EFFECTS OF PREFRONTAL CORTEX LESIONS ON SPONTANEOUS SLEEP-WAKE PATTERNS AND COMPENSATORY RESPONSE TO SLEEP LOSS IN RATS

by

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ABSTRACT

Recent evidence suggests a possible role for the prefrontal cortex (PFC) in sleep/wake regulation and sleep-related electroencephalogram (EEG) activity. This study investigated the effects of cell-specific ibotenic acid lesions to the PFC on sleep-wake patterns and the EEG under baseline conditions and during recovery from a 6 h period of sleep deprivation (SD) using gentle handling in rats. Control rats were injected with saline.

PFC lesions had no effects on overall amounts of wake, non rapid-eye movement (NREM) sleep, or rapid-eye movement sleep. However, lesioned animals had fewer wake and NREM sleep episodes and longer mean durations of these episodes particularly during the dark phase. Following SD, no significant lesion effects were observed in sleep rebound or homeostatic increase in NREM EEG delta power (a measure of sleep intensity).

These results suggest a role for the PFC in sleep-wake regulation, in particular behavioural state stability.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ANOVA	analysis of variance
DAB	diaminobenzidine
EEG	electroencephalogram
EMG	electromyogram
GABA	gamma-aminobutyric acid
Hz	hertz
IBO	ibotenic acid
LDT	laterdorsal tegmental nucleus
LH	lateral hypothalamus
MnPO	median preoptic nucleus
NeuN	neuronal nuclear antigen
NMDA	N-methyl-D-aspartate
NREM	non-rapid eye movement
PeF	perifornical region
PPT	pedunculopontine tegmental nucleus
PFC	prefrontal cortex
REM	rapid eye movement
SAL	saline treatment group
SCN	suprachiamastic nucleus
SD	sleep deprivation
TBS	Tris-buffered saline

VLPO	ventrolateral preoptic area
ZT	zeitgeber time

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When I embarked on the journey that is graduate school, I could have never imagined that these years would consist both the best three years of my life but also the toughest. I have learned and grown so much as an individual and I cannot begin to describe how thankful I am for the opportunities that I've had and everything associated with them.

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Chapter 1: Introduction

Every year around exam time, students around the world ignore sleep in order to get those few extra hours of studying. The reality is, the dreaded "all nighter" is no easy feat. While we can more or less abstain from many basic biological urges, the need for sleep will always take over barring the use of any serious intervention. At some point, despite the amounts of caffeine ingested the human body crashes. Wakefulness allows us to actively perceive the world around us and to fulfill other biological necessities. Sleep on the other hand remains more of a mystery for its function. However, our overall understanding of the sleep phenomenon has grown greatly. Despite the advances in our knowledge of sleep, some basic questions still remain, most notably how and why?

1.1. What is Sleep?

The understanding of sleep has progressed greatly in the past half-century as a result of many important findings made possible by advancement of research techniques. One such discovery described sleep as not a single process, but rather consisting of two unique behavioural stages: non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep (Aserinsky and Kleitman, 1953). Much like wake, NREM sleep and REM sleep are behavioural states with distinct patterns of cortical activity. NREM sleep is described as a stage largely lacking mobility and is accompanied electrophysiologically by low frequency (1-4 Hz), high amplitude electroencephalogram (EEG) delta wave recordings from the cortex (McCarley, 2007). REM sleep, on the other hand, is quite similar from an EEG standpoint to wake, consisting primarily of high frequency, low amplitude waves. Muscle atonia and rapid eye movements are characteristic behaviours displayed during REM sleep episodes; REM sleep is also the state in which dreaming, in particular vivid, dreaming is thought to occur (Siegel, 2004). Although the definitive function of sleep has yet to be identified, it is clear that it serves an important biological role as almost all animals have been known to display sleep-like behaviour, and most cannot exist without it (Siegel, 2005).

The function of sleep continues to be a hot topic of discussion in the field. Some of the currently accepted theories stipulate that sleep allows for the conservation of

energy, is involved in learning and memory processes, or helps to facilitate the biosynthesis of necessary macromolecules at the cellular level (Mignot, 2008). Each theory has its strengths and weaknesses; however, it is unlikely that such a complex process as sleep has a sole function. For instance, do we actually benefit from the proposed energy conservation thought to occur during sleep when it is well known that the brain remains quite active during sleep?

Many of the studies supporting the theories of sleep have used sleep deprivation (SD) techniques to observe the effects of SD on specific processes. Numerous studies support the notion of SD leading to cognitive impairments including decreases in learning (Saletin and Walker, 2012). Furthermore, SD has been linked to decreases in certain cellular processes such as gene expression (Lungato et al., 2013). As we continue to explore currently proposed and more possible theories of sleep function, it seems imperative to further our understanding of the regulation of sleep at the cellular level.

1.2. The Neurobiology of Sleep-Wake Regulatory Nuclei

The neurobiology of sleep and wake states has been studied using a multitude of different techniques including immunohistochemistry, microdialysis, electrophysiological recordings, and with the use of lesions (Jones, 2003). Numerous key studies have allowed us to piece together a greater understanding of the complex operation of the neuronal circuitry that is involved in the maintenance and switching between behavioural states. Figure 1 displays many of the known components of the wake-promoting system and the REM sleep-active system; these two states rely primarily on excitatory pathways which create widespread activation throughout the cortex. The wake-promoting areas make up the ascending arousal system, and include nuclei in the basal forebrain (cholinergic and GABAergic), the perifornical region of the hypothalamus (PeF-LH; orexinergic), the tuberomammillary nucleus (histaminergic), the dorsal raphe nucleus (serotonergic), and the locus coeruleus (noradrenergic) (Miller and O'Callaghan, 2006). Dopaminergic neurons located in the ventral periaqueductal gray have also been linked to the promotion of wake (Lu et al., 2006a). Cholinergic output from the basal forebrain and from subsets of the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT) are active during both wake and REM sleep periods. Furthermore, there are

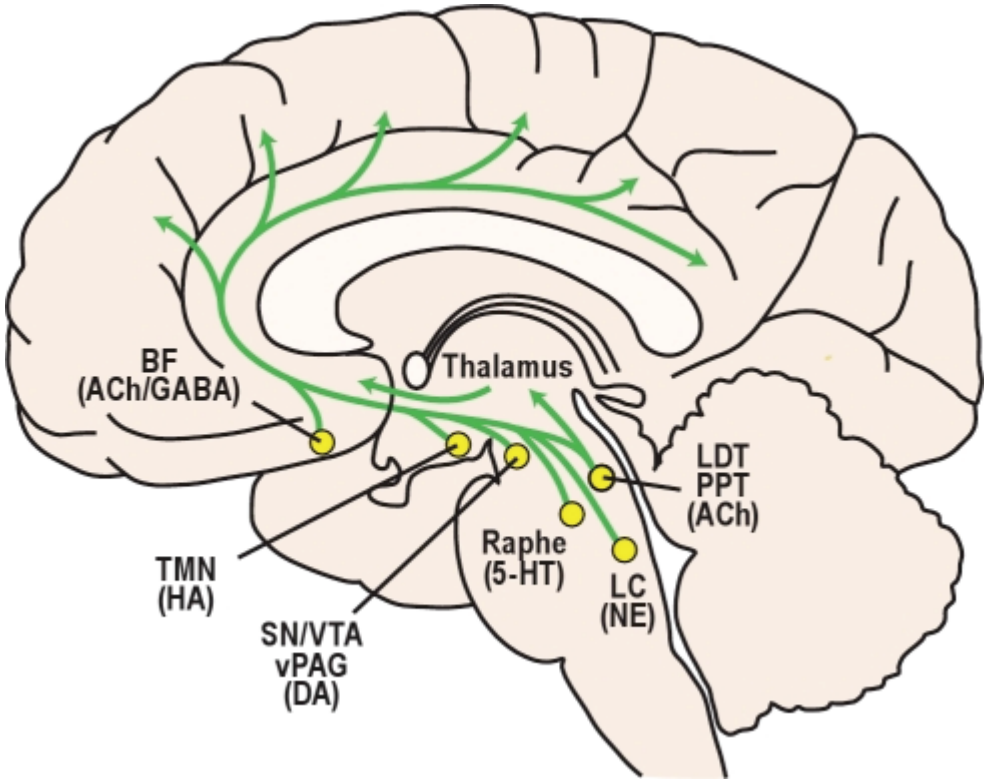


Figure 1: Wake and REM Sleep-Promoting Nuclei in the Human Brain. Specific nuclei (and their associated neurotransmitters) involved in wake, and REM sleep. Wake-promoting nuclei in the basal forebrain (acetylcholine, GABA), tuberomammillary nucleus (histamine), dorsal raphe nucleus (serotonin), ventrolateral periaqueductal gray and substantia nigra (dopamine), locus coeruleus (noradrenaline), and the perifornical region of the lateral hypothalamus (orexin – missing from the diagram) project to and activate the cortex. Cholinergic inputs from the laterodorsal tegmental and pedunculopontine tegmental nuclei to the thalamus are thought to be critical to the generation and maintenance of REM sleep (Adapted from Espana and Scammell, 2011).

populations of cholinergic cells in the LDT and PPT which are active strictly during REM sleep (McCarley, 2007). Other areas with REM sleep-active cells include those GABAergic neurons located in the sublaterodorsal nucleus adjacent to the locus coeruleus (Boissard et al., 2002), and melanin-concentrating hormone neurons in the PeF-LH (Verret et al., 2003).

NREM sleep-promoting nuclei act primarily via inhibitory inputs to many of the aforementioned wake related areas. The major sleep-promoting sites include the ventrolateral (VLPO) and median preoptic nuclei (MnPO) of the forebrain. GABAergic cells originating in VLPO, many of which also produce the inhibitory neuropeptide galanin, fire throughout sleep and target the aforementioned sleep-promoting areas of the brain (see Figure 2). Certain subsets of the VLPO neurons are more active during NREM sleep, and these are located in the core of the VLPO, while others fire more in REM sleep and these are located in the extended VLPO (Lu et al., 2002). Firing of GABAeric neurons in the MnPO increases during both NREM and REM sleep (Szymusiak et al., 2007). Further evidence has suggested the role of melanin-concentrating hormone from the PeF-LH as a factor involved in NREM and REM sleep regulation (Torterolo et al., 2011).

It is important to note that although most of these cell clusters fire in a predictable manner to help regulate and modulate behavioural state transitions and maintenance, the anatomical interconnectivity between most of these nuclei allows them to function reciprocally.

1.3. The Sleep- and Wake-promoting Nuclei Reciprocity and Behavioural State Stability

Many of the pathways involved in sleep- and wake-promotion have been described; it is also known that these systems interact to help maintain a single behavioural state at any given time. This notion of reciprocity for behavioural state control suggests that not only are any given set of neurons firing to produce a behavioural state, but they also work to suppress the underlying mechanisms that promote alternative behavioural states (Suntsova et al., 2007). These reciprocal connections compose part of

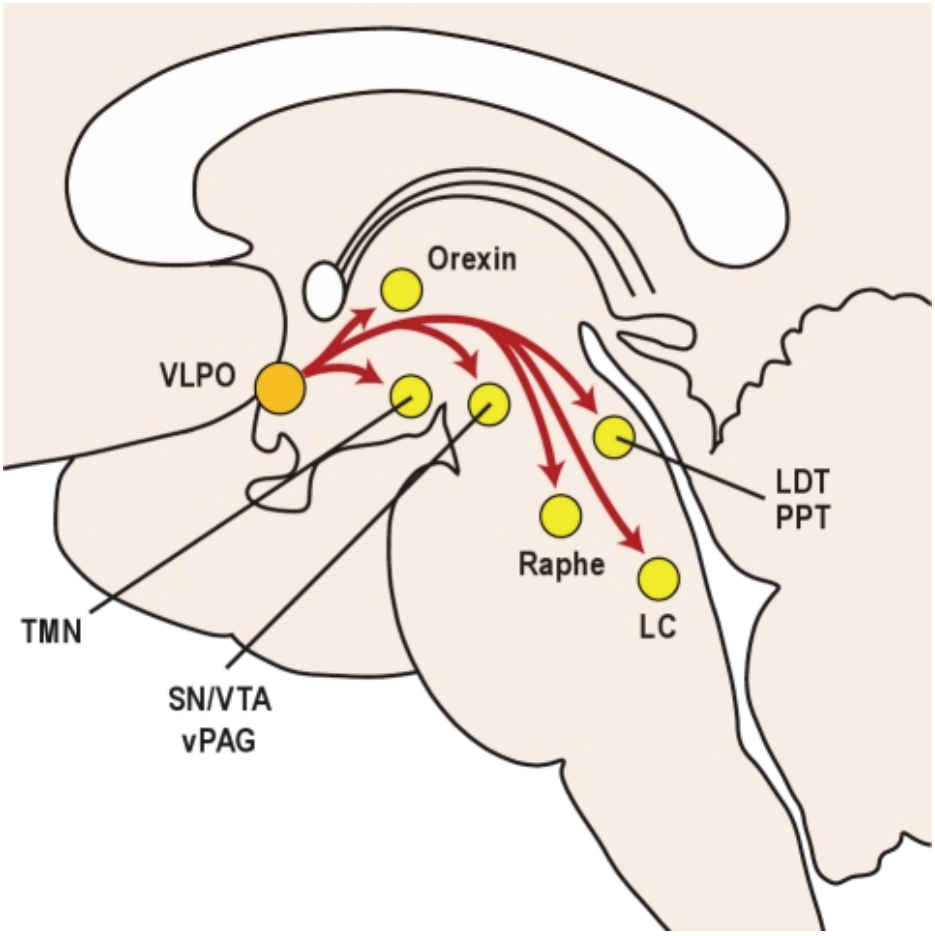


Figure 2: NREM Sleep-Promoting Nuclei in the Human Brain. It is thought that one of the major contributors to NREM sleep is the inhibitory action of ventrolateral preoptic (GABA, galanin) on the aforementioned wake-promoting areas of the brain (Shown in Figure 1) (Adapted from Espana and Scammell, 2011).

the mechanisms by which the "flip-flop switch" model of sleep regulation can operate (Saper et al., 2001).

The main postulate of the flip-flop switch is that the complex interaction of mutual inhibition between wake- and sleep-promoting nuclei will only allow the organism to actively display and be engaged in a single behavioural state at any given time. The VLPO is known to have subsets of projections to wake-promoting areas such as the tuberomammillary nucleus (Sherin et al., 1998), dorsal raphe nucleus and the locus coeruleus (Steininger et al., 2001). Another subset of VLPO projections targets the REM-promoting LDT and PPT (Saper et al., 2001). In the model, it is predicted that one subset of the VLPO neurons would fire and inhibit the other subset from activating, thus ensuring the maintenance of a single behavioural state. Orexin neurons from the PeF-LH have been suggested as prime modulators of this switch to help ensure behavioural state stability (Saper et al., 2005). Unfortunately the current model does not incorporate the role of the MnPO in behavioural state transitioning and maintenance or take into account other factors such a circadian rhythms or homeostatic processes (McCarley, 2007).

1.4. Sleep Modulation by Circadian and Homeostatic Processes

In 1982, Borbely proposed the two process model of sleep regulation. This theory postulates that sleep is modulated by an interaction between endogenously regulated circadian rhythms (process C) and homeostatic processes (process S). This theory stipulates that under normal conditions, circadian rhythms push a nocturnal rodent to spend more time awake during the dark phase and more time asleep in the light phase (process C). Throughout prolonged wake periods predominantly in the light phase, rats experience an increase in sleep pressure (process S). The buildup in sleep pressure would gradually oppose the wake-promoting influence of process C and, when sleep pressure reaches a threshold, it triggers sleep, which causes sleep pressure to dissipate as a function of time spent in sleep (as would the associated increase in EEG delta power) during the light phase (Borbely, 1982). Homeostatic pressure would be particularly increased following periods of SD. Levels of sleep propensity can be assessed by the amounts of NREM sleep and the associated EEG delta power following SD.

In mammals, process C is regulated by the principal molecular clock organized by circadian pacemaker cells of the suprachiamsmatic nucleus (SCN). The SCN is located in the ventral aspect of the preoptic area of the hypothalamus, just superior to the optic chiasm. These cells receive inputs from the retina which help maintain a fixed rhythm among themselves (entrain the endogenous rhythm) via external light cues. Ablation of the SCN resulted in the deregulation of behavioural rhythms and locomotor activity patterns in rats (reviewed in Moore, 2007). Anatomical studies revealed reciprocal connections between the SCN and most sleep-wake promoting nuclei, although the projections from the SCN to sleep-wake promoting nuclei are mostly indirect, with relays within the hypothalamus (Moga and Moore, 1997; Deurveilher and Semba, 2005). The interconnectivity between these systems suggests a role for circadian systems in the modulation of sleep-wake behaviour, as well as modulation of circadian time-keeping mechanisms in the SCN by the sleep-wake systems. Interestingly, the circadian influence of the SCN does not seem to affect overall amounts of sleep or wake in humans and rat and the changes observed after SCN lesions in rats seem to be manifested mostly in the timing of these behaviours. However, varied results were reported for squirrel monkeys (*Saimiri sciureus*)with SCN lesions, animals developed hypersomnia and it was postulated that the SCN may alternatively actively promote wakefulness while passively gating sleep (reviewed in Mistlberger, 2005).

1.4.1. SD Activates the Homeostatic Regulation of Sleep

The study of SD effects has become mainstream in helping to understand normal sleep-wake regulation. Homeostatic regulation of sleep dictates that SD should result in increased sleep amounts and sleep intensity (described as “rebound”) during the following period of sleep opportunity. SD can be achieved by using a number of techniques which can vary according to the required duration of deprivation. Rodents typically require some degree of stimulation to achieve proper degrees of SD in order to cause a buildup in sleep debt. Methodology of SD should be considered when interpreting results of SD studies.

Behavioural effects of SD may vary depending on the paradigm used; however, some consistencies exist. A six hour period of SD has been shown to result reliably in a

NREM sleep increase during the post-SD recovery period. Additionally, EEG delta power during NREM sleep has been strongly related to the total amount of SD time (Tobler and Borbely, 1990). These rebound responses were proportionally related to the pre-SD amounts of sleep (Vyazovskiy et al., 2007). Rodents are typically released from SD at the onset of the dark phase which is considered their active period; rebound effects suggest that the homeostatic response to SD may temporarily take precedence over the circadian drive for wake (Deboer et al., 2007).

Results pertaining to REM sleep rebound are not as consistent. Some studies with REM sleep specific deprivation displayed immediate rebounds following as little as two hours of deprivation (Shea et al., 2008). Six hour total SD led to statistically significant REM sleep rebound in some studies (Deboer et al., 2007); however, this was not always the case (Kaur et al., 2008). In contrast to brief total SD, 4 days of total SD led to a prolonged REM sleep rebound, which was more predominant than NREM sleep rebound, with peaks coming in the three to four days post deprivation (Rechtschaffen et al., 1999). It is unclear what the inconsistent results of short durations of SD pertaining to REM sleep mean, but NREM sleep rebound results suggest that in addition to circadian rhythms, sleep homeostatic processes are important to sleep-wake regulation.

1.5. Defining of the Prefrontal Cortex

It is a well known fact that sleep-wake architecture changes throughout the lifespan of humans. Along with the changes in sleep patterns that include decreases in the deeper stages of NREM sleep and more sleep fragmentation, a decline in cognitive functioning is also common in aging (Wilckens et al., 2012). The prefrontal cortex (PFC) is particularly sensitive to the effects of aging and has also been linked to NREM delta activity (Mander et al., 2013). These parallels to sleep-wake regulation as well as further lines of evidence that will be reviewed below suggest a possible role for the PFC in behavioural state regulation.

Anatomically, the PFC makes up a large portion of the frontal lobe in humans. It was originally described based on cytoarchitecture by Brodmann (1909) as the “region frontalis”, which covered Brodmann areas 8-13 and 44-47. It is directly rostral to the

motor cortices, and some portions can be distinguished by the presence of the granular layer 4 in their cytoarchitecture. Regions of the human PFC can be broken down into granular and agranular sections. Granular areas of the PFC include (with their associated Brodmann area or areas): caudal PFC (caudal lateral – 8); dorsomedial PFC (superior frontal gyrus and medial PFC – 9); lateral area (superior frontal gyrus – 9); mid-lateral PFC (middle frontal gyrus – 46); postero-lateral PFC (caudal middle frontal gyrus – 9/46); ventral PFC (inferior frontal gyrus – 45, 47); granular orbital PFC (orbital surface – 11, rostral 13/14); and, polar PFC (frontal pole – 10). Agranular regions include: medial agranular PFC (anterior cingulate – 24, infralimbic – 25, prelimbic – 32), and the lateral agranular PFC (caudal areas – 13/14, and agranular insular cortex) (Passingham and Wise, 2012).

There has been some debate regarding the existence of a rodent PFC (Uylings et al., 2003). The rodent mediodorsal thalamic nucleus has been shown to project to areas of the PFC in a similar fashion as observed in humans (Uylings and van Eden, 1990). This has led Uylings and colleagues (2003) to conclude that rodents do indeed have a PFC. It is thought that many of its areas are similar to those observed in humans; however, their anatomical delineation has not been made as clear. For the purpose of the present experiment, the Paxinos and Watson (1998) rat brain atlas was used to determine anatomical borders of PFC substructures (see Figure 6).

1.6. Connectivity of the PFC and Associated Functions

The overarching hypothesis for the function of the PFC involves the notion of executive functioning. The PFC receives numerous inputs from various areas of the brain which allow it to aid in the planning and directing of motor, cognitive, affective, and social behaviours over time (Kolb et al., 2012). It has a prolonged developmental period which is suggested to play a role in the mechanisms of plasticity resulting from experiences (Petanjek et al., 2011). This developmental process makes the PFC somewhat susceptible to potential stressors that may alter the normal development. Specific areas of the PFC are thought to specialize in their roles that are often related to the network of their connections.

Although there exist some minor discrepancies in the characterization of the medial PFC among species, it is accepted that the anterior cingulate cortex as well as the infralimbic and prelimbic cortices present in species ranging from rodents to humans (Passingham and Wise, 2012). The medial PFC projects both directly and indirectly to premotor areas, the anterior cingulate cortex being the significant contributor to these projections (Vogt and Pandya, 1987). Dense connections have also been shown to exist between the amygdala and the medial PFC (Morecraft et al., 2007). Furthermore, the hippocampus has been shown to have dense and reciprocal connections to the infralimbic and prelimbic areas of the medial PFC (Insausti and Munoz, 2001). The amygdala and hippocampus are involved in emotion and memory processing, respectively. Thus, the medial PFC can be described as the area of the brain responsible for choosing actions based on outcomes, which is supported by its connections to memory and emotion related brain structures (Passingham and Wise, 2012).

Other known connections of the PFC include reciprocal pathways with the claustrum (Tanne-Gariepy et al., 2002) and the basal ganglia (Nakano, 2000), which are thought to be involved with information integration processes. Connections to and from the thalamus are implicated with attention (Fries, 1984). The PFC receives dopaminergic inputs originating from areas of the midbrain (Schultz, 1998); this may support the system being involved in learning processes in relation to reward (Passingham and Wise, 2012). There is also evidence for a PFC-cerebellum loop, involving the basilar pontine nuclei and the thalamus; this network of connections likely aids in the integration of planning and controlling actions (Houk and Wise, 1995).

In the past few paragraphs we have explored the anatomical organization and some of the main functions of the PFC. Most of the anatomical connections have been identified using tracing techniques; however, new methods of studying neuronal interaction are consistently emerging, revealing new pathways. It has been mentioned that the PFC has a prolonged developmental stage and that it may be susceptible to negative influences during development. In the following section we will seek to address changes that may arise with the PFC, many of which may lead to irregular functional states.

1.7. The PFC and Psychopathology

In humans, the PFC is thought to undergo a number of developmental changes which typically continue until early adulthood (Spencer-Smith and Anderson, 2009). Throughout development, many neurobiological processes remain active in this area of the brain; however, environmental factors also influence the proper maturation of the PFC. Abnormalities affecting the PFC can often manifest themselves at the cognitive level and may often be accompanied by behavioural or emotional disturbances.

Patients who have been diagnosed with schizophrenia commonly display major impairments in PFC functioning. Schizophrenia is a psychological disorder characterized by a breakdown of thought and emotional processes that use representational knowledge (Arnsten, 2011). Symptoms typically include auditory hallucinations, psychosis, disorganization of speech or thinking, and these symptoms often make it difficult for the individual to integrate into society (Nuechterlein et al., 1992). Lower PFC activity levels measured by functional magnetic resonance imaging during a working memory task were highly correlated with symptoms of schizophrenia (Perlstein et al., 2001). A study using structural resonance imaging concluded that patients with schizophrenia tended to have a decreased gray matter volume density in the PFC (Eack et al., 2008). These studies suggest a marked disruption in the PFC neural circuitry as a strong factor related to schizophrenia.

The PFC has also been linked to depression as well as other mood related disorders. Clinical depression is characterized by episodes of general low-mood often related to a decrease in emotional self valuation which is accompanied by a degree of anhedonia. Depression is also related to higher levels of disability and mortality (Palazidou, 2012). Imaging studies have shown decreases in total volumes of the anterior cingulate and orbitofrontal PFC regions in depressed patients (Koolschijn et al., 2009). A positron emission tomography study showed decreased glucose metabolism in the anterior cingulate cortex in depressed patients (Drevets et al., 2002). There is also evidence suggesting the implication of the PFC in bipolar disorders (Townsend and Altshuler, 2012).

More recently, the PFC has begun to draw attention from researchers focusing on addiction behaviour. It is thought that the PFC may be implicated in such behaviours via regulation of the limbic reward regions as well as through involvement in other higher order executive functioning (Goldstein and Volkow, 2011). Although not always considered a psychopathological state, addiction can be associated with neurobiological changes and is now regularly approached as a mental disorder (Romer Thomsen et al., 2013).

Factors regulating PFC development are both genetic and environmental. The PFC may be subjected to various developmental changes which may alter its connectivity and proper functioning. As previously described, in many cases alteration of the PFC can lead to psychopathological conditions as well as cognitive declines. Interestingly, many of the disorders associated with the PFC tend to have a sleep component, that is to say there are changes in overall sleep architecture. This raises the question of what role, if any, the PFC may play in sleep-wake regulation.

1.8. The PFC and Sleep

The role of the PFC in sleep-wake regulation has not garnered much attention until recently. Connections between the PFC and sleep-wake promoting areas include the locus coeruleus (Robertson et al., 2013), dorsal raphe nucleus (Andrade, 2011), lateral hypothalamus (Lazarus et al., 2012) and ventral periaqueductal gray area (Lu et al., 2006b). This interconnectivity of sleep and wake-promoting nuclei with the PFC and the link with psychopathologies, combined with other lines of evidence discussed below, supports the notion of a potential role for the PFC in sleep-wake modulation.

1.8.1. The PFC and Caffeine

Adenosine buildup is known to play a role in sleep promotion. Caffeine has been shown to inhibit the A_1 and A_{2A} receptors in the brainstem and forebrain of the rat (Marks et al., 2003). Administration of an A_{2A} receptor antagonist (CGS) into the frontal associative portion of the PFC led to an increase in acetylcholine levels both locally and in the pontine reticular formation in mice (Van Dort et al., 2009). This suggests that blockade of adenosine receptors in the PFC may allow subsequent wake promotion via

cholinergic neurons located in the brainstem and basal forebrain. Van Dort and colleagues (2009) also reported that mice were quicker to recover from periods of anesthesia, and also displayed decreased EEG NREM delta power in response to CGS. Quicker recovery to anesthesia may further support a role for the PFC in wake promotion which could be regulated in response to adenosine buildup throughout the day. In fact, in the final experiment in that study, injections of an adenosine A_1 receptor antagonist into the PFC increased time spent in wake in unanesthetized animals. Furthermore, the decreased EEG NREM delta power may indicate that the animals experienced a decrease or down-regulation in homeostatic sleep pressure. These results suggest that the PFC may be involved in wake regulation through its local adenosine signaling and descending projections to the basal forebrain and brainstem reticular formation.

1.8.2. PFC Projections to the Basal Forebrain

It is well established that the basal forebrain plays an important role for cortical and behavioural activation (Kaur et al., 2008; Fuller et al., 2011). Sensory stimulation has been shown to increase acetylcholine release from sensory cortices in a modality- and region-specific manner, which reflects increased firing of cholinergic basal forebrain neurons selectively projecting to these cortical areas; however, inactivation of the PFC via $GABA_A$ receptor agonist (muscimol) abolished these effects (Rasmusson et al., 2007). Stimulation of the primary visual or somatosensory cortices triggered an evoked response in the PFC. Interestingly, stimulation to the PFC also led to an evoked response in the basal forebrain and vice-versa (Golmayo et al., 2003). These findings lend well to a proposed neural loop from the sensory cortices to the PFC, then to the basal forebrain. This proposed mechanism would lead to sensory activation of the basal forebrain and downstream throughout the cortex further promoting wake (Zaborszky et al., 1999).

The basal forebrain is also known to be involved in sleep homeostasis through nitric oxide-adenosine signaling. An increase of nitric oxide production in the basal forebrain was shown to occur in rats undergoing SD (Kalinchuk et al., 2006). These authors demonstrated that inhibiting the production of nitric oxide in the basal forebrain prevented NREM recovery sleep, while increasing nitric oxide production produced a NREM rebound-like effect similar to sleep rebound after prolonged wakefulness. It is

thought that these mechanisms underlying sleep homeostasis are influenced by indicators of energy metabolism (reviewed in Porkka-Heiskanen and Kalinchuk, 2011).

1.8.3. PFC Sensitivity to SD

More recently, studies have started to show that the PFC is particularly susceptible to SD. Decreased performance on a working memory task was reported following a 24 hour period of total SD in human subjects (Chee and Choo, 2004). Functional magnetic resonance imaging displayed increased activation of the PFC during this task. These authors suggested that the increased activation was a compensatory response to counteract the SD effect. However, overall PFC activation following prolonged SD seems to be decreased (Chee et al., 2006). Thus, the PFC seems to be negatively affected by SD, but is capable of increasing activation in response to higher cognitive demands. It is unclear to what extent this proposed compensatory activation is able to occur.

1.8.4. Slow EEG Oscillations Generated in the PFC

Massimini and colleagues (2004) reported that slow EEG oscillations predominantly originate from the PFC prior to travelling to more caudal regions of the cortex. These slow oscillations (< 1 Hz) are characteristic events of the deepest stage of NREM sleep, and represent the underlying neural activity, namely, the membrane potential fluctuations of cortical neurons (Steriade et al., 1993). Similar slow oscillations were identified in mice using a voltage sensitive dye (Mohajerani et al., 2010). The authors reported a high degree of bilateral synchronization of these oscillations. It is hypothesized that these slow waves may be implicated in neural mechanisms of synaptic plasticity (Steriade et al., 2001). Synaptic plasticity processes fall into a theory of sleep function, further supporting the potential link between PFC and sleep regulation.

1.9. The Current Study: Effects of PFC Lesions on Spontaneous Sleep-Wake Patterns and Compensatory Responses to Sleep Loss in Rats

In light of the aforementioned anatomical links between sleep-wake promoting areas with the PFC and several other lines of evidence indicating a role of the PFC in

sleep-related processes, the purpose of this thesis was to determine whether the PFC has any role in sleep-wake regulation by using excitotoxic lesions of the PFC in rats. The first goal was to determine whether the PFC regulates sleep-wake behaviour under baseline conditions, and the second goal was to determine if the PFC is involved in homeostatic sleep recovery following SD (six hours by gentle handling). Our specific hypotheses were as follow:

1. PFC lesions will reduce time spent in wake. It will also, somewhat paradoxically, reduce NREM sleep EEG delta activity.
2. PFC lesions will reduce recovery NREM sleep and recovery EEG delta activity (an index of sleep intensity) after 6 hours of sleep deprivation.

Chapter 2: Materials and Methods

2.1. Animals

Adult, male Wistar rats (weight 200-250 g upon arrival) were obtained from Charles River Canada (St. Constant, QC). The rats were maintained under a constant 12:12 light:dark cycle, with lights on at 7 am. Food and water were available ad libitum. The rats were housed in pairs and given a full week to acclimatize in the Carleton Animal Care Facility prior to surgery. All animal handling procedures were conducted in keeping with the guidelines of the Canadian Council on Animal Care and the protocol was approved by the University Committee on Laboratory Animals of Dalhousie University.

2.2. Surgery

A pilot study was conducted in order to determine the optimal concentration, volume, and placement of ibotenic acid injections into the PFC (n = 2 rats; 8 cortical injections total).

All of the rats in the main study underwent the same surgical procedure with the sole difference being the contents of the injections: ibotenic acid (n = 14) or saline (n = 5). Rats were anesthetized by intraperitoneal injection of a mixture of ketamine (60 mg/kg), acepromazine (0.6mg/kg), and xylazine (3.2 mg/kg) in a volume of 0.15 ml/100g. It took animals approximately 15 min to reach an appropriate level of surgical anesthesia. Rats were placed in a stereotaxic instrument and received two pairs of bilateral injections of ibotenic acid or saline into the prefrontal cortex at: (1) 4.2 mm anterior to bregma, ±2.2 mm lateral to the midline and 1.6 mm below the dura; and, (2) 2.7 mm anterior to bregma, ±1.0 mm lateral to the midline and 1.8 mm below the dura. A 5 µl Hamilton glass pipette was used to inject 0.7 µl of 100 mM ibotenic acid (Sigma-Aldrich Canada Ltd., Oakville, Ontario, CA) dissolved in physiological saline, or physiological saline alone, at a rate of 0.05 µl/10 s at each of the four sites. The glass pipette remained in place for 5 min following each injection.

Following the injections, three electroencephalography (EEG) and two electromyography (EMG) electrodes were implanted during the surgery. EEG electrodes,

made of small stainless steel screws, were implanted subdurally at the following locations: (1) parietal electrode, 2 mm posterior to bregma and 2 mm right to the midline; (2) occipital electrode, 6 mm posterior to bregma and 2 mm left to the midline; (3) ground electrode, 1 mm anterior to lambda and 2 mm right to the midline. EMG electrodes, made of stainless steel wires, were implanted bilaterally into the rat's acromiotrapezius muscle. All of the electrodes were connected to a miniature connector, which was fastened to the surface of the skull using dental cement. After surgery, rats were given subcutaneous injections of lactated Ringer's solution (5.0 ml), Baytril (2.5 mg/kg) and ketoprofen (5 mg/kg). Then, animals were housed individually, provided with mash and monitored closely to assure recovery.

2.3. Experimental Design and Data Acquisition

Upon recovery from surgery (7-10 days), the rats were transported into their respective recording chambers to habituate prior to recordings. Each chamber consisted of a plexiglass cage (37.5 x 37.5 x 29.5 cm^3) placed within a sound-proofed wooden cabinet (57.5 x 45.0 x 47.5 cm^3) equipped with a light, fan and front window. Rats were given three days to acclimatize to their new environment. Following this initial habituation period, each rat's skullcap was connected to a freely rotating recording cable, and the animals were given an additional three days to adapt prior to the beginning of any experimental recordings.

Sleep/wake baseline recordings began at 1 pm (ZT 6; Zeitgeber time, ZT 0 corresponding to the time of lights on) at the mid-point of the light phase and continued for 24 h. Baseline recordings were followed by 6 h of SD over the second half of the light phase (ZT 6-12).

Throughout the SD period, rats were carefully monitored visually as well as polygraphically and disturbed by "gentle handling" when they showed behavioural and/or electroencephalographic signs of sleep (relaxed posture with eyes closed, slowed frequency and increased amplitude EEG activity); rats were otherwise left undisturbed. Gentle handling was achieved by tapping on the cage, rustling the bedding within the

cage, or by introducing novel objects to the cage. Rats were not physically touched during this period.

Recordings concluded with a 24 h interval of recovery directly following the SD period (ZT 12). Animals were left undisturbed during this period. Figure 3 depicts a representation of the experimental timeline.

EEG and EMG signals were amplified and band pass-filtered (EEG: 0.3–100 Hz; EMG: 10–100 Hz; Grass Telefactor, West Warwick, RI, USA). Signals were digitized at 256 Hz and stored on a computer for off-line analysis (SleepSign, Kissei Comtec America, Irvine, CA, USA).

2.3.1. Sleep/Wake Scoring and Analysis

Recordings were automatically scored in 10 s epochs using SleepSign software. Each epoch was scored as one of the following three behavioural states: wake (low-voltage, fast EEG activity; moderate to high EMG activity), NREMS (high-voltage, slow EEG activity, mainly delta waves [0.5–4 Hz]; low EMG activity), or REMS (low-voltage EEG activity, mainly theta waves [4.5–8 Hz]; very low EMG activity with phasic muscle twitches). The automatic scoring was verified visually by individuals blind to the condition of animals to assure a minimal degree of error. Any discrepancies were manually corrected by the scorer.

EEG spectral analysis was also conducted for the sleep recordings. Total power, as well as power values in five frequency bands ranging from 0.5-50 Hz were analyzed for all behavioural states. Frequency bands analyzed were: delta [0.5-4 Hz], theta [4.5-8 Hz], sigma [8.5-13 Hz], beta [13.5-30 Hz] and gamma [30.5-50 Hz] (Deurveilher et al., 2012). The EEG power spectrum was expressed as a percentage of power over the 24 h baseline recording.

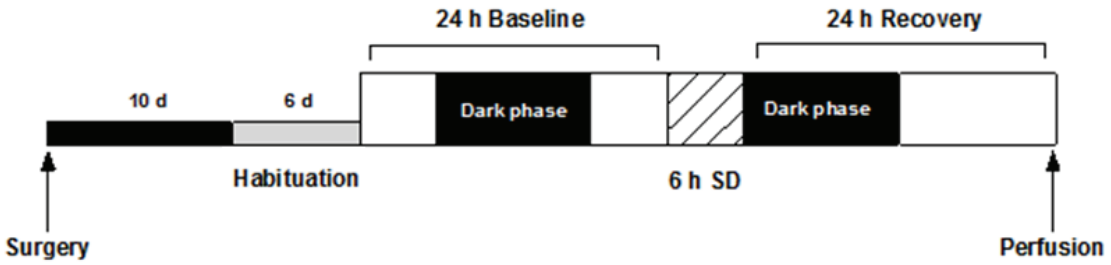


Figure 3: Experimental Timeline. Animals received either ibotenic acid or saline injections into the PFC and EEG/EMG implants. A recovery period of 10 days was given to the animals and they were monitored to assure proper recovery. Rats were habituated to the recording conditions for 6 days prior to the experimental recordings. The 24 hour baseline recording began in the mid-point of the light phase. Rats were sleep deprived for 6 hours by gentle handling, followed by a 24 hour recovery period. At the end of the recording, rats were perfused transcardially, and brains were collected and processed for histological analysis. A 12:12 light:dark cycle was maintained throughout the experiment.

2.4. Histology

2.4.1. Immunohistochemistry

Following the end of all sleep recordings, animals were given an overdose of an anaesthetic mixture (ketamine, 120 mg/kg; xylazine, 6.4 mg/kg; acepromazine, 1.2 mg/kg, i.p.) and perfused transcardially with a saline rinse followed by 4 % paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2 – 7.4). The brains were post-fixed in the same fixative solution, cryoprotected overnight in 30% sucrose in phosphate buffer, and later cut into five sets of 40 μm sections on a freezing microtome. Sections were cut approximately from 5.2-1.2 mm anterior to bregma (Paxinos and Watson, 1998).

One set of sections was processed immunohistochemically to label neuronal nuclear antigen (NeuN). NeuN is a non-specific mature neuron marker. Another set was Nissl stained using cresyl violet. A combination of these sections were used to delineate the ibotenic acid lesions.

For NeuN immunohistochemistry, brain sections were incubated overnight at room temperature in a mouse anti-NeuN monoclonal antibody (1:1000; clone A60, catalogue #MAB377; Millipore, Billeroca, Massachusetts, USA) diluted in 0.05 M Tris-buffered saline (TBS) containing 0.01 % sodium azide, 2 % normal donkey serum and 0.3 % Triton X-100. Following a rinse in TBS, sections were put into 3 % normal rat serum for 30 min. Sections were rinsed twice in TBS then incubated for 90 min in a secondary donkey anti-mouse IgG antibody (1:1000; Jackson Immunoresearch Laboratories) diluted in 0.05 M TBS containing 2 % normal donkey serum and 0.3 % Triton X-100. Tissue was rinsed three times in TBS. Sections were then placed in an avidin-biotin complex solution (1:200; ABC Elite Kit from Vector Laboratories, Burlingame, CA) for 60 min. Tissue was once again rinsed in TBS. Sections were incubated in a nickel diaminobenzidine (DAB) solution (0.02 % DAB, 0.6 % nickel ammonium sulphate in 0.05 M Tris buffer) for 10 mins. Hydrogen peroxide (0.006 % final concentration) was added to each well to allow visualisation of the labels. Sections were returned to TBS when an appropriate level of visualisation was achieved to prevent

further darkening. Sections were then mounted on glass slides. Slides were dried and coverslipped (Cytoseal 60; Richard-Allan Scientific, Kalamazoo, MI).

For the Nissl stain, sections were mounted and dried on glass slides. Sections on slides were stained in 0.1 % cresyl violet solution for 15 s, then quickly rinsed with distilled water. The slides were dipped into a differentiation solution for 20 s, then rinsed with distilled water. Sections were dehydrated using a series of ethanol and xylazine washes before being coverslipped.

2.4.2. Microscopy

Single section images labelled for NeuN, and Nissl bodies were collected on a Zeiss Axiovert 2 motorized microscope using a 5x objective. The microscope was coupled to an Axiocam Hrc camera (Zeiss). All images were collected under the same lighting conditions and were used in conjunction with Neurolucida (MBF Scientific) software to illustrate the lesions

2.4.3. Neurolucida Drawing

Sections ranging from approximately 4.7-2.7 mm anterior to bregma according to a brain atlas (Paxinos and Watson, 1998) that included lesions were individually traced visually using Neurolucida software. The total area of each lesion on individual sections was compiled and used for further analysis. Volume estimates of lesions were obtained using the Cavalieri formula (Coggeshall, 1992).

2.5. Statistics

Independent samples t-tests were used to compare means for behavioural data between the IBO and control conditions. Repeated measures ANOVAs were used to compare time course data for the two groups, followed by post-hoc tests where appropriate. Statistical significance was considered to be achieved if $p < 0.05$.

Chapter 3: Results

3.1. Histological Analyses

3.1.1. Histological Appearance of IBO Lesions

Immunostaining of NeuN, which selectively labels neurons, showed an almost complete neuronal loss around the injection sites in the PFC in 14 animals injected with IBO (Figure 4A). Small holes were also present at the sites of injection in a number of the IBO-treated animals (Figure 4A). Nissl-staining showed extensive gliosis at the lesion sites of the IBO-injected animals (Figure 5A),

Of 7 saline-injected animals, intact neurons were seen in the PFC (Figure 4B), with little or no gliosis (Figure 5B), in 4 animals. In the other 3 saline-injected animals, various degrees of lesion-like cell loss and/or tissue loss, presumably due to mechanical disturbance associated with the injection, were observed, and these 3 animals were excluded from the analyses, resulting in a total of 4 saline-injected animals.

3.1.2. Extents of Lesions

The extent of lesions in each of the 14 IBO-injected animals was drawn using the NeuN-stained sections, and the volume of lesions was estimated in each animal (see the Methods for procedures). The estimated volumes of lesions were ranked and grouped into: small (smallest 4), medium (middle 5), and large (largest 5; Figure 6).

The lesions were mainly restricted to levels from 4.7 mm anterior to 2.7 mm anterior to bregma. Cortical areas affected by the lesions varied somewhat among the IBO-injected animals, and included: the frontal associative cortex (FrA), primary (M1) and, secondary (M2) motor cortices, medial (MO), ventral (VO) and, lateral orbital (LO), prelimbic (PrL), infralimbic (IL) and anterior cingulate (Cg1) cortices (Figure 6A). Cortical areas in which 100 % of animals displayed significant cell loss were centered at the anterior cingulate cortex (Cg1), as shown in Figure 6E.

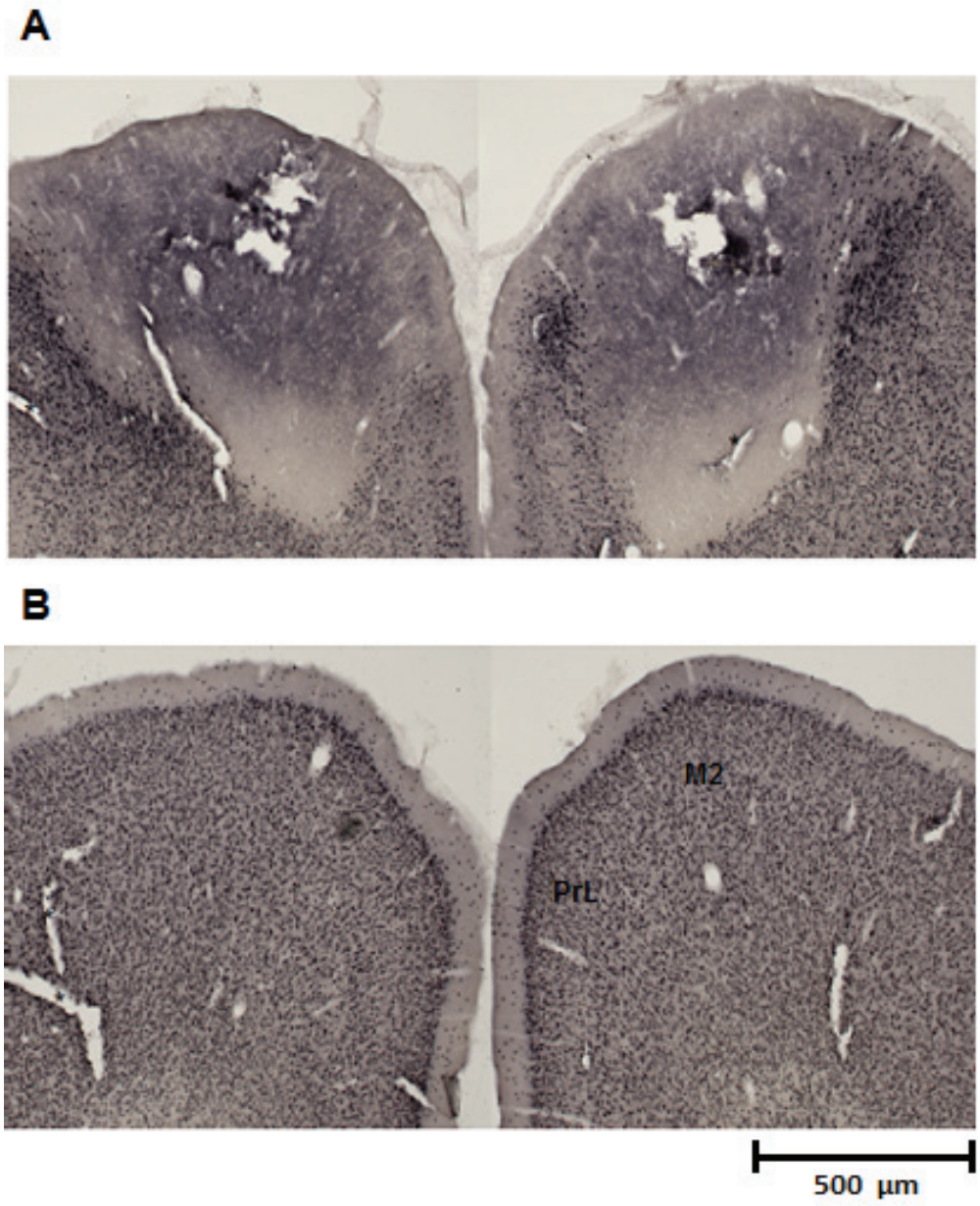


Figure 4: Examples of NeuN Staining. (A) NeuN staining of an IBO treated animal showing a loss of neurons to the M2 and PrL regions. (B) NeuN staining of a saline treated animal demonstrating no visible damage to the M2 and PrL regions.Sections display AP level +4.2.

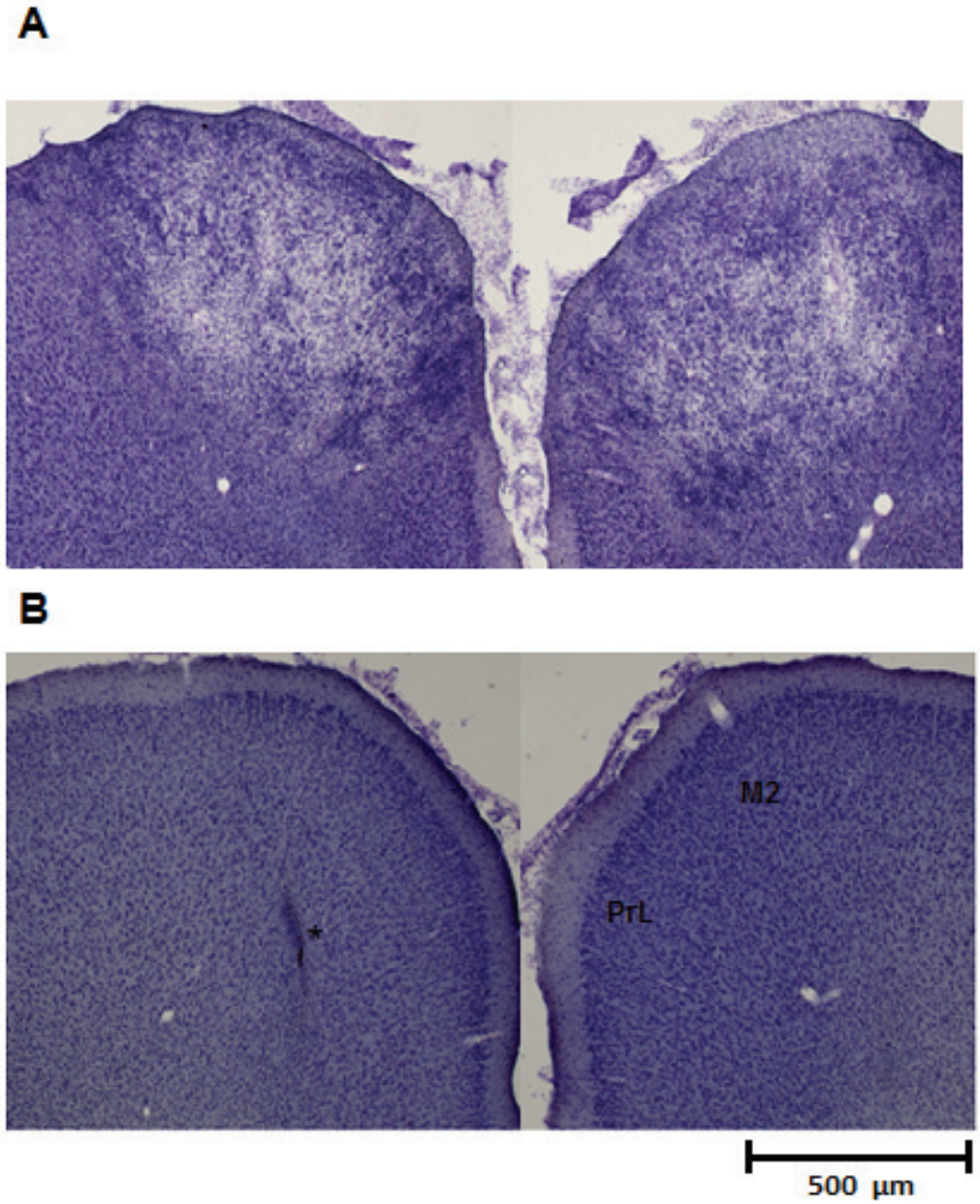


Figure 5: Examples of Nissl Staining. (A) Nissl staining of an IBO treated animal showing a loss of neurons and gliosis in the M2 and PrL regions. (B) Nissl staining of a saline treated animal demonstrating no visible damage. The injection needle track is indicated by an asterisk in the saline treated animal. Sections display AP level +4.2.

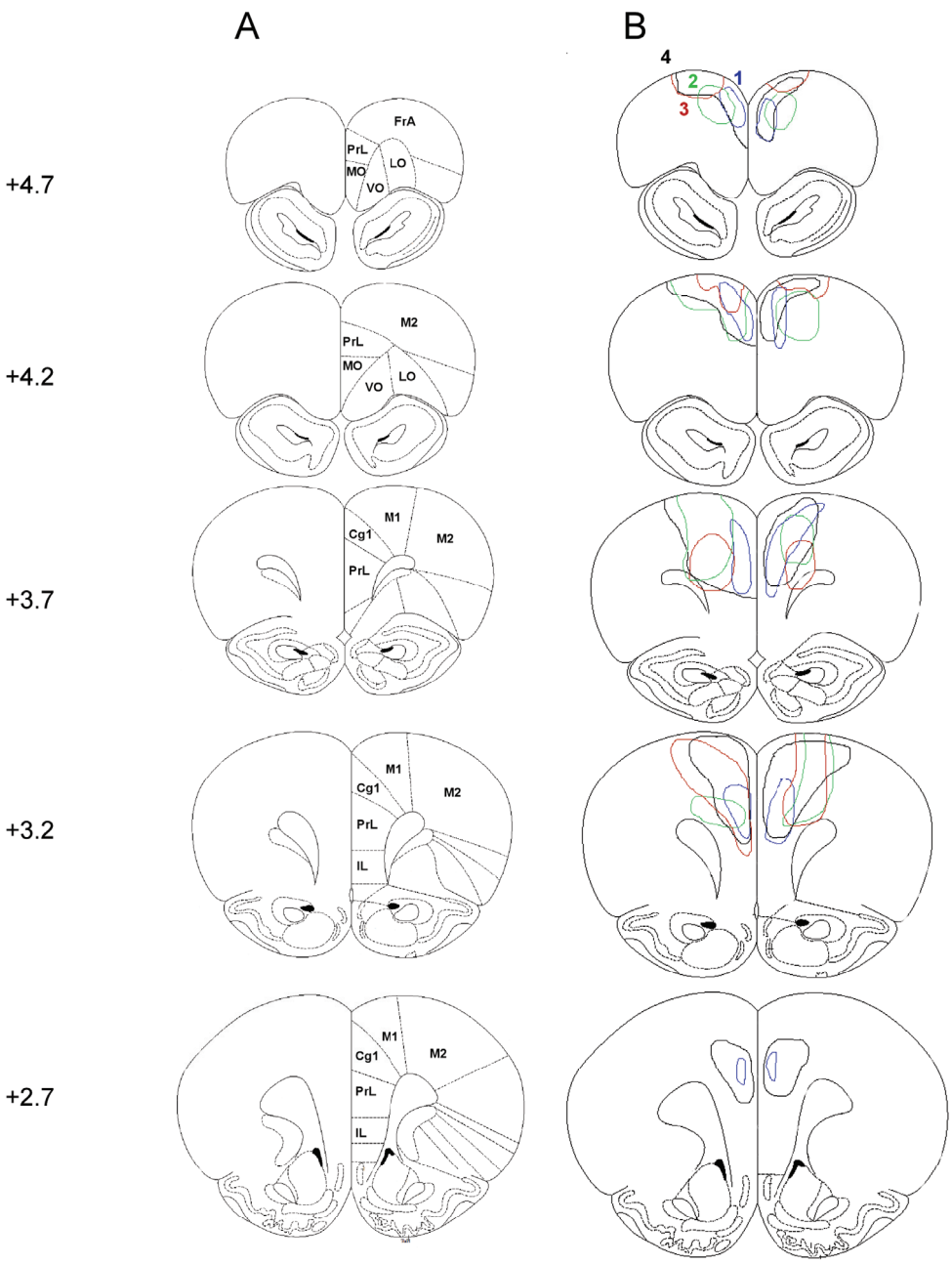


Figure 6: Extents of Ibotenic Acid Lesions. (A) Drawings of representative sections from a rat brain atlas by Paxinos and Watson (Paxinos and Watson, 1998) highlighting the areas of the PFC affected by the lesions. (B) The extents of lesions in IBO animals 1-4 represented by different colors. Lesions of 14 animals were ranked and grouped by total volume, and this set of drawings displays the smallest 4 of the lesions.

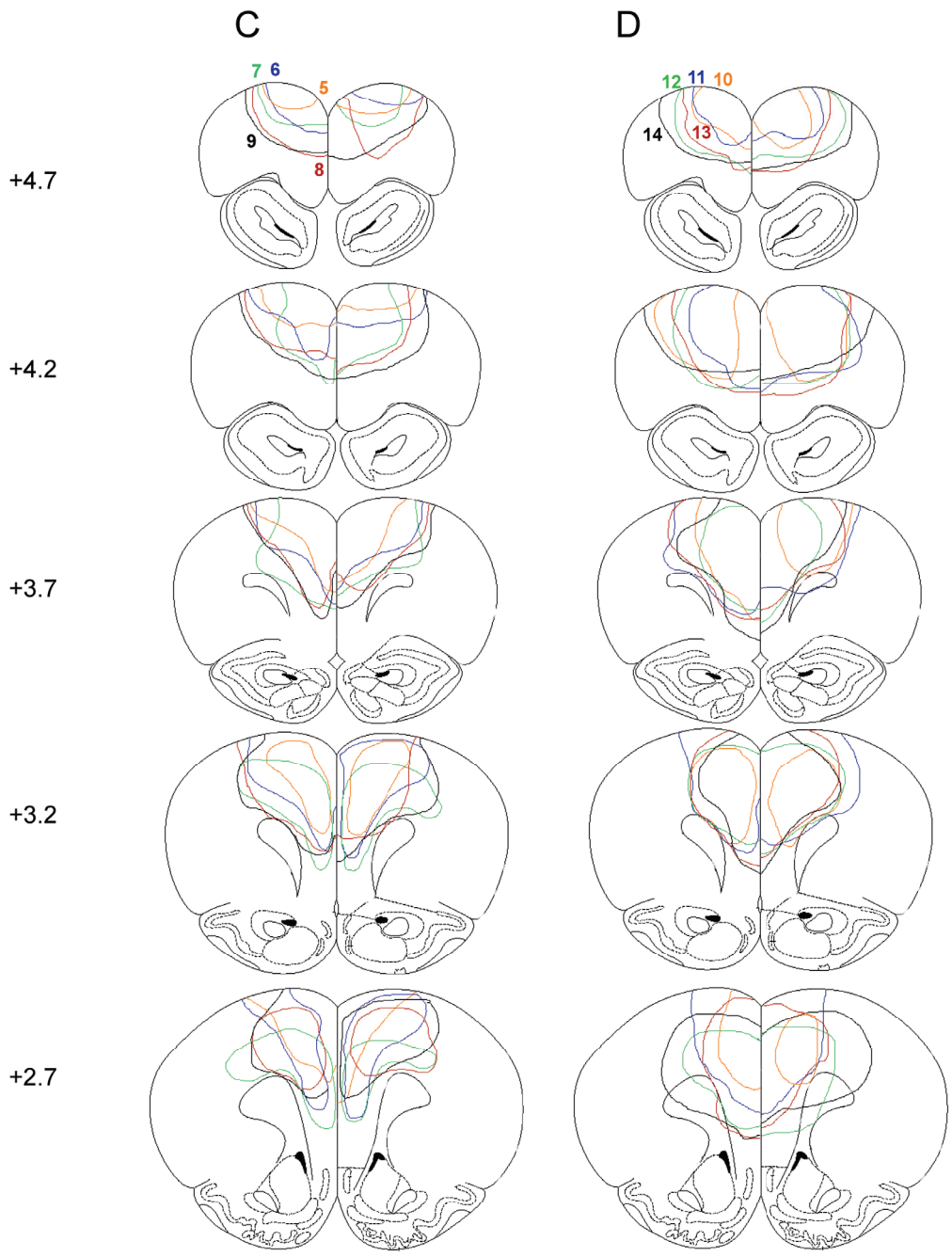


Figure 6 (Continued): Extents of Ibotenic Acid Lesions. (C) IBO animals 5-9 represented by different colors. This set of drawings displays the medium five lesions (D) IBO animals 10-14 represented by different colors. This set of drawings displays the largest five of the lesions.

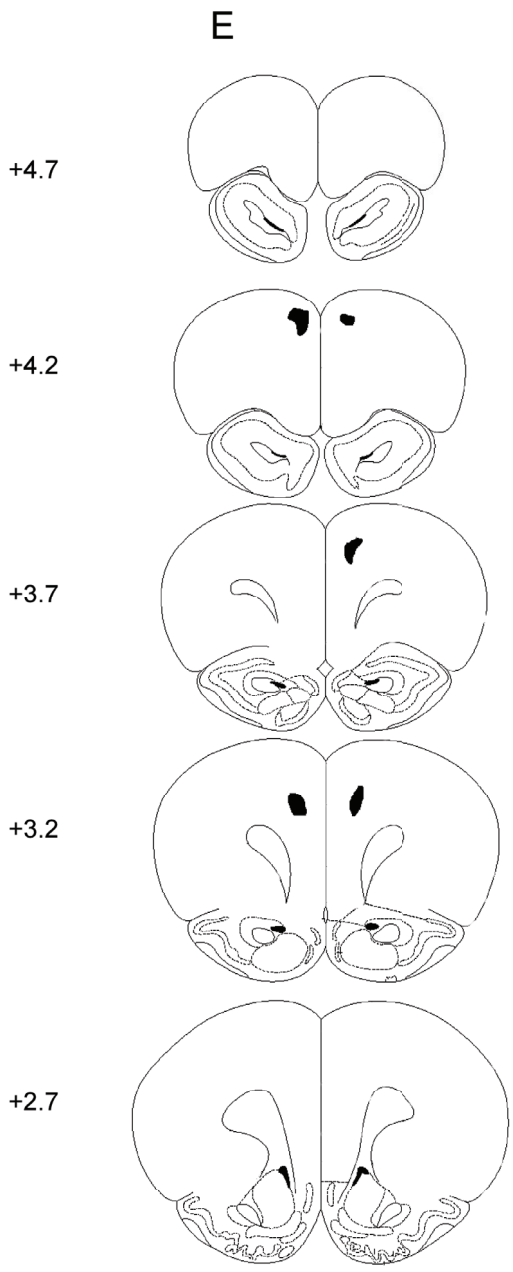


Figure 6 (Continued): Extents of Ibotenic Acid Lesions. (E) The black areas depict cortical regions where 100 % of animals displayed significant neuronal loss. These areas are located in the PrL and Cg1.

3.2. Baseline Behavioural States

PFC lesions did not cause any gross behavioural abnormalities. Furthermore, there were no apparent abnormalities in the EEG and EMG recordings of lesioned animals, and there were no obvious differences in the features of polygraphic recordings between the two groups. The three behavioural states (wake, NREM sleep, and REM sleep) were as readily distinguishable in the IBO group as they were for the saline group.

3.2.1.Wake

3.2.1.1.Total Amounts

The total amount of wake over the 24 h baseline period did not differ significantly between the lesioned and saline-injected animals (not shown). Furthermore, there was no difference between the treatment conditions in total amounts of wake during the 12 hour light or dark baseline periods (Figure 7A). The time course of wake amount in 2-h plots during the light and dark phases was also similar between the two treatment groups (Figure 7B). A repeated measures ANOVA indicated that there were no differences in wake amount between the saline and IBO treated animals during any of the 2-hour time blocks for the light or during the dark period (Figure 7B).

3.2.1.2. Numbers of Episodes

There was a significant difference between the groups during the dark phase with the IBO treated animals having fewer episodes of wake during this period ($t(16) = 3.3$, $p < 0.01$; Figure 8A). These animals also showed a similar but non-significant trend during the second, but not the first, half of the light phase (Figure 8B).

3.2.1.3. Mean Duration of Episodes

Mean duration of episodes differed between the IBO and control groups. IBO treated animals had longer durations per episode of wake in the dark phase of the baseline recording ($t(16) = 3.3$, $p < 0.01$; Figure 9A). This change accounts for the lack of difference in the total amount of wake despite fewer episodes. This suggests improved

consolidation of wake state. No further time-dependent differences between the groups were observed in a 2-hour plot analysis (Figure 9B).

Overall, total amounts of wake were unaffected by IBO lesions of the PFC. However, the IBO-treated animals had fewer wake episodes throughout the dark phase, and the mean duration of these episodes was longer, compared to saline-injected animals, indicating consolidation of baseline wake state in the dark phase in the PFC-lesioned animals.

3.2.2. NREM Sleep

3.2.2.1. Total Amounts

The total amounts of NREM sleep during the 12 hour light and dark period were not affected by the lesions (Figure 7C). Much like for wake, there were no differences in the amount of time rats spent in NREM sleep at any of the 2-hour intervals during the light and during the dark phase (Figure 7D).

3.2.2.2. Number of Episodes

IBO treated animals differed from the saline animals in terms of episode count during the dark phase, having fewer episodes of NREM sleep than the saline group ($t(16) = 3.3$, $p < 0.01$; Figure 8C). There was also a trend of a decrease in the second half of the light phase for the number of NREM episodes for the IBO treated rats (Figure 8D), but, these values did not reach statistical significance.

3.2.2.3. Mean Duration of Episodes

The mean duration of NREM sleep episodes was different between the groups. IBO treated animals had significantly longer NREM sleep episodes than their saline counterparts during the dark phase of baseline ($t(16) = 3.3$, $p < 0.01$; Figure 9C); there was no significant group difference in the light phase. A repeated measures ANOVA did not yield any significance at any time point in the 2-hour plots of NREM sleep episode duration (Figure 9D).

These results with NREM sleep are similar to the observed consolidation of wake. These results indicate that IBO treated animals entered individual wake and NREM sleep episodes less frequently but, once they were in either state, they stayed in that state for a longer period of time.

3.2.3. REM Sleep

3.2.3.1. Total Amounts

There were no differences between the two groups in regards to total amount of REM sleep during the light or the dark (Figure 7E) phases. Further analysis did not reveal any differences during any of the 2-hour intervals throughout the baseline recordings (Figure 7F).

3.2.3.2. Number of Episodes

REM sleep frequency was unaffected by the lesions during the baseline period (Figure 8E). The two groups did not differ throughout either the light or dark phases for the number of the REM sleep episodes (Figure 8F).

3.2.3.3. Mean Duration of Episodes

PFC lesions did not affect the mean duration of REM sleep episodes (Figure 9E). Further analyses revealed that the two groups did not differ significantly for any of the 2-hour time blocks (Figure 9F). These results are consistent with the total amounts and episode counts of REM sleep, which were not affected by the lesions.

Figure 7: Amounts of Wake, NREM, and REM Sleep During the 24 h Baseline Recording. Graphs on the left show the total amount of time that the saline and IBO treated rats spent in each behavioural state (wake, NREM sleep, REM sleep) during the 12-hour light and 12-hour dark phases of the baseline recording period. Graphs on the right show the amount of time, in two hour intervals, that the animals spent in each behavioural state. There were no differences in the amounts of time spent in wake (A), NREM (C) and, REM sleep (E). Further analyses did not yield any significant difference for each of the 2-hour time blocks for wake (B), NREM (D) or, REM sleep (F) amounts throughout the 24 hour baseline recording.

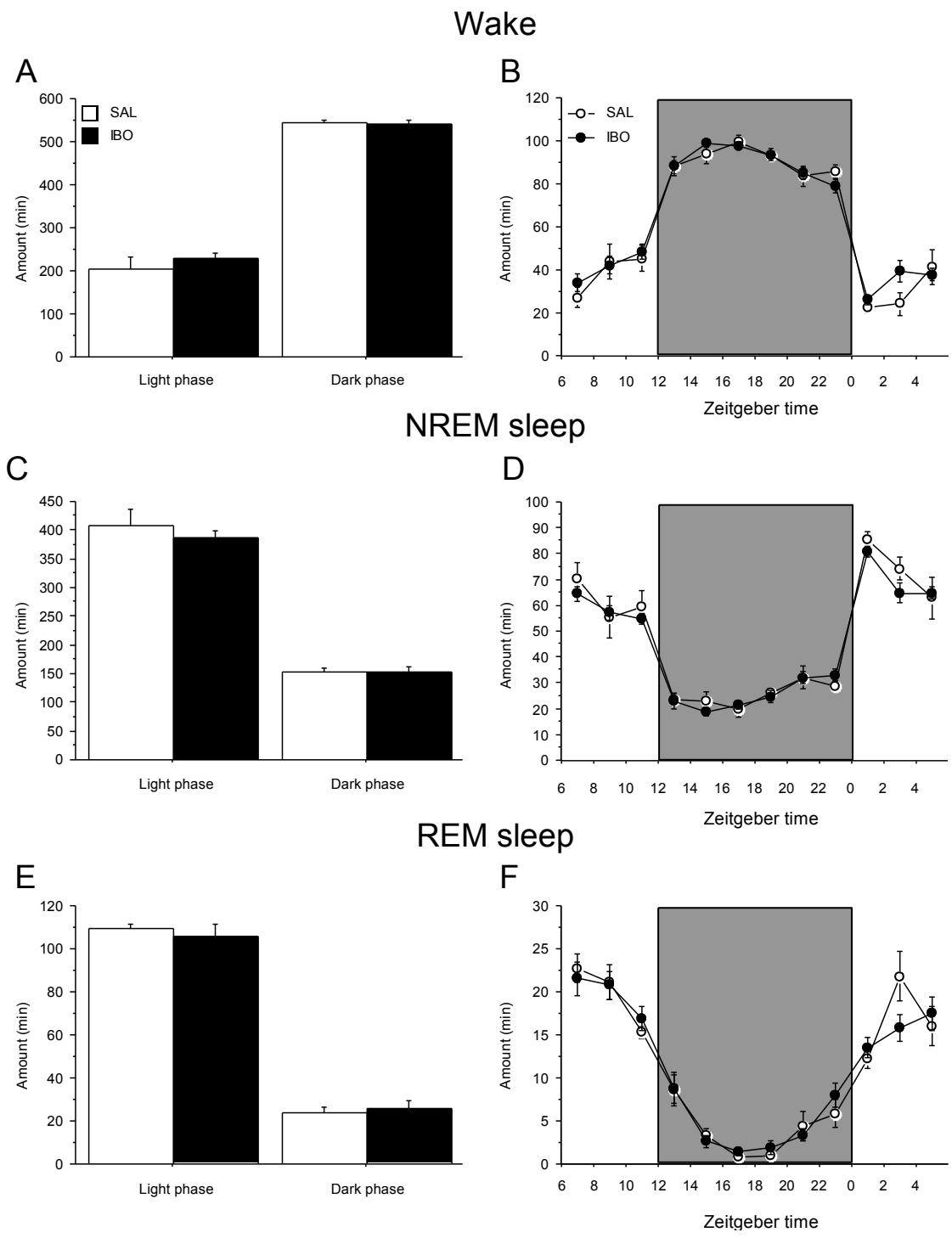

Wake
A
B
SAL
IBO
Amount (min)
Light phase
Dark phase
Zeitgeber time
NREM sleep
C
D
REM sleep
E
F

Figure 8: Numbers of Wake, NREM, and REM Episodes During Baseline. Graphs on the left show the number of bouts that the saline and IBO treated rats experienced for each behavioural state (wake, NREM sleep, REM sleep) during the 12-hour light and 12-hour dark baseline recording period. Graphs on the right show the number of episodes, in two hour intervals that the animals had for each behavioural state. Episode frequency for wake (A), and NREM sleep (C) were significantly decreased for the IBO treated animals compared to the saline treated animals during the dark phase (* $p < 0.05$). A similar trend was present for wake (B) and NREM sleep (D) during the second half of the light phase. No differences were present between the groups for frequency of REM sleep episodes (E, F).

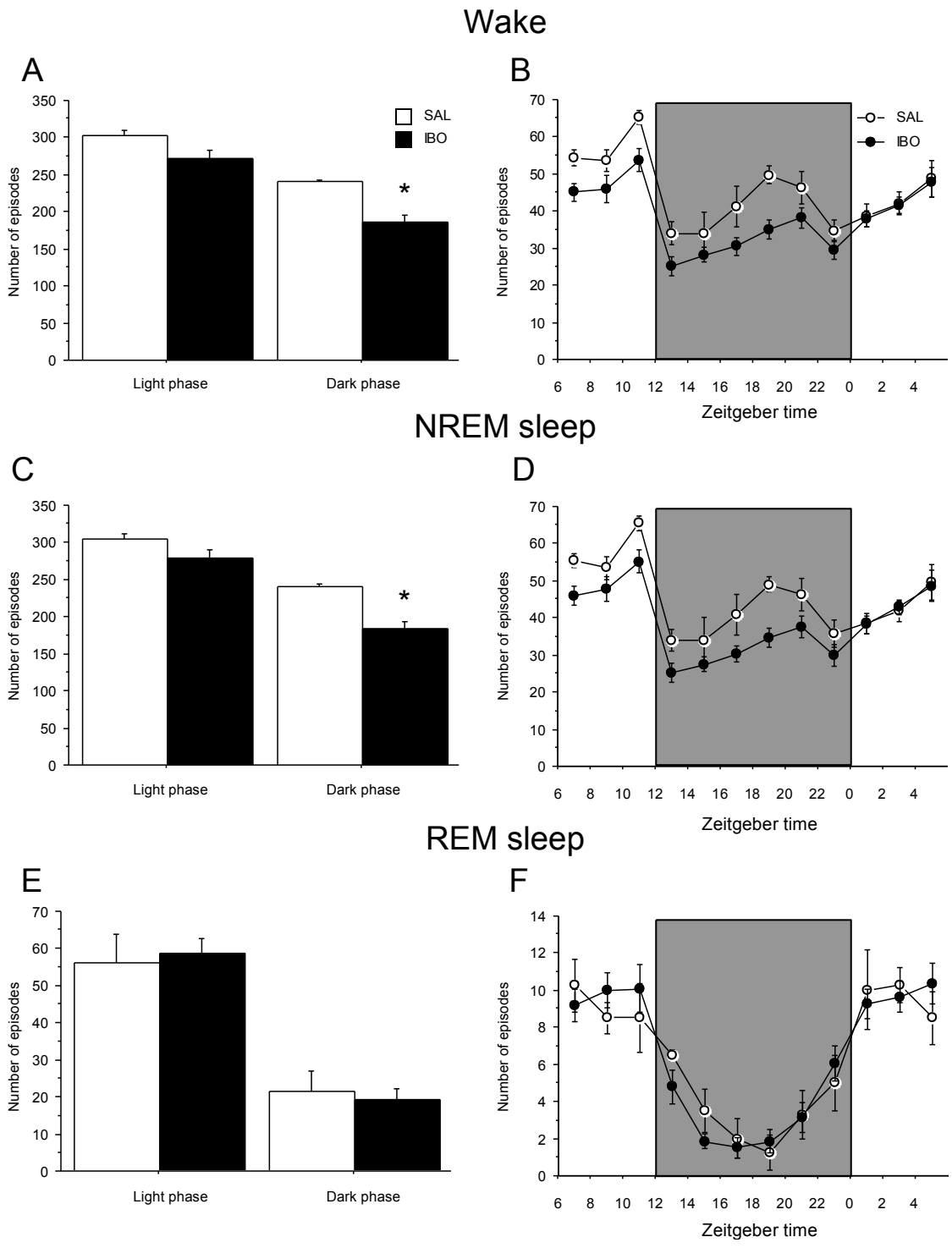
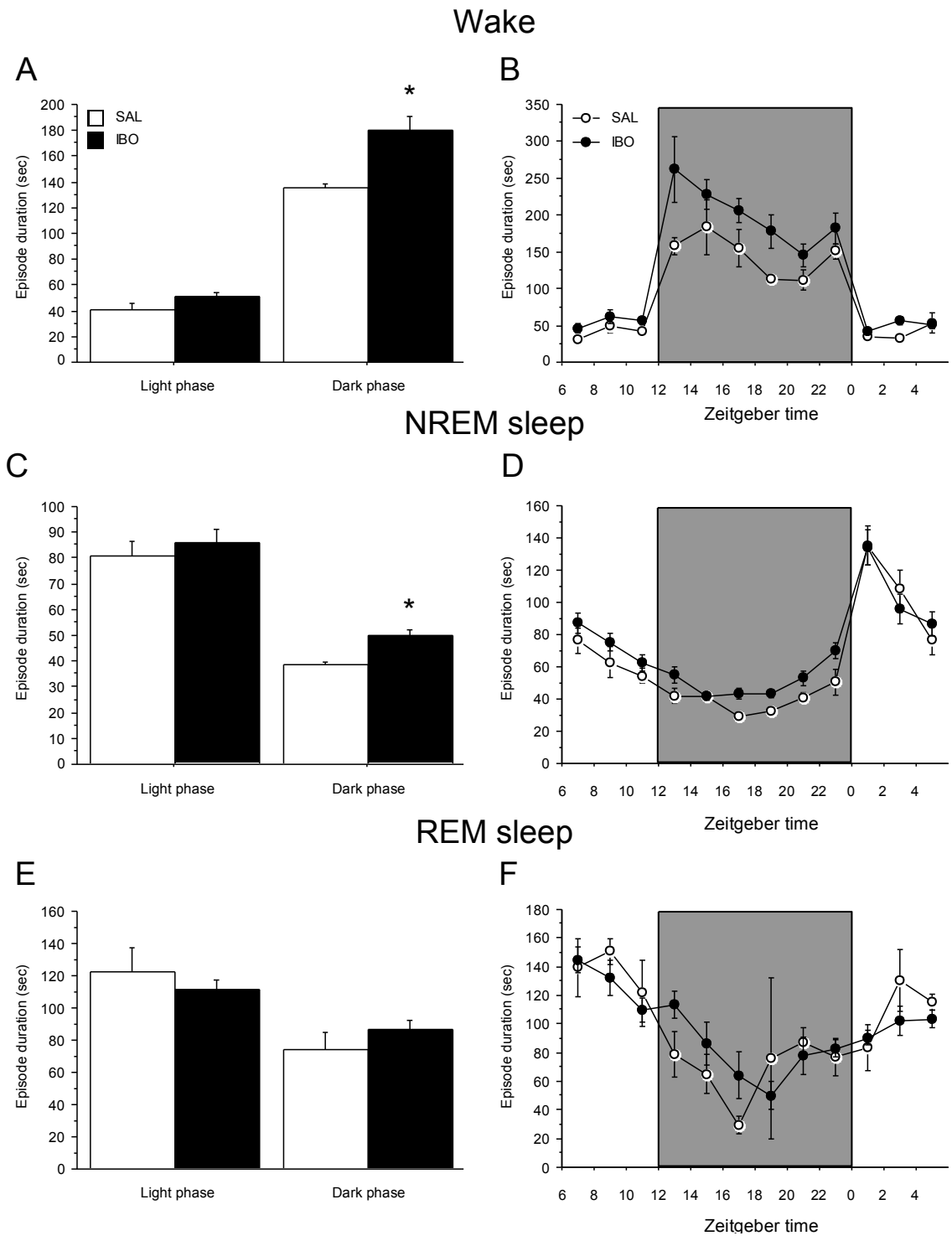

Wake
A
B
SAL
IBO
Number of episodes
Light phase
Dark phase
Zeitgeber time
NREM sleep
C
D
REM sleep
E
F

Figure 9: Mean Durations of Wake, NREM, and REM Episodes During the 24 h Baseline Recording. Graphs on the left show the mean duration of wake, NREM and, REM sleep episodes during the 12-hour light and 12-hour dark phases of the baseline recording periods. Graphs on the right show the mean duration of episodes, in two hour blocks. IBO treated animals had longer episodes of wake (A) and NREM sleep (C) during the dark phase than their saline counterparts (* $p < 0.05$). Wake (B) and NREM sleep (D) did not differ between the group for 2 hour time blocks throughout the baseline recording. No differences were present between the groups in the mean episode duration of REM sleep (E, F).


Wake
A
B
SAL
IBO
Episode duration (sec)
Light phase
Dark phase
Zeitgeber time
NREM sleep
C
D
REM sleep
E
F

3.2.4. Baseline Light-Dark Ratios

The light - dark ratio of time spent in each of the three behavioural states was examined to determine any possible effects of the IBO lesions on this ratio. Analyses conducted comparing the light-dark ratios for wake, NREM and, REM sleep during baseline recordings did not show any significant differences between the two treatment groups (Table 1).

3.2.5. Correlations Between Select Baseline Behavioural State Measures and Lesion Volume

Correlational analyses were conducted to examine whether lesion volume was correlated to any of the baseline behavioural state measures that showed significant lesion effects. There was no significant correlation between lesion volume and wake episode frequency (Figure 10A), or the duration of those episodes (Figure 10B). Similarly, there was no significant correlation between lesion volume and NREM sleep episode frequency (Figure 10C), or the duration of the episodes (Figure 10D). These data suggest no difference in the behavioural state measures during baseline recordings as a function of lesion size. Based on this result, the IBO group was kept as a single group, rather than dividing it according to the size of the lesion of each animal.

3.2.6. Baseline State Transition Analysis

3.2.6.1. Light Phase

The transition analysis was used as an alternative method to examine the dynamics between the three behavioural states. Mean numbers of transitions between the three states during the light phase of the baseline period are shown in Figure 11A. The treatment condition did not have an effect on any of these transitional frequencies.

3.2.6.2. Dark Phase

Mean numbers of the transitions during the dark phase of the baseline period are shown in Figure 11B. There were significantly less transitions for the IBO treated

Table 1: Light-Dark Ratios of Total Amounts of Behavioural States.

Behavioural State Treatment Group	L/D Ratio of Total Amount (Mean ± SD)	P Value
Wake		
IBO	.42 (.09)	.38
Saline	.38 (.10)	
NREM Sleep		
IBO	2.62 (.57)	.87
Saline	2.67 (.42)	
REM Sleep		
IBO	5.05 (2.66)	.82
Saline	4.72 (.99)	

IBO, n = 14
Saline, n = 4

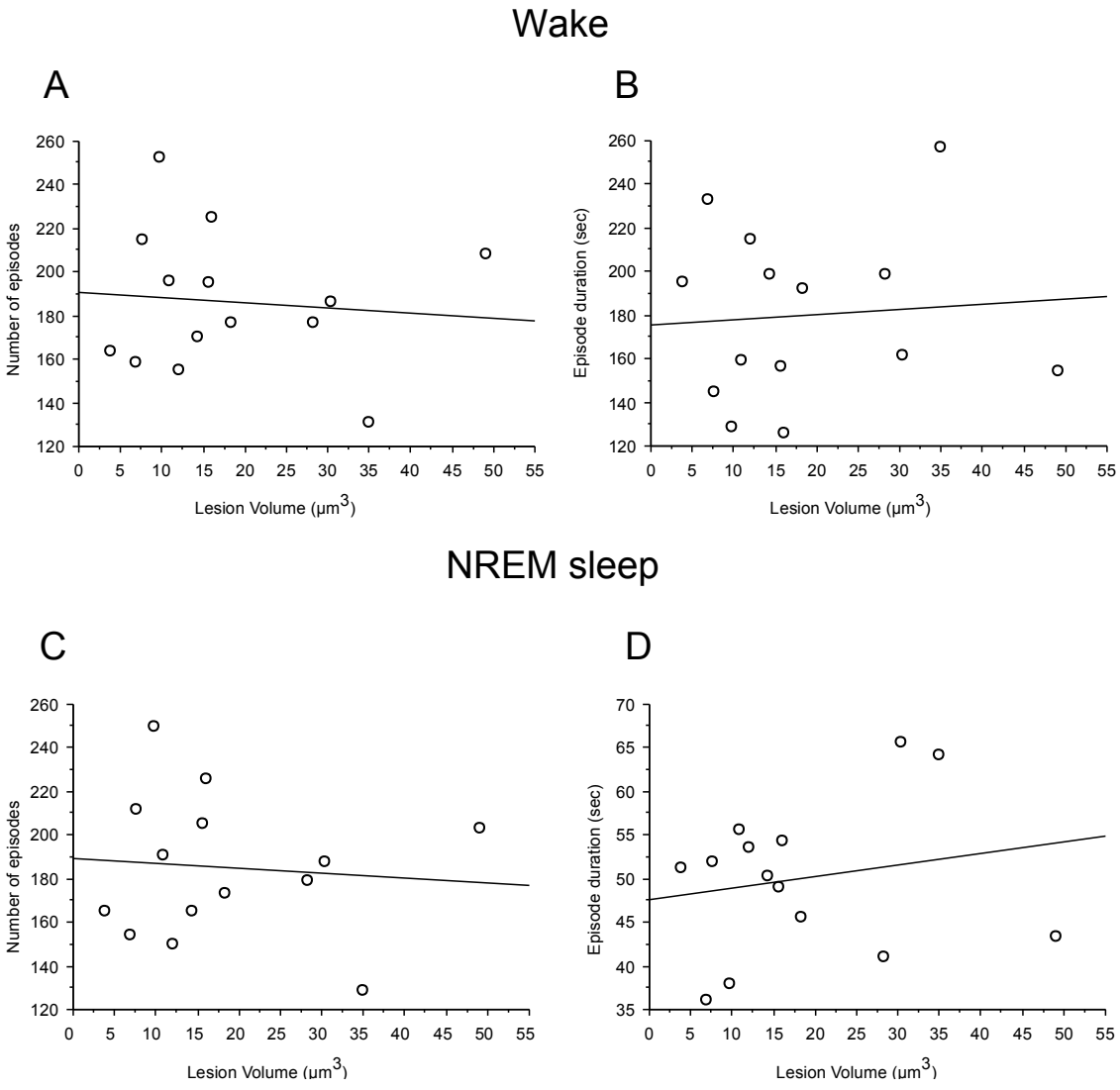


Figure 10: Correlations Between Lesion Volume and Select Baseline Behavioural State Measures. Correlations between total lesion volumes and behavioural measures that were significant were calculated to determine if lesion size had any effect. The number of episodes (A) as well as their mean duration (B) during wake showed no significant correlation with lesion volumes. Similarly, the number of episodes (C) and mean duration (D) during NREM sleep did not correlate significantly with lesion volumes.

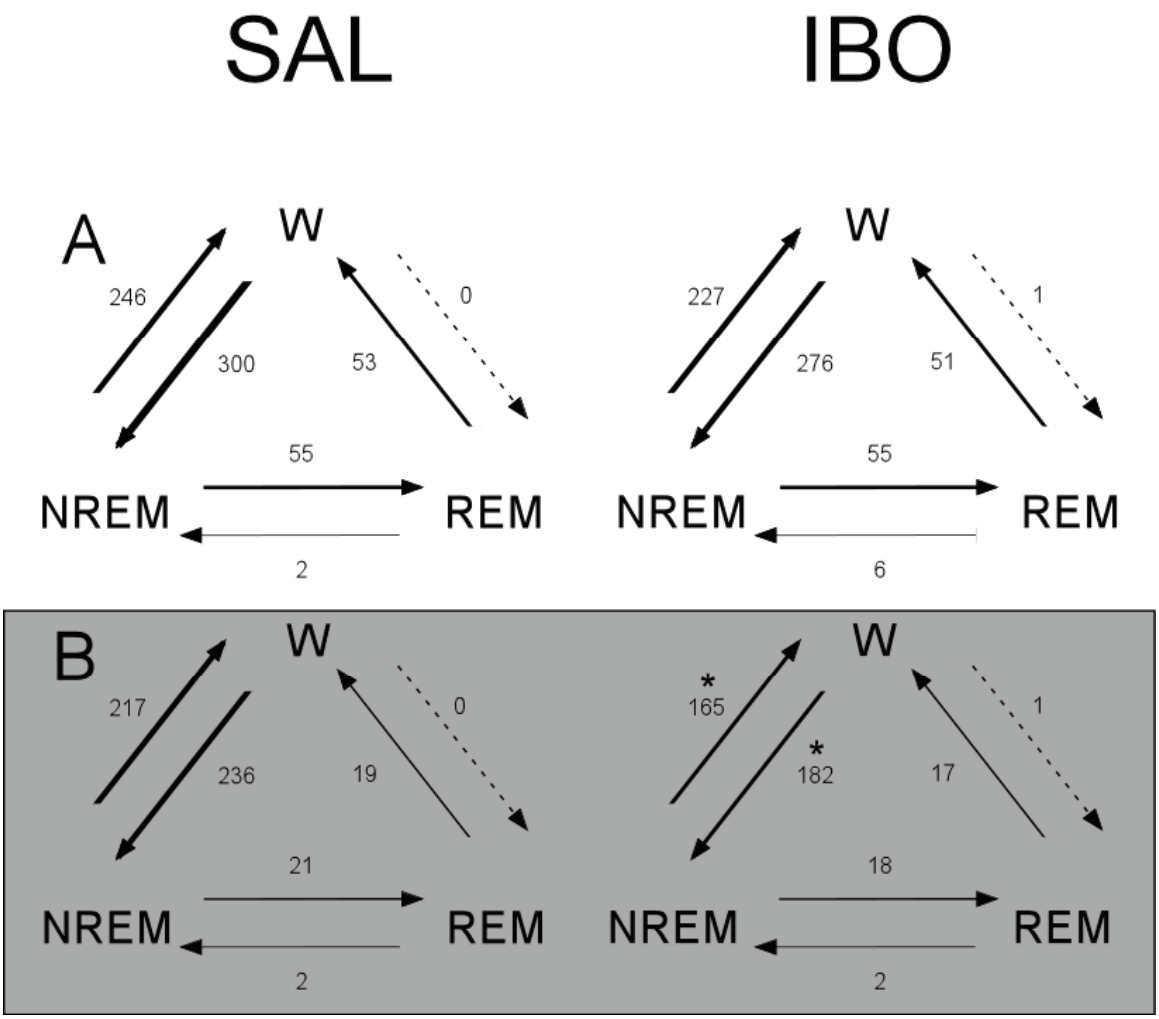


Figure 11: Baseline Transition Analysis. The diagrams displayed in the top row represent transitions during the 12-hour light period for both experimental groups. The diagrams in the second shaded row display transitions during the 12-hour dark phase for both groups. The mean number of transitions is displayed between each pair of behavioural states; arrow thickness approximates transition number. Treatment condition did not affect the number of transitions during the 12-hour light phase (A). For the dark phase, IBO treated animals transitioned significantly fewer times between wake to NREM, and NREM to wake than did the saline animals (B). * $p < 0.05$ compared to saline-injected animals.

animals from wake to NREM sleep ($t(16) = 3.3$, $p < 0.01$), and from NREM to wake ($t(16) = 3.0$, $p < 0.01$). These results further reinforce the data described above suggesting an increased consolidation of wake and NREM sleep states in the dark phase in the IBO treated animals

3.2.7. Baseline EEG Power Spectra

EEG power spectra were analyzed for all the three behavioural states. Frequency bands of interest included delta (0.5-4 Hz), theta (4.5-8 Hz), sigma (8.5-13 Hz), beta (13.5-30 Hz), and gamma (30.5-50 Hz). There were no significant differences between the two groups in power values for any of these bands during the baseline recordings. However, a non-significant, slight decrease in EEG power across the spectrum during the dark phase for the IBO treated group was present in wake (Figure 12A) and NREM sleep (Figure 12B) states. For the EEG during REM sleep, there were no significant differences between the groups in any frequency bands (Figure 12C). Similar results were observed during the light phase with a slight decrease in EEG power across the spectrum in wake and NREM sleep (data not shown).

3.3. Sleep Deprivation – The Number of Interventions

Interventions required to keep animals awake were counted throughout the 6-hour sleep deprivation period. The total number of interventions over the 6 h period was significantly reduced in the IBO treated animals compared to the saline-injected controls ($t(16) = 2.2$, $p < 0.05$; Figure 13A). An ANOVA did not show any significant interaction between the hour of deprivation and the treatment condition (Figure 13B).

3.4. Recovery Sleep

3.4.1. Total Amounts

Total wake (Figure 14A), NREM (Figure 14B) and REM sleep (Figure 14C) amounts during the first 12 hours (dark phase) of recovery after sleep deprivation were compared with total amounts during the dark-phase baseline recordings. There were significant differences in total amounts of wake, NREM and, REM sleep between the

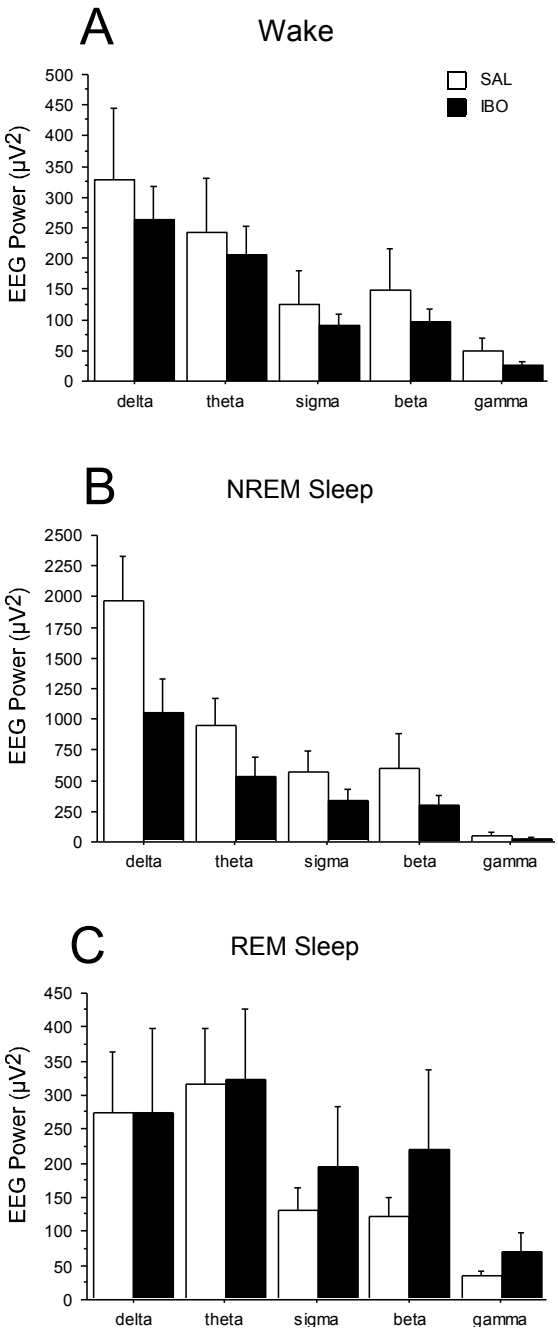


Figure 12: Baseline 12 h Dark Phase Total EEG Power. A non-significant trend of decreased EEG power across the spectrum can be seen during 12-hour dark phase for wake (A) and NREM sleep (B) recordings for IBO treated animals. No significant change in total EEG power during REM sleep (C) recordings.

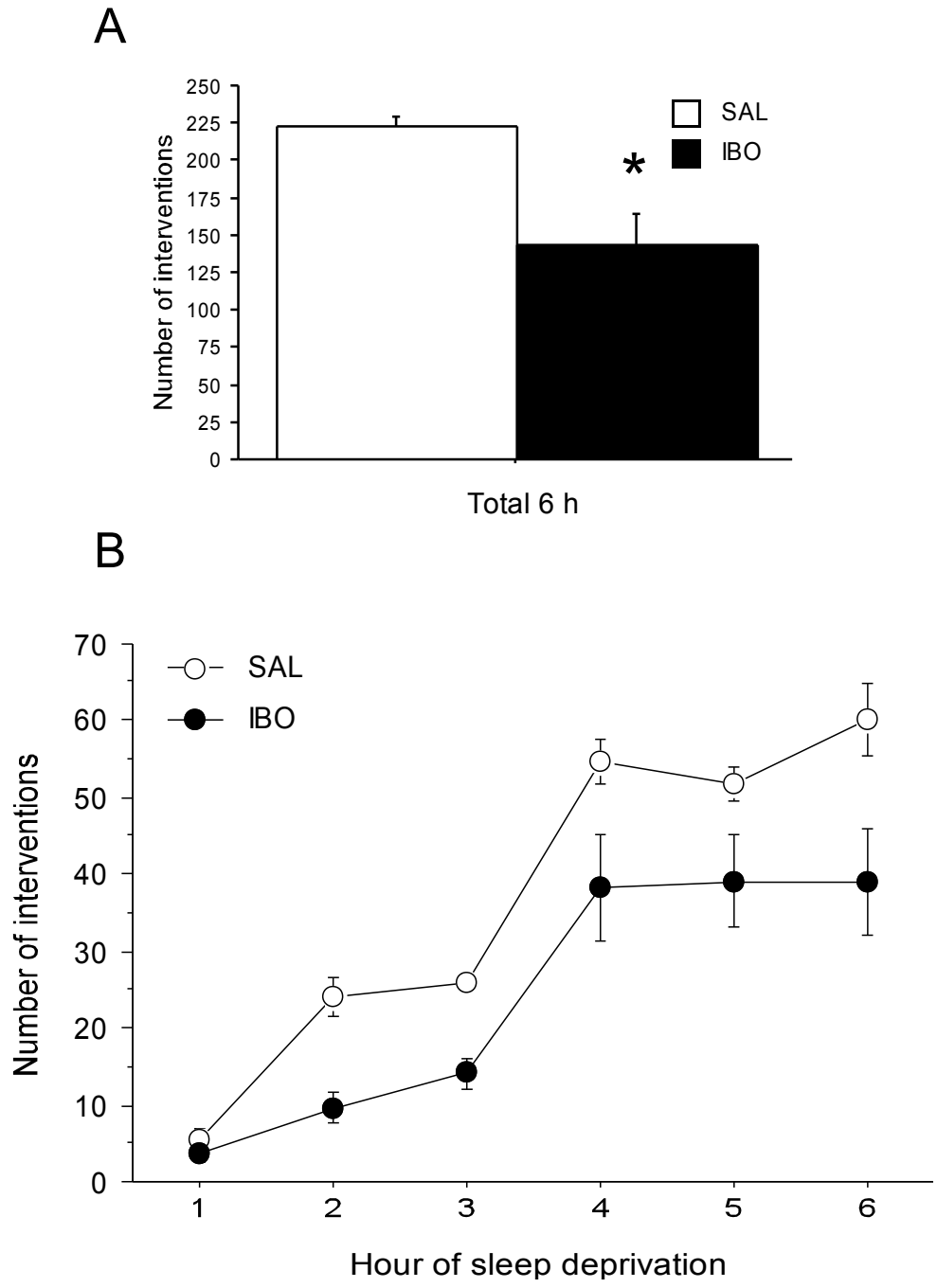
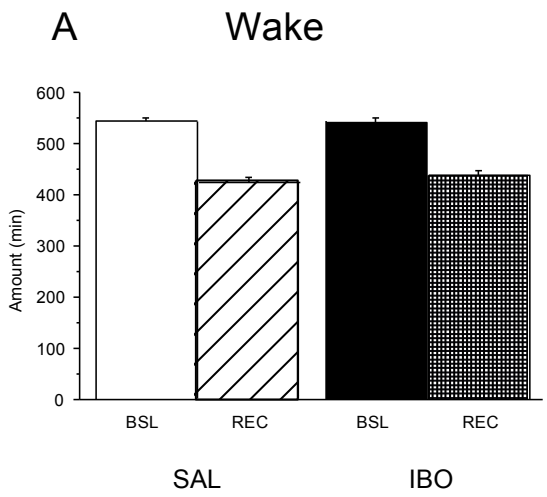
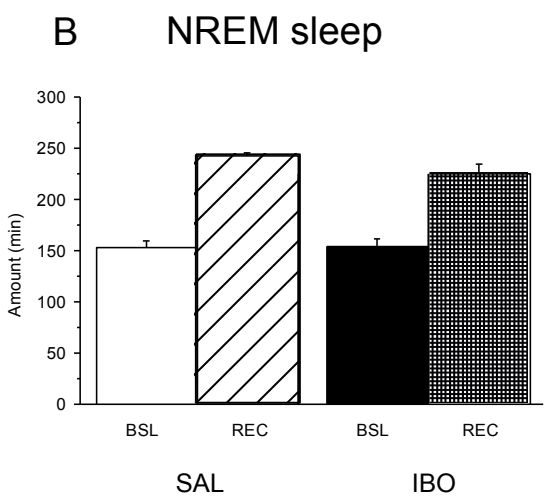
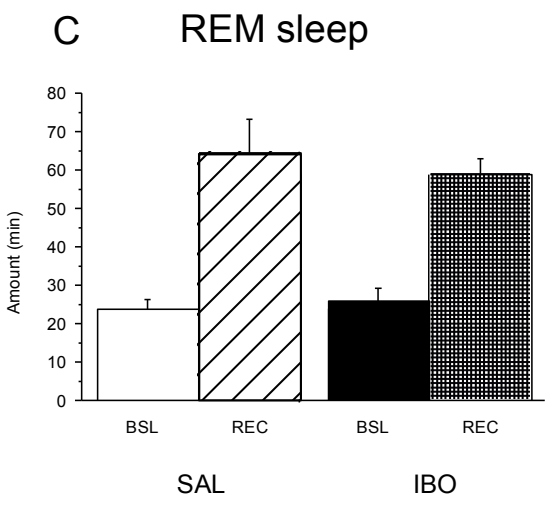


Figure 13: Number of Interventions During Sleep Deprivation. IBO-treated animals required significantly fewer interventions to keep awake during the 6-hour sleep deprivation period (A). Despite the overall differences between the two groups, there was no significant difference in the number of interventions between the groups at each 1-hour interval of deprivation (B). * $p < 0.05$ compared to saline-injected controls.

Figure 14: Amounts of Wake, NREM, and REM Sleep During the First 12 h (Dark Phase) of Recovery After Sleep Deprivation. Figures display a comparison between dark phase baseline (BSL) and recovery(REC) recordings between the two treatment groups. An ANOVA showed a main effect of time (BSL vs. REC; $p < 0.05$) for wake (A), NREM (B), and REM sleep (C). However, the two groups did not differ significantly on any of these measures, and there was no significant interaction.


A
Wake
600
500
400
300
200
100
0
Amount (min)
BSL
REC
BSL
REC
SAL
IBO


B
NREM sleep
300
250
200
150
100
50
0
Amount (min)
BSL
REC
BSL
REC
SAL
IBO


C
REM sleep
80
70
60
50
40
30
20
10
0
Amount (min)
BSL
REC
BSL
REC
SAL
IBO

baseline and recovery periods, including rebound increases in NREM and REM sleep amounts. However, an ANOVA showed no significant interaction between treatment condition and the recording condition. Similar results were observed during the light-phase recordings, and no significant group differences were found.

3.4.2. Time Course of Total Amounts of Wake, NREM, and REM Sleep

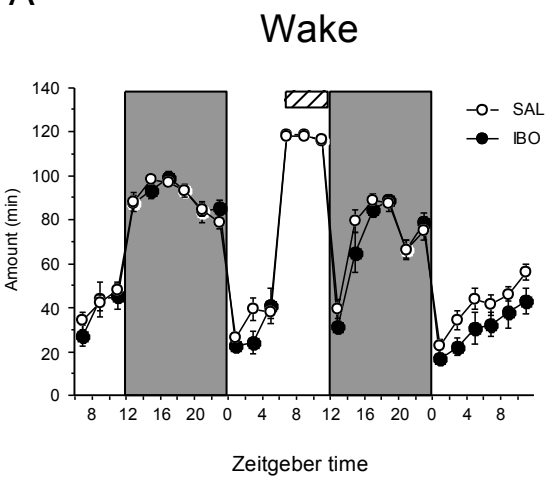
To further analyze the lack of differences observed for total amounts of wake, NREM and, REM sleep during baseline or recovery periods, full 54 hour analyses were conducted. Figure 15 displays the full experimental time course of behavioural states. Not surprisingly, there were no significant group differences observed for wake (A), NREM (B) or, REM sleep (C) in terms of total amounts. These plots show clearly that the lesions did not influence total amounts of wake, NREM or REM sleep, nor rebound NREM and REM sleep in these animals.

3.4.3. Time Course of EEG NREM Delta Power

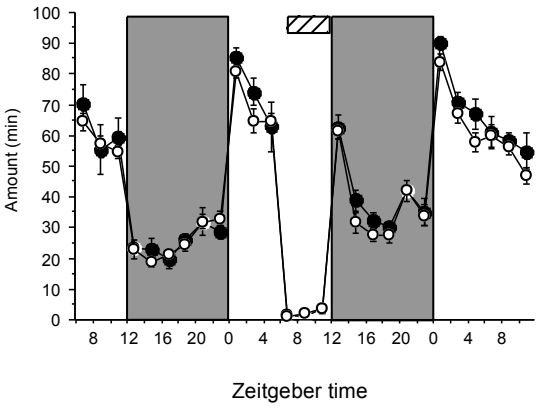
EEG NREM delta power was analyzed for the total time course (54 h) of the experiment (Figure 16). This measure is commonly used as an index of sleep intensity, and it was thought that any changes as a result of lesions would be most apparent in this measure. The two groups did not differ significantly throughout the entire experimental time course. There was a rebound in NREM EEG delta power immediately following SD, indicating a build-up in sleep pressure as a result of SD. There was a non-significant trend for the levels of NREM EEG delta power at the beginning of the recovery period to be lower in the IBO animals than in the control animals.

Figure 15: Amounts of Wake, NREM, and REM Sleep in 2 h Intervals During the Entire 54 h Period of the Experiment. Figures display a comparison between IBO treated animals, and their control counterparts. Dark phases are indicated by the shading and the 6-hour sleep deprivation period is marked by the hashed box. The two groups did not differ significantly on amounts of wake (A), NREM (B), or REM sleep (C) at 2 h intervals throughout the timecourse of the experiment, including 24-hour baseline, 6-hour sleep deprivation, and 24-hour recovery periods.

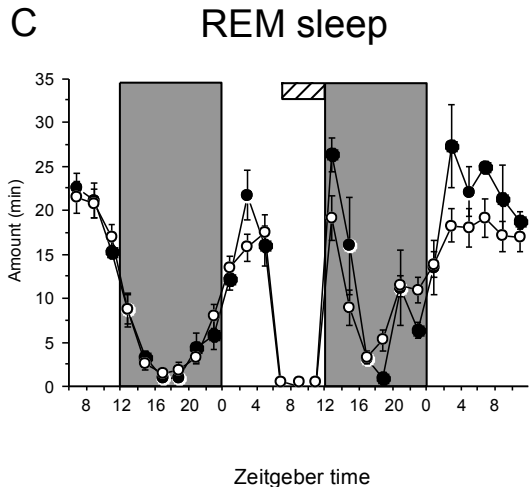
A


Wake
SAL
IBO
Amount (min)
Zeitgeber time

B


NREM sleep
Amount (min)
Zeitgeber time

C


REM sleep
Amount (min)
Zeitgeber time

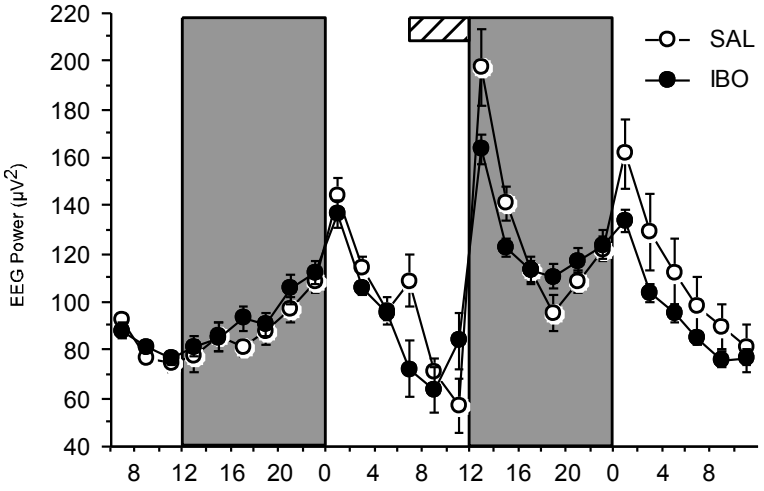


Figure 16: Time course of NREM EEG Delta Power During the Entire 54 h Period of the Experiment. Figures display a comparison between IBO treated animals, and their control counterparts at 2 h intervals. Dark phases are indicated by the shading and the 6-hour sleep deprivation period is marked by the hashed box. The two groups did not differ significantly in terms of NREM EEG power throughout the experiment. The spike in NREM EEG delta power observed immediately following SD indicates rebound caused by increased sleep pressure build-up during the SD period.

Chapter 4: Discussion

The present study sought to elucidate a possible role for the PFC in sleep-wake regulation. The major findings are as follows: 1) Under baseline conditions, lesions to the PFC caused changes in sleep-wake architecture particularly in the dark phase. Most notably, wake and NREM sleep episodes were less frequent in number but each episode lasted longer in duration, while the total amount of time spent in each behavioural state was unaffected by the lesions. Lesion volume did not affect any of the observed effects on sleep measures. The total EEG power during wake and NREM sleep periods tended to decrease for the IBO group compared to the SAL group particularly during the baseline dark phase, although this did not reach statistical significance probably due to a small group size for the SAL group. 2) After a brief (6 h) period of SD, there were no differences between the IBO and SAL groups in regards to NREM sleep rebound or NREM EEG delta wave activity (a well-established marker of sleep drive and intensity). Thus, PFC lesions seem to consolidate both wake and NREM episodes under baseline conditions particularly in the dark phase, but did not affect homeostatic responses to sleep loss.

This study was the first to look at the possible effects of PFC cell-specific lesions on sleep-wake regulation and in response to sleep deprivation in the adult rat. The main results, including somewhat unexpected results, will be discussed in the following sections.

4.1. Mechanism Supporting the Increased Consolidation of Wake/NREM Sleep After PFC Lesions

PFC lesions resulted in a behavioural phenotype in which total amounts of time spent in the three behavioural states was unaffected, whereas wake and NREM sleep periods seem to be more consolidated, as they were less frequent but lasted longer in duration, and transitions between wake and NREM sleep were less frequent. It is difficult to discern whether the described phenomenon is due to an improvement in consolidation or possibly a difficulty in transitioning between stages. However, to further understand

the observations, it is important to consider pathways which may link the PFC with the sleep-wake regulatory nuclei that were discussed in Chapter 1.

An interesting target to consider for the observed modulation of sleep-wake states lies in the orexinergic neurons of the PeF-LH. Saper and colleagues' (2001) flip-flop switch model suggests that activation of the orexin neurons plays an important role for the stabilization of behavioural states (i.e., prevention of excessive transitions between behavioural states). The PFC-lesioned animals transitioned less frequently compared to their behavioural controls. The output neurons of the PFC are thought to be glutamatergic. Therefore, a possible mechanism for this perceived consolidation may be a loss of glutamatergic inputs (as a result of lesions) to an unidentified downstream target which then projects inhibitory inputs to the orexin neurons of the LH to help increase state stability. For this mechanism to work, it would be necessary that increased activities of orexin neurons result in even greater behavioural state stablity, and this possibility requires confirmation.

Lazarus and colleagues (2012) described a neural pathway involved in sleep/wake regulation that includes excitatory inputs from the medial PFC to the nucleus accumbens. The nucleus accumbens has been known to have GABAergic projections to the PeF-LH (Yoshida et al., 2006). This pathway could act on orexin neurons which are thought to play an important role in behavioural state stabilization. However, a previous study reported that ibotenic acid lesions to the nucleus accumbens core increased the number of wake and NREM sleep episodes while decreasing the duration of NREM episodes particularly in the light phase; transitions between wake and NREM sleep were also increased (Qiu et al., 2010). These observations represent a sleep phenotype that is opposite to the sleep behaviours observed in PFC lesioned rats in the current study and, while interesting, this is inconsistent with the presumed excitatory nature of the PFC input to the nucleus accumbens. Thus, although the PFC-nucleus accumbens - orexin neurons link is attractive for explaining the present finding of behavioural state consolidation after PFC lesions, more detailed functional anatomical examination is necessary to fully explain the results through this link.

The PFC is also known to project directly to the PeF-LH (Hurley et al., 1991). Inhibitory interneurons are present in this area of the brain (Oomura et al., 1975). Thus, the inhibition of orexin neurons may occur indirectly via these neurons as a result of projections from the PFC to the PeF-LH which causes inhibitory interneurons to fire.

4.2 Decreased Sleep Pressure in IBO Animals?

The present experiment did not yield any significant differences between the two groups in terms of rebound NREM sleep amount. However, IBO treated animals demonstrated a trend of less NREM EEG delta power (a measure of sleep intensity and sleep pressure) following the SD period. Additionally, these animals required fewer interventions to remain awake during the 6 hours of SD. However, it is unclear whether the lower number of interventions was a direct result of a lower accumulation of sleep pressure over time, or due to the proposed transitional deficits, in the IBO animals.

The mechanisms underlying sleep debt and homeostatic sleep pressure remain unclear. However, accumulation of sleep pressure during wake occurs universally throughout a number of species including insects such as *Drosophila* (Bushey et al., 2011). Insects have a simple central nervous system and lack any structure paralleling the PFC. From a phylogenetic viewpoint it is apparent that the neural circuitry implicated in the accumulation of homeostatic pressure originates in a more primitive area of the brain than the PFC. This would suggest that the fewer required interventions with the IBO animals most likely resulted from their transitional issues, and that any trend in the EEG power was not a reflection of decreased sleep debt in these animals.

Alternatively, it is possible that the PFC may indeed serve a role related to process S. In the two-process model of sleep regulation proposed by Borbely (1982), homeostatic pressure continues to increase as a result of being awake until a threshold is met. Upon reaching this threshold, sleep pressure would be so great that the individual would fall asleep. However, it has been shown that the threshold can be altered (Daan et al., 1984). Thus, the fewer interventions necessary may have resulted from the IBO treated animals having a heightened threshold and being more resistant to the SD induced

sleepiness. This possibility is consistent with longer durations of baseline wake and NREM sleep episodes in PFC-lesioned animals.

4.3. Cell Body-Specific Lesions vs. Inhibition of the PFC

Ibotenic acid has been shown to cause cell body-specific neurotoxicity via N-methyl-D-aspartate (NMDA) receptor activation (Zinkand et al., 1992). Activation of the NMDA receptors causes an influx of Ca^{2+} ions into the cell soma. The calcium ion influx causes the release of a number of enzymes that in turn damage structural aspects of the cell. Furthermore, ibotenic acid lesions have been shown to be cell-specific and do not damage passing tracts and fibers (Kaur et al., 2008)

In the current study, lesions to the PFC were fairly consistent in terms of affected cortical areas (as seen in Figure 6). Areas in which all of the IBO treated animals displayed a lesion included the prelimbic area and the anterior cingulate. Only small portions of the PFC were lesioned in 100 % of the IBO animals, however, the effects on sleep measures remained consistent throughout the group. Given the time restraints, we were unable to analyze the lesions as to quantify the brain areas that were lesioned in a 80 % or more of the IBO animals, which would have likely provided larger portion of the PFC to objectively report as affected by ibotenic acid. This may suggest that the effects on sleep may be focused to these specific regions of the PFC that were commonly lesioned, or that these lesioned regions are connected indirectly in relation to the PFC's effect on sleep-wake regulation.

It is interesting to note that total volume of the lesion did not seem to influence the sleep measures examined in the current study. In fact, correlations between lesion volume and the sleep measures that were affected significantly by the lesion were examined, and none of them was significant. Further analysis showed that only a very limited area of the PFC was lesioned in all of the experimental animals. These results may suggest that a very specific region of the PFC mediates the behavioural changes. The cell body-specific lesions used in the study have the disadvantage of destroying the entire cell and thus abolishing any of its related functions. Alternatively, pharmacological inhibition is often used specifically to inhibit cell activity and is for the most part

reversible. Thus, there should not be any functional effects once the drug has been metabolized. This raises the question: would the experimental results have differed at all given the use of alternative techniques? Some clues to answering this question may be sought from considering other studies which manipulated PFC activities to observe possible changes associated to PFC lesions. Most of the studies inhibiting the PFC looked at more complex cognitive functions. For example, infusions of a GABA receptor agonist to the medial PFC markedly reduced the frequency and duration of social interaction in rats (van Kerkhof et al., 2013). A similar study showed that inhibition of the PFC severely hindered memory retrieval of an odor, particular in the presence of interference (Peters et al., 2013); in fact there is also evidence of a strong link between PFC activation and the dopamine system, and therefore the PFC is implicated in reward based situations (Lodge, 2011). These studies indicate that selective short-term inhibition of the PFC leads to a number of various deficits in behaviour; however, the effects of PFC lesions or prolonged inhibition can be much more severe.

4.4. The Role of the PFC in EEG Spectral Power

Despite not reaching significant values, there was a trend present throughout the experiment regarding a reduction in EEG spectral power in the IBO group. This trend was most apparent for NREM EEG delta power and under baseline conditions. Slow waves, characteristic of NREM sleep, are thought to originate in the PFC (Massimini et al., 2004). A recent study in humans displayed a strong correlation between NREM slow-wave activity and aging (Mander et al., 2013). The authors noted that PFC atrophy, which is common in aging, may be a contributing factor as older individuals experienced less slow-wave activity during sleep than did younger participants. This was supported by measurement of gray-matter density in the PFC. Areas of the PFC that showed the greatest reduction in gray-matter included the medial PFC and the posterior cingulate cortex. Interestingly, both of these regions have been shown to be implicated in slow-wave generation (Murphy et al., 2009). Mander and colleagues (2013) also showed that the decrease in slow-wave activity was linked to poorer cognitive performance on a memory task.

EEG spectral analysis allows us to measure voltage activity changes at the surface of the cortex. Given the reduction in slow-wave activity observed in aging in previous studies, and the trend seen in our study, it is plausible to suggest that the PFC may play a role in the generation of EEG activity. The electrophysiological processes underlying the generation of EEG activity may therefore also be affected in our IBO treated rats, including the generation of slow waves in the cortex and through the thalamocortical pathways (Steriade et al., 2001).

4.5. Sleep Function of the PFC – Lobotomy Cases

Due to ethical implications it is not possible to manipulate the PFC in human subjects to explore its potential role in sleep-wake regulation. However, past studies that employed a prefrontal lobotomy may offer some insight to its functionality pertaining to sleep. Does the observed phenotype display any parallels to human studies related to the PFC?

A large portion of schizophrenic patients experience a significant impairment for initiating and maintaining sleep (Cohrs, 2008). Typical treatment now consists of anti-psychotic medication, but treatment used half a century ago often consisted of a prefrontal lobotomy. A case-study that looked at sleep-wake patterns of individuals pre- and post-lobotomy reported that sleep architecture became more similar to that of control patients following recovery from the surgery (Hosokawa et al., 1968). In this study, lobotomized patients had spent significantly more time in REM sleep prior to surgery.

These results are interesting because they suggest a non-critical regulatory role of the PFC in sleep-wake regulation, suggesting that sleep-wake regulation may be regulated by, but is not dependent upon, the PFC. Abnormal functions in the PFC cause marked disturbances in sleep. Lobotomy of the PFC allows sleep-wake architecture to revert back almost to a baseline condition. Furthermore, lower-order species lacking a PFC typically have some sort of consistent sleep rhythms. This suggests that the PFC plays a role in sleep-wake regulation, but is likely not critical to basic sleep/wake states. The modulatory role of the PFC in sleep regulation is consistent with the results of the

experiment, as PFC lesions affected sleep-wake transitions but not the total amount of sleep or wake.

4.6. Methodological Considerations

One of the main concerns that need to be kept in mind while interpreting the results of the study pertains to the small number of saline-injected control animals (n = 4). Due to the fact that all of the sleep/wake scoring was conducted blindly, we did not know the number of animals in each group until statistical analyses began. Some saline-injected animals were required to be excluded due to poor EEG recordings, or signs of tissue damage at the injection sites. An additional group of several saline-injected animals were required to be euthanized due to technical problems prior to the experimental end point. In the original experimental plan, we had anticipated some variability in lesion size, and we had planned to divide lesioned animals into two or three groups according to lesion size. We, in fact, attempted to split the IBO group into small (n = 4), medium (n = 5) and, large (n = 5) based on total volume. However, further analyses did not yield any differences between these three groups in terms of their effects on sleep measures. Thus, we kept all the IBO treated animals as one group (n=14). We believe that if the group size was more balanced between control and IBO animals, more of the measures would have come out significant in terms of lesion effects, most notably the EEG power spectral values.

5.1. Future Directions

An interesting follow-up experiment would be to look at selective inhibition of the PFC in sleep-wake behaviour, e.g., taking a look to see if local administration of a GABA agonist to the PFC has any effect on the sleep-wake architecture, more specifically on the frequency and mean duration of wake and NREM sleep episodes. This could be accomplished over a single 24 hour baseline period with an appropriate number of injections to assure that the drug remain active based on its half-life (preferably muscimol). This technique would allow for a specific inhibition of the PFC that is reversible, thus manipulations could be made in a time-dependent manner to view if the PFC is responsible for the observed effects of lesions from the current experiment. Thus

the study could be conducted as a within-group design with random assignment of order (control then muscimol or muscimol then control), or individual animals could be used for each group. Assuming a similar behavioural phenotype was observed, further SD could be undertaken to observe the effects.

Another experiment to consider would be to replicate the lesion portion of the study and to examine any possible effects on memory consolidation and EEG spectral activity. This would look to further elucidate the role of the PFC in slow-wave activation and generation, and its implications for functions other than sleep. Based on previous studies, the IBO animals would experience some cognitive deficits (Petanjek et al., 2011). Furthermore, one would need to seek to explain if any changes in cognitive performance were associated with possible EEG changes or if they would be a function of PFC processing impairments.

5.2. Conclusions

The present study is one of the first to describe the role of the PFC in sleep-wake regulation of rats. Ibotenic acid allowed cell body-specific lesions of the PFC while maintaining the structural integrity of passing fibers and tracts. The results suggest that the PFC is implicated in sleep-wake regulation via a mechanism related to the transition and/or maintenance of wake and NREM sleep stages. There also seems to be a possible case made for the PFC being involved in EEG power generation. However, some of the lesion effects did not reach statistical significance, possibly due to the small group size of saline-injected control animals. Furthermore, many questions remain, and further work is necessary to elucidate the underlying mechanisms responsible for this behavioural phenotype. Nonetheless, these results help to further support a regulatory role for the PFC in sleep-wake behaviours.

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