared to saline-injected control animals. The NAc normally plays a role in reward and motivated behavior to both conditioned and unconditioned stimuli; thus it is widely believed that NAc dysfunction leads to anhedonia (53). Our finding that DMI activates the NAc via the vmPFC indicates that this circuit may be particularly important in antidepressant action. However, the NAc cells that are activated by the drug remain to be characterized. In contrast to our results following acute administration, chronic administration of clomimpramine (a tricyclic antidepressant) and fluoxetine were found to reduce neuronal firing rates in the NAc (54). As chronic administration is required to achieve improvement in human depression,

together these findings suggest that the transition from activation to reduced firing in the NAc may be important for the benefits of antidepressant treatment.

We did not detect drug-induced changes in cfos expression in the insular cortex, lateral septum or basolateral amygdala that receive projections from the vmPFC. It is possible that limitations in our methods and limited sample size did not allow the detection of cellular activation in these regions. For instance, there may be a neuronal subpopulation within a structure that is activated by the antidepressant drugs, but due to the size of the subpopulation an increase in cfos expression could not be detected compared to control animals. Another potential explanation is that a population of cells that is activated by the drugs does not express cfos, as expression of the protein has been associated with specific neuronal firing patterns in some cell groups (55).

Comparison to published literature

Two similar cfos-induction studies using antidepressant drugs have been completed in the past. In the first, Beck reported that neither desipramine (10 mg/kg) nor fluoxetine (15 mg/kg) increased cfos expression in the infralimbic cortex (the prelimbic cortex was not observed), and DMI did not increase cfos in the nucleus accumbens core (56). In addition, DMI did induce cfos expression in the anterior cingulate cortex compared to that of saline-injected animals. These discrepancies can be explained by a few factors: in the previous study, no apparent effort was made to control for circadian influences on cfos expression; and the same (0.5mm) counting box was used to count cells for every structure. The latter may contribute to the discrepancy because, as we have shown, some structures do not display uniform cfos expression following drug treatment and thus counting boxes that do not include the deep layers of the vmPFC are likely to

miss the changes in cfos expression. In addition, we only included animals that displayed cfos expression patterns characteristic of sleep, because awakened animals tend to have much greater cfos expression in the cortex and elsewhere. Therefore, we believe that by using sleeping animals we reduced variability.

In another recent paper by Miyata and colleagues, cfos expression was observed following fluoxetine administration (5 mg/kg and 10 mg/kg; 54). They reported that fluoxetine increased cfos expression in the NAc shell but not core, which is the opposite of our findings. In addition, they reported that cfos was not induced in the prelimbic cortex (which they call 'cingulate cortex area 3'). These discrepancies are most likely due to their counting method, as they used a small counting box (380um x 380um) that were apparently centered on each structure, thus making it very likely that cells in the deep layers of the prelimbic cortex were not counted. In addition, they also did not appear to control for circadian timing or the influence of wakefulness on cfos expression.

Conclusion

Although traditional antidepressant drugs such as tricyclic antidepressants and selective serotonin reuptake inhibitors require up to a few weeks to take effect, investigating their acute neural targets may allow us to understand the important circuitry involved in their mechanism of action. Our findings that three different classes of drugs with demonstrated antidepressant properties selectively target the vmPFC strengthen the notion that this region is important in antidepressant action and mood regulation. In the future, it will be imperative to investigate how the acute response develops into the mood-elevating chronic response in TCAs, SSRIs and other

drug classes, and whether their antidepressant effect is dependent on neuroplasticity, such as synaptic growth and changes in cell morphology.

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Chapter 3

Medial prefrontal cortex regulates depressive behavior and REM sleep

ABSTRACT

Major depressive disorder (MDD) is a debilitating disease that is diagnosed by its symptoms, such as persistent depressed mood and disturbances in sleep. The prefrontal cortex (PFC) has been implicated as an important structure in the neural circuitry of MDD, for there are pronounced abnormalities in blood flow and metabolic activity in PFC subregions, including the subgenual cingulate cortex (SGC, or Brodmann area 25). In addition, deep brain stimulation in the SGC has recently been shown to alleviate treatment-resistant depression. Depressed patients also show characteristic changes in sleep: insomnia, increased rapid-eye-movement (REM) sleep and shortened REM sleep latency. We hypothesized that sleep changes may be a consequence of the abnormal PFC activity in depressed patients. The rat ventromedial PFC (vmPFC) has been likened to the human subgenual cingulate cortex, so I made excitotic lesions in the vmPFC and the adjacent dorsal region (dmPFC). I found that both dmPFC and vmPFC lesions led to increased REM sleep, however only the vmPFC-lesioned animals displayed shortened REM latency and increased immobility in the forced swim test. These results support my hypothesis that the human homolog of the rat vmPFC, the SGC, may be a critical area for modulating both mood and REM sleep.

Introduction

Depression is the leading cause of disability worldwide, and continues to increase in prevalence (1). Known clinically as Major Depressive Disorder (MDD) in the U.S., it is diagnosed by the presence of at least five symptoms, which must include depressed mood or anhedonia (2). Examples of the other criteria are changes in appetite, loss of energy, and sleep disturbances. Despite the prevalence of MDD (and other mood disorders), its etiology remains unclear. It is generally believed that MDD is caused by dysfunction in a network of structures in the brain, rather than a single structure. This network is believed to include the prefrontal cortex, hippocampus, nucleus accumbens, and amygdala, based on their function and human imaging studies (3).

The prefrontal cortex (PFC) has particularly been of interest because of the marked changes in activity in depressed patients compared to healthy controls. For instance, the ventral regions demonstrate an increase in cerebral blood flow (4) and metabolic activity (5), whereas the dorsolateral PFC displays reduced activity (6–8). The subgenual cingulate cortex (SGC or Brodamann area 25), which is included in the PFC by many clinical researchers (9–12), shows increased glucose metabolism (13). Furthermore, responders to antidepressant treatment have significantly reduced activity in the SGC compared to their pre-treatment baseline levels (14).

Preclinical studies also provide supporting evidence for the importance of the medial prefrontal cortex (mPFC) in depression-associated behaviors. Human postmortem studies have shown reduced glial cell counts in MDD patients (15), and glial cell ablations in the rat mPFC resulted in behaviors that have been associated with depression, including decreased sucrose consumption in the sucrose preference test and increased immobility in the forced swim test (16). Excitotoxic neuronal lesions in the rat mPFC also led to ‘learned helplessness’ upon exposure to

inescapable footshocks (17). In addition, chronic stress protocols, which are believed to induce behaviors that are depression-like, led to decreased glial cell count (16) and dendritic atrophy (18). Interestingly, deep brain stimulation in the mPFC induced an antidepressant-like response in the forced swim test (19), and optogenetic stimulation in the mPFC of mice susceptible to social defeat stress reversed their avoidance of social interaction and increased their preference for sucrose (20). Altogether, these results suggest that inactivation of the rodent mPFC region is associated with depression-like outcome measures, whereas stimulating this region may produce antidepressant-like effects.

The role of insomnia with daytime fatigue as a diagnostic criterion for MDD is intriguing because it suggests a biological relationship between sleep and affect. Depressed patients complain of insomnia and demonstrate increases in REM sleep, along with decreased REM latency (the interval of time between sleep onset and REM sleep onset) (21–23). However, it is uncertain whether these sleep changes are related to the cause of MDD or are a consequence of it. Animals that undergo a chronic mild stress protocol demonstrate changes in sleep similar to those observed in depressed humans, suggesting these sleep changes may be secondary (24). However, most classes of antidepressant drugs suppress REM sleep, suggesting that treatment may be related to reversing the REM sleep changes (25). Even more perplexing, acute sleep deprivation has been found to effectively (albeit very temporarily) elevate mood, despite that most depressed patients suffer from insomnia (26). I was interested in exploring the depression-sleep relationship by investigating the neural circuitry of both depression and sleep, using the rat as a model of study.

I hypothesized that prefrontal cortex dysfunction may be key in producing depression-associated behaviors, including sleep disturbances. I made neuronal lesions in the ventral and

dorsal subdivisions of the rat medial prefrontal cortex and tested the animals for depressive-like behavior in an established rodent model of depression and sleep-wake behavior. I subsequently propose a model of sleep modulation via the prefrontal cortex.

The forced swim test (FST) was used to test depression-like behaviors (27; 28). The test was originally established to predict antidepressant efficacy, but it has been demonstrated that rat models of depression lead to increased immobility in the FST, such as the Flinders Sensitive Line (a genetic model of depression cite), and following chronic stress (29; 30) and social defeat protocols (31; 32). The FST is therefore generally believed to have validity, albeit limited, as an animal model of depression-associated behaviors (35).

Methods

Animal Surgery and EEG/EMG Implantation

Prior to surgery, animals were anesthetized with ketamine-xylazine (i.p., 800 mg/kg ketamine, 80 mg/kg xylazine, Med-Vet, Mettawa, IL) and then placed on a stereotaxic frame so that their head was fixed. Injections of ibotenic acid (IBO, Tocris, Ellisville, MO), 0.9% saline (Med-Vet, Mettawa, IL) or neural tracer (BD, Molecular Probes, Grand Island, NY or CTB, List Biological, Campbell, CA) were administered directly into the brain using a fine glass pipette (1 mm glass stock, tapering slowly to a 10-20um tip) connected to an air compression system. A series of 20-40psi puffs of air were used to deliver the compounds into and with the following coordinates (Paxinos and Watson, 2005) and volumes: ACC (dmPFC): AP+3.0 mm to 3.5 mm DV-1.4 mm RL+/-0.6 mm, 66nL 1% - 5% IBO; vmPFC: AP+3.0 mm to 3.5 mm, DV-3.4 RL+/-0.6 mm, 66-99nL 5% IBO, 16.5nL 1% CTB; vlPAG: AP-7.2 mm, DV-5.6 mm, RL-1.2 mm, 33nL 1% CTB; SLD: AP-9.4 mm, DV-6.3 mm, RL-1.2 mm, 16.5nL 1% CTB.

On the same day some of the animals received four 3.2mm EEG screw electrodes (Plastics One, Roanoke, VA) that were screwed into skull, one on each side of the frontal and parietal bones. Two flexible EMG wire electrodes (Plastics One, Roanoke, VA) were also placed on the left and right nuchal muscles. The free ends of the leads were placed in a plastic electrode pedestal (Plastics One, Roanoke, VA) that was cemented onto the skull using Jet Denture Repair Powder and Jet Liquid (Henry Schein, Melville, NY). Any animals that did not receive electrodes had their incision closed with wound clips. Upon completion of the procedure, the animal was given a subcutaneous injection of analgesic meloxicam (1.0 mg/kg, Med-Vet, Mettawa, IL) and allowed to recover on a warm plate until awakened from anesthesia.

Sleep Recordings and Analysis

After at least a week of post-surgical recovery, animals undergoing sleep recordings were placed in isolated recording chambers. Flexible cables (Plastics One, Roanoke, VA) that were mounted to fixed commutators were attached to the electrode pedestals, and the cages were placed such that the animals could move freely. As before, food and water were available ad libitum, ambient temperature was controlled, and the light:dark cycle was 12:12 with lights on at 8:00 am. Video cameras were placed to capture movement in the entire cage, and the animals were habituated without disturbance for at least two days and then recorded for 48h using VitalRecorder (Kissei Comtec Co., Nagano, Japan). Upon completion of the recordings, animals were detached from the cables and returned to the holding room.

The EEG/EMG recordings were analyzed using SleepSign (Kissei Comtec Co., Nagano, Japan). The recordings were divided into 12s epochs and each epoch scored manually as wake, REM, or NREM sleep. Wake was identified by high-frequency, desynchronized EEG accompanied by frequent EMG activity and observed behaviors on the video playback. NREM sleep was identified by the dominant presence of high-amplitude, low-frequency (<4 Hz) EEG activity and little muscle tone on the EMG recording. REM sleep was identified by theta waves (4-7 Hz) of consistent low amplitude on the EEG recording accompanied by very low EMG activity.

Sleep-wake percentages, bout numbers and bout durations were analyzed using unpaired t-test and adjusted using Bonferoni's correction, using a significance threshold $p<0.05$.

Spectral analysis

NREM and REM spectral analyses for control (n=5), dmPFCx (n=7), and vmPFCx (n=20) were conducted by re-scoring sleep 2-4p with 4s epochs, while wake was analyzed during 8-10p. Transitions and movement artifacts were removed from the analyses, and cases with high amounts of artifact were omitted. Waveforms in each vigilance state were grouped into frequency bands and analyzed using one-way ANOVA with the post-hoc Scheffe test for multiple comparisons, using a significance threshold $p<0.05$.

Statistical Analysis of Forced Swim Test

FST immobility between groups were analyzed using unpaired t-test and adjusted using Bonferoni's correction, using a significance threshold $p<0.05$. Both dmPFCx and vmPFCx lesion groups were included in the correlation analysis comparing FST and REM sleep latency.

Histology and Immunohistochemistry

Brains were sliced into four series of 40um sections using a freezing microtome. The sections were stored in PBS-0.02% azide in 20°C.

Immunohistochemical staining was completed as follows: tissue sections were rinsed in PBS three times, 3-5 min each. They were then incubated for 30 min in 0.3% H_2O_2 (Sigma, St. Louis, MO) in PBT (phosphate buffer with Triton X-100; Sigma, St. Louis, MO) to oxidize any remaining blood. The sections were again rinsed in PBS and then incubated in primary antibody diluted in PBT-Azide for 1-2 nights, depending on the antibody (NeuN, MAB377, mouse monoclonal, 1:20,000, Chemicon, Billerica, MA). Tissue were then rinsed in PBS three times, and incubated in secondary antibody (1:1000, biotin SP-conjugated against appropriate species IgG, Jackson ImmunoResearch Laboratories, West Grove, PA) for 60-90 min. Sections were

again rinsed in PBS and placed in ABC solution (1:1000 each Vectastain solutions A and B, Vector Laboratories, Burlingame, CA) for 60-90 min. Sections were rinsed in PBS and stained for 5 min in a solution consisting of: 1% DAB, 0.3% H_2O_2 (and 0.01% Ni, 0.005% $CoCl_2$ if desired a black stain). Staining procedure for BD started with ABC solution because the tracer is biotinylated. Tissue were then rinsed in PBS and mounted on microscope slides in gelatin.

Slides were counterstained by placing them in ddH_2O for 5 min, followed by 10-30 sec in 0.1% thionin (Sigma, St. Louis, MO). The slides were dehydrated step-wise by incubating in 50% EtOH, 70% EtOH, 95% EtOH, and 100% EtOH for 2 min each. Slides were then placed in xylene for several hours before covering with glass coverslips.

For CTB and BD double immunofluorescence staining, sections were rinsed and incubated in primary antibody as stated previously. Following rinses in PBS, sections were incubated in Alexa Fluor 488 (A11055, anti-goat, 1:1000, Molecular Probes, Grand Island, NY) and Cy3-conjugated streptavidin (016-160-084, 1:500, Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were rinsed and mounted on microscope slides under dim light. The fluorescent cells were imaged on a confocal microscope (Zeiss, Thornwood, NY) at 63x magnification, at a single optical layer.

Results

Cell body-specific lesions of the rat mPFC increase REM sleep and sleep fragmentation

Sleep-wake behavior was investigated after placing bilateral ibotenic acid lesions in the dorsal (anterior cingulate cortex, dmPFCx, n=9) or ventral mPFC (infralimbic and prelimbic cortex, vmPFCx, n=24) of adult male Sprague-Dawley rats. Photomicrographs of the lesions are shown in Fig. 3-1 and schematic drawings of lesions in a representative set of brains is shown in Fig. 3-2. Lesions that significantly extended into the premotor cortex (M2) or orbital frontal cortex were excluded from the analyses. Control animals were treated identically except they received injections of 0.9% saline (n=10).

Following placing the lesions, EEG and EMG electrodes were implanted into the skull and on the neck muscles, respectively, for recording sleep-wake behavior. After at least1 week for recovery, the animals were habituated to an isolated chamber and subsequently recorded for 48 hours. Neither lesion group exhibited statistically significant changes in total wake or NREM sleep over 24 hours, but both groups demonstrated a marked increase in REM sleep compared to control animals (dmPFCx: p=0.00035, adj. p=0.001, t(17)=3.95 and vmPFCx: p=0.016, adj. p=0.048, t(31)=2.05). Animals with lesions in the dmPFC had a 20.7% increase in REM, while vmPFCx animals had a 16% increase (Fig. 3-3B). Both groups also had increased sleep fragmentation. In particular, the vmPFCx animals demonstrated increased bout numbers of wake (32.1%, p=4.5*10^-5, adj. p=0.00014, t(31)=4.35) and NREM sleep (26.1%, p=8.1*10^-5, adj. p=0.00024, t(31)=4.14) during the light period (Fig. 3-3C). The vmPFCx animals also had shorter wake (-33.5%, p=0.00036, adj. p=0.0011, t(31)=3.61) and NREM sleep bouts (-26.0%, p=0.00018, adj. p=0.00054, t(31)=3.86) during the light period, and shorter NREM sleep bouts

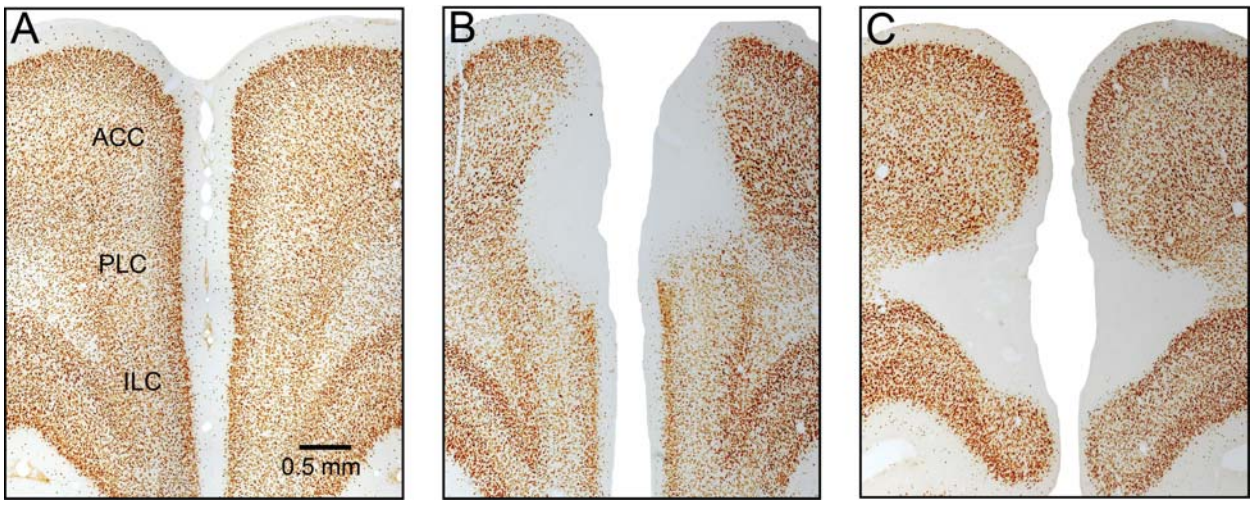

Figure 3-1. A mPFC histology of saline-injected control rat brain stained with NeuN. Ibotenic acid injections killed neurons in the dmPFC **B** and vmPFC **C**. Bregma +3.5mm.

Figure 3-2. Location of lesions in representative cases from each lesion group. Levels indicated on the left are with respect to Bregma.

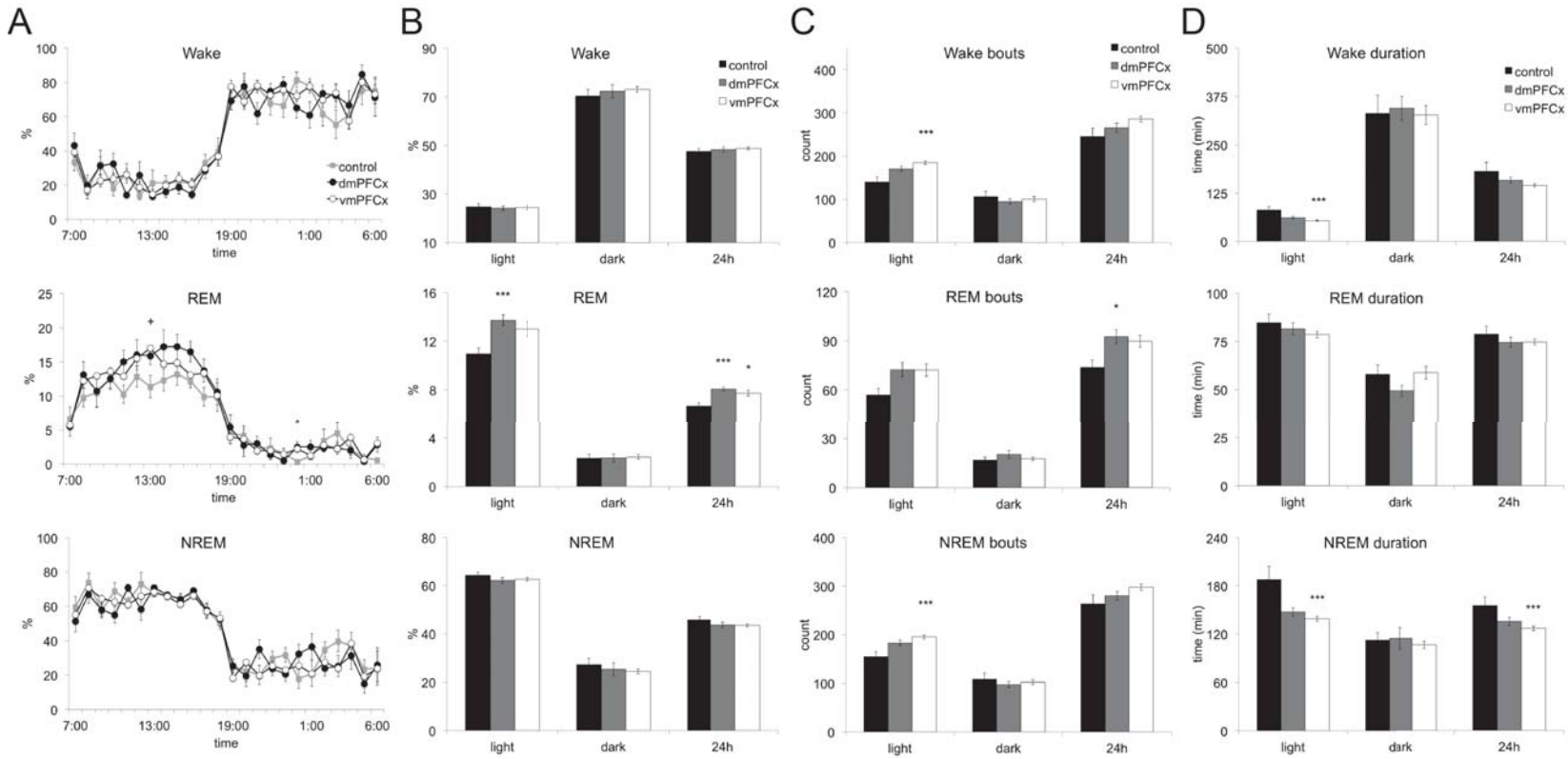

Figure 3-3. Summary of the sleep-wake behavior for the two experimental groups and control animals. **A**. The average wake, REM, and NREM sleep per hour over 24h shows a trend in increased REM sleep in both experimental groups. Symbols indicate significance using Bonferroni adjusted *p*-values: '*', adj. $p<0.05$ for dmPFCx group; '+', adj. $p<0.05$ for vmPFCx group. **B, C, D.** Percentage of time, bout numbers, and average bout duration of wake, REM, and NREM sleep are summarized for each the light phase, dark phase, and over 24h. Asterisks indicate adjusted *p*-values: '*', adj. $p<0.05$; '***', adj. $p<0.005$.

over the 24 hours (-18.3%, p=0.00097, adj. p=0.003, t(31)=3.23) (Fig. 3-3D). dmPFCx animals had 25.4% greater bout numbers of REM sleep over the 24 hours (p=0.0088, adj. p=0.026, t(17)=2.43). The increased sleep fragmentation during the light period can be observed in the hypnograms in Fig. 3-4.

The vmPFCx (-24.7%, p=0.0038, adj. p=0.012, t(31)=2.69), but not the dmPFCx (p>0.05, t(17)=1.04) animals, had significantly reduced REM latency (Fig 3-5). Altogether, the results from the sleep-wake analysis suggest that the ventral and dorsal mPFC both influence REM sleep amounts, but only lesions in the ventral region lead to a pronounced increase in sleep fragmentation and shortened REM sleep latency.

Spectral analysis of wake, REM sleep and NREM sleep demonstrated that the dmPFCx group had an increase in delta power during NREM sleep (30.8%, p=0.011, t(17)=2.85), along with decreases in alpha (-26.2%, p=0.029, t(17)=2.38) and beta+gamma power (-30.3%, p=0.022, t(17)=2.52). The vmPFCx group was not significantly different from controls in any of the vigilance states (Fig. 3-6).

Cell body-specific lesions of the rat vmPFC increase immobility in the FST

To investigate if the dmPFC and/or vmPFC may modulate depression-like behaviors, I tested a subset of the lesioned and sham-lesioned animals under the forced swim test (FST) paradigm (24). Briefly, an animal is placed in a cylinder of 25°C water for 15 minutes on Day 1, and 5 minutes on Day 2. An increase in the total length of time an animal spends immobile during the Day 2 test session is believed to indicate that an animal is exhibiting depression-like behaviors. The results showed that vmPFCx animals had 165.3% increased immobility (p=0.0044, adj. p=0.013, t(26)=2.66), while the dmPFCx animals were not statistically different from controls

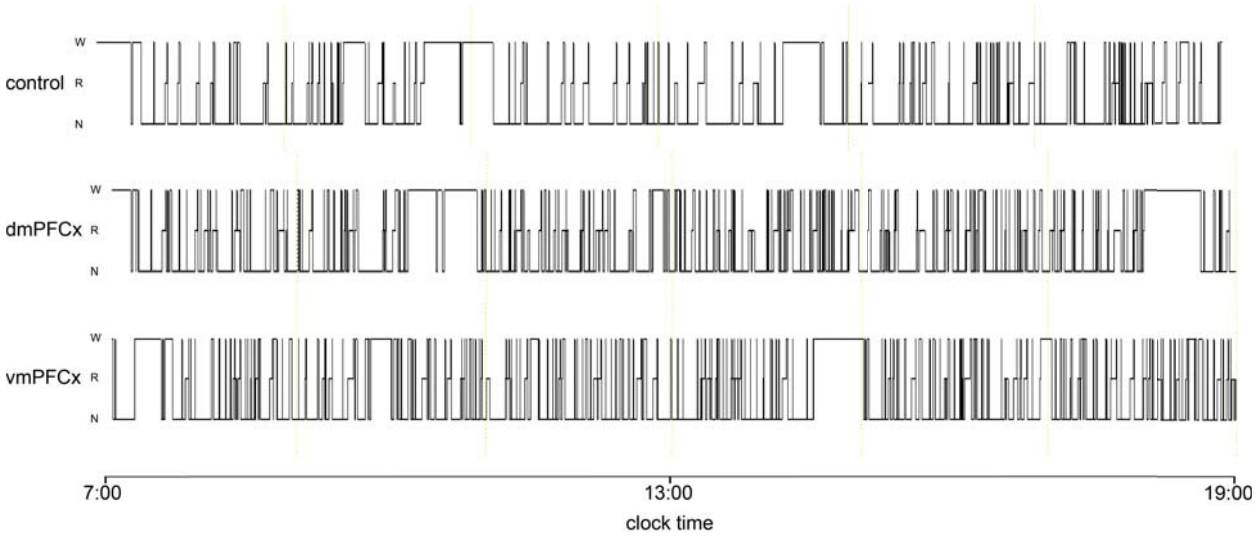

Figure 3-4. Hypnograms of individual cases from each group exemplify increased fragmentation (shorter and increased number of bouts) during the rest (lights on) period in the lesion groups compared to controls.

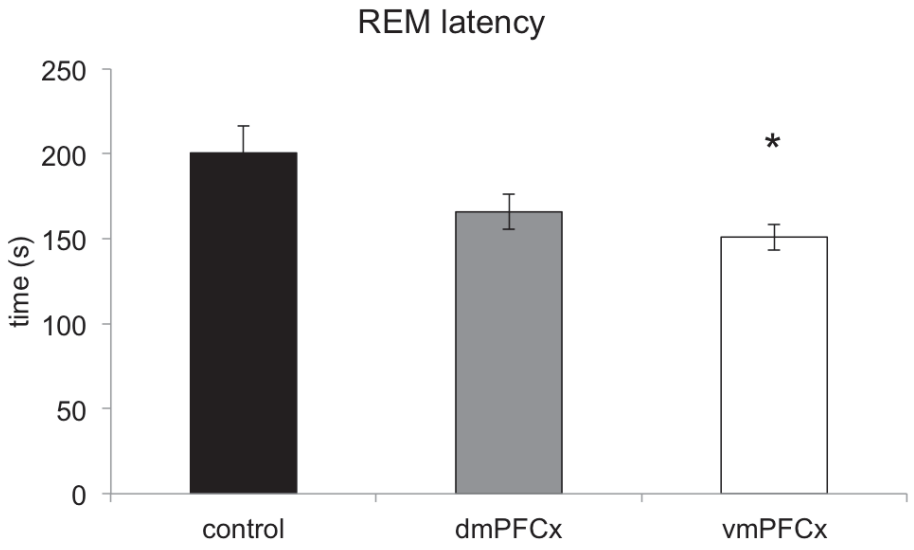

Figure 3-5. The vmPFCx, but not the dmPFCx, animals had shortened REM latency compared to controls. This measure was calculated by averaging the interval of time between the onset of NREM sleep and REM sleep for each sleep episode over 24h. '*', adj. p <0.05.

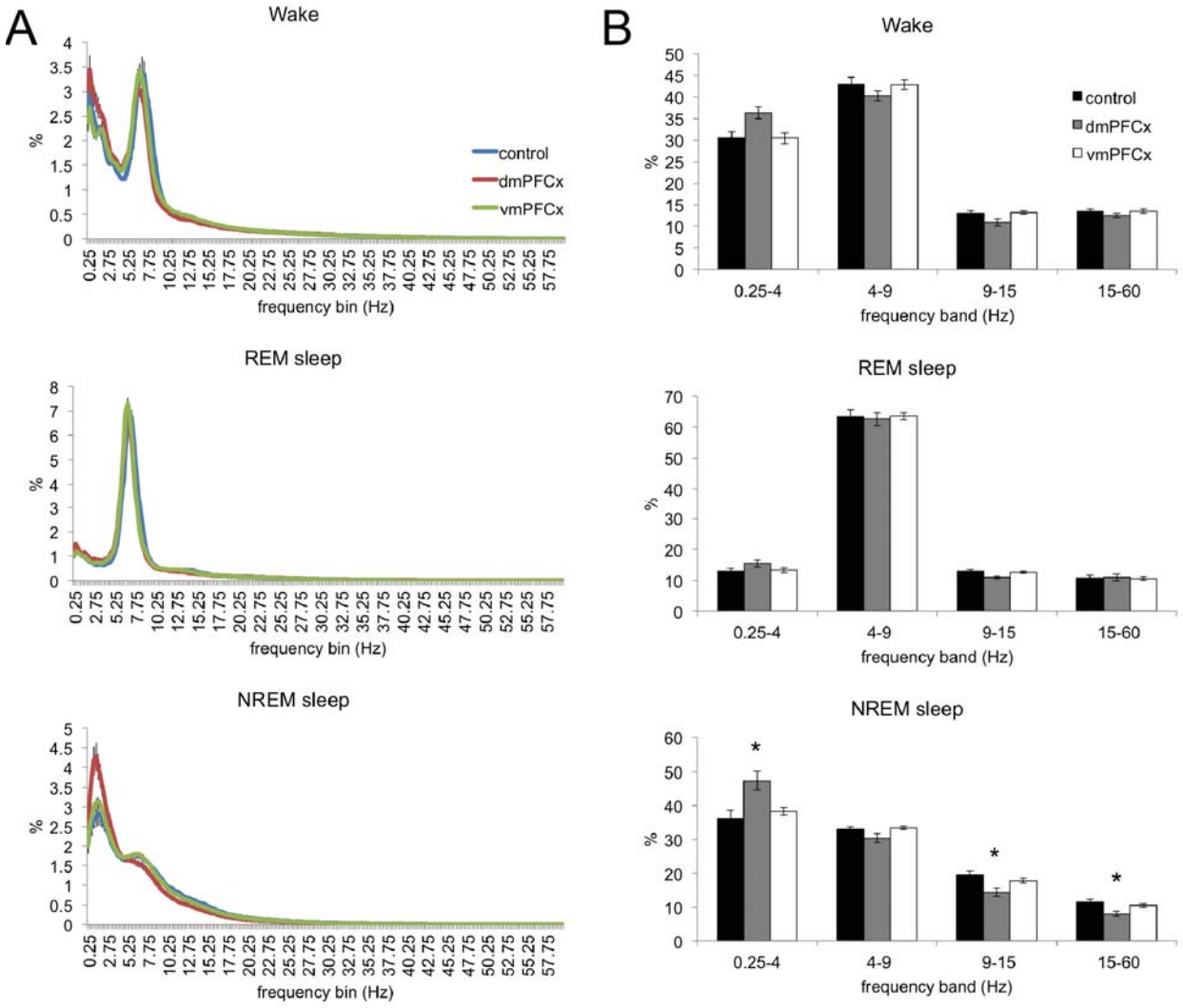

Figure 3-6. Spectral analysis of wake, REM and NREM sleep. **A** Frequency spectra of wake, REM sleep and NREM sleep of the three experimental groups. **B** Frequency data were grouped according to frequency bands correlating to delta (0.25-4 Hz), theta (4.0-9.0 Hz), alpha (9.0-15.0 Hz) and beta+gamma waves (15.0-60.0 Hz). Results were compared using one-way ANOVA with post-hoc Scheffe test: '*', adj. $p<0.05$. Sleep states were analyzed from 4s epochs scored between 2:00pm and 4:00pm. Wake was analyzed from 4s epochs scored between 8:00pm and 10:00pm.

(p>0.05) (Fig. 3-7). Fig. 3-8 shows immobility bouts during the 5-minute test session from representative animals in each group.

Immobility in the Forced Swim Test negatively correlated with REM sleep latency

Because vmPFCx animals exhibited shortened REM latency and increased immobility in the FST, both of which are characteristic of depression-like states, such as seen after chronic stress in rodents, I next asked whether these two behaviors were associated among all of the lesioned animals. A correlation analysis showed that the measures are significantly negatively correlated (R=-0.464, p=0.019; Fig. 3-9), suggesting that the two measures may depend upon the same substrate.

Neural circuit of mPFC regulating REM sleep

To determine what the substrate of the REM suppression may be, I examined the projections from the mPFC to the ventrolateral peiaqueductal gray matter (vlPAG). Lu and colleagues (38) had previously shown that the vlPAG and adjacent lateral pontine tegmentum (vlPAG-LPT) contains GABAergic neurons that inhibit the sublaterodorsal nucleus (SLD), which promotes REM sleep (38), suggesting that the vlPAG-LPT suppresses REM sleep. Other earlier tracing studies had shown that both vmPFC and dmPFC neurons send projections to the vlPAG-LPT (39; 40). To investigate whether each mPFC region may be modulating sleep via direct projections to this REM control site, I injected the anterograde tracer biotin dextran (BD) into the vmPFC or dmPFC and retrograde tracer cholera toxin B (CTB) into the SLD in the same animals (Figs. 3-10 and 3-12). I then examined the results of immunohistochemical staining for BD and CTB in the vlPAG-LPT.

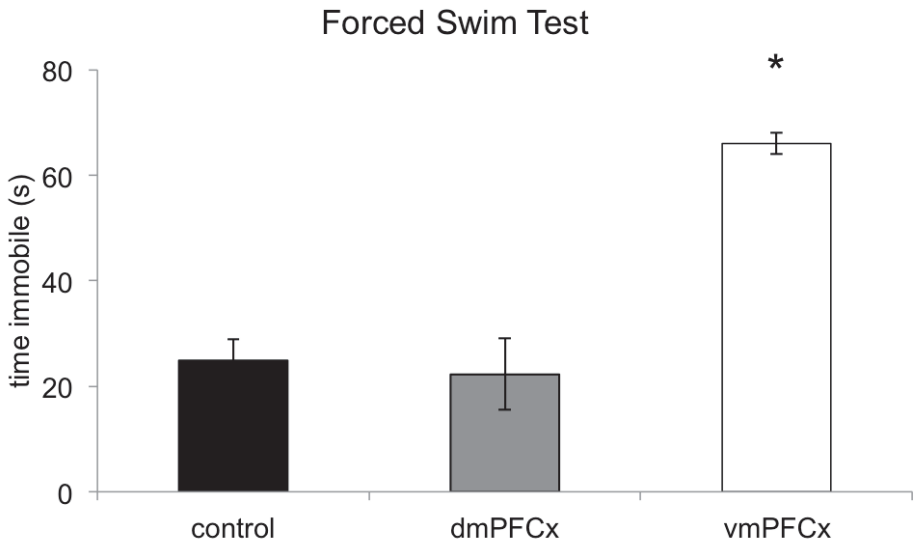

Figure 3-7. Average time animals in each experimental group were immobile during the 5min forced swim test. The vmPFCx, but not the dmPFCx animals had increased immobility compared to control animals. '*', adj. p <0.05.

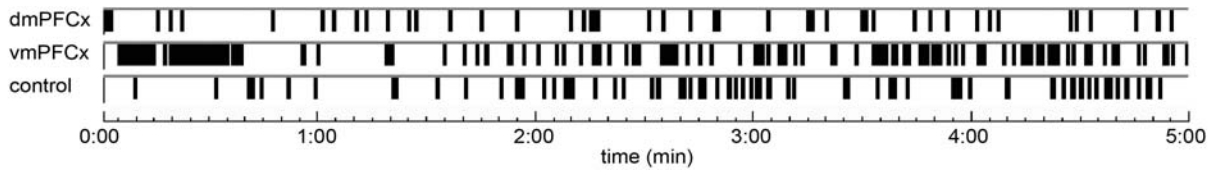

Figure 3-8. Example schematic of immobility bouts during the forced swim test as scored by Ethovision. The software compares dynamic pixel changes frame-to-frame, and if less than the threshold percentage of pixels differs between frames the animal is considered immobile. Parameters were validated by manual scorers (1).

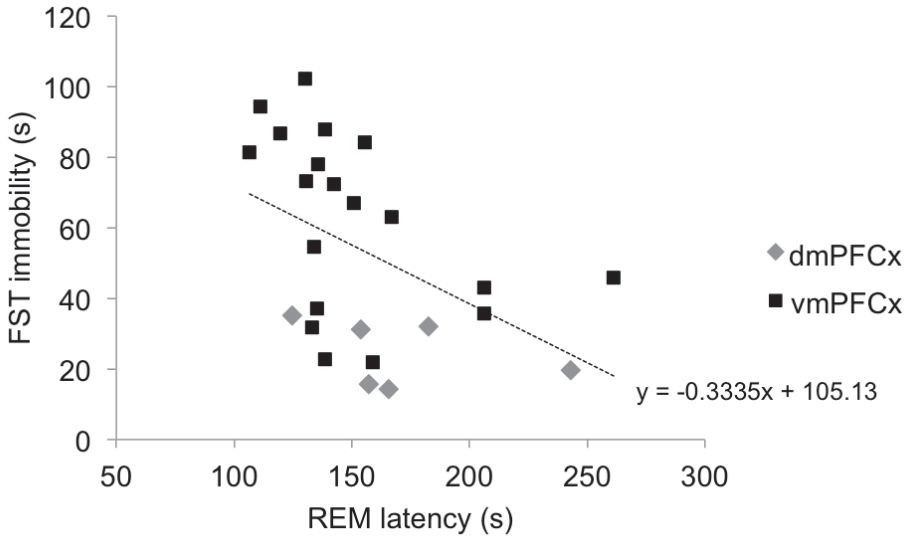

Figure 3-9. The time an animal was immobile during the FST was plotted v. their REM latency measure during sleep. A significant correlation between these measures was found (R=-0.464, p=0.019), suggesting a possible biological relationship between these two measures.

Of the retrogradely labeled cell bodies in the vlPAG-LPT, 24.2% had appositions from the dmPFC and 16.0% had appositions from the vmPFC. A series from each case was labeled with fluorescent antibodies and viewed under a confocal microscope to confirm the appositions (Figs. 2-11 and 2-13). The injection in Fig. 3-12B includes part of the parabrachial nucleus (PB). A retrograde tracing study where CTB was injected only into the PB demonstrated that some cells in the vlPAG do project to the PB, but there are none in the LPT (Fuller and Lu, unpublished observations). Therefore, some of the cells counted in the analysis may also include vlPAG->PB cells.

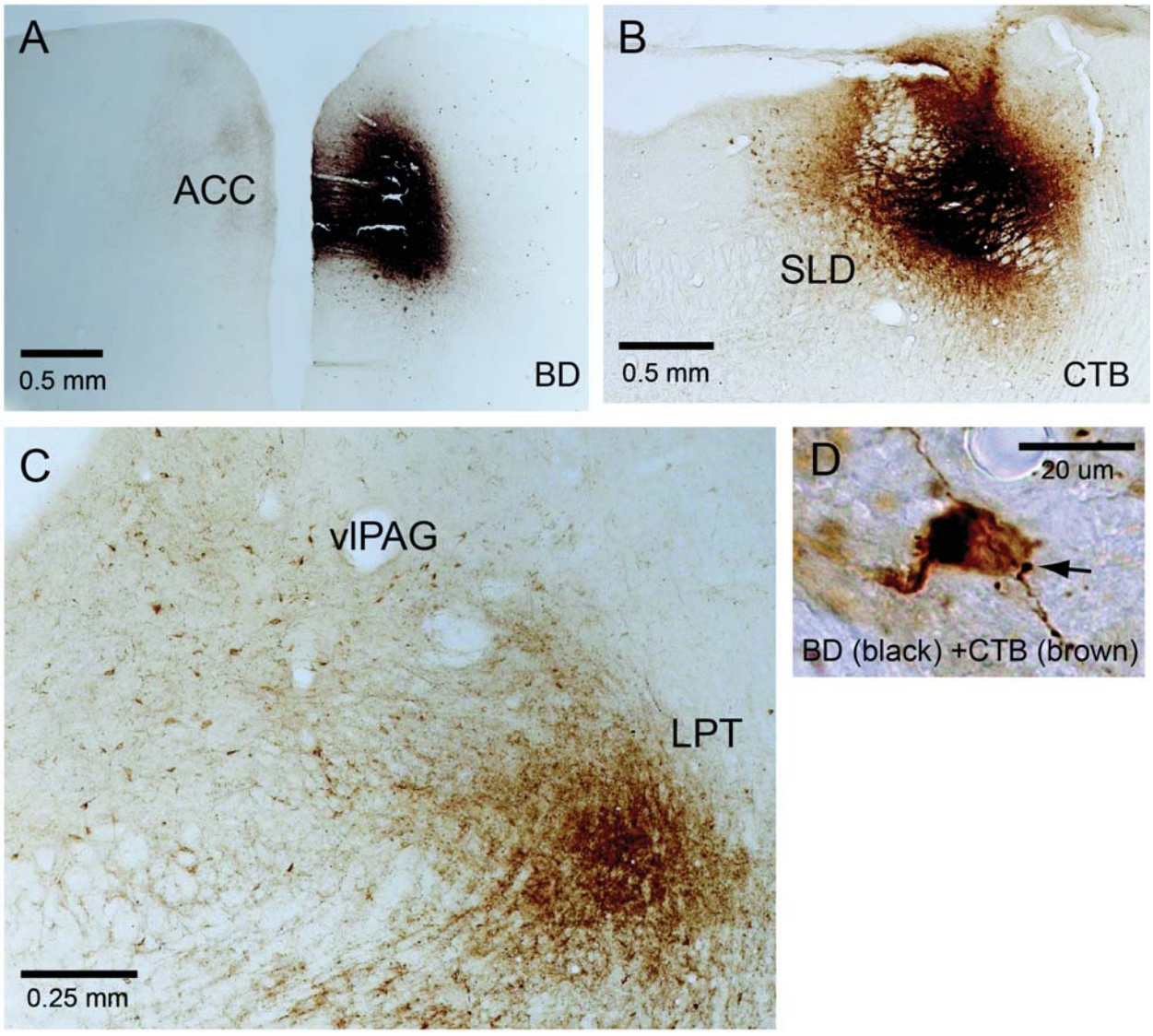

Figure 3-10. To investigate a possible pathway by which the dmPFC may be modulating REM sleep, two tracers were injected (both unilaterally) into an individual animal: **A** BD, an anterograde tracer, into the dmPFC (Bregma 3.5mm); **B** CTB, a retrograde tracer, into the REM-on SLD (Bregma -9.4mm). **C** Cells in the vlPAG-LPT (Bregma -7.2mm) were then sought that were stained for CTB (brown) and also had BD boutons (black) (D).

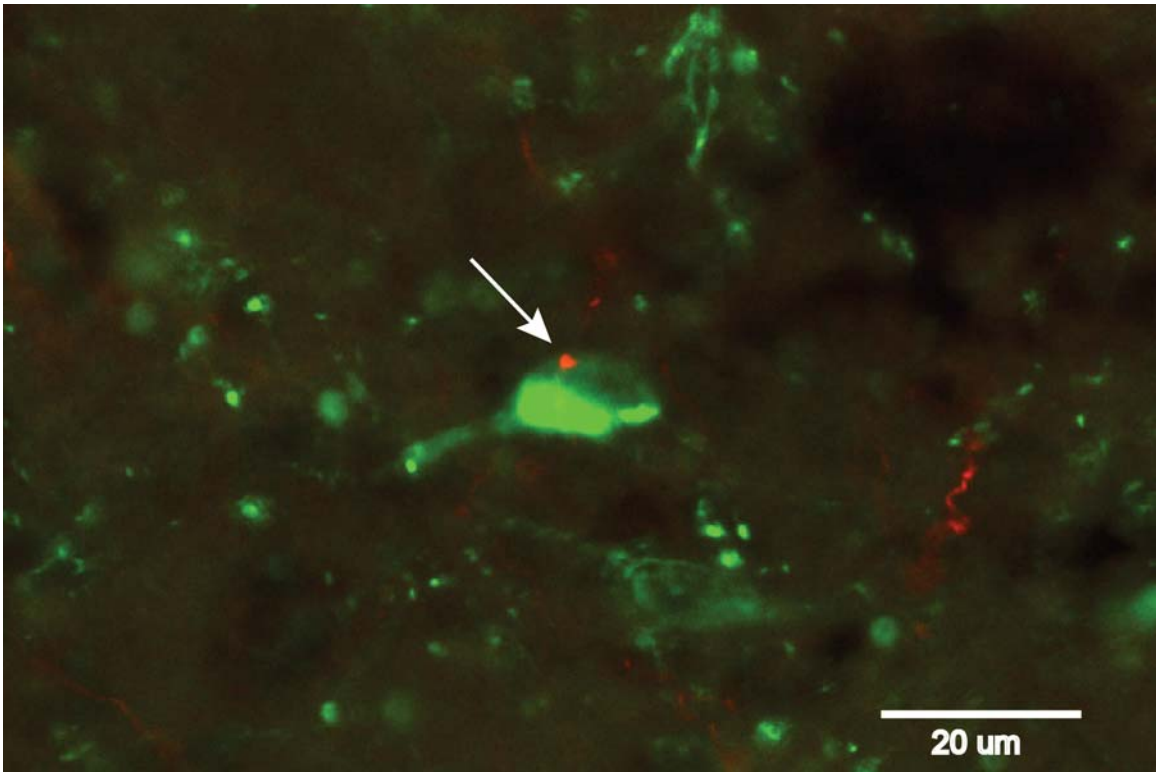

Figure 3-11. A series from the same case as in Fig. 3-10 was stained with AlexaFluor488 (green, CTB) and Cy3 (red, BD) and viewed under a confocal microscope (63x). A cell in the vlPAG is stained green, indicating it projects to the SLD area, and has a red bouton from the vmPFC (arrow). This image was taken in a single optical plane.

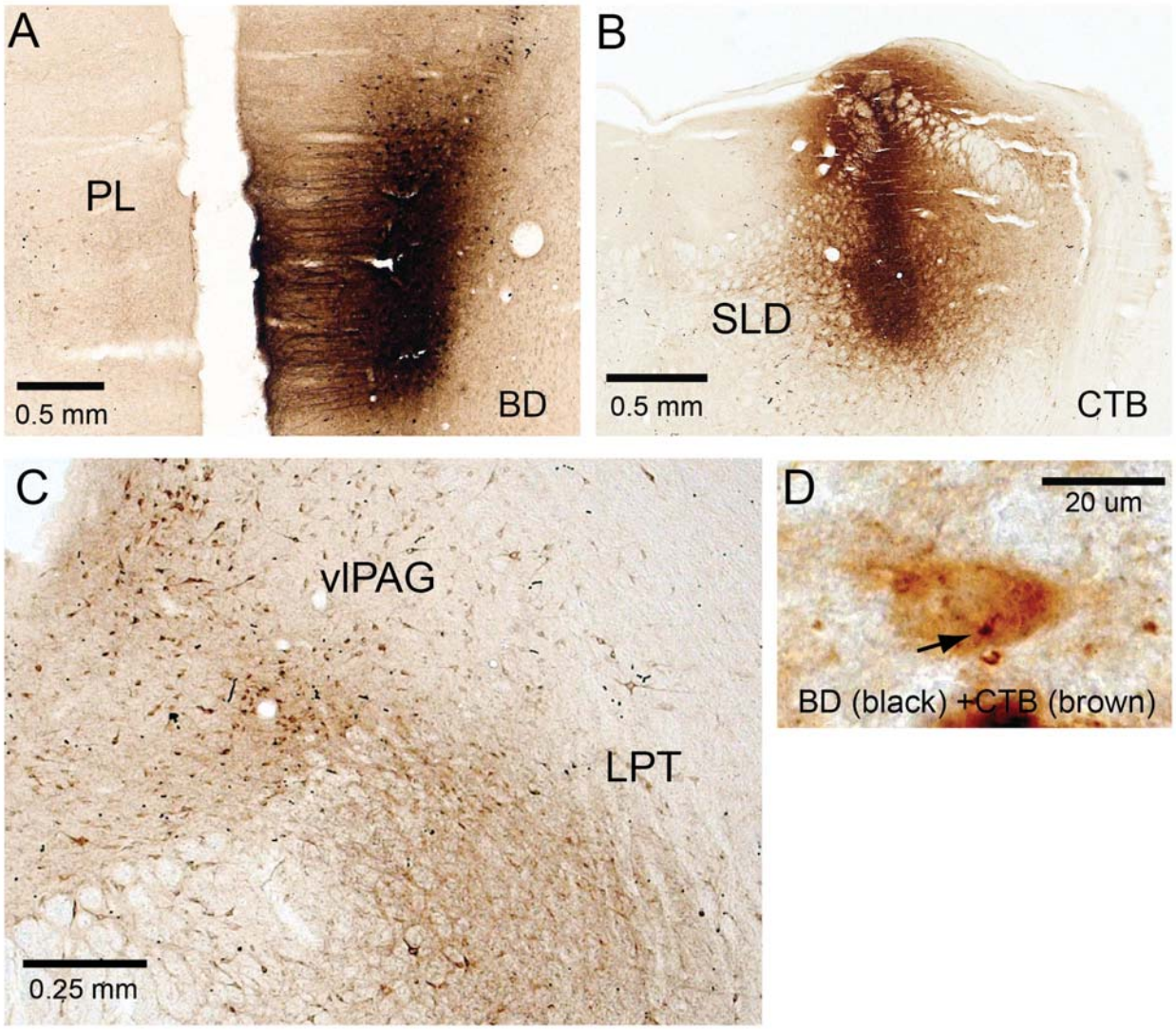

Figure 3-12. As in Fig. 3-10, two tracers were injected into an individual animal: **A** BD, an anterograde tracer, into the vmPFC (Bregma 3.5mm); **B** CTB, a retrograde tracer, into the REM-on SLD (Bregma -9.4mm). **C** Cell bodies in the vlPAG-LPT (Bregma -7.2mm) were then sought that were stained for CTB (brown) and also had BD boutons (black) (D).

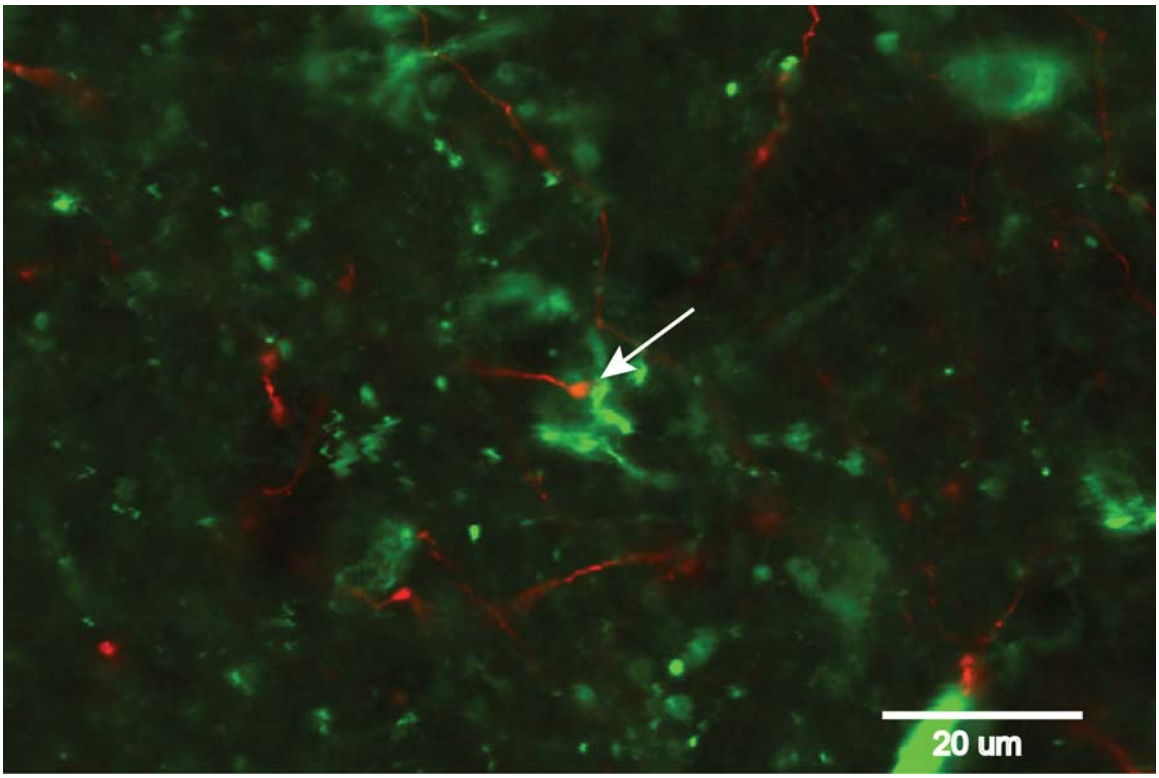

Figure 3-13. A series from the same case as in Fig. 3-12 was stained with AlexaFluor488 (green, CTB) and Cy3 (red, BD) and viewed under a confocal microscope (63x). A cell in the vlPAG is stained green, indicating it projects to the SLD area, and has a red bouton from the vmPFC (arrow). This image was taken in a single optical plane.

Discussion

While previous studies have identified the mPFC as an important region underlying MDD, how this region affected depression-associated behavior was unknown. In the present study, I investigated the roles of the dorsal and ventral subregions in the rat mPFC on sleep and depression-like behavior in the forced swim test. I found that ventral and dorsal mPFC lesions led to increased REM sleep, but only ventral mPFC lesions led to decreased REM latency, pronounced fragmentation, and increased immobility in the forced swim test. Although sleep in rodents is generally not as consolidated as in humans, nevertheless, increased fragmentation in rodent sleep may be analogous to a greater sleep-wake transitions seen in humans. Interestingly, the REM latency and FST measures were significantly correlated. Furthermore, neurons in the mPFC terminate on cells in the brainstem that are implicated in suppressing REM sleep, suggesting a possible mechanism of cortical REM modulation. Collectively, our results suggest that reduced activity in the vmPFC, distinct from the dmPFC, is important in causing the depression-associated behaviors of increased REM sleep, decreased REM latency, increased sleep fragmentation and increased immobility in the FST.

Our experimental methods had a few limitations. First, I only used one traditional rodent behavioral test for depression, the forced swim test (FST). This test may only be examining one aspect of a multifaceted disorder. However, as depressed patients and chronically stressed rodents have characteristic changes in sleep (24), sleep measures may be a useful addition to the standard battery of tests used. Second, in my spectral analysis I am unable to determine whether the significant changes in NREM power frequency bands were primarily due to an increase in slow-frequency bands or a decrease in high-frequency bands. Our method requires normalizing every epoch of each case (because of high EEG variability between animals), but as a

consequence the underlying reason for a shift in power distribution cannot be distinguished. Lastly, I used cholera toxin B (CTB) as a retrograde tracer, which strongly labels cell bodies but not dendrites. Descending projections from the cortex are likely excitatory and therefore dendritic, but I was unable to observe these contacts. Therefore I likely am underreporting the number of appositions. Nevertheless, the substantial numbers of appositions observed support our hypothesis that the mPFC excites neurons in the vlPAG-LPT.

Two papers published in the past several years have also examined the FST in mPFC-lesioned animals. Bissiere et al. (41) found that dmPFC-lesioned animals do exhibit a depression-like phenotype, contrary to our results. Given that their lesions were incomplete, particularly in the rostral dmPFC, it is surprising to find that they show an affect in the FST. One possibility is that their rats were tested over a 6-hour clocktime range, so the results may have reflected measurements at different circadian phases (42). I attempted to avoid any such effects by testing all our animals within the same 90-minute window of each day. Hamani et al. (19) lesioned the vmPFC and found that their animals did not show increased immobility in the FST. They primarily targeted the infralimbic cortex, as evidenced by their text as well as their coordinates. In addition, the single lesion histology section provided was located more caudally than our target. As I aimed to lesion both the prelimbic and infralimbic cortices, our larger dorsal-ventral range or more rostral location may account for a different result.

The rat mPFC and sleep circuitry

The dmPFC (anterior cingulate cortex) primarily projects to other neocortical regions, particularly premotor regions and the vmPFC, and parts of the medial and mediodorsal thalamus (43). The vmPFC (prelimbic and infralimbic cortices) primarily projects to limbic, hypothalamic,

and brainstem areas (44). The only region that both dorsal and ventral mPFC areas project to that has been shown to specifically modulate REM sleep is the vlPAG-LPT. Therefore, we hypothesized that REM sleep changes following dmPFC and vmPFC lesions were caused by loss of direct projections to the area. Descending cortical neurons are predominantly excitatory, so loss of excitatory projections to the REM-off vlPAG-LPT could have led to an increase in REM sleep. However, the finding that shortened REM sleep latency is unique to vmPFCx animals suggests that entry into REM sleep may be modulated by neural circuitry that is specific to this region. As REM sleep latency has been associated with affect disorders, it may be related to the finding that lesions here also lead to increased immobility in the FST.

I found a unique change in NREM sleep power distribution in the dmPFCx animal group. The delta frequency band was significantly increased, whereas the higher frequency bands were significantly decreased compared to control animals. These changes reflect a shift in distribution of power during NREM sleep. However, a potential source for this shift is unknown. The dmPFC has few projections to the basal forebrain that might influence sleep EEG (43). The role played by cortico-cortical connections of the dmPFC will be an important subject for future study.

Relationship between sleep and depression

An essential question in investigating the relationship between sleep disturbances and depression is whether one precedes the other (either in sequence or cause and effect), or they occur simultaneously (due to a common underlying biology). The presence of abnormal sleep patterns in depressed patients is well established, and there is evidence for causation in both directions.

Animals that have been subjected to chronic mild stress demonstrate significantly fragmented sleep and increased REM sleep (24; 45), as do animals that have been exposed to

regular inescapable footshocks (46). Stress is also associated with sleep changes in humans such as reduced sleep efficiency, however these subjects display decreased REM sleep unlike depressed patients (47). On the other hand, there is evidence that sleep changes are associated with increased vulnerability to depression: for instance, a persistent short REM sleep latency appears to increase the risk of relapse (48). Furthermore, healthy, never-depressed subjects with a strong family history of depression display specific sleep markers including higher REM density and decreased slow-wave sleep in the first NREM episode (49). Interestingly, patients in remission also show these characteristics (50). These markers may therefore indicate vulnerability to developing depression.

Several longitudinal studies confirm that poor sleep quality and decreased (or significantly increased) quantity increases the risk of developing an affective disorder (51–53), regardless of family history. In a European study, insomnia was found to precede MDD diagnosis in over 40% of initial episodes, and over 50% of relapses (54). Another group found that persistence of insomnia for one year increases the likelihood of becoming depressed by forty times (51). On the other hand, it is possible that in these studies the subjects have either preclinical depression or a pre-existing condition – for instance, persistent anxiety, a chronic medical condition etc. – that may give rise to depression, independent of its effect on sleep disturbances. Furthermore, as the diagnosis of depression is based on exhibiting a specific number of symptoms, and some of those are sleep symptoms, the existence of a sleep disorder may simply lower the threshold for other symptoms to meet the criteria for diagnosis. If one considers thatsome mood-related symptoms are closely associated with changes in sleep (for example if insomnia causes daytime fatigue), the effect of sleep disorders on reducing the threshold for diagnosing depression is magnified.

Although our results in this study do not shed light on the question of cause and effect, they do point to an explanation for why affective disorders and REM sleep changes often occur simultaneously. The rat vmPFC, a key structure in integrating information from the limbic system, is also a modulator of the REM-sleep system in the brainstem. Therefore change in activity in this region (or its human homologue, the SGC) that occurs as a result of negative life events (5) may influence sleep, which may be a component of predisposing the individual to depression.

Clinical Significance and Conclusion

The correlation between REM latency and FST immobility is intriguing. In humans several studies have found that REM latency is closely correlated with the Hamilton Depression Score (55–58). One possible interpretation is that the same population of neurons in the deep layers of the vmPFC reduces REM sleep and depression. However, not all studies have affirmed this correlation and decreased REM latency appears to be found in other psychiatric diseases as well (59–61). Therefore, the neural mechanism underlying early entry into REM sleep needs to be determined.

Our findings emphasize the importance of the vmPFC in modulating immobility in the FST, and reveal its role in modulating sleep. This structure is therefore a possible explanation for the sleep changes – increased fragmentation, increased REM sleep, and decreased REM latency – most frequently associated with major depressive disorder. This conjecture is strengthened by the knowledge that the prefrontal cortex of humans displays abnormal activity while in a depressed state. Although direction of causality between sleep and depression is still to be

determined, our study suggests that their neural circuitries overlap, yet may be distinct, as increases in REM sleep (without the other changes) are also observed in dmPFC lesions.

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Chapter 4

Conclusion

In this work, I addressed two questions related to major depressive disorder: 1) what are the targets of antidepressant action? and 2) what role does the ventromedial prefrontal cortex play in the induction of depression-associated behaviors? In the second chapter, I injected desipramine, fluoxetine, or ketamine systemically into rats and observed areas of the brain that were activated by the drugs using cfos immunohistochemical labeling. I found that within the PFC, the deep layers of the ventromedial PFC were selectively activated by all three drugs, while the vmPFC superficial layers were no different from saline-injected controls. However, KET also activated the dorsal region of the mPFC, whereas DMI and FLX did not. I also found that the nucleus accumbens was activated by DMI, and that this activation was dependent on the vmPFC. In addition, about 13% of the cells in the vmPFC that project to the NAc expressed cfos following DMI treatment. In the third chapter, I lesioned animals in the ventral and dorsal mPFC of rats and observed their response to tests of depression-like behavior. I found that REM sleep was increased in both lesion groups, but only the vmPFC had the following additional depression-like behaviors: reduced REM sleep latency, increased sleep fragmentation and increased immobility in the forced swim test. Lastly, I used tracer injections to propose how the subregions of the mPFC may be modulating REM sleep. I found that the dmPFC and vmPFC both project to cells in the REM-off vlPAG that subsequently project to the REM-on SLD, suggesting a possible pathway of REM sleep modulation. Altogether, my results suggest that the vmPFC may be a

critical region for both the etiology of major depressive disorder and its treatment via antidepressant drugs.

Antidepressant drugs and the ventromedial prefrontal cortex

Cellular mechanisms of antidepressant action

The result that DMI, FLX and KET selectively activate the deep layers of the vmPFC is intriguing because KET has a very distinct mechanism compared to the other two. The drugs may therefore share neural targets in bringing about their antidepressant effects on the depression circuitry. A current hypothesis of MDD is that it is a disease characterized by neuronal and glial atrophy, and antidepressants may work to increase cell growth and synaptic plasticity. For instance, chronic treatment of human glial cells with FLX or imipramine, a tricyclic antidepressant, led to increased levels of the sterol regulatory element-binding protein, which regulates cell lipogenesis and is important for cell growth (1). Mice that were administered chronic doses of FLX, DMI and the monoamine oxidase inhibitor tranylcypromine showed significantly increased levels of cAMP response element transcription (2), which is central to many cellular processes including growth. In addition, chronic FLX administration increased cellular proliferation in the rat PFC, as measured by mitotic cell counts (3). KET administration also leads to increased spine density in the PFC, and blocking a the mTOR cellular growth pathway via rapamycin prevented synaptogenesis and its ability to suppress immobility in the FST (4).

A striking difference between the action of KET and the typical antidepressants is their timing. While DMI and FLX require a few weeks of chronic administration before patient mood is alleviated, KET can act within a day (5). This difference may be due to variability in the time

required to achieve neuroplasticity, for the experiments supporting the idea that traditional antidepressants may cause plasticity required chronic administration. My cfos experiments do not reveal clues about the timing of the drugs, so different experimental methods will be necessary to investigate this particular aspect. For example, it will be interesting to observe the effect of chronic DMI on regional brain activity. However, a challenge of this experiment is that rodents develop tolerances to cfos expression, meaning that neurons may express less and less cfos if they are repeatedly activated. Therefore, an alternate method (such as using a different immediate early gene, or measuring mRNA instead of protein expression) will be required.

Administration of an NMDA receptor antagonist would be expected to result in decreased activation of its target cells. Therefore, it is surprising to observe that KET selectively activates areas of the rat brain at subanesthetic doses. One possible explanation is that the mPFC is normally inhibited by another neural locus, and KET acts to disinhibit the ventral and dorsal mPFC. Alternatively, KET may have a stronger affinity for the interneurons compared to pyramidal cells within the mPFC, and thus block the inhibiting effects of GABAergic interneurons on pyramidal cells. This explanation is supported by the finding that administration of MK801, another NMDA antagonist, into rats led to decreased firing of fast-spiking interneurons followed by increased pyramidal cell activity (6).

Role of the nucleus accumbens in action of desipramine

As mentioned, DMI also increased cfos expression in the NAc. This structure is believed to be important for both conditioned and unconditioned response to rewarding stimuli, and it has been proposed that dysfunction in this region may be responsible for anhedonia (7). I hypothesized that activation of the vmPFC leads to increased glutamatergic firing, and thus excitation of its

downstream targets. To determine whether this is true in the NAc, I administered DMI to vmPFCx animals and counted the cfos-labeled cells. The finding that the vmPFC lesions reduced the number of drug-activated cells in the NAc indicated that this may be an important pathway by which the drug acts on the limbic circuitry. However, additional studies need to be completed to elucidate the role of the NAc in antidepressant action. For instance, in a future study DMI can be administered during the forced swim test on animals with NAc neuronal lesions. This would demonstrate whether the NAc is essential for the effect DMI has on reducing FST immobility. Ideally, additional rodent tests for depression would be used, for it is possible that different aspects of depression are controlled by different neural circuitries. This is especially true since the NAc is reputed to play a role in anhedonia, so the sucrose preference test would be a good test for this aspect of depression.

Sleep, depression and the vmPFC

Circuitry of sleep changes in depression

In Chapter 3, I lesioned the dorsal and ventral regions of the rat mPFC and investigated the animals' sleep-wake behavior and immobility in the forced swim test. The finding that both dmPFCx and vmPFCx animals had increased REM sleep, while the vmPFCx animals had additional depression-like behavioral characteristics, suggests that the circuitry responsible for increased REM sleep may be distinct from that which is involved in REM latency and FST immobility. Injection of the retrograde tracer CTB into the vlPAG labels cells in both the dmPFC and vmPFC, so these cortical areas may suppress REM sleep via direct glutamatergic projection to the REM-off control area. Although I did not observe any other structures that potentially link

the REM-executive structures with the mPFC in my tracing studies, I cannot rule out the possibility that another pathway contributes to the mPFC-mediated REM sleep changes.

The neural pathway that caused sleep fragmentation in vmPFCx remains to be elucidated. The vmPFC projects to the ventrolateral preoptic area, which is an important sleep-promoting structure in the brain. Loss of vmPFC→VLPO projections in vmPFCx animals may be disrupting the sleep-wake switch, leading to increased fragmentation. Although additional experiments need to be performed to test this hypothesis, it will be difficult to determine whether this specific projection is responsible for fragmentation.

Spectral analysis of the EEG waveforms of dmPFCx and vmPFCx animals demonstrated that dmPFCx animals had a significantly different distribution of frequency bands compared to control animals during NREM sleep. That is, animals in this experimental group had proportionately greater delta waves and fewer high-frequency waves. This result is puzzling, and the implications of this distribution shift are unclear. The dorsal region of the mPFC has major projections to other neocortical regions, so loss of these projections may be affecting cortical EEG waveforms.

Sleep as a marker or cause of depression

In the past it has been suggested that shortened REM latency may be a marker for depression, for REM latency measures were found to be correlated with Hamilton Depression scores (8–10). In addition, REM latency was the sole sleep characteristic that displayed a relationship to depression severity and could be objectively measured (8). However, in the years following, the importance of REM latency in MDD was diminished because other psychiatric disorders – such as schizophrenia – were also found to be associated with REM latency decreases (11). My result

that REM latency and FST immobility are significantly negatively correlated appears to support the suggestion that REM latency may indeed have biological significance in affect. The face validity of FST immobility is a topic of debate, but if one assumes that it is a measure of depression-like behaviors and that immobility time may be correlated depression-like severity, then REM latency may be a marker of a specific dysfunction in the limbic system.

The neuroanatomical basis of shortened REM latency in these animals is unclear. Reduced REM latency is presumably a result of weakening of the REM-off switch or strengthening of the REM-on switch during sleep. As there are many neural structures that influence the vlPAG and SLD (as mentioned in Chapter 1), the neural circuitry of shortened REM latency needs to be investigated in future animal studies. Although my proposed model for REM sleep regulation by the mPFC may also be a factor in REM latency modulation, my model proposes similar circuitries for the dorsal and ventral regions of the mPFC. However, since shortened REM latency is unique to vmPFC-lesioned animals, I predict that a separate circuitry (such as the vmPFC projection to the lateral hypothalamus) is responsible for REM latency changes. Furthermore, as suggested above, the negative correlation between REM latency and FST immobility is indicative of the involvement of the limbic system in this result.

In general, my findings that lesions in the vmPFC cause increased REM sleep, decreased REM latency and increased immobility in the FST present a potential explanation for why sleep disturbances are closely linked to depression. The vmPFC in the rat, and its purported human homolog (BAs 25 and 32), may be a region of the brain where the limbic and sleep-modulating circuitries overlap. Therefore, dysfunction of this region may be related to the manifestation of all three behavioral outcomes. However, my findings do not address 1) what could be causing this dysfunction in depression and 2) whether sleep disturbances are a cause or effect of

depressed mood. Some published preclinical and clinical studies, as mentioned previously, suggest that persistent adverse conditions may lead to cellular changes in the PFC. For instance, chronic stress in rats leads to atrophy in the dendrites of cells in the mPFC (12; 13), and a postmortem study of depressed and healthy humans showed that the former had decreased glial densities and decreased neuron cell body sizes (14). Therefore it is possible that gray matter loss in the PFC of depressed patients (14–17) is a direct consequence of stress, anxiety, and other causes of persistent depressed mood. Therefore MDD, or its precursor, may be a disease of these cortical changes that could subsequently influence one's sleep.

However, there is also evidence that sleep changes are not directly caused by the cortical changes in MDD. Shortened REM latency and reduced sleep efficiency were found to remain in remitted subjects (18), despite it being reported elsewhere that remitted patients do not show gray matter volume differences compared to healthy controls (17). In addition, specific sleep characteristics – increased REM density (which has been reported in depressed patients) and decreased slow-wave sleep in the first NREM episode – were found to be present in healthy, never-depressed subjects with a strong family history of depression (19). Lastly, presence of insomnia for at least a year is a potent predictor of depression, suggesting that sleep disturbances may arise before the neurobiological changes that lead to MDD diagnosis (20).

Clinical implications of rat vmPFC lesion results

My results in Chapter 3 suggest that reduced activity in the rat vmPFC, and possibly human vmPFC, is associated with a number of characteristics of depression. This notion is supported by the findings that in MDD patients that were symptomatic at time of death, there is reduced expression of immediate early gene transcripts *zif268* and *arc* in the ventral anterior cingulate

cortex (21). However, these results are confusing considering that the subgenual cingulate region is found to be overactive in depressed patients (22; 23) and decreases in activity when a patient clinically responds to antidepressant treatment (24; 25). There are a few possible explanations for these apparently conflicting results. As my rodent lesion study, the human postmortem study and the imaging studies did not differentiate cellular subtypes, the different methods may be detecting abnormal activity in different cell types or cortical layers. For example, in MDD there may be subpopulations of the ventral PFC that display significantly increased and decreased activity. Alternatively, these studies may be demonstrating that there is an optimal level of cellular activity in this region that lies on an inverted U-shape curve. As a result, both too much and too little activity may lead to dysfunction and depression-associated behaviors. Thirdly, increased activity in human imaging studies may be compensatory, due to decreased neuronal body sizes and glial numbers in the ventral PFC (14). Lastly, a result of abnormal function in another structure in the depression circuitry may lead to disinhibition or hyperactivation of the ventral PFC. Further study in both humans and animals are required to unveil additional details in characterizing this region as it relates to depression.

Conclusion

In depressed patients, the prefrontal cortex displays abnormalities in metabolic activity, blood flow, and gray matter volume. There is evidence that some of these changes are reversed upon successful antidepressant therapy. I was motivated by these findings to investigate the importance of the prefrontal cortex in antidepressant action and the manifestation of depression. In my animal studies, I found that the ventromedial prefrontal cortex is a common target of three different drugs with antidepressant effects, in contrast to other regions of the cortex and brain. In

addition, lesions of this area give rise to depression-like behaviors in sleep and the forced swim test. Together, these results emphasize the importance of the rat vmPFC in modeling depression and antidepressants. However, the neural circuitry of each of these outcomes still needs to be investigated in detail to gain a better understanding of the vmPFC as a participant within the limbic network. For instance, it will be important to characterize the types of cells in the vmPFC that are activated by each drug to gain a deeper understanding of the local and downstream effects of their increased activation. As traditional antidepressants require chronic use for a few weeks before its effects are developed, the cellular events that occur during the transition from acute to chronic drug administration need to be elucidated. In addition, my hypothesis that PFC alterations in MDD may be related to the characteristic REM sleep changes observed in depressed patients needs to be tested in a clinical setting. These and future studies will be important for developing more effective antidepressant and possibly preventative measures for the disease that is predicted to soon become the greatest cause of health burden around the globe.

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