REGULATION OF AROUSAL BY ADENOSINE A_1 AND A_{2A} RECEPTORS IN THE PREFRONTAL CORTEX OF C57BL/6J MOUSE

by

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DEDICATION

To my family

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PREFACE

Appendix, entitled “Neurochemical Modulators of Sleep and Anesthetic States” was published in *Int Anesthesiol Clin*, 46: 75-104, 2008.

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°C	degrees centigrade
%	percent
ACh	acetylcholine
Ag^+	silver ion
ANOVA	analysis of variance
ATP	adenosine triphosphate
B6	C57BL/6J
CGS	2-*p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680; CGS)
CPA	N^6-Cyclopentyladenosine
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EEG	electroencephalogram
EMG	electromyogram
FFT	fast Fourier transform
FrA	frontal association area
GABA	γ-aminobutyric acid
G proteins	heterotrimeric guanine nucleotide binding proteins
h	hour(s)
HPLC/EC	high performance liquid chromatography with electrochemical detection
Hz	hertz (cycles per second)

I.V.	intravenous
LDT	laterodorsal tegmental
MAC	minimum alveolar concentration
min	minute(s)
μM	micromolar
mM	millimolar
NREM	non-rapid eye movement
P	probability
pmol	picomoles
PRF	pontine reticular formation
PPT	pedunculopontine tegmental
REM	rapid eye movement
s	second(s)
SD	standard deviation
SEM	standard error of the mean
SPA	N^6-p-Sulphophenyladenosine sodium salt
ZM	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385; ZM)

CHAPTER 1

INTRODUCTION AND STATEMENT OF RESEARCH QUESTION

The behavioral states of sleep and anesthesia are defined by specific traits. Sleep and anesthesia are also regulated by multiple neuromodulators in many brain regions. The two types of sleep, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep, are identified by analyzing specific properties of the electroencephalogram (EEG) and electromyogram (EMG). The state of general anesthesia is defined by the five characteristic traits of unconsciousness, amnesia, analgesia, immobility, and blunted autonomic responses. Although sleep and anesthesia are not the same state, anesthetics work at similar neural circuits that evolved to generate sleep (Franks, 2008, Kelz et al., 2008, Lydic and Biebuyck, 1994, Lydic, 1996, Tung et al., 2005) and this point is discussed in more detail in the Appendix. In order to further the mechanistic understanding of sleep and anesthesia (Lydic and Biebuyck, 1994), studies must characterize specific neuromodulators and brain regions. Therefore, this thesis work focused on two neuromodulators, acetylcholine (ACh) and adenosine, and two brain regions, the prefrontal cortex and the pontine reticular formation. The following introduction discusses the role of these neurotransmitters and brain regions in the context of sleep and anesthesia. First, is a discussion of the neurochemical regulation of sleep and anesthesia with focus on ACh and adenosine. The second section discusses the role

of the prefrontal cortex and pontine reticular formation in sleep and anesthesia. Third, is a discussion of the benefits and limitations of the techniques used to obtain the thesis findings. The Chapter concludes with an overview of the thesis research Aims and results.

NEUROCHEMICAL REGULATION OF SLEEP AND ANESTHESIA

Acetylcholine modulates states of sleep and wakefulness

ACh signaling is an important component of the sleep/wake regulatory system (Lydic and Baghdoyan, 2005, Semba, 1999) and ACh levels vary as a function of arousal state. For example, in the basal forebrain ACh release is highest during REM sleep compared to waking and lowest during NREM sleep (Vazquez and Baghdoyan, 2001). Similarly, frontal cortex (Marrosu et al., 1995), postcruciate cortex (Celesia and Jasper, 1966), and anterior suprasylvian and postcruciate cortex (Jasper and Tessier, 1971) ACh levels coming from cholinergic basal forebrain neurons are highest during wakefulness and REM sleep compared to NREM sleep. In the brainstem pontine reticular formation ACh release is highest during REM sleep compared to waking or NREM sleep (Kodama et al., 1990, Leonard and Lydic, 1995, Leonard and Lydic, 1997). In humans, systemic administration of the acetylcholinesterase inhibitor physostigmine during REM sleep caused the subjects to wake-up whereas systemic administration of physostigmine during NREM sleep induced REM sleep (Sitaram et al., 1976). These data are consistent with the interpretation that enhanced cholinergic neurotransmission increases cortically activated states of arousal.

Isoflurane anesthesia alters cholinergic neurotransmission

Isoflurane modulates nicotinic (Flood et al., 1997, Flood and Coates, 2002, Matsuura et al., 2002, Violet et al., 1997, Yamashita et al., 2005) and muscarinic (Do et al., 2001, Hudetz et al., 2003, Nietgen et al., 1998) cholinergic receptors and the release of ACh. Nicotinic receptors are comprised of five subunits and several studies have investigated the role of various nicotinic ACh receptor subunits in the mechanism of action of volatile anesthetics (Violet et al., 1997). Isoflurane at clinically relevant concentrations inhibits neuronal nicotinic ACh receptor currents expressed in vitro when the receptors contain the α4-β2 subunit combination (Yamashita et al., 2005). Isoflurane does not block the response of homomeric α7 nicotinic receptors to ACh when the anesthetic and the agonist are coadministered (Flood et al., 1997). However, clinically relevant concentrations of isoflurane do inhibit homomeric α7 nicotinic receptors when the anesthetic is applied prior to ACh, or when ACh is applied in high concentrations (Flood and Coates, 2002). These findings may be relevant in vivo, because α7 nicotinic receptors are present in the brain (Flood and Coates, 2002), and synaptic levels of ACh has been estimated to reach concentrations in the micromolar to millimolar range (Phillis, 2005). The effects of isoflurane on native (*i.e.*, non-recombinant) nicotinic receptors also have been investigated (Matsuura et al., 2002). Interestingly, both isoflurane and a structurally similar halogenated molecule, 1,2-dichlorohexafluorocyclobutane, that does not cause immobility but does have amnestic properties inhibit native neuronal nicotinic ACh receptors in rat medial habenula neurons (Matsuura et al., 2002). Another structurally similar agent, 2,3-dichlorooctafluorobutane, with neither immobilizing or amnestic properties does not block nicotinic receptor-mediated currents (Matsuura et al.,

2002). These data suggest that the amnestic effects of isoflurane may be mediated, in part, by nicotinic receptors in the medial habenula. More in vivo studies are needed to determine if nicotinic ACh receptors are relevant for the production of anesthesia by isoflurane.

There are five muscarinic cholinergic receptor subtypes, and isoflurane has been shown to inhibit M3 but not M1 muscarinic receptors (Nietgen et al., 1998). M1 and M3 receptors are structurally quite similar, thus different effects of the same anesthetic on these two subtypes implies that the binding site of action is quite specific. More recently, the same investigators showed that isoflurane-induced inhibition of M3 receptor signaling is mediated by an increase in protein kinase C activity, but the site of action on the M3 receptor has not yet been localized (Do et al., 2001). Another study found that intracerebroventricular administration of the acetylcholinesterase inhibitor neostigmine or the muscarinic agonist oxotremorine to isoflurane anesthetized rats increases spontaneous limb and orofacial exploratory movements, indicating increased arousal (Hudetz et al., 2003). These data are consistent with the interpretation that activation of central cholinergic neurotransmission can reverse some aspects of isoflurane anesthesia.

Studies of intact brain using in vivo microdialysis report that isoflurane causes a dose dependent decrease in ACh release in rat cerebral cortex (Dong et al., 2006, Shichino et al., 1997), rat striatum (Shichino et al., 1997), and cat pontine reticular formation (Keifer et al., 1996). The effect of isoflurane on ACh release varies with age. Isoflurane causes a significantly larger decrease in prefrontal cortex ACh release in old versus young rats (Jansson et al., 2004). Future studies are needed to determine whether aged rats show performance or memory deficits following isoflurane anesthesia. Such a

finding would support the interpretation that isoflurane-induced decreases in prefrontal cortex ACh release may contribute to increased post-operative delirium in the elderly. A discussion of the role of cholinergic neurotransmission on other anesthetics (pg 99-101, 106-108, 117-118, and 121-122) and of isoflurane on other neurotransmitters (pg 110-119) can be found in the Appendix.

Adenosinergic modulation of sleep and wakefulness

Adenosine is a sleep promoting neuromodulator that increases in the basal forebrain and cortex during prolonged wakefulness (Porkka-Heiskanen et al., 2000). The breakdown product of adenosine triphosphate, adenosine is ubiquitously distributed throughout the brain (Dunwiddie and Masino, 2001). Extracellular adenosine levels are regulated by a bidirectional nucleoside transporter (Latini and Pedata, 2001). There are four G-protein coupled receptor subtypes A_1, A_{2A}, A_{2B}, and A_3 of adenosine receptors (Ribeiro et al., 2002). Caffeine, the most commonly used psychoactive stimulant, promotes wakefulness by antagonizing adenosine A_1 and A_{2A} receptors (Fredholm et al., 1999).

Adenosine decreases isoflurane anesthesia requirements

Peroperative adenosine infusion in humans undergoing breast surgery decreases isoflurane requirement and decreases postoperative analgesic requirement (Segerdahl et al., 1995). A similar study of patients undergoing shoulder surgery showed that adenosine reduces the requirement for isoflurane but has no effect on postoperative analgesic requirement (Segerdahl et al., 1996). Although adenosine is well recognized to

have antinociceptive effects (Boison, 2008), few studies have examined the possible role of adenosine contributing to the anesthetic effects of isoflurane. In rat, isoflurane-induced reductions in focal cerebral ischemia are blocked by an adenosine A_1 receptor antagonist, indicating that this neuroprotective effect of isoflurane may be mediated by adenosine A_1 receptors (Liu et al., 2006). Isoflurane-induced activation of adenosine A_1 receptors in primary cultures of rat hippocampal neurons also suppresses spontaneous calcium oscillations (Tas et al., 2003). This study suggests that another mechanism by which isoflurane may be neuroprotective is by increasing adenosine levels (Tas et al., 2003). A discussion of the role of adenosinergic neurotransmission on other anesthetics can be found in the Appendix (pg 101-102, 108, 119, and 122).

BEHAVIORAL STATE MODULATION BY THE PREFRONTAL CORTEX AND THE PONTINE RETICULAR FORMATION

The prefrontal cortex is deactivated during sleep and anesthesia

The prefrontal cortex regulates executive functions such as working memory, a sense of time, controlling inhibition (Fuster, 2001), and attention (Sarter et al., 2006). Sleep deprivation strongly impairs prefrontal cortex functions such as psychomotor vigilance (Muzur et al., 2002). In the elderly, prefrontal cortex slow wave activity during NREM sleep is correlated with better performance (Anderson and Horne, 2003). During the unconsciousness states of sleep (Stickgold et al., 2001) and anesthesia (Andrade, 1996) the prefrontal cortex is deactivated. However, during REM sleep where much of the cortex is reactivated, the dorsolateral prefrontal cortex remains deactivated, and this deactivation likely accounts for the different sense of self awareness and time that occurs

in dreams (Muzur et al., 2002).

Further support that the prefrontal cortex contributes to arousal state control comes from studies demonstrating changes in prefrontal cortex neurotransmitters across the sleep wake cycle. For example, extracellular levels of serotonin in the prefrontal cortex are higher during wakefulness compared to NREM sleep (de Saint Hilaire et al., 2000) whereas prefrontal cortex dopamine levels are highest during wakefulness and REM sleep compared to NREM sleep in rat (Lena et al., 2005). Cortical ACh levels also varies across the sleep wake cycle being highest during the activated states of wakefulness and REM sleep (Marrosu et al., 1995), (Celesia and Jasper, 1966), (Jasper and Tessier, 1971).

The pontine reticular formation contributes to the regulation of sleep and anesthesia

Early brain transection studies by Jouvet (Jouvet, 1962) demonstrated that the pontine reticular formation was necessary for the generation of NREM and REM sleep. Both lesions of the pontine reticular formation in humans (Autret et al., 1988, Gironell et al., 1995, Kimura et al., 2000, Kushida et al., 1991, Lavie et al., 1984) and lesions of the cholinergic dorsolateral pontomesencephalic tegmentum in non-human animals (Webster and Jones, 1988) cause disruption of REM sleep. In the pontine reticular formation, ACh release is highest during REM sleep compared to wakefulness and NREM sleep (Kodama et al., 1990, Leonard and Lydic, 1995, Leonard and Lydic, 1997). Cholinomimetics delivered to the pontine reticular formation induce a REM sleep-like state in cat (Baghdoyan et al., 1984), rat (Bourgin et al., 1995), and mouse (Lydic et al., 2002). The pontine reticular formation also contributes to regulation of attention. When humans

perform attention demanding tasks, the pontine reticular formation is activated as measured by positron emission tomography (Kinomura et al., 1996). Anesthetics modulate pontine reticular formation neurotransmission as demonstrated by a decrease in ACh release within the pontine reticular formation caused by halothane in cat (Keifer et al., 1996).

TECHNICAL CONSIDERATIONS

The mouse model: a tool for studies of arousal state control

The C57BL/6J (B6) mouse genome has been sequenced and shares 99% homology with the human genome (Mouse Genome Sequencing Consortium, 2002). This similarity with the human genome and known genomic sequence provides the rationale for use of mice in the current thesis studies. This remarkable homology implies that studies using mice have the potential to establish links, relevant for humans, between physiological and behavioral phenotypes and their underlying causal genotype. Considerable evidence supports the view that understanding genetic modulation of normal phenotypes will ultimately make it possible to develop molecular therapy targeted at specific human diseases (Balicki and Beutler, 2002, Green et al., 2000, Hollams et al., 2002, Sakamoto, 2004). Thus, these thesis studies used B6 mice to investigate the role of prefrontal cortex adenosine A_1 and A_{2A} receptors in arousal state control.

Knowledge of the mouse genome has been useful for the development of knockout models where a specific gene can be deleted and the resulting phenotype analyzed. An acknowledged limitation of knockout models is that the absence of the

gene during development can cause compensatory changes and lead to differences in the adult animal that are not due to the gene of interest (Robert, 1998). One way to circumvent this problem is to use conditional knockout mice with promoters such as CAMKII that do not eliminate the gene until after primary development (Wang et al., 2003). In a study related to this thesis work, adenosine A_1 or A_{2A} receptor knockout mice were given caffeine and only the adenosine A_{2A} receptor knockout mice showed no enhancement of wakefulness by caffeine (Huang et al., 2005). The authors of this study interpreted this result to suggest that caffeine promotes wakefulness through the adenosine A_{2A} receptor and not the adenosine A_1 receptor. In contrast to this finding in knockout mice, the results of the present thesis work using intact B6 mice revealed that both adenosine A_1 and A_{2A} receptors in the prefrontal cortex contribute to the regulation of cortical and behavioral arousal.

Measures of behavioral arousal

EEG Analysis: The behavioral arousal state of human and non-human animals can be determined by analyzing the EEG. The present thesis work used fast Fourier transform (FFT) analysis to quantify the power at a given frequency of the EEG waveform (Pigeau et al., 1981). This analysis yields a graphical representation of the characteristics of the EEG. During the cortically activated states of wakefulness and REM sleep, many neurons fire in a fast desynchronized pattern resulting in a low amplitude high frequency EEG (Campbell et al., 2006, Subasi, 2005, Sussman et al., 1979, Zhang et al., 2008). During the cortically deactivated states of NREM sleep and anesthesia, neuronal firing is synchronized resulting in a large amplitude slow frequency

EEG (Campbell et al., 2006, Subasi, 2005, Sussman et al., 1979, Zhang et al., 2008). The EEG waveform and power at a given EEG frequency provide standard tools for objectively classifying behavioral states as wakefulness, NREM sleep, or REM sleep. These EEG and FFT characteristics are homologous to those observed in the human EEG. This excellent homology makes it possible for pre-clinical studies of behavioral state control using non-human animals to elucidate the cellular and molecular mechanisms regulating sleep in humans.

Recovery of righting response: The righting response is a well established measure of recovery from various hypnotics ranging from anesthetics (Straw and Mitchell, 1967) to drugs of abuse such as ethanol (Melchior and Allen, 1993) and opioids (Sakai et al., 2002). The righting response after inhaled anesthetics in mice (DeMarco et al., 2004) and rats (Ishizawa, 2000) has been used to further delineate the cholinergic mechanisms of anesthetic action. The present thesis work used the righting response after isoflurane anesthesia in mouse as a measure of behavioral arousal in response to adenosine receptor agonists and antagonists.

In vivo microdialysis

Microdialysis has revolutionized the study of brain neurochemistry because it permits brain region specific measurement of neurotransmitters (Benveniste and Huttemeier, 1990, Jin et al., 2008, Robinson and Justice, 1991, Watson et al., 2006) as well as pharmacological manipulation of local environments (Hocht et al., 2007, Li et al., 2006, Pan et al., 2007, Yu et al., 2007). Microdialysis probes consist of a semipermeable membrane where molecules, such as neurotransmitters or drugs, diffuse across their

concentration gradient both in and out of the probe. CMA/7 dialysis probes used in the present thesis research are estimated to deliver about 5% of the dialysate drug concentration. This estimate comes from in vitro analysis of the probes ability to recovery a known concentration of acetylcholine. Many factors affect diffusion across the dialysis probe membrane including dialysate composition, dialysate flow rate, the molecular weight cut off of the membrane, temperature, and the environment surrounding the probe membrane (Watson et al., 2006). The following discusses these factors in relation to differences between in vitro and in vivo studies. The dialysate (Ringer's) is isotonic with cerebral spinal fluid to minimize ion and fluid diffusion across the membrane. The dialysate flow rate was held constant at 2 μl/min, a commonly used flow rate for in vivo microdialysis studies (Coleman et al., 2006). The molecular weight cut off of the membrane (6 kD) is factory determined and stays constant across all experiments. The dialysate composition, flow rate, and membrane cut off are the same for both in vitro and in vivo studies. One difference between in vitro and in vivo studies is the temperature. In vitro probe recoveries are collected at room temperature (~21 degrees C) whereas the in vivo samples are collected at (~37 degrees C). This increase in temperature will slightly increase diffusion across the probe membrane compared to our in vitro probe recovery conditions. However, as discussed below, this slight increase in diffusion does not increase diffusion greater than the inhibitory factors present by placing the probe in brain tissue. For example, the environment surrounding the probe affects diffusion and also differs between in vitro and in vivo studies. In vivo, there are neurons and glial cells surrounding the probe that impede direct diffusion (tortuosity) to the dialysis membrane (Benveniste and Huttemeier, 1990, Watson et al., 2006), whereas, in

vitro, the probe is surrounded by a homogenous solution. The homogenous solution gives the best case diffusion scenario across the probe membrane. While in vitro recovery of acetylcholine measures the ability of a molecule outside the probe to get into the probe, and in vivo drug delivery is the ability of a drug inside the probe to get out of the probe, the same membrane properties determine the outcome of diffusion in or out of the probe. Despite the many variables that affect microdialysis, the drugs tested in the present thesis studies caused significant changes in cortical and pontine reticular formation ACh release, anesthesia recovery time, and EEG delta power, demonstrating the robust nature of the results.

OVERVIEW OF RESEARCH GOALS AND FINDINGS

The unifying goal for this thesis research was to characterize cholinergic and adenosinergic mechanisms in the prefrontal cortex of B6 mouse that contribute to arousal state control. The prefrontal cortex has multiple regulatory roles including the temporal organization of behavior (Fuster, 2001), working memory (Gabrieli et al., 1998, Goldman-Rakic, 1996), regulation of behavioral arousal (Horne, 1993, Muzur et al., 2002), controlling inhibition (Fuster, 2001) and autonomic regulation (Groenewegen and Uylings, 2000). The rationale for focusing on the prefrontal cortex as an important region for arousal state control is provided by evidence that sleep deprivation impairs many functions subserved by the prefrontal cortex, and the prefrontal cortex is deactivated during sleep (Stickgold et al., 2001) and anesthesia (Andrade, 1996). Prefrontal cortex ACh is essential for normal cognitive function (Sarter and Bruno, 1997,

Yu and Dayan, 2002) and prefrontal cortex ACh levels increase during tasks that require attention (Kozak et al., 2006, Passetti et al., 2000). Cognitive performance decreases with prolonged wakefulness (Thomas et al., 2000) and during prolonged wakefulness, adenosine levels rise within the basal forebrain and cortex to promote sleep (Porkka-Heiskanen et al., 2000). Further evidence that adenosine promotes sleep is supported by the fact that the most commonly used psychoactive stimulant caffeine, is an adenosine A_1 and A_{2A} receptor antagonist (Fredholm et al., 1999). There is debate in the field as to which adenosine receptor subtype is more important for the role of adenosine in arousal state control. Available evidence supports both adenosine A_1 (Basheer et al., 2001, Benington et al., 1995, Yanik and Radulovacki, 1987) and A_{2A} (Huang et al., 2005, Stenberg et al., 2003, Urade et al., 2003) receptors in the regulation of behavioral arousal by adenosine. The exact role of specific receptor subtypes vary as a function of brain region (Fredholm et al., 2005, Ribeiro et al., 2002). Although data make clear that adenosine promotes sleep and ACh promotes arousal, the interaction between these two neurotransmitter systems in the prefrontal cortex remain incompletely understood. The following aims of this thesis research were designed to provide insight into the mechanisms through which adenosinergic and cholinergic neurotransmission in the prefrontal cortex modulate behavioral arousal.

Specific Aims

Specific Aim 1 tested the hypothesis that adenosine A_1 and A_{2A} receptors in the prefrontal cortex of B6 mouse modulate cortical ACh release, behavioral arousal, and EEG delta power. Aim 1 results are reported in Chapter 2. Aim 1 used in vivo microdialysis to deliver adenosine A_1 and A_{2A} receptor agonists and antagonists to the prefrontal cortex of isoflurane anesthetized B6 mice. Prefrontal cortex ACh release, anesthesia recovery time, and EEG delta power were quantified. The adenosine A_{2A} receptor agonist CGS was delivered to the prefrontal cortex by microdialysis. These studies made it possible to evaluate the extent to which prefrontal cortex ACh release was modulated by adenosine A_{2A} receptors. CGS caused a concentration dependent increase in prefrontal cortex ACh release and decrease in anesthesia recovery time. These CGS-induced alterations in ACh release and anesthesia recovery time were blocked by coadministration of the adenosine A_{2A} receptor antagonist ZM. CGS also decreased cortical EEG delta power. The results showed for the first time that activation of adenosine A_{2A} receptors in the prefrontal cortex promotes waking phenotypes (Van Dort CJ et al., 2007a). In contrast, microdialysis delivery of the adenosine A_1 receptor agonist SPA to the prefrontal cortex caused a concentration dependent decrease in prefrontal cortex ACh release and delayed anesthesia recovery time. Coadministration of SPA with the adenosine A_1 receptor antagonist DPCPX blocked the SPA-induced decrease in ACh release and increase in anesthesia recovery time (Van Dort CJ et al., 2006a). Consistent with the interpretation that activation of adenosine A_1 receptors in the prefrontal cortex decreases waking phenotypes, SPA also increased cortical EEG delta power. Having

demonstrated that activation of prefrontal cortex adenosine A_1 and A_{2A} receptors by the exogenous agonists altered ACh release, anesthesia recovery time, and EEG delta power, the next set of experiments was designed to determine if the endogenous molecule, adenosine, also modulates these waking phenotypes. Dialysis delivery of the adenosine A_1 and A_{2A} receptor antagonist caffeine or the adenosine A_1 receptor antagonist DPCPX caused a concentration dependent increase in ACh release and decrease in anesthesia recovery time (Van Dort CJ et al., 2006b). EEG delta power was decreased by both caffeine and DPCPX, whereas the adenosine A_{2A} receptor antagonist ZM did not alter ACh release, anesthesia recovery time, or EEG delta power. The results are consistent with the interpretation that endogenous adenosine in the prefrontal cortex inhibits waking phenotypes through adenosine A_1 receptors. Taken together, these data from Aim 1 support the conclusion that adenosine A_1 receptors in the prefrontal cortex of B6 mouse modulate arousal and suggest that caffeine promotes arousal, in part, through prefrontal cortex adenosine A_1 receptor blockade.

Specific Aim 2 tested the hypothesis that blockade of adenosine A_1 receptors in the prefrontal cortex increases wakefulness and decreases sleep. The results of Aim 2 are shown in Chapter 2. Acetylcholine and adenosine levels vary with arousal state of the animal, therefore the Aim 1 studies described above used isoflurane anesthesia to hold arousal state constant. In order to determine if prefrontal cortex adenosine A_1 receptors also modulate the naturally occurring states of sleep and wakefulness, Aim 2 used unanesthetized intact behaving mice implanted with EEG and EMG electrodes and a guide tube aimed for the prefrontal cortex. The states of sleep and

wakefulness were quantified for 2 h following microinjection of DPCPX or saline (vehicle control). DPCPX caused a significant increase in wakefulness and decrease in NREM sleep but did not change REM sleep (Van Dort CJ et al., 2007b). These data from intact freely behaving mice further support the Aim 1 conclusion that activation of prefrontal cortex adenosine A_1 receptors by endogenous adenosine not only modulates the waking phenotypes of increased ACh release, decreased anesthesia recovery time, and decreased EEG delta power but also inhibits wakefulness.

Specific Aim 3 tested the hypothesis that adenosine A_1 and A_{2A} receptors in the prefrontal cortex modulate ACh release in the pontine reticular formation. Aim 3 findings are reported in Chapter 3. Early brain transection studies (Jouvet, 1962) revealed that rhythmic oscillations between the physiological signs of NREM sleep and REM sleep persist after the forebrain is removed. These studies helped localize the central pattern generator for REM and NREM sleep to the pontine brainstem. Aim 1 and 2 results showed that adenosine receptors in the prefrontal cortex modulate EEG, behavioral arousal, and sleep. In view of early studies showing that rostral brain regions are not necessary for generating the ultradian rhythm of REM and NREM sleep, the Aim 1 and 2 results imply that the prefrontal cortex modulates states of sleep and wakefulness by way of descending input to caudal arousal control centers. Therefore, Aim 3 was designed to determine if prefrontal cortex adenosine receptors modulate pontine reticular formation ACh release. The Aim 3 results indicate that microdialysis delivery of the adenosine A_{2A} receptor agonist CGS to the prefrontal cortex increased pontine reticular formation ACh release whereas microdialysis delivery of the adenosine A_1 receptor

agonist SPA to the prefrontal cortex decreased pontine reticular formation ACh release. These findings are the first to demonstrate that pharmacological manipulation of the prefrontal cortex can alter neurotransmitter release in the pontine reticular formation. This finding also suggests that one mechanism by which prefrontal cortex adenosine A_1 and A_{2A} receptors modulate behavioral arousal is by altering pontine reticular formation ACh release. The next set of studies was designed to determine if endogenous adenosine also modulates behavioral arousal through pontine reticular formation ACh release. Blocking a receptor with an antagonist permits the inference that the endogenous neurotransmitter for that receptor modulates the dependent measure being quantified. Therefore, the adenosine A_1 and A_{2A} receptor antagonist caffeine or the adenosine A_1 receptor antagonist DPCPX were delivered by microdialysis to the prefrontal cortex. Both drugs caused a significant increase in pontine reticular formation ACh release. Taken together, these data are consistent with the interpretation that endogenous adenosine in the prefrontal cortex modulates arousal, in part, by altering pontine reticular formation ACh release.

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CHAPTER 2

ADENOSINE A_1 AND A_{2A} RECEPTORS IN MOUSE PREFRONTAL CORTEX MODULATE ACETYLCHOLINE RELEASE AND BEHAVIORAL AROUSAL

SUMMARY

During prolonged intervals of wakefulness brain adenosine levels rise within the basal forebrain and cortex. The view that adenosine promotes sleep is supported by the corollary that N-methylated xanthines such as caffeine increase brain and behavioral arousal by blocking adenosine receptors. The four subtypes of adenosine receptors are distributed heterogeneously throughout the brain, yet the neurotransmitter systems and brain regions through which adenosine receptor blockade causes arousal are incompletely understood. The prefrontal cortex contributes to the regulation of sleep and wakefulness. This study used in vivo microdialysis to quantify prefrontal cortex acetylcholine release with simultaneous recording of the cortical electroencephalogram and time to waking after anesthesia. In additional experiments, sleep and wakefulness were quantified after microinjection of an adenosine A_1 receptor antagonist into the prefrontal cortex. This chapter reports the results from Aims 1 and 2 demonstrating that adenosine A_1 and A_{2A} receptors in the prefrontal cortex modulate cortical acetylcholine release, behavioral arousal, electroencephalographic delta power, and sleep. The results provide novel evidence that adenosine A_1 receptors within the prefrontal cortex comprise part of a descending system that inhibits wakefulness.

INTRODUCTION

States of electroencephalographic (EEG) and behavioral arousal are modulated by adenosinergic and cholinergic neurotransmission (Steriade and McCarley, 2005). During prolonged intervals of wakefulness, adenosine levels increase within the basal forebrain and cortex (Benington and Heller, 1995, Huston et al., 1996, Porkka-Heiskanen et al., 2000, Radulovacki, 2005). This rise in adenosine inhibits sub-cortical cholinergic neurons known to promote arousal (Basheer et al., 2004, Porkka-Heiskanen et al., 2002). Caffeine, the most widely used psychoactive drug (Fredholm et al., 1999), is an adenosine A_1 and A_{2A} receptor antagonist but the mechanisms through which caffeine promotes behavioral arousal are incompletely understood.

There is good agreement that adenosine A_1 and A_{2A} receptors in the brainstem of mouse (Coleman et al., 2006), rat (Marks et al., 2003), and cat (Tanase et al., 2003) contribute to the regulation of arousal. The finding that systemically administered caffeine increased wakefulness in adenosine A_1, but not A_{2A}, receptor knockout mice was interpreted to demonstrate that caffeine promotes wakefulness by antagonizing adenosine A_{2A} but not A_1 receptors (Huang et al., 2005). No studies have determined whether cortical adenosine A_1 and/or A_{2A} receptors modulate arousal in wild-type mice.
The prefrontal cortex contributes to arousal state control in humans (Muzur et al., 2002) and modulates EEG activation in C57BL/6J (B6) mouse (Douglas et al., 2002). The present study tested the hypothesis that adenosine A_1 and A_{2A} receptors in the prefrontal cortex of B6 mouse modulate cortical acetylcholine (ACh) release, behavioral arousal, EEG delta power, and sleep. Adenosine A_1 and A_{2A} receptor agonists and antagonists were delivered by microdialysis to the prefrontal cortex while measuring prefrontal

cortex ACh release, EEG delta power, and time required to resume wakefulness after anesthesia. Finally, this study determined whether microinjecting an adenosine A_1 receptor antagonist into the prefrontal cortex increases wakefulness and decreases sleep.

MATERIALS AND METHODS

Overview of Experimental Design

All experiments adhered to the Public Health Service Policy on Humane Use and Care of Laboratory Animals (NIH Publication 80-23, National Academy of Sciences Press, Washington, DC, 1996) and were reviewed and approved by the University of Michigan Committee on Use and Care of Animals. Experiments used adult male C57BL/6J (B6) mice (n=101; Jackson Laboratory, Bar Harbor, ME, USA). Mice had *ad libitum* access to food and water. Each mouse was used for only one dialysis experiment, and only one drug concentration was tested per mouse.

The time course of the dialysis experiments and collection and quantification of ACh from the frontal association area (FrA) is schematized by Figure 2.1A. The FrA is the mouse homologue of the prefrontal cortex (Fuster, 2001). These studies measured prefrontal cortex ACh release during isoflurane anesthesia. Control ACh release was determined during dialysis with Ringer's prior to dialysis delivery of adenosine A_1 and A_{2A} receptor agonists and antagonists. An additional dependent measure included the time (min) required for resumption of righting after offset of isoflurane anesthesia. A second set of experiments conducted during isoflurane anesthesia recorded the cortical EEG while measuring prefrontal cortex ACh release and time to waking after anesthesia. The dependent measures of time to waking after anesthesia (Tung et al., 2002, Tung et

al., 2005), ACh release in the prefrontal cortex (Kozak et al., 2006), and decreased EEG delta power (Douglas et al., 2002) are accepted indicators of behavioral arousal. A third set of experiments was conducted using intact, unanesthetized mice implanted with a guide tube aimed for the prefrontal cortex and with EEG and EMG electrodes, making it possible to record states of wakefulness, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep.

Experimental Procedures

Microdialysis and high performance liquid chromatography with electrochemical detection (HPLC/EC). Mice were anesthetized with 2.0% isoflurane in O_2 and placed in a Kopf Model 962 small animal stereotaxic instrument with a Kopf Model 923-B mouse anesthesia mask (David Kopf Instruments, Tujunga, CA). Delivered isoflurane concentration was measured using a Cardiocap/5 monitor (Datex-Ohmeda, Madison, WI) and decreased to 1.6% for the surgery. Core body temperature was monitored and held constant between 36.5-37.5°C throughout the experiment. A skull craniotomy of 1 mm diameter was made at stereotaxic coordinates 3.0 mm anterior to bregma and 1.6 mm lateral to the midline (Paxinos and Franklin, 2001) for placement of a CMA/7 dialysis probe (6 kDa cutoff; 1.0 mm length, 0.24 mm diameter cuprophane membrane; CMA/Microdialysis, Stockholm, Sweden) in the prefrontal cortex (Fig. 1B). After the craniotomy was made, delivered isoflurane concentration was reduced and maintained at 1.3%, corresponding to the EC_{50} or minimum alveolar concentration (1 MAC) for B6 mouse (Sonner et al., 1999).

The microdialysis probe was perfused continuously (2 µl/min) with Ringer's solution (control) comprised of 147 mM NaCl, 2.4 mM $CaCl_2$, 4 mM KCl, and 10 µM

neostigmine. Salts used in the Ringer's solution were purchased from Fisher Scientific (Pittsburgh, PA). Drugs delivered to the prefrontal cortex by microdialysis included the adenosine A_{2A} receptor agonist 2-*p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680; CGS), the adenosine A_{2A} receptor antagonist 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385; ZM), the adenosine A_1 receptor agonist N^6-p-Sulfophenyladenosine sodium salt (SPA), and the adenosine A_1 receptor antagonist 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX). Prefrontal cortex ACh was also measured before and during delivery of the adenosine receptor antagonist caffeine. DPCPX and ZM were dissolved in dimethylsulfoxide (DMSO) and diluted in Ringer's to a final DMSO concentration of 1.0% and 0.07% respectively. CMA/7 dialysis probes are estimated to deliver about 5% of any given drug concentration. Thus, estimated drug concentrations that significantly changed the dependent measures ranged from 500 nM to 50 μM. SPA, CGS, DPCPX, caffeine, and neostigmine bromide were purchased from Sigma-Aldrich (St. Louis, MO). ZM was purchased from Tocris (Ellisville, MO).

A CMA/110 liquid switch was used to dialyze with Ringer's followed by Ringer's containing drug (Fig. 1A). Dialysis samples were collected every 12.5 min. Six samples were collected during dialysis with Ringer's (control) then six samples were collected during dialysis with drug. Each 25 μl dialysis sample was injected into a HPLC/EC system (Bioanalytical Systems, (BAS), West Lafeyette, IN). The sample was carried through the system in 50 mM Na_2HPO_4 mobile phase (pH 8.5) at a flow rate of 1 ml/min. ACh and choline were separated by an analytical column and then broken down into hydrogen peroxide by an immobilized enzyme reaction column. The hydrogen peroxide

was detected by a platinum electrode (0.5 V applied potential) in reference to an Ag^{+}/AgCl electrode. Chromgraph software (Bioanalytical Systems Inc.) was used to digitize and quantify the resulting chromatograms (Fig. 1C) compared to a series of ACh standards. The percentage of a known ACh solution recovered by the probe was determined before and after every experiment. If the pre-and post-experiment probe recoveries were significantly different by t-test, the data from that experiment were discarded.

Time to Resumption of Wakefulness after Anesthesia. At the end of the experiment when microdialysis sample collection was completed, the dialysis probe was removed, the scalp incision was closed, and anesthesia was discontinued. Mice were removed from the stereotaxic frame and placed in dorsal recumbency under a heat lamp. The time to righting and resumption of normal weight-bearing posture was recorded. The resumption of righting response provides an established measure of the time for the onset of wakefulness after cessation of anesthesia (Bignall, 1974, DeMarco et al., 2004, Kelz et al., 2008, Kubota et al., 1999, Tung et al., 2002, Tung et al., 2005).

EEG Recording and Power Spectral Analysis. Electrodes for recording the EEG were constructed from 0.13 mm diameter stainless steel wire (California Fine Wire, Grover City, CA) and were implanted above the cortex, contralateral to the microdialysis probe. The stereotaxic coordinates for the 2 EEG electrodes were 2.8 mm anterior to bregma and 2.4 mm posterior to bregma with both at -1.5 mm lateral to the midline. The EEG was recorded during dialysis with Ringer's (control; 75 min) and during dialysis with drug (75 min). The EEG signal was amplified and filtered at 0.3 and 30 Hz using a Grass (West Warwick, RI) Model P511K amplifier. The amplified and filtered EEG was

digitized and recorded using Spike2 (Cambridge Electronic Design, Cambridge, UK) software at a sampling rate of 128 Hz. Fast Fourier transform (FFT) analysis was used to compute EEG power (V^2) in 0.5 Hz increments for EEG frequencies ranging from 0.5 to 25.0 Hz. Sample bins were 10 s in duration. During each experiment the 10 s bins were sampled every 250 s and EEG power was computed for 18 bins during Ringer's dialysis and 18 bins during dialysis drug delivery.

Recording Sleep in the Freely Behaving Mouse. Adult male B6 mice were stereotaxically implanted under isoflurane anesthesia with EEG and EMG recording electrodes and a guide tube aimed for the prefrontal cortex as described above. The guide tube was placed at a 45 degree angle at coordinates 3 mm anterior to bregma and 0.5 mm lateral to the midline (Paxinos and Franklin, 2001). The EEG recording electrodes were constructed as described above and the EMG recording electrodes were constructed from AS632 biomed wire (Cooner Wire Company, Chatsworth, CA). The guide tube, two stainless steel anchor screws (Small Parts Inc., Miami Lakes, FL), EEG and EMG electrodes, and a plastic pedestal (Plastics One, Roanoke, VA) containing the electrodes were adhered to the skull using dental acrylic. The mice were allowed to recover for one week and conditioned to handling and being placed in a Plexiglas recording chamber (BAS Raturn system; Bioanalytical Systems Inc., West Lafayette, IN).

The day before the experiment, mice were placed in the recording chamber between 16:00 and 17:00 h and left overnight. On the day of the experiment between 9:00 and 10:00 h, a 50 nL microinjection of 100 μM (1.5 ng) DPCPX or saline + 1% DMSO (vehicle) was performed followed by a 2 h recording of sleep and wakefulness. The EEG and EMG signals were amplified with a Grass Instruments model 15RXi

amplifier (West Warwick, RI). Signals were filtered (EEG: 0.1-30 Hz; EMG: 10-100 Hz), digitized at 128 Hz, and recorded using Icelus Acquisition software (Opp, 1998). Each mouse received 2 microinjections that were separated by 1 week. States of wakefulness, NREM sleep, and REM sleep were quantified in 10 s bins. The EEG power spectra were quantified using FFT analysis.

Statistics

ACh release was expressed as percent of ACh measured during dialysis with Ringer's (control condition). Drug main-effects on prefrontal cortex ACh release and time to waking after anesthesia were analyzed using one-way analysis of variance (ANOVA). Post-hoc analyses included Dunnett's statistic, Tukey-Kramer multiple comparisons test, and Student's t-test (GBStat^{TM} v6.5.6, Dynamic Microsystems, Inc., Silver Spring, MD). Drug effects on EEG power were evaluated using two-way repeated measures ANOVA on treatment, frequency and the interaction, and a post hoc analysis to test the effect of treatment on a given frequency (SAS v9.1.3, SAS Institute Inc., Cary, NC). Non-linear regression analysis of the concentration response data provided coefficients of determination (r^2) for each drug (GraphPad Prism software v4.0a for Macintosh, GraphPad Software Inc., San Diego, CA). Sleep and wake data were analyzed using Student's t-test. Data are reported as mean ± standard error of the mean (SEM). A probability value of $P \leq 0.05$ was considered to be statistically significant.

Histology

Seven days after microdialysis experiments and 3 days after microinjection experiments, mice were deeply anesthetized and decapitated. The brains were removed, frozen, and coronally sectioned (40 μm thick). The sections were fixed with hot (80°C)

paraformaldehyde vapor and stained with cresyl violet. The location of a glial scar caused by the microdialysis probe or microinjector was determined by comparing stained sections with a mouse brain atlas (Paxinos and Franklin, 2001). Results were included in the group data only for experiments in which histology confirmed that the microdialysis probe or microinjector was placed in the prefrontal cortex.

RESULTS

Figure 2.1A illustrates the within-subjects design used for the microdialysis experiments. ACh release was quantified during 75 min of dialysis with Ringer's (control) followed by an additional 75 min of dialysis with Ringer's (negative control) or Ringer's containing drug. Results of the negative control experiments confirmed that ACh release was stable during 150 min of isoflurane anesthesia. One-way ANOVA revealed no significant difference in total anesthesia time (mean ± SEM = 284 ± 2 min) across all microdialysis experiments. The results summarize data obtained from 101 mice. Post-experiment histological analysis using the B6 mouse brain atlas (Paxinos and Franklin, 2001) confirmed that all microdialysis and microinjection sites were within the FrA of the prefrontal cortex.

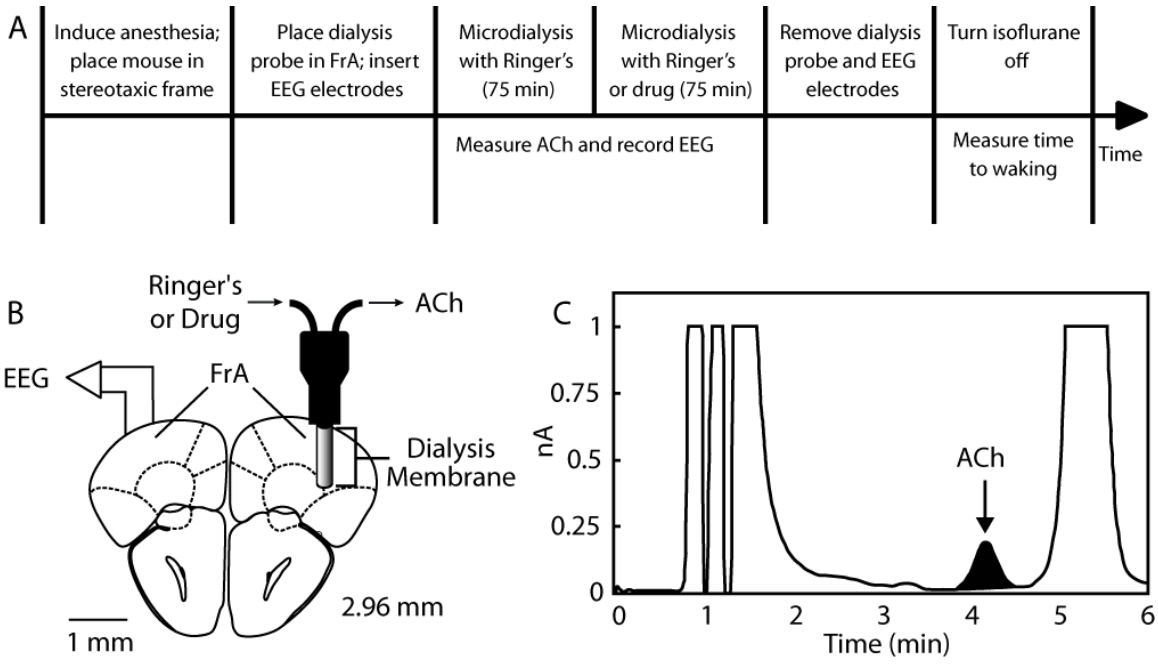

Figure 2.1. Microdialysis experimental design. (A) Schematic time-line of microdialysis experiments. The top row outlines the experimental manipulations and the bottom row indicates when the dependent measures were obtained. (B) The coronal diagram modified from a mouse brain atlas (Paxinos and Franklin, 2001) illustrates placement of a microdialysis probe in the frontal association area (FrA), mouse homologue of the prefrontal cortex (Fuster, 2001). The dialysis probe membrane (1 mm length, 0.24 mm diameter) is drawn to scale and EEG recording electrodes are schematized above the contralateral prefrontal cortex. (C) The representative chromatogram shows an acetylcholine (ACh) peak obtained by microdialysis of the prefrontal cortex.

An adenosine A_{2A} receptor agonist increased waking phenotypes.

The first set of studies was designed to determine whether microdialysis delivery of the adenosine A_{2A} receptor agonist CGS to the prefrontal cortex increased waking phenotypes. For these mice (n = 21) waking phenotypes included increased cortical ACh release, decreased time to waking after anesthesia, and cortical EEG activation. CGS caused a significant drug main effect on ACh release within the prefrontal cortex ($F_{(5,12)}$ = 12.8, P = 1.9 x 10^{-4}) (Fig. 2.2A) and time required for resumption of wakefulness after isoflurane anesthesia ($F_{(5,12)}$ = 6.4, P = 0.0042) (Fig. 2.2B). The right-most point in Fig. 2.2A and B (10 + ZM) shows that CGS (10 μM) coadministered with the adenosine A_{2A} receptor antagonist ZM (0.1 μM) blocked the effects of CGS on ACh release and anesthesia recovery time. Figure 2.2C illustrates that CGS also decreased delta power in the cortical EEG. FFT analyses confirmed a decrease in EEG delta power (0.5 to 4 Hz) caused by CGS. Two-way repeated measures ANOVA revealed no frequency by drug interaction but post hoc analyses comparing the means at each frequency between treatments revealed that delta power from 0.5 to 1.0 Hz was significantly reduced by CGS. The ANOVA included 8 frequency conditions (0.5 to 4.0 Hz every 0.5 Hz) and 2 treatment conditions (Ringer's or CGS).

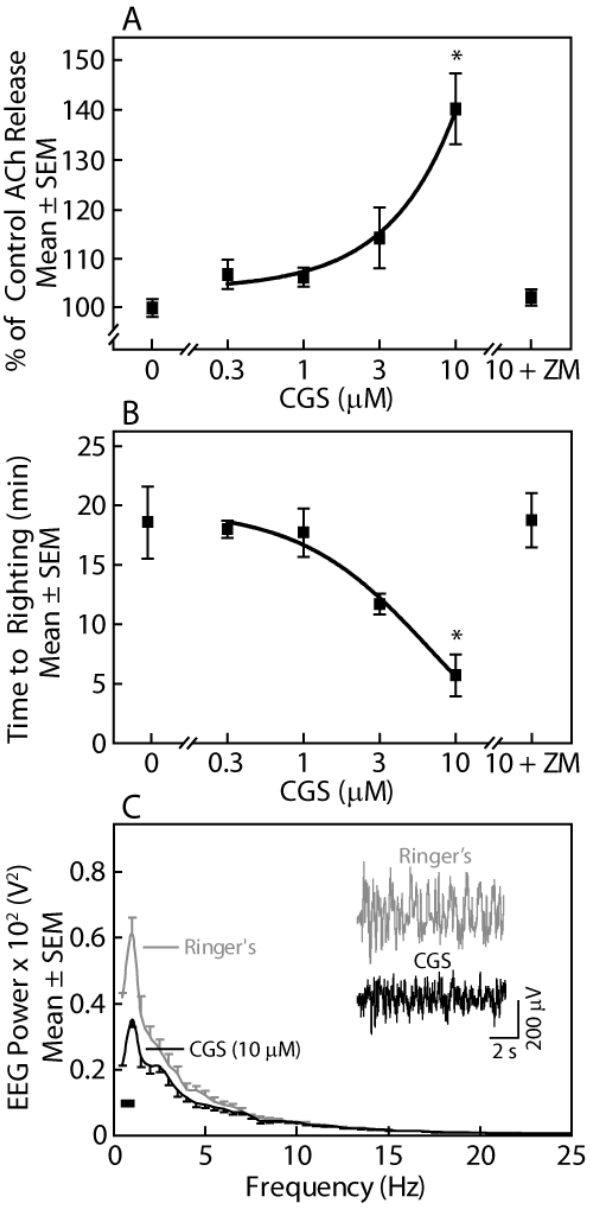

Figure 2.2. Microdialysis delivery of CGS to the prefrontal cortex increased all measures of arousal. CGS caused a concentration dependent increase in ACh release (A), decrease in time to waking after anesthesia (B), and decrease in EEG delta power (C). Each point in A and B plots data from 3 mice. Post-hoc Dunnett's statistic showed that CGS (10 μM) significantly (* $P < 0.01$) increased ACh release and decreased time to righting after anesthesia, and that coadministration of CGS plus the antagonist (10 + ZM) blocked these effects. Comparison of the two functions shown in A and B by regression analysis indicates that ACh release in the prefrontal cortex accounted for 99.9% of the variance in time to waking after anesthesia. The EEG power spectra in C represent an average from 3 additional mice. The inset in C shows representative 10 s EEG recordings during dialysis with Ringer's or Ringer's containing CGS. The black bar (lower left of C) indicates significantly different EEG delta frequencies between Ringer's and CGS dialysis.

Waking phenotypes were decreased by an adenosine A_1 receptor agonist.

Additional experiments in another group of mice (n = 21) determined whether microdialysis delivery of the adenosine A_1 receptor agonist SPA to the prefrontal cortex reduced ACh release and arousal. SPA caused a significant drug main effect on prefrontal cortex ACh release ($F_{(6,14)} = 25.0$, $P = 1.03 \times 10^{-6}$) (Fig. 2.3A) and the amount of time required for resumption of waking after anesthesia ($F_{(6,14)} = 46.5$, $P = 1.8 \times 10^{-8}$) (Fig. 2.3B). Coadministration of the adenosine A_1 receptor antagonist DPCPX (100 μM) with SPA (10 mM) blocked the effects of SPA (10 + DPCPX in Fig. 2.3A and B). Figure 2.3C illustrates that SPA increased EEG delta power. Two-way repeated measures ANOVA identified a significant frequency by drug interaction ($F_{(7,14)} = 9.3$, $P = 0.0002$) and post hoc analyses revealed that SPA significantly increased EEG delta power in the 0.5 to 2.5 Hz band.

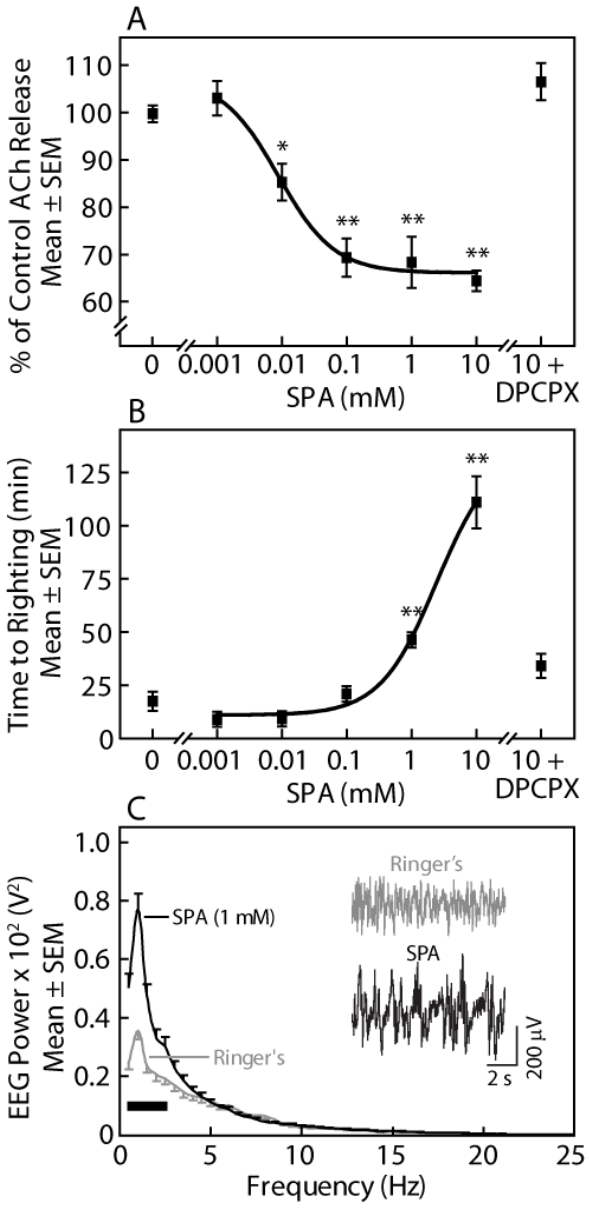

Figure 2.3. Dialysis administration of SPA to the prefrontal cortex decreased dependent measures of arousal. SPA caused a concentration dependent decrease in ACh release in the prefrontal cortex (A) and an increase in time required for resumption of wakefulness after anesthesia (B). Asterisks (* $P < 0.05$, ** $P < 0.01$) indicate concentrations of SPA that caused a significant difference compared to Ringer's control (0 mM SPA) by post hoc Dunnett's statistic. Coadministration of SPA plus the antagonist (10 + DPCPX) blocked the effects of SPA on both ACh release and time to resumption of righting. Comparison by regression analysis of data shown in A and B indicate that ACh release accounted for 90.2% of the variance in wake-up time. Each point in A and B summarizes results from 3 mice. C illustrates EEG power spectra averaged for 3 additional mice during dialysis with Ringer's (control) or Ringer's containing 1 mM SPA. The inset (C) shows two 10 s EEG recordings and the black bar indicates the significant SPA-induced increase in the 0 to 4 Hz delta range.

Caffeine and an adenosine A_1 receptor antagonist but not an adenosine A_{2A} receptor antagonist enhanced waking phenotypes.

Having demonstrated that exogenous adenosine A_1 and A_{2A} receptor agonists delivered to the prefrontal cortex alter waking phenotypes, 42 additional mice were used to determine whether endogenous adenosine acting at adenosine A_1 and/or A_{2A} receptors modulates the same arousal phenotypes. Dialysis delivery of caffeine caused a significant, concentration dependent increase in ACh release within the prefrontal cortex during anesthesia ($F_{(4,10)} = 3.5$, $P = 0.049$) (Fig. 2.4A) and decrease in the time required for resumption of waking after anesthesia ($F_{(4,10)} = 6.9$, $P = 0.0061$) (Fig. 2.4B). Dialysis delivery of the adenosine A_1 receptor antagonist DPCPX also caused a significant, concentration dependent increase in prefrontal cortex ACh release ($F_{(5,12)} = 201.7$, $P = 3.8 \times 10^{-11}$) (Fig. 2.4A) and a significant, concentration dependent decrease in time required for resumption of waking after anesthesia ($F_{(5,12)} = 8.2$, $P = 0.0014$) (Fig. 2.4B). In contrast, dialysis delivery of five concentrations of the adenosine A_{2A} receptor antagonist ZM to the prefrontal cortex did not alter prefrontal cortex ACh release (Fig. 2.4A) or time to waking after anesthesia (Fig. 2.4B). In order to maintain selectivity for the A_{2A} receptor (Linden et al., 1999, Lopes et al., 1999), greater concentrations of ZM were not tested.

Adenosine A_1 receptors in prefrontal cortex modulate ACh release and arousal: Efficacy and potency comparisons.

Derivation of the concentration response data for selective antagonist of the adenosine A_1 receptor (DPCPX), the adenosine A_{2A} receptor (ZM), and the mixed

antagonist (caffeine) made it possible to gain insight into their relative efficacy and potency for altering ACh release and arousal. Figure 2.4A shows that dialysis delivery of DPCPX produced the greatest increase in ACh release, ZM did not alter ACh release, and caffeine caused a significant increase in ACh release. The percent of variance in ACh release accounted for by the three antagonists was 100% for caffeine, 99% for DPCPX, and 30% for ZM. Figure 2.4B suggests that DPCPX and caffeine are equally efficacious in shortening the time required for resumption of wakefulness, and DPCPX is more potent than caffeine. Across the range of concentrations examined, ZM did not shorten time to resumption of righting. The percent of variance in anesthesia recovery time accounted for by the three antagonists was 97% for DPCPX, 89% for caffeine, and 15% for ZM.

Caffeine and an adenosine A_1 receptor antagonist but not an adenosine A_{2A} receptor antagonist decreased EEG delta power.

Caffeine significantly decreased EEG delta power (Fig. 2.5A). Two-way repeated measures ANOVA revealed no frequency by drug interaction but post hoc analyses comparing the means at each frequency between treatments indicated that caffeine depressed EEG power at 1.0 Hz. The DPCPX-induced decrease in EEG delta power is shown in Fig. 2.5B. Two-way repeated measures ANOVA revealed significant frequency by drug interaction ($F_{(7, 14)} = 43.2$, $P < 0.0001$) and post hoc analyses showed that DPCPX depressed EEG power from 0.5 to 3.5 Hz. Figure 2.5C illustrates that the adenosine A_{2A} receptor antagonist ZM did not alter EEG delta power.

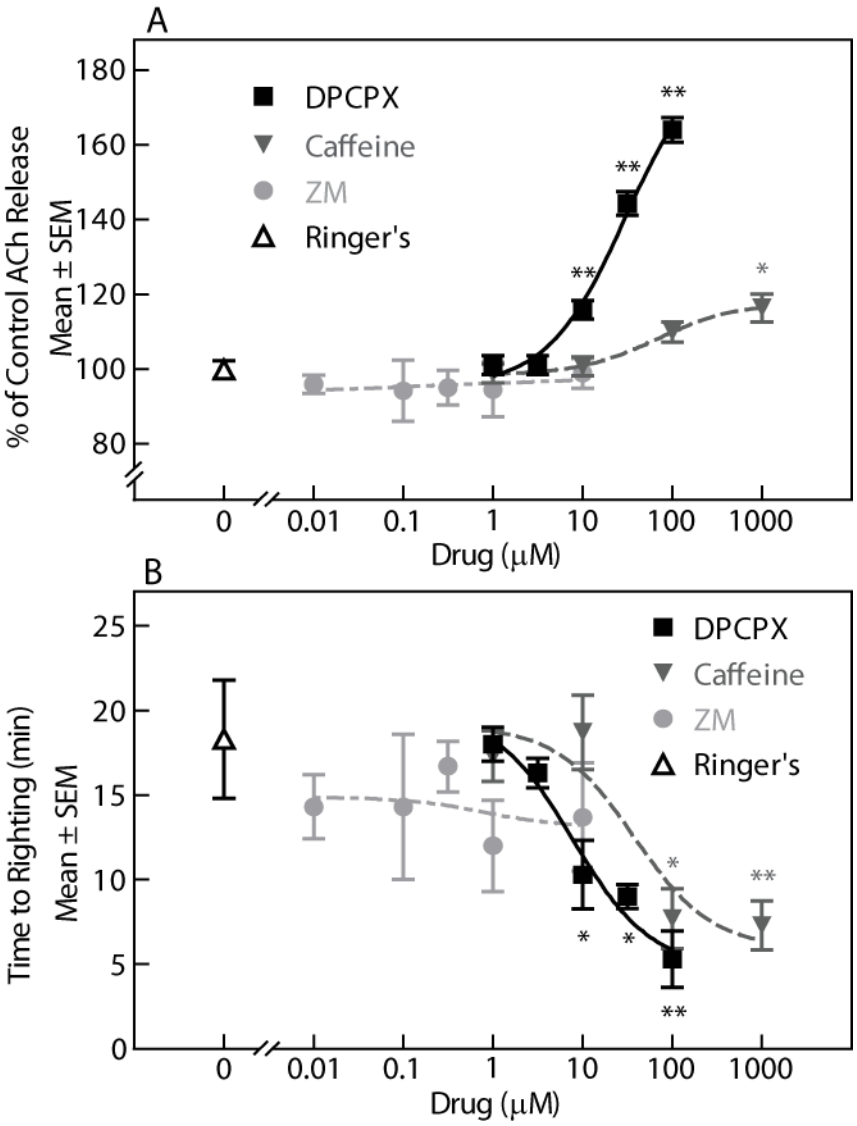

Figure 2.4. Caffeine and DPCPX promote arousal. Caffeine and DPCPX (**A**) increased prefrontal cortex ACh release and (B) decreased time to waking after anesthesia. Each point in A and B summarizes mean ± standard error of the mean (SEM) from 3 mice. Post-hoc Dunnett's analysis identified concentrations of caffeine and DPCPX that caused a significant (* $P < 0.05$, ** $P < 0.01$) change from Ringer's control. Application of regression analyses to compare ACh release (A) and time to righting (B) revealed that ACh release in the prefrontal cortex accounted for 93% of the variance in time to righting after anesthesia for caffeine, 92% for DPCPX, and 0.4% for ZM. Dialysis administration of ZM to the prefrontal cortex did not alter either dependent measure of arousal.

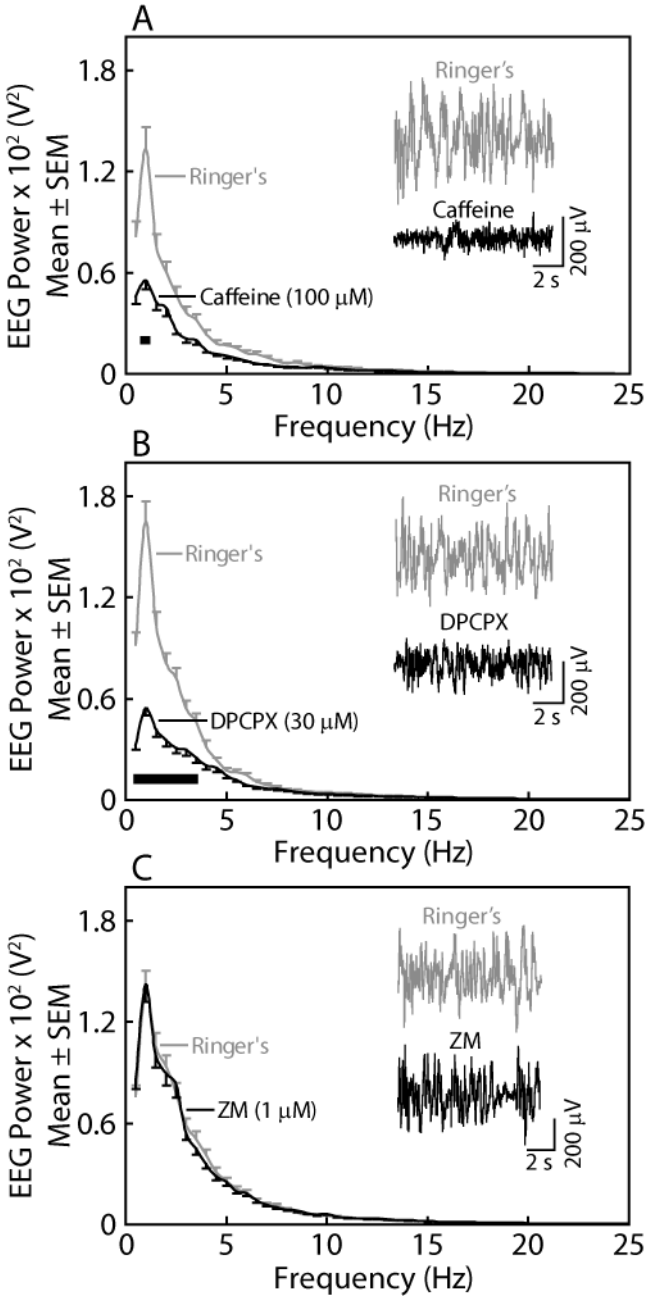

Figure 2.5. Power spectral analysis of the cortical EEG revealed that caffeine (A) and DPCPX (B) significantly decreased EEG delta power. Dialysis delivery of ZM (C) did not decrease EEG delta power. The insets show 10 s EEG recordings from the same mouse during dialysis with Ringer's or Ringer's containing drug. Black bars (lower left of A and B) indicate delta frequency bands that showed significantly decreased power during dialysis with caffeine and DPCPX. Each graph represents data obtained from 3 mice.

Microinjecting an adenosine A_1 receptor antagonist into the prefrontal cortex of unanesthetized B6 mice increased wakefulness and decreased NREM sleep.

The foregoing results encouraged a final set of experiments using intact, unanesthetized B6 mice (n = 8) to determine whether prefrontal cortex administration of the adenosine A_1 receptor antagonist DPCPX altered sleep and wakefulness. Compared to microinjection of saline (control), (Fig. 2.6A) DPCPX increased wakefulness by 25.0% ($t_{(14)} = 13.1$, $P = 1.5 \times 10^{-9}$) and decreased NREM sleep by 40.3% ($t_{(14)} = 13.4$, $P = 1.1 \times 10^{-9}$). Figure 2.6B, C, and D summarize the averaged EEG power spectra during wakefulness, NREM sleep, and REM sleep. Insets show representative EEG and EMG recordings from the same mouse after microinjection of DPCPX. Wakefulness was characterized by a mixed frequency EEG and high amplitude EMG. NREM sleep was characterized by high delta power (0.5 - 4 Hz) in the EEG and a lower amplitude EMG. REM sleep was characterized by a high theta power (5 - 8 Hz) in the EEG and a low amplitude EMG. Figure 2.6B, C, and D demonstrate that EEG power spectra were not different following microinjection of saline or DPCPX. Sleep architecture was normal after microinjection of DPCPX, and REM sleep always followed NREM sleep.

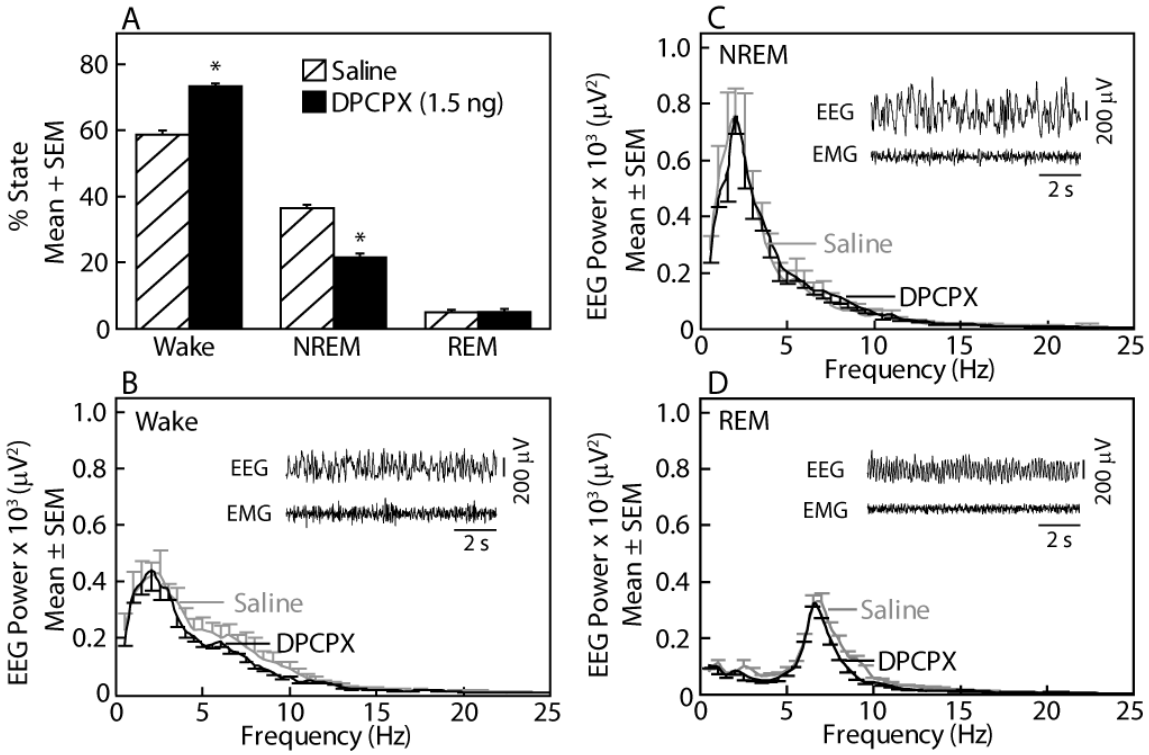

Figure 2.6. Microinjection of the adenosine A_1 receptor antagonist DPCPX into the prefrontal cortex caused a significant (* $P < 0.0001$ by Student's t-test) (A) increase in wakefulness and decrease in NREM sleep. The EEG power spectra did not differ between saline and DPCPX for (B) waking, (C) NREM sleep, or (D) REM sleep. EEG data were analyzed in 0.5 Hz increments and represent the average of 8 mice. The insets show 10 s EEG and EMG recordings after microinjection of DPCPX.

DISCUSSION

Two novel findings emerged from this study. First, microdialysis delivery of adenosinergic agonists and antagonists to the prefrontal cortex showed that adenosine receptors in the prefrontal cortex exert descending modulation of behavioral arousal. Top-down modulation from prefrontal cortex to basal forebrain is important for cholinergically mediated focused attention (Sarter et al., 2006). Antagonism of adenosine A_1 receptors with DPCPX and caffeine suggests the possibility that adenosine in the prefrontal cortex can also inhibit caudal brain centers that generate behavioral arousal. Second, the results indicate a definitive role for prefrontal cortex adenosine A_1 receptors in the modulation of ACh release, behavioral arousal, EEG delta power, and sleep. Previous studies of adenosine A_1 receptor knockout mice suggested that caffeine modulates arousal solely via adenosine A_{2A} receptors (Huang et al., 2005). As discussed below, the present data show that in the prefrontal cortex, endogenous adenosine acting at adenosine A_1 receptors decreases arousal, and that caffeine blocks the effects of endogenous adenosine on arousal by antagonizing prefrontal cortex adenosine A_1 receptors.

Adenosine A_1 and A_{2A} receptor agonists had opposing effects on waking phenotypes.

Dialysis administration of the adenosine A_{2A} receptor agonist CGS caused a concentration dependent increase in ACh release (Fig. 2.2A) and decrease in the time to waking after anesthesia (Fig. 2.2B). The increase in prefrontal cortex ACh release parallels findings in the pontine reticular formation of B6 mouse where dialysis delivery of similar concentrations of CGS also caused an increase in ACh release (Coleman et al.,

2006). This same study found that CGS caused a biphasic concentration response curve for ACh release by activating adenosine A_1 receptors at higher concentrations (Coleman et al., 2006, Halldner et al., 2004, Klotz, 2000, Lopes et al., 2004). In the present study, microdialysis with 30 μM CGS caused no change in ACh release or anesthesia recovery time, suggesting that at dialysis concentrations greater than 10 μM, CGS loses specificity for the A_{2A} receptor and starts to bind the adenosine A_1 receptor. The findings that the CGS effects were concentration dependent and blocked by the antagonist ZM (Fig. 2.2A and B) support the interpretation that adenosine A_{2A} receptors in the prefrontal cortex respond to exogenous agonists by increasing ACh release and behavioral arousal, and decreasing EEG slow wave activity.

Dialysis administration of the adenosine A_1 receptor agonist SPA caused a concentration dependent decrease in ACh release (Fig. 2.3A) within the prefrontal cortex and an increase in time required for resumption of waking after anesthesia (Fig. 2.3B). The decrease in ACh release and the increase in time to waking after anesthesia were blocked by coadministering the adenosine A_1 receptor antagonist DPCPX. These findings are consistent with previous data showing that dialysis delivery of SPA to cat pontine reticular formation caused a decrease in ACh release and an increase in the time required to awaken after anesthesia (Tanase et al., 2003). These data demonstrate that adenosine A_1 receptors in the prefrontal cortex can decrease ACh release, as well as behavioral and EEG arousal.

Endogenous adenosine in the prefrontal cortex decreases arousal by actions at adenosine A_1 receptors.

The adenosine A_{2A} receptor antagonist ZM blocked the CGS-induced enhancement of waking phenotypes, but ZM alone did not alter prefrontal cortex ACh release (Fig. 2.4A), time to waking after anesthesia (Fig. 2.4B), or EEG delta power (Fig. 2.5C). These results suggest that in B6 mouse endogenous adenosine does not alter arousal through adenosine A_{2A} receptors in the prefrontal cortex. In contrast, the adenosine A_1 receptor antagonist DPCPX caused a significant, concentration dependent increase in ACh release within the prefrontal cortex (Fig. 2.4A), decrease in time for resumption of waking after anesthesia (Fig. 2.4B), and decreased delta power in the EEG (Fig. 2.5B). The DPCPX data show, for the first time, that behavioral and EEG arousal are reduced by endogenous adenosine acting at adenosine A_1 receptors in the prefrontal cortex of B6 mouse. This interpretation is also supported by the caffeine data. Caffeine caused a significant, concentration dependent increase in ACh release within the prefrontal cortex (Fig. 2.4A), decrease in time to waking after anesthesia (Fig. 2.4B), and decreased EEG delta power (Fig. 2.5A).

The recordings of sleep and wakefulness also demonstrate that adenosine A_1 receptors in the prefrontal cortex contribute to the regulation of EEG and behavioral arousal. Microinjection of the adenosine A_1 receptor antagonist DPCPX into the prefrontal cortex increased wakefulness and decreased NREM sleep (Fig. 2.6A). Following microinjection of saline and DPCPX, the mice exhibited normal behavioral phenotypes of grooming and locomotor behavior. Quantitative EEG analyses (Fig. 2.6B, C, and D) revealed no significant differences following saline control and DPCPX

microinjections. Considered together with the microdialysis results, the microinjection data indicate that DPCPX enhanced wakefulness and decreased sleep by increasing prefrontal cortex ACh release. Thus, multiple lines of evidence support the interpretation that endogenous adenosine acting through adenosine A_1 receptors in the prefrontal cortex inhibits wakefulness.

Conclusions

The prefrontal cortex contributes to the regulation of sleep and wakefulness (Muzur et al., 2002), and the present results show for the first time that adenosine A_1 and A_{2A} receptors in the prefrontal cortex of B6 mouse modulate behavioral arousal, cortical ACh release, EEG delta power, and sleep. Caffeine is an adenosine A_1 and A_{2A} receptor antagonist and data from humans and rodents indicate that caffeine promotes arousal by enhancing cortical cholinergic transmission. For example, caffeine enhances psychomotor vigilance, in part, by activating the human prefrontal cortex (Higashi et al., 2004) and systemic administration of caffeine increases ACh release in the prefrontal cortex of rat (Acquas et al., 2002). Attention demanding tasks increase ACh release in the prefrontal cortex (Kozak et al., 2006) and morning consumption of caffeine is an effective countermeasure for cognitive deficits known to be associated with sleep inertia (Van Dongen et al., 2001). Microdialysis delivery of caffeine, DPCPX, and ZM in the present study demonstrates that ACh release in the prefrontal cortex is significantly increased by blocking adenosine A_1 receptors. The present results suggest that adenosine A_1 receptors in the prefrontal cortex activate a descending system that decreases arousal, likely via extensive projections to caudal brain regions (Gabbott et al., 2005, Morgane

and Mokler, 2006). The ability of caffeine to antagonize descending pathways that inhibit wakefulness comprise one mechanism by which the prefrontal cortex can override the enhanced drive to sleep associated with sleep restriction or prolonged intervals of wakefulness.

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CHAPTER 3

ADENOSINE A_1 AND A_{2A} RECEPTORS IN MOUSE PREFRONTAL CORTEX MODULATE PONTINE RETICULAR FORMATION ACETYLCHOLINE RELEASE

SUMMARY

Prefrontal cortex adenosine receptors modulate cortical acetylcholine release, electroencephalographic and behavioral arousal, as well as sleep in the C57BL/6J mouse (Van Dort CJ et al., 2008). These results, and early brain transection studies demonstrating that the forebrain is not needed to generate the non-rapid eye movement (NREM)/ rapid eye movement (REM) sleep cycle (Jouvet, 1962), suggest that the prefrontal cortex modulates arousal states via descending input to caudal arousal control centers. Therefore this study tested the hypothesis that dialysis delivery of adenosine A_1 and A_{2A} receptor agonists and antagonists to the prefrontal cortex of B6 mouse modulates pontine reticular formation acetylcholine release. Results from Aim 3 are reported in this Chapter. Microdialysis delivery of an adenosine A_{2A} receptor agonist to the prefrontal cortex increased simultaneously collected pontine reticular formation acetylcholine (ACh) release, whereas administering an adenosine A_1 receptor agonist decreased pontine reticular formation acetylcholine release. These findings are the first to demonstrate that pharmacological manipulation of the prefrontal cortex can alter neurotransmitter release in the pontine reticular formation. The adenosine A_1 and A_{2A} receptor antagonist, caffeine and an adenosine A_1 receptor antagonist delivered by microdialysis to the

prefrontal cortex increased pontine reticular formation ACh release. These data are consistent with the interpretation that endogenous adenosine in the prefrontal cortex can modulate behavioral arousal states, in part, via a descending pathway that alters acetylcholine release in the pontine reticular formation.

INTRODUCTION

Adenosine promotes sleep and caffeine, the most commonly used psychoactive stimulant (Fredholm et al., 1999), promotes wakefulness by antagonizing adenosine A_1 and A_{2A} receptors. The specific brain regions and receptor subtypes important for the regulation of behavioral arousal by adenosine remain incompletely understood. Data support a role for both adenosine A_1 (Basheer et al., 2001, Benington et al., 1995, Elmenhorst et al., 2007, Yanik and Radulovacki, 1987) and A_{2A} (Huang et al., 2005, Urade et al., 2003) receptors in promoting sleep. Prefrontal cortex adenosine A_1 and A_{2A} receptors have recently been shown to modulate behavioral arousal, ACh release in the prefrontal cortex, and sleep (Van Dort CJ et al., 2008). Although the prefrontal cortex helps regulate wakefulness, early brain transection studies showed that the forebrain is not necessary for generating the NREM and REM sleep cycle (Jouvet, 1962). One mechanism through which the prefrontal cortex might alter behavioral arousal is via descending input to caudal brain regions known to regulate sleep and wakefulness (Steriade and McCarley, 2005).

Several lines of evidence provide justification for choosing the pontine reticular formation as one region through which the prefrontal cortex alters behavioral arousal. First, the classic studies of Moruzzi and Magoun (Moruzzi and Magoun, 1949) showed

that stimulation of the pontine reticular formation activates the cortex via the ascending reticular activating system. Second, there are projections from the prefrontal cortex to the pontine reticular formation in Wistar rat (Allen and Hopkins, 1998, Brodal and Bjaalie, 1992). In addition to neuroanatomical studies, neurochemical data show that cholinergic neurotransmission in the pontine reticular formation can alter ACh release in the prefrontal cortex (DeMarco et al., 2004).

Despite the known descending projections from the prefrontal cortex to the pontine reticular formation, no previous studies have investigated whether these projections modulate neurotransmitter release in the pontine reticular formation. Therefore, the present experiments were designed to test the hypothesis that dialysis delivery of adenosine A_1 and A_{2A} receptor agonists and antagonists to the prefrontal cortex of B6 mouse modulates pontine reticular formation acetylcholine release.

MATERIALS AND METHODS

All experiments adhered to the Public Health Service Policy on Humane Use and Care of Laboratory Animals (NIH Publication 80-23, National Academy of Sciences Press, Washington, DC, 1996) and were reviewed and approved by the University of Michigan Committee on Use and Care of Animals. Adult male C57BL/6J (B6) mice (n=12; Jackson Laboratory, Bar Harbor, ME, USA) were kept under constant illumination and had *ad libitum* access to food and water. Each mouse was used for only one experiment.

Experimental Procedure. Figure 3.1 illustrates the experimental design including the location of the 2 dialysis probes, drug delivery to the prefrontal cortex, and

quantification of ACh release from the pontine reticular formation. Mice were anesthetized with 2.0% isoflurane in O_2 and placed in a Kopf Model 962 small animal stereotaxic instrument with a Kopf Model 923-B mouse anesthesia mask (David Kopf Instruments, Tujunga, CA). Delivered isoflurane concentration was measured using a Cardiocap/5 monitor (Datex-Ohmeda, Madison, WI) and decreased to 1.6% for the surgery. Core body temperature was monitored and held constant between 36.5-37.5°C throughout the experiment. Two craniotomies were made, one at stereotaxic coordinates 3.0 mm anterior to bregma and 1.6 mm lateral to the midline and a second at 4.72 mm posterior to bregma and 0.7 mm lateral to the midline (Paxinos and Franklin, 2001). Delivered isoflurane concentration was then reduced and maintained at 1.3%, corresponding to the EC_{50} or minimum alveolar concentration for B6 mouse (Sonner et al., 1999).

In vivo microdialysis and high performance liquid chromatography with electrochemical detection (HPLC/EC). Two CMA/7 dialysis probes (6 kDa cutoff; 1.0 mm length, 0.24 mm diameter cuprophane membrane; CMA/Microdialysis, Stockholm, Sweden) were used for each experiment. The microdialysis probes were perfused continuously (2 µl/min) with Ringer's solution (control) comprised of 147 mM NaCl, 2.4 mM $CaCl_2$, 4 mM KCl, and 10 µM neostigmine. Salts used in the Ringer's solution were purchased from Fisher Scientific (Pittsburgh, PA). Drugs delivered to the prefrontal cortex by microdialysis included the adenosine A_{2A} receptor agonist 2-*p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680; CGS), the adenosine A_1 receptor agonist N^6-p-Sulfophenyladenosine sodium salt (SPA), the adenosine A_1 receptor antagonist 8-Cyclopentyl-1,3-dipropylxanthine

(DPCPX), and the adenosine A_1 and A_{2A} receptor antagonist caffeine. DPCPX was dissolved in dimethylsulfoxide (DMSO) and diluted in Ringer's to a final DMSO concentration of 1.0%. SPA, CGS, DPCPX, caffeine, and neostigmine bromide were purchased from Sigma-Aldrich (St. Louis, MO).

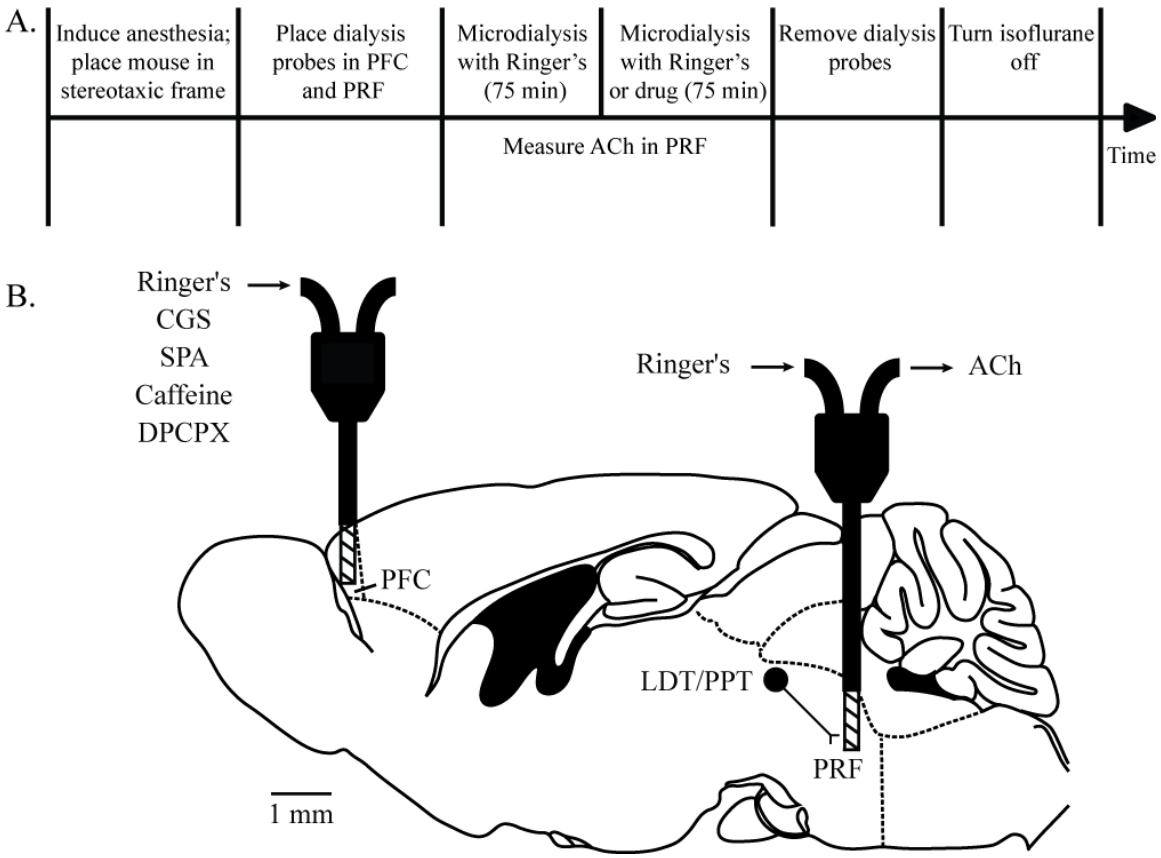

Figure 3.1. Timeline of a microdialysis experiment. (A) Shows the sequence of events with the top row outlining the experimental manipulations and the bottom row showing the dependent measures. (B) The sagittal diagram modified from the mouse brain atlas (Paxinos and Franklin, 2007) shows placement of one microdialysis probe in the prefrontal cortex (PFC) and a second microdialysis probe in the pontine reticular formation (PRF). The dialysis probe membranes are drawn to scale (1 mm length, 0.24 mm diameter). The distance between the two probe tips is 8.56 mm. The laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei provide cholinergic input to the PRF.

A CMA/110 liquid switch was used to dialyze the prefrontal cortex with Ringer's followed by Ringer's containing drug (Fig. 3.1A) and to dialyze the pontine reticular formation with Ringer's. Dialysis samples were collected every 12.5 min. Six samples were collected during dialysis with Ringer's (control) then six samples were collected during dialysis with drug. Each 25 µl dialysis sample was injected into a HPLC/EC system (Bioanalytical Systems, (BAS), West Lafeyette, IN). The sample was carried through the system in 50 mM Na_2HPO_4 mobile phase (pH 8.5) at a flow rate of 1 ml/min. ACh and choline were separated by an analytical column and then broken down into hydrogen peroxide by an immobilized enzyme reaction column. The hydrogen peroxide was detected by a platinum electrode (0.5 V applied potential) in reference to an Ag^+/AgCl electrode. Chromgraph software (Bioanalytical Systems Inc.) was used to digitize and quantify the resulting chromatograms compared to a series of ACh standards. The percentage of a known ACh solution recovered by the probe was determined before and after every experiment. If the pre-and post-experiment probe recoveries were significantly different by t-test, the data from that experiment were discarded.

Data Analysis. ACh release was expressed as percent of ACh measured during dialysis with Ringer's (control condition). Drug effects on pontine reticular formation ACh release were analyzed using Students t-test (GBStat™ v6.5.6, Dynamic Microsystems, Inc., Silver Spring, MD). Data are reported as mean ± standard error of the mean (SEM). A probability value of $P \leq 0.05$ was considered to be statistically significant. Seven days after the experiment mice were deeply anesthetized and decapitated. The brains were removed, frozen, and coronally sectioned (40 µm thick). The sections were fixed with hot (80°C) paraformaldehyde vapor and stained with cresyl

violet. The location of a glial scar caused by the microdialysis probe was determined by comparing stained sections with a mouse brain atlas (Paxinos and Franklin, 2001). Results were included in the group data only for experiments in which histology confirmed that the microdialysis probes had been placed within the prefrontal cortex and the pontine reticular formation.

RESULTS

Time course of ACh release in the pontine reticular formation

Pontine reticular formation ACh release (Fig. 3.2A) was collected during dialysis with Ringer's in both the pontine reticular formation and the prefrontal cortex (75 min; control) followed by continuous dialysis with Ringer's in the pontine reticular formation and dialysis with Ringer's plus drug to the prefrontal cortex (75 min). The ACh release from the pontine reticular formation dialysis samples was averaged across each condition (Ringer's or prefrontal cortex drug) (Fig. 3.2B). Dialysis delivery of the adenosine A_{2A} receptor agonist CGS to the prefrontal cortex significantly increased pontine reticular formation ACh release ($t = 5.9$; $df = 10$; $p < 0.0001$).

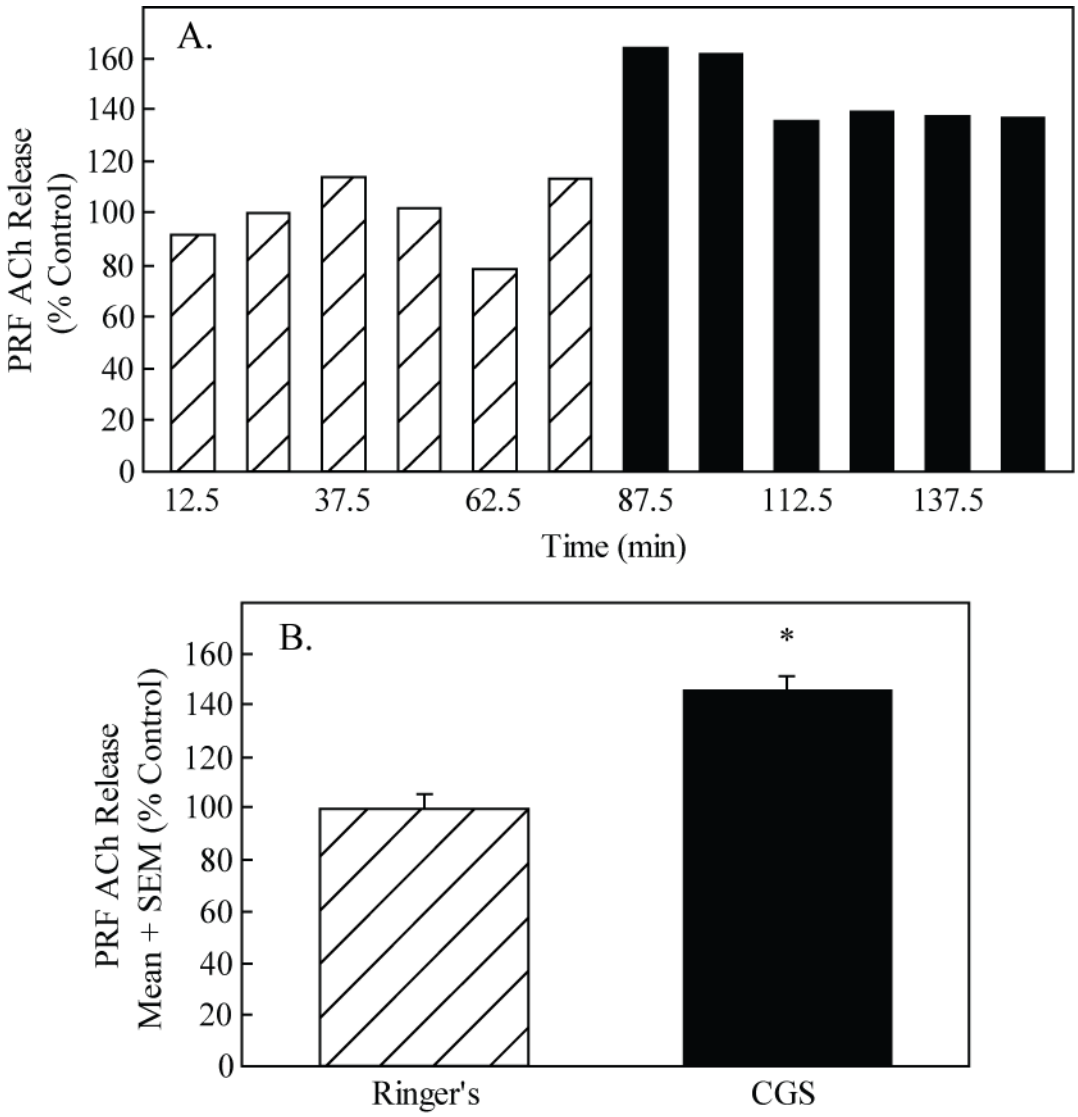

Figure 3.2. ACh release in the pontine reticular formation (PRF) during a single representative experiment. (A) illustrates an experiment time course. Bars show 6 sequential PRF ACh samples collected during dialysis with Ringer's (control, hatched bars) followed by 6 samples collected during dialysis of the prefrontal cortex with Ringer's plus CGS (solid bars). Each bar represents 1 ACh sample from the same mouse. (B) Dialysis delivery of CGS to the prefrontal cortex caused a significant increase in pontine reticular formation ACh release (49.9%).

Dialysis delivery of adenosine A_1 and A_{2A} receptor agonists and antagonists to the prefrontal cortex altered pontine reticular formation ACh release

Analysis of the group data shows that CGS, an adenosine A_{2A} receptor agonist, delivered to the prefrontal cortex increased pontine reticular formation ACh release (Fig. 3.3A) ($t = 9.8$; $df = 4$; $p < 0.001$). Microdialysis delivery of the adenosine A_1 receptor agonist SPA to the prefrontal cortex decreased pontine reticular formation ACh release (Fig. 3.3B) ($t = 8.1$; $df = 4$; $p < 0.001$). Dialysis delivery of caffeine (Fig. 3.3C) ($t = 21.4$; $df = 4$; $p < 0.0001$) or the adenosine A_1 receptor antagonist DPCPX (Fig. 3.3D) ($t = 16.0$; $df = 4$; $p < 0.0001$) to the prefrontal cortex increased pontine reticular formation ACh release. Figure 3.4 illustrates the locations of the dialysis probes (A and B) and representative cresyl violet stained histological sections (C and D) showing the glial scar produced by the dialysis probe.

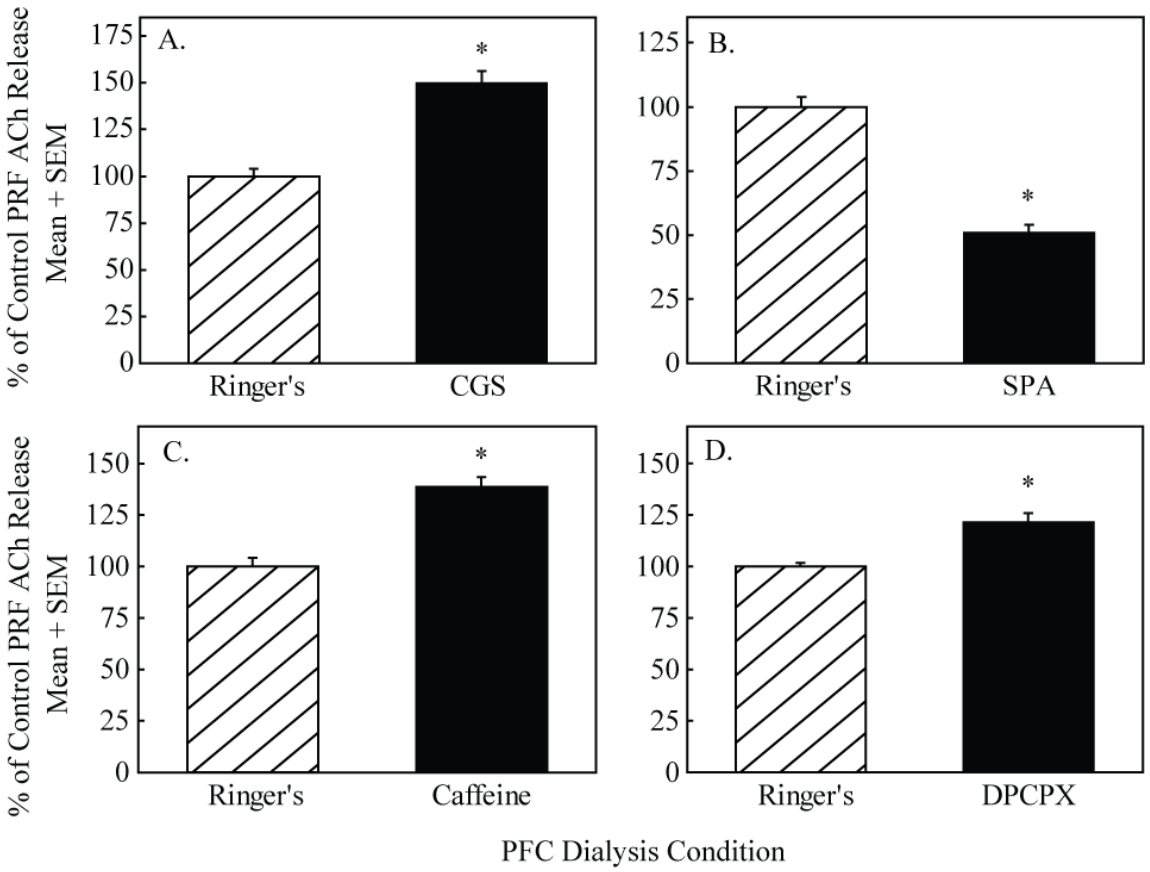

Figure 3.3. Adenosine A_1 and A_{2A} receptor agonists and antagonists delivered to the prefrontal cortex (PFC) significantly alter ACh release in the pontine reticular formation (PRF). (A) Dialysis administration of the adenosine A_{2A} receptor agonist CGS to the prefrontal cortex caused a significant increase in pontine reticular formation ACh release, whereas (B) prefrontal cortex SPA, an adenosine A_1 receptor agonist, caused a significant decrease in pontine reticular formation ACh release. Pontine reticular formation ACh release was also increased by prefrontal cortex (C) caffeine and the (D) adenosine A_1 receptor antagonist DPCPX. Each drug was tested in 3 mice.

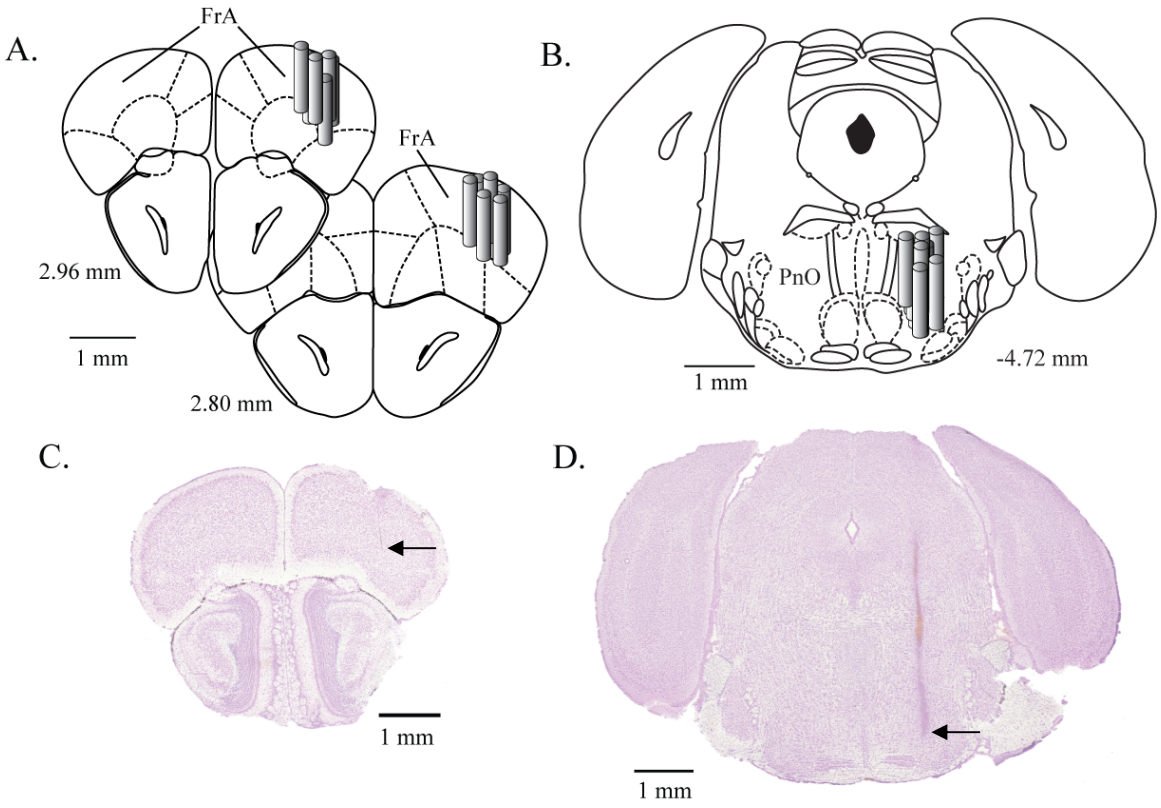

Figure 3.4. The locations of (A) 12 prefrontal cortex and (B) 12 pontine reticular formation microdialysis sites are illustrated on coronal diagrams from a mouse brain atlas (Paxinos and Franklin, 2007). Dialysis probe membranes are represented by cylinders and are drawn to scale (1 mm length, 0.24 mm diameter). Average stereotaxic coordinates (mean ± SEM) for the prefrontal cortex probe sites were 2.89 ± 0.08 mm anterior to bregma and 1.75 ± 0.05 mm lateral to the midline and for the pontine reticular formation were 4.72 ± 0.15 mm posterior to bregma and 0.7 ± 0.12 mm lateral to the midline. The cresyl violet-stained coronal sections (C and D) show typical prefrontal cortex and pontine reticular formation microdialysis sites (arrow). In relation to bregma, the prefrontal cortex section is approximately 2.96 mm anterior and the pontine reticular formation section is approximately 4.72 mm posterior.

DISCUSSION

The present data show that adenosine A_1 and A_{2A} receptor agonists and antagonists delivered by microdialysis to the prefrontal cortex alter pontine reticular formation ACh release. This is the first study to demonstrate that pharmacological manipulation of prefrontal cortex adenosine A_1 and A_{2A} receptors alters cholinergic neurotransmission in the pontine reticular formation. The results are discussed relative to related studies and the role of the prefrontal cortex in regulating behavioral arousal.

Prefrontal cortex adenosine A_1 and A_{2A} receptors modulate behavioral arousal by altering pontine reticular formation ACh release.

Dialysis delivery of the adenosine A_{2A} receptor agonist CGS to the prefrontal cortex increased pontine reticular formation ACh release (Fig. 3.3A). This finding is consistent with an increase in ACh release caused by local administration of CGS to the pontine reticular formation in B6 mouse (Coleman et al., 2006). Prefrontal cortex SPA, an adenosine A_1 receptor agonist, decreased pontine reticular formation ACh release (Fig. 3.3B) similar to a study in cat where local administration of SPA to homologous regions of the pontine reticular formation also decreased ACh release and significantly delayed resumption of wakefulness following anesthesia (Tanase et al., 2003). The Fig. 3 data are consistent with the interpretation that prefrontal cortex adenosine A_1 and A_{2A} receptors in B6 mouse modulate behavioral arousal, in part, via a descending pathway that modulates ACh levels in a pontine component of the ascending reticular activating system.

Endogenous adenosine within the prefrontal cortex modulates behavioral arousal by varying ACh release within the pontine reticular formation.

Dialysis delivery of caffeine (Fig. 3.3C) and the adenosine A_1 receptor antagonist DPCPX (Fig. 3.3D) caused a significant increase in pontine reticular formation ACh release. These data, taken together with the earlier finding that prefrontal cortex adenosine A_1 and A_{2A} receptors modulate ACh release, behavioral arousal, and sleep (Van Dort CJ et al., 2008) suggest that prefrontal cortex adenosine modulates behavioral arousal, in part, by altering pontine reticular formation ACh release. Cholinergic neurotransmission in the pontine reticular formation is important for the regulation of behavioral arousal (Lydic and Baghdoyan, 2005, Steriade and McCarley, 2005). Pontine reticular formation ACh levels vary with states of sleep and wakefulness. Pontine reticular formation ACh levels are the highest during REM sleep, followed by wakefulness, and the lowest during NREM sleep (Kodama et al., 1990, Leonard and Lydic, 1995, Leonard and Lydic, 1997). Enhancing cholinergic neurotransmission in the pontine reticular formation by local delivery of the acetylcholinesterase inhibitor neostigmine or the cholinergic agonist carbachol causes a REM sleep-like state in mouse (Lydic et al., 2002), rat (Bourgin et al., 1995), and cat (Baghdoyan et al., 1984). Dialysis delivery of carbachol to the pontine reticular formation of mouse significantly decreased ACh release in the prefrontal cortex (DeMarco et al., 2004). The present results extend Moruzzi and Magoun's 1949 concept of an ascending arousal activating system (Moruzzi and Magoun, 1949) by providing functional evidence for a descending pathway from the prefrontal cortex to the pontine reticular formation. Given the known role of the prefrontal cortex in executive function (Fuster, 2001) the results suggest one pathway for

top-down, volitional modulation of arousal. The ability of the executive cortex to recruit input from the ascending cortical activating system may provide one mechanism allowing sleep-deprived individuals to cope with attention demanding tasks (Chee et al., 2008).

Limitations and Conclusions

The finding that the prefrontal cortex alters ACh release in the pontine reticular formation does not imply a single mechanism. The prefrontal cortex projects to many caudal brain regions known to regulate behavioral arousal (Gabbott et al., 2005, Morgane and Mokler, 2006). The finding that the prefrontal cortex can alter neurotransmitter release in the pontine reticular formation, also fits with the concept of "top-down" processing used to describe the ability of the prefrontal cortex to control attention (Buschman and Miller, 2007). Although the role of the prefrontal cortex in the volitional control of attention has been demonstrated by many studies (Reviewed in (Miller and D'Esposito, 2005, Sarter et al., 2006)), the present results are the first to suggest a top-down regulatory control system for behavioral arousal. The present data suggest that the ability to consciously decide to stay awake despite homeostatic and circadian drive to sleep may be mediated, in part, by prefrontal cortex adenosine A_1 and A_{2A} receptors modulating pontine reticular formation ACh release.

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CHAPTER 4

SUMMARY AND CONCLUSION

This Ph.D. thesis research used a pharmacological approach to elucidate the mechanisms by which prefrontal cortex adenosine receptors modulate the waking phenotypes of acetylcholine (ACh) release, anesthesia recovery time, electroencephalographic (EEG) delta power, and sleep. The results summarized in this thesis are consistent with the interpretation that adenosine A_1 and A_{2A} receptors in the prefrontal cortex of B6 mouse modulate cortical ACh release, behavioral and EEG arousal (Van Dort CJ et al., 2006a, Van Dort CJ et al., 2006b, Van Dort CJ et al., 2007a), and sleep (Van Dort CJ et al., 2007b), in part, by altering pontine reticular formation ACh release. These results are discussed below in relation to possible mechanisms for prefrontal cortex modulation of arousal, relevant recent studies, and potential clinical relevance.

A systematic review of the literature shows the present research to be the first to characterize adenosinergic and cholinergic interactions in the prefrontal cortex of B6 mouse. Functions of the prefrontal cortex, such as working memory (Gabrieli et al., 1998) and attention (Chee et al., 2008, Drummond et al., 2001, Mander et al., 2008, Muzur et al., 2002, Sarter et al., 2006), are negatively affected by sleep deprivation. This thesis focused on the frontal association area of the prefrontal cortex, the mouse homolog

of the human and primate prefrontal cortex (Muzur et al., 2002, Uylings and van Eden, 1990, Uylings et al., 2003).

The Chapter 2 data support the hypotheses of Aims 1 and 2 and demonstrate that prefrontal cortex adenosine A_1 and A_{2A} receptors modulate the waking phenotypes of ACh release, anesthesia recovery time, EEG delta power, and sleep. Unilateral dialysis delivery of CGS, an adenosine A_{2A} receptor agonist, to the prefrontal cortex of B6 mouse increased cortical ACh release (Fig. 2.2). This increase in ACh release is consistent with previous data showing that administration of CGS to the pontine reticular formation of B6 mouse increased ACh release (Coleman et al., 2006). Dialysis delivery of the adenosine A_1 receptor agonist SPA to the prefrontal cortex significantly decreased waking phenotypes including a decrease in prefrontal cortex ACh release and increase in anesthesia recovery time (Fig. 2.3). The present finding that dialysis delivery of SPA to the prefrontal cortex delayed resumption of wakefulness from anesthesia is consistent with the finding that dialysis administration of SPA to the pontine reticular formation of cat decreased ACh release and increased anesthesia recovery time (Tanase et al., 2003). The good agreement in results obtained from two different species supports the interpretation that adenosine A_1 and A_{2A} receptors modulate ACh release and anesthesia recovery time. The effects of both CGS and SPA were concentration dependent and blocked by their respective selective antagonist suggesting that the CGS and SPA induced changes in ACh release and anesthesia recovery time were receptor mediated. The opposing effects of the adenosine A_{2A} versus A_1 receptor agonist discovered in the present thesis results fit with the known signaling mechanisms of adenosine receptors.

Adenosine A_{2A} receptors are excitatory G-protein coupled whereas adenosine A_1 receptors are inhibitory G-protein coupled (Dunwiddie and Masino, 2001).

The decrease in mouse prefrontal cortex ACh release caused by SPA is consistent with findings in rat cerebral cortex where the adenosine A_1 receptor agonist N^6-Cyclopentyladenosine (CPA) also decreased basal ACh levels (Phillis et al., 1993). In contrast to the present study, the adenosine A_{2A} receptor agonist CGS did not alter basal ACh levels in rat cerebral cortex (Phillis et al., 1993). Additional data show that electrically stimulated ACh release from rat cortical slices was decreased by the adenosine A_1 receptor agonist CPA, whereas the adenosine A_{2A} receptor agonist CGS, did not alter ACh release (Broad and Fredholm, 1996). In urethane anesthetized rats, no adenosine A_1 or A_{2A} receptor agonist or antagonist tested altered basal ACh release from the barrelfield of the somatosensory cortex (Materi et al., 2000). Therefore, the Materi et al. study examined the effects of adenosine receptor agonists and antagonists on evoked cortical ACh release induced by electrical stimulation of the pedunculopontine tegmental nucleus (Materi et al., 2000). Local dialysis delivery of adenosine or the adenosine A_1 receptor agonist CPA to the somatosensory cortex decreased evoked ACh release in the somatosensory cortex and this decrease in evoked ACh release was blocked by the adenosine A_1 receptor antagonist DPCPX (Materi et al., 2000). Both the Materi et al. study and the current thesis data agree that adenosine A_1 receptor agonists decrease cortical ACh release. In contrast to the present thesis results, neither the adenosine A_{2A} receptor agonist CGS, the adenosine A_1 receptor antagonist DPCPX, or the adenosine A_1 and A_{2A} receptor antagonist caffeine delivered to the somatosensory cortex alone, altered evoked ACh release (Materi et al., 2000). The discrepancies between the Materi et al.

findings and the present research may have resulted from the fact that the two studies used different species, different brain regions, and different anesthetics. In contrast to the present studies of ACh release from the prefrontal cortex of isoflurane anesthetized B6 mouse, the Materi et al. data were derived from the somatosensory cortex of urethane anesthetized Wistar rat. Urethane has been shown to exert non-specific effects on muscarinic and nicotinic cholinergic, as well as γ-aminobutyric acid (GABA)ergic, glycinergic, and N-methyl-D-aspartate (NMDA) receptors (Hara and Harris, 2002).

Having demonstrated that exogenous adenosine A_1 and A_{2A} receptor agonists delivered to the prefrontal cortex alter waking phenotypes, additional experiments were designed to determine whether endogenous adenosine acting at adenosine A_1 and/or A_{2A} receptors in the prefrontal cortex also modulates ACh release in the pontine reticular formation. The adenosine A_1 and A_{2A} receptor antagonist caffeine or the adenosine A_1 receptor antagonist DPCPX were delivered to the prefrontal cortex by microdialysis and both significantly increased waking phenotypes (Fig. 2.4 and 2.5). Similar to the increase in prefrontal cortex ACh release caused by local administration of DPCPX in the present thesis study, systemic administration of the adenosine A_1 receptor antagonist 8-dicyclopropylmethyl-1,3-dipropylxanthine to rat increased cerebral cortex ACh release (Kurokawa et al., 1996). Interestingly, the present thesis results demonstrated that the adenosine A_{2A} receptor antagonist ZM did not alter ACh release, anesthesia recovery time, or EEG delta power (Fig. 2.4 and 2.5). The finding that the A_{2A} receptor antagonist did not alter waking phenotypes was novel because a study using adenosine A_1 and A_{2A} receptor knockout mice demonstrated that caffeine was able to promote wakefulness in adenosine A_1 but not A_{2A} receptor knockout mice (Huang et al., 2005). The authors

interpreted these data to suggest that caffeine promotes wakefulness through blockade of adenosine A_{2A} receptors and not adenosine A_1 receptors (Huang et al., 2005). In contrast to the study of adenosine receptor knockout mice, the good agreement between the present caffeine and DPCPX data clearly indicate a role for prefrontal cortex adenosine A_1 receptors in the regulation of behavioral arousal in normal B6 mice. The differences between the Huang et al. study and the thesis data may be due to the type of mouse model used, the route of drug administration, or the concentration of caffeine. An acknowledged limitation of knockout mice in which a gene has been ablated is compensation by other genes (Crawley, 2000, Robert, 1998). The Huang et al. study administered caffeine systemically by intraperitoneal injection whereas the drugs tested in the present thesis research were delivered locally to the prefrontal cortex. Alternatively, the concentration of caffeine used in the Huang et al. study has been shown to induce locomotor activity (Fredholm et al., 1999, Lindskog et al., 2002). Therefore, the increase in wakefulness may be secondary to the increase in activity.

There is debate as to which adenosine receptor subtype, A_1 or A_{2A}, is more important for the role of adenosine in arousal state control. Interestingly, studies of intact rats, cats, and humans, using pharmacology and sleep deprivation, show that adenosine A_1 receptors mediate the sleep promoting effects of adenosine. In contrast, studies using adenosine receptor knockout mice suggest that adenosine A_{2A} receptors mediate the sleep promoting effects of adenosine. For example, systemic or intracerebroventricular administration of the adenosine A_1 receptor agonist CPA to rat causes a concentration dependent increase in slow wave activity during NREM sleep, mimicking the electroencephalographic changes that occur with sleep deprivation (Benington et al.,

1995). In cat, microdialysis delivery of the adenosine A_1 receptor antagonist cyclopentyltheophylline to the cholinergic basal forebrain increases wakefulness and decreases NREM and REM sleep and the adenosine A_1 receptor agonist cyclohexyladenosine decreases wakefulness and increases sleep (Strecker et al., 2000). Sleep deprivation in rat causes a significant increase in adenosine A_1 receptor mRNA (Basheer et al., 2001) and protein (Basheer et al., 2007) in the basal forebrain whereas adenosine A_{2A} receptor mRNA decreases (Basheer et al., 2001). These results suggest that basal forebrain adenosine A_1 receptors contribute, in part, to the sleep inducing action of increased basal forebrain adenosine during prolonged wakefulness. Adenosine A_1 receptor binding is also up regulated in the cortex and corpus striatum after REM sleep deprivation in rat (Yanik and Radulovacki, 1987). In humans, sleep deprivation causes a global brain increase in adenosine A_1 receptor binding demonstrated by positron emission tomography, with the highest binding in the orbitofrontal cortex (Elmenhorst et al., 2007). These studies support the interpretation that cortical adenosine A_1 receptors contribute to sleep homeostasis in humans as well as non-human animals. The thesis data demonstrating that adenosine A_1 receptors in the prefrontal cortex mediate, in part, the sleep promoting effect of adenosine, fit well with data from intact rat, cat, and human.

Given the striking effects of adenosine A_1 and A_{2A} receptor agonists and antagonists on sleep and wakefulness, it was surprising that adenosine A_1 and A_{2A} receptor knockout mice did not have a more pronounced phenotype. Both adenosine A_1 (Stenberg et al., 2003) and adenosine A_{2A} (Urade et al., 2003) receptor knockout mice survive development with no major defects. These knockout mice also show normal circadian sleep wake cycles compared to wildtype mice (Stenberg et al., 2003, Urade et

al., 2003). In view of pharmacological studies of intact animals showing clear functions for adenosine A_1 and A_{2A} receptors, it will be interesting to determine if differences can be found in sleep and wakefulness if future studies use conditional (where the gene can be knocked out after development) and regional (where the gene can be knockout out in specific brain regions) gene knockouts. One difference between the adenosine A_1 and A_{2A} receptor knockout mice is their response to a pharmacological challenge of caffeine. Caffeine promotes wakefulness in adenosine A_1 but not A_{2A} receptor knockout mice, suggesting that in these genetically altered mice caffeine promotes arousal by blocking adenosine A_{2A} and not A_1 receptors (Huang et al., 2005).

Cortical ACh levels vary with the arousal state of the animal (Jasper and Tessier, 1971, Marrosu et al., 1995) therefore, the foregoing studies used anesthesia to hold state constant during the intensive concentration response curve and antagonist blocking data collection. These results encouraged another set of experiments using intact, unanesthetized mice to determine whether prefrontal cortex adenosine A_1 receptors also modulate sleep and wakefulness. Microinjection of the adenosine A_1 receptor antagonist DPCPX into the prefrontal cortex increased wakefulness, decreased NREM sleep, and did not change REM sleep. These data are consistent with the interpretation that prefrontal cortex adenosine, acting through adenosine A_1 receptors, promotes sleep and inhibits wakefulness, in addition to inhibiting the waking phenotypes discussed above. These results are supported by a recent study demonstrating that DPCPX reversed ethanol induced sleep when microinjected into the basal forebrain of rat (Thakkar et al., 2008). In cat, microdialysis delivery of a different adenosine A_1 receptor antagonist,

cyclopentyltheophylline, to the basal forebrain also increased wakefulness and decreased sleep (Strecker et al., 2000).

Early brain transection studies (Jouvet, 1962) revealed that rhythmic oscillations between traits characteristic of NREM sleep and REM sleep persist after the forebrain is removed. These studies helped localize the central pattern generator for REM and NREM sleep to the pontine brainstem (Jouvet, 1962). The data reported in Chapter 2 showed that adenosine A_1 and A_{2A} receptors in the prefrontal cortex modulate ACh release, behavioral and EEG arousal, and sleep. In view of early studies demonstrating that rostral brain regions are not necessary for generating REM and NREM sleep, the Chapter 2 results imply that the prefrontal cortex modulates states of sleep and wakefulness by way of descending input to caudal arousal control centers. Therefore, Chapter 3 experiments were designed to determine if pontine reticular formation ACh release was one mechanism by which prefrontal cortex adenosine A_1 and A_{2A} receptors modulate behavioral arousal. Microdialysis delivery of an adenosine A_{2A} receptor agonist, adenosine A_1 receptor antagonist, or caffeine to the prefrontal cortex increased pontine reticular formation ACh release whereas an adenosine A_1 receptor agonist decreased pontine reticular formation ACh release. These findings are the first to demonstrate that pharmacological manipulation of the prefrontal cortex can alter neurotransmitter release in the pontine reticular formation. These results also suggest that one mechanism through which prefrontal cortex adenosine A_1 and A_{2A} receptors modulate behavioral arousal is by altering pontine reticular formation ACh release.

Adenosinergic compounds have been used to manipulate arousal and pain in humans. The most common adenosinergic compound used in humans is the stimulant

caffeine (Fredholm et al., 1999). Adenosine also has antinociceptive properties (Foster et al., 1994, Sawynok, 1998, Sawynok and Liu, 2003, Sollevi, 1997) but only a few studies have investigated the antinociceptive properties of adenosine in humans (Segerdahl et al., 1995, Segerdahl et al., 1996). In subjects undergoing breast (Segerdahl et al., 1995) or shoulder surgery (Segerdahl et al., 1996), peroperative systemic adenosine infusion decreased postoperative analgesic requirement (Segerdahl et al., 1995) as well as the level of isoflurane needed to maintain unconsciousness during surgery (Segerdahl et al., 1995, Segerdahl et al., 1996).

Further evidence that adenosinergic neurotransmission is involved in human sleep homeostasis stems from studies of polymorphisms in adenosine related genes in humans. Adenosine deaminase plays a role in adenosine metabolism by breaking down adenosine into inosine. A polymorphism in the adenosine deaminase gene that decreases the breakdown of adenosine, thereby increasing adenosine levels, causes an increase in deep slow waves during NREM sleep (Retey et al., 2005). A different polymorphism in the adenosine A_{2A} receptor gene is correlated with anxiety induced by caffeine (Alsene et al., 2003). This polymorphism in the adenosine A_{2A} receptor is also associated with interindividual differences in sensitivity to caffeine and the ability of caffeine to disrupt sleep quality (Retey et al., 2007). These differential arousal phenotypes associated with genetic polymorphisms in adenosine related genes suggests that adenosine plays a role in sleep homeostasis in humans.

In conclusion, four novel findings emerged from this thesis research in B6 mouse: 1) prefrontal cortex adenosine A_1 and A_{2A} receptors modulate cortical ACh release, behavioral and EEG arousal, 2) caffeine promotes wakefulness, in part, by blocking

prefrontal cortex adenosine A_1 receptors, 3) adenosine A_1 receptors in the prefrontal cortex modulate sleep and wakefulness, and 4) prefrontal cortex adenosine A_1 and A_{2A} receptors modulate behavioral arousal, in part, by altering pontine reticular formation ACh release. Taken together, these results are consistent with the interpretation that adenosine A_1 and A_{2A} receptors in the prefrontal cortex of B6 mouse play an important role in regulating behavioral arousal and sleep.

The present thesis results encourage further studies to elucidate the mechanism of caffeine action on wakefulness as well as prefrontal cortex control of behavioral arousal. From Aims 1 and 2, a number of potential future studies arise, including addition of concentration response curve data for the effects of DPCPX microinjected into the prefrontal cortex on sleep and wakefulness or microinjecting other adenosine receptor agonists and antagonists like CGS, SPA, and caffeine into the prefrontal cortex and recording their effects on sleep and wakefulness. Concentration response and antagonist blocking data from these sleep studies would help characterize the role of adenosine A_1 and A_{2A} receptors in the prefrontal cortex on sleep and wakefulness. Another approach to determining the wakefulness promoting mechanism of caffeine is to combination systemic and local drug delivery. Systemic administration of caffeine increases wakefulness (Huang et al., 2005). If this increase in wakefulness by caffeine can be blocked by local administration of the adenosine A_1 receptor agonist to the prefrontal cortex, this would suggest that one mechanism by which caffeine promotes wakefulness is by blockade of adenosine A_1 receptors in the prefrontal cortex. Aim 3 results support further investigation of the role of the prefrontal cortex on behavioral arousal. One way to determine what brain regions are activated and deactivated by pharmacological

manipulation of the prefrontal cortex would be by imaging studies. New rodent magnetic resonance imaging technology makes it possible to measure regional brain activity in the live animal. Another way to look at where the prefrontal cortex is changing activity would be by injecting an adenosine receptor agonist or antagonist into the prefrontal cortex, slicing the brain, and staining for immediate early gene c-fos expression, a marker of neuronal activity. Brain regions that might show changes in activity after prefrontal cortex stimulation would be the cholinergic basal forebrain or the lateraldorsal tegmental/ pedunculopontine tegmental nuclei. Once candidate brain regions are identified, dual dialysis experiments could be performed to determine the neurochemical phenotype of the region involved. Another useful tool for studying adenosine receptor signaling is knockout mice. As discussed earlier, adenosine A_1 and A_{2A} receptor knockout mice have been made and could be used for further pharmacological analysis of the receptor systems involved in the regulation of behavioral arousal, sleep, and wakefulness.

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APPENDIX

NEUROCHEMICAL MODULATORS OF SLEEP AND ANESTHETIC STATES

The regulation of consciousness is a fundamental question that has a long and storied association with philosophy. Today, consciousness studies command a central position in contemporary neuroscience (Blackmore, 2004, Gallagher, 2000, Koch, 2004). The complexities of consciousness studies and the pressing demands of clinical care have led most anesthesiologists to focus on research problems with pragmatic outcomes. Yet the ability to accurately assess and manipulate states of consciousness with anesthetic drugs is the ultimate concern for every surgical patient and anesthesia provider. This volume recognizes consciousness studies as a legitimate and accessible concern for anesthesiology. This chapter considers the relationship between molecules known to regulate the loss of consciousness during anesthesia and molecules that regulate the loss of consciousness during physiological sleep. Sleep neurobiology has been shown to provide unique insights into the study of consciousness (Hobson et al., 1999, Hobson et al., 2000, Hobson and Pace-Schott, 2002). The proposal (Lydic, 1996, Lydic and Baghdoyan, 1998) that neuronal networks that evolved to generate states of sleep and wakefulness also contribute to the generation of anesthetic states has been supported by many laboratories (Fiset, 2003, Franks, 2008, Hudetz et al., 2003, Kelz et al., 2008, Lydic and Baghdoyan, 2005a, Meuret et al., 2000, Nelson et al., 2002b, Nelson et al., 2003,

Pain et al., 2000, Tung et al., 2005, Yasuda et al., 2003). There is evidence that the loss of consciousness during sleep is caused, in part, by the loss of functional connectivity and information processing (Massimini et al., 2005). Functional connectivity is critically dependent on neurochemical transmission. Therefore, this chapter focuses on intravenous and volatile anesthetics that have been shown to alter endogenous neurotransmitters known to regulate states of consciousness. Reviews on sleep from an anesthesiology perspective are available elsewhere (Lydic and Baghdoyan, 1998, Lydic and Baghdoyan, 2005b, Lydic and Baghdoyan, 2005c, Lydic and Baghdoyan, 2007, Tung and Mendelson, 2004).

The present overview is derived from a September 2007 PubMed title search of peer-reviewed papers linking 10 commonly used anesthetics with 11 endogenous molecules known to regulate states of consciousness. The list of intravenous anesthetics includes propofol, pentobarbital, ketamine, etomidate, and midazolam. The list of volatile anesthetics includes isoflurane, sevoflurane, nitrous oxide, xenon, and desflurane. The 11 endogenous molecules known to regulate sleep/wake states (Steriade and McCarley, 2005) include acetylcholine (ACh), γ-aminobutyric acid (GABA), glutamate, adenosine, dopamine, histamine, serotonin, norepinephrine, hypocretin/orexin, glycine, and galanin. This 10 by 11 matrix was searched from 1950 to 2007 and identified 660 references. A search of the last 10 years (1997 to 2007) revealed a total of 192 references (Tables 1 and 2).

I. INTRAVENOUS ANESTHETICS ALTER NEUROTRANSMITTERS THAT REGULATE SLEEP AND WAKEFULNESS. The endogenous neurotransmitters and neuromodulators ACh, GABA, glutamate, the monoamines (dopamine, histamine, serotonin, and norepinephrine), and adenosine contribute to generating and maintaining states of sleep and wakefulness. The following section selectively highlights studies investigating the effects of the most cited agents (Table 1), propofol and ketamine, on these neurotransmitter systems.

Table A.1. Intravenous Anesthetic Agents and Sleep-Related Neurotransmitters. The matrix summarizes the total number of peer reviewed publications obtained from a PubMed search spanning from 1997 to 2007, in which the title listed both the intravenous (I.V.) agent (rows) and the endogenous neurotransmitter molecule (columns). The first column lists the five I.V. agents and columns 2 through 6 list the neurotransmitters studied. Monoamines include serotonin, norepinephrine, dopamine, and histamine. Column 7 summarizes the total number of citations for each I.V. agent.

I.V. Anesthetics	Monoamines	GABA	Glutamate	Acetylcholine	Adenosine	Total
Propofol	12	7	6	4	6	35
Ketamine	13	4	5	11	1	34
Pentobarbital	4	8	2	1	2	17
Midazolam	2	4	4	1	2	13
Etomidate	1	6	1	1	0	9
Total	32	29	18	18	11	108

1. Propofol

Propofol was successfully introduced into clinical practice during the late 1980s. Propofol is a small hydrophobic alkylphenol derivative and its anesthetic actions are mediated primarily via the $GABA_A$ receptor (Reves et al., 2005). Propofol also alters the actions of other sleep-related neuromodulators. As reviewed below, the anesthetic effects of propofol also may be mediated by its effects on monoaminergic, GABAergic, glutamatergic, cholinergic, and adenosinergic neurotransmission in brain regions known to regulate sleep and wakefulness.

1.1 Monoamines: A consistent finding from studies of sleep neurobiology is that monoamines promote wakefulness (reviewed in Ref. (Steriade and McCarley, 2005)). Serotonin containing neurons in the dorsal raphe, norepinephrine containing neurons in the locus coeruleus, and histaminergic neurons in the tuberomammillary nucleus discharge at their fastest rates during wakefulness, slow their discharge rates during non-rapid eye movement (NREM) sleep, and are silent during rapid eye movement (REM) sleep. This wake-on/sleep-off discharge pattern is consistent with a role for these monoaminergic neurotransmitters in promoting wakefulness. Interestingly, although dopaminergic neurons in the ventral tegmental area do not change their discharge rates across the sleep-wake cycle, a large body of evidence demonstrates that dopamine also promotes wakefulness (reviewed in Ref. (Lydic and Baghdoyan, 2005a)).

The cell groups described above provide monoaminergic input to the prefrontal cortex, which contributes to the regulation of behavioral arousal (Muzur et al., 2002). Serotonin levels in rat frontal cortex decrease during sleep compared to wakefulness (Bjorvatn et al., 2002), as would be predicted by the wake-on/sleep-off discharge pattern

of serotonergic neurons. Similarly, norepinephrine levels decrease during REM sleep compared to wakefulness in rat medial prefrontal cortex (Lena et al., 2005). Dopamine levels in rat medial prefrontal cortex also vary across the sleep wake cycle such that dopamine levels are greater during the electroencephalographically (EEG) activated states of wakefulness and REM sleep compared to the EEG deactivated state of NREM sleep (Lena et al., 2005). In the locus coeruleus and amygdale, the release of norepinephrine and serotonin decreases with sleep whereas dopamine release does not change, demonstrating that neurochemical changes during sleep are neurotransmitter and brain region dependent (Shouse et al., 2000).

The elimination of waking consciousness by propofol may be due, in part, to suppression of monoaminergic transmission in multiple arousal promoting brain regions. Dopamine levels in the nucleus accumbens are greater during wakefulness and REM sleep (Lena et al., 2005) than during NREM sleep, and propofol decreases dopamine release in the nucleus accumbens (Pain et al., 2002, Schulte et al., 2000). However, propofol increases dopamine and serotonin metabolites in rat somatosensory cortex (Shyr et al., 1997). This finding suggests that propofol increases the release of these transmitters in rat somatosensory cortex. Dopamine and serotonin each can cause excitation or inhibition, depending upon the type of receptor they activate. Thus, it will be important to combine electrophysiological and neurochemical studies to provide a complete understanding of the effects of anesthetics on monoaminergic neurotransmission in specific brain regions.

Systemic administration of epinephrine, norepinephrine, and dopamine decreases arterial blood propofol concentrations and increase cardiac output in sheep under

continuous propofol infusion, suggesting that monoamines can reverse propofol anesthesia by altering circulation (Myburgh et al., 2001). Systemic administration of the α2 receptor agonist clonidine to rats increases the duration of anesthesia produced by propofol and decreases prefrontal cortex norepinephrine release (Kushikata et al., 2002). This same study also found that systemic administration of the α2 receptor antagonist yohimbine decreases the duration of propofol anesthesia and increases cortical norepinephrine release. Given the important role of monoamines in promoting wakefulness, a productive area for future studies will be to determine whether propofol inhibits monoaminergic neurotransmission in the prefrontal cortex and the locus coeruleus. The mechanisms of anesthetic action, similar to the neurobiology of sleep, will be better understood as the effects of anesthetics on neurotransmission are elucidated on a brain region-by-region basis.

1.2 GABA: GABA is an inhibitory amino acid involved in actively generating sleep (Steriade and McCarley, 2005) and anesthesia (Orser, 2006). The $GABA_A$ receptor is made up of 5 subunits. The α, β, and γ subunits all have been shown to be involved in the regulation of sleep and anesthesia. Most $GABA_A$ receptors are composed of 2α, 2β, and 1γ subunit (Bormann, 2000). The combination of receptor subtypes that comprise the $GABA_A$ receptor varies in different brain regions and may account for differential effects of drugs in each region (reviewed in Ref. (Mody and Pearce, 2004)). For example, the ability of propofol to activate $GABA_A$ receptors varies with the type of α subunit (α1 versus α6) (Krasowski et al., 1997). The endogenous molecule GABA and the anesthetic propofol act at different subtypes of the $GABA_A$ receptor β2 subunit

(molecular weight 54 versus 56 kDa) to produce differential effects on naturally occurring sleep versus anesthesia (Bjornstrom and Eintrei, 2003).

Administration of $GABA_A$ receptor agonists or antagonists to brain regions regulating states of consciousness can either increase or decrease wakefulness, depending on the brain region into which the drugs are administered. For example, enhancing GABAergic inhibition in brain regions that promote arousal, such as the posterior hypothalamus, locus coeruleus, and dorsal raphe nucleus, produces sleep (reviewed in Ref. (Lydic and Baghdoyan, 2005a)). In contrast, administering GABAmimetics into the pontine reticular formation increases wakefulness and decreases sleep (Sanford et al., 2003, Watson et al., 2008, Xi et al., 1999). Anesthetics enhance GABAergic neurotransmission by increasing chloride ion conductance and causing neuronal hyperpolarization (reviewed in Ref. (Mody and Pearce, 2004)). In the brain stem locus coeruleus and dorsal raphe nucleus, GABA levels are highest during REM sleep (Nitz and Siegel, 1997a) and, as noted above, electrophysiological data show that locus coeruleus and dorsal raphe neurons cease firing during REM sleep (reviewed in Ref. (Steriade and McCarley, 2005)). Propofol acting at $GABA_A$ receptors inhibits the firing of locus coeruleus neurons (Chen et al., 1999). The time to propofol-induced loss of righting, used as a measure of sedation, is reduced by microinjecting a $GABA_A$ receptor antagonist into the tuberomammillary nucleus of the hypothalamus, a wakefulness promoting brain region (Nelson et al., 2002b). This finding suggests that propofol causes its sedative effects, in part, by potentiating GABAergic inhibition of hypothalamic neurons that promote wakefulness. Similarly, intravenous administration of gabazine and picrotoxin, which block transmission at $GABA_A$ receptors, causes large increases in the

ED_{50} for propofol induced immobility in rat (Sonner et al., 2003). These findings support the interpretation that immobility caused by propofol is mediated by $GABA_A$ receptors.

Propofol decreases regional cerebral glucose metabolism in rat (Dam et al., 1990) and human (Alkire et al., 1995), and the extent of this depression varies by brain region. In humans, regional cerebral glucose metabolism in the cortex showed greater depression than in subcortical areas and the greatest depression within the cortex occurred in the left anterior cingulate and inferior colliculus (Alkire et al., 1995). Propofol enhances GABAergic neurotransmission (Peduto et al., 1991) and the glucose metabolism data correlate with the high benzodiazepine receptor density in human cerebral cortex (Braestrup et al., 1977). The brain regions in which propofol selectively alters cerebral metabolism provide targets for further localization of function studies aiming to identify the mechanisms by which propofol produces anesthesia.

1.3 Glutamate: There is considerable evidence that excitatory amino acids contribute to the regulation of both sleep- and anesthesia-induced losses of waking consciousness. Glutamatergic transmission in many brain regions is important for sleep and anesthesia. In the brainstem, the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei contain neurons that contribute to REM sleep generation (reviewed in Ref. (Lydic and Baghdoyan, 2005a)). Microinjection of glutamate into the PPT induces waking and/or REM sleep depending on the concentration of injected glutamate (Datta et al., 2001). Glutamate levels in the PPT are greater during wakefulness than during NREM sleep or REM sleep (Kodama and Honda, 1999), consistent with the interpretation that glutamate in the LDT and PPT promotes arousal. Further evidence that glutamate promotes wakefulness is shown by systemic

administration of the glutamate receptor antagonist riluzole which increases NREM sleep and REM sleep in rats (Stutzmann et al., 1988). However, intracerebroventricular administration of the glutamate N-methyl-D-aspartate (NMDA) receptor antagonists MK-801 and AP5 decreases REM sleep but does not change NREM sleep or wakefulness (Prospero-Garcia et al., 1994). The present search of the literature identified no studies that quantified the effect of propofol on brainstem levels of glutamate.

Available data demonstrate that the role of glutamate in the regulation of consciousness also varies as a function of brain region. In one study, glutamate levels in the orbitofrontal cortex of rat were highest during REM sleep, decreased during wakefulness, and lowest during NREM sleep (Lopez-Rodriguez et al., 2007). These data are consistent with the interpretation that glutamate promotes an activated cortical EEG. Glutamate levels did not change in the prefrontal cortex during NREM sleep and REM sleep compared to levels during wakefulness (Lena et al., 2005), suggesting that cortical glutamate is not involved in sleep regulation. REM sleep deprivation in the rat increased cortical glutamate levels (Bettendorff et al., 1996) alternatively implying that glutamate in the cortex is somehow involved in the regulation of sleep. In the basal forebrain, microinjection of the glutamate receptor agonists NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) increased wakefulness and γ (30-60 Hz) EEG activity and decreased delta (1-4 Hz) EEG activity (Cape and Jones, 2000). Microinjection of NMDA and AMPA into the cholinergic cell area of the basal forebrain also caused C-fos activation, a widely used marker of neuronal activity (Cape and Jones, 2000). These results are consistent with the interpretation that glutamate in the basal forebrain promotes waking consciousness.

Data from many species and brain regions demonstrate that propofol decreases presynaptic, sodium channel-dependent, glutamate release in cortical, striatal, and hippocampal synaptosomes isolated from rats, mice, and guinea pigs (Lingamaneni et al., 2001, Ratnakumari and Hemmings, 1997). Only one study showed that propofol did not change presynaptic glutamate uptake, binding, or transport in rat cortical isolated nerve terminal preparations (Westphalen and Hemmings, 2003b). Another study by the same group demonstrated that propofol did inhibit calcium dependent evoked glutamate release in rat cortical synaptosomes (Westphalen and Hemmings, 2003a). Considered together, these data suggest that propofol depresses glutamate neurotransmission but the exact mechanism has yet to be elucidated.

Both NMDA and AMPA receptors are important for the regulation of sleep, but only NMDA receptors contribute to the inhibition of currents in cultured mouse hippocampal neurons by propofol (Orser et al., 1995). This inhibition occurs by allosteric modulation of the channel versus blockade of the channel. The literature search did not reveal any studies that have characterized the effect of propofol on metabotropic glutamate receptors. An exciting opportunity for future studies is to elucidate the role of glutamate in the neurochemical regulation of consciousness.

1.4 ACh: There is a long-standing appreciation that cholinergic neurotransmission contributes to the loss of consciousness associated with sleep and anesthesia (Lydic and Baghdoyan, 1998)' (Backman et al., 2004). Pontine ACh contributes to the generation of REM sleep and ACh release within the pontine reticular formation is greater during REM sleep than during NREM sleep and wakefulness (Leonard and Lydic, 1997). The clinical finding that the acetylcholinesterase inhibitor

physostigmine reverses propofol sedation suggests that propofol produces unconsciousness, in part, by disrupting cholinergic neurotransmission (Meuret et al., 2000). REM sleep, like wakefulness, is a brain activated state that is characterized by increases in cholinergic neurotransmission. Brain activated states of consciousness, such as wakefulness and REM sleep, are promoted by drugs that enhance cholinergic neurotransmission. Thus, the finding that physostigmine causes arousal from propofol sedation in humans (Meuret et al., 2000) is consistent with data showing that administering neostigmine or carbachol into the pontine reticular formation causes a REM sleep-like state in mouse (Lydic et al., 2002), rat (Bourgin et al., 1995), and cat (Baghdoyan et al., 1984).

The foregoing gain-of-function data illustrated by manipulations that increase ACh are complimented by loss-of-function studies that chemically eliminate cholinergic neurons. For example, the immunotoxin 192 IgG-Saporin selectively destroys basal forebrain cholinergic neurons. In rat, intracerebroventricular administration of 192 IgG-Saporin caused a reduction in ACh levels in frontoparietal cortex and hippocampus and a decrease in propofol-induced locomotor inhibition (Pain et al., 2000). These findings suggest that inhibition of basal forebrain cholinergic neurons contributes to the hypnotic effect of propofol (Pain et al., 2000). Results from this study are also consistent with the notion that anesthetics act in a brain site specific manner, because acetylcholine levels in the striatum and cerebellum were not reduced by treatment with 192 IgG-Saporin. The cerebellum and striatum are not brain regions with primary arousal state regulating functions.

Both muscarinic and nicotinic ACh receptors are important for the regulation of sleep and wakefulness. In vitro studies show that propofol blocks ACh-induced muscarinic M1 receptor currents (Nagase et al., 1999). Propofol also inhibits nicotinic ACh receptor mediated currents when the nicotinic receptor is composed of the α4 β2 subunit but not the α7 homomeric subunit (Flood et al., 1997, Furuya et al., 1999). ACh release within the cerebral cortex and dorsal hippocampus is greater during wakefulness and REM sleep than during NREM sleep (Marrosu et al., 1995). Propofol decreased ACh release in rat frontal cortex and hippocampus (Kikuchi et al., 1998), providing additional support for the conclusion that propofol causes sedation, in part, by inhibiting cholinergic neurotransmission in brain regions that regulate arousal.

1.5 Adenosine: Consistent with the idea that the neuronal circuits controlling sleep are preferentially modulated by anesthetics (Lydic, 1996), local administration of propofol to the medial preoptic area, a region known to promote sleep (Steriade and McCarley, 2005), decreased latency to sleep onset, increased NREM sleep, and increased total sleep time (Tung et al., 2001). The idea that neuronal networks that generate sleep also regulate anesthesia suggests the possibility that prior sleep history may affect anesthetic action. However, no clinical studies have demonstrated any difference in anesthetic requirement that is dependent on sleep deprivation. Preclinical studies have shown that sleep deprivation can enhance the sedative effects of propofol. Rats were sleep deprived for 24 h and then administered propofol anesthesia. Although loss of righting response is not identical to sleep, sleep deprivation decreased the latency for and prolonged the duration of loss of righting response caused by propofol (Tung et al.,

2002). These results also support the view that propofol acts at circuits involved in sleep regulation.

Adenosine is a sleep promoting neuromodulator that acts through 4 G protein coupled receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3. During prolonged wakefulness brain adenosine levels increase within the basal forebrain and cortex (Basheer et al., 2004). The ability of sleep deprivation to decrease loss of righting response in rat was partially reversed by administration of adenosine A_1 and A_{2A} receptor antagonists (Tung et al., 2005). Adenosine is known to inhibit excitatory neurotransmission through adenosine A_1 receptors (Tohdoh et al., 2000). In another study, rats were sleep deprived for 24 h and then allowed to have 6 h of ad libitum sleep or 6 h of propofol anesthesia. Rats that experienced ad libitum sleep and propofol anesthesia had similar amounts of NREM sleep and REM sleep (Tung et al., 2004). The authors interpreted these results to suggest that propofol anesthesia provides some of the restorative processes that take place during naturally occurring sleep.

2. Ketamine

Ketamine was first tested clinically in the mid 1960s (Corssen and Domino, 1966, Domino et al., 1965). The term dissociative anesthetic was introduced at that time, and referred to the findings that during treatment with ketamine, sensory information appeared to reach the sensory cortex but was not accurately perceived due to depression of cortical association areas (Corssen and Domino, 1966). Thus, ketamine caused a dissociation between nociceptive input and the subjective experience of nociception. Ketamine is a phencyclidine derivative that produces analgesia and amnesia without

causing a complete loss of consciousness (Lydic and Baghdoyan, 2002, Reves et al., 2005, White et al., 1982). The primary mechanism of ketamine anesthesia is by antagonism of NMDA receptors (Kohrs and Durieux, 1998). Through NMDA receptor blockade, ketamine alters the actions of several arousal state related neurotransmitters, as outlined below.

2.1 Monoamines: Emergence from ketamine anesthesia is often characterized by confusion, agitation, visual hallucinations, and delirium (Kohrs and Durieux, 1998). This psychomimetic response has been well-investigated in the context of schizophrenia research (Aalto et al., 2005, Kegeles et al., 2000, Moghaddam et al., 1997, Smith et al., 1998). Psychotic-like symptoms occurring during ketamine emergence may result from ketamine induced increases in dopamine release, particularly in the cerebral cortex. Subanesthetic doses of ketamine increase the release of dopamine and serotonin in rat prefrontal cortex (Lindefors et al., 1997, Lorrain et al., 2003) and increase dopamine release in rat nucleus accumbens (Masuzawa et al., 2003). Increased dopamine release in the nucleus accumbens may contribute to the addictive properties of ketamine (Masuzawa et al., 2003). Brain imaging of healthy human volunteers showed that subanesthetic doses of ketamine decreased the binding of a dopamine D2/D3 receptor agonist in the posterior cingulate and retrosplenial cortices (Aalto et al., 2005). This decrease in agonist binding was most likely due to a ketamine induced increase in dopamine release, as ketamine increases cortical dopamine release in rat (Aalto et al., 2005, Moghaddam et al., 1997).

Dopamine receptor subtypes contribute to emergence responses to ketamine. Subanesthetic doses of ketamine in rodents cause hyperlocomotor activity characterized

by staggering and stereotypic head-wagging. These behavioral responses to ketamine are reduced in dopamine D1A receptor knockout mice, suggesting a role for the dopamine D1 receptor in mediating emergence responses to ketamine (Miyamoto et al., 2001). Studies using in vitro expression systems for dopamine D2 receptors or rat membrane preparations have found conflicting results. One study showed that ketamine has high affinity for dopamine D2 receptors (Kapur and Seeman, 2001), whereas another study found that ketamine does not cause functional responses via the dopamine D2 receptor (Jordan et al., 2006). In vivo binding studies in humans using PET imaging have suggested that subanesthetic doses of ketamine increase dopamine D2 receptor binding in the striatum but not the cerebellum (Smith et al., 1998), whereas similar studies using a different ligand in monkeys showed no ketamine induced change in D2 receptor binding in the basal ganglia or cerebellum (Nader et al., 1999). As noted repeatedly throughout this chapter, the brain region where anesthetic induced changes in neurotransmission occur is key to determining the response and the relevance to anesthetic mechanisms of action. Changes in dopamine levels in the cerebral cortex are much more likely to alter mental state than are changes in dopamine levels within the striatum or cerebellum.

Studies using in vitro expression systems of monoamine transmitters indicated that ketamine causes a direct inhibition of monoamine transporters, which would enhance monoaminergic effects and provide an additional mechanism underlying the ketamine emergence reaction (Nishimura and Sato, 1999). Dopamine transporters, but not norepinephrine or serotonin transporters, are selectively inhibited by the S(+)-isomer of ketamine (Nishimura and Sato, 1999). In vivo studies have shown that ketamine increases norepinephrine release in rat medial prefrontal cortex (Kubota et al., 1999).

Norepinephrine release is normally greater during wakefulness than during physiological sleep, thus the ketamine-induced increase in norepinephrine may account for some of the dissociative properties of ketamine.

2.2 GABA: Ketamine has been shown to have some effects on GABAergic neurotransmission, but as of yet it is unclear if ketamine-induced alterations in GABAergic transmission are related to the mechanism of ketamine anesthesia. In vivo studies report that systemic administration of the $GABA_A$ receptor agonist muscimol potentiates ketamine-induced loss of righting response, and coadministration of the $GABA_A$ receptor antagonist bicuculline with ketamine antagonizes ketamine-induced loss of righting (Irifune et al., 2000). Data from other studies suggest that these responses are mediated indirectly. For example, studies using in vitro expression systems have shown that at clinically relevant concentrations, ketamine does not modulate recombinant $GABA_A$ receptor activity (Flood and Krasowski, 2000). Furthermore, the $GABA_A$ receptor antagonists picrotoxin and gabazine cause only a small increase in the ED_{50} for ketamine induced inhibition of mobility in response to a noxious stimulus, suggesting an indirect role of $GABA_A$ receptors (Sonner et al., 2003). Finally, acute and chronic systemic administration of ketamine does not change GABA levels in rat medial prefrontal cortex (Lindefors et al., 1997).

2.3 Glutamate: Ketamine is a noncompetitive NMDA receptor antagonist and the anesthetic effects of ketamine are thought to result primarily from NMDA receptor blockade. For example, the ketamine induced increase in dopamine release (discussed above in the section on monoamines (Aalto et al., 2005, Lindefors et al., 1997, Lorrain et al., 2003, Moghaddam et al., 1997)) is mediated by NMDA receptors. Studies measuring

glutamate show that chronic treatment with ketamine decreases cerebrospinal fluid glutamate levels in rats (Tomiya et al., 2006). During cerebral ischemia glutamate transporters reverse and release glutamate into the extracellular space causing neuronal damage (Sakai and Amaha, 2000). Intravenous anesthetics are thought to be neuroprotective by decreasing this glutamate release into the extracellular space (Sakai and Amaha, 2000). In Chinese hamster ovary cells transfected with a cloned human glial glutamate transporter, ketamine decreased glutamate-induced outward currents suggesting that ketamine modulates glutamate transporters in vitro (Sakai and Amaha, 2000). Many studies have investigated the role of glutamatergic signaling and the dissociative state that is produced by ketamine anesthesia. Ketamine increases glutamate release in the nucleus accumbens (Razoux et al., 2007) and prefrontal cortex of rat (Lorrain et al., 2003). Ketamine also increases anterior cingulate glutamate activity in humans (Rowland et al., 2005). These region-specific increases in glutamate likely account for some of the dissociative properties produced by ketamine anesthesia.

2.4 ACh: Ketamine modulates cholinergic neurotransmission in multiple brain regions and at both muscarinic and nicotinic cholinergic receptors. Many studies have demonstrated that ketamine is a noncompetitive inhibitor at nicotinic ACh receptors (Coates and Flood, 2001, Furuya et al., 1999). The inhibition of neuronal nicotinic ACh receptors by ketamine is subunit dependent, and nicotinic receptors that contain the β1 versus β2 subunit are more sensitive to ketamine (Yamakura et al., 2000). Additionally, the presence of a single amino acid in the extracellular transmembrane region of the α7 subunit of nicotinic receptors determines whether ketamine can inhibit nicotinic receptor currents (Ho and Flood, 2004). These data are consistent with the interpretation that

ketamine produces anesthesia, in part, by modulating nicotinic ACh receptors. The story is complicated, however, because the S enantiomer of ketamine is three times more potent than the R enantiomer, yet in vitro the two enantiomers inhibit neuronal nicotinic ACh receptor currents equally (Sasaki et al., 2000). In contrast to the prior study, these data suggest that the anesthetic effects of ketamine are unlikely to be mediated primarily through nicotinic receptor signaling (Sasaki et al., 2000). Another mechanism by which ketamine might modulate cholinergic neurotransmission is through muscarinic ACh receptors. Fewer studies have investigated the effects of ketamine on the five subtypes of muscarinic cholinergic receptors. Ketamine has been shown to inhibit M1 muscarinic ACh receptor currents in vitro (Durieux, 1995). Consistent with the idea that ketamine anesthesia modulates ACh neurotransmission, repeated administration of ketamine causes up regulation of muscarinic ACh receptors in the forebrain (Morita et al., 1995).

Several studies have determined the effect of ketamine on brain ACh release in vivo. Two studies demonstrated that systemically administered ketamine increased frontal (Kikuchi et al., 1997) and prefrontal (Nelson et al., 2002a) ACh release in rat. These data are difficult to interpret relative to the present chapter because cortical ACh promotes waking consciousness. As noted above, ketamine is a dissociative anesthetic and the increase in cortical ACh release may contribute to the ability of ketamine to activate the bispectral index (Mashour, 2006a). Intravenous delivery of ketamine as well as local ketamine administration to cat pontine reticular formation decreases ACh release and inhibits REM sleep (Lydic and Baghdoyan, 2002). The finding that ketamine increases ACh release in cortex (Kikuchi et al., 1997), (Nelson et al., 2002a) and decreases ACh release in the reticular formation (Lydic and Baghdoyan, 2002) again

emphasizes that efforts to elucidate the neurochemical regulation of sleep and anesthesia can anticipate results to vary as a function of brain region. Thus, we consider the postulate of a single "anesthesia center" in the brain to be an unhelpful throwback to the hope for a single, unifying mechanism.

2.5 Adenosine: Only one study was identified that investigated the role of adenosine in the mechanism of action of ketamine. That study found that an adenosine A_{2A} receptor agonist blocked ketamine induced hyperactivity, suggesting that adenosine or adenosine receptors may somehow contribute to ketamine induced locomotor activation (Mandryk et al., 2005). Adenosine is an important modulator of physiological sleep and alertness during wakefulness, and future studies examining the effects of ketamine on the actions of adenosine in the basal forebrain and prefrontal cortex are likely to contribute to a mechanistic understanding of ketamine induced alterations in arousal state.

II. VOLATILE ANESTHETICS ALTER NEUROTRANSMITTERS THAT REGULATE SLEEP AND WAKEFULNESS. This section selectively highlights the effects of isoflurane and sevoflurane, the most studied and widely used inhaled anesthetics (Table 2), on endogenous sleep-related molecules that include monoamines, ACh, GABA, glutamate, and adenosine.

Table A.2. Volatile Anesthetic Agents and Sleep-Related Neurotransmitters.
The number of peer reviewed publications derived from a PubMed search ranging from 1997 to 2007 in which the title listed both the volatile anesthetic (first column) and the endogenous neurotransmitter molecule (columns 2 through 6) is presented in this table. The right-most column summarizes the total number of citations for each agent. The bottom right cell gives the total number of studies found that investigated the effects of volatile anesthetics on neurotransmitters.

Volatile Anesthetics	Monoamines	GABA	Glutamate	Acetylcholine	Adenosine	Total
Isoflurane	14	13	12	8	7	54
Sevoflurane	3	3	2	3	0	11
Nitrous	3	2	3	2	0	10
Xenon	3	1	1	2	1	8
Desflurane	0	0	0	0	1	1
Total	23	19	18	15	9	84

3. Isoflurane

Ether, nitrous oxide, and chloroform were among the first molecules recognized for their anesthetic properties during the 1840s. Isoflurane, a halogenated ether, was developed in 1965 and entered clinical practice in the late 1970s (Eger, 1981). Isoflurane binds to a specific site on the $GABA_A$ receptor to enhance neuronal inhibition. Additionally, isoflurane alters neurotransmission by varying the effects of many other sleep-regulating neuromodulators. Whether the effects of isoflurane on the transmitter systems discussed below are direct or indirect remains to be determined.

3.1 Monoamines: Few studies have investigated the role of serotonergic neurotransmission in isoflurane anesthesia. The medullary hypoglossal nucleus innervates the genioglossal muscles of the tongue and genioglossal muscles can obstruct patency of the airway. Endogenous serotonin excites hypoglossal neurons (Berger et al., 1992) and isoflurane depresses the excitatory effect of serotonin on hypoglossal motoneurons in dogs (Brandes et al., 2007). Isoflurane also decreases hippocampal serotonin levels in wild type and serotonin transporter knockout mice (Whittington and Virag, 2006). These results indicate that the mechanism by which isoflurane decreases hippocampal serotonin levels is independent of the serotonin transporter. Decreases in serotonin levels persisted for several hours after cessation of isoflurane (Whittington and Virag, 2006). Hippocampal serotonin contributes to cognition and affect, and these data encourage additional studies to determine if isoflurane-induced decreases in hippocampal serotonin cause subsequent behavioral consequences.

Nitrous oxide and isoflurane are commonly coadministered and nitrous oxide produces analgesia, in part, by altering norepinephrine release in the spinal cord.

Electrophysiological data show that isoflurane and norepinephrine each enhanced inhibitory postsynaptic currents in rat substantia gelatinosa neurons (Georgiev et al., 2006). Coadministration of isoflurane and norepinephrine produced a greater increase in inhibitory postsynaptic currents than either drug alone, suggesting that isoflurane may produce analgesia, in part, by modulating norepinephrine neurotransmission at the level of the spinal cord dorsal horn (Georgiev et al., 2006).

The preoptic area of the hypothalamus contains NREM sleep promoting neurons and is important for thermoregulation (reviewed in Ref. (Kumar et al., 2007)). General anesthetics disrupt thermoregulatory control by neural mechanisms that remain unclear. Isoflurane increases preoptic area norepinephrine release in rat brain slices, suggesting that enhanced norepinephrine signaling in the hypothalamus may contribute to hypothermia during isoflurane anesthesia (Kushikata et al., 2005).

Histaminergic neurons in the tuberomammillary nucleus of the posterior hypothalamus are an important component of wakefulness promoting neuronal systems (reviewed in Ref. (Passani et al., 2007)). However, few studies have investigated the effects of volatile anesthetics on histaminergic neurotransmission. One study investigated histamine metabolism in rat hypothalamus and found that isoflurane altered histamine turnover differentially in the anterior versus the posterior hypothalamus (Hashimoto et al., 1998). Isoflurane was shown to increase histamine levels by inhibiting histamine degradation in both the anterior and posterior hypothalamus. However, histamine degradation was increased during the post-isoflurane recovery period only in the posterior hypothalamus (Hashimoto et al., 1998). The post-anesthesia increase in histamine turnover within the posterior hypothalamus is consistent with the wakefulness

promoting role of both histamine and the posterior hypothalamus. In contrast, the anterior hypothalamus contains sleep promoting GABAergic neurons that do not respond to histamine (Gallopin et al., 2000). These data showing brain region specific effects of isoflurane on histamine metabolism (Hashimoto et al., 1998) encourage future studies examining the effects of isoflurane on synaptic transmission within the anterior and posterior hypothalamus. Such studies can be expected to yield mechanistic insights into how anesthetics alter states of consciousness.

Dopamine is wakefulness promoting in animals and in humans (Lydic and Baghdoyan, 2005a). For example, intracerebroventricular administration of dopamine D1 and D2 receptor agonists during physiological sleep in rats causes an increase in wakefulness, an increase in motor activity, and a decrease in sleep (Isaac and Berridge, 2003). Isoflurane increases basal dopamine release and dopamine metabolites in rat striatum in vivo (Adachi et al., 2005, Adachi et al., 2008, Keita et al., 1999) and in rat striatal slices ex vivo (Keita et al., 1999). Dopamine transporter knock out mice show an increase in wakefulness and a decrease in NREM sleep (Wisor et al., 2001). Positron emission tomography studies in rhesus monkey (Votaw et al., 2003) and in human (Votaw et al., 2004) demonstrated that dopamine transporter binding in the striatum decreases during isoflurane anesthesia. These studies suggest that isoflurane increases dopamine levels by inhibiting dopamine reuptake. In vitro experiments confirm that isoflurane causes internalization of the dopamine transporter (Byas-Smith et al., 2004).

Emergence from anesthesia can be associated with an excitatory agitation phase, particularly in preschool children (Bortone et al., 2006). Striatal dopamine may contribute to this emergence reaction. In mice, recovery from isoflurane anesthesia is

characterized by increased locomotor activity and increased dopamine turnover in the nucleus accumbens and striatum (Irifune et al., 1997). The importance of investigating these mechanisms in multiple brain regions is demonstrated by the fact that dopamine levels within the cortex and nucleus accumbens are greater during the activated states of wakefulness and REM sleep (Lena et al., 2005), whereas dopamine levels in the locus coeruleus and amygdala do not change across the sleep wake cycle (Shouse et al., 2000).

3.2 GABA: $GABA_A$ receptors are an important target for inhalation anesthetics and contain a binding site for isoflurane (Mashour et al., 2005). Clinically relevant concentrations of isoflurane reduce the amplitude and extend the decay of GABA evoked currents by slowing the rate of GABA unbinding from recombinant $GABA_A$ receptors (Hapfelmeier et al., 2001a). Isoflurane enhances $GABA_A$ receptor mediated currents in cultured rat cerebral cortical neurons (Ming et al., 2001) and, at clinically relevant concentrations, inhibits both the release and reuptake of GABA in mouse cortical brain slices (Liachenko et al., 1999). Isoflurane also increases the binding of the benzodiazepine receptor antagonist ^{11}C-flumazenil to $GABA_A$ receptors in human cortex and cerebellum, as demonstrated by positron emission tomography (Gyulai et al., 2001). Further evidence that isoflurane modulates GABAergic neurotransmission is demonstrated by the ability of an intrathecally administered $GABA_A$ receptor antagonist to increase the minimum alveolar concentration (MAC) value of isoflurane by 47% in rats (Zhang et al., 2001b).

There have been few studies characterizing changes in endogenous GABA levels during states of sleep, wakefulness, or general anesthesia. GABA levels during sleep are increased above waking levels in cat dorsal raphe nucleus (Nitz and Siegel, 1997a), locus

coeruleus (Nitz and Siegel, 1997b), and posterior hypothalamus (Nitz and Siegel, 1996). These findings support the interpretation that GABAergic inhibition of these wakefulness promoting monoaminergic nuclei contributes to the generation of physiological sleep. Compared to waking levels, isoflurane has been shown to decrease GABA levels in rat basal forebrain and somatosensory cortex (Dong et al., 2006). Preliminary data from cat also show that GABA levels in the substantia innominata region of the basal forebrain are lower during isoflurane anesthesia than during wakefulness (Vanini et al., 2007). GABAergic input to the cortex from the basal forebrain can cause excitation by inhibiting cortical inhibitory interneurons (Freund and Gulyas, 1991, Freund and Meskenaite, 1992, Manns et al., 2000). Thus, the isoflurane induced decrease in cortical GABA (Dong et al., 2006) is consistent with the fact that isoflurane decreases cortical activation and slows the cortical EEG. Interestingly, isoflurane caused no change in posterior hypothalamic GABA levels (Dong et al., 2006). Thus, even during states of general anesthesia there are brain site specific changes in GABAergic transmission.

The capability of isoflurane to produce anesthesia is dependent on the composition of $GABA_A$ receptor subunits. For example, a mutation in the α1 subunit of the $GABA_A$ receptor makes the receptor insensitive to isoflurane (Borghese et al., 2006). The ability of isoflurane to enhance GABA evoked $GABA_A$ receptor currents in cultured Sf9 cells is dependent on the presence of the γ2s subunit (Yamashita et al., 1999). GABA-mediated currents have been studied using an in vitro expression system transfected with recombinant $GABA_A$ receptors, and dual effects were reported (Neumahr et al., 2000). At clinically relevant concentrations isoflurane was shown to potentiate GABA-mediated currents, and at higher concentrations isoflurane inhibited

GABA currents (Neumahr et al., 2000). The potentiating effects that predominate at lower concentrations are thought to be relevant for the mechanism of isoflurane action. Studies in rat indicate that spinal $GABA_A$ receptors can contribute to immobility caused by isoflurane (Zhang et al., 2001a). The role of $GABA_A$ receptors in mediating immobility is not straight forward. For example, pharmacological blocking studies from this same group conclude that the immobilizing effect of isoflurane is not mediated by $GABA_A$ receptors (Sonner et al., 2003). Transgenic mice have been used in an effort to clarify which components of the $GABA_A$ receptor mediate immobility caused by isoflurane. Mice with a knock-in mutation in the β3 subunit of the $GABA_A$ receptor are less sensitive to the immobilizing action of isoflurane (Lambert et al., 2005).

3.3 Glutamate: One mechanism by which isoflurane has been proposed to cause anesthesia is by inhibiting excitatory neurotransmission. Glutamate is the major excitatory amino acid transmitter in the brain, and the effects of glutamate can be reduced by decreasing its release, increasing its uptake, or blocking its receptors. Glutamate transporters are located on neurons and glia, and take up extracellular glutamate to regulate synaptic glutamate levels. Uptake is the major inactivation mechanism for glutamate, as it is not enzymatically degraded.

Isoflurane has been shown to reduce glutamate release in isolated nerve terminals derived from rat cortex, hippocampus, and striatum (Larsen et al., 1998, Lingamaneni et al., 2001). Isoflurane decreases glutamate release in rat hippocampal (Winegar and MacIver, 2006) and cerebral cortex slices (Liachenko et al., 1999). At greater concentrations, however, isoflurane also inhibits glutamate uptake (Liachenko et al., 1999). The authors suggest that the effects of isoflurane depend upon a balance between

inhibition of release and inhibition of reuptake (Liachenko et al., 1999). Several other studies have shown that isoflurane increases glutamate uptake. In cultured rat glial cells, isoflurane increases glutamate uptake via glutamate transporters (Zuo, 2001) and in vivo inhibitors of glutamate transporters increase MAC for isoflurane in rat (Cechova and Zuo, 2006). Isoflurane also increases glutamate uptake in rat cerebral cortex synaptosomes (Larsen et al., 1997). Five types of glutamate transporters have been identified, and isoflurane increases the expression and activity of glutamate type 3 transporters in cultured rat glioma cells (Huang and Zuo, 2003). Isoflurane causes phosphorylation of a serine residue to activate the glutamate type 3 transporter and redistribute it to the plasma membrane (Huang et al., 2006).

Glutamate causes excitation by activating NMDA receptors, and NMDA receptor activation requires the binding of both glutamate and glycine. Isoflurane has recently been shown to inhibit NMDA receptors by binding to the glycine site (Dickinson, 2007). This finding suggests that blocking the excitatory effects of glutamate at NMDA receptors may be one mechanism underlying the anesthetic and neuroprotective effects of isoflurane.

The above studies were performed using reduced preparations such as cell cultures, synaptosomes, or brain slices. Few in vivo studies using intact animals have determined the effects of isoflurane on glutamatergic transmission. In vivo microdialysis work using rat demonstrated that isoflurane differentially alters glutamate levels depending on brain region (Dong et al., 2006). Compared to wakefulness, isoflurane causes a concentration dependent increase in glutamate levels in the basal forebrain, an increase in somatosensory cortex glutamate levels at one concentration only, and no

effect on glutamate levels in the posterior hypothalamus (Dong et al., 2006). The mechanistic implications of the surprising finding that isoflurane increases basal forebrain glutamate levels are not yet clear.

3.4 ACh: Isoflurane modulates nicotinic and muscarinic cholinergic receptors, and the release of ACh. Nicotinic receptors are comprised of five subunits, and several studies have investigated the role of various nicotinic ACh receptor subunits in the mechanism of action of volatile anesthetics (Violet et al., 1997). Isoflurane at clinically relevant concentrations inhibits neuronal nicotinic ACh receptor currents expressed in vitro when the receptors contain the α4-β2 subunit combination (Yamashita et al., 2005). Isoflurane does not block the response of homomeric α7 nicotinic receptors to ACh when the anesthetic and the agonist are coadministered (Flood et al., 1997). However, clinically relevant concentrations of isoflurane do inhibit homomeric α7 nicotinic receptors when the anesthetic is applied prior to ACh, or when ACh is applied in high concentrations (Flood and Coates, 2002). These findings may be relevant in vivo, because α7 nicotinic receptors do occur in brain (Flood and Coates, 2002), and synaptic levels of neurotransmitters have been estimated to reach concentrations in the micromolar to millimolar range. The effects of isoflurane on native (i.e., non-recombinant) nicotinic receptors also have been investigated (Matsuura et al., 2002). Interestingly, both isoflurane and a structurally similar halogenated molecule that does not cause immobility but does have amnestic properties inhibits native neuronal nicotinic ACh receptors in rat medial habenula neurons (Matsuura et al., 2002). Another structurally similar agent with neither immobilizing nor amnestic properties does not block nicotinic receptor-mediated currents (Matsuura et al., 2002). These data suggest that the amnestic effects of

isoflurane may be mediated, in part, by nicotinic receptors in the medial habenula. More in vivo studies are needed to determine if nicotinic ACh receptors are relevant for the production of anesthesia by isoflurane.

There are five muscarinic cholinergic receptor subtypes, and isoflurane has been shown to inhibit M3 but not M1 muscarinic receptors (Nietgen et al., 1998). M1 and M3 receptors are structurally quite similar, thus different effects of the same anesthetic on these two subtypes implies that the site of action is quite specific. More recently, the same investigators showed that isoflurane-induced inhibition of M3 receptor signaling is mediated by an increase in protein kinase C activity, but the site of action on the M3 receptor has not yet been localized (Do et al., 2001). Another study found that intracerebroventricular administration of the acetylcholinesterase inhibitor neostigmine or the muscarinic agonist oxotremorine to isoflurane anesthetized rats increases spontaneous limb and orofacial exploratory movements, indicating increased arousal (Hudetz et al., 2003). Cholinergic activation during isoflurane anesthesia also activates the cortex, as indicated by an increase in cross-approximate entropy of the bihemispheric frontal EEG (Hudetz et al., 2003). These data are consistent with the interpretation that activation of central cholinergic neurotransmission can reverse some aspects of isoflurane anesthesia.

Studies of intact brain using in vivo microdialysis report that isoflurane causes a dose dependent decrease in ACh release in rat cerebral cortex (Dong et al., 2006, Shichino et al., 1997), rat striatum (Shichino et al., 1997), and cat pontine reticular formation (Keifer et al., 1996). The effect of isoflurane on ACh release varies with age. Isoflurane causes a significantly larger decrease in prefrontal cortex ACh release in old versus young rats (Jansson et al., 2004). Future studies are needed to determine whether

aged rats show performance or memory deficits following isoflurane anesthesia. Such a finding would support the interpretation that isoflurane-induced decreases in prefrontal cortex ACh release may contribute to increased post-operative delirium in the elderly.

3.5 Adenosine: Peroperative adenosine infusion in humans undergoing breast surgery decreases isoflurane requirement and decreases postoperative analgesic requirement (Segerdahl et al., 1995). A similar study of patients undergoing shoulder surgery showed that adenosine reduces the requirement for isoflurane but has no effect on postoperative analgesic requirement (Segerdahl et al., 1996). Although adenosine is well recognized to have antinociceptive effects (Boison, 2008), few studies have examined the possible role of adenosine in mediating the anesthetic effects of isoflurane. In rat, isoflurane-induced reductions in focal cerebral ischemia are blocked by an adenosine A_1 receptor antagonist, indicating that this neuroprotective effect of isoflurane may be mediated by adenosine A_1 receptors (Liu et al., 2006). Isoflurane-induced activation of adenosine A_1 receptors in primary cultures of rat hippocampal neurons also suppresses spontaneous calcium oscillations (Tas et al., 2003). This study suggests that another mechanism by which isoflurane may be neuroprotective is by increasing adenosine levels (Tas et al., 2003).

4. Sevoflurane

Sevoflurane is a nonflammable halogenated ether. Sevoflurane is the newest inhalation anesthetic to be used in humans, and was introduced into clinical practice in the late 1980s . The effects of sevoflurane on sleep-related neurotransmitters and neuromodulators are discussed below.

4.1 Monoamines: Few studies have investigated the role of monoaminergic neurotransmission in sevoflurane anesthesia. Sevoflurane produces a higher incidence of agitation during emergence from anesthesia in children than other general anesthetics (Bortone et al., 2006, Vlajkovic and Sindjelic, 2007). Alpha2 adrenergic agonists, such as dexmedetomidine, decrease the frequency of emergence agitation by sevoflurane (Vlajkovic and Sindjelic, 2007), and α2 agonists inhibit the firing of noradrenergic locus coeruleus neurons (Aghajanian and VanderMaelen, 1982). Noradrenergic neurons in the locus coeruleus promote wakefulness (reviewed in Ref. (Steriade and McCarley, 2005)), and sevoflurane directly excites noradrenergic locus coeruleus neurons in rat (Yasui et al., 2007). Sevoflurane increases norepinephrine release in rat preoptic area (Anzawa et al., 2001). Taken together, these data support the interpretation that noradrenergic neurotransmission contributes to emergence agitation produced by sevoflurane. The monoamines dopamine and histamine also promote wakefulness (reviewed in Ref. (Steriade and McCarley, 2005)), and sevoflurane increases cortical dopamine release in rat brain slices by modulating dopamine transporters (Silva et al., 2007). Sevoflurane also increases hypothalamic histamine levels in rat by inhibiting histamine metabolism (Hashimoto et al., 1998).

4.2 GABA: Sevoflurane, similar to many anesthetic molecules, enhances transmission at $GABA_A$ receptors. Using recombinant α1, β2, γ2 $GABA_A$ receptors, isoflurane has been shown to increase the affinity of GABA and cause an open-channel block at the $GABA_A$ receptor (Hapfelmeier et al., 2001b). These data suggest sevoflurane increases GABAergic transmission by binding to at least two different sites on $GABA_A$ receptors (Hapfelmeier et al., 2001b). Sevoflurane also promotes GABA

evoked $GABA_A$ receptor chloride currents in isolated rat hippocampal neurons and modulates the GABA response by altering activation and decay phases of the current (Kira et al., 1998). The modulation of hippocampal GABA receptors by sevoflurane is dependent on norepinephrine signaling, as coadministration of sevoflurane and norepinephrine have a large additive effect on inhibitory post synaptic currents and prolong the decay of the current (Nishikawa et al., 2005). These results suggest that sevoflurane anesthesia is mediated, in part, by enhanced GABAergic neurotransmission. This interpretation awaits confirmation from in vivo studies.

4.3 Glutamate: General anesthetics are thought to produce their effects by the dual actions of increasing inhibitory GABAergic neurotransmission and inhibiting excitatory glutamatergic neurotransmission. Sevoflurane has been shown to decrease calcium dependent glutamate release using synaptosomes isolated from human cerebral cortex (Moe et al., 2002). Another study using rat cortical neurons also demonstrated that sevoflurane decreases glutamate release (Vinje et al., 2002). These data encourage additional experiments designed to determine whether the sevoflurane-induced decrease in glutamate release contributes to the anesthetic effects of sevoflurane in vivo.

4.4 ACh: Cholinergic neurotransmission is important for the regulation of sleep and waking consciousness, as well as anesthesia (Lydic and Baghdoyan, 1998, Lydic and Baghdoyan, 2005a). Nicotinic receptors are inhibited by several anesthetics (Flood et al., 1997, Violet et al., 1997), including sevoflurane (Scheller et al., 1997). In humans, thalamocortical connectivity is suppressed when anesthetics induce loss of consciousness (White and Alkire, 2003). Nicotinic receptors are densely expressed throughout the human thalamus (Gallezot et al., 2005), and microinjection of nicotine into rat central

medial thalamus reverses the sevoflurane induced loss of righting response (Alkire et al., 2007). These data suggest that suppression of midline thalamic cholinergic neurons contribute to sevoflurane induced unconsciousness.

4.5 Adenosine: The PubMed review revealed no studies examining the role of adenosinergic neurotransmission in sevoflurane anesthesia.

III. CONCLUSIONS

A recurring theme that emerges from any consideration of anesthetic alterations in brain neurochemistry is that the direction and magnitude of chemical change varies as a function of brain region and anesthetic agent. There is no evidence that any single mechanism or brain region regulates the loss of waking consciousness during sleep or anesthesia. Even within seemingly homogenous states of consciousness, the brain reveals widely disparate levels of activation (reviewed in Ref. (Mashour, 2006b, Nofzinger, 2006, Tononi, 2005)). Such data make clear that understanding anesthetic alterations in consciousness will be limited unless the cellular and molecular mechanisms are elucidated on a brain region-by-region basis. Progress can be made if future studies include local delivery of anesthetic molecules along with measurement of endogenous neurotransmitters in specific brain regions. Another limitation concerns the lack of rigor regarding how anesthetic states of consciousness are classified. The terms loss of righting reflex, sleep, sedation, hypnosis, and sleep-time are often used casually to reach desired conclusions regarding anesthesia-induced alterations in consciousness. This lack of terminological rigor is particularly problematic for speculative extrapolations seeking to establish a link between pre-clinical studies and clinical implications. A formal and

consistent classification of states based on physiological and behavioral traits (Hobson, 1978, Plum and Posner, 1980) is essential and clinically relevant (McGuire et al., 2000). Drugs which selectively suspend consciousness are a logical path to understanding the neurological substrate of consciousness (Paton, 1984) and anesthesiology has unique potential to contribute to the clinical neuroscience of consciousness studies (Mashour, 2004, Mashour et al., 2005, Mashour, 2006b). Finally, judgments concerning the status of consciousness studies for anesthesiology should be tempered by expectations that incorporate an evolutionary perspective. Neurochemical networks that evolved to generate the loss of waking consciousness during sleep are the most logical substrates through which anesthetic molecules eliminate waking consciousness. Continuing efforts by anesthesiologists to understand consciousness will be promoted by research paradigms that incorporate the specific brain regions and neurochemical modulators of sleep.

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