$GABA_A$ RECEPTORS IN THE PONTINE RETICULAR FORMATION OF C57BL/6J MOUSE MODULATE NEUROCHEMICAL, ELECTROGRAPHIC, AND BEHAVIORAL PHENOTYPES OF WAKEFULNESS

by

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DEDICATION

To my family

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As I come to the completion of one chapter of my life and prepare to begin the next, I reflect upon a quote I have carried with me throughout my graduate studies from the African-American educator and author Booker T. Washington - "Success is to be measured not so much by the position that one has reached in life as by the obstacles by which s(he) has overcome while trying to succeed". As I prepare to complete my PhD and leave The University of Michigan after six years, I reflect on the journey that brought me here, the experiences that shaped my time as a PhD student, and most importantly the people who were there along the way. I have been fortunate to have many people guiding and supporting me, and I am FOREVER indebted to them all.

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LIST OF ABBREVIATIONS

˚C	degrees Celsius
%	percent
ACh	acetylcholine
ANOVA	analysis of variance
ARAS	ascending reticular activating system
B6	C57BL/6J
BDZ	benzodiazepine
BRA	benzodiazepine receptor agonist
df	degrees of freedom
EEG	electroencephalogram
EMG	electromyogram
FFT	Fast Fourier transform
GABA	γ-aminobutyric acid
h	hour(s)
HPLC/ED	high performance liquid chromatography with electrochemical detection
Hz	hertz
i.p.	intraperitoneal
kDa	kilo Dalton

KO	knockout
LDT	laterodorsal tegmental
min	minute(s)
μm	micromolar
mM	millimolar
ng	nanogram
nl	nanoliter
NREM	non-rapid eye movement
p	probability
pmol	picomoles
PnO	pontine reticular nucleus, oral part
PPT	pedunculopontine tegmental
PRF	pontine reticular formation
REM	rapid eye movement
s	second(s)
SEM	standard error of the mean

CHAPTER 1

INTRODUCTION AND STATEMENT OF RESEARCH QUESTION

States of sleep and anesthesia are defined by specific traits. Sleep is a *naturally* occurring behavioral state characterized by a decrease in voluntary body movement, an unawareness of surroundings, and an increased threshold to sensory stimulation. Anesthesia on the other hand is a *pharmacologically induced* reversible state with characteristics of amnesia, analgesia, unconsciousness, immobility, and blunted autonomic responses. One commonality between sleep and anesthesia is that some of the drugs that are used to produce sleep, sedation, or general anesthesia act to enhance transmission at $GABA_A$ receptors. Understanding the cellular and molecular mechanisms through which both sleep and anesthesia occur can provide insights into each of their specific traits (Lydic, 2001; Lydic and Baghdoyan, 2006; Watson et al., 2009). Teasing out these mechanisms can provide relief for the burdens that arise from the disorders associated with sleep or the complications that arise from anesthesia through the rational design of therapeutically selective drugs.

Insomnia is a sleep disorder that affects 10-15% of the United States population (Drake et al., 2003) and is characterized by difficulty initiating and/or maintaining sleep despite ample time spent in bed. Insomnia can have severe consequences including fatigue, social impairment, and daytime accidents. These consequences result in large

direct and indirect costs occurring from such things as medical care and absenteeism from work. The economic burden of insomnia in the US based on direct costs alone in the 1990s was over 13.9 billion dollars, and indirect costs added in during this time period led to totals exceeding 35 billion dollars, with the cost continuing to rise sharply in the last decade (Walsh and Engelhardt, 1999; Ozminkowski et al., 2007). Current pharmacological treatments for insomnia include benzodiazepine receptor agonists (BRAs), which enhance transmission at $GABA_A$ receptors. Although these drugs improve sleep by increasing overall sleep time, some BRAs produce undesirable side effects such as reduced slow wave sleep and decreased rapid eye movement (REM) sleep (Mendelson, 2005), making the need for rational drug design increasingly important.

Anesthesia is administered to millions of patients each year in the United States, despite little being known about the mechanisms by which these drugs produce their effects or side effects. Postoperative nausea and vomiting is the most common anesthetic complication, and it occurs in 30-50% of all surgical patients (Gundzik, 2008; Conway, 2009). In the elderly population, postoperative delirium following anesthesia is a common occurrence, observed in anywhere from 5-15% of patients (Silverstein et al., 2007; Sieber, 2009). It is also estimated that each year between 0.007% and 0.13% of patients administered general anesthesia experience some type of awareness (Sebel et al., 2004; Pollard et al., 2007), and 1 in 250,00 die as a direct result of anesthesia (Lydic, 2001). Further understanding of the mechanisms through which both sleep and anesthesia produce their effects can contribute to the safer use of drugs to treat sleep disorders and produce anesthesia (Lydic, 2001).

Both sleep and anesthesia are modulated by a number of neurotransmitters in different brain regions. Even though the two states have their own distinctive traits, anesthetics act through some of the same neural circuitry and brain regions that generate sleep (Lydic and Baghdoyan, 2006; Ishizawa, 2007; Franks, 2008; Watson et al., 2009). Studies have demonstrated the brain region selective effects of γ-aminobutyric acid (GABA), and increase of GABAergic transmission in the pontine reticular formation (PRF) has been shown to promote wakefulness and suppress sleep (Xi et al., 1999; Vanini et al., 2008; Watson et al., 2008). In an attempt to characterize how multiple brain regions and neurotransmitters work together to regulate states of arousal, it is necessary to examine the individual contributions of these neurotransmitters and brain regions in the regulation of arousal states. This dissertation research focused on the PRF and two of the neurotransmitters in the PRF associated with arousal state control, GABA and acetylcholine (ACh). The introduction begins with a discussion of the PRF and its role in both sleep and anesthesia. There is consideration of neurotransmitter regulation of state, with a focus on GABA and ACh. After that is a brief examination of the technical approaches and limitations of the dissertation studies in the context of the results. The chapter closes with an overview of the dissertation objectives and results.

THE ROLE OF THE PONTINE RETICULAR FORMATION IN AROUSAL STATE CONTROL

The pontine reticular formation contributes to the generation of arousal states

The functional concept of the PRF became apparent with the pioneering work of Moruzzi and Magoun in 1949, who proposed the existence of a neuronal network responsible for the control of arousal states that they named the ascending reticular activating system (ARAS) (Moruzzi and Magoun, 1949). This system of neurons and neural fibers, including the reticular formation, contributes to the regulation of states of arousal such as sleep, wakefulness, and anesthesia. The PRF, one of the phylogenetically oldest portions of the brain, is made up of cells of varying sizes and shapes and is surrounded by networks of fibers that run in all directions (Rossi and Brodal, 1956). Within the PRF there are two main divisions, the pontine reticular nucleus, oral part (PnO) and the pontine reticular nucleus, caudal part (PnC). The PRF contains $GABA_A$ receptors, GABA inter-neurons, and glutamatergic neurons, and receives cholinergic input from the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei (Kosaka et al., 1988; Mitani et al., 1988; Shiromani et al., 1988; Jones, 1990; Ford et al., 1995; Pirker et al., 2000; de la Roza and Reinoso-Suarez, 2006; Rodrigo-Angulo et al., 2008). Work by Jouvet and others highlighted the pons as a brain region that contributes to arousal state control and is key for REM sleep generation (Jouvet, 1962; Lavie et al., 1984; Webster and Jones, 1988; Reinoso-Suarez et al., 2001).

Studies of the pontine reticular formation enhance the overall knowledge of sleep and anesthesia

The fields of sleep and anesthesia have benefited tremendously from research aimed at understanding the different brain regions involved in the regulation of arousal states. Early studies focused at the level of the PRF used brain transections to establish the role of this region in the generation of non-REM (NREM) sleep and REM sleep (Jouvet, 1962; Carli and Zanchetti, 1965; Jouvet, 1965). In addition, the Reciprocal Interaction Model conceptualized the neural mechanisms generating states of wakefulness and sleep using both structural and mathematical models (McCarley and Hobson, 1975). This model hypothesized a shift between NREM sleep and REM sleep based on the reciprocal connections of pontine structures (Hobson et al., 1975). The mathematical model is unique in that it has predictive value. Pontine lesion studies in both humans (Lavie et al., 1984; Webster and Jones, 1988; Kushida et al., 1991; Gironell et al., 1995; Kimura et al., 2000; Reinoso-Suarez et al., 2001) and animals (Webster and Jones, 1988) demonstrate a disruption in REM sleep, validating Jouvet's findings. These studies prompt further research examining the role of pontine neurotransmitters in the generation of arousal states, particularly REM sleep.

NEUROTRANSMITTER REGULATION OF SLEEP AND WAKEFULNESS

GABAergic modulation of sleep and wakefulness

The main inhibitory neurotransmitter in the mammalian central nervous system is GABA, which plays a vital role in regulating neuronal excitability throughout the entire nervous system. GABA was discovered in large amounts in the brain in 1950 by three different groups of researchers (Awapara et al., 1950; Roberts and Frankel, 1950; Udenfriend, 1950). Studies for the next 20 years studied the inhibitory actions of GABA as it related to physiological processes in the brain, until the 1970s and 1980s when the focus shifted to identifying and characterizing the receptors upon which GABA acts (Krnjevic, 2004; Bowery and Smart, 2006). There are two types of transmembrane receptors with GABA as their endogenous ligand, $GABA_A$ and $GABA_B$, with $GABA_A$ receptors also having a subclass of receptors known as $GABA_A$-ρ receptors (formerly $GABA_C$ receptors) (Olsen and Sieghart, 2008).

The focus for these dissertation studies was on $GABA_A$ receptors because of their direct link to both sleep and anesthesia. $GABA_A$ receptors were discovered toward the end of the 1980s and classified as ionotropic receptors based on their ability to open their channel upon binding of the ligand GABA. These receptors consist of five protein subunits that surround a central pore preferentially permeable to negative chloride ions, and there are multiple subunit combinations and binding sites for the receptor. Generally speaking, drugs acting as agonists or allosteric modulators of $GABA_A$ receptors typically

have anticonvulsant, anxiolytic, amnesic, sedative, or hypnotic effects. These properties are useful for both anesthesia and sleep.

GABA and its inhibitory neurotransmitter actions have been defined and characterized extensively over the last 60 years, and more recently studies have begun to investigate the role GABA plays in specific physiological processes such as arousal state. Administration of $GABA_A$ receptor agonists to brain regions such as the posterior hypothalamus, locus coeruleus, medial preoptic area, and the dorsal raphe nucleus increases sleep (Mallick et al., 2001; Tung et al., 2001; Baghdoyan and Lydic, 2002), whereas in the PRF increasing GABAergic transmission increases wakefulness (Camacho-Arroyo et al., 1991; Xi et al., 1999; Sanford et al., 2003). Increasing GABA levels in the PRF with the GABA reuptake inhibitor nipecotic acid increases wakefulness (Watson et al., 2008). These studies provide support for analyzing neurotransmitter effects on arousal states in a brain region specific manner. Doing so will allow for a greater understanding of the mechanisms involved in regulating both sleep and anesthesia and may lead to rational therapeutic drug design.

$GABA_A$ receptors as binding sites for drugs that produce sleep and anesthesia

Some anesthetics and pharmacological treatments for insomnia are known to act on $GABA_A$ receptors to produce their effects, as mentioned above. Anesthetics are used to produce a reversible state characterized by five traits: analgesia, amnesia, hypnosis, paralysis, and obtundation of reflexes. Anesthetics act on a multitude of receptors, most importantly for these dissertation studies are those working at $GABA_A$ receptors (Bonin and Orser, 2008). Most of these anesthetics act as allosteric modulators which potentiate

the effects of GABA at $GABA_A$ receptors and include barbiturates, etomidate, propofol, and volatile anesthetics. Benzodiazepines (BDZs), which will be discussed in further detail below, are also routinely used in the practice of anesthesia. In clinical settings anesthetics act in numerous ways to induce anesthesia, maintain anesthesia, or produce conscious sedation (Grasshoff et al., 2006). Many studies have been done analyzing how these drugs work when administered systemically and distribute throughout the brain. However, it is also necessary to examine the brain region specific effects of these anesthetics in order to elucidate their mechanisms of action. Injection of propofol or pentobarbital into the medial preoptic area of the rat elicited significant decreases in sleep latency and increases in NREM sleep (Mendelson, 1996; Tung et al., 2001), suggesting potential sites of action for these drugs. More pre-clinical studies investigating the role of anesthetics that modulate GABAergic transmission are needed to further understand the brain regions and mechanisms that regulate anesthetic effects.

To produce the anxiolysis and hypnosis important for anesthesia, BDZs are often employed. These drugs are also widely used in the treatment of conditions ranging from seizures to muscle spasms, and most importantly for these dissertation studies, as a pharmacological treatment for anxiety and insomnia. Similar to the anesthetics described above, BDZs act as allosteric modulators of the $GABA_A$ receptor. Non-BDZs, which are structurally different from the classic BDZs, also act as allosteric modulators at the same binding site as BDZs, referred to as BRAs, and are commonly used to treat insomnia (Gottesmann, 2002). There are numerous BDZs used today in the practice of anesthesia, midazolam, diazepam, and lorazepam being the most common (White and Eng, 2009). For the treatment of insomnia, many drugs are prescribed "off-label", but currently

approved drugs for the treatment of the disorder include the BDZs estazolam, flurazepam, quazepam, temazepam, and triazolam, and the BRAs zaleplon, zolpidem, and eszopiclone (2005).

In the treatment of insomnia BRAs work to decrease the latency to sleep onset, decrease the time spent awake after sleep onset, and increase total sleep time. As with anesthetics, studies have been performed investigating the effects of systemic BRA administration. Intraperitoneal (i.p.) injections of lorazepam in mice significantly increase NREM sleep while reducing motor activity (Tang et al., 2009). Eszopiclone and zolpidem administered i.p. to guinea pigs also produce significant increases in NREM sleep (Xi and Chase, 2008). Pre-clinical studies have begun studying the role of BRAs in various brain regions. Interestingly, microinjection of triazolam into the ventrolateral preoptic area of rat produced no significant effect on sleep (Mendelson, 1999), in contrast to the medial optic area where triazolam produced a significant increase in NREM sleep (Mendelson et al., 1989; Mendelson and Martin, 1992) and the dorsal raphe nucleus where triazolam increased sleep latency and decreased NREM sleep (Mendelson et al., 1987). This suggests a possible role for the medial optic area in the generation of NREM sleep following BDZ administration and further supports the need for brain region specific studies in efforts to understand where anesthetic and arousal state effects are mediated.

Acetylcholine modulates arousal states

Another neurotransmitter important in the PRF and for this dissertation work is ACh. ACh is found in both the peripheral and central nervous systems. This neurotransmitter holds great significance as the first to be identified (1914) and confirmed (1921) by Henry Dale and Otto Loewi. This discovery led them to share the 1936 Nobel Prize in Physiology or Medicine. After over 60 years of research aimed at understanding this neurotransmitter and chemical transmission, focus in the 1980s turned to the identification and characterization of the two types of acetylcholine receptors, nicotinic and muscarinic (Brown, 2006). Nicotinic receptors are ionotropic receptors that are stimulated by both ACh and nicotine, whereas muscarinic receptors are metabotropic receptors stimulated by both ACh and muscarine. There are five molecularly distinct types of muscarinic acetylcholine receptors which are G protein-coupled, M_1-M_5 (Fukuda et al., 1987; Brown, 2006). M_1, M_3, and M_5 receptors are $G_{q/11}$-coupled and M_2 and M_4 are $G_{i/o}$-coupled (Caulfield and Birdsall, 1998). ACh and its receptors play a key role in regulating states of arousal (Lydic and Baghdoyan, 2005, 2008).

Within the brain there are two main populations of cholinergic projection neurons, those in the basal forebrain and those in the LDT/PPT (Kimura et al., 1981; Woolf and Butcher, 1986), the latter and its contribution to arousal being important for this dissertation work. The LDT/PPT provides cholinergic input to the PRF (Jones and Beaudet, 1987). Administration of a muscarinic antagonist to normal humans increases REM sleep latency (Sagales et al., 1969). Pre-clinical and clinical studies using cholinergic agonists and acetylcholinesterase inhibitors decrease the latency to REM sleep, increase the duration of REM sleep, and increase total REM sleep time (Domino et

al., 1968; Sitaram et al., 1976; Sitaram et al., 1978), supporting the role of ACh in REM sleep.

Brain region specific studies have helped to identify areas playing a role in cholinergic regulation of REM sleep. Microinjecting cholinomimetics into the PRF produced a REM sleep-like state (Baghdoyan and Lydic, 2002; Lydic et al., 2002; Coleman et al., 2004b; Douglas et al., 2005), and microdialysis studies demonstrated significant increases in ACh release during REM sleep (Leonard and Lydic, 1997) and the REM sleep-like state (Lydic et al., 1991). Studies using specific agonists and antagonists for muscarinic receptors have supported the role of ACh in REM sleep. M_1, M_2, and M_3 type muscarinic receptors have been localized to the PRF (Baghdoyan et al., 1994a; Baghdoyan et al., 1994b; Mallios et al., 1995; Baghdoyan, 1997), and animal studies have implicated M_2 and possibly M_3 type receptors in the PRF contributing to REM sleep (Imeri et al., 1994; Sakai and Onoe, 1997; Baghdoyan and Lydic, 1999; Coleman et al., 2004b, a). All of these data are consistent with the interpretation that cholinergic transmission regulates REM sleep.

TECHNICAL APPROACHES AND CONSIDERATIONS

The mouse model

It is known that 99% of mouse genes have homologues in humans (Waterston et al., 2002), making the mouse an excellent resource for identifying neuropharmacological mechanisms and molecular targets for rational drug design (Watters and McLeod, 2002). Understanding the relationship between genotype and sleep phenotype (Valatx et al.,

1972; Toth, 2001; Tafti and Franken, 2002) is essential to the future understanding and treatment of sleep disorders. This dissertation work used the C57BL/6J (B6) mouse as a tool to elucidate brain region specific effects of GABAergic transmission on arousal states (Lydic et al., 2002; Coleman et al., 2004b; Douglas et al., 2005; Coleman et al., 2006; Van Dort et al., 2009). The major advantage of the B6 mouse is that it is the most widely used inbred mouse strain, and it is the first to have its genome sequenced (Waterston et al., 2002). Research studies using this mouse strain have studied everything from behavior and learning to drug addiction and cancer (Bursch et al., 2004; Singh et al., 2007).

The present dissertation work provides background for future sleep studies utilizing genetically altered mouse strains in an attempt to identify specific receptors and neurotransmitters involved in the physiological processes underlying sleep. Future studies could involve conditional knockout (KO) mice, which have a specific gene, neuron, or channel inactivated, and would allow for functional studies of targets involved in regulating arousal states based on comparisons of observed differences from this dissertation work. Conditional KO mice have provided novel insights into cancer, cardiovascular disease, anxiety, and obesity, and have provided advancement in both the biomedical and pharmaceutical arenas (Rudmann and Durham, 1999).

The field of sleep has also been furthered by the use of conditional KO mice. Conditional KO mice are a powerful tool for understanding sleep as well as its disorders, providing insight into the function of specific neurons and neuronal systems. Orexin/ataxin-3 transgenic mice (Chou et al., 2001; Hara et al., 2001) have been used to study orexinergic involvement in the circadian control of sleep and wakefulness (Kantor

et al., 2009) as well as narcoleptic sleep/wake fragmentation in relation to metabolism (Zhang et al., 2007). The role of calcium channels in the blockade of arousal signal transmission was studied in thalamic-$Ca_v3.1$ KO mice (Anderson et al., 2005). The present dissertation work used intracranial drug administration to examine the neurochemical mechanisms modulating states of arousal. These studies set the stage for future work using these same methods in different transgenic mouse models.

Measures of arousal

Electroencephalogram: The electroencephalogram (EEG) is established as the primary tool used in evaluating sleep in both humans and animals. An EEG is a recording of the electrical activity of the brain resulting from neuronal firing, which can be analyzed to determine sleep stages based on classically characterized traits. Neurons fire relatively fast during the active states of wakefulness and REM sleep. This firing produces desynchronized waves that are low in voltage and not consistent in pattern. During NREM sleep, also known as slow wave sleep, neurons fire at slower rates. Brain activity during NREM sleep is slow in frequency with large amplitude waves which are synchronized and more consistent in pattern (McCormick and Bal, 1997; Steriade, 2006; Miller, 2007).

In addition to the EEG signal, states of arousal can also be characterized by analyzing a breakdown of the EEG signal into its many waveform components using a Fast Fourier transform (FFT). An FFT is a mathematical algorithm designed to extract information from signals, in this case EEG signals, and transform the signals from the time domain to the frequency domain (Mager and Abernethy, 2007). The application of

the FFT to sleep studies allows for the EEG signals to be broken down into different waveforms with varying frequencies (Dumermuth and Fluhler, 1967). These waveform components are correlated with various states of arousal when quantified as power for a given frequency (Muthuswamy and Thakor, 1998; Campbell, 2009). Behavioral EEG characteristics are the same in both animals and humans, making it possible to correlate behavioral arousal in animals to arousal in humans. The present dissertation studies used EEG signals recorded from mice to objectively characterize states of wakefulness, NREM sleep, and REM sleep following drug administration. The FFT was analyzed along with the EEGs to help in characterizing arousal states and to study the effects of drug on quality of sleep. There were two main waveforms analyzed in this dissertation, delta waves and theta waves. Delta waves occur during NREM sleep (restorative sleep) and are between the frequencies of 0.5 and 4 Hz. REM sleep has dominant theta waves with frequencies between 4 and 9 Hz.

Recovery of Righting Response: The recovery of righting response is widely used as an established measure of wakefulness following administration of commonly used drugs such as anesthetics, opioids, and alcohol in humans (Straw and Mitchell, 1967; Kissin et al., 1993; York and Chan, 1993; Ajadi et al., 2009) as well as rodents (Bignall, 1974; Tung et al., 2002; Demarco et al., 2004; Kelz et al., 2008; Van Dort et al., 2009). For these dissertation studies mice were placed in dorsal recumbency under a heated lamp and the time (in minutes) required for a mouse to resume a normal upright posture was recorded. This recovery of righting response was used as a measure of wakefulness following microdialysis delivery of the $GABA_A$ receptor antagonist bicuculline to the PnO.

OVERVIEW OF RESEARCH OBJECTIVES

The unifying goal of this dissertation research was to elucidate the neurochemical mechanisms by which $GABA_A$ receptors in the PRF of B6 mouse modulate wakefulness and sleep. The PRF is part of the ARAS and plays an important role in generating REM sleep (Steriade and McCarley, 2005). Administration of GABAergic drugs to the PRF produces alterations in sleep and wakefulness (Lydic and Baghdoyan, 2005). Administration of the $GABA_A$ receptor agonist muscimol into cat PRF or the oral portion of rat PRF (PnO) increases wakefulness and decreases sleep (Camacho-Arroyo et al., 1991; Xi et al., 1999; Sanford et al., 2003). Microinjection of the $GABA_A$ receptor antagonist bicuculline into the same brain region of cat or rat causes an increase in REM sleep (Xi et al., 1999; Sanford et al., 2003). Microinjection of the GABA uptake inhibitor nipecotic acid (NPA) or the GABA synthesis inhibitor 3-mercaptopropionic acid (3-MPA) into rat PnO, respectively, increases or decreases wakefulness (Watson et al., 2008). GABA levels in cat PRF decrease during the isoflurane-induced loss of wakefulness, and microinjection of NPA or 3-MPA into rat PnO increases or decreases, respectively, isoflurane induction time (Vanini et al., 2008). Collectively, these data support the interpretation that GABAergic transmission in the PRF promotes wakefulness. Results from the mouse can be compared to results from other species in order to determine how the mouse is similar to or different from previous work. The studies done in mouse can help to provide further insight into the mechanisms regulating arousal.

Within the PRF, GABAergic and cholinergic neurons interact to modulate sleep and wakefulness. The PRF contains GABAergic neurons inter-neurons (Ford et al.,

1995) and $GABA_A$ receptors (Heldt and Ressler, 2007). The PRF is also cholinoceptive (Baghdoyan, 1997), receiving ACh from the more rostrally and dorsally located LDT and PPT nuclei (Steriade and McCarley, 2005). Glutamatergic neurons that project to the LDT/PPT originate in the PRF (Steininger et al., 1992). Electrical stimulation of LDT/PPT neurons increases both REM sleep (Thakkar et al., 1996) and ACh release in the PRF (Lydic and Baghdoyan, 1993). The discharge rate of LDT/PPT neurons increases prior to and during REM sleep (Kayama et al., 1992). In the opposite manner, neurotoxic lesions of the LDT/PPT decrease REM sleep (Webster and Jones, 1988). Cholinomimetics administered to the PRF increase REM sleep (Baghdoyan and Lydic, 1999) by activating M_2 muscarinic receptors (Coleman et al., 2004b) putatively localized to GABAergic neurons in the PRF (Brown et al., 2008). Muscarinic autoreceptors also modulate ACh release presynaptically in the PRF (Baghdoyan et al., 1998; Coleman et al., 2004a). Bicuculline administered to the PRF increases ACh release within the PRF (Vazquez and Baghdoyan, 2004) and ACh in the PRF increases during REM sleep (Leonard and Lydic, 1997). The mechanisms by which enhancing transmission at GABA receptors in the PRF increases wakefulness are largely unknown but may involve cholinergic transmission from the LDT/PPT and GABAergic inhibition in the PRF. The following dissertation research aims were designed to provide insight into the mechanisms by which GABAergic and cholinergic neurotransmission in the PRF modulate sleep and wakefulness.

Specific Aims

Specific Aim 1 tested the hypotheses that microinjection of the $GABA_A$ receptor agonist muscimol into the PnO concentration dependently increases wakefulness and decreases sleep, and that co-administration of the $GABA_A$ receptor antagonist bicuculline blocks the increase in wakefulness and decrease in sleep caused by muscimol alone. Aim 1 also tested the hypothesis that bicuculline microinjected into the PnO causes a concentration dependent decrease in wakefulness and increase in sleep. The results of Aim 1 are presented in Chapter 2. Aim 1 used mice implanted with microinjection guide tubes stereotaxically aimed for the PnO as well as EEG and electromyogram (EMG) recording electrodes. Muscimol, bicuculline, or muscimol and bicuculline were microinjected into the PnO of the freely moving mouse. EEG and EMG signals were recorded for 4 h post-injection prior to analysis of dependent measures of sleep and wakefulness. In addition, EEG power was analyzed during states of wakefulness, NREM sleep, and REM sleep. Four concentrations (0.5, 5, 50, and 500 pmol/50 nl) of muscimol were studied along with the vehicle control saline. These studies were done in order to determine if $GABA_A$ receptors in the PnO modulate states of wakefulness and sleep.

Microinjection of muscimol into the PnO of B6 mouse caused a significant, concentration dependent increase in the amount of wakefulness and decrease in the amount of NREM sleep and REM sleep compared to the vehicle control saline. Administration of muscimol also caused a significant, concentration dependent increase in the latency to the onset of both NREM sleep and REM sleep and decrease in the

number of episodes of wakefulness, NREM sleep, and REM sleep. Muscimol caused a significant, concentration dependent decrease in the number of transitions between states of wakefulness, NREM sleep, and REM sleep, and an increase in the average duration of wakefulness episodes. Muscimol increased EEG delta power during wakefulness and EEG theta power during REM sleep, with no effect on EEG power during NREM sleep.

Co-administration of muscimol (50 pmol/nl) and bicuculline (5 pmol/50 nl) was performed to determine whether the muscimol-induced effects on sleep could be antagonized. Co-administration of bicuculline with muscimol blocked the muscimol-induced increase in the amount of wakefulness, the decrease in the amount of NREM sleep, and the decrease in the amount of REM sleep. Muscimol co-administered with bicuculline blocked the increase in NREM sleep latency and partially blocked the increase in REM sleep latency. Co-administration of bicuculline and muscimol blocked the muscimol-induced decrease in the number of episodes of wakefulness, NREM sleep, and REM sleep, as well as the decrease in the number of transitions and the increase in the average duration of wakefulness. The results support the interpretation that $GABA_A$ receptors in the PRF promote wakefulness.

In order to determine whether endogenous GABA within the PnO modulates sleep, EEG and EMG recordings were obtained after microinjecting three concentrations (0.5, 5, and 50 pmol/50 nl) of bicuculline. Bicuculline caused a significant, concentration dependent decrease in wakefulness and increase in both NREM sleep and REM sleep when compared with the vehicle control saline. There were no alterations in EEG power during any state following PnO administration of bicuculline. The results are consistent with the interpretation that $GABA_A$ receptors in the PnO promote wakefulness. Taken

together, these Aim 1 results demonstrate that PnO $GABA_A$ receptors promote wakefulness.

Specific Aim 2 tested the hypotheses that $GABA_A$ receptors in the PnO of B6 mouse modulate ACh release in the PnO, respiratory rate, and time to recovery of righting following isoflurane anesthesia. The results of Aim 2 are reported in Chapter 2. The behavioral effects of GABA are known to be brain region specific, and selectively increasing endogenous GABA levels in the PRF promotes wakefulness and suppresses sleep (Vanini et al., 2008; Watson et al., 2008). In both cat and rat, blockade of $GABA_A$ receptors in the PRF causes an increase in REM sleep (Xi et al., 1999; Sanford et al., 2003; Marks et al., 2008). In cat, blockade of $GABA_A$ receptors in the PRF also causes an increase in PRF ACh release (Vazquez and Baghdoyan, 2004). ACh in the PRF may trigger REM sleep by inhibiting GABAergic wakefulness promoting neurons via M_2 muscarinic receptor activation (Baghdoyan and Lydic, 1999; Coleman et al., 2004b; Brischoux et al., 2008; Brown et al., 2008). Cholinergic transmission in the PRF promotes REM sleep, and the increase in REM sleep caused by antagonizing $GABA_A$ receptors can be blocked by antagonizing muscarinic cholinergic receptors (Marks et al., 2008). Taken together, these data suggest that GABA in the PRF inhibits REM sleep by inhibiting ACh release in the PRF (Vazquez and Baghdoyan, 2004).

The Aim 1 results reviewed above support the interpretation that GABAergic transmission in the PnO promotes wakefulness. In order to analyze the mechanisms by which $GABA_A$ receptors in mouse PnO modulate states of arousal, Aim 2 examined ACh release in the PnO. Aim 2 studies were performed using microdialysis delivery of

bicuculline to the PnO of the isoflurane-anesthetized B6 mouse while simultaneously measuring ACh release in the PnO. Dependent measures were ACh release in the PnO, respiratory rate during anesthesia, and time to recovery of righting following isoflurane anesthesia. A concentration response curve was generated for the effects of bicuculline on ACh release, respiratory rate, and recovery time using the vehicle control Ringer's and five concentrations of bicuculline (0.1, 0.3, 1, 3, and 10 mM).

Bicuculline caused a significant, concentration dependent increase in ACh release in the PnO and increase in anesthesia recovery time compared to Ringer's. Respiratory rate was concentration dependently decreased during dialysis with bicuculline. Taken together with the results from Aim 1, the present data supports the interpretation that $GABA_A$ receptors in the PnO of B6 mouse modulate sleep and wakefulness, ACh release in the PnO, behavioral arousal, and breathing rate. The finding that ACh release in the PnO is modulated by $GABA_A$ receptors in the PnO is consistent with the interpretation that the blockade of GABAergic transmission in the PnO leads to decreases in wakefulness and increases in sleep, in part, by increasing ACh release.

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CHAPTER 2

$GABA_A$ RECEPTORS IN THE PONTINE RETICULAR FORMATION OF C57BL/6J MOUSE MODULATE NEUROCHEMICAL, ELECTROGRAPHIC, AND BEHAVIORAL PHENOTYPES OF WAKEFULNESS

SUMMARY

Drugs that potentiate transmission at gamma-aminobutyric $acid_A$ ($GABA_A$) receptors are widely used to produce sleep and general anesthesia. The mechanisms underlying these effects are unknown. This study tested the hypothesis that $GABA_A$ receptors in mouse pontine reticular nucleus, oral part (PnO) modulate five phenotypes of arousal: sleep and wakefulness, cortical electroencephalogram (EEG) activity, acetylcholine (ACh) release in the PnO, breathing rate, and recovery time from general anesthesia. PnO microinjections of saline (vehicle control), muscimol, muscimol with bicuculline, and bicuculline alone were performed in C57BL/6J (B6) mice (n = 33) implanted with EEG recording electrodes. Muscimol caused a significant increase in wakefulness and decrease in rapid eye movement (REM) sleep and non-REM (NREM) sleep. These effects were reversed by co-administration of bicuculline. Bicuculline alone caused a significant decrease in wakefulness and increase in NREM sleep and REM sleep. Muscimol significantly increased EEG power in the delta range (0.5-4 Hz) during wakefulness and in the theta range (4-9 Hz) during REM sleep. Dialysis delivery of bicuculline to the PnO of isoflurane-anesthetized B6 mice (n = 18) caused a significant increase in ACh release in the PnO, breathing rate, and anesthesia recovery time. All

drug effects were concentration dependent. These data support the conclusion that one function of $GABA_A$ receptors in the PnO of B6 mouse is to promote wakefulness. Increasing GABAergic transmission in the PnO may be one mechanism by which some hypnotics produce state dissociations.

INTRODUCTION

Sleep is a fundamental biological process and disorders of sleep and wakefulness are now recognized to have a major negative impact on human health and productivity (Walsh and Engelhardt, 1999; Leger et al., 2002; Ting and Malhotra, 2005; Wilson, 2005; Colten et al., 2006). Because sleep is characterized by similar physiological traits in most mammals, animal models are of key importance in understanding the neurochemical mechanisms underlying normal as well as disordered human sleep. Sleep is a heritable phenotype in human (Katzenberg et al., 1998; Toh et al., 2001) and mouse (Franken et al., 1999; Franken et al., 2001), and all human genes have mouse homologues (O'Brien et al., 1999; Bradley, 2002). The mouse facilitates identifying genetic mechanisms underlying mammalian physiology and pathophysiology, and permits direct manipulation of the genome to create disease phenotypes (Paigen and Eppig, 2000).

Sleep and wakefulness are generated by complex interactions between many brain regions, neurotransmitters, and neuromodulators (Hobson and Pace-Schott, 2002; Lydic and Baghdoyan, 2005; Datta and Maclean, 2007; Stenberg, 2007). In humans, drugs that enhance the actions of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) cause sleep (Winsky-Sommerer, 2009), sedation, or general anesthesia (Franks, 2008). Preclinical studies using intracranial drug administration demonstrate

that GABAergic drugs promote either sleep or wakefulness, depending upon site of drug administration within the brain (Lin et al., 1989; Sallanon et al., 1989; Sastre et al., 1996; Kaur et al., 1997; Nitz and Siegel, 1997; Ali et al., 1999; Xi et al., 1999; Manfridi et al., 2001; Boissard et al., 2002; Pollock and Mistlberger, 2003; Sanford et al., 2003; Vanini et al., 2007; Watson et al., 2008; Pal and Mallick, 2009; Sapin et al., 2009).

The pontine reticular formation is a component of the ascending reticular activating system and contributes to the generation of cortical electroencephalogram (EEG) activation and rapid eye movement (REM) sleep (Lydic and Baghdoyan, 2005; Steriade and McCarley, 2005). Pharmacological enhancement of GABAergic transmission in the pontine reticular formation of cat and rat increases wakefulness and decreases sleep (Camacho-Arroyo et al., 1991; Xi et al., 1999; Sanford et al., 2003; Marks et al., 2008; Watson et al., 2008), whereas blocking pontine reticular formation $GABA_A$ receptors decreases time spent in wakefulness and increases time spent in REM sleep (Xi et al., 1999; Sanford et al., 2003; Marks et al., 2008). Increasing GABAergic transmission in the PnO also lengthens anesthesia induction time, and endogenous GABA levels in the PnO are greater during wakefulness than during anesthesia (Vanini et al., 2008)] or sleep (Vanini et al., 2009). Acetylcholine (ACh) in the pontine reticular formation promotes REM sleep (Lydic and Baghdoyan, 2008), and direct administration of bicuculline to cat pontine reticular formation increases ACh release and triggers the onset of REM sleep (Vazquez and Baghdoyan, 2004). Taken together, these pharmacological data support the interpretation that GABAergic transmission within the pontine reticular formation promotes wakefulness, inhibits ACh release, and inhibits REM sleep.

No previous studies have determined whether GABAergic transmission in mouse PnO modulates states of behavioral arousal or traits that characterize these states. The present study used in vivo microinjection and microdialysis to test the hypothesis that $GABA_A$ receptors in the PnO of C57BL/6J (B6) mouse modulate sleep and wakefulness, cortical EEG activity, ACh release in the PnO, rate of breathing, and recovery time from general anesthesia. Microinjection experiments conducted in awake mice were designed to determine whether PnO microinjection of muscimol increases wakefulness and decreases sleep, and whether bicuculline increases REM sleep and decreases wakefulness. These experiments also investigated whether administering muscimol and bicuculline directly into the PnO alters EEG power. Microdialysis experiments performed with isoflurane anesthetized mice aimed to determine whether PnO delivery of bicuculline increases ACh release in the PnO and alters breathing and anesthesia recovery time. All responses were predicted to be concentration dependent. The B6 mouse was selected for these studies because its genome has been sequenced (Waterston et al., 2002), it is one of the strains that has been recommended for phenotyping by the Phenome Committee of the Jackson Laboratory (www.jax.org/phenome) (Paigen and Eppig, 2000), and it serves as a background strain for many genetically modified mice (Silver, 1995). Preliminary reports of these data have been published (Flint et al., 2007; Flint et al., 2007; Flint et al., 2008; Flint et al., 2009; Flint et al., 2009).

METHODS

Animals. Experiments were approved by the University of Michigan Committee on Use and Care of Animals and conducted in accordance with the U.S. Department of Agriculture Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Institutes of Health Publication 80-23, National Academy of Sciences Press, Washington DC, 1996). Adult male B6 mice (25-30 g, n = 51) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed in a humidity controlled facility under constant light. Mice had ad libitum access to food and water, and were kept for a minimum of one week before being used for experiments.

Surgical procedures for implantation of microinjection guide tubes and recording electrodes. Mice were anesthetized with 2-3% isoflurane (Abbott Laboratories, North Chicago, IL, USA) delivered in 100% oxygen at a flow rate of 1 l/min. Once unconscious, mice were placed in a stereotaxic frame (Model 962, David Kopf, Tujunga, CA, USA) fitted with a mouse adaptor (Model 921) and mouse anesthesia mask (Model 907). Delivered isoflurane concentration was reduced to 1.5% and a flow rate of 0.5 l/min. The concentration of isoflurane delivered to the anesthesia mask was measured continuously by spectrophotometry (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA). Core body temperature was maintained at 36-37°C using a heating pad filled with continuously circulating hot water (TP400 T/Pump Heat Therapy System, Gaymar, Orchard Park, NY, USA). One 26 gauge stainless steel guide tube (Cannula Guide # C315GS-4-SPC, Plastics One, Roanoke, VA, USA) occluded by a stylet (Dummy Cannula # C315DCS-4-SPC, Plastics One) was implanted 3 mm dorsal to the PnO at

stereotaxic coordinates 4.24 mm caudal to bregma, 0.8 mm lateral to the midline, and 1.5 mm ventral to bregma (Paxinos and Franklin, 2001). Three electrodes (H-Formvar Wire, 0.005" diameter, California Fine Wire Company, Grover City, CA, USA) for recording the cortical EEG were placed directly under the skull approximately 0.5 mm caudal and 1.5 mm lateral to bregma, 2.0 mm caudal and 1.5 mm lateral to bregma, and 1.0 mm rostral and 1.5 mm lateral to bregma. One pair of electrodes for recording the electromyogram (EMG) (Biomed Bare Braided Wire, 0.12" overall diameter, Cooner Wire, Chatsworth, CA, USA) were inserted into the neck muscles. Recording electrodes terminated in gold pins (# E363-0, Plastics One) that were gathered together and placed into a plastic connector (6-pin Collector Pedestal # MS363, Plastics One). Two stainless steel anchor screws (# 39052, Plastics One) were inserted into the skull and the plastic connector, screws, and guide tube were secured to the skull with dental acrylic (Jet Acrylic Self Curing Resin and Liquid, Lang Dental Manufacturing Co., Inc., Wheeling, IL, USA). Isoflurane delivery was discontinued and mice were kept warm and under continuous observation until ambulatory. Mice were housed individually and allowed one week to recover from surgery.

Intracranial microinjection procedure. Mice were conditioned to being housed in a Raturn recording chamber (Bioanalytical Systems Inc., West Lafayette, IN, USA) where they had ad libitum access to food and water. The conditioning period included handling, removing the stylet from the guide tube, reinserting the stylet to simulate a microinjection, and tethering the mice to a recording cable (Connector Cable System # 363-441/6-150cm-6TC, Plastics One). After one week of conditioning, mice entered the

microinjection protocol. Before each microinjection and recording session, mice spent 18 h in the recording environment to ensure a normal sleep cycle (Tang et al., 2005).

Solutions of muscimol (Sigma Chemical Co., St. Louis, MO, USA) and bicuculline methiodide (Sigma Chemical Co.) were prepared immediately prior to use. Unilateral microinjections (50 nl) were made using a 1 μl Hamilton syringe (Thomas Scientific, Swedesboro, NJ, USA) mounted in a manual microdrive and connected to a 33 gauge microinjector (Internal Cannula # C315IS-4-SPC, Plastics One) via PE-20 tubing (Fisher Scientific, Pittsburgh, PA, USA). Microinjections occurred between 8:00 and 10:00 a.m. and were followed immediately by 4 h of continuous recording. Each mouse received one microinjection of saline (0.9%, vehicle control) and either four concentrations of muscimol (0.5, 5, 50, and 500 pmol/50 nl, corresponding to 0.057, 0.571, 5.71, and 57.1 ng) or three concentrations of bicuculline (0.5, 5, and 50 pmol/50 nl, corresponding to 0.25, 2.5, and 25 ng) for a total of five or four microinjections, respectively, per mouse. A second group of mice each received one microinjection of saline, muscimol (50 pmol), and muscimol (50 pmol) co-administered with bicuculline (5 pmol), for a total of three microinjections per mouse. Microinjection duration was one min. The order of the microinjections was randomized and microinjections in the same mouse were separated by one week.

Recording and objectively identifying states of sleep and wakefulness. EEG and EMG signals were amplified, filtered, digitized at 128 Hz, and scored as previously described (Watson et al., 2007) using Icelus Data Acquisition and Analysis software (Opp, 1998). States of sleep and wakefulness for each 4 h recording were scored in 10 s epochs

according to the following criteria. Wakefulness was characterized by a mixed frequency EEG (0.5-25 Hz) and a relatively high amplitude EMG containing periods of movement artifact. Non-REM (NREM) sleep was scored according to the presence of slow waves in the EEG (0.5-4 Hz), lower EMG amplitude than wakefulness, and lack of movement artifact. REM sleep was identified by a dominant EEG theta rhythm (6-8 Hz) and EMG hypotonia. Sixty five percent of all sleep records were scored by two investigators, one of which was blinded to the treatment condition. Inter-rater reliability between the two scorers was 93%. To construct plots of average EEG power (V_{RMS}), five 1 min intervals of recording time were analyzed for each of the three behavioral states from selected recordings. Fast Fourier Transform analysis identified dominant EEG frequencies in 2 s bins. Frequencies ranging from 0.5 to 25 Hz were analyzed in increments of 0.5 Hz, and five consecutive 2 s bins were averaged for each 10 s epoch.

In vivo microdialysis experiments and quantification of ACh. Microdialysis experiments were performed using isoflurane anesthetized mice (one per experiment) and procedures that have been described in detail (Van Dort et al., 2009). Briefly, mice were placed in a Kopf stereotaxic frame and a CMA/7 microdialysis probe (CMA Microdialysis, Solna, Sweden) was aimed unilaterally for the PnO at coordinates 4.7 mm posterior, 0.8 mm lateral, and 5.4 mm ventral to bregma (Paxinos and Franklin, 2001). The probe was perfused continuously (2 µl/min) with Ringer's solution (147 mM NaCl, 2.4 mM $CaCl_2$, 4 mM KCl, 10 µM neostigmine) followed by Ringer's containing bicuculline methiodide (0, 0.1, 0.3, 1, 3, or 10 mM). Dialysis samples (25 µl) were collected sequentially every 12.5 min. Core body temperature and breathing rate were recorded each time a dialysis

sample was collected. For each experiment, five samples were obtained during dialysis with Ringer's (control) and five samples were acquired during dialysis administration of bicuculline. Only one concentration of bicuculline was tested per experiment, and each concentration of bicuculline was tested in three mice. After collecting the last sample, the dialysis probe was removed from the brain, the scalp incision was closed, isoflurane delivery was discontinued, and mice were placed on their backs under a heating lamp. The time (min) from cessation of isoflurane delivery until the mice righted themselves was recorded. Recovery of righting response is a well recognized measure of time to wakefulness following general anesthesia (Van Dort et al., 2009). Total anesthesia time (min) was defined as the time from onset of induction to cessation of isoflurane delivery and was recorded for each experiment.

Each 25 µl dialysis sample was injected into a high performance liquid chromatography system (Bioanalytical Systems Inc.) immediately after collection. ACh was separated and quantified as previously described (Van Dort et al., 2009). Chromatograms were digitized using ChromGraph software (Bioanalytical Systems) and the amount of ACh in each dialysis sample was calculated using a standard curve based on seven known amounts of ACh ranging from 0.05 to 1.0 pmol.

Recovery of ACh by all dialysis probes was determined in vitro before and after each experiment by placing the probe in a known concentration of ACh and collecting five dialysis samples. There was no significant difference in pre- and post-experimental probe recoveries. This information is important for two reasons. First, changes in ACh measured in vivo can be ascribed to the effects of bicuculline and did not result from intra-experimental changes in the dialysis membrane. Second, the percent probe

recovery can be used to estimate the amount of drug delivered to the brain (Watson et al., 2006). The average ACh recovery by the dialysis membranes used for this study was 5.5%, indicating that the concentrations of bicuculline delivered to the PnO ranged from approximately 5.5 to 550 μM.

Histological analysis of microinjection and microdialysis sites. Following completion of the last microinjection and 4 h recording or following each microdialysis experiment, mice were deeply anesthetized and decapitated. Brains were rapidly removed, frozen, and sectioned coronally at 40 μm in thickness. Slide mounted tissue sections were dried, fixed in paraformaldehyde vapor (80°C), and stained with cresyl violet. Tissue sections were digitized and compared with a mouse brain atlas (Paxinos and Franklin, 2001) to assign stereotaxic coordinates to each microinjection or microdialysis site. Only data obtained from sites localized to the PnO were included in the statistical analyses.

Quantitative analysis of drug effects. All dependent measures were tested and found to be normally distributed. Data were analyzed using descriptive and inferential statistics. Dependent measures of sleep and wakefulness included the percent of total recording time spent in wakefulness, NREM sleep, and REM sleep, the average duration and number of episodes of each state, the number of transitions between states, and the latency to onset of NREM sleep and REM sleep. Drug effects on dependent measures of sleep and wakefulness were analyzed by repeated measures one-way analysis of variance (ANOVA) or ANOVA using a linear mixed model adjusted for incomplete block design. Each ANOVA was followed by a Dunnett's multiple comparisons test. ACh release

during dialysis administration of bicuculline is reported as the percent of ACh measured during dialysis with Ringer's (control). The effect of bicuculline on ACh release in the PnO was analyzed using ANOVA and a post hoc Dunnett's test. Drug effects on EEG power were assessed using repeated measures two-way ANOVA and post hoc comparison of treatment at each frequency. The effects of bicuculline on recovery of righting response, core body temperature, and rate of breathing were analyzed by ANOVA and Dunnett's test. Statistical programs used included GBStat v.6.5.6 for Macintosh, GraphPad Prism 5.0a for Windows, and SAS 9.1.2 for Windows. A probability (p) value of ≤ 0.05 was considered significant. Data are reported as mean ± standard error of the mean (SEM).

RESULTS

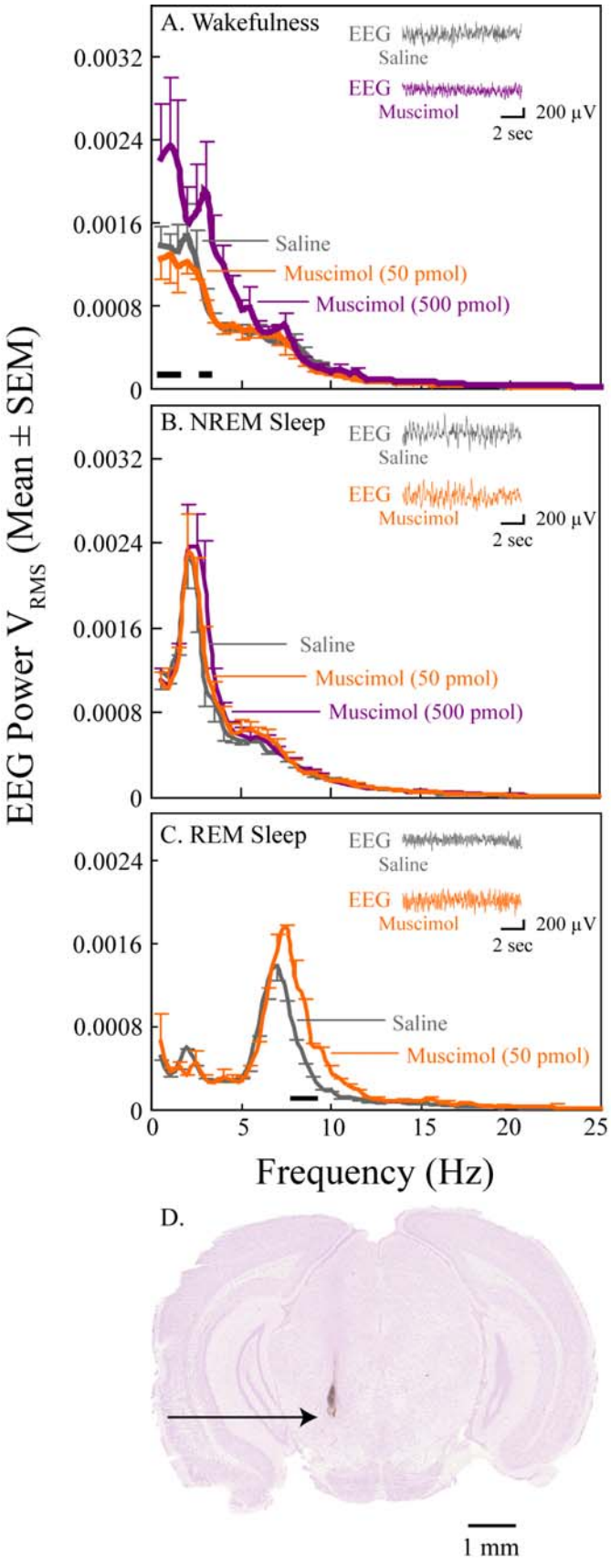
Muscimol microinjected into the PnO increased EEG power and caused a concentration dependent increase in wakefulness and decrease in sleep

The effects of muscimol on sleep and wakefulness are summarized by **Figures 2.1, 2.2, and 2.3**. The **Figure 2.1** power spectral analyses revealed that PnO microinjection (**Fig. 2.1D**) of muscimol significantly increased EEG power during wakefulness (**Fig. 2.1A;** $F = 20.54$; $df = 2, 6$; $p = 0.0021$). During REM sleep there was a significant treatment by EEG frequency interaction (**Fig. 2.1C**; $F = 4.72$; $df = 12, 12$; $p = 0.0059$). Muscimol did not alter EEG power during NREM sleep (**Fig. 2.1B**).

Figure 2.2 plots the temporal distribution of sleep and wakefulness for two mice following microinjection of saline and muscimol. These figures, which are representative of the group data, illustrate that muscimol increased the time spent in wakefulness, decreased the time spent in NREM sleep, and decreased (**Figs. 2.2B and 2.2E**) or

abolished (**Figs. 2.2C and 2.2F**) REM sleep compared to control (**Figs. 2.2A and 2.2D**). Normal aspects of the sleep cycle following microinjection of muscimol included the findings that NREM sleep was always preceded by wakefulness, and REM sleep was always preceded by NREM sleep.

Figure 2.1. Microinjection of muscimol into the PnO increased EEG power during wakefulness and REM sleep. Data are from four mice. Black bars directly above the abscissa in parts **A** and **C** indicate the frequencies at which muscimol caused a significant increase in EEG power relative to saline. **A**, during wakefulness muscimol (500 pmol) significantly increased cortical EEG power between 0.5-1.5 Hz and at 3 Hz. **B**, muscimol did not alter EEG power in NREM sleep. **C**, during REM sleep muscimol (50 pmol) caused a significant frequency-by-treatment interaction. Post hoc analyses comparing the means at each frequency revealed that muscimol increased EEG power between 7.5-9.5 Hz. The insets in parts **A - C** show representative pairs of 10 s EEG recordings obtained from the same mouse following microinjection of saline and muscimol. **D**, this digitized image of a cresyl violet-stained coronal section shows a representative microinjection site (arrow) in the PnO, located approximately 4.2 mm posterior to bregma.


A. Wakefulness
EEG
Saline
EEG
Muscimol
200 µV
2 sec
Saline
Muscimol (50 pmol)
Muscimol (500 pmol)
B. NREM Sleep
C. REM Sleep
EEG Power V_{RMS} (Mean ± SEM)
0.0032
0.0024
0.0016
0.0008
0
0
5
10
15
20
25
Frequency (Hz)
D.
1 mm

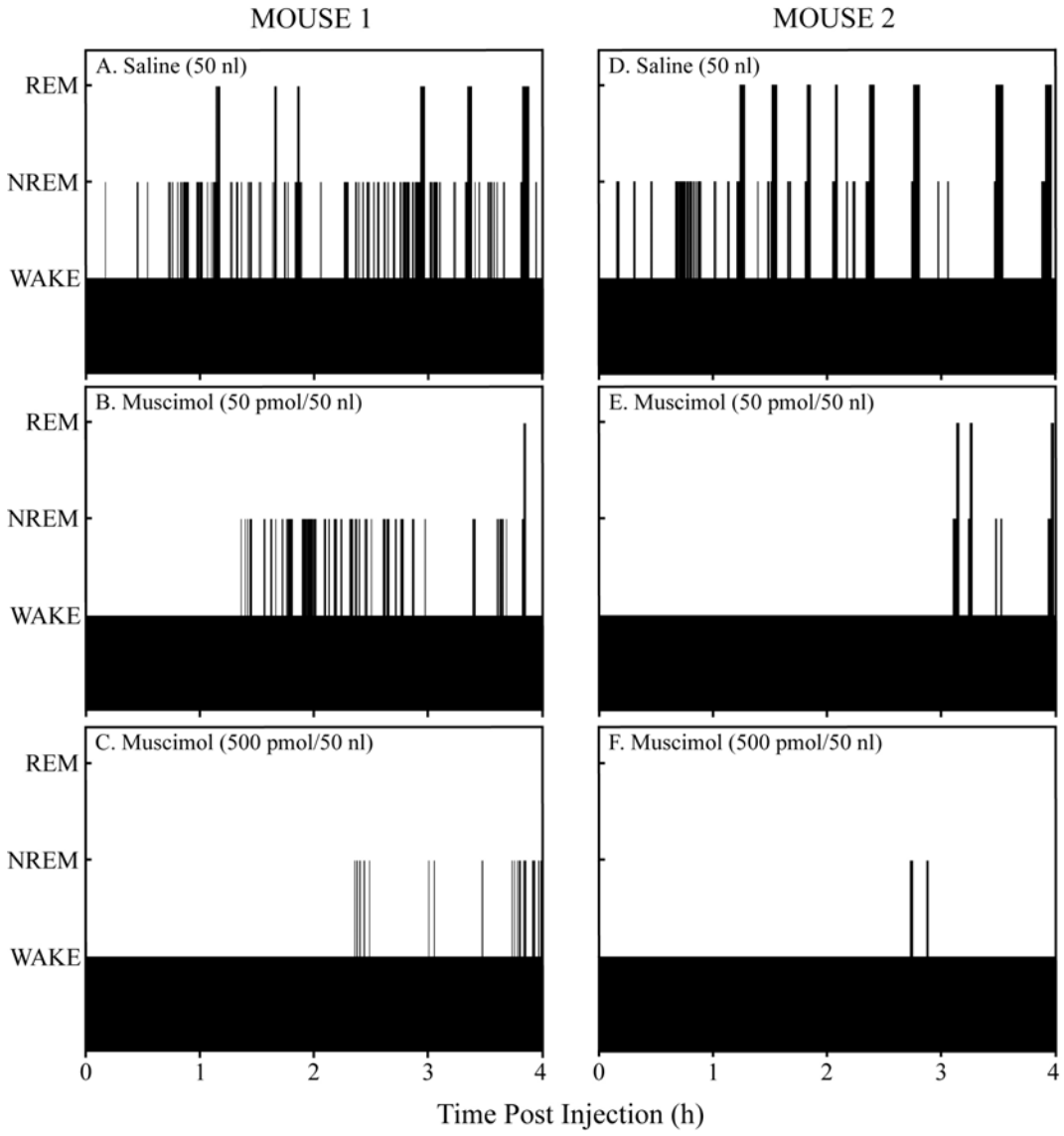
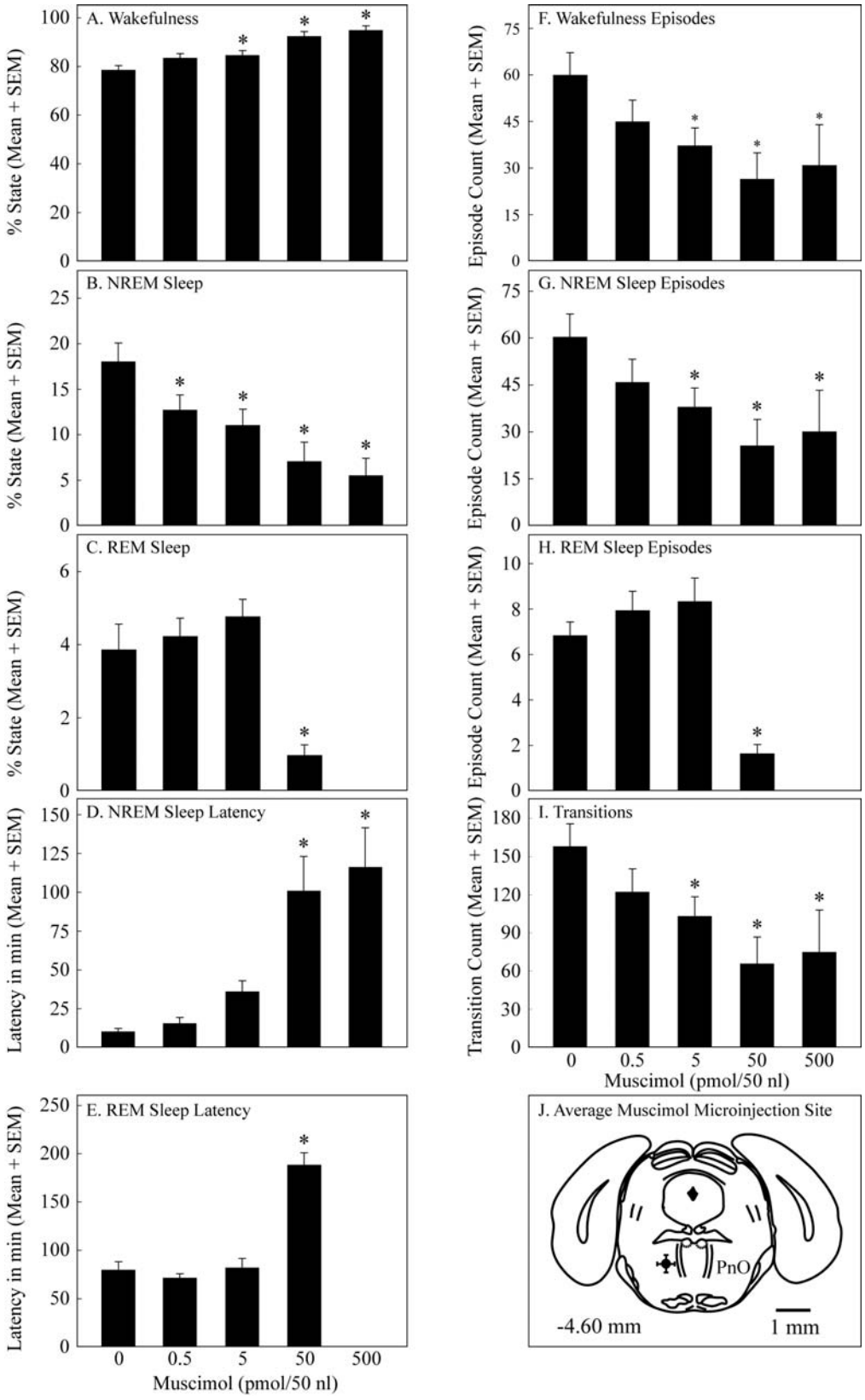


Figure 2.2. PnO microinjection of muscimol disrupted sleep architecture by increasing wakefulness. Black bars plot the time course of sleep and wakefulness recorded from two mice. The height of the bars corresponds to arousal state, with the lowest bars indicating wakefulness, intermediate bars representing NREM sleep, and highest bars showing the occurrence of REM sleep.

Figure 2.3 summarizes the group data for the quantitative effects of muscimol on sleep and wakefulness in 10 mice. Because the highest concentration of muscimol (500 pmol/50 nl) eliminated REM sleep, each ANOVA for dependent measures of REM sleep was run using four rather than five concentrations of muscimol. Muscimol increased the percent of time spent in wakefulness (**Fig. 2.3A**; $F = 27.4$; $df = 4, 36$; $p < 0.0001$) and decreased the time spent in both NREM sleep (**Fig. 2.3B**; $F = 18.9$; $df = 4, 36$; $p < 0.0001$) and REM sleep (**Fig. 2.3C**; $F = 16.2$; $df = 3, 27$; $p < 0.0001$). Microinjection of muscimol increased the latency to onset of NREM sleep (**Fig. 2.3D**; $F = 15.5$; $df = 4, 36$; $p < 0.0001$) and REM sleep (**Fig. 2.3E**; $F = 39.0$; $df = 3, 27$; $p < 0.0001$), and decreased the number of episodes of wakefulness (**Fig. 2.3F**; $F = 6.4$; $df = 4, 36$; $p = 0.0005$), NREM sleep (**Fig. 2.3G**; $F = 7.2$; $df = 4, 36$; $p = 0.0002$), and REM sleep (**Fig. 2.3H**; $F = 14.9$; $df = 3, 27$; $p < 0.0001$). Muscimol also decreased the number of transitions between states of wakefulness, NREM sleep, and REM sleep (**Fig. 2.3I**; $F = 8.4$; $df = 4, 36$; $p < 0.0001$). Muscimol significantly increased the average duration of wakefulness ($F = 7.04$; $df = 4, 36$; $p = 0.0003$) and had no effect on the average duration of NREM sleep or REM sleep (data not shown). All microinjection sites were localized to the PnO (**Fig. 2.3J**). Mean ± SEM stereotaxic coordinates for the PnO microinjection sites were 4.6 ± 0.1 mm posterior to bregma, 0.9 ± 0.1 mm lateral to the midline, and 4.6 ± 0.1 mm below the skull surface (Paxinos and Franklin, 2001).

Figure 2.3. Muscimol caused a concentration dependent increase in wakefulness and decrease in sleep. **A-I,** asterisks indicate a significant ($p < 0.05$) difference from control (0 pmol muscimol). **J,** a coronal diagram modified from a mouse brain atlas (Paxinos and Franklin, 2001) illustrates the mean ± SEM location of the 10 microinjection sites (black dot and bars in left PnO).


A. Wakefulness
% State (Mean + SEM)
B. NREM Sleep
% State (Mean + SEM)
C. REM Sleep
% State (Mean + SEM)
D. NREM Sleep Latency
Latency in min (Mean + SEM)
E. REM Sleep Latency
Latency in min (Mean + SEM)
0 0.5 5 50 500
Muscimol (pmol/50 nl)
F. Wakefulness Episodes
Episode Count (Mean + SEM)
G. NREM Sleep Episodes
Episode Count (Mean + SEM)
H. REM Sleep Episodes
Episode Count (Mean + SEM)
I. Transitions
Transition Count (Mean + SEM)
0 0.5 5 50 500
Muscimol (pmol/50 nl)
J. Average Muscimol Microinjection Site
PnO
-4.60 mm
1 mm

Co-administration of bicuculline and muscimol to the PnO blocked the increase in wakefulness and decrease in both NREM sleep and REM sleep caused by muscimol

Figures 2.4 and 2.5 summarize the effects on sleep and wakefulness of co-administering muscimol and bicuculline to the PnO. **Figure 2.4** shows the time course of sleep and wakefulness during three experiments performed in two mice. These results are representative of the group data and illustrate the findings that bicuculline blocked the muscimol-induced increase in wakefulness and decrease in NREM sleep and REM sleep. The group data obtained by microinjecting saline, muscimol, and muscimol + bicuculline into the PnO of 12 mice are shown in **Figure 2.5**. ANOVA revealed a significant treatment main effect on wakefulness (**Fig. 2.5A**; $F = 87.2$; $df = 2, 22$; $p < 0.0001$), NREM sleep (**Fig. 2.5B**; $F = 85.8$; $df = 2, 22$; $p < 0.0001$), REM sleep (**Fig. 2.5C**; $F = 12.0$; $df = 2, 22$; $p = 0.0003$), NREM sleep latency (**Fig. 2.5D**; $F = 20.0$; $df = 2, 22$; $p < 0.0001$), REM sleep latency (**Fig. 2.5E**; $F = 7.6$; $df = 2, 22$; $p = 0.003$), and the number of episodes of wakefulness (**Fig. 2.5F**; $F = 30.3$; $df = 2, 22$; $p < 0.0001$) and NREM sleep (**Fig. 2.5G**; $F = 30.6$; $df = 2, 22$; $p < 0.0001$). There was also a drug main effect on the number of REM sleep episodes (**Fig. 2.5H**; $F = 10.8$; $df = 2, 22$; $p = 0.0005$), and a significant treatment effect on the number of transitions (**Fig. 2.5I**; $F = 19.8$; $df = 2, 22$; $p < 0.0001$) between states of wakefulness, NREM sleep, and REM sleep. Mean ± SEM stereotaxic coordinates (Paxinos and Franklin, 2001) for the antagonist blocking study microinjection sites (**Fig. 2.5J**) were 4.6 ± 0.1 mm posterior to bregma, 1.0 ± 0.1 mm lateral to the midline, and 4.6 ± 0.1 mm below the skull surface.

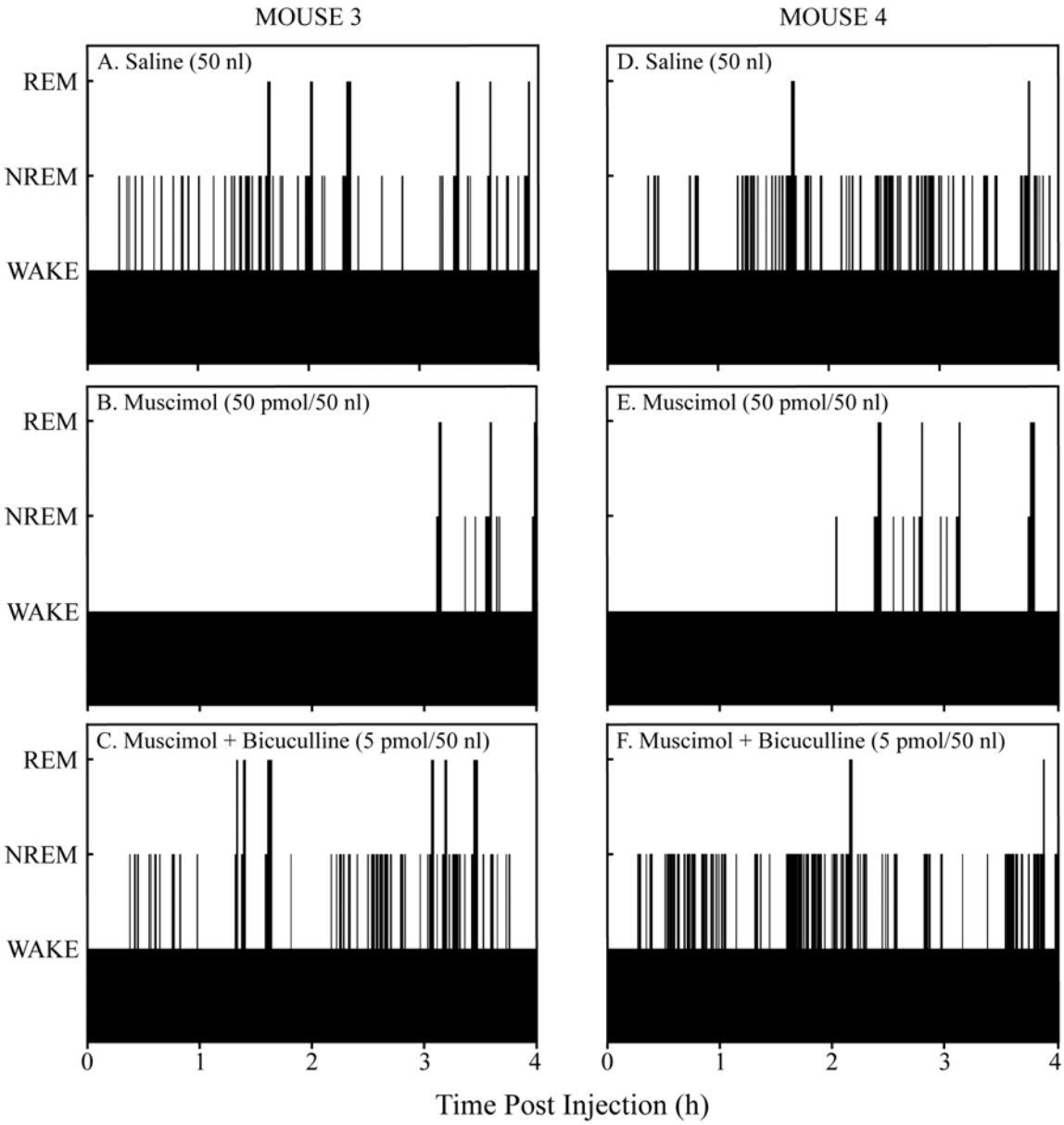
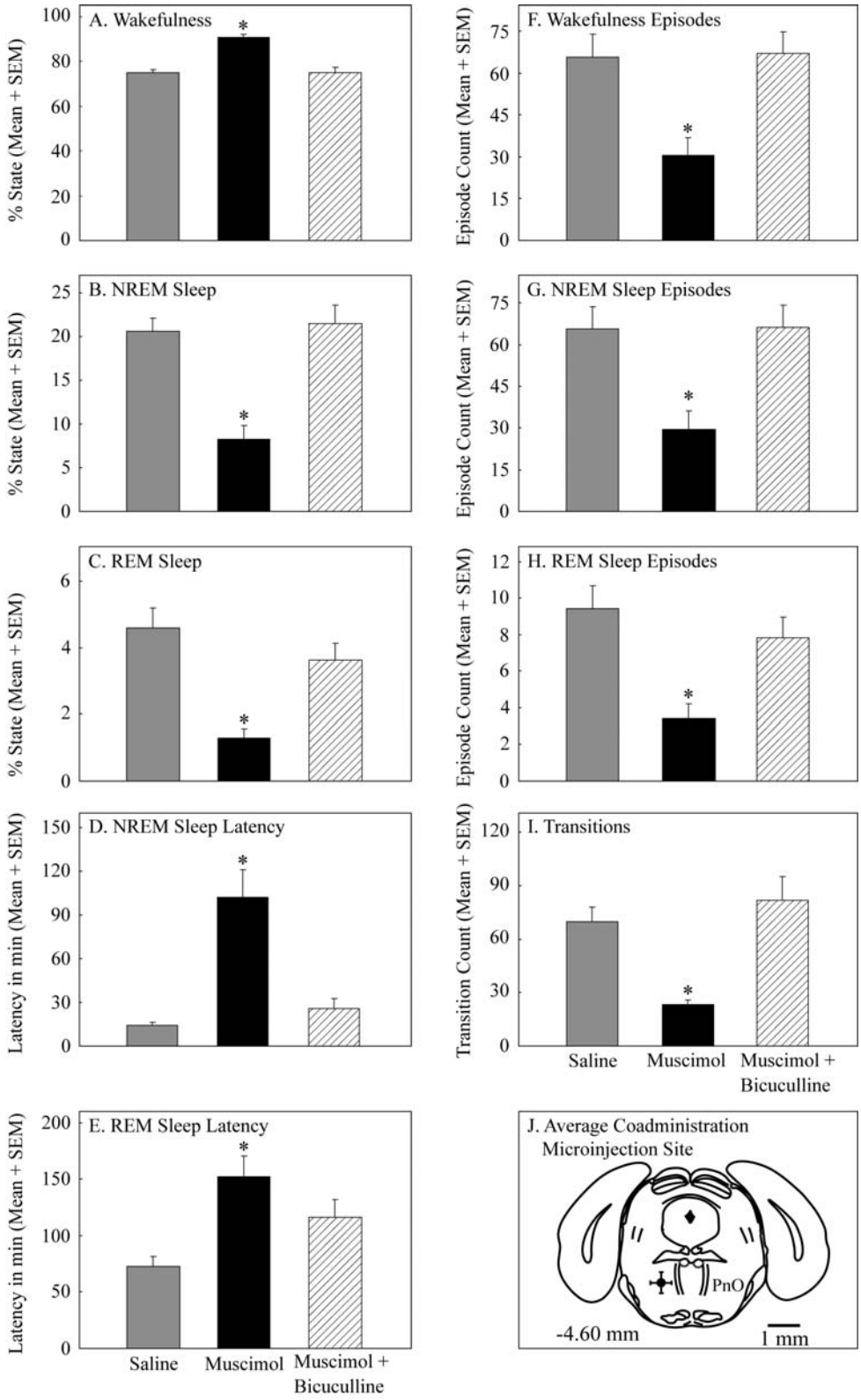


Figure 2.4. Bicuculline blocked the sleep inhibition caused by muscimol. Plots show the time course of sleep and wakefulness recorded from two representative mice. Arousal state is indicated by the height of the bars. The sequence, frequency, and duration of wakefulness, NREM sleep, and REM sleep are represented by low, intermediate, and high bars, respectively.

Figure 2.5. PnO microinjection of bicuculline (5 pmol/50 nl) blocked the increase in wakefulness, decrease in NREM sleep, and decrease in REM sleep caused by muscimol (50 pmol/50 nl). **A-I,** asterisks indicate a significant ($p < 0.05$) difference from control (saline). **J,** a coronal diagram at 4.60 mm posterior to bregma was modified from a mouse brain atlas (Paxinos and Franklin, 2001) to indicate the mean ± SEM location of 12 histologically confirmed microinjection (black dot and bars in left PnO).


A. Wakefulness
% State (Mean + SEM)
B. NREM Sleep
% State (Mean + SEM)
C. REM Sleep
% State (Mean + SEM)
D. NREM Sleep Latency
Latency in min (Mean + SEM)
E. REM Sleep Latency
Latency in min (Mean + SEM)
Saline
Muscimol
Muscimol + Bicuculline
F. Wakefulness Episodes
Episode Count (Mean + SEM)
G. NREM Sleep Episodes
Episode Count (Mean + SEM)
H. REM Sleep Episodes
Episode Count (Mean + SEM)
I. Transitions
Transition Count (Mean + SEM)
Saline
Muscimol
Muscimol + Bicuculline
J. Average Coadministration Microinjection Site
PnO
-4.60 mm
1 mm

Microinjection of bicuculline into the PnO significantly decreased wakefulness and increased sleep

Having demonstrated that PnO muscimol causes a concentration dependent and reversible increase in wakefulness and decrease in sleep, this study next aimed to determine whether endogenous GABA in the PnO of the B6 mouse modulates sleep and wakefulness. The results are summarized by **Figures 2.6, 2.7, and 2.8. Figure 2.6** shows that bicuculline (50 pmol/50 nl) did not alter EEG power at any frequency during wakefulness (**Fig. 2.6A**), NREM sleep (**Fig. 2.6B**), or REM sleep (**Fig. 2.6C**). The **Figure 2.6** insets show typical EEG recordings (10 s each) following PnO microinjection of saline and bicuculline (50 pmol/50 nl). Visual inspection of EEG recordings from each mouse also showed that states of sleep and wakefulness appeared normal following PnO microinjection of all bicuculline concentrations.

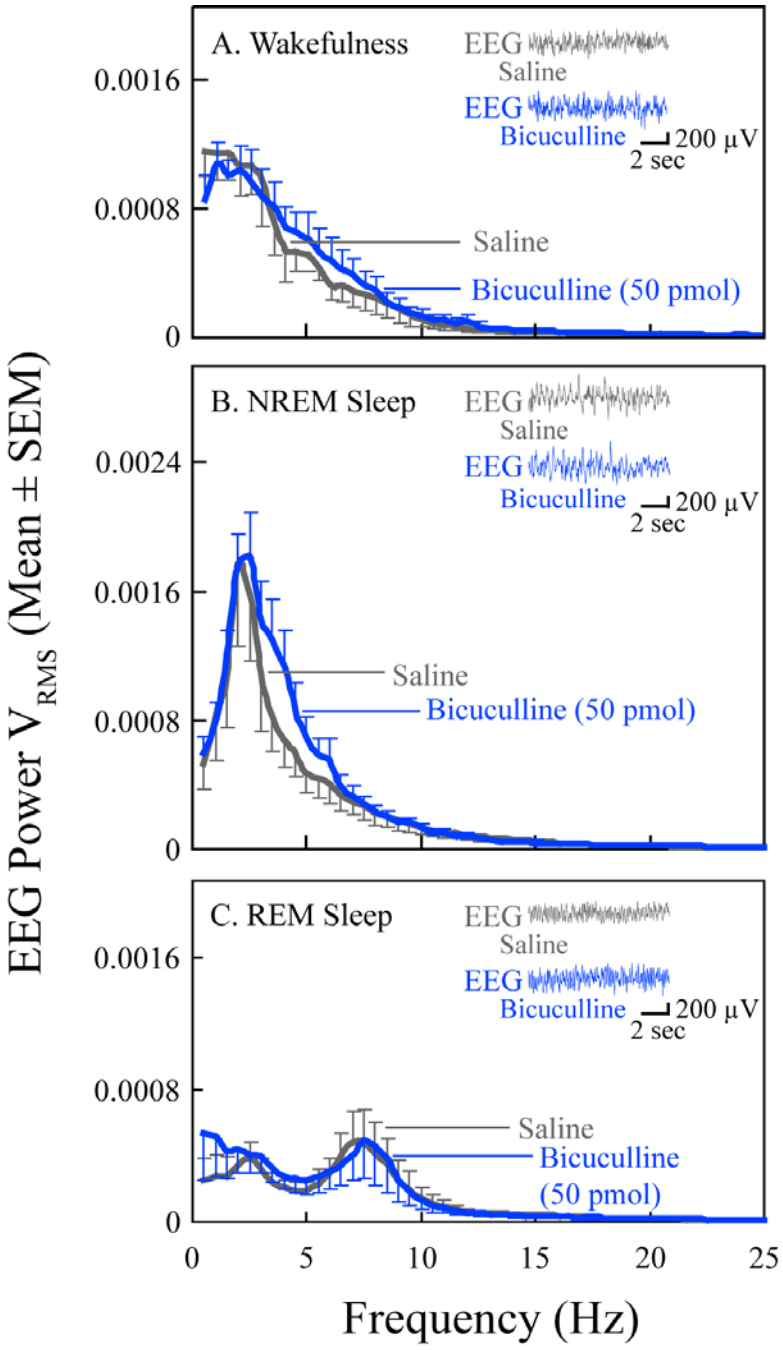


Figure 2.6. EEG power was not altered by PnO microinjection of bicuculline. Graphs plot average (n = 6 mice) EEG power during **A,** wakefulness, **B,** NREM sleep, and **C,** REM sleep following PnO microinjection of saline (gray line) and bicuculline (blue line). Cortical EEG power did not differ following PnO microinjection of bicuculline or saline. The insets show representative pairs of EEG recordings from the same mouse and illustrate the indistinguishable signals following microinjection of bicuculline and saline.

Plotting the temporal distribution of sleep and wakefulness from two representative mice following microinjection of saline (**Fig. 2.7A and 2.7C**) and bicuculline (50 pmol/50 nl; **Fig. 2.7B and 2.7D**) shows that bicuculline decreased the amount of wakefulness and increased the amount of NREM sleep and REM sleep. The group data (n = 11 mice) are summarized by **Figure 2.8**. Bicuculline caused a significant, concentration dependent decrease in the amount of wakefulness (**Fig. 2.8A;** $F = 3.0$; $df = 3, 27$; $p = 0.05$) and increase in the amount of NREM sleep **(Fig. 2.8B;** $F = 2.9$; $df = 3, 27$; $p = 0.05$**)** and REM sleep (**Fig. 2.8C;** $F = 7.9$; $df = 3, 27$; $p = 0.0006$). Histological analyses revealed that all bicuculline microinjections were made within the PnO. Mean ± SEM stereotaxic coordinates for the bicuculline microinjection sites (**Fig. 2.8D**) were 4.6 ± 0.1 mm posterior to bregma, 0.9 ± 0.04 mm lateral to the midline, and 4.3 ± 0.1 mm below the skull surface (Paxinos and Franklin, 2001).

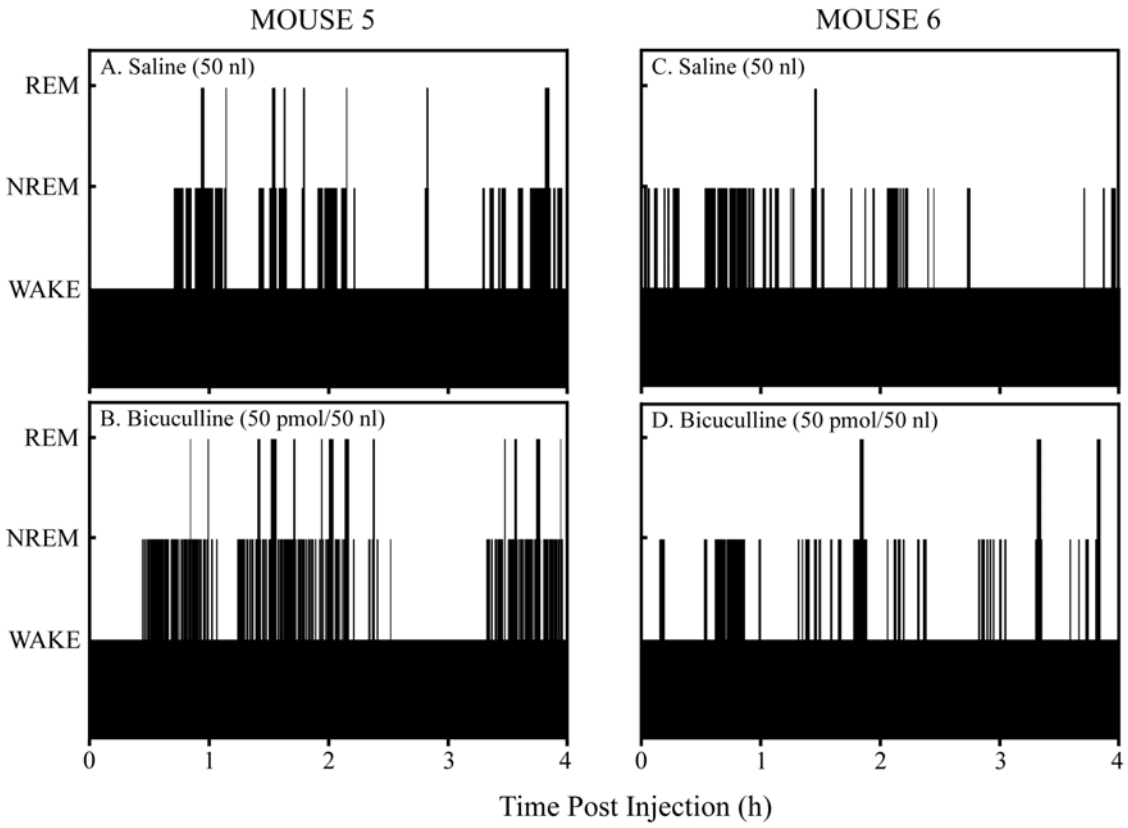
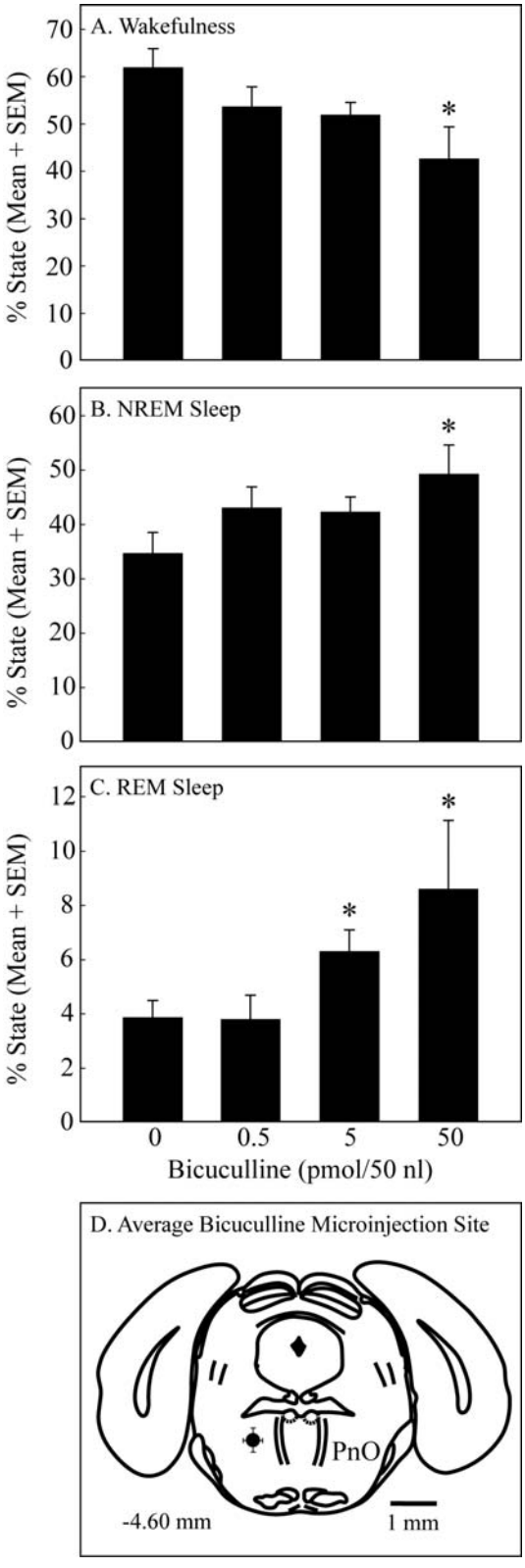


Figure 2.7. PnO microinjection of bicuculline altered sleep architecture by increasing sleep. Black bars illustrate the time course of sleep and wakefulness recorded from two mice for 4 h following PnO microinjection of **A,** saline and **B,** bicuculline (50 pmol). Bicuculline decreased the time spent in wakefulness and increased the time spent in NREM sleep and REM sleep.

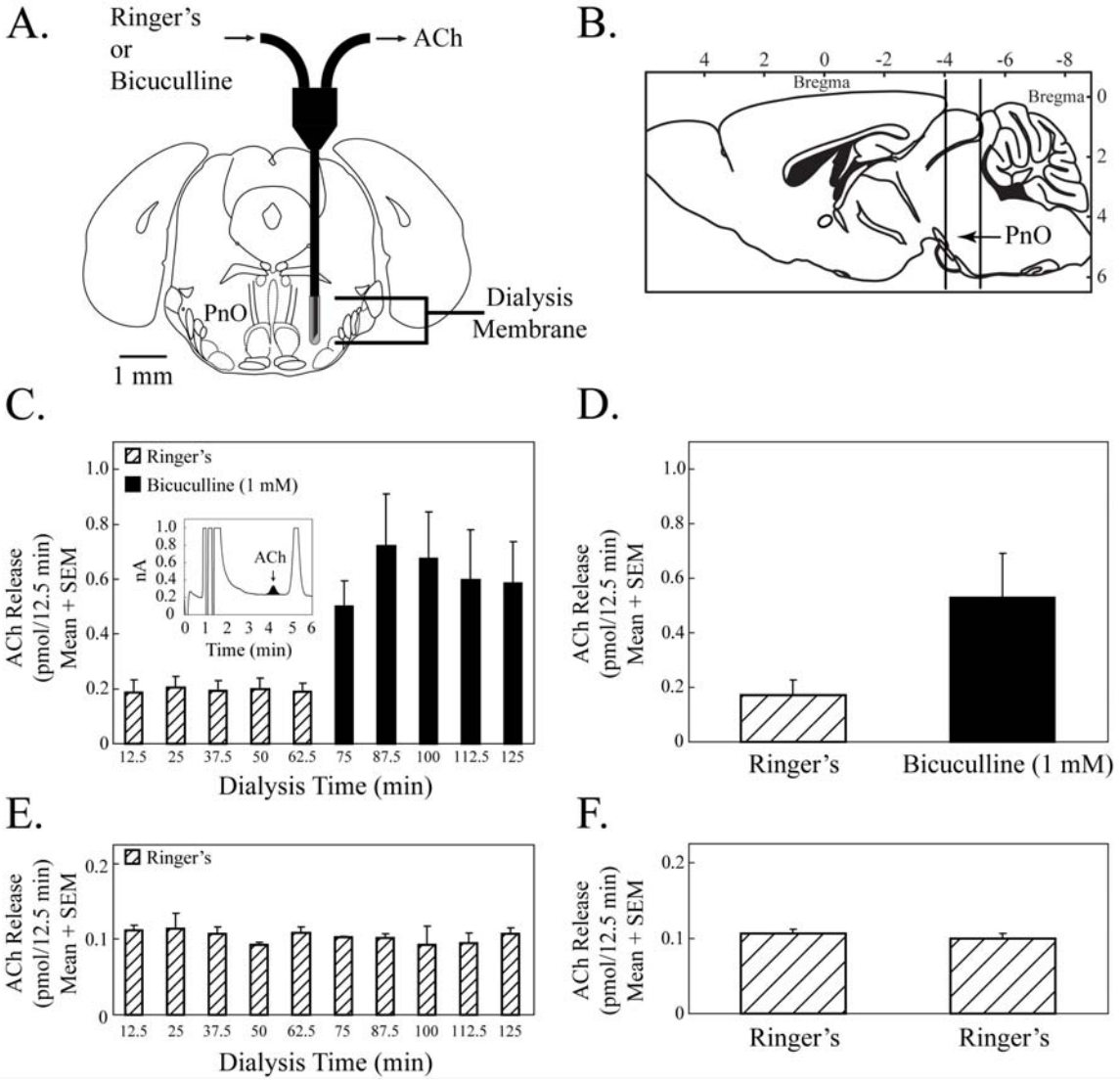
Figure 2.8. Microinjection of bicuculline into the PnO caused a concentration dependent decrease in wakefulness and increase in NREM sleep and REM sleep. **A-C,** asterisks indicate a significant ($p < 0.05$) difference from control (0 pmol bicuculline). **D,** a coronal diagram at 4.60 mm posterior to bregma was modified from a mouse brain atlas (Paxinos and Franklin, 2001) with a black dot and bars indicating the mean ± SEM location of the 11 histologically confirmed PnO microinjection sites.


A. Wakefulness
% State (Mean + SEM)
70
60
50
40
30
20
10
0
*
B. NREM Sleep
% State (Mean + SEM)
60
50
40
30
20
10
0
*
C. REM Sleep
% State (Mean + SEM)
12
10
8
6
4
2
0
*
*
0
0.5
5
50
Bicuculline (pmol/50 nl)
D. Average Bicuculline Microinjection Site
PnO
-4.60 mm
1 mm

Microdialysis delivery of bicuculline to the PnO caused a concentration dependent increase in ACh release in the PnO, decrease in respiratory rate, and increase in anesthesia recovery time

Figure 2.9 illustrates the use of in vivo microdialysis to determine whether $GABA_A$ receptors in the PnO of the B6 mouse modulate ACh release, rate of breathing, and anesthesia recovery time. Dialysis probes were aimed for the PnO **(Fig. 2.9A)** and placement was confirmed histologically. Mean ± SEM stereotaxic coordinates of the dialysis sites in the PnO (**Fig. 2.9B**) were 4.8 ± 0.1 mm posterior to bregma, 1.0 ± 0.1 mm lateral to the midline, and 4.9 ± 0.1 mm below the skull surface (Paxinos and Franklin, 2001). Dialysis with Ringer's followed by dialysis with Ringer's containing 1 mM bicuculline **(Figs. 2.9C and 2.9D)** revealed that bicuculline caused a 210% increase in ACh release in the PnO. Control experiments (**Fig. 2.9E and 2.9F**) measured ACh during dialysis with Ringer's and demonstrated that ACh release was stable across the 125 min sampling period. **Figure 2.10** summarizes the group data showing that bicuculline caused a significant, concentration dependent increase in ACh release in the PnO ($F = 16.73$; $df = 5, 12$; $p < 0.0001$).

Figure 2.9. ACh release in mouse PnO. **A**, a coronal diagram modified from a mouse brain atlas (Paxinos and Franklin, 2001) schematizes a microdialysis probe in the PnO. The dialysis membrane is drawn to scale (1 mm length and 0.24 mm diameter). **B**, vertical lines on the sagittal diagram of the mouse brain (Paxinos and Franklin, 2001) show the anterior-posterior range of the histologically confirmed microdialysis sites in the PnO. **C**, time course plots of ACh release in the PnO. Each bar represents the average ACh release from 3 mice. Dialysis administration of bicuculline (black bars) increased ACh release within the first 12.5 min of administration. The inset shows a chromatogram with a representative ACh peak obtained during control dialysis of the PnO. **D**, demonstrates that ACh measures were collapsed across time. For each experiment the first five dialysis samples shown in part C were averaged to provide control levels of ACh release (Ringer's) and the last five samples were averaged to determine the effect of bicuculline on ACh release. **E**, control experiments confirmed that ACh release did not change as a function of time. Each bar represents the average ACh release from 3 mice. **F**, ACh release was stable during isoflurane anesthesia.


A.
Ringer's
or
Bicuculline
ACh
PnO
Dialysis
Membrane
1 mm
B.
4 2 0 -2 -4 -6 -8
Bregma
Bregma
0 2 4 6
PnO
C.
Ringer's
Bicuculline (1 mM)
ACh Release
(pmol/12.5 min)
Mean + SEM
nA
ACh
Time (min)
12.5 25 37.5 50 62.5 75 87.5 100 112.5 125
Dialysis Time (min)
D.
ACh Release
(pmol/12.5 min)
Mean + SEM
Ringer's
Bicuculline (1 mM)
E.
Ringer's
ACh Release
(pmol/12.5 min)
Mean + SEM
12.5 25 37.5 50 62.5 75 87.5 100 112.5 125
Dialysis Time (min)
F.
ACh Release
(pmol/12.5 min)
Mean + SEM
Ringer's
Ringer's

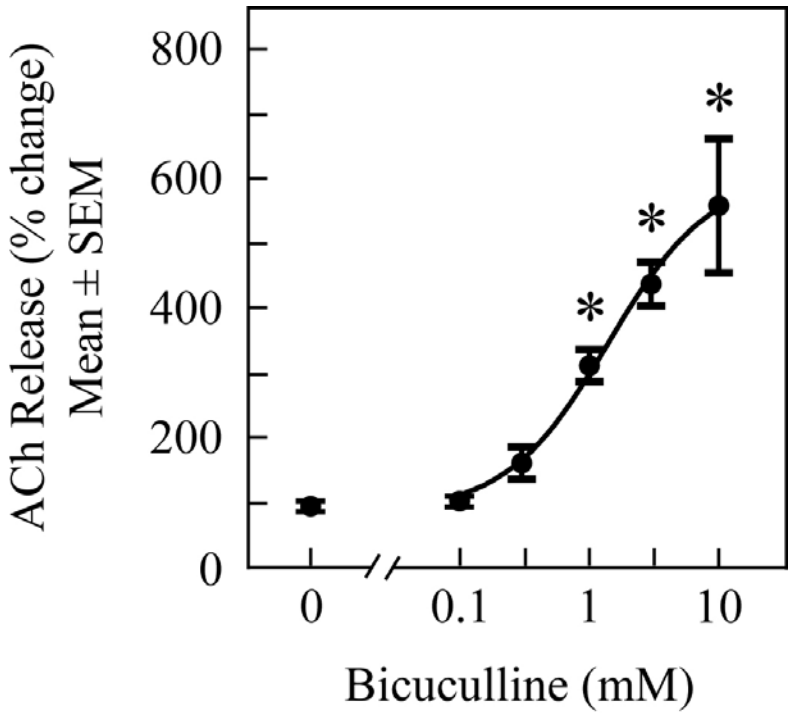


Figure 2.10. Blocking $GABA_A$ receptors in the PnO caused a concentration dependent increase in ACh release in the PnO. Asterisks indicate a significant difference from control (0 mM bicuculline, 100% ACh release). The concentration of bicuculline accounted for 87% of the variance in ACh release. Data are from 3 mice per concentration, for a total of 18 mice.

Figure 2.11 shows that bicuculline caused a significant, concentration dependent decrease in rate of breathing ($F = 6.04$; $df = 5, 12$; $p = 0.005$). There was no significant effect of bicuculline on core body temperature, which averaged 37.3 ± 0.06 ˚C across all dialysis experiments. Upon completion of dialysis sample collection, the same mice were used to determine the effect of PnO bicuculline on time to recovery from isoflurane anesthesia. **Figure 2.12** shows that blocking $GABA_A$ receptors in the PnO caused a significant, concentration dependent increase in anesthesia recovery time ($F = 8.67$; $df = 3, 8$; $p = 0.007$). The two highest concentrations of bicuculline caused seizures followed by death, thus measures of recovery time following PnO dialysis with 3 mM (n = 3 mice) or 10 mM (n = 3 mice) bicuculline were not included in the final data sets. Duration of exposure to anesthetics can influence recovery time, and the duration of isoflurane administration was held constant at 234 ± 1 min across all experiments.

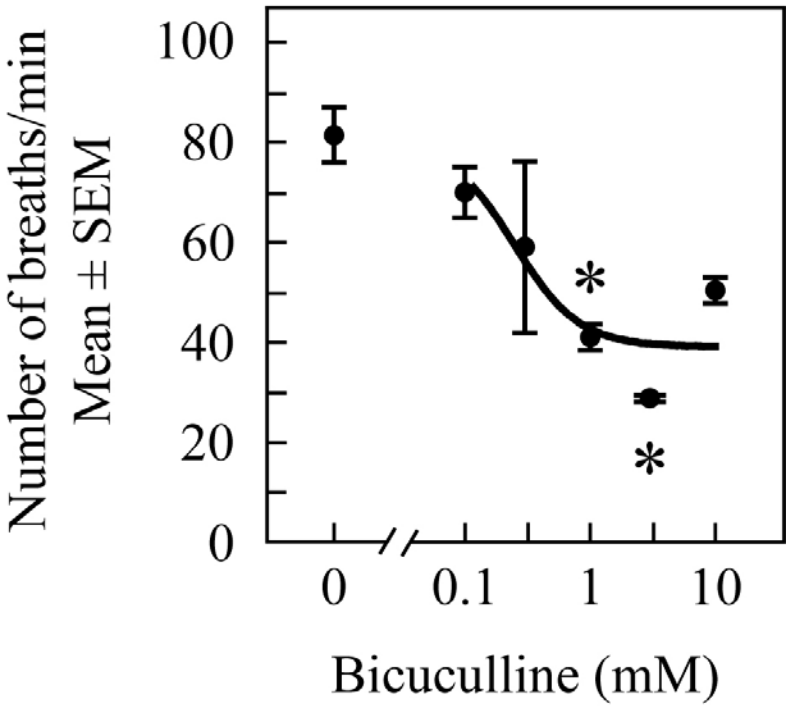


Figure 2.11. Bicuculline caused a concentration dependent decrease in breathing rate. Asterisks indicate a significant difference from control (0 mM bicuculline). The concentration of bicuculline accounted for 76% of the variance in breathing rate. Data are from 3 mice per concentration, for a total of 18 mice.

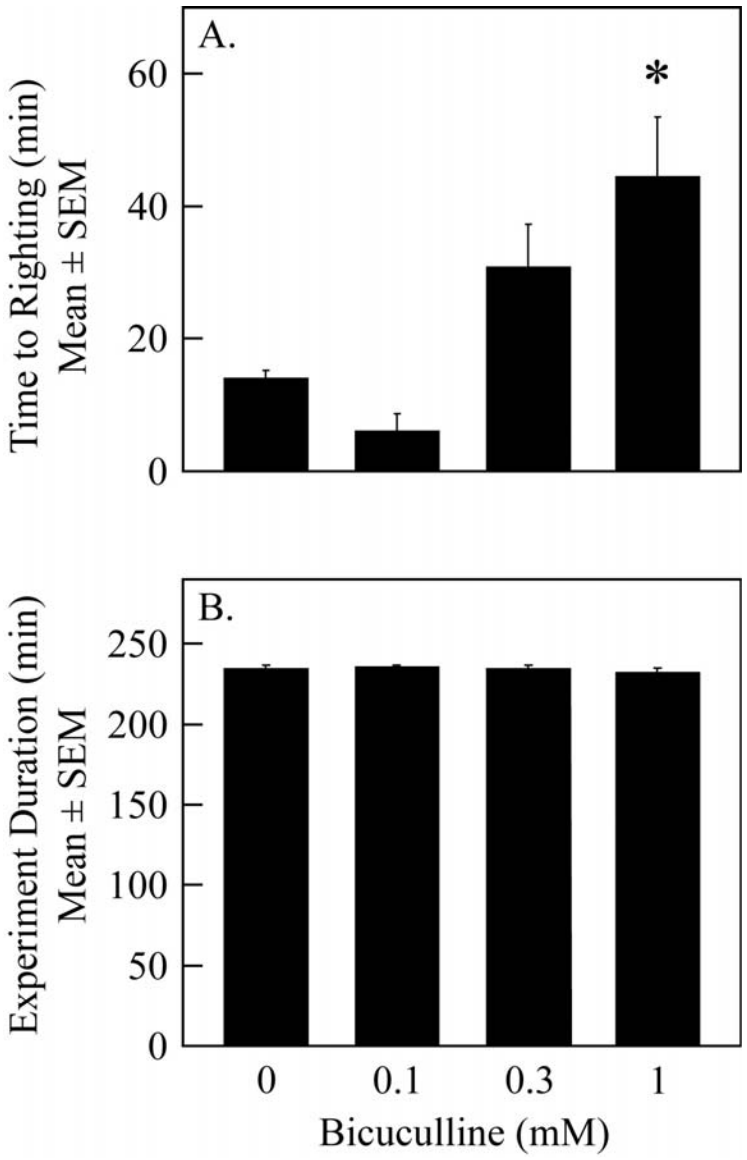


Figure 2.12. Bicuculline administered to the PnO caused a concentration dependent increase in anesthesia recovery time. Asterisks indicate a significant difference from control (0 mM bicuculline). Data are from 3 mice per concentration, for a total of 12 mice. **A**, the concentration of bicuculline accounted for 76% of the variance in recovery time. **B**, shows that total anesthesia time was held constant across all experiments.

DISCUSSION

Drugs that increase transmission at $GABA_A$ receptors are used extensively in clinical practice to produce states of sleep (Winsky-Sommerer, 2009) or anesthesia (Franks, 2008). Selectively altering GABAergic transmission in specific brain regions, however, has widely varying effects on arousal states (Vanini et al., 2010; Watson et al., 2010). Studies using cat and rat have shown that GABAergic transmission in the PRF contributes to generating states of sleep, wakefulness, and general anesthesia (Lydic and Baghdoyan, 2005; Steriade and McCarley, 2005). The present study is the first to characterize the effects of GABAergic transmission in mouse PnO on states of arousal and traits characterizing those states. The findings demonstrate that $GABA_A$ receptors in PnO of the B6 mouse modulate occurrence of sleep/wake states, activity of the cortical EEG, release of ACh in the PnO, breathing rate, and time needed to regain wakefulness following isoflurane anesthesia. The ensuing discussion considers the results in relation to clinical implications of brain region-specific effects of GABAergic transmission on arousal states, and evidence that endogenous GABA in the PRF promotes wakefulness.

Technical considerations

Limitations of intracranial microinjection as an approach for elucidating the behavioral state-related roles of neurotransmitter receptors have been reviewed (Myers, 1966; Routtenberg, 1972; Nicholson, 1985). The small size of the mouse brain presents particular challenges for the microinjection approach because distances between structures are short and microinjected drugs diffuse away from their injection sites into neighboring brain regions (Edeline et al., 2002). Compensatory measures include use of

small injection volumes and selection of relatively large brain nuclei as targets. With these approaches, the B6 mouse has been used successfully for microinjection studies to characterize drug effects on behavioral states and their physiological traits (Lydic et al., 2002; Coleman et al., 2004b; Douglas et al., 2005; Van Dort et al., 2009).

Limitations of microdialysis for drug delivery (Boschi and Scherrmann, 2000) and recovery of endogenous neurotransmitters (Watson et al., 2006) are well known. One disadvantage of microdialysis compared to microinjection is the inability to determine exactly how much drug is administered to the brain. This limitation can be addressed by demonstrating concentration response relationships for the effects of receptor agonists and antagonists on transmitter levels, as has been done successfully in the PnO (Douglas et al., 2001; Coleman et al., 2004a; Coleman et al., 2006) and prefrontal cortex (Van Dort et al., 2009) of B6 mouse.

GABAergic transmission causes brain region specific effects on sleep and wakefulness

This study is the first to demonstrate that microinjecting muscimol into the PnO of B6 mouse caused a concentration-dependent increase in wakefulness and decrease in sleep (**Figs. 2.2 & 2.3**) that was blocked by co-administration of bicuculline **(Figs. 2.4 & 2.5**). The data indicate that these changes in sleep and wakefulness are mediated by $GABA_A$ receptors localized to the PnO. Additionally, by showing that microinjection of bicuculline caused a concentration dependent decrease in wakefulness and increase in sleep (**Figs. 2.7 & 2.8**), this study provides the first in vivo evidence that endogenous GABA in B6 mouse PnO promotes wakefulness and inhibits sleep.

Preclinical studies using intracranial drug administration have revealed that the effects of $GABA_A$ receptor agonists and antagonists on sleep and wakefulness vary significantly as a function of brain region. Within cat hypothalamus, the $GABA_A$ receptor agonist muscimol induces sleep onset with short latency and increases sleep time when microinjected into posterior hypothalamus, but increases wakefulness and decreases sleep time when microinjected into preoptic and anterior hypothalamic areas (Lin et al., 1989). Opposite effects of muscimol on sleep and wakefulness also can be evoked from within the pontine portion of the brainstem. REM sleep is increased by microinjecting muscimol into cat dorsal raphé nucleus (Nitz and Siegel, 1997) and inhibited by delivering muscimol to the PRF of cat (Xi et al., 1999), rat (Camacho-Arroyo et al., 1991), and mouse (**Figs. 2.2-2.5**). Studies using intracranial administration of drugs that block transmission at $GABA_A$ receptors, such as bicuculline (**Figs. 2.7 & 2.8**) (Camacho-Arroyo et al., 1991; Xi et al., 1999; Sanford et al., 2003), gabazine (Marks et al., 2008), and picrotoxin (Kaur et al., 1997; Nitz and Siegel, 1997; Ali et al., 1999; Pal and Mallick, 2009), provide convincing evidence that endogenous GABA differentially alters sleep and wakefulness depending upon brain site of action. One unifying explanation for these findings is that in brain regions that function to generate wakefulness, GABAergic transmission causes sleep, whereas in brain regions that generate sleep GABAergic transmission increases wakefulness.

The discovery that effects of GABAergic transmission on arousal states are anatomically site-specific within the brain provides insight into how systemically administered benzodiazepines produce sleep architecture that differs from that of spontaneously occurring sleep. Benzodiazepine hypnotics increase total sleep time and

shorten sleep latency, but some of these drugs increase REM sleep latency (Borbély and Achermann, 1991). Drug actions at the level of the PRF, where GABAergic transmission increases wakefulness, decreases sleep, and inhibits ACh release may account for the increased REM latency caused by some benzodiazepine hypnotics.

Muscimol but not bicuculline caused state-trait dissociations

States of wakefulness, NREM sleep, and REM sleep can occur in a humbling complexity of admixtures that combine traits characteristic of one state with traits typical of a completely different state. These mixed states are referred to as dissociated states and represent a significant clinical concern (Mahowald and Schenck, 1992, 2001). The present study found that direct administration of muscimol into the PnO caused a dissociation between waking behavior and EEG activity. During the increase in wakefulness caused by muscimol (500 pmol) mice showed a marked increase in running activity yet significantly greater EEG power in the delta range (0.5-5 Hz) (**Fig. 2.1**). Increased delta activity normally occurs during NREM sleep which is characterized by decreased motor activity and a loss of consciousness. A previous study that administered muscimol to B6 mice by intraperitoneal injection reported periods of vigorous wheel running activity during which the frontal EEG was dominated by high amplitude slow waves characteristic of NREM sleep (Vyazovskiy et al., 2007). The finding that the slowing of the EEG caused by systemic administration of muscimol also occurs following muscimol delivery directly into the PnO supports the interpretation that the increase in delta power caused by intraperitoneal muscimol is mediated, at least in part, by $GABA_A$ receptors in the PnO. This interpretation is consistent with data showing that

the benzodiazepine receptor agonist hypnotics zolpidem and diazepam increase EEG delta power when administered to the PnO of isoflurane anesthetized rat (Hambrecht et al., 2009).

PnO microinjection of muscimol significantly decreased the amount of time spent in REM sleep, and the REM sleep that did occur following muscimol (50 pmol) administration was characterized by increased EEG power in the theta range (4-9 Hz) (**Fig. 2.1**). Theta activity normally is prominent during REM sleep and increases during wakefulness when animals are exploring their environment (Poe et al., 2009). Although the mechanisms underlying GABA-induced state-trait dissociation are not known, the phenomenon of paradoxical activation by benzodiazepines has been documented by multiple case reports (Paton, 2002; Brefel-Courbon et al., 2007; Schiff and Posner, 2007; Hall et al., 2008). Efforts to synthesize these case reports have led to a network model by which the benzodiazepine receptor agonist zolpidem might produce EEG and behavioral activation (Schiff, 2008, 2010). The present results expand this network model by showing that increasing GABAergic transmission in the PRF may be another mechanism through which hypnotics produce state dissociations.

$GABA_A$ receptors in the PRF function to inhibit local ACh release, stimulate breathing, and decrease anesthesia recovery time: implications for arousal state control

Dialysis delivery of bicuculline to the PnO caused a concentration dependent increase in ACh release (**Fig. 2.10**). Bicuculline also increases ACh release in cat PRF (Vazquez and Baghdoyan, 2004), and these findings support the conclusion that one functional role

of endogenous GABA in the PnO is to inhibit ACh release. Extensive evidence demonstrates that cholinergic transmission within the PRF promotes REM sleep (Lydic and Baghdoyan, 2008), and the increase in REM sleep caused by blocking $GABA_A$ receptors in the PnO can be prevented by pretreating the PnO with the muscarinic receptor antagonist atropine (Marks et al., 2008). Considered together, these findings suggest that GABAergic tone in the PRF normally suppresses REM sleep, in part by inhibiting PRF ACh release.

The finding that human rate of breathing declines significantly during the loss of wakefulness caused by anesthesia (Fink, 1961) or sleep (Phillipson and Sullivan, 1978) initiated ongoing efforts to identify cellular and molecular substrates comprising a wakefulness stimulus for breathing. Although the PRF does not contain neurons with respiratory related discharge patterns, breathing is significantly altered by PRF neurons that regulate EEG and behavioral arousal (Lydic and Baghdoyan, 1993). Blockade of $GABA_A$ receptors in the PnO with bicuculline decreased rate of breathing (**Fig. 2.11**). This finding implies that endogenous GABA in the PRF stimulates breathing, and suggests that GABAergic transmission in the PnO contributes to the wakefulness stimulus for breathing. Consistent with this interpretation is the finding that bicuculline delivered to the PnO delayed recovery time from isoflurane anesthesia (**Fig. 2.12A**). In rat, administering drugs that decrease or increase GABAergic transmission in the PnO shorten or lengthen, respectively, the time required for isoflurane to induce loss of consciousness (Vanini et al., 2008). Induction of and emergence from general anesthesia are mechanistically distinct processes (Kelz et al., 2008), and the present data are the first in any species to show that inhibition of $GABA_A$ receptors in the PnO increases

anesthesia recovery time. Taken together, the findings of increased ACh release, decreased breathing rate, and increased anesthesia recovery time caused by blocking PnO $GABA_A$ receptors provide novel evidence that GABAergic transmission in the PnO promotes wakefulness.

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CHAPTER 3

SUMMARY AND CONCLUSIONS

Many drugs that produce both sleep and anesthesia act on $GABA_A$ receptors, but the mechanisms underlying their effects remain largely unknown. The pontine reticular nucleus, oral part (PnO) is a brain region long known for contributing to arousal state control (Jouvet, 1962; Lydic and Baghdoyan, 2005). This dissertation research utilized a pharmacological approach to investigate the role of $GABA_A$ receptors in the PnO in the regulation of five arousal phenotypes: sleep and wakefulness, cortical EEG activity, ACh release in the PnO, respiratory rate, and recovery from anesthesia. The results of this research are consistent with the interpretation that $GABA_A$ receptors in the PnO of B6 mouse promote wakefulness (Flint et al., 2007; Flint et al., 2007; Flint et al., 2008; Flint et al., 2009; Flint et al., 2009). Involvement of $GABA_A$ receptors in the PnO in regulating arousal are discussed in the context of these results using a proposed synaptic model.

The studies in Chapter 2 were designed to study the effects of GABAergic transmission in the PnO on ACh release in the PnO. The data demonstrated that administration of the $GABA_A$ receptor agonist muscimol to the PnO increased cortical EEG activity during wakefulness and REM sleep (Fig. 2.1). Administration of muscimol to the PnO also increased wakefulness and decreased sleep (Figs. 2.2 and 2.3). Co-administration of the $GABA_A$ receptor antagonist bicuculline reversed the effects of

muscimol on wakefulness and sleep (Figs. 2.4 and 2.5). Bicuculline administered to the PnO decreased wakefulness and increased sleep (Figs. 2.7 and 2.8). Microdialysis delivery of bicuculline to the PnO increased ACh release in the PnO (Fig. 2.10), decreased respiratory rate (Fig. 2.11), and increased the recovery of righting following isoflurane anesthesia (Fig. 2.12A). The data in Chapter 2 are novel in that they characterize for the first time, the role of $GABA_A$ receptors in the phenotypes of arousal in the mouse PnO. These data support the conclusion that GABA in the pontine reticular formation (PRF) promotes wakefulness, and is consistent with studies done in cat (Xi et al., 1999; Vazquez and Baghdoyan, 2004) and rat (Camacho-Arroyo et al., 1991; Sanford et al., 2003; Marks et al., 2008). These consistencies across species suggest that the wakefulness promoting effects of $GABA_A$ receptors in the PRF are somewhat conserved, making it possible to ultimately make comparisons between species as they relate to arousal state.

Both anatomical and functional studies have determined that cholinergic projections to the PRF originate in the LDT/PPT (Mitani et al., 1988; Shiromani et al., 1988; Jones, 1990; Lydic and Baghdoyan, 1993; Semba, 1993) (Table 3.2). Electrical stimulation of LDT neurons increases REM sleep (Thakkar et al., 1996), and ACh release in the PRF increases during REM sleep (Kodama et al., 1990; Lydic et al., 1991; Leonard and Lydic, 1995, 1997). The PRF also contains GABAergic inter-neurons (Ford et al., 1995) (Table 3.1). Studies in cat and rat show that increasing GABAergic transmission in the PRF increases wakefulness and decreases sleep (Camacho-Arroyo et al., 1991; Xi et al., 1999; Sanford et al., 2003; Watson et al., 2008). One interpretation for the results in Chapter 2 showing that muscimol administered to the PnO increased wakefulness and

decreased sleep is that GABAergic transmission in the PRF inhibits the release of ACh from LDT/PPT terminals (Fig. 3.1A&B), and therefore produces wakefulness. In contrast, the blockade of $GABA_A$ receptors in the PRF with bicuculline allows for the release of ACh from LDT/PPT terminals (Fig 3.1A&B) and leads to REM sleep. The PRF also contains glutamatergic neurons that project to the LDT/PPT (Kaneko et al., 1989; Steininger et al., 1992; Lai et al., 1993) (Table 3.3). GABAergic transmission in the PRF could inhibit the ability of LDT/PPT cholinergic neurons to be excited by glutamate (Fig. 3.1A&C), thereby decreasing ACh release in the PRF and decreasing REM sleep. Blockade of $GABA_A$ receptors in the PRF would allow for glutamatergic excitation of ACh neurons in the LDT/PPT (Fig. 3.1A&C) to cause ACh release in the PRF, and REM sleep.

In Chapter 2, PnO dialysis with bicuculline was shown to increase ACh release in the PnO. Along with data in cat demonstrating that the blockade of $GABA_A$ receptors in the PRF increases REM sleep (Vazquez and Baghdoyan, 2004) (Table3.1), this finding is consistent with the interpretation that GABA in the PRF suppresses REM sleep, at least in part, by inhibiting ACh release in the PRF. Two possible mechanisms could be contributing to the increase in ACh release caused by blocking $GABA_A$ receptors in the PRF. First, bicuculline could block GABA inhibition of cholinergic terminals (Fig. 3.1B) and thus result in an increase of ACh release in the PRF. Second, the normal inhibition of glutamate neurons in the PRF by GABA (Fig. 3.1C) could be prevented by bicuculline, and glutamatergic excitation of LDT/PPT ACh neurons (Fig. 3.1A&C) would lead to an increase in ACh release in the PRF.

Breathing is altered by PRF neurons that regulate arousal despite the fact that the PRF contains no neurons with respiratory related discharge patterns (Lydic and Baghdoyan, 1993). Electrical stimulation of the PPT increases ACh release in the PRF and causes respiratory rate depression similar to PRF microinjection of carbachol (Lydic and Baghdoyan, 1993) (Table 3.2). Increasing GABA levels in the PnO increases respiration rate (Vanini et al., 2008) (Table 3.1). The finding in Chapter 2 that respiratory rate decreased following bicuculline administration to the PRF implies that GABA in the PRF stimulates breathing. Based on these findings, one interpretation for the decrease in breathing is that bicuculline prevents the inhibition of GABA on glutamate release (Fig. 3.1B), allowing glutamate to excite ACh neurons in the LDT/PPT (Fig. 3.1C) and depress breathing.

Drugs that alter GABA levels by inhibiting GABA uptake (NPA) or GABA synthesis (3-MPA) have been studied in relation to states of arousal. Microinjection of NPA into the PnO increases wakefulness and decreases sleep, whereas 3-MPA delivered to the PnO increases sleep and decreases wakefulness (Watson et al., 2008) (Table 3.1). Increasing or decreasing GABA levels in the PnO increases or decreases, respectively, time to loss of righting (Vanini et al., 2008) (Table 3.1). Bicuculline delivered to the PnO increases time to recovery of righting after anesthesia (Fig. 2.12A). Taken together, these data also support the interpretation that GABA in the PnO promotes wakefulness. Based on this interpretation, the bicuculline-mediated increase in recovery time could occur by blockade of the wakefulness promoting effects of GABA in the PRF (Fig. 3.1B&C).

GABA

These dissertation results support the interpretation that GABA in the PRF promotes wakefulness. Within the PRF are GABA neurons and receptors that play a key role in arousal state control (Table 3.1). The PRF has been shown to contain local and distant projecting GABAergic neurons (Ford et al., 1995; de la Roza and Reinoso-Suarez, 2006; Brown et al., 2008; Rodrigo-Angulo et al., 2008; Liang and Marks, 2009) as well as $GABA_A$ receptors (Waldvogel et al.; Fritschy and Mohler, 1995; Pirker et al., 2000; Heldt and Ressler, 2007) (Fig. 3.1B&C). GABAergic neurons are also present in the LDT/PPT (Jia et al., 2003; Boucetta and Jones, 2009; Wang and Morales, 2009). Facilitation of GABAergic transmission in the PRF increases wakefulness and decreases sleep (Camacho-Arroyo et al., 1991; Xi et al., 1999, 2001; Sanford et al., 2003; Watson et al., 2008; Fig. 2.3). Furthermore, elevating GABA levels in the PnO increases time to loss of righting as well as respiratory rate (Vanini et al., 2008). Dialysis delivery of bicuculline to the PRF increases ACh release (Vazquez and Baghdoyan, 2004; Fig. 2.10) and REM sleep (Xi et al., 1999, 2001; Sanford et al., 2003; Vazquez and Baghdoyan, 2004), supporting the role of both GABA and ACh in the PRF in REM sleep.

Table 3.1. The PRF contains GABA inter-neurons and $GABA_A$ receptors. GABA in the PRF promotes wakefulness and respiratory rate.

KEY FINDING	SPECIES	METHODS	CITATION
Within the PRF there are local and distant projecting GABAergic neurons	Cat, Rat, Mouse	Immunocytochemistry, Immunohistochemistry, Retrograde transport, Electrophysiology	Ford et al., 1995, de la Roza and Reinoso-Suarez, 2006, Brown et al., 2008, Rodrigo-Angulo et al., 2008, Liang and Marks, 2009
The PRF contains $GABA_A$ receptors	Human, Rat, Mouse	Immunocytochemistry, Immunohistochemistry, In situ hybridization	Fritschy and Mohler, 1995, Pirker et al., 2000, Heldt and Ressler, 2007, Waldvogel et al., 2010
PRF GABAergic transmission increases wakefulness and decreases sleep	Cat, Rat	Microinjection, Sleep recording	Camacho-Arroyo et al., 1991, Xi et al., 1999, Xi et al., 2001, Sanford et al., 2003, Watson et al., 2008
Increasing PRF GABA levels increases time to loss of righting and respiratory rate	Rat	Microinjection	Vanini et al., 2008
Blockade of PRF $GABA_A$ receptors increases REM sleep	Cat, Rat	Microdialysis, Microinjection, Sleep recording	Xi et al., 1999, Xi et al., 2001, Sanford et al., 2003, Vazquez and Baghdoyan, 2004
GABAergic neurons are present in the LDT/PPT	Cat, Rat	Immunocytochemistry, In situ hybridization, Unit recordings	Jia et al., 2003, Boucetta and Jones, 2009, Wang and Morales, 2009

ACETYLCHOLINE

As seen in Table 3.2, cholinergic neurons and receptors provide ACh to the PRF and play an important role in regulating sleep and wakefulness. The PRF is cholinoceptive, receiving ACh from LDT/PPT cholinergic neurons (Jones and Beaudet, 1987; Mitani et al., 1988; Shiromani et al., 1988; Lydic and Baghdoyan, 1993; Semba, 1993) (Fig. 3.1A). Stimulation of the PPT increases both ACh release and respiratory depression (Lydic and Baghdoyan, 1993). ACh release in the PRF is also modulated by muscarinic receptors. Muscarinic receptors proposed to be localized either presynaptically on LDT/PPT cholinergic terminals (Fig. 3.1B) or postsynaptically on glutamatergic neurons (Fig. 3.1C), decrease ACh release (Baghdoyan et al., 1998; Coleman et al., 2004a). M_2 muscarinic receptors shown to be localized postsynaptically on GABAergic neurons in the PRF (Brischoux et al., 2008; Brown et al., 2008) (Fig. 3.1B) contribute to the generation of REM sleep (Baghdoyan and Lydic, 1999; Coleman et al., 2004b). Muscarinic receptors also modulate ACh release in the PRF (Baghdoyan et al., 1998; Coleman et al., 2004). Taken together, the findings are consistent with the Chapter 2 results that blockade of GABAergic transmission in the PRF increases ACh release.

Table 3.2. ACh neurons in the LDT/PPT project to the PRF, which contains muscarinic receptors. ACh in the PRF modulates REM sleep.

KEY FINDING	SPECIES	METHODS	CITATION
The PRF receives cholinergic input from LDT/PPT nuclei	Cat, Rat	Immunocytochemistry, Immunohistochemistry, Anterograde/Retrograde tracing, Microdialysis, Microinjection, Electrical stimulation	Jones and Beaudet, 1987, Mitani et al., 1988, Shiromani et al., 1988, Lydic and Baghdoyan, 1993, Semba, 1993
PPT stimulation increases PRF ACh release and respiratory depression	Cat	Microdialysis and Electrical stimulation	Lydic and Baghdoyan, 1993
M_2 muscarinic receptors in the PRF modulate REM sleep	Cat, Mouse	Microinjection, Sleep recording	Baghdoyan and Lydic, 1999, Coleman et al., 2004
M_2 muscarinic receptors are on GABA neurons	Rat, Mouse	Immunohistochemistry, Electrophysiology	Brown et al., 2008, Brischoux et al., 2008
Muscarinic receptors modulate PRF ACh release	Cat, Mouse	Microdialysis	Baghdoyan et al., 1998, Coleman et al., 2004

GLUTAMATE

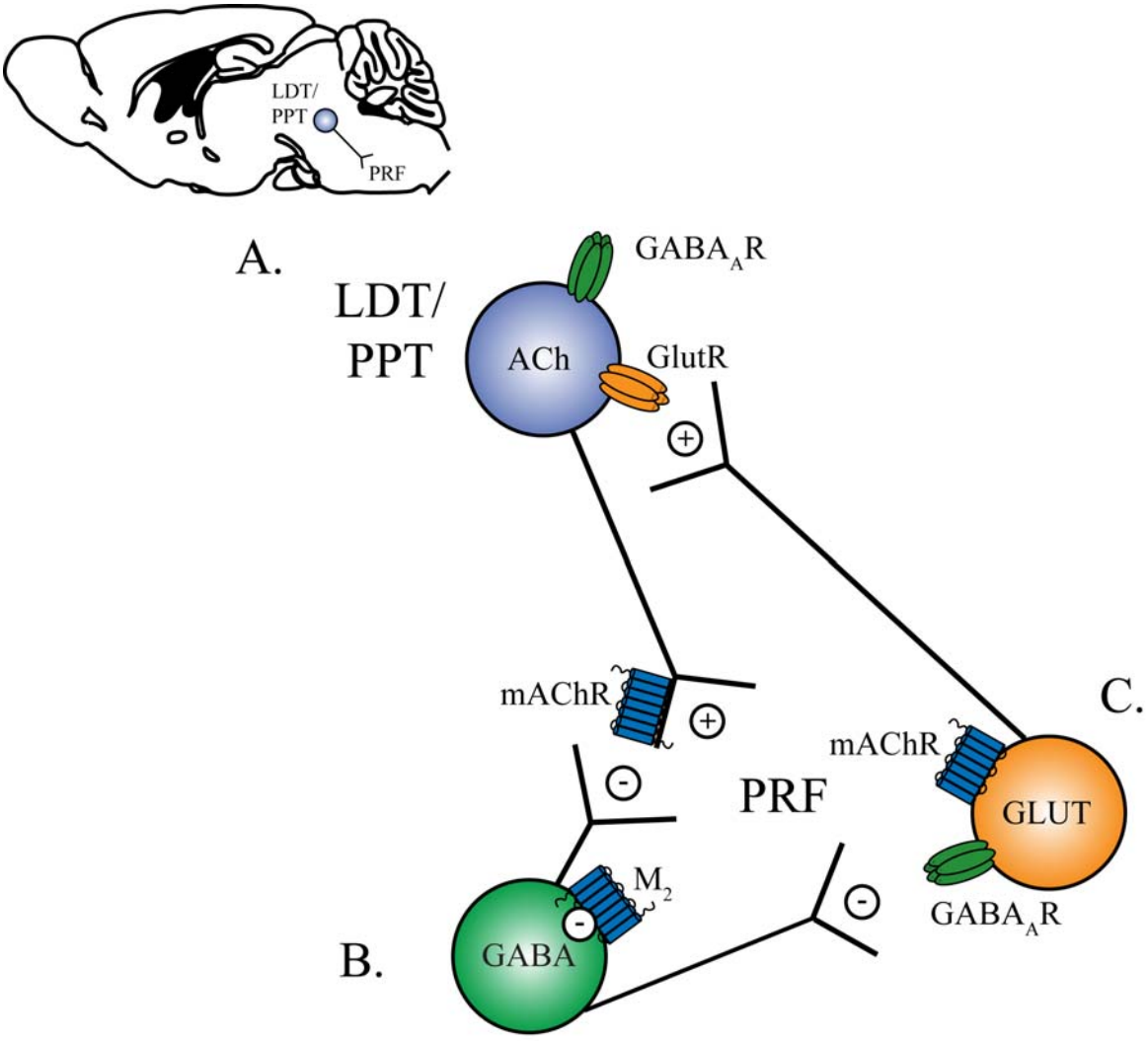
As the major excitatory neurotransmitter in the mammalian central nervous system, glutamate acts on ionotropic NMDA, AMPA, or kainate receptors and metabotropic glutamate receptors (Lodge, 2009). The involvement of glutamate in sleep and wakefulness has not been well characterized, although glutamatergic neurons and receptors have been localized to brain regions implicated in sleep-wake control such as the PRF and LDT/PPT (Table 3.3). Within the PRF, there are glutamatergic neurons that project to the LDT/PPT (Kaneko et al., 1989; Steininger et al., 1992; Lai et al., 1993) (Fig. 3.1C), which also has glutamatergic neurons (Boucetta and Jones, 2009; Wang and Morales, 2009). In addition, glutamate kainate receptors have been localized in the PPT (Fig. 3.1A), where local glutamate produces REM sleep (Datta, 2002; Datta et al., 2002). $GABA_A$ receptors on glutamate neurons in the PRF may inhibit glutamate excitation of LDT/PPT cholinergic neurons and lead to a decrease in ACh release in the PRF and an increase in wakefulness, consistent with the Chapter 2 results that GABAergic transmission increases wakefulness and the interpretation that GABA promotes wakefulness.

Table 3.3. Glutamate neurons in the PRF project to the LDT/PPT. Glutamate receptors in the PPT induce REM sleep.

KEY FINDING	SPECIES	METHODS	CITATION
The PRF contains glutamatergic neurons that project to the LDT/PPT	Cat, Rat	Immunocytochemistry, Immunohistochemistry, Retrograde tracing	Kaneko et al., 1989, Steininger et al., 1992, Lai et al., 1993
PPT kainate receptors induce REM sleep	Rat	Microinjection, Sleep recording	Datta, 2002, Datta et al., 2002
Glutamatergic neurons are present in the LDT/PPT	Rat	Immunocytochemistry, In situ hybridization, Unit recordings	Boucetta and Jones, 2009, Wang and Morales, 2009

Figure 3.1 is a synaptic model of the PRF depicting possible GABAergic, cholinergic, and glutamatergic interactions regulating states of arousal.

Figure 3.1. Synaptic model illustrating neurons in the pontine reticular formation (PRF) of B6 mouse through which GABA may promote wakefulness. **A**, Laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) cholinergic neurons (blue) provide ACh to the PRF (Jones and Beaudet, 1987; Mitani et al., 1988; Shiromani et al., 1988; Lydic and Baghdoyan, 1993; Semba, 1993). **B**, The PRF contains locally projecting GABA (green) neurons (Ford et al., 1995; de la Roza and Reinoso-Suarez, 2006; Brown et al., 2008). **C**, PRF glutamate neurons (orange) project to the LDT/PPT (Kaneko et al., 1989; Steininger et al., 1992; Lai et al., 1993). The findings in Chapter 2 that muscimol delivered to the PRF increases wakefulness and bicuculline administered to the PRF increases sleep and ACh release are consistent with the effects of muscimol and bicuculline on GABA neurons to inhibit or allow, respectively, ACh release from the LDT/PPT (B) and/or inhibit or allow, respectively, glutamate excitation of LDT/PPT cholinergic neurons (C). Inhibition or excitation of LDT/PPT cholinergic nerve terminals or neurons would result in either a decrease or increase in ACh release in the PRF and lead to wakefulness or REM sleep. The Chapter 2 results reporting an increase in recovery of righting following bicuculline administration in the PRF are consistent with the idea that blocking GABA neurons in the PRF causes ACh release by blocking inhibition of LDT/PPT ACh neurons (B) or exciting LDT/PPT ACh through PRF glutamate neurons (C). Both scenarios could lead to REM sleep and support the interpretation that GABA promotes wakefulness. One interpretation of the Chapter 2 finding that bicuculline delivered to the PRF decreases respiratory rate is that bicuculline is acting to block the inhibitory actions of GABA in the PRF on LDT/PPT ACh neurons (B), and in turn allowing ACh to be released into the PRF. When PPT neurons are stimulated they release ACh in the PRF and can cause respiratory rate depression. Plus signs (+) indicate excitatory effects and minus signs (-) indicate inhibitory effects. Abbreviations in model: ACh, cholinergic neuron; GABA, GABAergic neuron; $GABA_AR$, $GABA_A$ receptor; GLUT, glutamate neuron; GlutR, glutamate receptor; LDT/PPT, laterodorsal tegmental and pedunculopontine tegmental; M_2, M_2 subtype muscarinic receptor; mAChR, muscarinic cholinergic receptor; PRF, pontine reticular formation


LDT/
PPT
PRF
A.
LDT/
PPT
ACh
GABA$_A$R
GlutR
mAChR
PRF
C.
mAChR
GLUT
GABA$_A$R
M$_2$
GABA
B.

In conclusion, five novel findings were reported in this dissertation pertaining to the effects of GABA in the PnO of B6 mouse on states and traits of arousal. These findings showed that $GABA_A$ receptors in the PnO of B6 mouse alter states of sleep and wakefulness, cortical EEG activity, ACh release in the PnO, respiratory rate, and recovery of righting from anesthesia. Taken together, these results demonstrate that $GABA_A$ receptors in the PnO of B6 mouse regulate arousal phenotypes, and that GABA acting at $GABA_A$ receptors in the PnO promotes wakefulness.

$GABA_A$ receptors remain a major target in the pharmacological treatment of sleep disorders such as insomnia. Despite the emergence of insomnia treatments that act on other receptors such as melatonin, the clinical development of drugs acting to enhance GABAergic transmission remains viable for research programs at pharmaceutical companies such as Neurocrine Biosciences, Evotec, and Neurogen (Winsky-Sommerer, 2009). All three companies have reached at least Phase II trials with their potential drugs, and the early clinical results encourage further exploration of their use as therapies for insomnia in hopes that they may prevent some of the negative side effects patients experience with current drug treatments (Lemon et al., 2009; Walsh et al., 2009). Insomnia patients currently exhibit increases in total sleep time following systemic BDZ administration, but often times at the expense of slow wave sleep and REM sleep (Feinberg et al., 1979; Borbély and Achermann, 1991). Pharmacologically-induced state dissociations can also occur (Mendelson, 2005) when the characteristic traits which make up individual states of wakefulness, NREM sleep, and REM sleep are observed in a completely different state (Mahowald and Schenck, 1992, 2001). Interestingly, my research showed that increasing GABAergic transmission in the PnO produced decreases

in NREM sleep and REM sleep as well as state dissociations (Chapter 2). Potential effects of increasing GABAergic neurotransmission in the PnO may be to decrease REM sleep and produce state dissociations similar to the actions of current treatments for insomnia. Future investigations into the roles of other brain regions and neurotransmitters, in addition to genetically modified mouse strains, may lead to the rational design of drugs to treat the sleep loss associated with insomnia, without affecting other stages of sleep or producing state dissociations.

The present dissertation results demonstrate the use of pharmacological manipulations in the mouse to investigate arousal state control. The main focus of this dissertation research was to investigate the role of $GABA_A$ receptors in waking phenotypes in the PnO, and these data supported the finding that GABA promotes wakefulness. These dissertation results encourage the use of mouse strains, such as those that selectively knockout GABA subunits (Tobler et al., 2001; Wisor et al., 2002; Kopp et al., 2003; Winsky-Sommerer et al., 2007; Jia et al., 2008; Winsky-Sommerer et al., 2008; Rau et al., 2009), in order to uncover the synaptic mechanisms involved in arousal state control. Additional microinjection and microdialysis studies examining the role that other neurotransmitters in the PnO, such as glutamate, play in regulating states of sleep and wakefulness might be helpful in filling in the gaps of the current synaptic model (Fig. 3.1). Understanding synaptic mechanisms in the PRF as they relate to sleep and wakefulness would provide insight into the regulation of sleep and lead to a greater understanding of both sleep disorders and anesthetic action.

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