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REGULATION OF CORTICAL ACETYLCHOLINE RELEASE IN THE RAT AS STUDIED BY IN VIVO MICRODIALYSIS

by

Leticia M. Materi

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 2000

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Regulation of Cortical Acetylcholine Release in the Rat as Studied by *in vivo* Microdialysis"

by Leticia M. Materi

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: November 20, 2000

External Examiner

Research Supervisor

Examining Committee

DALHOUSIE UNIVERSITY

DATE: November 20, 2000

AUTHOR: LETICIA M. MATERI

TITLE: REGULATION OF CORTICAL ACETYLCHOLINE RELEASE IN THE RAT AS STUDIED BY IN VIVO MICRODIALYSIS

DEGREE: Ph.D. **CONVOCATION:** **YEAR:**

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This thesis is dedicated to my parents, Tony and Cheryl Materi. I am eternally grateful for their unwavering and unconditional love, support, and encouragement. I love you very much, mom and dad.

TABLE OF CONTENTS

INDEX OF FIGURES AND TABLES

ABSTRACT

Cortical acetylcholine (ACh) has been implicated in diverse cognitive processes such as learning, memory, and attention. The release of ACh in the cortex is greatest during periods of high-frequency, low-voltage electroencephalographic (EEG) activity which occurs naturally during wakefulness and rapid-eye-movement sleep. Anatomical studies have demonstrated that cortical ACh in the rat is primarily released from the axon terminals of cortically projecting cholinergic neurons located in the nucleus basalis magnocellularis of the basal forebrain. Thus, factors that modulate the activity of the basal forebrain cholinergic neurons or act presynaptically at intracortical cholinergic terminals may alter cortical ACh release and influence cortical EEG arousal.

One factor that may act to regulate cortical ACh efflux and promote sleep is the purine nucleoside adenosine. The effects of intracortical administration of adenosine and selective adenosine receptor agonists and antagonists on cortical ACh release evoked by electrical stimulation of the pedunculopontine tegmental nucleus was tested using *in vivo* microdialysis in urethane anesthetized Wistar rats. The results demonstrated that cortical ACh release was inhibited by activation of intracortical A_1 adenosine receptors but unaffected by infusion of an A_{2A} adenosine receptor agonist.

A second factor that may regulate cortical ACh outflow is the excitatory amino acid glutamate. The cortex receives dense glutamatergic input from a number of subcortical structures and there is increasing evidence for extrasynaptic spillover of glutamate. Thus, glutamate may act at cholinergic terminals to regulate the release of ACh in the cortex. Using *in vivo* microdialysis, the effects of glutamate and selective ionotropic glutamate receptor agonists and antagonists were examined. It was determined that glutamate regulates cortical ACh outflow via an indirect pathway involving GABAergic neurons.

To determine if a similar circuit existed within the basal forebrain to regulate cortical ACh release and EEG activity, selective ionotropic glutamate receptor agonists were applied to the basal forebrain of urethane anesthetized rats. Activation of these receptors elicited a significant increase in cortical ACh outflow but had only minor effects on cortical EEG activity. Specifically, ionotropic glutamate receptor agonists evoked modest increases in the relative power of high-frequency EEG activity with no change to low-frequency activity.

Together, these data suggest that changes in cortical ACh outflow may be regulated at the level of the axon terminal by adenosine and, indirectly, glutamate. Activation of basal forebrain ionotropic glutamate receptors also influences cortical ACh release as well as EEG activity. Such regulation of cortical ACh release and EEG arousal may contribute to behavioural state regulation, synaptic plasticity, and attentional processes.

LIST OF ABBREVIATIONS

ACh	acetylcholine
aCSF	artificial cerebral spinal fluid
ADP	adenosine dephosphate
AMG	amygdala
AMP	5´-adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPS	adenylosuccinate
ATP	adenosine triphosphate
AV	anteroventral thalamic nucleus
°C	degrees Celsius
cAMP	cyclic 3´,5´-adenosine monophosphate
CGS 21680	2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine hydrochloride
ChAT	choline acetyltransferase
CN	cranial nerve nuclei
CNG	cingulate cortex
CPA	N^6-cyclopentyladenosine
CPP	(±)-3-(2-carboxypiperazin-4yl)-propyl-1-phosphonic acid
8-CPT	8-cyclopentyltheophylline
DB	diagonal band nuclei
DCN	deep cerebellar nuclei

DMSO	dimethylsulfoxide
DNQX	6,7-dinitroquinoxaline-2,3-dione
DPCPX	8-cyclopentyl-1, 3-dipropylxanthine
DR	dorsal raphe nucleus
E1	first evoked acetylcholine sample
E2	second evoked acetylcholine sample
EEG	electroencephalogram
FC	frontal cortex
g	gram
GABA	γ-aminobutyric acid
GTP	guanosine triphosphate
HIP	hippocampus
HPLC	high-performance liquid chromatography
Hz	hertz
IMP	inosine monophosphate
IP_3	inositol 1, 4, 5-trisphosphate
L	lateral dorsal and lateral posterior thalamic nuclei
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
LGN	lateral geniculate nucleus
LH	lateral hypothalamic area
L-*trans*-2,4-PDC	L-*trans*-pyrrolidine-2,4-dicarboxylic acid
MD	mediodorsal thalamic nucleus

MGN	medial geniculate nucleus
min	minute
ml	milliliter
µl	microliter
mM	millimolar
µM	micromolar
MPA	medial preoptic area
MPFCTX	medial prefrontal cortex
MRF	medullary reticular formation
MS	medial septal nucleus
muscimol	3-hydroxy-5-aminomethylisoxazole hydrobromide
mV	millivolt
NADPH	nicotinamide adenine dinucleotide phosphate
NBM	nucleus basalis magnocellularis
NBTI	S-(4-nitrobenzyl)-6-thioinosine
NMDA	N-methyl-D-aspartic acid
OB	olfactory bulb
PACPX	1, 3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine
PB	phosphate buffer
PBS	phosphate buffered saline
PC	parietal cortex
PFA	paraformaldehyde
PIR	piriform cortex

PLSD	protected least significant difference test
pmol	picomole
PPT	pedunculopontine tegmental nucleus
PRF	pontine reticular formation
PT	pretectal area
RT	reticular thalamic nucleus
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SC	superior colliculus
sec	second
S.E.M.	standard error of the mean
SI	substantia innominata
SN	substantia nigra
SSC	sodium citrate buffer
ST	subthalamus
TC	temporal cortex
V	ventrobasal complex
VIS	visual cortex
vs	versus
VST	vestibular nuclei

LIST OF PUBLICATIONS

Published papers:

Materi, L. M., Rasmusson, D. D., and Semba, K. (2000) Inhibition of synaptically evoked cortical acetylcholine release by adenosine: An *in vivo* microdialysis study in the rat. *Neurosci.*, **97**, 219-226.

Papers submitted for publication:

Materi, L. M. and Semba, K. (submitted) Inhibition of synaptically evoked cortical acetylcholine release by intracortical glutamate: Involvement of GABAergic neurons. *Eur. J. Neurosci.*

Abstracts:

Materi, L. M. and Semba, K. (1999) Presynaptic inhibition of synaptically evoked cortical acetylcholine release by glutamate as determined by in vivo microdialysis in urethane anesthetized rats. *Soc. Neurosci. Abstr.*, **25**, 452.

Materi, L. M., Rasmusson, D. D., and Semba, K. (1997) Inhibition of synaptically evoked acetylcholine release by adenosine: An in vivo microdialysis study. *Purines and their Receptors: Ann. Neuropharmacol. Conference Abstr.*, **5**, 18.

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Materi, L. M., Kirk, I. J., Oddie, S. D., Sainsbury, R. S., and Bland, B. H. (1995). The influence of the thalamic nucleus reuniens on hippocampal theta and sharp-wave field activity in urethane anesthetized rats. *Soc. Neurosci. Abstr.*, **21**, 1205.

ACKNOWLEDGEMENTS

This work is the culmination of many years of effort and I am greatly indebted to numerous people for helping me bring this project to completion. Firstly, I would like to thank my supervisor and mentor, Kazue Semba, for her gentle guidance, helpful advice, patience, and encouragement. I am truly grateful, Kazue, for the opportunity to work with you and I thank you for the abundant support and help you gave me during my time at Dalhousie.

I also wish to thank my other mentor, Doug Rasmusson, for his generous help and invaluable critiques of my work. Thank you, Doug, for challenging my ideas and keeping the red ink flowing! I am genuinely grateful for your enthusiasm, support, and even your sense of humour.

Adenosine analysis was performed in the laboratory of Thomas White and I am very appreciative of his generosity and the helpful discussions concerning my work.

In addition to the excellent supervision I received at Dalhousie, I was also fortunate enough to meet many people who showed me endless support and constant encouragement. I would like to express my boundless gratitude to my colleagues, peers, and Friends: Niki Boyd, Susan Dick, Xin Lu, Monika Fejtek, Jessica Wyles, and Bill Fortin. Many thanks, Niki, for your Friendship and for not letting me walk home alone at night. Thank you for teaching me about grace, determination, and professionalism, Sue. Thank you, Xin, for keeping me focused on what is important and for sharing your wisdom. Most heartfelt thanks, Monika, for your encouragement and for reminding me to dance. Many thanks, Jessica, for your kindness and generosity. Bill, I am

unfortunately limited by language and wish there was a word big enough to express all my thanks. I am eternally grateful for all of the help you have given me, your pep talks and encouragement, your confidence in my abilities even when I doubted myself, your sense of humour, and, especially, your Friendship. There are many others (Raja Abdel-Majid, Janet Hankins, Adam Baker, Jena Pitman - to name just a few!) who helped and encouraged me in many ways and to each of you: Thank you, thank you, thank you!

Working in a lab necessarily implies working with a team and I would like to express my most sincere thanks to those who helped me in ways too numerable to list and made work just a little too fun. Many thanks to Joan Burns who kept the lab running smoothly, shared her jokes, and never let me run out of rats. Thank you to Julie Jordan who taught me how to rebuild an HPLC using nothing more than a few straws and some duct tape. Sincere thanks to Jessica Pastorius who helped with my histology and never complained (at least not out loud). The in situ hybridization studies were performed in the Vision 2000 laboratory and I am grateful to the students, post-docs (especially George Robertson), and staff there who assisted me on this project.

Moral support was supplied (in limitless quantities) by my sisters, Sandy Kon and Crystal Preston. I am infinitely grateful for your love and encouragement.

Financial support was provided by the Medical Research Council of Canada, who provided funding for equipment, and the Natural Sciences and Engineering Research Council and the Department of Anatomy and Neurobiology, who supported me while I pursued my research at Dalhousie University.

Pharmacological support was provided by the staff at Tim Hortons. I hope that one day you find my work with caffeine to be as helpful to you as I found your work with caffeine to be helpful to me.

CHAPTER I. INTRODUCTION

The physiological functions of acetylcholine (ACh) have been known for nearly a century. The classic study of Loewi on the release of Vagusstoff from perfused frog heart following vagal stimulation, the subsequent identification of this substance as ACh by Loewi and Navratil (cited in Dale, 1935), and the demonstration that ACh is released at the neuromuscular junction (Dale et al., 1936) established this substance as a neurotransmitter capable of influencing physiological processes.

ACh in the central nervous system has been implicated in cortical activation. The level of cortical activation is often defined according to electroencephalographic (EEG) patterns that are thought to reflect the activity levels of cortical neurons. These EEG patterns fluctuate from high-frequency, low-voltage activity, which is observed during wakefulness and rapid-eye-movement (REM) sleep, to large-amplitude, slow, synchronized activity, which occurs during slow-wave sleep. It has been reported that high-frequency, synchronized EEG activity known as gamma rhythm can be recorded from several cortical areas during wakefulness (Jefferys et al., 1996). However, for the purposes of the present discussion, the phrase 'synchronized activity' will be used to refer to the low-frequency EEG activity observed during slow-wave sleep. Thus the cortex can be considered to be in an activated, or aroused, state when the EEG is characterized by high-frequency, low-voltage activity.

The role of cortical activation in cognitive processes and how this activation is generated and maintained are the subject of much discussion. Embedded in these debates remain many unanswered questions: How is cortical activation modified? Why does the cortex cycle through periods of quiescence and arousal? What physiological mechanisms control this event? Since ACh may influence cortical EEG activation, the

regulation of the release of this neurotransmitter may influence both cognition and behaviour.

1. THE CHOLINERGIC SYSTEM

Original theories concerning cortical arousal postulated that sensory input was the main contributing factor to cortical EEG activation. Thus, when the cerebrum was separated from the spinal cord and brainstem, through which sensory input travels, the result was cortical EEG synchronization and sleep-like behaviour (Kleitman and Camille, 1932). However, this hypothesis was rejected as a result of the work of Moruzzi and Magoun (1949). These researchers demonstrated that electrical stimulation of the brainstem reticular formation, not sensory pathways, elicited cortical desynchronization in an otherwise sleeping preparation. Lesions of the reticular formation in cats resulted in decreased spontaneous behavioural activity and an EEG characterized by large, slow waves (Lindsley et al., 1950). Further examination of cortical EEG activation evoked by reticular formation stimulation revealed the involvement of both thalamic and extrathalamic relay areas (Starzl et al., 1951). Activation of the reticular formation by electrical stimulation resulted in increased neuronal activity in various subcortical structures including the ventromedial thalamus and, more rostrally, the anterior limb of the internal capsule and globus pallidus (Starzl et al., 1951). However, at that time, no specific neurochemical substrate was linked to cortical EEG activation induced by activation of the reticular formation. Subsequent anatomical studies by Shute and Lewis (1963, 1967), as discussed below, suggested that ACh containing neurons and fibers were ideally situated to facilitate cortical arousal.

1.1. Early anatomical studies

Cholinergic neurons and fibers within the brain were originally localized using a histochemical technique to visualize acetylcholinesterase (AChE), the enzyme that breaks down ACh. Using this method, Shute and Lewis (1963, 1967) identified two presumably cholinergic fiber tracts that originated from the vicinity of the reticular formation of the mesopontine tegmentum. The first tract projected dorsally to the tectum, pretectal area, and thalamus and was termed the dorsal tegmental pathway. The second AChE-containing fiber tract, termed the ventral tegmental pathway, was found to project to the subthalamus, hypothalamus, globus pallidus, and lateral preoptic area. Since the termination of these fibers corresponded to the thalamic and extrathalamic relay regions of the ascending reticular activating system described by Starzl et al. (1951), Shute and Lewis (1967) speculated that AChE-expressing fibers formed the "anatomical basis of the ascending reticular activating system which is responsible for electrocortical arousal". In addition to the ascending AChE fibers from the brainstem, Shute and Lewis (1967) demonstrated the presence of AChE-containing fibers that projected to the neocortex from neurons in the globus pallidus and lateral preoptic area. These authors speculated that this represented a more rostral extension of the cholinergic reticular activating system.

1.2. Early physiological studies

The hypothesis that ACh regulates arousal was further substantiated by physiological studies that examined cortical neurotransmitter efflux during reticular

formation stimulation. Kanai and Szerb (1965) demonstrated that cortical EEG activation observed during electrical stimulation of the mesencephalic reticular formation was correlated with increased cortical ACh release in the cat. When the postsynaptic effects of ACh were blocked by atropine, reticular formation stimulation no longer elicited cortical EEG arousal despite increased cortical ACh efflux (Kanai and Szerb, 1965). By blocking ACh hydrolysis locally, Celesia and Jasper (1966) induced cortical EEG activation that could be antagonized by intravenous atropine. These data suggest that ACh may regulate experimentally induced cortical EEG activation. Wakefulness and rapid-eye-movement sleep are natural states that are characterized by cortical EEG arousal. Jasper and Tessier (1971) demonstrated, in freely moving cats, that these two states are also characterized by high levels of cortical ACh compared to periods of slow-wave sleep.

These initial studies helped establish the hypothesis that central cholinergic systems contribute to the generation of low-voltage, high-frequency cortical EEG activity. However, it has been suggested that other neurotransmitter systems are also capable of regulating cortical arousal and that this activity does not necessarily reflect ACh release. By activating various subcortical structures using electrical stimulation, Szerb (1967) demonstrated several instances of discrepancy between ACh release and cortical activation. Specifically, stimulation of the septum greatly increased cortical ACh efflux but had little effect on the EEG recorded from the cortex. In addition to this effect, low-frequency (30 Hz) stimulation of the reticular formation increased cortical ACh efflux without inducing cortical activation. This suggests that cortical EEG activation does not necessarily reflect increased cortical ACh release. To gain a better

understanding of the effects of ACh on cortical EEG activity, the response of individual cortical neurons to ACh has been studied.

1.3. Single unit studies

Initial studies examining the effects of ACh on the activity of individual cortical neurons noted that ACh application often elicited an excitatory response (Krnjevic and Phillis, 1963; Spehlmann, 1963). ACh has also been shown to hyperpolarize a subpopulation of cortical neurons but it was subsequently determined that this effect was mediated by γ-aminobutyric acid (GABA; McCormick and Prince, 1986). ACh-induced excitation of cortical neurons is due to tonic depolarization of the cell accompanied by an increase in membrane resistance (Krnjevic et al., 1971) due to decreased potassium conductance (for a review, see McCormick, 1990). The effects of ACh on neuronal firing stand in contrast to those elicited by the excitatory amino acid glutamate. Spehlmann et al. (1971) demonstrated in cats that glutamate enhanced the firing rate of virtually every neuron tested while exogenously applied ACh increased the spontaneous firing rate of only a subset of cortical neurons. In addition to this, it was noted that the effect of ACh was not observed until 10 to 30 seconds after the onset of ACh application. In comparison, glutamate altered neuronal responses within one second of application (Spehlmann et al., 1971). The enhanced firing rate induced by ACh returned to baseline values within 10 to 60 seconds after ACh application ended (Spehlmann et al., 1971).

These data suggest that ACh can regulate the excitability of individual neurons over a prolonged period of time. The decreased potassium conductance

induced by ACh would depolarize postsynaptic neurons and, possibly, increase the probability of action potential generation in response to additional inputs, including those transmitting sensory information.

1.4. Sensory information processing

In general, ACh increases the response of cortical neurons to specific sensory input without affecting spontaneous activity. Sillito and Kemp (1983) examined the effect of exogenously applied ACh on the receptive field properties of neurons in the cat visual cortex. These authors demonstrated that iontophoretically applied ACh increased the magnitude of response of visual cortex neurons to optimal stimuli with only minor changes to spontaneous activity or to responses to non-optimal stimuli. This suggests that the enhanced response was not due to an overall increase in the excitability of the neuron. Sillito and Kemp (1983) also noted that the maximum effect observed occurred up to three minutes following ACh application and recovery generally took five minutes or longer. This ACh-induced facilitation in neuronal response to sensory stimuli has also been shown in the somatosensory cortex of cats (Metherate et al., 1987, 1988) and rats (Donoghue and Carroll, 1987). Within the auditory cortex, however, the role of ACh in sensory information processing is less clear. Metherate and Weinberger (1990) demonstrated a decrease in neuronal response in auditory cortex when the best frequency tone was paired with ACh administration, and an enhanced response to surrounding frequencies. The reason for this discrepancy is not immediately clear but may indicate that ACh affects different populations of cells within each cortical region.

The long lasting changes in neuronal response produced by ACh application also occur when this neurotransmitter is paired with glutamate (Metherate et al., 1987). Specifically, the firing rate of neurons exposed to exogenous glutamate is enhanced when ACh is concurrently administered (Metherate et al., 1987). When ACh is delivered alone prior to glutamate application, neuronal responses to subsequent glutamate application are unchanged (Metherate et al., 1987).

1.5. ACh mediated plasticity

These ACh induced changes in the responsiveness of cortical neurons to sensory inputs have been suggested to underlie the induction of neuronal plasticity. Plasticity is defined here as long-lasting, relatively permanent changes in the excitability of neurons to a particular stimulus. Recently, it has been shown that pairing electrical stimulation of the basal forebrain, which elicits cortical ACh release (Rasmusson et al., 1992), with an auditory stimulus results in a reorganization of the receptive fields in the rat auditory cortex (Kilgard and Merzenich, 1998). Specifically, a greater proportion of neurons in the primary auditory cortex responded to a particular tone when that tone had been repeatedly paired with basal forebrain stimulation compared to naïve animals. Reductions in cortical ACh levels reduce or prevent neuronal plasticity. Using a whisker-pairing paradigm, Baskerville et al. (1997) and Sachdev et al. (1998) have shown that depletion of cortical ACh by immunotoxic lesion of cholinergic basal forebrain neurons prevented cortical reorganization in rats. When all whiskers except D_2 and D_3 were trimmed daily, the response property of cortical barrel neurons corresponding to the D_2 whisker changed

considerably (Baskerville et al., 1997). Specifically, the mean firing rate of neurons within the D_2 barrel increased substantially in response to deflection of the D_2 whisker compared to controls. In addition to this effect, neurons within the D_2 barrel showed increased activity in response to deflection of the D_3 whisker. These changes in the response attributes of D_2 barrel neurons were reduced or prevented by selective lesioning of cortically projecting cholinergic neurons (Baskerville et al., 1997). This suggests that ACh facilitates synaptic plasticity by producing a state in which the response characteristics of neurons are more likely to be altered by additional inputs (for a review, see Dykes, 1997).

Together, these data suggest that mechanisms that regulate cortical ACh release could have a profound effect on cognition, attention, and memory. Alterations in ACh efflux could produce global changes in the level of cortical activation as well as more regionally selective changes in synaptic connectivity. The following discussion focuses on what is currently known about the neuropharmacology of ACh and the anatomical distribution of cholinergic systems within the brain.

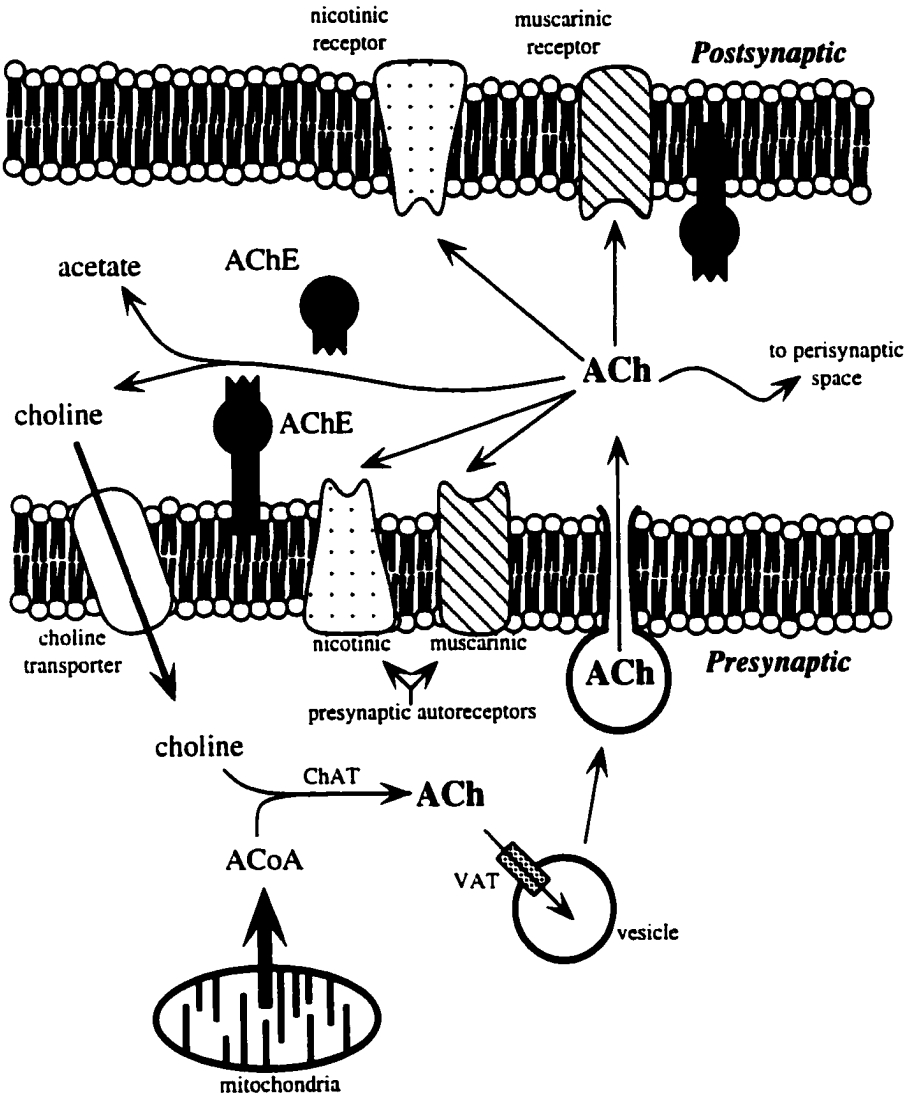
1.6. ACh metabolism

ACh is synthesized within the axon terminals of cholinergic neurons when the enzyme choline acetyltransferase (ChAT) catalyzes the transfer of the acetyl group from acetyl-coenzyme A to choline (Fig.1; for a review, see Cooper et al., 1996). Acetyl-coenzyme A is derived from pyruvate generated by glucose metabolism within mitochondria. In order to participate in ACh synthesis, acetyl-coenzyme A must be transported across the mitochondrial membrane to the cytosol. In contrast, choline is

actively transported into the neuron from the extracellular environment by high-affinity, sodium-dependent choline transporters. Once ACh is produced, it is actively transported into vesicles by vesicular ACh transporters. After ACh is released into the synaptic cleft, the primary mode of inactivation is by enzymatic degradation to acetate and choline. This process is mediated by the enzyme AChE.

Figure 1. A schematic representation of the release and metabolism of the neurotransmitter ACh. Synaptically released ACh may bind to pre- and postsynaptic membrane bound receptors to regulate neurotransmitter release and neuronal activity, respectively. See text for details.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ACoA, acetyl coenzyme A; ChAT, choline acetyltransferase; VAT, vesicular acetylcholine transporter.


nicotinic
receptor
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Postsynaptic
acetate
AChE
ACh
to perisynaptic
space
choline
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choline
transporter
nicotinic
muscarinic
ACh
Presynaptic
presynaptic autoreceptors
choline
ChAT
ACh
ACoA
VAT
vesicle
mitochondria

Figure 1

1.7. ACh receptors

ACh influences neuronal activity by acting at membrane bound receptors (for a review, see Taylor and Brown, 1999). Two classes of acetylcholine receptors have been identified: ionotropic nicotinic receptors and metabotropic muscarinic receptors. Nicotinic receptors have been divided into three subtypes based on anatomical distribution, pharmacology, and subunit composition. One type of nicotinic receptor is the muscle type found on muscle tissue and is formed by a combination of two $\alpha 1$ subunits and one each of the $\beta 1$, γ, and δ subunits. The remaining two types of nicotinic receptors, the neuronal types, are found within the central nervous system and are differentiated based on their sensitivity to α-bungarotoxin. The neuronal nicotinic receptors are formed by various combinations of five of the following subunits: $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\alpha 9$, $\beta 2$, $\beta 3$, and $\beta 4$. In addition to nicotinic receptors, ACh also binds to muscarinic receptors. These receptors contain seven transmembrane-spanning domains and relay signals by regulating second messenger pathways. To date, five subtypes of muscarinic receptors have been identified (M_1 - M_5). The M_1, M_3, and M_5 muscarinic receptors modulate the activity of the second messengers inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol through GTP-binding proteins. IP_3 is known to increase the concentration of intracellular calcium while diacylglycerol increases the activity of protein kinase C. The M_2 and M_4 receptors also act via GTP-binding proteins but these receptors regulate inwardly rectifying potassium channels and inhibit adenylyl cyclase thereby decreasing the amount of cAMP formed. This latter effect will reduce protein kinase A activation.

1.8. Anatomical organization of the cholinergic system

As described above, the initial anatomical studies of the cholinergic system by Shute and Lewis used histochemical techniques to identify AChE expressing neurons and fibers within the brain. However, it has been shown that several non-cholinergic neurons express AChE (Satoh et al., 1983). Unlike AChE, the enzyme ChAT is thought to be a definitive anatomical marker for cholinergic neurons. Neurons expressing ChAT have been shown to be present in the olfactory tubercle, striatum, basal forebrain, habenula, mesopontine tegmentum, and in motor nuclei of the cranial nerves (Satoh et al., 1983; Vincent et al., 1986). Of these various cholinergic regions, only the mesopontine tegmentum and the basal forebrain have been implicated in cortical EEG desynchronization and behavioural state control.

1.8.1. Mesopontine tegmentum

Within the mesopontine tegmentum, a conspicuous group of ChAT-positive cells can be identified. These cholinergic cells were originally associated with a number of nuclei of the midbrain and upper pontine tegmentum including the substantia nigra (Gould and Butcher, 1986), cuneiform nucleus (Shute and Lewis, 1967), the pedunculopontine tegmental nucleus (PPT), and the laterodorsal tegmental nucleus (LDT; Vincent, Satoh, and Fibiger, 1986; Woolf, 1991; Wainer and Mesulam, 1990). Careful examination of tissue immunostained for ChAT has led to the conclusion that cholinergic cells of the mesopontine tegmentum are localized within the PPT and LDT (Woolf and Butcher, 1986; for a review, see Inglis and Winn, 1994).

The first description of the PPT was made by Jacobsohn in 1909 based on results from Nissl-stained human tissue (cited in Semba and Fibiger, 1989). It was described as consisting of a collection of large, darkly staining neurons that extends from the caudal pole of the red nucleus to the parabrachial nucleus in close association with the ascending limb of the superior cerebellar peduncle. The LDT was originally described in 1926 by Castaldi (cited in Semba and Fibiger, 1989). The LDT is situated lateral to the dorsal tegmental nucleus, and rostromedial to the locus coeruleus.

Within the boundaries of the PPT and LDT exist a heterogeneous population of cells. Besides the presence of large cholinergic cells, a subpopulation of smaller neurons has been described (Honda and Semba, 1995). Neurons expressing glutamate-like (Clements and Grant, 1990; Lavoie and Parent, 1994) and glutamic acid decarboxylase (GAD)-like (Jones, 1991) immunoreactivity have been described in the PPT and LDT. In both cholinergic and non-cholinergic neurons, a wide range of peptides such as substance P, corticotropin-releasing factor, and bombesin/gastrin-releasing peptide have also been localized (Vincent et al., 1986). Using a histochemical procedure based on reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity, Vincent et al. (1983a) demonstrated that in the mesopontine tegmentum, NADPH-diaphorase is present only in the cholinergic cells of the PPT and LDT. The afferent and efferent fibers of these nuclei have been examined previously. Since part of the work presented in this thesis used electrical stimulation of the PPT to elicit cortical ACh release (as outlined in the Materials and Methods section in chapters 2 and 3), the connectivity of this nucleus is summarized below.

Afferents to the PPT have been described as originating from such diverse neural regions as the frontal cortex, lateral hypothalamus, subthalamic nucleus, dorsal and median raphe, locus coeruleus and medullary reticular formation (Moon Edley and Graybiel, 1983; Woolf, 1991; Semba and Fibiger, 1992; Steininger et al., 1992). The dendrites of ChAT immunostained neurons of the PPT have been described as extending perpendicularly into several fiber pathways including the medial lemniscus, lateral lemniscus, superior cerebellar peduncle, dorsal tegmental bundle, central tegmental tract, and medial longitudinal fasciculus (Rye et al., 1987). This suggests that the PPT may receive additional input from sensory and motor fiber tracts that do not ordinarily terminate on the cell bodies contained within this nucleus.

The efferent projections of the PPT have also been described (see Fig. 2). By injecting retrograde tracers into various regions of the brainstem and processing the tissue for ChAT immunocytochemistry, Woolf and Butcher (1989) described the targets of descending cholinergic fibers originating from the PPT. These authors noted that fibers from cholinergic neurons in the PPT terminated in various cranial nerve nuclei, dorsal and median raphe nuclei, locus coeruleus, deep cerebellar nuclei, pontine nuclei, and regions of the medullary and pontine reticular formation (Woolf and Butcher, 1989). Iontophoretic injections of the anterograde tracer *Phaseolus vulgaris*-leukoagglutinin (PHA-L) into the PPT resulted in labelled ascending fibers and varicosities within the midline and intralaminar thalamic nuclei, central and medial nuclei of the amygdala, lateral hypothalamus, nucleus basalis of Meynert, and septum (Hallanger and Wainer, 1988). In order to ascertain whether these ascending projections were indeed cholinergic, a retrograde tracer was injected into regions of the

hypothalamus, septum, ventral pallidum, and amygdala, and the tissue was processed for ChAT immunoreactivity. The retrogradely traced PPT neurons innervating the nucleus basalis of Meynert or amygdala were predominantly non-cholinergic. However, over 20% of cells retrogradely labelled from the lateral hypothalamus and over 90% of cells retrogradely labelled from the septum were cholinergic neurons of the mesopontine tegmentum. The efferent fibers from the PPT project to their respective targets via a number of pathways. The dorsal medial projections travel through the dorsal tegmental bundle, the dorsal lateral projections travel through the lateral tegmental bundle, and fibers travelling in more ventral areas project to their targets via the medial forebrain bundle (Hallanger and Wainer, 1988). The medial prefrontal cortex is the only cortical region known to receive direct projections from the cholinergic mesopontine tegmentum (Vincent et al., 1983b).

Figure 2. Schematic illustrating the major ascending and descending cholinergic pathways originating from the pedunculopontine tegmental and laterodorsal tegmental nuclei. Abbreviations: AV, anteroventral thalamic nucleus; CN, cranial nerve nuclei; DB, diagonal band nuclei; DCN, deep cerebellar nuclei; DR, dorsal raphe nucleus; L, lateral dorsal and lateral posterior thalamic nuclei; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; LGN, lateral geniculate nucleus; LH, lateral hypothalamic area; MD, mediodorsal thalamic nucleus; MGN, medial geniculate nucleus; MPA, medial preoptic area; MPF CTX, medial prefrontal cortex; MRF, medullary reticular formation; MS, medial septal nucleus; PPT, pedunculopontine tegmental nucleus; PRF, pontine reticular formation; PT, pretectal area; RT, reticular thalamic nucleus ; SC, superior colliculus; SN, substantia nigra; ST, subthalamus; V, ventrobasal complex; VST, vestibular nuclei.

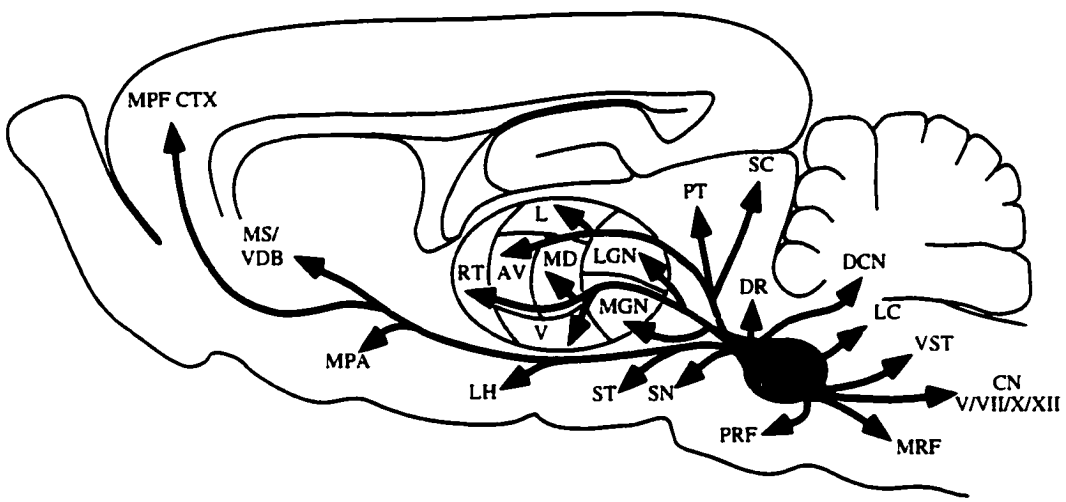

MPF CTX
MS/
VDB
MPA
LH
L
RT
AV
MD
LGN
MGN
V
ST
SN
PT
SC
DR
DCN
LC
VST
CN
V/VII/X/XII
PRF
MRF

Figure 2

1.8.2. Basal forebrain

The basal forebrain is a heterogeneous collection of cell groups situated within the ventral and medial aspects of the cerebral hemispheres. The nuclei associated with this region include the nucleus accumbens, olfactory tubercle, septum, diagonal band nuclei, ventral pallidum, bed nucleus of the stria terminalis, substantia innominata, magnocellular preoptic nucleus, nucleus basalis of Meynert, and regions of the amygdala and hypothalamus (Bigl et al., 1982; Heimer and Alheid, 1991). Butcher and Semba (1989) noted that the nomenclature associated with the basal forebrain nuclei has not been used consistently. For example, various names have been applied to the most caudal region of the basal forebrain including nucleus basalis of Meynert and nucleus basalis magnocellularis. In light of this, all discussions regarding the anatomy of the basal forebrain presented here will refer to the nuclei using the same nomenclature chosen by the authors of the original work.

Throughout various subregions of the basal forebrain exist large neurons that stain intensely for AChE and ChAT (Bigl et al., 1982). While these neurons have been described as forming a distinct nucleus (see Saper, 1984), they are usually considered to be distributed throughout several separate nuclei. Specifically, the cholinergic basal forebrain neurons are found within the medial septal nucleus, the vertical and horizontal limbs of the diagonal band, the magnocellular preoptic area, the substantia innominata, and the nucleus basalis of Meynert (Butcher and Semba, 1989). Both the afferent and efferent connectivities of these cholinergic cells are discussed below.

Detailed studies examining projections to the magnocellular basal forebrain have not only identified the origins of these afferent fibers but also their possible

neurotransmitter content. Semba et al. (1988) used retrograde tracing, immunocytochemistry, and extracellular recordings to determine which areas project to the cholinergic regions of the basal forebrain. The dorsal raphe nucleus was shown to send dense projections to the nucleus basalis magnocellularis, horizontal limb of the diagonal band, and the magnocellular preoptic area. However, few of the retrogradely labelled cells of the dorsal raphe were immunoreactive for serotonin. In contrast, Jones and Cuello (1989) demonstrated the presence of serotonin containing fibers within the vicinity of basal forebrain areas known to contain cholinergic neurons. Jones and Cuello (1989) also reported that the majority of dorsal raphe neurons retrogradely labelled from the basal forebrain expressed serotonin. Although few retrogradely labelled cells were present in the locus coeruleus, all of these were double labelled for tyrosine hydroxylase, indicating that they are noradrenergic (Semba et al., 1988). Many neurons in the ventral tegmental area and substantia nigra that projected to the basal forebrain also expressed tyrosine hydroxylase thus suggesting that they were dopaminergic (Semba et al., 1988; Jones and Cuello, 1989). Approximately 40% of retrogradely labelled cells in the LDT were immunoreactive for ChAT (Semba et al., 1988). Within the PPT, neurons double labelled for retrograde tracer and ChAT were observed in conjunction with numerous cells labelled only with retrograde tracer. This finding stands in agreement with the work of Woolf and Butcher (1986) who found that most of the ChAT-positive cells projecting to the magnocellular preoptic/ventral pallidal area from the mesopontine tegmentum were mainly confined to the LDT while only a few such cells were observed in the posterior portion of the PPT. However, both Jones and Cuello (1989) and Hallanger and Wainer (1988) found few retrogradely labelled

cholinergic neurons within the PPT and LDT when the tracer was injected into the nucleus basalis. This discrepancy may be due to placement of the retrograde tracer within the basal forebrain. Most of the sites of injection chosen by Jones and Cuello (1989) and Hallanger and Wainer (1988) were in basal forebrain regions more caudal and lateral to the sites used by Semba et al. (1988) and Woolf and Butcher (1986).

Most research examining the efferent projections of the basal forebrain has identified four major efferent cholinergic fiber systems (see Fig. 3; Wainer and Mesulam, 1990; Butcher and Semba, 1989). The first originates from the medial septal nucleus and the vertical limb of the diagonal band and projects to the hippocampal formation. The second arises from the horizontal limb of the diagonal band and projects primarily to the olfactory bulb and cortical areas associated with the limbic system. The third cholinergic fiber pathway arises from neurons located in the magnocellular preoptic area and substantia innominata and projects to the amygdala and limbic cortex. The final cholinergic pathway from the basal forebrain originates from the nucleus basalis of Meynert and projects to the amygdala and neocortex. Since the work presented in this thesis concentrates on factors that regulate cortical ACh release, this latter projection system will be discussed in further detail.

Figure 3. Schematic depicting the major cholinergic pathways originating from the basal forebrain. Abbreviations: AMG, amygdala; CNG, cingulate cortex; DB, diagonal band nuclei; FC, frontal cortex; HIP, hippocampus; MPA, medial preoptic area; MS, medial septal nucleus; NBM, nucleus basalis magnocellularis; OB, olfactory bulb; PC, parietal cortex; PIR, piriform cortex; RT, reticular thalamic nucleus; SI, substantia innominata; TC, temporal cortex; VIS, visual cortex.

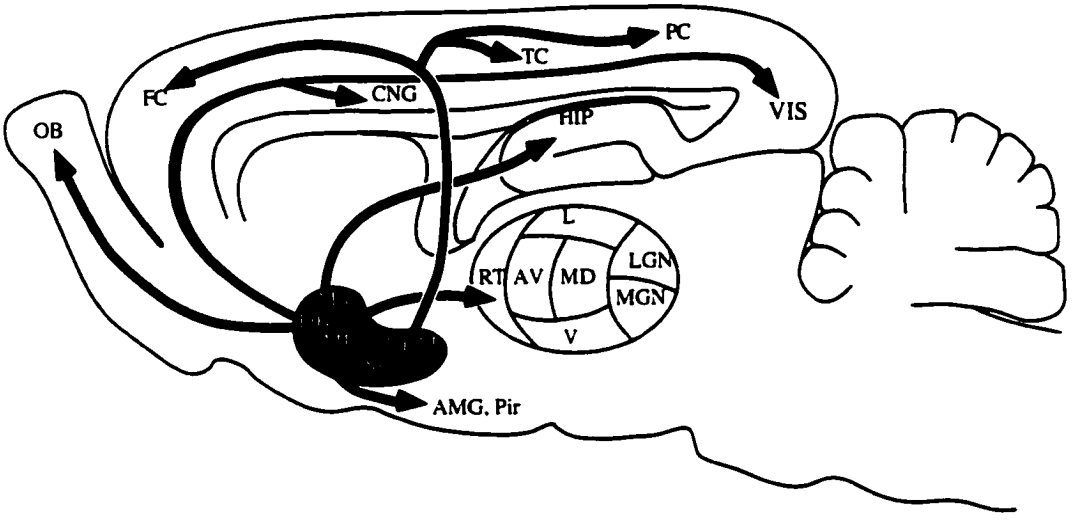


Figure 3

1.8.3. Cortically projecting cholinergic basal forebrain neurons

Through the use of anterograde autoradiographic transport, Saper (1984) demonstrated that fibers from the basal forebrain reach the cerebral cortex primarily via two pathways. The first pathway, described as the medial pathway, was characterized by axons innervating the medial aspect of the cortex that arose primarily from the medial septal and diagonal band nuclei and the medial portions of the substantia innominata and globus pallidus. These fibers travelled dorsally over the genu of the corpus callosum to proceed either rostrally into the medial frontal cortex or through the cingulate bundle to the cingulate cortex, visual cortex, subiculum, or CA fields of the hippocampus. The second cortically directed pathway originating from the cholinergic basal forebrain followed a more lateral route. While part of this path began in the medial septal, diagonal band, and the magnocellular preoptic nuclei, the majority of fibers in the lateral pathway were shown to originate from the lateral and caudal regions of the substantia innominata and globus pallidus. These fibers projected through the external capsule and terminated in the lateral aspect of the neocortex. These results also demonstrate the topographic nature of the cortically projecting basal forebrain neurons. Saper (1984) described a laminar specific distribution of the fiber terminals in the cerebral cortex that was relatively constant across cortical areas. The highest concentration of labelled axons was observed in cortical layer V and the most ventral area of layer VI, with more moderate projections to layers I and III. This stands in contrast to the results of Lysakowski et al. (1989). These authors examined the laminar distribution of ChAT immunoreactive fibers in regions of the rat cortex defined by cytoarchitecture and demonstrated that cortical regions with similar function displayed

similar patterns of innervation. With respect to laminar distribution of cortical cholinergic inputs, at least 13 general patterns were identified (Lysakowski et al., 1989).

Not only do the projections from the basal forebrain show a laminar pattern of distribution, but the termination of individual cells appears to be restricted to a very small portion of the cortex in rats. Bigl et al. (1982) performed double retrograde tracing combined with immunocytochemistry to examine the extent of collateralization of axons originating from cholinergic basal forebrain neurons in the cerebral cortex. The only evidence for collateralization was obtained from infusions of retrograde tracer to the visual and cingulate cortices. Of the total number of AChE-positive basal forebrain neurons that contained retrograde tracer from either the visual or cingulate cortex, only 3.2% were double labelled. These findings were later confirmed by Price and Stern (1983) who demonstrated that the terminal field of individual cells originating from the nucleus basalis-diagonal band complex has a diameter of no greater than 1-1.5 mm.

1.8.4. Cortically projecting non-cholinergic basal forebrain neurons

The basal forebrain does not consist of a homogenous population of cells. In addition to cholinergic neurons that project to the cortex, the basal forebrain also contains non-cholinergic, cortically projecting cells (Rye et al., 1984). Using double labelling with a retrograde tracer and immunocytochemistry, Gritti et al. (1997) have demonstrated that both glutamic acid decarboxylase (GAD) containing neurons, which are presumably GABAergic, and neurons of unknown neurotransmitter content within the basal forebrain send projections to the neocortex. Cortically projecting GABAergic

basal forebrain neurons have been shown to innervate cortical inhibitory interneurons (Freund and Meskenaite, 1992) and could, therefore, regulate neuronal activity in the cortex through disinhibitory processes.

The basal forebrain innervates a wide area of cortex and this suggests that it is well situated to regulate global cortical activity. However, the limited field of innervation by each cholinergic fiber implies that ACh efflux from a single neuron may also regulate cortical activity within discrete areas of cortex. Regulation of the release of ACh in the cortex would influence neuronal activity and ultimately behaviour. The following section examines the nature of neurotransmission and how it can be modified.

2. NEUROTRANSMITTER RELEASE

2.1. Historical overview

During the late nineteenth century, the nature of the connections between neurons was under intense investigation. At that time, two competing views were postulated (for a review, see Strata and Harvey, 1999). One hypothesis, proposed by Camillo Golgi, suggested that the neurons within the brain formed a continuous network, or syncytium, of fibers. This was known as the reticular theory. In contrast, the competing view, the neuron doctrine, suggested that neurons within the central nervous system were independent, discontinuous units. The work of Ramón y Cajal, using a modified version of Golgi's staining method to label individual cells, provided strong anatomical evidence for the latter theory, as did the embryological studies by His, and the work of Forel on nerve cell responses to injury (cited in Ramón y Cajal, 1908). During the 1950s, electron microscopy provided conclusive evidence that

neurons were indeed discrete elements (DeRoberts, 1958). While a few neurons do display cytoplasmic continuity due to the presence of gap junctions, the vast majority of neurons within the central nervous system are thought to be physically separate units.

The view of neurons as discrete individual elements implied that a mechanism for the transmission of information from one neuron to another must exist. Sherrington coined the word "synapse" (from the Greek, to clasp) to describe the specialized points of contact between neurons (for a review, see Eccles, 1982). Two competing theories regarding the mechanisms for interneuronal communication at synapses existed. The first theory postulated that communication between neurons was due to electrical signals. This view was derived, in part, from observations that neurons both generate and respond to electrical currents. The second theory suggested that transmission of information between neurons was a result of chemical signals. One of the first experiments to suggest that neurons responded to chemical stimulation was performed by Elliott (1904) who demonstrated that, even in the absence of nerves, application of exogenous adrenaline mimicked sympathetic nerve stimulation and produced contraction of smooth muscle. Following this, Dixon (cited in Dale, 1935) demonstrated that application of samples obtained from a heart preparation exposed to vagus nerve stimulation reduced the rate of contraction of a second heart. However, the experiment credited for yielding definitive proof that neurons interact via chemical neurotransmission was performed in 1921 by Otto Loewi (cited in Eccles, 1982). He demonstrated that stimulation of the vagus nerve innervating one heart would decrease the contraction rate of a second heart in a

connected perfusion chamber. Thus, secretion of a chemical from the first vagus nerve-heart tissue preparation diffused through the perfusion medium to affect the response of the second heart.

Once chemical neurotransmission was identified as the process governing communication between neurons, research began to focus on the mechanisms involved in neurotransmitter release. It was soon recognized that neurons could not only be described based on anatomical location but also on neurotransmitter content (Dale, 1933). Dale (1935) speculated that a chemically distinct neuron released the same chemical transmitter(s) at all of its axon terminals. The hypothesis postulated by Dale was later promoted by Eccles et al. (1954) who referred to it as Dale's principle.

2.2. Processes involved in neurotransmitter release

Since these early reports, the mechanisms involved in neurotransmission have been studied extensively. Using a tetrodotoxin exposed neuromuscular preparation obtained from frog, Katz and Miledi (1967) demonstrated that neurotransmitter release only occurred when calcium was present immediately before a depolarizing pulse was applied. If calcium was applied following the depolarizing pulse, no transmitter was released. It is now known that neurotransmitters are packaged within synaptic vesicles that fuse with the membrane of the synaptic terminal and allow the neurotransmitter to be released into the synaptic cleft. Within the membrane of these vesicles exist two types of functionally distinct proteins (Südhof and Jahn, 1991). The first class directs the movement of synaptic vesicles

within the terminal. The second class regulates the uptake of neurotransmitters from the cytosol.

The release of neurotransmitter is the end result of a series of intracellular events that occur within the synaptic terminal (Fig. 4; for a review, see Südhof, 1995). In the presynaptic terminal, vesicle membrane proteins known as synapsins anchor the neurotransmitter filled vesicle to cytoskeletal elements. When the synapsins are phosphorylated the vesicle is released from the cytoskeleton and transported by rab proteins to the active zone, a specialized region of the presynaptic membrane where exocytosis occurs. Once the synaptic vesicle has docked at the appropriate site at the presynaptic membrane close to calcium channels, it then undergoes priming. This step involves the partial fusing of the vesicle membrane to the presynaptic terminal membrane that allows for the rapid release of neurotransmitter following calcium influx. Priming occurs when integral proteins within the membrane of the vesicle bind to specific target proteins in the membrane of the terminal at the release site. Specifically, synaptobrevin/VAMP in the vesicle membrane binds to a protein complex in the presynaptic membrane formed by the union of syntaxin and SNAP-25. Once the trimeric core (synaptobrevin:synataxin:SNAP-25) is formed, both N-ethylmaleimide sensitive factor (NSF) and SNAP attach. NSF, which is an ATP-ase, destabilizes the trimeric core and may cause fusion of one layer of the lipid bilayer. When the nerve terminal is depolarized, calcium enters and triggers the release of neurotransmitter by interacting with calcium-sensor proteins, synaptotagmins, on the membrane of the vesicle. This leads to complete fusion of the vesicle membrane with the presynaptic membrane and release of neurotransmitter into the synaptic cleft. The

released neurotransmitter then interacts with specific receptors located on the pre- or postsynaptic membrane. The actions of the released neurotransmitter are terminated when it is degraded enzymatically, taken up via neuronal or glial cells, or diffuses away from the synaptic cleft. Clathrin-mediated endocytosis of the synaptic vesicles occurs following neurotransmitter release.

Figure 4. Steps involved in the release of neurotransmitter into the synaptic cleft. Following detachment from the cytoskeleton, neurotransmitter filled vesicles are translocated to the presynaptic membrane. Following docking, vesicle-associated membrane proteins interact with proteins located on the intracellular side of the presynaptic membrane. Fusion and release of the neurotransmitter is triggered by the influx of calcium ions. See text for details.

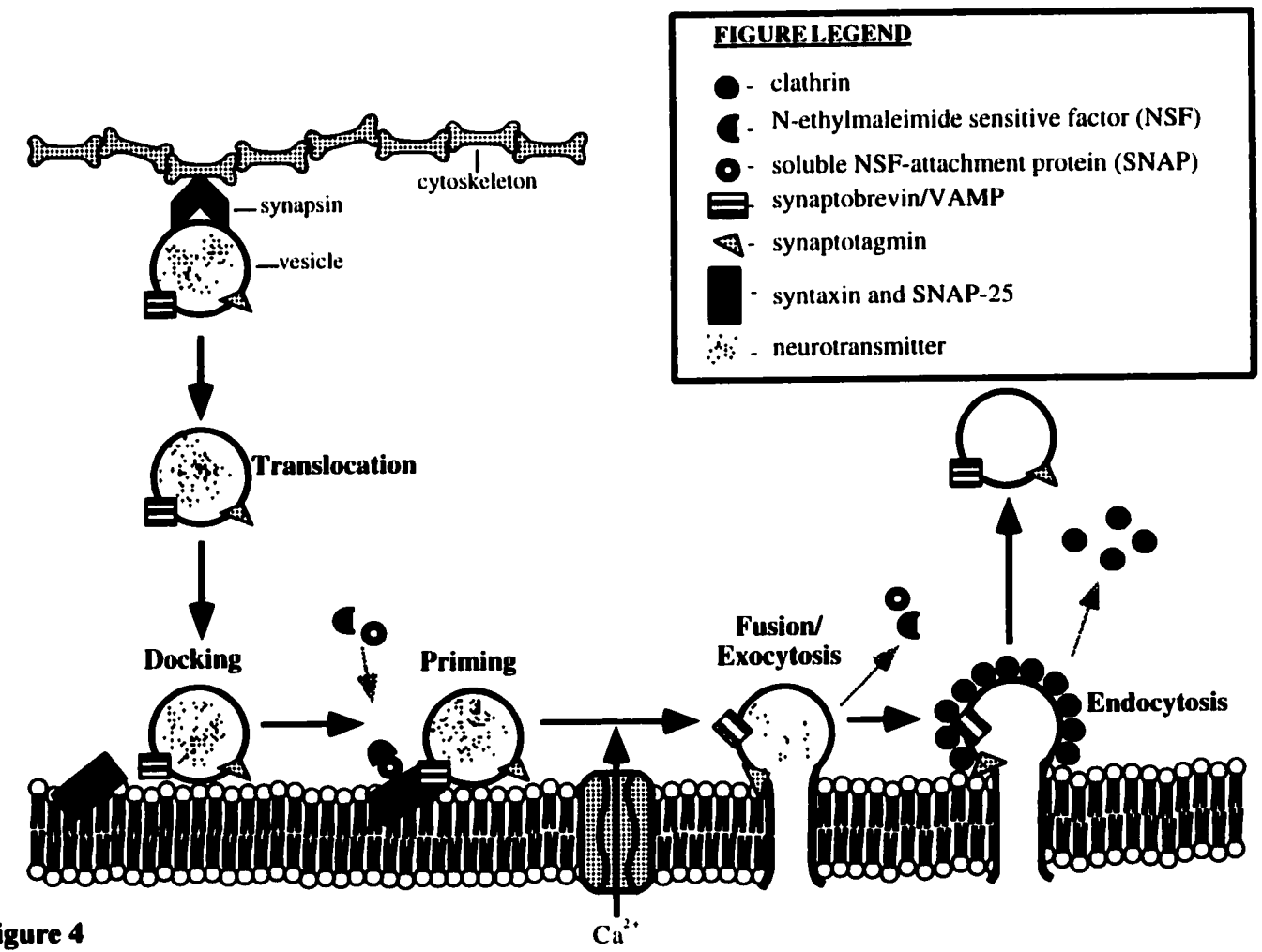


FIGURE LEGEND
- clathrin
- N-ethylmaleimide sensitive factor (NSF)
- soluble NSF-attachment protein (SNAP)
- synaptobrevin/VAMP
- synaptotagmin
- syntaxin and SNAP-25
- neurotransmitter
cytoskeleton
synapsin
vesicle
Translocation
Docking
Priming
Fusion/
Exocytosis
Endocytosis
Ca2+

Figure 4

2.3. Modulation of transmitter release

As stated above, neurotransmitters are released from presynaptic terminals, diffuse across the synaptic cleft, and bind to specific receptors. These receptors are located in various regions including the postsynaptic target. Often neurotransmitters bind to receptors located on the membrane of the terminal from which the transmitter itself was released. These receptors are known as presynaptic autoreceptors. Neurotransmitters will also diffuse away from the original site of release and bind to presynaptic heteroreceptors that reside on the membrane of presynaptic terminals of other neurons. By acting at presynaptic receptors, neurotransmitters regulate their own release or the release of other transmitters.

Since neurotransmitter release depends on a series of steps, the amount of release can be modulated by factors that regulate any one of these events. The active release of transmitter is dependent upon the arrival of an action potential and subsequent depolarization of the synaptic terminal. Thus, the activation of postsynaptic receptors on the dendrites or soma of a neuron will alter the membrane potential, contribute to the generation of an action potential, and ultimately regulate transmitter release. The release of transmitters can also be regulated at the level of the synaptic terminal. One mechanism for controlling neurotransmitter efflux is via interaction with proteins involved in the mobilization, docking, or priming of vesicles (Wu and Saggau, 1997). Calcium channels also regulate transmitter release since calcium influx is required for release (Fossier et al., 1999). By altering the activation of presynaptic ion channels, modulators of neurotransmitter release could either

enhance or reduce action potential-induced depolarization of the terminal (Meir et al., 1999). This suggests that neurotransmission is a graded event.

2.4. Regulation of cortical ACh release

ACh release can be regulated by exogenously applied neurotransmitters and analogues. As described above, cholinergic neurons located in the basal forebrain send projections to the neocortex. This suggests that cortical ACh release can be regulated at two levels. The first is through direct modulation of the activity of cholinergic neurons within the basal forebrain. Activation of cholinergic neurons by direct electrical stimulation (Rasmusson et al., 1992), stimulation of afferents to the basal forebrain (Rasmusson et al., 1994), or infusion of transmitters or analogs into the basal forebrain (Casamenti et al., 1986; Kurosawa et al., 1989; Bertorelli et al., 1991) can alter cortical ACh release *in vivo*. Examination of the neurotransmitter content of the terminals synapsing on identified cholinergic neurons would suggest that the activity of these cells could be modified by a variety of transmitters. Previous studies have shown that terminals containing GABA (Leranth and Frotscher, 1989), substance P (Bolam et al., 1986), enkephalin (Chang et al., 1987), and somatostatin (Záborszky, 1989) synapse on identified cholinergic neurons. This suggests that many different neurotransmitter systems may regulate cortical ACh release by directly influencing the activity of cholinergic basal forebrain neurons. Physiological studies suggest that the amino acid glutamate regulates the activity of neurons within the basal forebrain. Glutamate infusions into the nucleus basalis of Meynert increased cortical ACh levels in rats (Kurosawa et al., 1989). Rasmusson et al. (1994) have

shown that application of a non-selective glutamate receptor antagonist to the basal forebrain reduced both cortical ACh efflux and EEG desynchronization evoked by electrical stimulation of the PPT. These authors suggested that glutamatergic inputs from the PPT regulated the activity of cortically projecting cholinergic neurons. Further testing demonstrated that PPT-stimulation induced cortical ACh release could be reduced by a combination of antagonists to the ionotropic glutamate receptors selective for N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) while cortical EEG desynchronization was reduced by a selective AMPA receptor antagonist (Rasmusson et al., 1996). This suggests that both NMDA and AMPA receptors in the basal forebrain regulate cortical ACh efflux but differentially regulate EEG activity. However, electrical stimulation of the PPT may also activate other subcortical structures that regulate cortical EEG activity, such as the thalamus. Direct infusion of glutamatergic analogues into the basal forebrain may provide better insight into the role of specific ionotropic glutamate receptors located in the basal forebrain on cortical ACh release and EEG activity.

A second mechanism for modulating ACh efflux is through activation of presynaptic receptors located on intracortical cholinergic terminals. Previous *in vivo* studies have suggested that a number of transmitters and modulators regulate ACh efflux presynaptically, including serotonin (Crespi et al., 1997), neurotensin (Lapchak et al., 1990), noradrenaline (Tellez et al., 1997), and ACh (Marchi & Raiteri, 1985; Vannucchi & Pepeu, 1995). Two additional factors that may regulate ACh release by acting at receptors on intracortical cholinergic terminals are adenosine and glutamate. It is well established that adenosine regulates neurotransmitter release by activating

presynaptic A_1 adenosine receptors on axonal terminals (Dunwiddie and Fredholm, 1997). Studies examining the effects of adenosine on cortical ACh release have not yielded consistent results. While *in vitro* studies have shown that adenosine reduces cortical ACh release due to electrical stimulation (Pedata et al., 1983; Broad and Fredholm, 1996), *in vivo* studies have shown no effect of adenosine on potassium evoked ACh release (Phillis et al., 1993a). Studies examining the effects of activating intracortical glutamate receptors on ACh release are also surprisingly discrepant. *In vivo* studies suggest that activation of intracortical NMDA receptors decreases ACh release (Hasegawa et al., 1993) while *in vitro* studies demonstrate that activation of ionotropic glutamate receptors in the cortex elicits ACh efflux (Lodge and Johnston, 1985; Ulus et al., 1992). Since the cortex receives dense glutamatergic inputs from a variety of subcortical structures, including the thalamus, and extrasynaptic spillover of glutamate can regulate the release of other neurotransmitters (Mitchell and Silver, 2000), the regulation of ACh release by intracortical glutamate is worth examining.

3. THESIS OVERVIEW

The work presented in this thesis examined the effects of the neuromodulator adenosine and the neurotransmitter glutamate on cortical ACh release. The location of the A_1 and A_{2A} adenosine receptors was confirmed using *in situ* hybridization. The effects of intracortical adenosine on ACh release evoked by electrical stimulation of the PPT were tested using *in vivo* microdialysis in urethane-anesthetized rats. The effects of intracortical delivery of glutamate were examined using the same protocol. In addition to this, the effects of selective ionotropic

glutamate receptor agonists delivered to the basal forebrain on cortical ACh release and EEG activity were also tested.

3.1. Hypotheses

Based on the discussion above regarding the anatomical distribution of cholinergic neurons, the physiological importance of this system, and modulation of neurotransmitter release, the work presented in this thesis focused primarily on testing the following general hypothesis:

General hypothesis: Cortical acetylcholine release can be regulated by factors acting within the cortex on intracortical cholinergic terminals and by factors acting on the cell bodies of cholinergic neurons within the basal forebrain.

From this general hypothesis, the following specific hypotheses were derived and tested:

Study 1. The effects of intracortical adenosine on synaptically evoked cortical ACh release.

A. Intracortical delivery of the purine nucleoside adenosine will inhibit evoked cortical ACh release.

B. Intracortical delivery of a selective A_1 receptor agonist will inhibit evoked cortical ACh release

C. Intracortical delivery of a selective A_{2A} receptor agonist will have no effect on evoked cortical ACh release.

Study 2. Distribution of the mRNA for A_1 and A_{2A} adenosine receptors in the brain.

A. The mRNA for the A_1 adenosine receptor will be found primarily within the cortex, cerebellum, hippocampus, and thalamus.

B. The mRNA for the A_{2A} adenosine receptor will be found primarily within the striatum.

Study 3. The effects of intracortical glutamate on synaptically evoked cortical ACh release.

A. Intracortical delivery of the excitatory amino acid glutamate will enhance evoked cortical ACh release.

B. Glutamate induced enhancement of cortical ACh release will be mediated by ionotropic glutamate receptors.

Study 4. The effects of delivery of ionotropic glutamate receptor agonists into the basal forebrain on cortical ACh levels and cortical EEG.

A. Application of the ionotropic glutamate receptor agonist NMDA to the basal forebrain will enhance ACh release in the cortex with little to no change in cortical EEG activation.

B. Application of the ionotropic glutamate receptor agonist AMPA to the basal forebrain will result in enhanced ACh release and increased cortical EEG activation.

CHAPTER II. REGULATION OF CORTICAL ACETYLCHOLINE RELEASE BY ADENOSINE

Preface

Adenosine is a purine nucleoside that has traditionally been regarded as functioning intracellularly to influence cell replication and energy metabolism. Drury and Szent-Gyorgyi (1929) were the first to recognize that adenosine may also be released extracellularly to influence the activity of the nervous system. It is now known that adenosine may be released directly into the extracellular space from either neurons or glia via a bidirectional nucleoside transporter or may be formed due to the breakdown of released ATP (Fig. 5; Linden, 1999). The actions of adenosine are terminated when this purine is removed by reuptake into cells or degraded enzymatically (Linden, 1999). Following reuptake, adenosine may be degraded to inosine by adenosine deaminase or may be reincorporated into the nucleotide pool upon phosphorylation by adenosine kinase.

Figure 5. Schematic representation of adenosine release and metabolism. Abbreviations: ADP, adenosine dephosphate; AMP, 5′-adenosine monophosphate; AMPS, adenylosuccinate; ATP, adenosine triphosphate; cAMP, cyclic 3′,5′-adenosine monophosphate; GTP, guanosine triphosphate; IMP, inosine monophosphate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine. Enzymes catalyzing the various reactions are as follows:

1. adenosine deaminase
2. inosine nucleosidase *and* purine nucleoside phosphorylase
3. xanthine oxidase
4. S-adenosylhomocysteine hydrolase
5. S-adenosylhomocysteine nucleosidase
6. nucleoside ribosyltransferase
7. adenosine nucleosidase *and* nucleoside ribosyltransferase
8. hypoxanthine-guanine phosphoribosyltransferase
9. inosinate nucleosidase *and* hypoxanthine phosphoribosyltransferase
10. adenosine kinase
11. 5′-nucleotidase
12. adenylosuccinate lyase
13. adenylosuccinate synthetase
14. diphosphohydrolase
15. adenine phosphoribosyltransferase
16. AMP nucleosidase

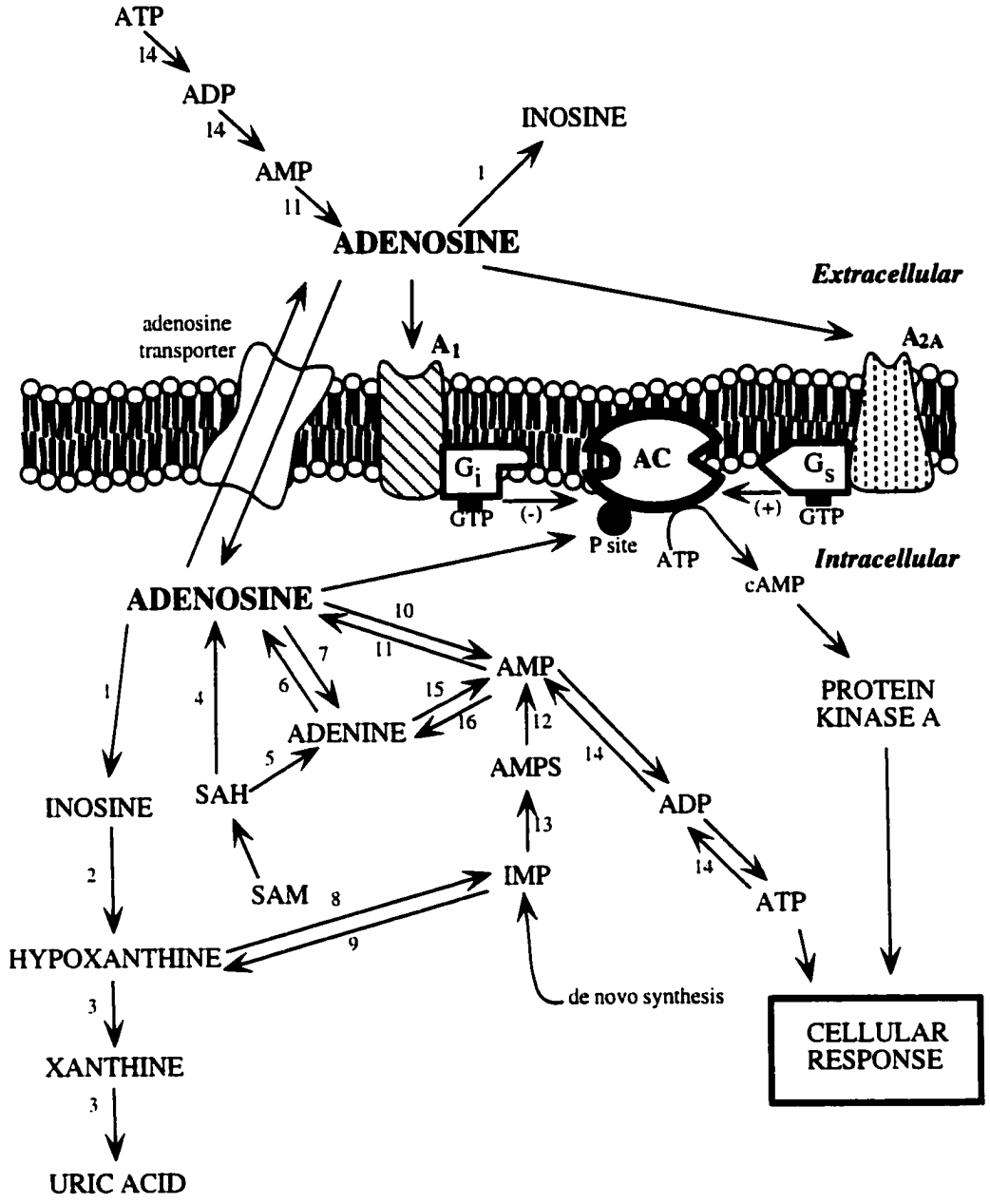

ATP
14
ADP
14
AMP
11
ADENOSINE
INOSINE
1
Extracellular
adenosine
transporter
A1
A2A
Gi
AC
Gs
GTP
(-)
(+)
GTP
P site
ATP
Intracellular
cAMP
PROTEIN
KINASE A
ADENOSINE
10
11
7
6
AMP
15
16
ADENINE
12
AMPS
14
ADP
1
4
5
SAH
INOSINE
2
SAM
8
IMP
13
14
ATP
9
HYPOXANTHINE
de novo synthesis
3
CELLULAR
RESPONSE
XANTHINE
3
URIC ACID

Figure 5

Within the central nervous system, the dominant effect of extracellular adenosine is to inhibit neuronal activity by acting at membrane bound receptors. To date, four adenosine receptor subtypes have been identified based upon agonist pharmacology and DNA/RNA sequences. These include the A_1, A_{2A}, A_{2B}, and A_3 receptors, all of which are coupled to G-proteins. Of these, the A_1 and A_{2A} receptors are most prevalent within the central nervous system. Both regulate the activity of adenylyl cyclase and, thus, modulate cyclic AMP. While the A_1 receptor has been demonstrated to inhibit adenylyl cyclase, the A_{2A} receptor activates this enzyme (Ralevic and Burnstock, 1998). Adenosine may also regulate adenylyl cyclase activity directly by binding to the intracellular P site (Londos and Wolff, 1977). However, compared to 2′,5′-dideoxyadenosine, adenosine is a weak agonist at this region.

Endogenous levels of adenosine in the brain have been reported to increase in response to periods of high metabolic activity in pathological conditions such as stroke and ischemia (Miller and Hsu, 1992; Rudolphi et al., 1992). Wakefulness is a non-pathological state that is associated with increased neuronal activity. Adenosine is hypothesized to act as a somnogenic factor for several reasons. Firstly, the most commonly consumed psychostimulant in the world, caffeine, has been shown to produce arousal primarily by antagonizing adenosine receptors in the brain (Snyder et al., 1981). Secondly, various species of animals, including dogs (Haulica et al., 1973), birds (Marley and Nistico, 1972), rats (Radulovacki et al., 1984), and cats (Portas et al., 1997) have been shown to be susceptible to the hypnogenic effects of adenosine. Adenosine has also been shown, *in vitro*, to inhibit the firing rate of neurons in brain regions associated with wakefulness such as the laterodorsal tegmental nucleus and the

diagonal band of Broca (Rainnie et al., 1994) and the locus coeruleus (Shefner and Chiu, 1986). Finally, studies that have examined endogenous adenosine within the brain have noted circadian fluctuations (Huston et al., 1996; de Sanchez et al., 1993). Porkka-Heiskanen et al. (1997) have shown that the measured adenosine level in the cat basal forebrain was higher during wakefulness compared to periods of spontaneous sleep. During sleep deprivation, adenosine levels rose steadily in the basal forebrain and delivery of NBTI, an adenosine transport blocker, to the basal forebrain, but not the thalamus, resulted in a significant decrease in the time spent awake and a significant increase in both REM and slow-wave sleep (Porkka-Heiskanen et al., 1997).

As described in the previous chapter, cortical ACh levels are highest during wakefulness or rapid-eye-movement sleep and lowest during slow-wave sleep. The effects of adenosine on neurotransmission have been repeatedly demonstrated both *in vitro* and *in vivo*. During wakefulness there is a trend for adenosine levels to rise and it is therefore possible that adenosine may inhibit cortical ACh release and act as a slow wave sleep-inducing factor.

The first study presented in this chapter examined the effects of adenosine and selective agonists and antagonists on cortical ACh release using *in vivo* microdialysis in urethane-anesthetized rats. The second study confirmed the distribution of mRNA for the A_1 and A_{2A} adenosine receptors using *in situ* hybridization.

Study 1: Inhibition of Synaptically Evoked Cortical Acetylcholine Release by Adenosine: An *in vivo* Microdialysis Study in the Rat

Acetylcholine (ACh) release in the cortex is associated with both behavioural activation and cortical activation as determined by electroencephalographic (EEG) activity (Kanai and Szerb, 1965; Celesia and Jasper, 1966; Semba, 1991; Jones, 1993). The rate of efflux of ACh in the cortex is greatest during periods of desynchronized, high frequency EEG activity such as that observed during waking and paradoxical sleep, and lowest during slow-wave sleep (Jasper and Tessier, 1971). Cortical ACh is derived primarily from the axon terminals of cholinergic neurons located in the nucleus basalis magnocellularis (NBM) of the basal forebrain (Bigl et al., 1982; Rye et al., 1984; Saper, 1984; Baskerville et al., 1993). Direct electrical or chemical stimulation of the NBM results in the release of ACh in the neocortex of rats (Casamenti et al., 1986; Kurosawa et al., 1989; Rasmusson et al., 1992). Previous studies have demonstrated a correlation between the activity of neurons within the basal forebrain and cortical and behavioural arousal (Szymusiak and McGinty, 1986; Détári and Vanderwolf, 1987; Szymusiak and McGinty, 1989; Détári et al., 1997, 1999). Factors that modulate the activity of cholinergic basal forebrain neurons could, therefore, alter cortical ACh release and have a major effect on cortical arousal and behavioural state.

Increasing evidence suggests that the purine nucleoside adenosine is a somnogenic factor (Benington and Heller, 1995). Delivery of adenosine and its agonists promote sleep in a variety of species including dogs (Haulica et al., 1973), birds (Marley and Nistico, 1972), rats (Radulovacki et al., 1985), and cats (Portas et al., 1997). Caffeine, probably the most universally consumed psychostimulant, is believed to enhance wakefulness by blocking adenosine receptors (Snyder et al.,

1981; Nehlig et al., 1992). Examinations of endogenous extracellular adenosine levels within the central nervous system and the correlation between these levels and behaviour have provided further support for the role of adenosine as a sleep-promoting factor. For example, Huston et al. (1996) have shown that increases in extracellular adenosine levels in hippocampus but not neostriatum are followed by a significant increase in sleep-like behaviour in freely behaving rats. Similarly, the amount of extracellular adenosine within the basal forebrain of cats increases during prolonged wakefulness and declines during sleep (Porkka-Heiskanen et al., 1997). Diurnal fluctuations in extracellular adenosine levels have also been noted in cortex (Chagoya de Sánchez et al., 1993).

The precise mechanisms by which adenosine increases sleep propensity are yet to be elucidated. Adenosine modifies neuronal activity by acting at membrane bound, G-protein-coupled receptors: A_1, A_{2A}, A_{2B}, and A_3. Of these, only the A_1 and the A_{2A} are highly expressed within the rat brain (Olah and Stiles, 1995). The A_1 receptor is negatively coupled to adenylyl cyclase activity, while the A_{2A} receptor is positively coupled to this enzyme (Fredholm et al., 1994). There is increasing evidence that adenosine promotes sleep, at least in part, by activating A_1 receptors located on neurons in the basal forebrain. Previous *in vitro* studies have shown that adenosine inhibits the firing rates of unidentified basal forebrain neurons by activating A_1 receptors (Rainnie et al., 1994). Adenosine administered into the basal forebrain of freely moving cats reduced time spent in waking in a concentration-dependent manner (Portas et al., 1997). Using local perfusion of an adenosine transporter inhibitor, Porkka-Heiskanen et al. (1997) demonstrated that an increase in endogenous

adenosine levels in the basal forebrain is associated with a significant decrease in the time spent awake and a significant increase in time spent sleeping in the cat. This behavioural effect was not observed following perfusion of the same transporter inhibitor into the thalamus despite a similar elevation of local extracellular adenosine levels (Porkka-Heiskanen et al., 1997). The thalamus, like the basal forebrain, sends projections to the cortex, but these projections are non-cholinergic. Together, these results suggest that the somnogenic action of adenosine is site-specific, and that in the basal forebrain, adenosine promotes sleep by inhibition of cholinergic and/or non-cholinergic neurons.

Adenosine may also act presynaptically at cholinergic terminals in the cortex to inhibit ACh release and increase sleep propensity. It is well established that adenosine inhibits neurotransmitter release by activating presynaptic A_1 receptors (Dunwiddie and Fredholm, 1997). However, previous results on the effects of adenosine on cortical ACh release are surprisingly discrepant. *In vitro* studies using rat cortical slices have shown that adenosine can reduce, in a concentration-dependent manner, electrical stimulation evoked ACh release and that this effect is mediated by activation of the A_1 adenosine receptor (Pedata et al., 1983; Broad and Fredholm, 1996). In contrast, using the cortical cup technique on halothane anesthetized rats, Phillis et al. (1993) demonstrated that basal ACh efflux, but not potassium evoked release, could be inhibited by an A_1 receptor agonist. Furthermore, Kurokawa et al. (1996) have shown that delivery of a selective A_1 agonist either by intraperitoneal injection or via a microdialysis probe in the cortex of freely moving rats did not alter basal cortical ACh efflux. Studies examining the effects of adenosine receptor antagonists have also

yielded contradictory results. Using rat cortical slices, low concentrations of the non-selective adenosine receptor antagonist caffeine have been shown to enhance evoked ACh release while higher concentrations inhibit evoked ACh efflux (Pedata et al., 1984). Other studies using similar methods have demonstrated that selective A_1 adenosine receptor antagonists had no effect on evoked ACh release in the cortex (Broad and Fredholm, 1996). In view of the important role adenosine might play in the modulation of cortical ACh release and, possibly, behavioural state, these discrepancies require clarification.

Towards this goal, the effect of adenosine on synaptically evoked cortical ACh release was examined *in vivo* using a well established model of brainstem stimulation-induced cortical ACh efflux (Rasmusson et al., 1994). The present study examined the presynaptic adenosinergic modulation of cortical ACh release evoked by synaptically activating basal forebrain neurons through electrical stimulation of the pedunculopontine tegmental nucleus (PPT).

Materials and Methods

All animals used in this study were handled in accordance with the guidelines of the Canadian Council on Animal Care. Recipes for all of the solutions used in the present experiment as well as those presented in the subsequent chapters are listed in Appendix A.

Data were obtained from 128 male Wistar rats (Charles River, St. Constant, Quebec) weighing between 250 and 500g. Each animal was anesthetized with 1.4 g/kg (i.p.) urethane and a supplemental dose, if necessary, to maintain

areflexia. No additional injections were given for the remainder of the experiment. The animal was placed in a stereotaxic frame with bregma and lambda on a horizontal plane. The body temperature of the animal was maintained within one degree of 37.2°C using a rectal thermometer connected to a feedback-controlled heating pad (Shurite, New Haven, Connecticut, USA). Surgery was carried out on the right side of the brain. Unless otherwise indicated, all stereotaxic measurements were in reference to bregma. A microdialysis probe (2 mm membrane length, 0.5 mm outer diameter; 30 kDa molecular cutoff; Bioanalytical Systems, BAS, West Lafayette, Indiana, USA) was inserted in the barrelfield of the somatosensory cortex (coordinates: posterior 1.4 mm and lateral 5.0 mm) and the probe tip was positioned 2.8 mm ventral to the dural surface. Thus, the entire length of the membrane of the probe was positioned diagonally across the cortical layers within the gray matter. The probe was continuously perfused at a rate of 2 µl/minute with artificial cerebrospinal fluid (3 mM KCl, 125 mM NaCl, 1.3 mM $CaCl_2$, and 1 mM $MgSO_4$) to which atropine and neostigmine methylsulfate (Sigma, Oakville, Ontario, Canada) were added to yield a final concentration of 10 µM each. A concentric bipolar stimulating electrode (250 µm tip diameter; Frederick Haer and Co., Brunswick, Maine, USA) was lowered into the PPT (coordinates: posterior 8.4 mm and lateral 2.0 mm; 6.6 -6.8 mm ventral to the pial surface). Sample collection commenced following a 90-minute equilibration period. A schematic representation of this experiment is depicted in Figure 6.

Each dialysis sample was collected over a 20-minute period. Three baseline samples were collected to establish basal ACh levels. Following this, one sample was collected during delivery of a one second stimulus train (0.2 ms, 100 Hz,

400 μA) applied to the PPT once per minute for 20 minutes (Rasmusson et al., 1994). This was followed by the collection of 4 or 5 samples without stimulation, during which cortical ACh levels returned to baseline levels. The dialysis perfusate was switched to perfusate containing the drug to be tested at the beginning of the sample collected immediately prior to presentation of the second stimulus train. The switching of the perfusate was timed so as to account for the dead volume in the tubing leading to and from the microdialysis probe. The drug remained in the perfusate for the remainder of the experiment. One sample was taken in the presence of the drug alone without stimulation. This was subsequently followed by a sample collected during stimulation of the PPT using the same parameters employed in the first stimulation. Two or three post-stimulation samples were then collected. No drug was added for control animals.

Acetylcholine analysis

Each sample was analyzed for its content of ACh using high-performance liquid chromatography with electrochemical detection (Waters, Mississauga, Ontario, Canada). An isocratic pump delivered the mobile phase (50 mM Na_2HPO_4, 0.1 mM EDTA, 0.005% ProClin (BAS), pH adjusted to 8.5 with orthophosphoric acid) at a rate of 0.1 ml/min. A UniJet Microbore column (BAS) was used to separate ACh from choline. In the postcolumn immobilized enzyme reactor containing AChE and choline oxidase (BAS), ACh was converted to hydrogen peroxide and betaine. The hydrogen peroxide was then oxidized by a platinum working electrode (oxidation potential: +650 mV) and detected amperometrically. Data were gathered and analyzed using Powerchrome software (Castle Hill, New South Wales, Australia). The system was

calibrated for each experiment using standard solutions containing 1, 2, and 4 pmol of ACh.

Histology

Following completion of the experiment, the animal was further anesthetized with excess sodium pentobarbital and perfused transcardially with 50 ml of 0.9% saline followed by 400 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed and postfixed overnight at 4°C and then transferred to either 15% or 30% sucrose in 0.1 M phosphate buffer and stored at 4°C. Brain regions containing the tracks of the microdialysis probe and the stimulating electrode were cut into 60 μm coronal sections on a vibratome and collected in 0.05 M Tris buffer saline (TBS; pH 7.4). Sections containing the track of the microdialysis probe were mounted immediately on chrome-alum-gelatinized slides and allowed to air dry before staining with cresyl violet. Brain sections that contained the track of the stimulating electrode were processed histochemically for nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity, a selective marker for cholinergic neurons in the PPT (Vincent et al., 1983). Briefly, sections were rinsed in 0.05 M TBS and then incubated at 37°C for 45 to 60 minutes in 0.05 M TBS containing 0.1 mg/ml nitroblue tetrazolium, 1 mg/ml β-NADPH, and 0.3% Triton X-100 (Sigma). Following completion of the reaction, the tissue was rinsed in 0.025 M TBS, mounted, air dried, and coverslipped.

Data analysis

Differences between experimental and control groups were assessed using a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test (PLSD) for post-hoc comparisons. Data obtained from the control group are represented as a black bar on each histogram. To evaluate differences between two groups, Student's t-test was used. To determine evoked release, the absolute amount of ACh detected in the sample collected immediately prior to PPT stimulation was subtracted from the ACh detected during the stimulation period for both stimulation trials (E1 and E2). Other specific comparisons are described in the results section. All histograms depict the mean plus the standard error of the mean.

Materials

The following chemicals were used: adenosine (Sigma), caffeine (BDH Chemicals), 2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680) (Research Biochemicals International, RBI), N^6-cyclopentyladenosine (CPA; RBI), dipyridamole (Sigma), S-(4-nitrobenzyl)-6-thioinosine (NBTI; RBI), 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), and 1, 3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine (PACPX; RBI). Each of these drugs was dissolved in perfusate except for dipyridamole, NBTI, DPCPX, and PACPX which were first dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) and then diluted to the desired concentration with perfusate yielding a final DMSO concentration of 1%. Control data were obtained in the presence of 1% DMSO alone to determine the effects of DMSO on cortical ACh release. A few experiments using the A_1 receptor antagonist 8-cyclopentyltheophylline (8-CPT) were performed but were excluded from analysis

because 8-CPT produced a peak on the chromatogram that interfered with identification of the ACh peak.

All tables and graphs of the results are presented at the end of the results section. The values obtained from the control group are represented in each graph for comparison.

Figure 6. Schematic depicting the experimental protocol. Both spontaneous and PPT stimulation evoked cortical ACh was collected from urethane-anesthetized rats using a microdialysis probe. For details, see text.

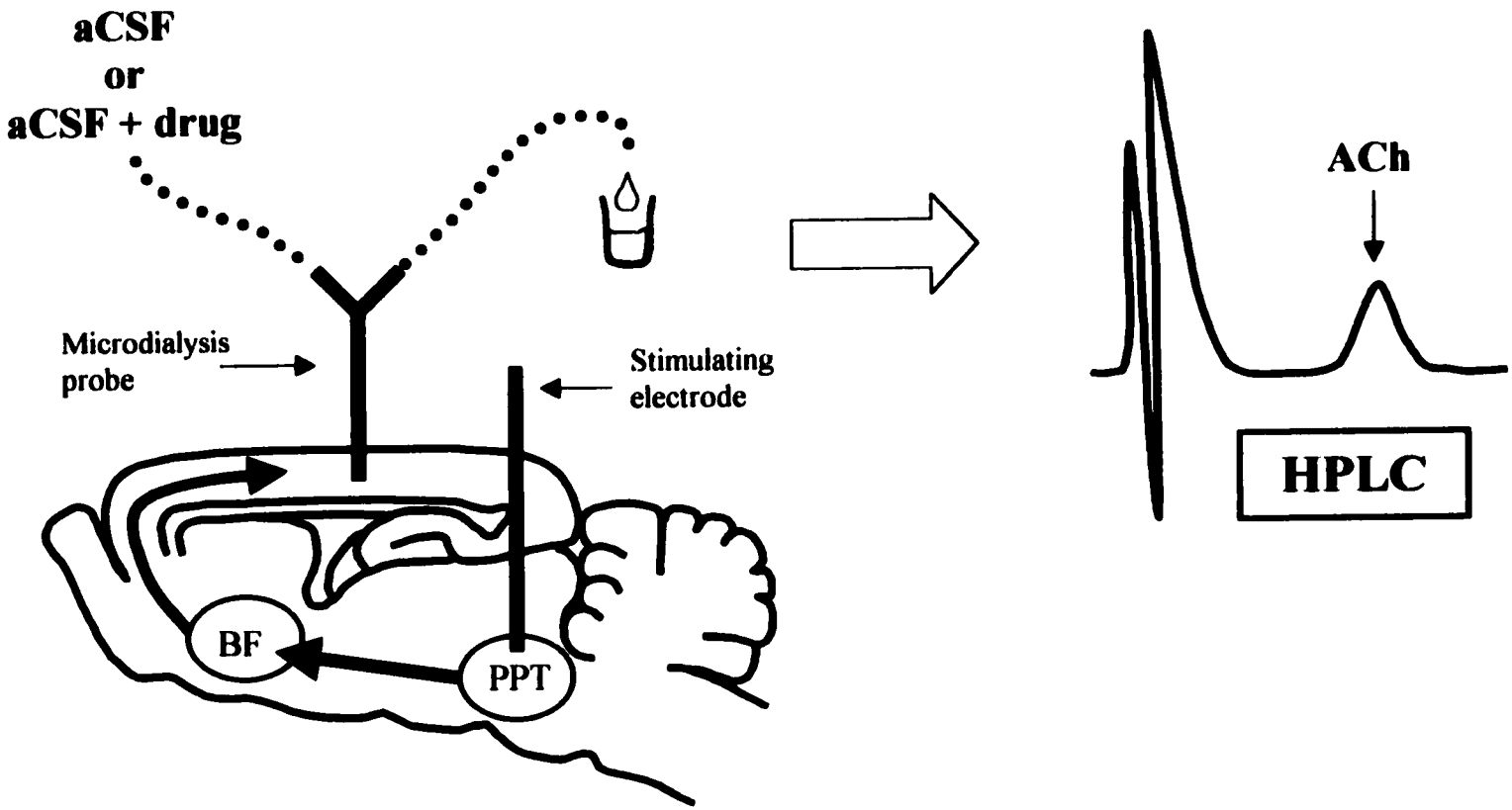

aCSF
or
aCSF + drug
Microdialysis
probe
Stimulating
electrode
BF
PPT
ACh
HPLC

Figure 6

Results

Basal vs. evoked cortical ACh release

The mean basal amount of ACh collected from the neocortex prior to the first PPT stimulation was 0.57 pmol per 20-minute sample (n = 128; Table 1), which is comparable to previous reports (Rasmusson et al., 1994). It has been shown in earlier work that most of the ACh released spontaneously in the cortex of urethane-anesthetized rats is not tetrodotoxin-sensitive and therefore is not a result of the activity of cholinergic intracortical or basal forebrain neurons (Rasmusson et al., 1994). Electrical stimulation of the PPT was highly effective in eliciting ACh release in the cortex (see Fig 7). The mean amount of cortical ACh collected increased more than four-fold from the baseline value during electrical stimulation of the PPT (Table 1). This increase was statistically significant as determined by paired-sample t-test ($t_{127} = 11.95$, $p<0.0001$).

The effects of repeated PPT stimulation were evaluated in the seven control animals. The amount of ACh released during the second stimulation was slightly, but not significantly, higher than the amount released during the first stimulus presentation. The average E2/E1 ratio for the control group was 1.15 ± 0.10 (S.E.M.; n = 7). This suggests that changes in evoked cortical ACh efflux observed in the subsequent experiments were the result of the local effects of the drugs tested and not due to alterations in the ability of basal forebrain cholinergic neurons to release ACh in response to the second PPT stimulation or to damage of the PPT or basal forebrain cholinergic neurons.

The effects of adenosine and specific receptor agonists and antagonists

None of the drugs tested in the present study significantly affected basal ACh release. Since DMSO was used to dissolve some of the drugs tested, the effects of 1% DMSO on cortical ACh release were examined. The average E2/E1 ratio obtained from the DMSO group was 1.13 ± 0.18 (S.E.M.). This was not significantly different from the control ratio described above.

In order to evaluate the effects of adenosine on evoked ACh release, the E2/E1 values calculated from the group exposed to adenosine were compared to the same ratio calculated from the control group. As shown in Figure 8, adenosine at a concentration of 0.04 mM in the perfusate had no significant effect on synaptically evoked cortical ACh release. However, 0.2, 1, and 5 mM adenosine produced a statistically significant, concentration-dependent reduction of the E2/E1 ratio ($F_{4, 22} = 9.68$, $p<0.001$; Fisher's PLSD: control vs. 0.2, 1, or 5 mM adenosine, $p< 0.05$; 5 mM adenosine vs. 0.04, 0.2, and 1 mM adenosine, $p<0.05$).

To determine if activation of the A_1 adenosine receptor alone could inhibit synaptically evoked cortical ACh release, the effects of the A_1 receptor agonist CPA were tested. As shown in Figure 9A, addition of 0.1 μM CPA to the perfusate had no significant effect on cortical ACh release. However, addition of 1, 100, and 500 μM CPA to the perfusate reduced the E2/E1 ratio by 32%, 36%, and 33%, respectively. These values were significantly lower than controls ($F_{4, 22}= 3.40$; Fisher's PLSD: control vs. 1, 100, or 500 μM CPA, $p < 0.05$) but they were not significantly different from each other.

The A_1 receptor antagonist DPCPX was delivered simultaneously with adenosine in the perfusate to determine if the inhibitory effect of adenosine was mediated by the A_1 adenosine receptor. As shown in Figure 9B, the inhibitory effect of 200 μM adenosine was antagonized by DPCPX. These data strongly suggest that the inhibitory action of adenosine on synaptically evoked cortical ACh release is mediated by the A_1 receptor.

To determine if activation of the adenosine A_{2A} receptor influenced evoked cortical ACh release, the A_{2A} receptor agonist CGS 21680 was tested. As shown in Figure 10, the E2/E1 values obtained following perfusion of CGS 21680 through the microdialysis probe was not significantly different from the E2/E1 ratio calculated from the control group. It is interesting tc note that the highest concentration of CGS 21680 tested (100 μM) appeared to produce an inhibitory response although this was not statistically significant. While CGS 21680 has a higher affinity for the A_{2A} receptor, it may also bind to the A_1 adenosine receptor at higher concentrations (Hutchison et al., 1989). To ensure that the inhibitory effects of the A1 adenosine receptor were not masking any possible excitatory A2A receptor effects, CGS 21680 was applied in the presence of the A_1 receptor antagonist DPCPX (Fig. 10). This did not result in any significant change in evoked ACh release in the cortex.

The role of endogenous adenosine

To determine whether endogenous adenosine suppressed cortical ACh release in the present study, the effects of adenosine receptor and transporter

antagonists were tested. The amount of evoked ACh release was not altered by the selective A_1 receptor antagonist DPCPX (1, 10, and 50 μM) or by the administration of the non-selective adenosine receptor antagonist caffeine (10, 50, and 500 μM; data not shown). This suggests that tonic inhibition of evoked ACh release by basal amounts of extracellular adenosine is negligible under the current experimental condition.

In contrast, combined delivery of the nucleoside transporter inhibitors NBTI (50 μM) and dipyridamole (50 μM), which presumably increased extracellular adenosine levels (Dunwiddie and Diao, 1994), produced a significant decrease in evoked ACh release ($F_{1,10}$= 15.67, $p<0.01$; Fig. 11). This effect of the nucleoside transport inhibitors on evoked ACh release was antagonized by the simultaneous delivery of 500 μM caffeine (Fig. 11).

Histology

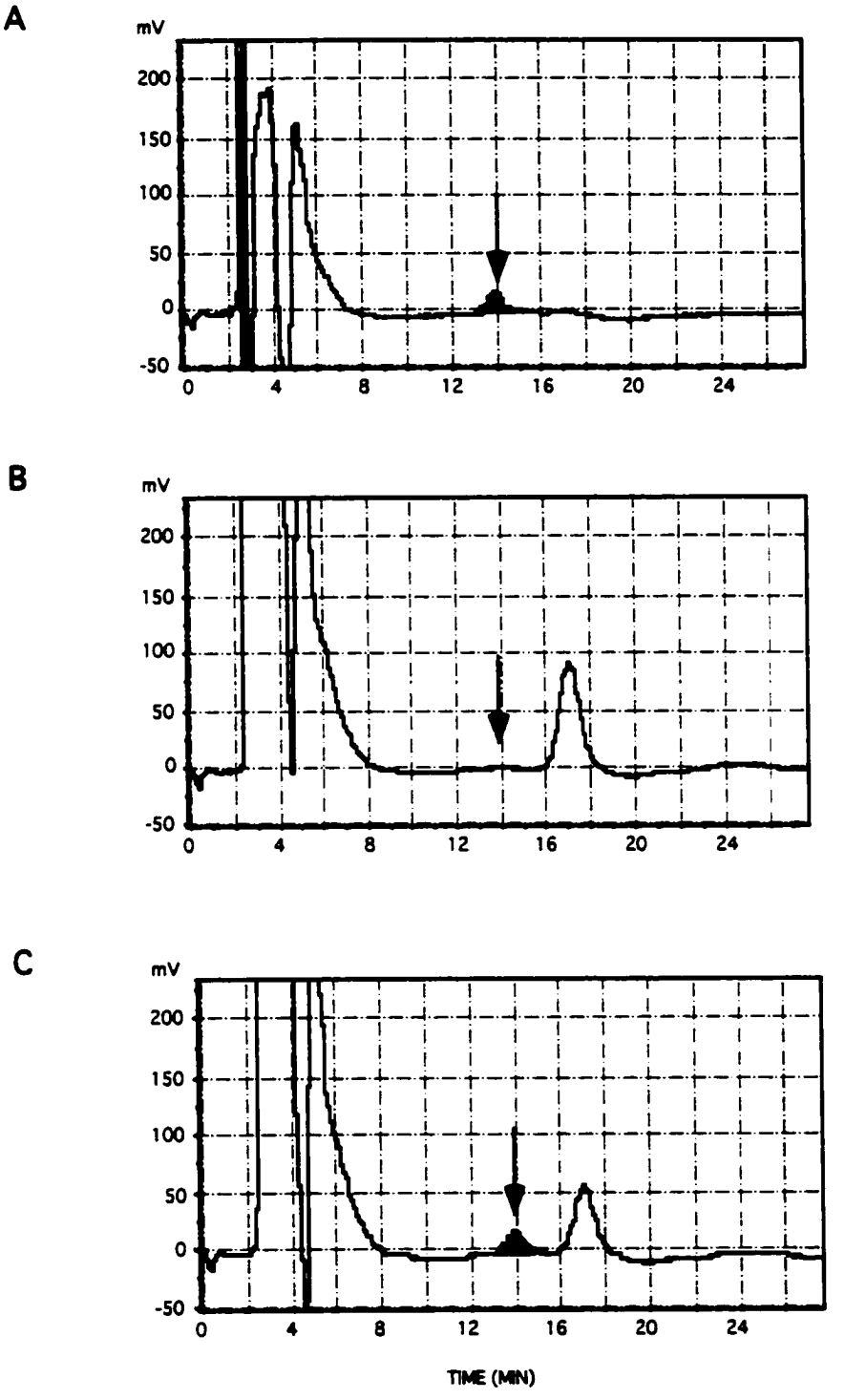
Histological examination confirmed in every animal the placement of the microdialysis probe within the gray matter of the barrel region of the somatosensory cortex (Fig. 12A), and the tip of the stimulating electrode within 0.3 mm of the PPT as identified by the presence of NADPH-diaphorase (Fig. 12B).

Table 1. Acetylcholine release (pmol per 20-minute sample collected at 2 µl/min) in the cortex before and during the first PPT stimulation (E1).

	n	mean	S.E.M.	median	min.	max.
basal	128	0.57	0.07	0.29	0	5.66
E1	128	2.44*	0.17	2.12	0.23	12.88

*$p<0.0001$ versus basal mean.

Figure 7. Chromatograms depicting the 4 pmol standard (top), spontaneous cortical ACh release prior to PPT stimulation (middle), and cortical ACh release in response to electrical stimulation of the PPT (bottom). The arrows indicate the peaks representing ACh, and the second peaks in the biological samples represent choline. Note that PPT stimulation enhances cortical ACh release.


A
mV
200
150
100
50
0
-50
0
4
8
12
16
20
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B
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0
-50
0
4
8
12
16
20
24
C
mV
200
150
100
50
0
-50
0
4
8
12
16
20
24
TIME (MIN)

Figure 7

Figure 8. The effect of adenosine in the perfusate on cortical acetylcholine released by electrical stimulation of the PPT. Adenosine inhibits evoked acetylcholine release in a concentration dependent manner. Mean ± S.E.M.; $^{*}P < 0.05$ vs control (black bar); $^{\#}P < 0.05$ vs 0.4, 0.2, and 1 mM adenosine.

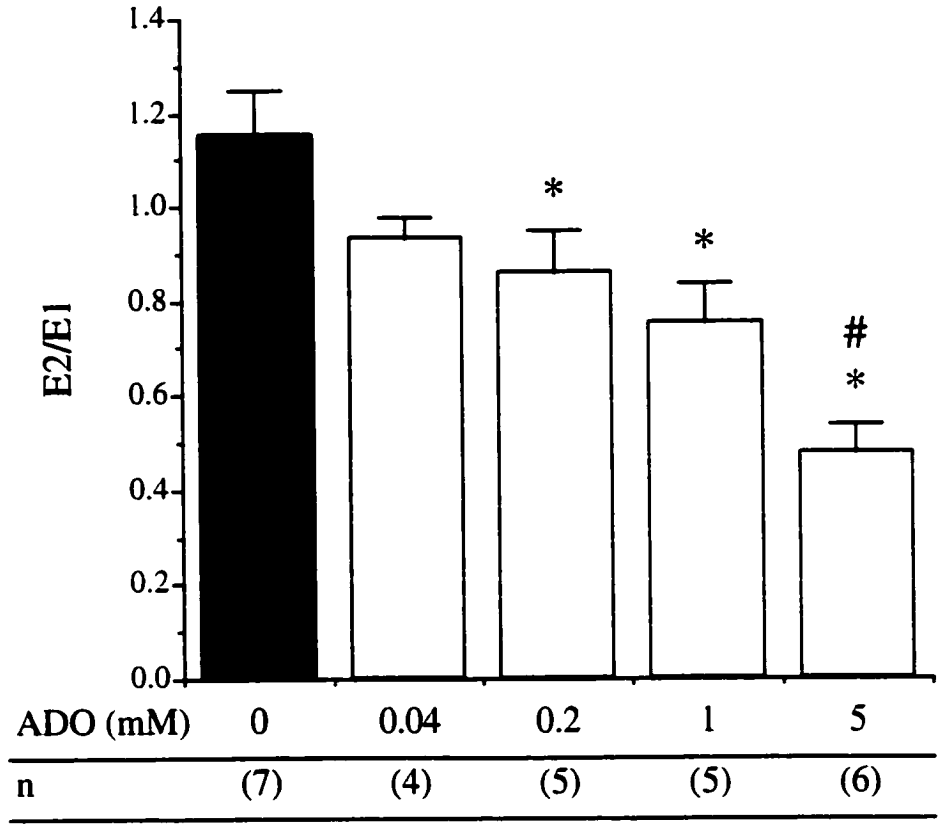
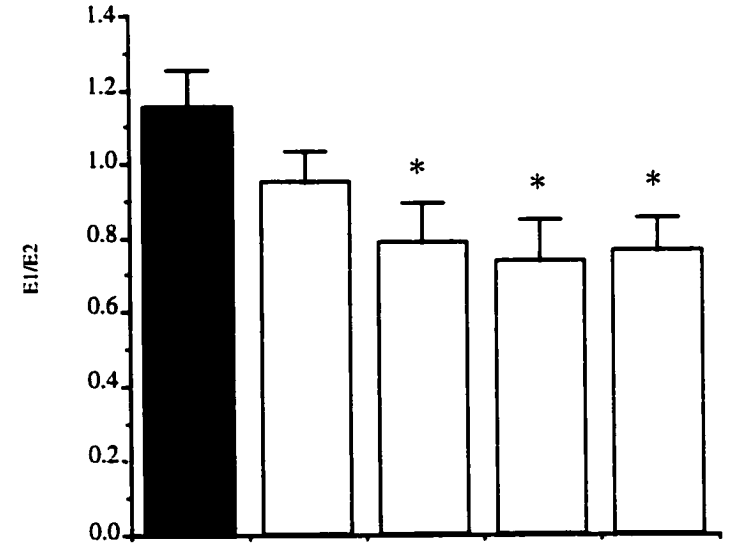


Figure 8

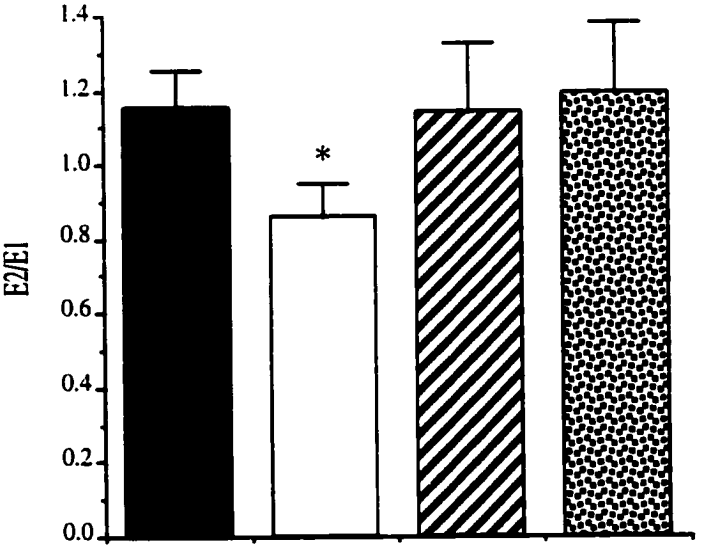
Figure 9. The effect of (A) the A_1 adenosine receptor agonist CPA and (B) the A_1 adenosine receptor antagonist DPCPX on acetylcholine release in the cortex. Mean ± S.E.M.; $*P < 0.05$ vs control (black bar).

A.



CPA (μM)	0	0.1	1	100	500
n	(7)	(5)	(5)	(5)	(5)

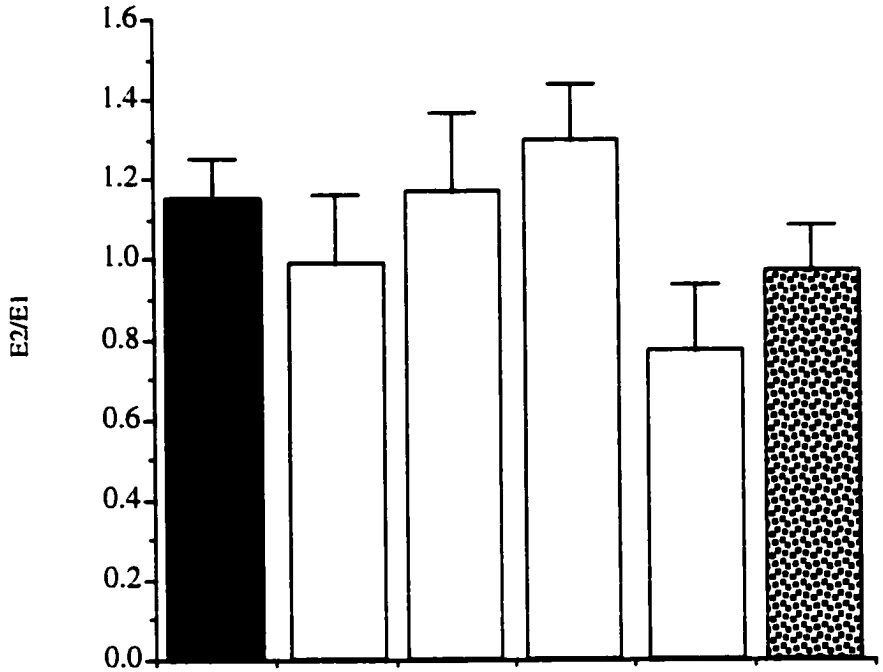
B.



ADO (μM)	0	200	0	200
DPCPX (μM)	0	0	50	50
n	(7)	(5)	(4)	(5)

Figure 9

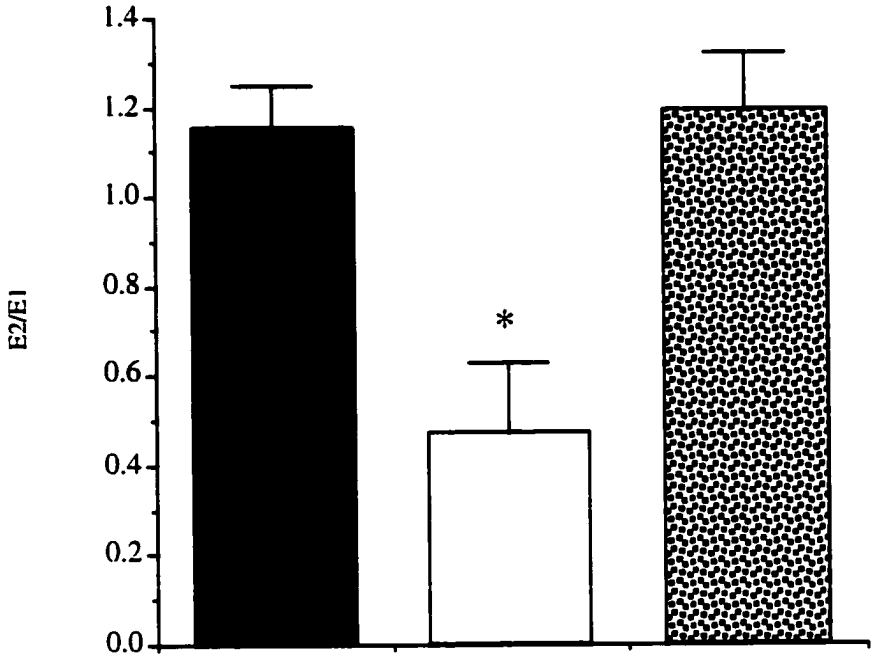
Figure 10. Evoked acetylcholine release in the presence of the A_{2A} adenosine receptor agonist CGS 21680 alone or in combination with the A_1 adenosine receptor antagonist DPCPX. Application of CGS 21680 had no effect on evoked ACh release even when the inhibitory effects of the A_1 adenosine receptor were blocked.



CGS 21680 (μM)	0	0.1	1	10	100	10
DPCPX (μM)	0	0	0	0	0	50
n	(7)	(5)	(5)	(5)	(6)	(5)

Figure 10

Figure 11. The effect of adenosine transporter inhibitors on evoked acetylcholine release in the absence and presence of caffeine. Mean ± S.E.M.; $*P < 0.05$ vs control group (black bar) and dipyridamole, NBTI, and caffeine group (stippled bar).

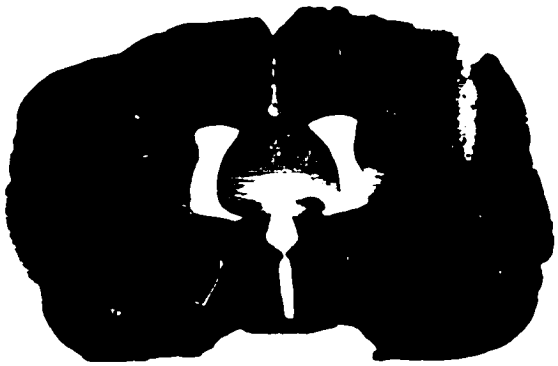


Dipyridamole (μM)	0	50	50
NBTI (μM)	0	50	50
Caffeine (μM)	0	0	500
n	(7)	(5)	(5)

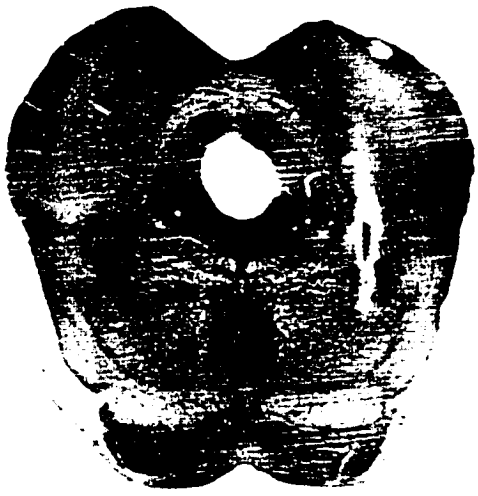
Figure 11

Figure 12. Photomicrographs of (A) the barrel field of the somatosensory cortex stained with cresyl violet (magnification: 5.3x) and (B) the mesopontine tegmentum following histochemistry for NADPH diaphorase (magnification: 5.5x) to show the position of the microdialysis probe and stimulating electrode, respectively. (C) Higher magnification (45x) photomicrograph of the PPT demonstrating the general morphology of the tissue surrounding the tip of the stimulating electrode and the presence of NADPH diaphorase positive, and therefore cholinergic, neurons.

A



B



C

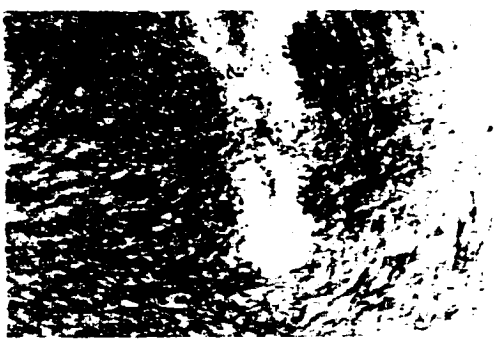


Figure 12

Discussion

Using *in vivo* microdialysis in urethane-anesthetized rats, the present study demonstrated that exogenous adenosine administered locally by reverse dialysis produced a significant, concentration-dependent decrease in PPT stimulation evoked ACh efflux in the cortex. This effect was mimicked by perfusion of the selective A_1 receptor agonist CPA, and blocked by the selective A_1 receptor antagonist DPCPX. Activation of A_{2A} adenosine receptors did not affect evoked cortical ACh release, even in the presence of DPCPX. The application of either DPCPX or caffeine, a non-selective adenosine receptor antagonist, alone did not influence resting or evoked ACh release in the cortex suggesting that there is no tonic inhibition of ACh release by endogenous adenosine in the present experimental condition. However, the nucleoside transporter inhibitors dipyridamole and NBTI inhibited evoked ACh release and this effect was antagonized by caffeine.

Technical considerations

The present results should be interpreted in light of several important technical issues. The first issue concerns the source of cortical ACh. Most of the ACh collected from the cortex in response to PPT stimulation is likely to be released from the intracortical terminals of basal forebrain cholinergic neurons. Previous anatomical studies have shown that most of the AChE- or choline acetyltransferase-positive fibers in the cortex originate in the NBM, whereas some may belong to cortical interneurons (Semba and Fibiger, 1989; Woolf, 1991). A subset of cholinergic neurons in the mesopontine tegmentum, in particular the laterodorsal tegmental nucleus, also projects

to the cortex but this innervation is limited to the medial prefrontal cortex (Vincent et al., 1983). Lesions of cholinergic basal forebrain neurons with a selective immunotoxin result in a nearly complete loss of cholinergic markers in the cerebral cortex (Heckers et al., 1994). Physiologically, Rasmusson et al. (1994) demonstrated that blocking the activity of cells within the NBM by local infusion of 3 μM tetrodotoxin into the NBM produced a decrease of up to 85% of PPT stimulation induced cortical ACh efflux. These findings collectively suggest that most of the evoked cortical ACh release which occurs during electrical stimulation of the PPT is a result of increased firing of cortically projecting cholinergic NBM cells.

The second technical issue relates to the concentration of drugs used in the present study. These concentrations were roughly ten times greater than those reported in previous *in vitro* studies measuring neurotransmitter release from brain slices. The seemingly high concentrations of drugs that are typically used in microdialysis studies are necessary for two reasons: the incomplete permeability of the microdialysis membrane, and the poor diffusion of some analytes through tissue (Shippenberg and Thompson, 1997).

A third issue is the use of urethane-anesthetized rats in the present study. While urethane is known to reduce basal levels of ACh efflux in the cortex, the advantage of using this preparation is that a steady baseline of ACh release can be maintained without fluctuations associated with awake preparations. Thus, a standardized condition was maintained which allowed for the examination of acetylcholine release free of any confounding effects resulting from spontaneous or PPT stimulation-induced behavioural state changes.

Finally, in order to block the breakdown of ACh, it was necessary to include the AChE inhibitor neostigmine in the perfusate. In addition, the muscarinic receptor antagonist atropine was also added in order to obtain detectable levels of baseline ACh release, since activation of presynaptic muscarinic autoreceptors is known to decrease cortical ACh efflux (Dudar and Szerb, 1969; Suzuki et al., 1988). It is unlikely that the addition of these drugs confounded the present results for the following reasons. Previous work using hippocampal synaptosomes has suggested that presynaptic adenosine receptors located on cholinergic terminals and cholinergic autoreceptors interact using a common mechanism to modulate ACh release since the effects of adenosine on ACh release were reduced by the simultaneous addition of a muscarinic agonist (Pedata et al., 1986). However, it is possible that this type of interaction is specific to cholinergic terminals within the hippocampus because in the present study, even when the presynaptic muscarinic receptors are blocked by atropine, evoked cortical ACh efflux was inhibited by adenosine. Together, these data suggest that, under urethane anesthesia, any interactions between presynaptic adenosine receptors and muscarinic autoreceptors are negligible with the concentration of atropine used in the present experiment.

Presynaptic inhibition of evoked ACh release is mediated by A_1 adenosine receptors

The inhibition of cortical ACh release by activation of A_1 receptors is consistent with previous anatomical and functional studies. The A_1 adenosine receptor has been shown to be widely distributed throughout the rat brain with highest concentrations in the cerebral cortex, cerebellum, thalamus, and hippocampus

(Goodman et al., 1982; Mahan et al., 1991, Reppert et al., 1991; Rivkees et al., 1995). The inhibitory effect of the A_1 receptor-selective agonist CPA on synaptically evoked ACh release and the ability of DPCPX to antagonize the adenosine-mediated inhibition of evoked ACh release suggest that a proportion of the A_1 receptors present in the cortex reside on the terminals of cholinergic fibers. However, the inhibitory effect of CPA did not appear to match the degree of inhibition produced by adenosine itself. This added inhibition by adenosine may be the result of direct interaction of adenosine with the intracellular P-site on the adenylyl cyclase enzyme (Londos and Wolff, 1977). Physiologically, the presynaptic inhibition of neurotransmitter release by adenosine has been shown previously to be mediated mostly by activation of A_1 receptors. For example, the inhibitory presynaptic effects of adenosine on ACh release have been demonstrated at neuromuscular junctions (Sawynok and Jhamandas, 1976; Nitahara et al., 1995) as well as in hippocampus (Pedata et al., 1986; Carter et al., 1995). Adenosine also inhibits electrically evoked ACh release from cortical slices through activation of the A_1 receptor subtype (Pedata et al., 1983; Broad and Fredholm, 1996). Although the A_1 receptor-mediated inhibition of ACh release by adenosine in the present study is most likely due to presynaptic mechanisms, the use of *in vivo* microdialysis does not permit direct assessment of the possibility of the additional involvement of indirect postsynaptic mechanisms.

Unlike the present study which demonstrated an inhibitory effect of A_1 receptor activation on synaptically evoked ACh release, Phillis et al. (1993) did not find a similar inhibitory effect of adenosine on potassium-evoked cortical ACh release in anesthetized rats. The discrepancy is likely due to the method of evoking ACh release.

Potassium has been shown to increase adenosine release both *in vitro* from rat brain synaptosomes (MacDonald and White, 1985) and *in vivo* from rat cerebral cortex (Phillis et al., 1993). This suggests that the effects of any further addition of adenosine could be masked. Thus, K^+-evoked ACh release would not be suitable for examining the neuromodulatory effects of adenosine.

A_{2A} adenosine receptors do not modulate cortical ACh release

The results of the present study suggest that activation of A_{2A} adenosine receptors does not influence cortical ACh release. Anatomical studies have shown that the distribution of A_{2A} receptors in the cortex is limited compared to the striatum, olfactory tubercle, and cerebellum (Jarvis et al., 1989; Johansson and Fredholm, 1995; Svenningsson et al., 1997). Physiological studies have shown that activation of the adenosine A_{2A} receptor can decrease ischemia-induced cortical γ-aminobutyric acid (GABA) release (O'Regan et al., 1992a) and increase ischemia-induced cortical aspartate and glutamate release (O'Regan et al., 1992b). However, based on the present findings as well as those reported previously (Pedata et al., 1986; Broad and Fredholm, 1996), A_{2A} receptors do not appear to influence cortical ACh release. The A_1 and A_{2A} adenosine receptors have been shown to be co-expressed by neurons of the hippocampus (Cunha et al., 1994) and the nodose ganglion (Castillo-Meléndez et al., 1994). Since these receptors have opposing actions on adenylyl cyclase, it is possible that any effects of A_{2A} receptor activation on neurotransmission in the present study may have been masked by activation of the A_1 receptor. However, perfusion of the A_{2A} agonist in the presence of the A_1 antagonist failed to affect cortical ACh release. This

suggests that such colocalization does not occur on the axonal terminals of cholinergic basal forebrain neurons. Thus, A_{2A} adenosine receptors do not appear to play a role in modulating cortical ACh release.

The role of endogenous adenosine in modulating cortical ACh release.

Endogenous extracellular adenosine within the brain is thought to be derived from multiple sources. Adenosine may be released from presynaptic terminals or from postsynaptic sites, it may be released directly into the extracellular environment as a nucleoside via nucleoside transporters, or it may be derived from the breakdown of nucleotides that are either synaptically released or released via nucleotide transporters (for a review, see Dunwiddie, 1985). All of these are potential sources of endogenous adenosine in the present study. The lack of effect of caffeine and DPCPX suggests that evoked cortical ACh release is not under tonic inhibitory control by endogenous adenosine in urethane-anesthetized rats. This stands in contrast to the previous demonstration that spontaneous ACh efflux in neocortex could be enhanced following oral administration of a selective A_1 antagonist in unanesthetized rats (Kurokawa et al., 1996). The discrepancy may be a result of the method of drug delivery. It is possible that the orally delivered antagonist produced the increase in cortical ACh release by acting at a site other than the cerebral cortex. It is also possible that endogenous adenosine levels may be higher in unanesthetized animals due to higher rates of metabolism and may therefore produce an inhibitory tone over cortical ACh release. However, the present results suggesting that endogenous adenosine does not tonically inhibit ACh release in the cortex are consistent with previous findings which

demonstrated that the direct delivery of an A_1 receptor antagonist to isolated cortical slices *in vitro* did not affect electrically evoked ACh release (Broad and Fredholm, 1996).

In order to produce maximal inhibition of the nucleoside transport system and thereby raise the extracellular level of endogenous adenosine, both NBTI and dipyridamole were delivered simultaneously (Dunwiddie and Diao, 1994). Application of the transport inhibitors produced a significant decrease in evoked ACh release. It is possible that the inhibition of ACh release by the transporter inhibitors may not have been mediated by adenosine receptors but rather from other pharmacological effects of the drugs. This, however, seems unlikely since the effect of the transporter inhibitors was antagonized by simultaneous application of the adenosine receptor antagonist caffeine. Although caffeine is a known inhibitor of phosphodiesterase activity and can mobilize intracellular calcium, the concentrations used in the present study are likely acting only at adenosine receptors for a number of reasons. Firstly, similar to the results obtained with DPCPX, caffeine did not have any effect on evoked ACh release. Caffeine has been shown to bind to adenosine receptors in the micromolar range but inhibition of phosphodiesterase activity and changes in intracellular calcium release would require millimolar concentrations of caffeine if delivered by microdialysis (Nehlig et al., 1992). Finally, in the present study, caffeine antagonized the effects of the A_1 receptor agonist CPA in a concentration dependent manner (data not shown). Therefore, a more likely explanation is that perfusion of the adenosine transporter inhibitors produced localized increases in endogenous extracellular adenosine levels which inhibited evoked ACh release via the A_1 receptor.

Adenosinergic sleep homeostasis: A cholinergic link.

The present results, in combination with previous studies, suggest that adenosine may play an important role in regulating cortical excitability, and possibly behavioural state, by acting within both the basal forebrain and the cortex to inhibit cholinergic transmission. In the basal forebrain, adenosine postsynaptically inhibits presumably cholinergic, and possibly non-cholinergic, neurons by acting at A_1 receptors located on their somata. This would lead to a decrease in cortical ACh release. In the cortex, adenosine acts presynaptically at A_1 receptors to inhibit cortical ACh release, as demonstrated by the present study. Previous research has shown that ACh can inhibit specific potassium currents in cortical neurons which would increase neuronal excitability and contribute to the cortical desynchronization observed during wakefulness and rapid eye movement sleep (McCormick, 1990). Thus, reductions in cortical ACh levels, such as those observed during slow wave sleep, could diminish the potassium current blockade, resulting in a decrease in the excitability of cortical neurons. Inhibition of cortical ACh efflux as a result of both postsynaptic inhibition in the basal forebrain and presynaptic inhibition in the cortex would therefore regulate cortical arousal and behavioural state. This inhibition of cortical ACh release as a result of an increase in extracellular adenosine is likely to occur during prolonged wakefulness since extracellular adenosine levels selectively increase within the basal forebrain and the cortex during sleep deprivation (Strecker et al., 1999). Thus, the A_1 receptors located at the terminals, and probably cell bodies, of the cortically projecting

cholinergic basal forebrain neurons are likely to be one link between adenosinergic homeostatic sleep regulatory mechanisms and arousal-promoting systems of the brain.

Study 2: The Distribution of mRNA for the A_1 and A_{2A} Adenosine Receptors in the Rat Brain: An *In Situ* Hybridization Study

Substantial evidence suggests that adenosine regulates neuronal activity and neurotransmitter efflux within the central nervous system (Williams, 1987). Adenosine acts as a neuromodulator by binding to membrane bound G-protein coupled receptors. Within the brain, the A_1 and A_{2A} adenosine receptors are the most prevalent. These receptors have distinct pharmacological profiles and physiological effects. The A_1 adenosine receptor has been shown to be negatively coupled to adenyly cyclase and found throughout the brain with highest expression in the cortex, cerebellum, hippocampus, and thalamus (Reppert et al., 1991). In contrast, A_{2A} adenosine receptors activate adenylyl cyclase and are found in the striatum, nucleus accumbens, and the olfactory tubercle (Stehle et al., 1992).

This study attempted to confirm the anatomical distribution of the mRNA for the A_1 and A_{2A} adenosine receptors in the rat brain using *in situ* hybridization. Specifically, this study attempted to determine if mRNA for the A_1 adenosine receptor existed within cholinergic neurons of the basal forebrain. Based on the results of previous studies, it was hypothesized that mRNA for the A_1 adenosine receptor is most prevalent within the cortex of the rat brain but also present in the basal forebrain, while the mRNA for the A_{2A} adenosine receptor is highly expressed within the striatum.

Materials and Methods

Adult male Wistar rats (300 to 400 g; Charles River, Quebec, Canada) were given an overdose of pentobarbital and perfused transcardially with one of the following: (a) 50 ml 0.1 M phosphate buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB); (b) 50 ml of 0.1 M PBS

followed by 400 ml of 4% PFA in 0.1 M PB; or (c) 50 ml 0.1 M PBS followed by 200 ml of 0.05 M PBS containing 2% PFA and 0.2% glutaraldehyde. Brains were rapidly removed and either frozen immediately using crushed dry ice and then placed in a -80°C freezer or were postfixed in 4% PFA at 4°C for 3 to 5 hours and then transferred to 15% sucrose overnight at 4°C. Coronal sections (10 µm) were cut on a cryostat and thaw-mounted onto positively charged microscope slides (Superfrost Plus, Fisher Scientific) and stored for up to one week at -80°C.

The oligonucleotide probes for the A_1 adenosine receptor mRNA, 5′-CCCGTAGTACTTCTGGGGGTCACCGGAGGAGGCTGACACCTTTTTGTT-3′ (Mahan et al., 1991), and A_{2A} adenosine receptor mRNA, 5′-CGAGCCCGCTCCCCAGGCAGAGGCTGGCTCTCCATCTGCTTCAG-3′ (Stehle et al., 1992), were prepared by Genosys (Texas, U.S.A.). The probes were radiolabelled using materials obtained from a 3'-end labelling kit prepared by Amersham. Specifically, the hybridization probes were prepared by incubating diluted (2 pmol/ml) oligonucleotide probes (1.0 µl) with 5 µl [P^{33}]-ATP and 2.5 µl terminal transferase enzyme in 14 µl sterilized deionized water with 2.5 µl terminal transferase reaction buffer (Amersham) for 2 hours at 37°C. The reaction was terminated by diluting with 75 µl sterilized deionized water. Microspin G-25 columns (Pharmacia) were used to separate labelled from non-labelled probes.

The tissue was then prepared for hybridization with the radiolabelled oligonucleotide probe. Where appropriate, solutions were prepared from autoclaved deionized water pretreated with 0.1% diethylpyrocarbonate (DEPC; Sigma). The tissue was processed in slide containers which had been soaked in 1 N NaOH for at

least 30 minutes and then rinsed at least 4 times with deionized water and at least twice with 1 x PBS. Mounted sections were rinsed 3 x 5 min in 1 x PBS and then 4 x 5 min in 1 x sodium citrate buffer (SSC). The slides were then air-dried and then incubated overnight at 37°C with the radiolabelled probe that had been diluted to 1 x 10^6 cpm per ml hybridization buffer. The hybridization buffer consisted of deionized formamide (50%), 4 x SSC, 25 mM sodium phosphate, acid alkaline hydrolyzed salmon sperm DNA (0.02 mg/ml), dextran sulfate (0.1 g/ml), yeast tRNA (0.25 mg/ml), and 1% Dernhardt's reagent (0.02% bovine serum albumin, 0.02% polyvinvylpyrolidone and 0.02% Ficoll; Sigma).

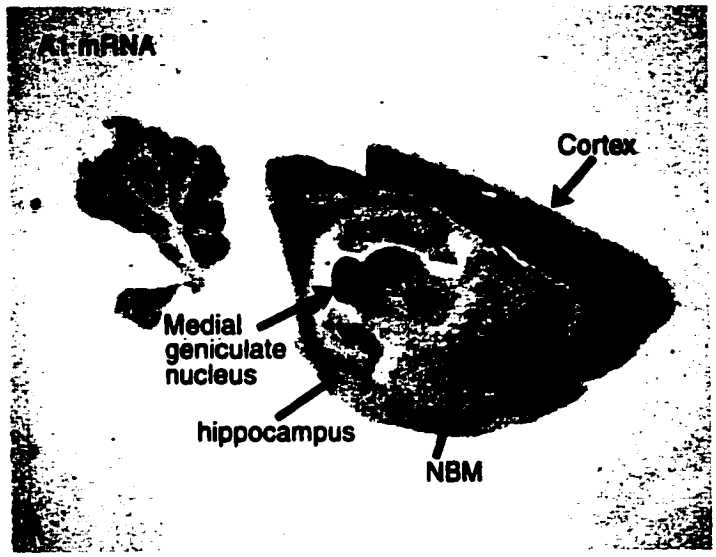
The following day, the slides were washed 4 x 15 min at 58°C in each of the following buffers: 1 x SSC with 1% sodium thiosulphate (Sigma), 0.5 x SSC with 0.5% sodium thiosulphate; and finally 0.25 x SSC with 0.25% sodium thiosulphate. Slides were then allowed to cool to room temperature and then quickly dipped in DEPC treated water to remove excess salt. The slides were air dried overnight at room temperature. The labelled slides were placed within an X-ray cassette and photographic film (Kodak) was placed over the slides with the emulsion side contacting the tissue. The cassette was then stored at -80°C for up to 4 weeks before the film was developed.

Results

Using radiolabelled antisense oligonucleotide probes, mRNA for the A_1 and A_{2A} adenosine receptors were shown to be widely distributed throughout the rat brain. The highest levels of mRNA for the A_1 adenosine receptor were found in the cortex,

cerebellum, hippocampus, and thalamus (Fig. 13A and 13B). Within the cortex, hybridization appeared in all cortical layers with slightly stronger labelling observed in layers 3 and 4 (Fig. 13B). Intense labelling in the hippocampus was found throughout the CA1, CA2, and CA3 fields and the dentate gyrus. Moderate hybridization was found in the basal forebrain (Fig 13A). In contrast, the distribution of the mRNA for the A_{2A} adenosine receptor was restricted to the caudate and putamen (Fig. 14).

Figure 13. Film autoradiograph showing the distribution of A_1 adenosine receptor mRNA in rat brain. (A) Sagittal and (B) coronal sections show the distribution of mRNA for the A_1 adenosine receptor. These autoradiographs demonstrate that mRNA for the A_1 adenosine receptor are found predominantly in the cortex, hippocampus, and cerebellum.


A1 mRNA
Cortex
Medial
geniculate
nucleus
hippocampus
NBM

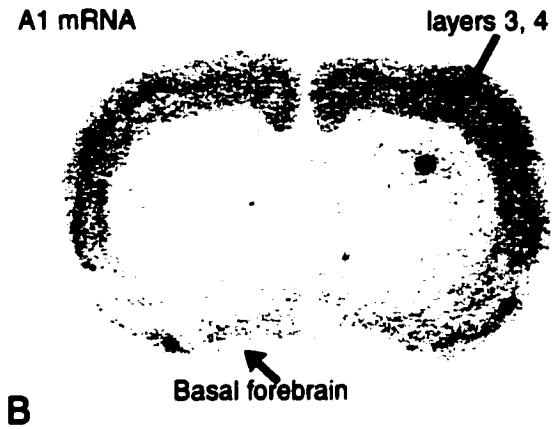

A1 mRNA
layers 3, 4
Basal forebrain
B

Figure 13

Figure 14. Film autoradiograph showing the distribution of A_{2A} adenosine receptor mRNA in rat brain. Coronal section shows the distribution of mRNA for the A_{2A} adenosine receptor. This autoradiograph demonstrates that mRNA for the A_{2A} adenosine receptor is found predominantly in the striatum.

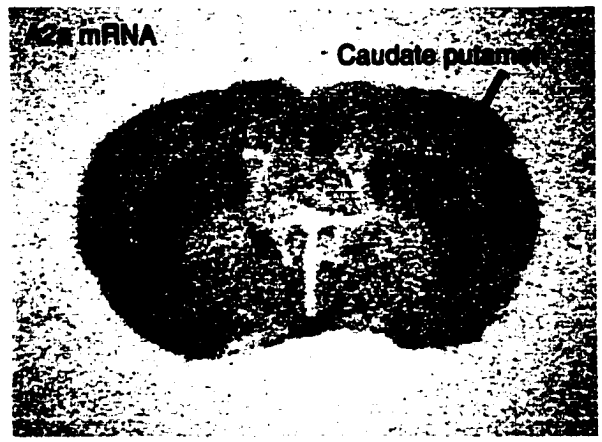


Figure 14

Discussion

Using *in situ* hybridization, the present study examined the distribution of the mRNA for both the A_1 and A_{2A} adenosine receptors. The A_1 receptor was found in a variety of brain regions. Specifically, A_1 adenosine receptor mRNA was found preferentially in the cortex, cerebellum, hippocampus, and thalamus. Moderate amounts of mRNA for the A_1 adenosine receptor were found in the basal forebrain. A_{2A} adenosine receptor mRNA was limited in distribution to the striatum and olfactory tubercle. The results of this study confirmed those reported previously (Mahan et al., 1991; Reppert et al., 1991; Stehle et al., 1992).

The original purpose of this study was to determine if mRNA for the A_1 and A_{2A} adenosine receptors was expressed by cholinergic neurons in the basal forebrain. This was attempted using both *in situ* hybridization and immunocytochemistry for ChAT. Unfortunately, this effort was unsuccessful due to technical difficulties.

General Discussion

Original hypotheses:

Study 1.

A. Intracortical delivery of the purine nucleoside adenosine will inhibit evoked cortical ACh release.

B. Intracortical delivery of a selective A_1 receptor agonist will inhibit evoked cortical ACh release

C. Intracortical delivery of a selective A_{2A} receptor agonist will have no effect on evoked cortical ACh release.

Study 2.

A. The mRNA for the A_1 adenosine receptor will be found primarily within the cortex, cerebellum, hippocampus, and thalamus.

B. The mRNA for the A_{2A} adenosine receptor will be found primarily within the striatum.

The results described in this chapter demonstrate that intracortical infusion of adenosine by reverse dialysis inhibits evoked cortical ACh release and that this effect is mediated by the A_1 adenosine receptor. The in situ hybridization study demonstrated that mRNA for the A_1 adenosine receptor is highly expressed within the neocortex, hippocampus, thalamus, and cerebellum of the rat and moderately expressed in the basal

forebrain. Expression of mRNA for the A_{2A} adenosine receptor is highest within the striatum. These results support my original hypotheses.

The results presented in study 1 suggest that adenosine regulates neurotransmitter release. Adenosine may attenuate neurotransmitter release by directly affecting the intracellular machinery involved in exocytosis, activating K^+ channels, and/or inhibiting Ca^{2+} channels (for a review see Dunwiddie and Fredholm, 1997). Using cutaneous pectoris nerve-muscle preparations from frog, Silinsky (1984) examined the spontaneous release of ACh evoked by Ca^{2+} containing liposomes or lanthanum, both of which produce release independent of Ca^{2+} channel activation. It was determined that the spontaneous ACh efflux observed under these conditions could be antagonized by the adenosine receptor agonist 2-chloroadenosine. Results from rat hippocampal slice culture preparations have shown that adenosine application decreases the frequency of miniature excitatory postsynaptic currents but not the distribution of the recorded amplitudes (Scanziani et al., 1992). Further experiments determined that this result could not be antagonized by the Ca^{2+} channel blocker Cd^{2+} (Scanziani et al., 1992). Thus, the inhibition of spontaneous neurotransmitter release mediated by adenosine may be due to interference of the intracellular secretory process (Silinsky, 1986).

The inhibitory action of adenosine may also involve the activation of presynaptic K^+ currents that would hyperpolarize nerve terminals, shorten the length of the action potential, and decrease neurotransmitter release. Using rat brain synaptosomes, Michaelis et al. (1988) demonstrated that adenosine analogues could enhance K^+ conductance across the plasma membrane. *In vivo* studies using K^+

sensitive microelectrodes in the rat caudate nucleus demonstrated that application of adenosine elevated the extracellular K^+ concentration (Michaelis et al., 1988). However, it is difficult to conclude from this study whether the modulation of K^+ channels is occurring at presynaptic or postsynaptic membranes. Silinsky et al. (1990) have shown that adenosine analogues do not alter presynaptic K^+ currents in frog motor nerve endings. Adenosine has been shown to modulate K^+ conductance at postsynaptic sites but not at presynaptic terminals in the rat hippocampus (Trussell and Jackson, 1987).

Finally, adenosine mediated presynaptic inhibition of neurotransmitter release may be a direct result of alterations to Ca^{2+} channel activity (Mogul et al., 1993; Scholz and Miller, 1991; Umemiya and Berger, 1994). Activation of presynaptic A_1 receptors has been shown to be associated with decreased Ca^{2+} currents in hippocampal neurons of the rat (Wu and Saggau, 1994) and chick ciliary ganglion (Bennett and Ho, 1991; Yawo and Chuhma, 1993). These data suggest that the mechanisms by which adenosine acts to modulate neurotransmission may vary depending upon the tissue studied and may involve more than one intracellular signaling system. Further studies may determine the mechanism by which adenosine inhibits cortical ACh release.

CHAPTER III: REGULATION OF CORTICAL ACETYLCHOLINE RELEASE BY INTRACORTICAL IONOTROPIC GLUTAMATE RECEPTORS

Preface

As discussed in the previous chapter, factors such as adenosine may act directly on cholinergic terminals to regulate ACh release. Other neurotransmitters may modulate the release of ACh indirectly. Several neuromodulators have been shown to regulate cortical ACh release by a tetrodotoxin-sensitive mechanism including neurotensin (Lapchak et al., 1990), thyrotropin releasing hormone (Giovannini et al., 1991), serotonin (Consolo et al., 1994), and histamine (Blandina et al., 1996). Because these effects were sensitive to tetrodotoxin, the involvement of a polyneuronal circuit is likely. Activation of cortical noradrenergic receptors has been shown to decrease ACh efflux and facilitate GABA release in freely moving guinea pigs (Moroni et al., 1983). Beani et al. (1986) further characterized the inhibitory effects of noradrenergic receptor activation on ACh release *in vitro*. These authors demonstrated that norepinephrine regulates ACh efflux either by acting directly at noradrenergic receptors located on cholinergic terminals or indirectly via GABAergic neurons. Serotonin has also been shown to regulate cortical ACh release via a GABAergic intermediary. Ramírez et al. (1996) determined that $GABA_A$ receptor antagonists potentiated the release of cortical ACh induced by 5-HT_3 receptor blockade. This suggests that the two receptors act synergistically to regulate ACh efflux.

Studies examining the effects of the amino acid glutamate have yielded inconsistent results. Application of ionotropic glutamate receptor agonists has been shown to enhance ACh release from rat cortical slices (Lodge and Johnston, 1985; Ulus et al., 1992). However, Hasegawa et al. (1993) have shown *in vivo* that local

delivery of N-methyl-D-aspartate (NMDA) to the cortex reduced ACh release induced by systemic MK-801, an NMDA receptor antagonist. This suggests that activation of intracortical glutamate receptors inhibits ACh release. The discrepancy between *in vitro* and *in vivo* studies requires further investigation.

The study presented in this chapter attempted to examine and pharmacologically characterize the effect of glutamate on cortical ACh release evoked by electrical stimulation of the PPT.

Study 3: Inhibition of Synaptically Evoked Cortical Acetylcholine Release by Intracortical Glutamate: Involvement of GABAergic Neurons

Cortical acetylcholine (ACh) has been implicated in attention (for a review, see Sarter and Bruno, 2000; Acquas et al., 1996), plasticity (Baskerville et al., 1997), facilitation of sensory information processing (Rasmusson, 1993), and cortical activation during wakefulness and rapid eye movement sleep (Jasper and Tessier, 1971). Increases in cortical ACh efflux alter the properties of postsynaptic neurons and their responses to other inputs. Local delivery of ACh enhances the response of cortical neurons to appropriate modality-specific stimuli in somatosensory (Donoghue and Carroll, 1987; Metherate et al., 1987), auditory (Metherate and Weinberger, 1990), and visual cortices (Sillito and Kemp, 1983). This suggests that factors that regulate cortical ACh efflux could influence cognitive processes and behaviour.

Cortical ACh is derived from the terminals of cholinergic neurons whose cell bodies are located within the basal forebrain (for a review, see Semba and Fibiger, 1989). Therefore, the release of cortical ACh can be modulated at two levels. First, cortical ACh release depends on the activity of cholinergic cells within the basal forebrain. Activation of cholinergic neurons by direct electrical stimulation (Rasmusson et al., 1992), stimulation of afferents to the basal forebrain (Rasmusson et al., 1994), or infusion of transmitters or analogs into the basal forebrain (Casamenti et al., 1986; Kurosawa et al., 1989; Bertorelli et al., 1991) can alter cortical ACh release *in vivo*. A second mechanism for modulating ACh efflux is through activation of presynaptic receptors located on intracortical cholinergic terminals. Previous *in vivo* studies have suggested that a number of transmitters and modulators regulate ACh efflux presynaptically, including serotonin (Crespi et al., 1997), adenosine (Materi et al., 2000), neurotensin (Lapchak et al., 1990), noradrenaline (Beani et al.,

1986; Tellez et al., 1997), GABA (Giorgetti et al., 2000), and ACh (Marchi and Raiteri, 1985; Vannucchi and Pepeu, 1995).

In light of the fact that the cortex receives dense glutamatergic inputs from a number of subcortical regions including the thalamus, and the increasing evidence for extrasynaptic spillover of glutamate (for a review, see Bergles et al., 1999; Mitchell and Silver, 2000), the possibility that this amino acid modulates cortical ACh release is worth considering. Studies using *in vivo* microdialysis in unanesthetized rats demonstrated that intraperitoneal delivery of an NMDA receptor antagonist increased cortical ACh release, and this increase was antagonized by intracortical infusion of N-methyl-D-aspartate (NMDA; Hasegawa et al., 1993). This result suggests that activation of cortical NMDA receptors might inhibit ACh release. However, *in vitro* studies using rat cortical slices have demonstrated that NMDA administration evokes ACh release (Lodge and Johnston, 1985; Ulus et al., 1992). Since the level of cortical ACh efflux could have profound effects on behaviour, this discrepancy requires further investigation.

To understand the role of glutamate in presynaptic modulation of cortical ACh release, the present study examined the effects of locally applied glutamate and specific receptor agonists and antagonists on ACh efflux using an established model of brainstem stimulation-induced cortical ACh release (Rasmusson et al., 1994). Preliminary results have been reported in abstract form (Materi and Semba, 1999).

Materials and Methods

All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care. Data were collected from 125 male Wistar rats (300 to 500 g) anesthetized with 1.4 g/kg (i.p.) urethane. The experimental protocol employed was outlined in our previous study (Materi et al., 2000) with slight modifications. Briefly, a microdialysis probe (membrane length: 2 mm; 0.5 mm outer diameter; molecular cutoff: 30 kDa; Bioanalytical Systems (BAS), West Lafayette, Indiana, USA) was lowered vertically into the barrel field of somatosensory cortex (coordinates: 1.4 mm posterior and 5.0 mm lateral to bregma; 2.8 mm ventral to the dural surface). The probe was continuously perfused at a rate of 2 μl/min with artificial cerebrospinal fluid to which atropine (10 μM: Sigma, Oakville, Ontario, Canada) and neostigmine methylsulfate (10 μM; Sigma) were added. The tip of a concentric bipolar stimulating electrode (250 μm tip diameter; Frederick Haer and Co., Brunswick, Maine, USA) was positioned within the PPT (coordinates: 8.4 mm posterior and 2.0 mm lateral to bregma; 6.7 mm ventral to the pial surface). Sample collection commenced following a 90 min equilibration period.

As in our previous study (Materi et al., 2000), each sample was collected over a 20-min period. Following three or four baseline samples, one sample was collected during electrical stimulation of the pedunculopontine tegmental nucleus (PPT). The PPT was stimulated using a one second stimulus train (0.2 ms, 100 Hz, 400 μA) applied once per min for 20 min (Rasmusson et al., 1994). Following this, four samples were collected during which the amount of ACh present in the cortex returned to baseline levels. The dialysis perfusate was then switched to perfusate

containing the drug being tested and this perfusate remained present for the remainder of the experiment. One sample collected in the presence of the drug was used to determine the effect of the drug on spontaneous ACh release. Following this, the PPT was stimulated using the same parameters as outlined above. This sample was used to determine the effect of the drug on synaptically evoked ACh release. Two or three post-stimulation samples were then collected. Control animals were not exposed to any drugs.

Acetylcholine assay

Each sample was analyzed for ACh content using high-performance liquid chromatography (HPLC) with electrochemical detection (Waters, Mississauga, Ontario, Canada) as described previously (Materi et al., 2000). Data were collected and analyzed using Powerchrome software (Castle Hill, New South Wales, Australia). The system was calibrated for each experiment using standard solutions containing 1, 2, and 4 pmol of ACh.

Adenosine assay

The samples derived from experiments examining the effects of glutamate alone or the simultaneous delivery of glutamate and caffeine were assayed for both ACh and adenosine. To obtain a sufficient volume for each assay, these samples were diluted with artificial cerebrospinal fluid (1:1 v/v), mixed vigorously, and then divided equally into two halves. One half was assayed for ACh content as outlined above. The second half was assayed for adenosine content using the protocol outlined by Pazzagli et al.

(1994) with modifications. Briefly, for each microliter of sample to be analyzed for adenosine, 0.125 µl of chloroacetaldehyde (4.5%) was added. The adenosine/chloroacetaldehyde sample was then tightly sealed and placed in boiling water for 20 min. Following this, the samples were stored for up to one week at -20°C. Each sample was assayed using HPLC with a Nova-Pak C_{18} column (4 µm; Waters) and fluorescence detection (Waters). The mobile phase consisted of a 50 mM acetate buffer (pH adjusted to 4.5 with acetic acid) containing 10% (v/v) acetonitrile and 2.4 mM 1-octanesulfonic acid sodium salt (Sigma) and was delivered at a rate of 0.7 ml/min. The amount of adenosine present in each sample was analyzed by comparison of peak heights to adenosine standards of known concentration.

Histology

The placements of the microdialysis probe and stimulating electrode were examined histologically as described previously (Materi et al., 2000). Forebrain sections containing the microdialysis probe track were stained with cresyl violet, and brainstem sections containing the stimulating electrode track were processed histochemically for nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity, a selective marker for cholinergic neurons within the mesopontine tegmentum (Vincent et al., 1983).

Data analysis

Values were expressed as means ± S.E.M., and probabilities of less than 0.05 were considered statistically significant. Changes in spontaneous ACh release

due to drugs delivered intracortically by reverse dialysis were examined by comparing the first sample collected during drug delivery without PPT stimulation to the sample immediately preceding it. Differences in the amount of ACh collected were compared using a paired Student's *t*-test.

To examine the amount of evoked release, the absolute amount of ACh detected in the sample immediately prior to PPT stimulation was subtracted from the absolute amount of ACh detected during stimulation for both stimulation trials (E1 and E2), and the mean ratio of E2 to E1 was calculated for each group. The effect of drug application on synaptically evoked ACh release was investigated by comparing the E2/E1 ratio of the control group, which was not exposed to any drug, to the same ratio calculated from each experimental group. The differences in the ratios between the control and experimental groups were assessed by analysis of variance. Post-hoc comparisons were performed using Fisher's protected least significant difference test (PLSD).

Differences in cortical adenosine levels before and after delivery of glutamate, with or without caffeine, were determined using Student's *t*-test. Other specific comparisons are described in the results section.

Chemicals

The following chemicals were used: L-glutamic acid (Sigma); caffeine (BDH Chemicals, Toronto, Ontario, Canada); L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L-*trans*-2,4-PDC; Tocris, Ballwin, MO, U.S.A.); N-methyl-D-aspartic acid (NMDA; Research Biochemicals International (RBI), Natick, MA, U.S.A.); α-amino-

3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA; Cambridge Research Biochemicals, Wilmington, DE, U.S.A.); 6,7-dinitroquinoxaline-2,3-dione (DNQX; RBI); (±)-3-(2-carboxypiperazin-4yl)-propyl-1-phosphonic acid (CPP; RBI); (-)-bicuculline methiodide (RBI); phaclofen (RBI); and 3-hydroxy-5-aminomethylisoxazole hydrobromide (muscimol; RBI). All of these drugs were dissolved in perfusate with the exception of DNQX. This drugwas dissolved initially in dimethylsulfoxide (DMSO; Sigma) and then diluted to the desired concentration with perfusate yielding a final DMSO concentration of 1%. We demonstrated in a previous study (Materi et al., 2000) that 1% DMSO has no effect on either spontaneous or PPT-stimulation evoked cortical ACh release. As in previous studies using microdialysis (e.g., Materi et al., 2000), concentrations used were approximately ten times greater than those reported in *in vitro* studies measuring neurotransmitter release from brain slices. This was necessary due to the incomplete permeability of the microdialysis membrane, and the poor diffusion of some analytes through tissue.

Results

Spontaneous vs. PPT stimulation evoked cortical acetylcholine release

The mean basal amount of ACh present in the cortex prior to the first PPT stimulation was 0.49 ± 0.06 pmol per 20-min sample. During stimulation of the PPT, the amount of ACh collected from the cortex increased significantly (t_{124} = 12.21, P<0.0001) to an average of 2.41 ± 0.20 pmol per 20-min sample. These values were comparable to those reported in previous studies (Rasmusson et al., 1994; Materi et

al., 2000). This confirms that electrical stimulation of the PPT is a highly effective method for increasing cortical ACh release *in vivo*.

The effects of repeated PPT stimulation on cortical ACh release were examined in six control animals. The amount of cortical ACh released during the second stimulation was slightly, but not significantly, greater than the amount released during the first stimulation (E2/E1 = 1.16 ± 0.12). This suggests that repeated exposure to electrical stimulation does not damage the PPT or alter the ACh releasing ability of basal forebrain neurons. Any observed changes to the E2/E1 ratio in the experimental groups must therefore be due to local effects of the drugs delivered by reverse dialysis.

Glutamate inhibits evoked acetylcholine efflux in the cortex

To examine the effect of the glutamate on spontaneous ACh release, the first sample collected during drug delivery was compared to the sample obtained immediately prior to it. Glutamate (0.1, 1, or 10 mM) had no significant effect on spontaneous cortical ACh release.

The effects of glutamate on PPT stimulation evoked cortical ACh release were determined by comparing the E2/E1 ratios calculated from the groups exposed to different concentrations of glutamate to the same ratio calculated from the control group. As shown in Figure 15, glutamate produced a concentration-dependent decrease in evoked cortical ACh release. While application of 0.1 mM glutamate did not alter evoked ACh efflux, delivery of 1 mM and 10 mM glutamate yielded mean E2/E1 ratios that were 62% and 32% of the control value, respectively, representing a

significant decrease in evoked ACh release ($F_{3, 19} = 6.89$, $P<0.01$; Fisher's PLSD: control vs. 1 mM and 10 mM glutamate respectively, $P<0.05$). This suggests that exogenously applied glutamate acts intracortically to inhibit evoked ACh release.

Inhibition of endogenous glutamate uptake reduces evoked ACh release

A previous microdialysis study demonstrated that infusion of the glutamate transport blocker L-*trans*-2, 4-PDC increased cortical glutamate levels *in vivo* (Semba and Wakuta, 1998). We found that infusion of L-*trans*-2,4-PDC into the cortex did not alter spontaneous ACh release, but significantly reduced PPT stimulation induced ACh release (t_8=3.21, $P<0.05$; Fig. 15). The mean E2/E1 ratio calculated from the group exposed to the glutamate uptake blocker was 47% of the control value, comparable to the effects of exogenous glutamate described above. Thus, the effect of exogenous glutamate on cortical ACh efflux was mimicked by the glutamate uptake inhibitor L-*trans*-2,4-PDC.

Ionotropic glutamate receptors mediate the inhibitory effects of glutamate on evoked ACh release

To determine which receptors mediate glutamate-induced inhibition of ACh release, the effects of selective ionotropic glutamate receptor agonists and antagonists were examined. None of these drugs affected spontaneous cortical ACh efflux. As shown in Figure 16A, delivery of 1mM NMDA (but not 0.025, 0.1, or 0.3 mM) by reverse dialysis significantly reduced evoked cortical ACh release ($F_{4, 21}$ = 4.97, $P<0.01$; Fisher's PLSD: control vs. 1 mM NMDA, $P< 0.01$). AMPA (100 μM,

but not 10 μM) also significantly reduced cortical ACh release evoked by PPT stimulation ($F_{2, 12} = 5.56$, $P<0.05$; Fisher's PLSD: control vs. 100 μM AMPA, $P<0.01$; Fig. 16B). This suggests that both NMDA and AMPA receptors are involved in the glutamate-induced inhibition of evoked ACh release.

To test this possibility further, the effect of glutamate (1 mM) on ACh efflux was tested in the presence of the non-NMDA receptor antagonist DNQX or the NMDA receptor antagonist CPP. Infusion of DNQX (0.1 mM) or CPP (0.1 mM) alone did not affect spontaneous or evoked ACh release (Fig. 17).

Simultaneous delivery of DNQX and 1 mM glutamate produced a mean E2/E1 ratio that was 25% greater than the same ratio calculated from the group that received glutamate alone (Fig. 17). While this difference was not statistically significant, this ratio was also not significantly different from the ratio calculated from the control group. Higher concentrations of DNQX were not used due to the possibility of non-specific effects (Drejer and Honoré, 1988). These results suggest that infusion of 0.1 mM DNQX only partially antagonized the inhibitory effect of glutamate

As shown in Figure 17, CPP fully antagonized the inhibitory effects of 1 mM glutamate on evoked cortical ACh release. The E2/E1 ratio obtained from the group exposed to both CPP and glutamate was significantly greater than the same ratio obtained from the group exposed to glutamate alone, but not significantly different from the control value ($F_{2, 14} = 3.78$, $P<0.05$; Fisher's PLSD: 1 mM glutamate vs. 1 mM glutamate plus 0.1 mM CPP, $P<0.05$).

The inhibitory effects of glutamate are not mediated by activation of adenosine receptors

Glutamate is known to increase extracellular adenosine concentrations in the cortex (Hoehn and White, 1989; Bennett et al., 1999), and we recently demonstrated that adenosine inhibits evoked cortical ACh release in urethane-anesthetized rats (Materi et al., 2000). To determine if the inhibitory response to glutamate was due to an increase in extracellular adenosine levels and subsequent activation of adenosine receptors, the effect of glutamate in the presence of the broad spectrum adenosine receptor antagonist caffeine was examined. These samples were assayed for both ACh and adenosine content.

In addition to inhibiting evoked ACh release as described above, glutamate (1 and 10 mM) produced a significant increase in extracellular adenosine levels (Fig. 18A). Specifically, glutamate (1 mM) increased basal adenosine levels from 19.2 pmol to 50.9 pmol per 20-min sample ($t_4 = 4.72$, $P<0.01$). Similarly, samples collected prior to delivery of 10 mM glutamate contained an average of 19.8 pmol per 20-min sample and this increased significantly to 193.5 pmol per 20-min sample following drug delivery ($t_5 = 3.88$, $P<0.05$).

Caffeine (0.5 mM) did not antagonize the inhibitory effects of glutamate (1 and 10 mM) on evoked ACh release (see Fig. 18B). The E2/E1 ratios calculated from the groups exposed to both caffeine and glutamate were similar to those calculated from the groups exposed to glutamate alone (Fig. 15). Administration of caffeine did not block the significant increase in extracellular adenosine levels caused

by glutamate (caffeine plus 1 mM glutamate: $t_4 = 5.04$, $P<0.01$; caffeine plus 10 mM glutamate: $t_3 = 5.55$, $P<0.05$; data not shown).

Glutamate mediated inhibition of evoked ACh release involves GABAergic systems

Local GABAergic inhibition of cortical ACh release was reported recently (Giorgetti et al., 2000). To determine if extracellular GABA in the cortex is involved in the inhibition of ACh release, the effects of glutamate (1 mM) on evoked cortical ACh release were tested in the presence of the $GABA_A$ receptor antagonist bicuculline or the $GABA_B$ receptor antagonist phaclofen.

The $GABA_A$ receptor antagonist bicuculline (0.05 mM) affected spontaneous ACh release. Application of bicuculline (0.05 mM) significantly increased spontaneous cortical ACh release from an average basal release of 0.35 ± 0.05 pmol per 20-min to 0.91 ± 0.12 pmol per 20-min sample ($t_4 = 3.42$, $P<0.05$). This suggests the presence of tonic inhibition of cortical ACh release by $GABA_A$ receptor activation under the present experimental conditions.

The simultaneous delivery of bicuculline and glutamate did not alter spontaneous ACh release but did reverse the inhibitory effects of glutamate on evoked cortical ACh release (Fig. 19A). The ratio obtained from the group exposed to both bicuculline and glutamate was significantly greater than the ratio obtained from the 1 mM glutamate alone group ($F_{3, 18} = 3.57$, $P<0.05$; Fisher's PLSD: 1 mM glutamate vs. 1 mM glutamate and 0.05 mM bicuculline, $P<0.05$) but not significantly different from the control group ratio.

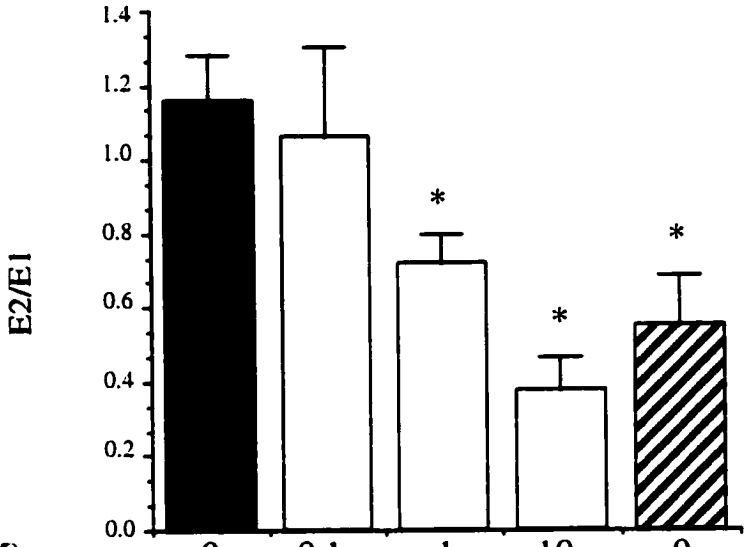
Delivery of the $GABA_B$ receptor antagonist phaclofen (0.05 mM) alone or in combination with 1 mM glutamate did not have any effect on basal ACh release. Simultaneous delivery of phaclofen and glutamate yielded a mean E2/E1 ratio that was 35% greater than that of the group exposed to glutamate alone but this was not statistically significant (Fig. 19A).

To examine the involvement of $GABA_A$ receptors further, the effects of the $GABA_A$ receptor agonist muscimol were tested. Application of muscimol by reverse dialysis did not alter spontaneous cortical ACh release. However, as shown in Figure 19B, 100 μM muscimol (but not 20 μM) significantly reduced synaptically evoked ACh efflux in the cortex ($F_{2, 14} = 4.44$, $P<0.05$; Fisher's PLSD: control vs. 100 μM muscimol, $P<0.05$).

Histology

The placement of the microdialyisis probe within the grey matter of the barrel field of the somatosensory cortex, and the tip of the stimulating electrode within 0.2 mm of the PPT was confirmed in every animal by histological examination.

Figure 15. Evoked cortical acetylcholine release in the presence of glutamate and the glutamate transport blocker L-*trans*-2, 4-PDC. Glutamate reduced PPT stimulation evoked acetylcholine release in a concentration dependent manner. Mean ± S.E.M.; $*P < 0.05$ vs control (black bar).

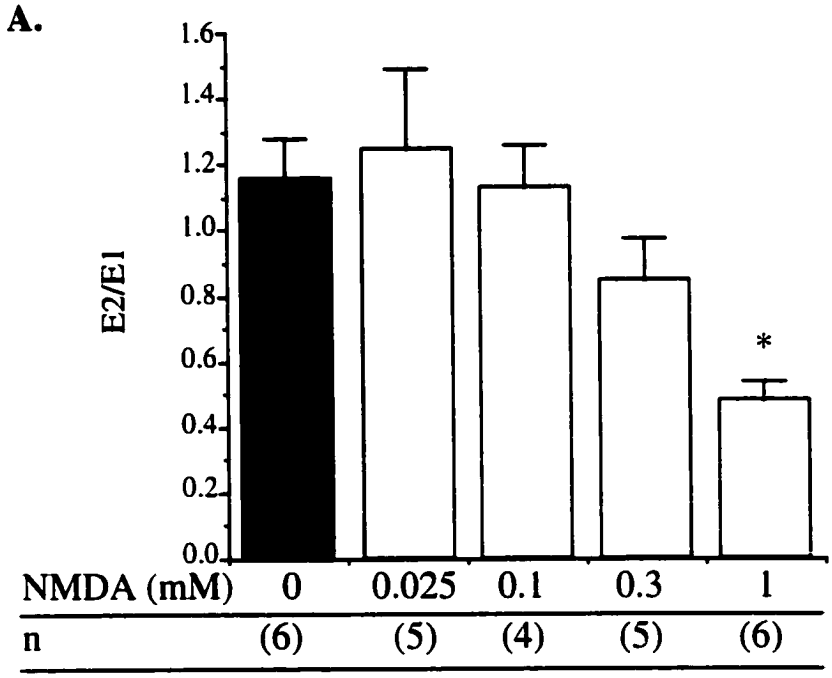


Glutamate (mM)	0	0.1	1	10	0
L-trans-2, 4-PDC (mM)	0	0	0	0	1
n	(6)	(5)	(6)	(6)	(4)

Figure 15

Figure 16. The effect of the selective ionotropic glutamate receptor agonists (A) NMDA and (B) AMPA on evoked cortical acetylcholine release. The inhibitory effects of glutamate were mimicked by both NMDA and AMPA. Mean ± S.E.M.; *$P < 0.05$ vs control (black bar).

A.


E2/E1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NMDA (mM) 0 0.025 0.1 0.3 1
n (6) (5) (4) (5) (6)

B.

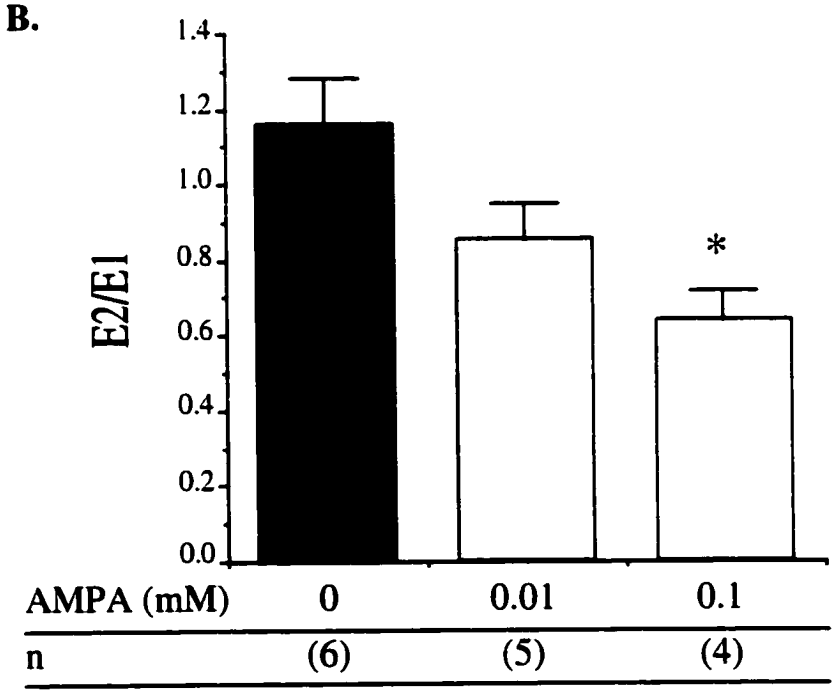
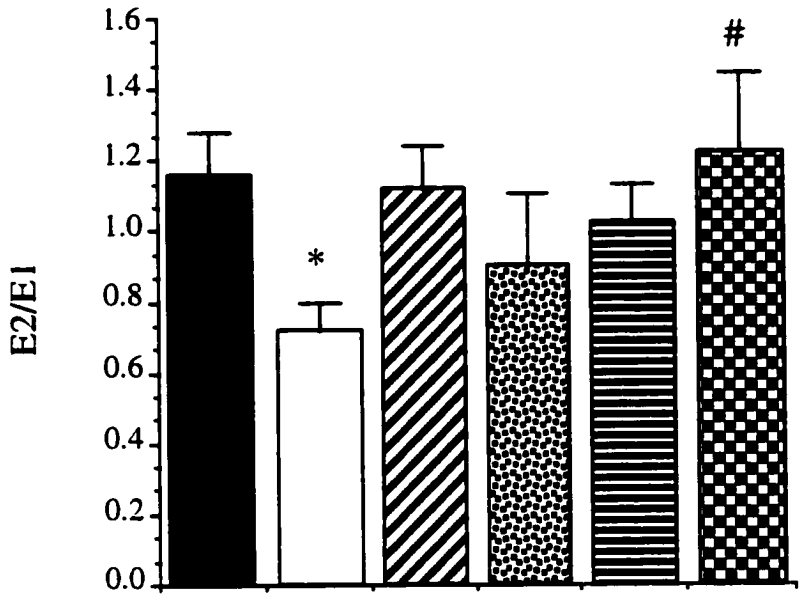

E2/E1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
AMPA (mM) 0 0.01 0.1
n (6) (5) (4)

Figure 16

Figure 17. The effect of selective ionotropic glutamate receptor antagonists alone or in combination with glutamate on acetylcholine release in the cortex. Mean ± S.E.M.; $^{*}P < 0.05$ vs control (black bar); $^{\#}P < 0.05$ vs 1 mM glutamate (white bar).

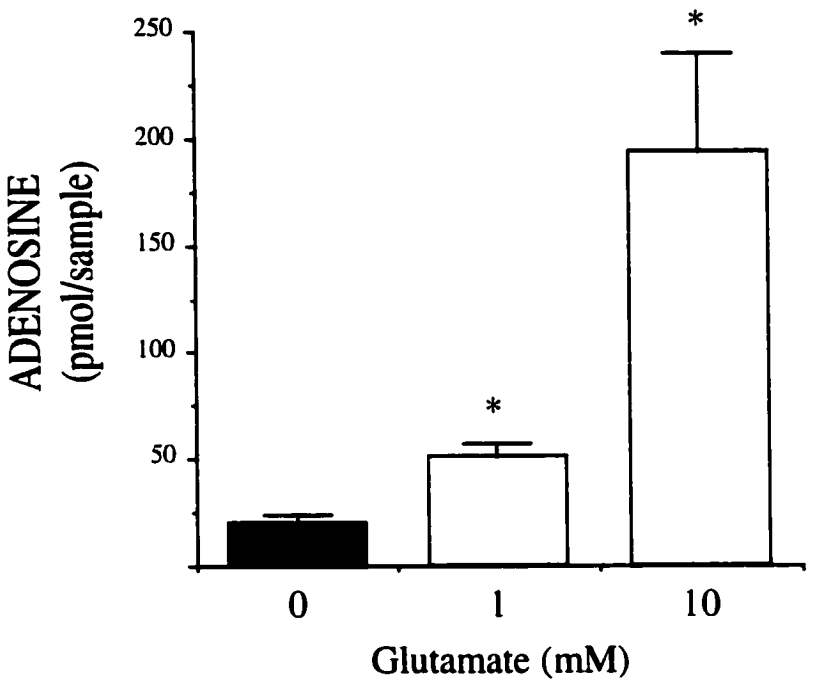


Glutamate (mM)	0	1	0	1	0	1
DNQX (mM)	0	0	0.1	0.1	0	0
CPP (mM)	0	0	0	0	0.1	0.1
n	(6)	(6)	(5)	(6)	(5)	(5)

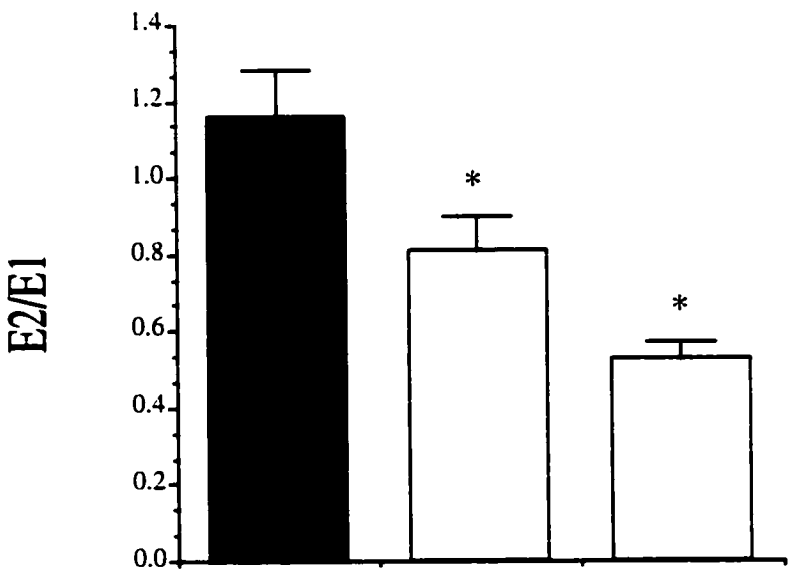
Figure 17

Figure 18. The effect of local delivery of glutamate on extracellular levels of adenosine in the cortex (A). Cortical acetylcholine release in the presence of the simultaneous infusion of glutamate and the non-selective adenosine receptor antagonist caffeine (B). Antagonizing adenosine receptors did not reverse the inhibitory effect of glutamate on evoked acetylcholine release. Mean ± S.E.M.; $*P < 0.05$ vs control (black bar).

A.



B.

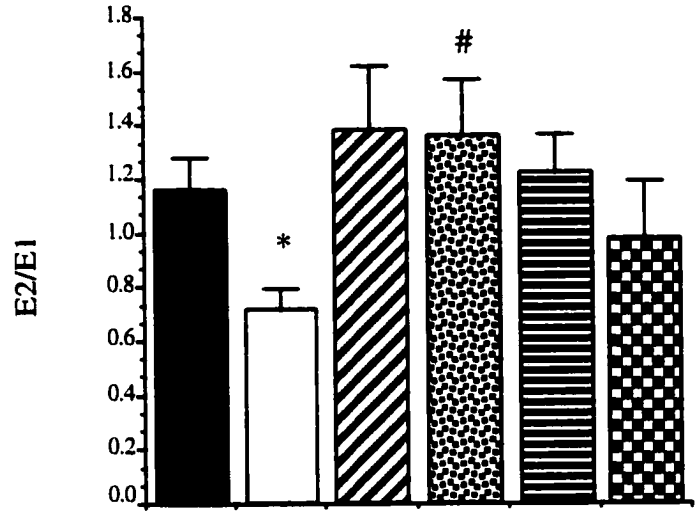


Glutamate (mM)	0	1	10
Caffeine (mM)	0	0.5	0.5
n	(6)	(5)	(4)

Figure 18

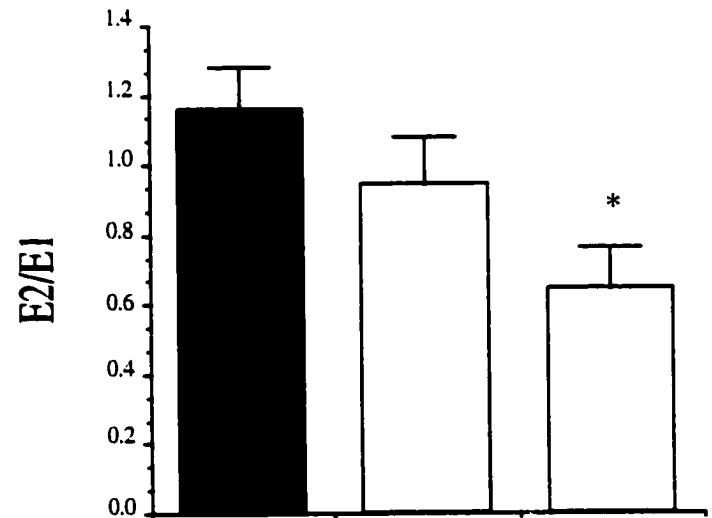
Figure 19. Cortical acetylcholine release in the presence of (A) glutamate alone or in combination with selective GABA receptor antagonists. (B) The effect of local administration of the $GABA_A$ receptor agonist muscimol on synaptically evoked acetylcholine release in the cortex. These results suggest that the inhibitory effect of glutamate on evoked acetylcholine release was mediated by GABAergic receptors. Mean ± S.E.M.; $^{*}P < 0.05$ vs control (black bar); $^{\#}P < 0.05$ vs 1 mM glutamate (white bar).

A.



Glutamate (mM)	0	1	0	1	0	1
Bicuculline (mM)	0	0	0.05	0.05	0	0
Phaclofen (mM)	0	0	0	0	0.05	0.05
n	(6)	(6)	(5)	(5)	(5)	(4)

B.



Muscimol (μM)	0	20	100
n	(6)	(5)	(6)

Figure 19

Discussion

The main findings of this study are: (1) glutamate has no effect on spontaneous cortical ACh release, but inhibits evoked ACh release in a concentration-dependent manner; (2) this inhibition can be mimicked by the glutamate transporter blocker L-*trans*-2, 4-PDC, as well as by the ionotropic glutamate receptor agonists NMDA and AMPA, and can be antagonized partially by DNQX and fully by CPP; and (3) glutamate induced inhibition of evoked cortical ACh efflux was fully antagonized by the $GABA_A$ receptor antagonist bicuculline and partially by the $GABA_B$ receptor antagonist phaclofen. These findings suggest that glutamate can inhibit evoked cortical ACh release via an indirect circuit involving GABAergic receptors.

Technical considerations

Advantages and limitations of the microdialysis technique and the use of urethane-anesthetized animals were discussed in the previous chapter. One additional concern is the potential excitotoxic damage of intracortical neurons by glutamate and its analogues. However, Vanický et al. (1998) examined rat cerebral cortex 24 hours following 20 minute delivery by reverse dialysis and found that NMDA caused lesions when administered at concentrations of 10 mM or higher. The highest concentration of NMDA used in the present study was ten-fold less and, therefore, damage due to NMDA is likely to be negligible.

Comparison with previous studies using ionotropic glutamate receptor agonists

As stated above, activation of cortical AMPA and NMDA receptors reduced synaptically evoked, but not spontaneous, cortical ACh efflux. These results are consistent with those reported by Hasegawa et al. (1993) who demonstrated in unanesthetized rats that local administration of NMDA to the cortex inhibited ACh efflux evoked by the systemic delivery of an NMDA receptor antagonist. In contrast, Lodge and Johnston (1985) reported that application of NMDA, quisqualate, and kainate enhanced basal ACh efflux from cortical slices. Ulus et al. (1992) also reported the enhancement of basal ACh release from rat cortical slices following NMDA application. It is not immediately clear why the results should vary depending upon whether ACh is collected from cortical slices, or from the cortex of an intact animal regardless of anesthesia. However, it is possible that the absence of subcortical neuronal systems and other factors associated with *in vitro* conditions contributed to the discrepancy.

The inhibitory effect of glutamate on evoked ACh release is not mediated by adenosine

We tested the possibility that intracortical glutamate inhibited evoked ACh release via an indirect mechanism that involved other neuromodulatory agents, namely adenosine and GABA. Glutamate is known to increase extracellular cortical adenosine levels (Hoehn and White, 1989; Bennett et al., 1999) and our previous study (Materi et al., 2000) demonstrated that adenosine inhibits evoked cortical ACh release in a concentration dependent manner in urethane-anesthetized rats via activation of the A_1 adenosine receptor. In the present study, however, despite

increased extracellular adenosine levels, simultaneous delivery of the broad-spectrum adenosine receptor antagonist caffeine did not reverse the effects of glutamate on PPT-stimulation evoked cortical ACh release. We have demonstrated previously that administration of 0.5 mM caffeine by reverse dialysis has no effect on spontaneous or PPT stimulation evoked cortical ACh release but was able to antagonize the inhibitory effects of adenosine transporter inhibitors on evoked ACh release (Materi et al., 2000). In addition to this effect, caffeine antagonized the inhibitory actions of the selective A_1 receptor agonist N^6-cyclopentyladenosine (1 μM; unpublished observations) on evoked ACh release. The activation of the A_1 adenosine receptor is known to inhibit adenylyl cyclase activity as well as calcium entry, and increase potassium conductance (for a review, see Williams, 1987). The fact that caffeine did not have any effect on glutamate induced inhibition of cortical ACh release suggests that glutamate induced inhibition is independent of, and more potent than, adenosine receptor-mediated inhibition.

Inhibition of ACh release by glutamate: Involvement of GABAergic neurons

The observed inhibitory effect of glutamate on evoked cortical ACh release is likely to involve intracortical GABAergic receptors. This conclusion is based on the observation that the inhibitory effects of glutamate on evoked ACh release could be completely reversed by the simultaneous delivery of the $GABA_A$ receptor antagonist bicuculline. Furthermore, administration of bicuculline enhanced spontaneous ACh release suggesting the presence of a tonic, GABA-mediated inhibitory tone. These results are consistent with the findings of Giorgetti et al.

(2000) who demonstrated GABA-induced inhibition of cortical ACh release in freely moving rats. Since glutamate had no effect on evoked ACh release in the presence of bicuculline, this suggests that glutamate does not act directly on intracortical cholinergic terminals.

The sensitivity of glutamate-induced inhibition of ACh release to GABA receptor antagonists suggests that the inhibition may be due to the effects of GABA released in response to activation of ionotropic glutamate receptors on GABAergic cortical neurons. Previous studies have shown that application of ionotropic glutamate receptor agonists increases GABA release *in vitro* from cultured cortical interneurons (Drejer et al., 1987) and *in vivo* from the hippocampus and striatum of freely moving rats (Hata et al., 1997). In the cortex, GABA may originate from intracortical interneurons or from the GABAergic basal forebrain projection neurons (Freund and Meskenaite, 1992). Therefore, it is likely that in the present study, infusion of ionotropic glutamate receptor agonists increased cortical GABA levels by activating GABAergic cortical neurons, and GABA in turn inhibited ACh release via presynaptic $GABA_A$, and possibly $GABA_B$, receptors on cholinergic terminals. Activation of the presynaptic $GABA_A$ receptors could reduce neurotransmitter efflux by hyperpolarizing nerve terminals via increased Cl^- conductance that, in turn, would reduce the probability of neurotransmitter release (for a review, see MacDermott et al., 1999). $GABA_B$ receptors might also be present on cholinergic terminals, and activation of these receptors by GABA could open potassium channels and/or suppress calcium channel activation, thus inhibiting ACh release. However, this inhibition does not appear to be as powerful as $GABA_A$ receptor-mediated inhibition

because the $GABA_B$ antagonist only partially blocked glutamate-evoked inhibition of ACh release. Activation of $GABA_B$ receptors on GABAergic neurons has been suggested to participate in autoinhibition of GABA release from intracortical neurons and result in increased cortical ACh release (Giorgetti et al., 2000). This mechanism is unlikely to play a crucial role in the present experimental condition, however, because administration of the $GABA_B$ receptor antagonist phaclofen partially reversed, rather than enhanced, the glutamate-induced inhibition of ACh release.

Previous *in vitro* studies reported that GABA has a facilitatory effect on cortical ACh release (Bianchi et al., 1982; Bonanno et al., 1991). However, our *in vivo* results demonstrate that GABA mediates not only a tonic inhibitory tone on cortical ACh release but is responsible for the reduction in evoked cortical ACh release. This is consistent with the recent report by Giorgetti et al. (2000). The reason for the discrepancy between the *in vitro* and *in vivo* results is unclear.

That GABA can mediate the indirect inhibitory actions of other neurotransmitters on cortical ACh efflux has been suggested. Previous studies have shown that the inhibitory effects of serotonin (Ramírez et al., 1996), histamine (Giorgetti et al., 1997), and norepinephrine (Beani et al., 1986) on cortical ACh release are mediated by GABA. It has also been demonstrated that glutamate can regulate neurotransmitter efflux via a polysynaptic circuit involving GABAergic neurons. Becquet et al. (1990), using a push-pull cannula, demonstrated that glutamate induced reduction in serotonin release from the caudate nucleus of rats was antagonized by bicuculline. A glutamatergic-GABAergic circuit within the septum of the rat has also been shown to regulate cortical ACh release (Giovannini et al., 1997).

Specifically, increased cortical ACh efflux due to local administration of CPP to the septum was blocked by the simultaneous delivery of muscimol (Giovannini et al., 1997). The present results suggest that a similar circuit is present within the cerebral cortex at the terminal level of basal forebrain cholinergic neurons.

Functional implications

Within the cortex, the effect of ACh is to increase overall excitability as demonstrated by changes in electroencephalographic activity and increases in the signal to noise ratio (for a review, see McCormick, 1990). ACh has been shown to induce long lasting changes in the response properties of cortical neurons to a variety of sensory inputs, including touch (Metherate et al., 1987), audition (Metherate and Weinberger, 1990), and vision (Sillito and Kemp, 1983). ACh is also suggested to mediate cortical plasticity (Sachdev et al., 1998) and reorganization (Kilgard and Merzenich, 1998). However, cortical ACh release and the associated plasticity would also need to be regulated and optimized for adaptive behaviour. Such regulation might be mediated by the glutamatergic inputs to GABAergic neurons from pyramidal neurons, excitatory intrinsic neurons, and/or thalamocortical afferents (Hensch et al., 1998; Douglas and Martin, 1998). Ludvig et al. (1992) have shown that administration of NMDA by reverse dialysis at concentrations equivalent to the ones used in the present study resulted in the depression of both behavioural and local cortical EEG activity. This effect may have, in part, been a result of changes to cortical ACh release. GABAergic interneurons in the cortex might therefore be at a

strategic site for interplay of various inputs to influence cortical ACh release indirectly.

General Discussion

Original hypotheses:

Study 3.

A. Intracortical delivery of the excitatory amino acid glutamate will enhance evoked cortical ACh release.

B. Glutamate induced enhancement of cortical ACh release will be mediated by ionotropic glutamate receptors.

The results described in this chapter demonstrate that exogenous glutamate applied locally within the somatosensory cortex of the urethane-anesthetized rat decreases ACh outflow. This was shown to be due to a polysynaptic pathway involving ionotropic glutamate receptors and GABAergic neurons. This finding stands in contrast to my original hypotheses.

The finding that glutamate application inhibited evoked cortical ACh release was unexpected. Mitchell (1963) demonstrated that sciatic nerve stimulation enhanced cortical ACh outflow from the somatosensory cortex. Sensory systems are known to project heavily to specific regions of the thalamus. Collier and Mitchell (1967) demonstrated that stimulation of the thalamic lateral geniculate nucleus increased ACh release from the visual cortex of rabbits. Since it is now known that thalamocortical projections are glutamatergic and not cholinergic, it was reasonable to suggest that enhanced glutamate release due to stimulation of the thalamocortical fibers may have enhanced cortical ACh outflow by acting at presynaptic glutamatergic

receptors on cholinergic terminals. In light of the fact that intracortical glutamate inhibits cortical ACh release, it is likely that the enhanced cortical ACh release observed by Mitchell (1963) and Collier and Mitchell (1967) may have been a result of antidromic activation of cholinergic basal forebrain neurons or indirect activation of basal forebrain afferents, which would indirectly increase cortical ACh release.

As mentioned above, GABAergic neurons may regulate the effects of a variety of neurotransmitters on cortical ACh release. Since ACh plays a role in regulating neuronal activity, glutamate-induced reductions in the outflow of this neurotransmitter may influence cortical EEG arousal. Using microdialysis combined with EEG recording, Ludvig et al. (1992) demonstrated that intracortical infusion of NMDA decreased EEG amplitude and reduced the power of all recorded frequency bands. In addition to these effects, NMDA decreased the occurrence of fast EEG activity and enhanced slow frequency rhythms in a concentration dependent manner (Ludvig et al., 1992). These effects may have been mediated by GABAergic neurons. Further testing may elucidate the mechanism by which intracortical ionotropic glutamate receptors regulate cortical spreading depression.

The data presented thus far suggest that various modulatory factors can regulate ACh release by direct interaction with receptors located on cholinergic synaptic terminals or via a polysynaptic circuit. Depending upon whether the cholinergic terminal is affected directly or following a series of synaptic events, the timing of the effect of the neuromodulator will vary. This may ultimately affect the activity of postsynaptic neurons and the level of cortical EEG activation.

Cortical ACh release may also be regulated at the level of the basal forebrain. To determine if a similar polysynaptic circuit involving GABAergic neurons exists at the level of the basal forebrain, the effect of ionotropic glutamate receptor activation within the basal forebrain was examined in the following chapter.

CHAPTER IV: MODULATION OF CORTICAL EEG AND ACETYLCHOLINE RELEASE BY ACTIVATION OF IONOTROPIC GLUTAMATE RECEPTORS IN THE BASAL FOREBRAIN

Preface

The excitatory effects of the amino acid glutamate have been known for decades. Curtis et al. (1959) demonstrated that glutamate elicits an excitatory response in spinal neurons. These findings were confirmed and extended in subsequent studies. However, glutamate was shown to evoke excitation in virtually all neurons tested which led some to speculate that this amino acid was inducing nonspecific effects (for a review, see Weinberg, 1999). Despite these initial misgivings, glutamate is now regarded as the primary excitatory neurotransmitter within the central nervous system. Glutamate found within the central nervous system is derived from glucose metabolism since this amino acid is unable to cross the blood-brain barrier (Deutch and Roth, 1999). Glutamate influences neuronal activity by binding to membrane-bound receptors. Similar to cholinergic receptors, both ionotropic and metabotropic glutamate receptors exist. The ionotropic glutamate receptors will be the focus of the present discussion.

To date, three classes of ionotropic glutamate receptors have been identified based upon pharmacological and genetic studies. These are the kainate, NMDA, and AMPA receptors. Of these, the effects of activation of AMPA and NMDA receptors within the basal forebrain on cortical EEG and ACh release were examined in the following study.

AMPA receptors are found throughout the central nervous system and mediate fast excitatory synaptic transmission following activation by glutamate (for a review, see Waxham, 1999). Various subunits (GluR1 - GluR4) coassemble to form functional AMPA receptor proteins that allow for the conductance of sodium and

potassium ions. Calcium ions are also conducted through these receptors if GluR2 subunits are present. Anatomical studies suggest that AMPA receptors are present within the basal forebrain. Immunocytochemical studies indicate that a large proportion of cholinergic neurons, identified by the presence of low affinity nerve growth factor receptor immunoreactivity, expressed the GluR4 subunit but the GluR1, GluR2, or GluR3 subunits were only moderately expressed in cholinergic basal forebrain neurons (Page and Everitt, 1995). Physiological studies have shown that AMPA receptors mediate an excitatory response in individual basal forebrain neurons. Using patch-clamp recording, Waters and Allen (1998) demonstrated that application of AMPA induced a pronounced inward current that exhibited rapid desensitization. Page et al. (1993) demonstrated that AMPA infusions into the basal forebrain induced *c-fos* in 71.5% of cholinergic neurons. This suggests that a large proportion of cholinergic neurons express functional AMPA receptors.

Three families of NMDA subunits (NR1, NR2A - NR2D, and NR3A) have been identified. Homomeric receptors comprised solely of the NR1 subunit possess the full complement of pharmacological features of an NMDA receptor (for a review, see Hollmann, 1999). This subunit is expressed throughout the brain while the NR2A - NR2D and NR3A subunits are differentially distributed. Activation of the NMDA receptor is tightly regulated since it has at least six distinct binding sites for endogenous ligands that influence the probability of channel opening (for a review, see Dingledine and McBain, 1999). Physiological studies suggest that this receptor is expressed by cholinergic neurons within the basal forebrain. Bath application of NMDA has been shown to modulate basal forebrain neuronal activity

(Khateb et al., 1995). Specifically, NMDA induced voltage-dependent rhythmic bursting in cholinergic neurons identified immunohistochemically for the ACh synthesizing enzyme ChAT (Khateb et al., 1995).

These data suggest that activation of ionotropic glutamate receptors by their agonists affects the activity of basal forebrain neurons. This activation may, therefore, influence both cortical EEG activity and ACh release. It has recently been reported that administration of both NMDA and AMPA into the basal forebrain increases the occurrence of high-frequency EEG activity (Cape and Jones, 2000). However, cortical ACh outflow was not measured. Since the basal forebrain contains a large number of non-cholinergic cortically projecting neurons that may also regulate cortical EEG activation, a comparison between ionotropic glutamate receptor mediated changes in EEG arousal and ACh release would shed light on this issue.

The following study examined the effect of intra-basal forebrain application of both NMDA and AMPA on cortical EEG activation and ACh outflow.

Study 4: Effects of AMPA and NMDA Infusions into the Basal Forebrain on Cortical Acetylcholine Release and EEG Activity

Cortical acetylcholine (ACh) has been linked to processes such as attention (Acquas et al., 1996), cortical reorganization (Baskerville et al., 1997), sensory information processing (Rasmusson, 1993), and the cortical activation observed during the behaviourally distinct states of waking and rapid-eye-movement sleep (Jasper & Tessier, 1971). ACh released in the cortex is derived primarily from the terminals of cholinergic neurons located within the nucleus basalis magnocellularis (NBM) of the basal forebrain (for a review, see Semba & Fibiger, 1989). Previous studies have shown that activation of the NBM by direct electrical stimulation (Rasmusson et al., 1992), infusion of neurotransmitters or analogues (Casamenti et al., 1986), or stimulation of afferents (Rasmusson et al., 1994, 1996) results in ACh efflux in the cortex.

Low-voltage, high-frequency electroencephalographic (EEG) activity has been considered to reflect cortical activation, and has been linked with increased ACh efflux in the cortex (Kanai & Szerb, 1965; Szerb, 1967). This suggests that basal forebrain cholinergic neurons contribute significantly to cortical EEG arousal. Immunohistochemically identified cholinergic neurons in the basal forebrain have been shown to increase their discharge rate and shift their pattern of discharge from tonic to burst firing during tail pinch-induced cortical activation which is characterized by the presence of both gamma and theta activity (Manns et al., 2000). Removal of cholinergic inputs to the cortex should presumably result in slowing of the EEG. This has been shown to occur. Immunotoxic lesions of cholinergic basal forebrain neurons have been shown to result in increased power to lower frequency band activity and reduced power to higher frequency band activity in freely moving

rats (Holschneider et al., 1999). However, these changes were relatively minor compared to the extent to which cholinergic input to the cortex was diminished.

The excitatory amino acid glutamate has been shown to regulate the activity of basal forebrain neurons. Iontophoretic application of glutamate strongly excited cortically projecting basal forebrain neurons *in vivo* (Lamour et al., 1986). Glutamate regulates neuronal activity by binding to membrane bound receptors including the ionotropic NMDA and AMPA receptors. Both NMDA and AMPA receptors have been shown to be present within the basal forebrain (Martin et al., 1993; Wenzel et al., 1995) but the precise localization of these receptors on neurochemically-identified neurons has not been determined. Nevertheless, results obtained from the following physiological studies suggest that some of these receptors are expressed by cholinergic neurons. Expression of the immediate early gene c-*fos*, which is used as a marker for neuronal activation, in cholinergic and non-cholinergic neurons of the basal forebrain can be induced by local infusions of either AMPA or NMDA (Page et al., 1993). Cholinergic basal forebrain neurons have been shown to be relatively susceptible to the toxic effects of high concentrations of AMPA (Page et al., 1991). Application of NMDA has also been shown to modulate the activity of cholinergic neurons. Low-threshold bursting activity in hyperpolarized cholinergic neurons can be induced by NMDA application *in vitro* (Khateb et al., 1995). In the absence of a hyperpolarizing current injection, NMDA application produced tonic firing in basal forebrain cholinergic neurons (Khateb et al., 1997).

Together, these data suggest that activation of ionotropic glutamate receptors within the basal forebrain modulates the activity of neurons within this

region. This change in activity at the neuronal level may induce measurable modifications in cortical EEG activity and ACh levels. Microinjections of glutamate into the NBM have been shown in anesthetized rats to elicit ACh release in the ipsilateral neocortex (Kurosawa et al., 1989). Cortical EEG activation and ACh release induced by electrical stimulation of the brainstem is reduced by infusion of a broad-spectrum glutamate receptor antagonist into the NBM (Rasmusson et al,. 1994). To characterize the glutamate receptors mediating this response, Rasmusson et al. (1996) examined the effects of selective ionotropic glutamate receptor antagonists applied to the NBM on brainstem stimulation-induced cortical activation and ACh release. It was demonstrated that application of an NMDA receptor antagonist preferentially inhibited ACh release with no effect on cortical activation while a non-NMDA receptor antagonist reduced cortical activation without significantly altering ACh release (Rasmusson et al., 1996). These results suggest that cortical activation is not exclusively dependent upon ACh release. However, electrical stimulation of the brainstem may induce cortical activation via other subcortical structures including the thalamus. It has been reported recently that application of AMPA and NMDA directly to the basal forebrain in freely moving rats increased occurrence of both theta and gamma activity while reducing low-frequency delta EEG activity (Cape and Jones, 2000). Cape and Jones (2000) also reported the expression of c-Fos protein in both cholinergic and non-cholinergic basal forebrain neurons following microinjections of NMDA. Cortical ACh release was not measured in that study.

To gain further insight into the role of ionotropic glutamate receptors in the basal forebrain in cortical ACh release and EEG activation, we infused selective

glutamate receptor agonists into the basal forebrain by reverse dialysis in urethane-anesthetized rats. Cortical EEG and ACh outflow were measured simultaneously. This allowed for the direct comparison between changes in cortical EEG and ACh release induced by NMDA and AMPA.

Materials and Methods

Subjects

All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care. Data were collected from 31 male Wistar rats (Charles River, St. Constant, Quebec. Canada) weighing between 250 to 500 g. Each animal was anesthetized with 1.4 g/kg urethane (i.p.) and given a supplemental dose, if necessary, to maintain areflexia prior to the equilibration period. No additional injections of anesthetic were required for the remainder of the experiment. The animal was placed in a stereotaxic frame with bregma and lambda on a horizontal plane and held there for the duration of the experiment. Surgery was carried out on the right side of the brain. The experimental setup used in this experiment is depicted in Figure 20.

Microdialysis

A microdialysis probe (2 mm membrane length, 0.5 mm outer diameter; Bioanalytical Systems (BAS), Indiana, USA) was lowered into the somatosensory cortex (coordinates: 1.4 mm posterior and 5 mm lateral to bregma) with the tip positioned 2.8 mm ventral to the dural surface. The probe was continuously perfused

at a rate of 2 μl/min with artificial cerebral spinal fluid (aCSF; 3 mM KCl, 125 mM NaCl, 1.3 mM $CaCl_2$, and 1 mM $MgSO_4$) to which atropine (10 μM; Sigma, Ontario, Canada) and neostigmine methylsulfate (10 μM; Sigma) were added.

A second microdialysis probe, identical in structure to the cortical probe, was inserted into the NBM at a 45° angle. It was positioned such that the tip of the probe rested 1.0 mm posterior, 1.8 mm lateral, and 7.8-8.0 mm ventral relative to bregma. This probe was continuously perfused with aCSF without atropine and neostigmine.

EEG recording and analysis

Cortical EEG activity was recorded using a transcortical bipolar silver wire electrode with a tip separation of approximately 2 mm. The electrode was positioned within 1 mm anterior to the cortical microdialysis probe. The EEG signal was amplified and filtered between 0.1 - 500 Hz using an A-M Systems AC amplifier (Washington, USA) and stored at a sampling rate of 136 Hz. Fifteen-second samples of EEG were collected every min using DataWave software (Colorado, USA) and analyzed off-line by fast-Fourier transform analysis.

Total power (0.5 - 45 Hz) was calculated as was the power for the delta (0.5 - 4 Hz), theta/alpha (4 - 12 Hz), low beta (12 - 20 Hz), and high beta (20 - 45 Hz) bands from artifact-free samples. Since large differences in absolute power between animals existed, a ratio of the absolute power of each frequency band to the total power was calculated for each 15-second sample to obtain a relative measure. These relative powers were averaged over each 20-minute microdialysis collection period so

that direct comparisons could be made between ACh release and cortical EEG activity.

Experimental schedule

A 60 min equilibration period was allowed to pass following insertion of the microdialysis probes and cortical EEG recording electrode. Two 20-min samples (baseline 1 and 2) were collected to measure spontaneous cortical ACh release and EEG activity. Then the NBM microdialysis probe perfusate was switched to aCSF containing a specific concentration of either AMPA (Tocris Cookson Inc., Bristol, UK) or NMDA (Research Biochemicals Inc., Massachusetts, USA) and the third sample was collected. Following this 20-minute period, the perfusate was switched back to aCSF that remained present for the duration of the experiment (recovery samples; additional 60 min).

Acetylcholine assay

Each sample was analyzed for ACh content using high-performance liquid chromatography with electrochemical detection (Waters, Ontario, Canada; Materi et al., 2000; see Chapter 2 (study 1), Materials and Methods). Mobile phase (50 mM Na_2HPO_4, 0.1 mM EDTA, 0.005% ProClin (BAS), pH adjusted to 8.5 with orthophosphoric acid) was delivered at a rate of 0.1 ml/min using an isocratic pump. ACh was separated from choline using a UniJet Microbore column (BAS), converted to hydrogen peroxide by a postcolumn immobilized enzyme reactor, and detected amperometrically by a platinum working electrode (oxidation potential: +650 mV).

Data were collected and analyzed using Powerchrome software (New South Wales, Australia). For each experiment, the system was calibrated using standards of known concentrations of ACh.

Statistical analysis

To determine the amount of change in cortical ACh release evoked by infusion of AMPA or NMDA into the basal forebrain, a ratio of the average amount of ACh collected during the third and fourth samples to the average amount of ACh collected during the two 20-min baseline samples was calculated for each animal. The third sample represents the period of drug delivery in the experimental groups and the fourth sample represents the first 20-min recovery period. Control animals were not exposed to any drugs. Averages over two sampling periods were used due to the apparently long lasting effects of the drugs that reflected diffusion and clearing of the drug. Ratios calculated from the experimental groups were compared to those calculated from the control group using a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test (PLSD).

To determine the effect of drug application to the basal forebrain on cortical EEG activity, statistics were performed on the average amount of relative power for each 20-min sample following square root transformation. This transformation was used due to the large difference in-group variance that was proportional to the group means. Changes across the 20-min sampling periods were assessed using a one-way ANOVA followed by Fisher's PLSD for each group.

Correlation between changes in ACh release and cortical EEG was assessed using simple linear regression. The change in ACh release was calculated by dividing the average amount of ACh release during the third (drug infusion) and fourth (first recovery) samples by the average amount of ACh release during the first two samples (baseline 1 and 2). The change in EEG was calculated by dividing the average relative power for each frequency bin during the third (drug infusion) and fourth (first recovery) samples by the average of the relative power of the appropriate frequency bin for the first two samples (baseline 1 and 2).

Histology

Following completion of the experiment, the animal was further anesthetized with an excess amount of sodium pentobarbital (i.p.) and perfused transcardially with 50 ml of 0.9% saline followed by 400 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed and postfixed overnight at 4°C and then transferred to 30% sucrose in 0.1 M phosphate buffer and stored at 4°C. Vibratome sections (60 μm) were cut throughout the regions containing the tracks of the EEG electrode and microdialysis probes and collected in 0.05 M Tris buffered saline (pH 7.4). Sections were mounted immediately on chrom-alum-gelatinized slides and allowed to air dry before staining with cresyl violet. Slides were then air-dried and coverslipped. For each animal, the locations of the EEG electrode and the two microdialysis probes were confirmed by comparison to the atlas of Paxinos and Watson (1998).

Figure 20. Schematic depicting the experimental design used in Study 4. Drugs were infused directly into the basal forebrain by reverse dialysis and cortical ACh was collected using a second microdialysis probe. Cortical EEG activity was recorded throughout the experiment. For details, see text.

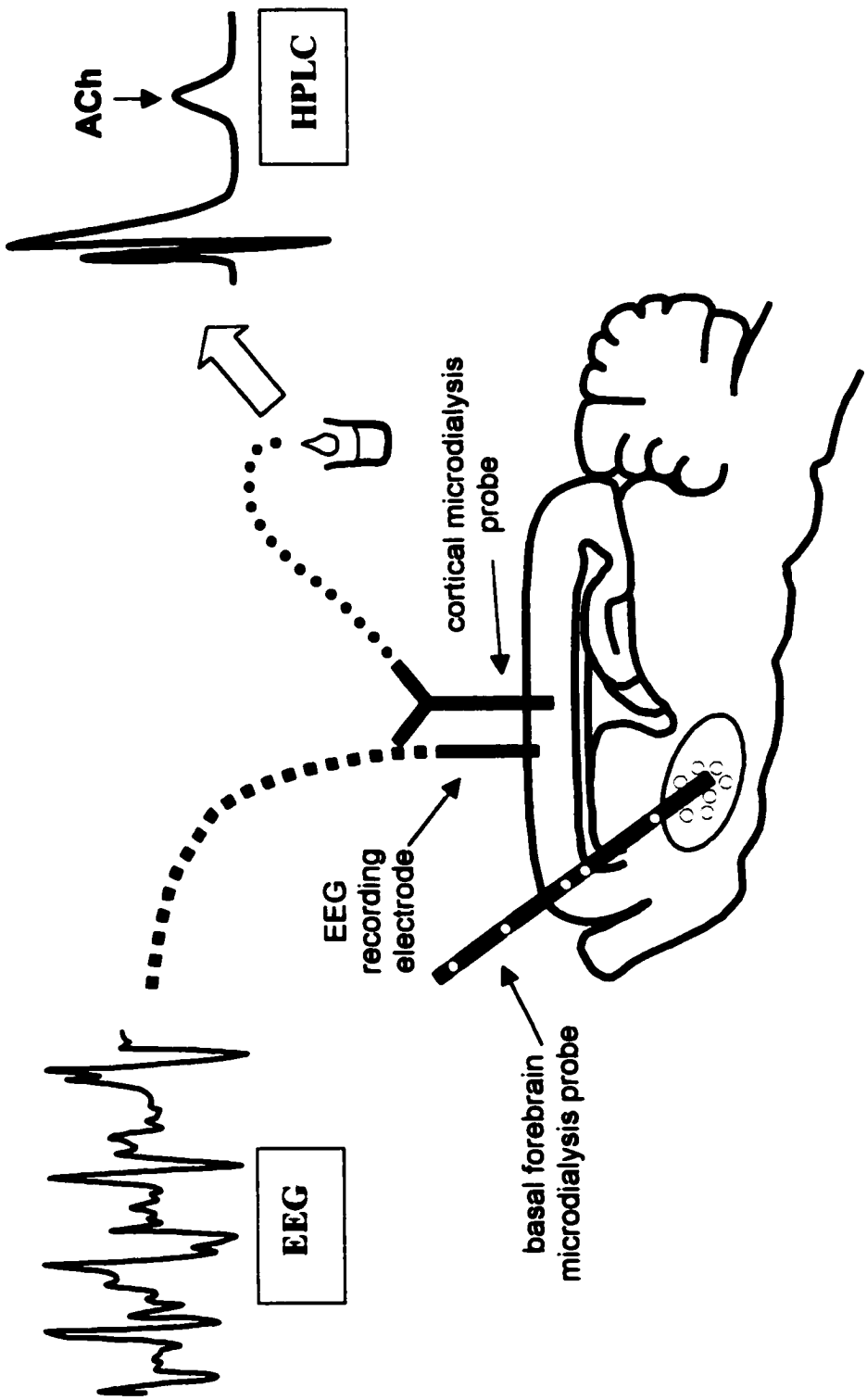

ACh
HPLC
cortical microdialysis probe
EEG recording electrode
basal forebrain microdialysis probe
EEG

Figure 20

Results

Baseline cortical ACh release and EEG activity

The average spontaneous cortical ACh release for the first two 20-min baseline samples was 0.20 ± 0.03 pmol per sample (mean ± S.E.M., $n = 30$). The average amount of ACh collected from control animals during the third sampling period was slightly, but not significantly, less (0.17 ± 0.04 pmol; $n = 6$). The ratio of the third sample to the average of the two previous baseline samples was 0.83 for the control group. During the remaining three samples, the amount of spontaneous cortical ACh release in the control group remained relatively stable (0.13 ± 0.03, 0.12 ± 0.03, and 0.24 ± 0.14 pmol/20 min, respectively). Statistical analysis using a one-way ANOVA did not reveal any difference among the six samples over the course of the experiment. These results suggest that spontaneous cortical ACh release was detectable and was relatively stable for the duration of the experiment in urethane-anesthetized rats.

EEG activity was also stable throughout the entire recording period for animals in the control group (Fig. 21). Delta activity (0.5 - 4 Hz) dominated the EEG during the two 20-min baseline samples for each animal (Figs. 21 and 22). Epochs dominated by high-frequency EEG activity were rarely observed, if at all.

Effects of AMPA on cortical ACh release and EEG

Infusion of 1 μM AMPA into the basal forebrain by reverse dialysis slightly, but not significantly, increased cortical ACh release. Application of AMPA (1μM) to the basal forebrain had no effect on cortical activation. However, the

overall trend of low and high beta activity appeared to mimic that of ACh release (Fig. 23).

Delivery of 10 μM AMPA to the basal forebrain by reverse dialysis significantly increased ACh release but did not significantly alter cortical EEG activation (Fig. 24). Again, the overall trend in increased ACh release and increased relative power of higher frequency activity were similar.

Cortical ACh outflow was significantly increased by infusion of 100 μM AMPA to the basal forebrain. Application of this drug also had a profound effect on the recorded cortical EEG. Specifically, infusion of 100 μM AMPA into the basal forebrain elicited low-voltage, high-frequency EEG (Fig. 25). Both low and high beta activity increased in response to infusion of 100 μM AMPA (Fig. 26). Similar to the observed ACh release, both low and high beta activity continued to rise following drug delivery. Compared to the amount of activity during the initial 40 min baseline period, the relative power of low beta activity during the second and third post-drug samples was significantly increased ($F_{5, 24} = 2.56$, Fisher's PLSD: baseline 1 vs recovery 1 and 2, baseline 2 vs recovery 1 and 2, $P < 0.05$). High beta activity increased significantly during the third post-drug sample ($F_{5, 24} = 1.73$, Fisher's PLSD: baseline 1 vs recovery 2, baseline 2 vs recovery 2, $P < 0.05$). There was no statistically significant change in theta/alpha or delta activity with 100 μM AMPA application.

Effects of NMDA on cortical ACh release and EEG

Infusion of 0.1 mM NMDA did not significantly affect cortical ACh outflow or EEG activity (Fig. 27). Application of 1 mM NMDA to the basal forebrain significantly increased cortical ACh release. Cortical EEG activity was not significantly altered by infusion of 1 mM NMDA despite the large increase in ACh release (Fig. 28).

Concentration-response relationships of the AMPA and NMDA effects

Changes in cortical ACh outflow evoked by drug application to the basal forebrain were determined by comparing the ratios of the average amount of ACh collected during the third and fourth samples to the average amount of ACh collected during the two 20-min baseline samples from the control group to those calculated from experimental groups. Infusion of AMPA into the basal forebrain by reverse dialysis increased cortical ACh release in a concentration-dependent manner (Fig. 29A). Application of either 10 μM or 100 μM AMPA resulted in a significant increase in cortical ACh efflux ($F_{3, 16} = 5.46$; Fisher's PLSD: control vs.10 or 100 μM AMPA, $P < 0.05$).

The application of NMDA to the basal forebrain increased cortical ACh efflux in a concentration-dependent manner. Cortical ACh release was significantly increased by infusion of 1 mM NMDA ($F_{2, 13} = 35.55$; Fisher's PLSD: control vs.1 mM NMDA, $P < 0.0001$) but not 0.1 mM NMDA (Fig. 29B).

A summary of the effects of AMPA on cortical EEG can be seen in Figure 30. While infusion of AMPA had little effect on lower-frequency band activity, this drug tended to increase the relative magnitude of both low and high beta activity.

The effects of NMDA on cortical EEG arousal are summarized in Figure 31. Similar to the effects observed during AMPA infusion, NMDA applied to the basal forebrain had little effect on delta or theta/alpha but tended to increase the relative power of both low and high beta activity.

Correlation between ACh release and the low and high frequency EEG activities

Regression analysis was conducted using the changes in ACh release and relative power of each frequency band collapsed across different drug treatment groups and controls. The results demonstrated the presence of a significant correlation between cortical ACh outflow and both low and high beta activity (low beta: $r = 0.52$, $P < 0.01$; high beta: $r = 0.66$, $P < 0.01$; Fig 32). No significant correlation was found between ACh release and delta or theta/alpha.

Histology

The placements of the EEG recording electrode (Fig. 33A) and cortical microdialysis probe (Fig. 33B) within the grey matter of the somatosensory cortex, and the tip of the second microdialysis probe within 0.2 mm of the nucleus basalis were confirmed in every animal by histological examination (Fig. 33B).

Figure 21. EEG traces taken from the cortex of a control animal not exposed to any drugs. These traces were taken during (A) the first sample, (B) the third sample, and (C) the final (sixth) sample of the experiment. The exact time of the recording is noted under each trace. Calibrations: 1 sec, 1 mV.

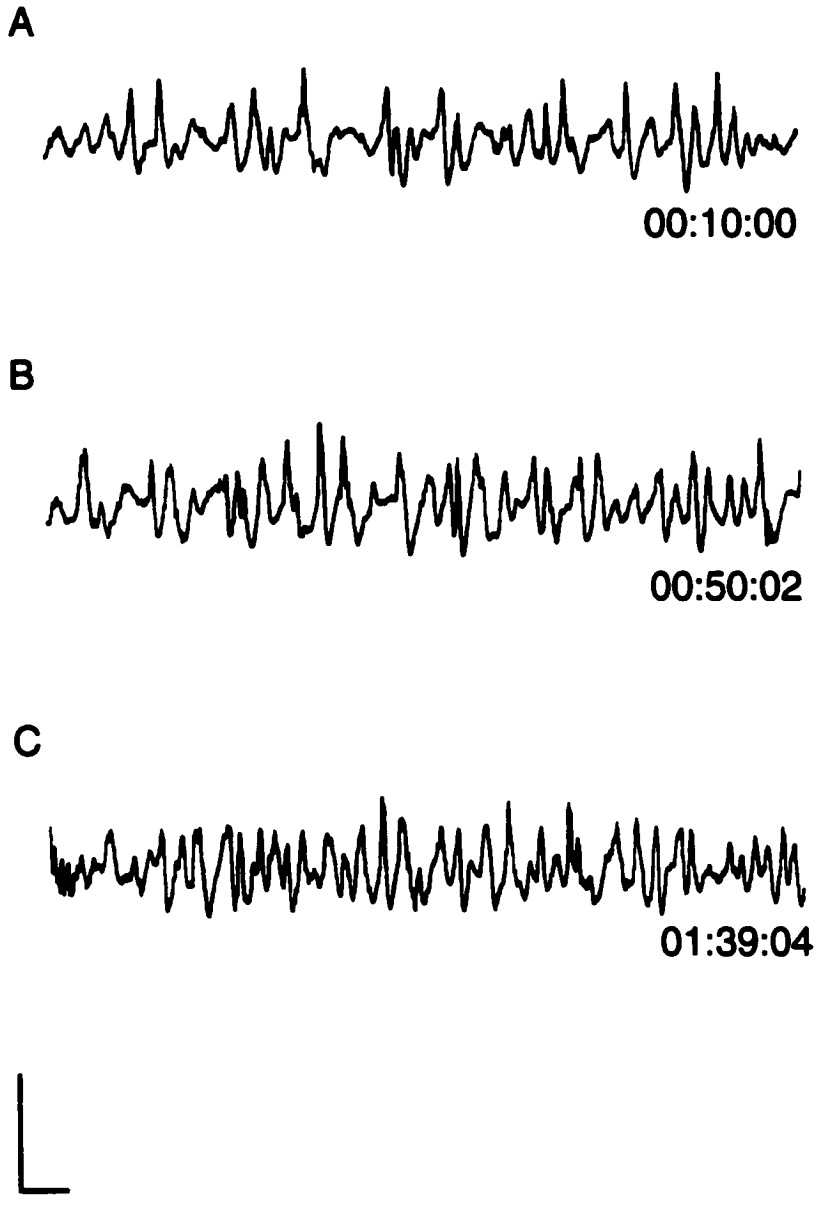


Figure 21

Figure 22. ACh release and the relative power of delta, theta/alpha, low beta, and high beta activity determined from spontaneous cortical EEG activity in urethane-anesthetized rats not exposed to any drugs (n = 6). Mean ± S.E.M. Abbreviations: B, baseline sample.

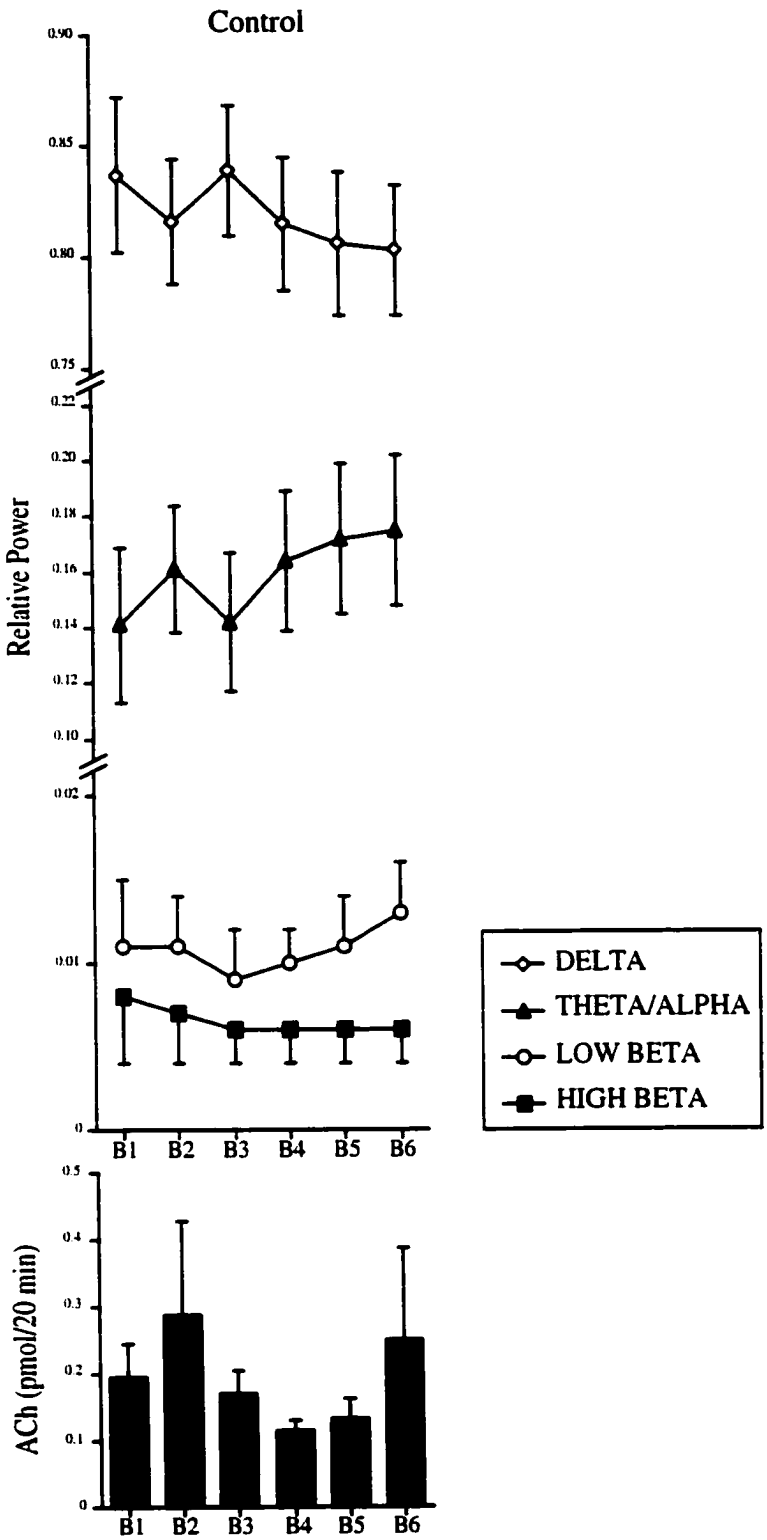

Control
Relative Power
0.90
0.85
0.80
0.75
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.02
0.01
0
B1
B2
B3
B4
B5
B6
DELTA
THETA/ALPHA
LOW BETA
HIGH BETA
ACh (pmol/20 min)
0.5
0.4
0.3
0.2
0.1
0
B1
B2
B3
B4
B5
B6

Figure 22

Figure 23. The effects of 1 μM AMPA on the relative power of delta, theta/alpha, low beta and high beta activity recorded from rat cortex and cortical ACh release (n = 4). Mean ± S.E.M. Abbreviations: B1, first 20-min baseline; B2, second 20-min baseline; D, drug infusion; R1, R2, and R3, the first, second, and third 20-min recovery samples, respectively (no drug present).

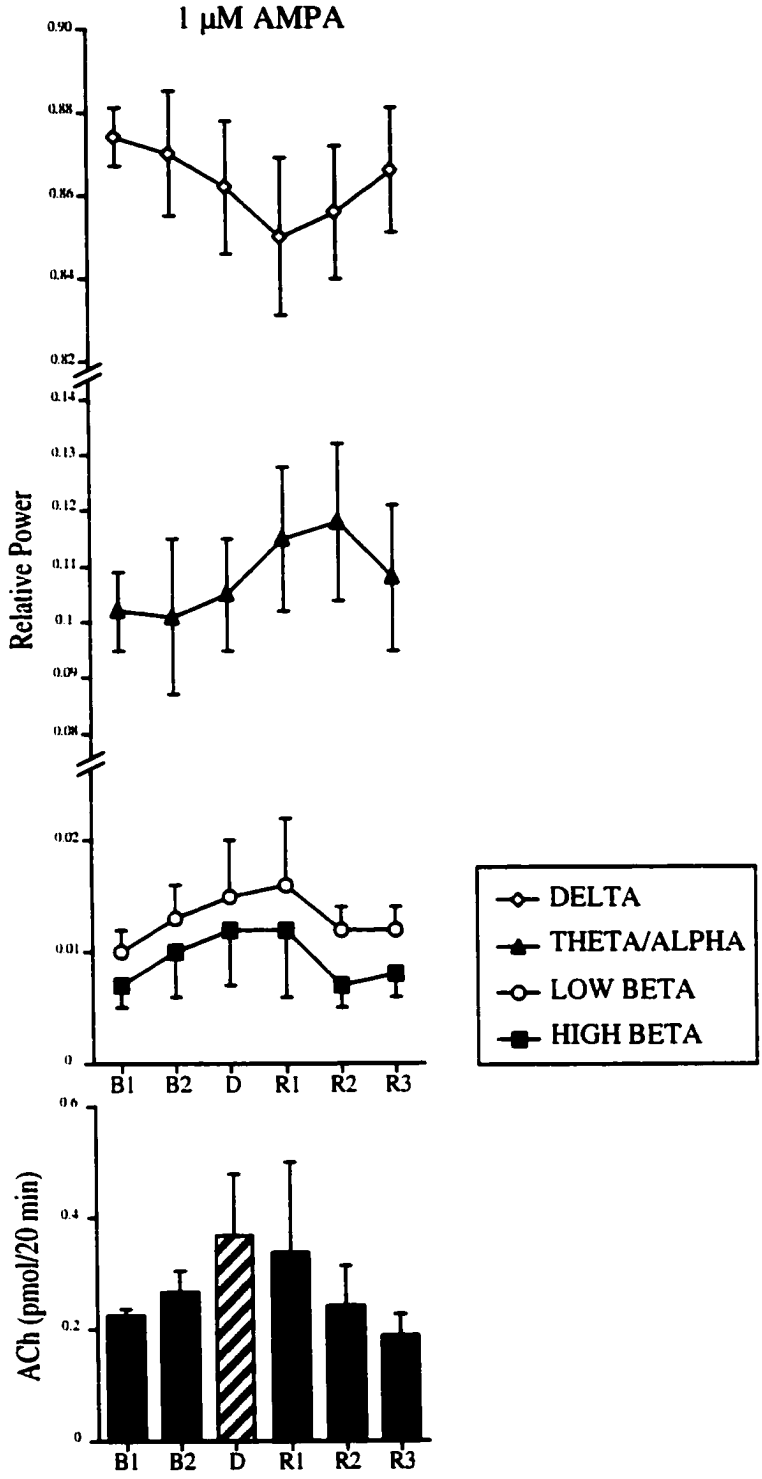

1 µM AMPA
Relative Power
DELTA
THETA/ALPHA
LOW BETA
HIGH BETA
B1
B2
D
R1
R2
R3
ACh (pmol/20 min)

Figure 23

Figure 24. The effects of 10 μM AMPA on the relative power of delta, theta/alpha, low beta and high beta activity and cortical ACh release (n = 5). Mean ± S.E.M. Abbreviations: B1, first 20-min baseline; B2, second 20-min baseline; D, drug infusion; R1, R2, and R3, the first, second, and third 20-min recovery samples, respectively (no drug present).

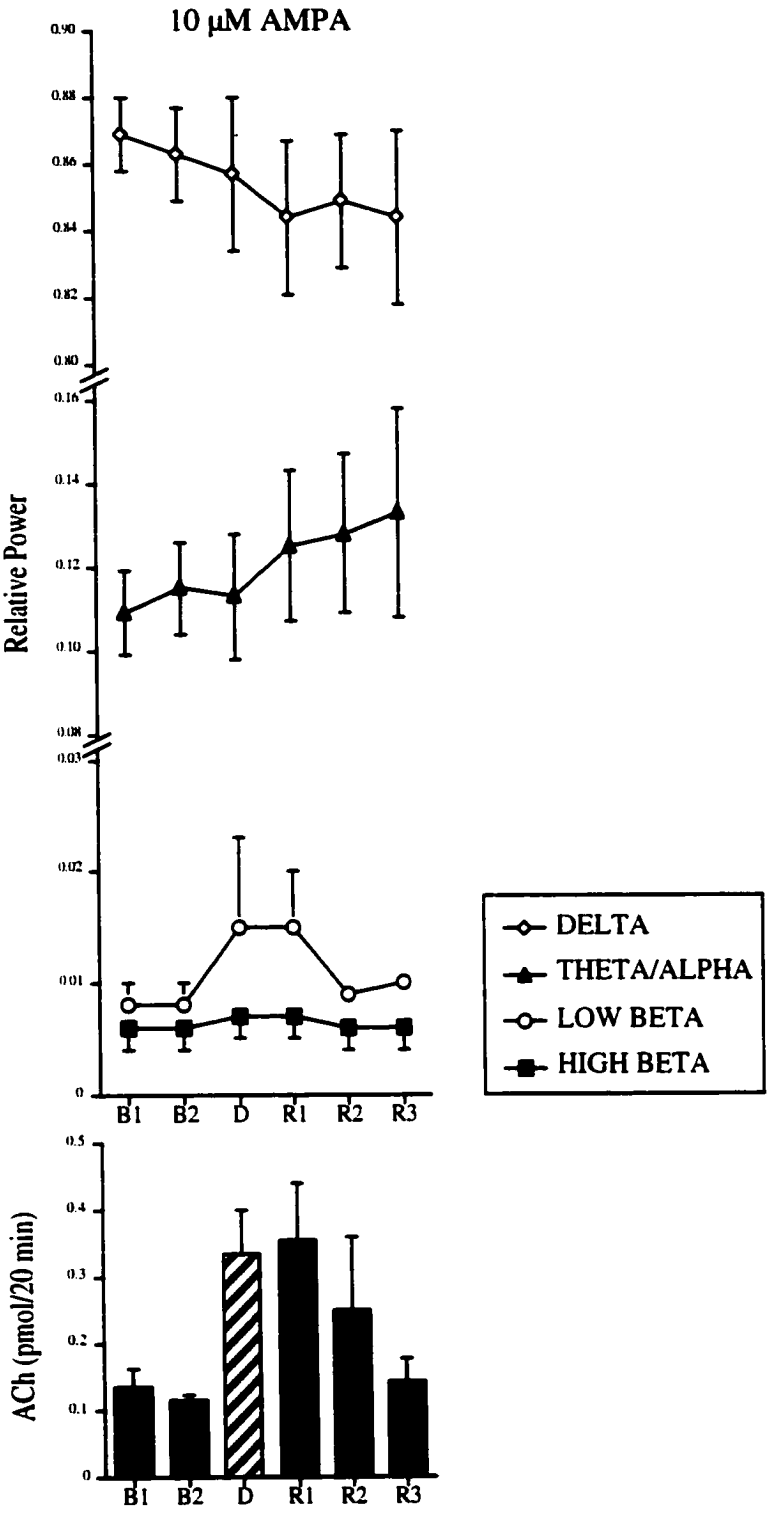
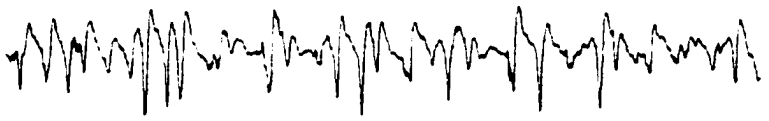

10 μM AMPA
Relative Power
0.90
0.88
0.86
0.84
0.82
0.80
0.16
0.14
0.12
0.10
0.08
0.03
0.02
0.01
0
B1
B2
D
R1
R2
R3
DELTA
THETA/ALPHA
LOW BETA
HIGH BETA
ACh (pmol/20 min)
0.5
0.4
0.3
0.2
0.1
0
B1
B2
D
R1
R2
R3

Figure 24

Figure 25. The effects of 100 μM AMPA application to the basal forebrain on cortical EEG activity. (A) Prior to AMPA infusion, large slow waves dominated the cortical EEG. (B) Following AMPA application, the cortical EEG was dominated by low-voltage, high-frequency activity. Calibrations: 1 sec, 1 mV.

A



B

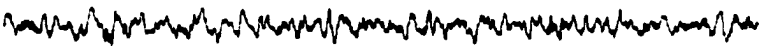


Figure 25

Figure 26. The effects of infusion of 100 μM AMPA on the relative power of delta, theta/alpha, low beta and high beta activity and ACh release from the cortex of urethane-anesthetized rats (n = 5). Mean ± S.E.M.; for both low and high beta: $*P <$ 0.05 vs B1 and B2. Abbreviations: B1, first 20-min baseline; B2, second 20-min baseline; D, drug infusion; R1, R2, and R3, the first, second, and third 20-min recovery samples, respectively (no drug present).

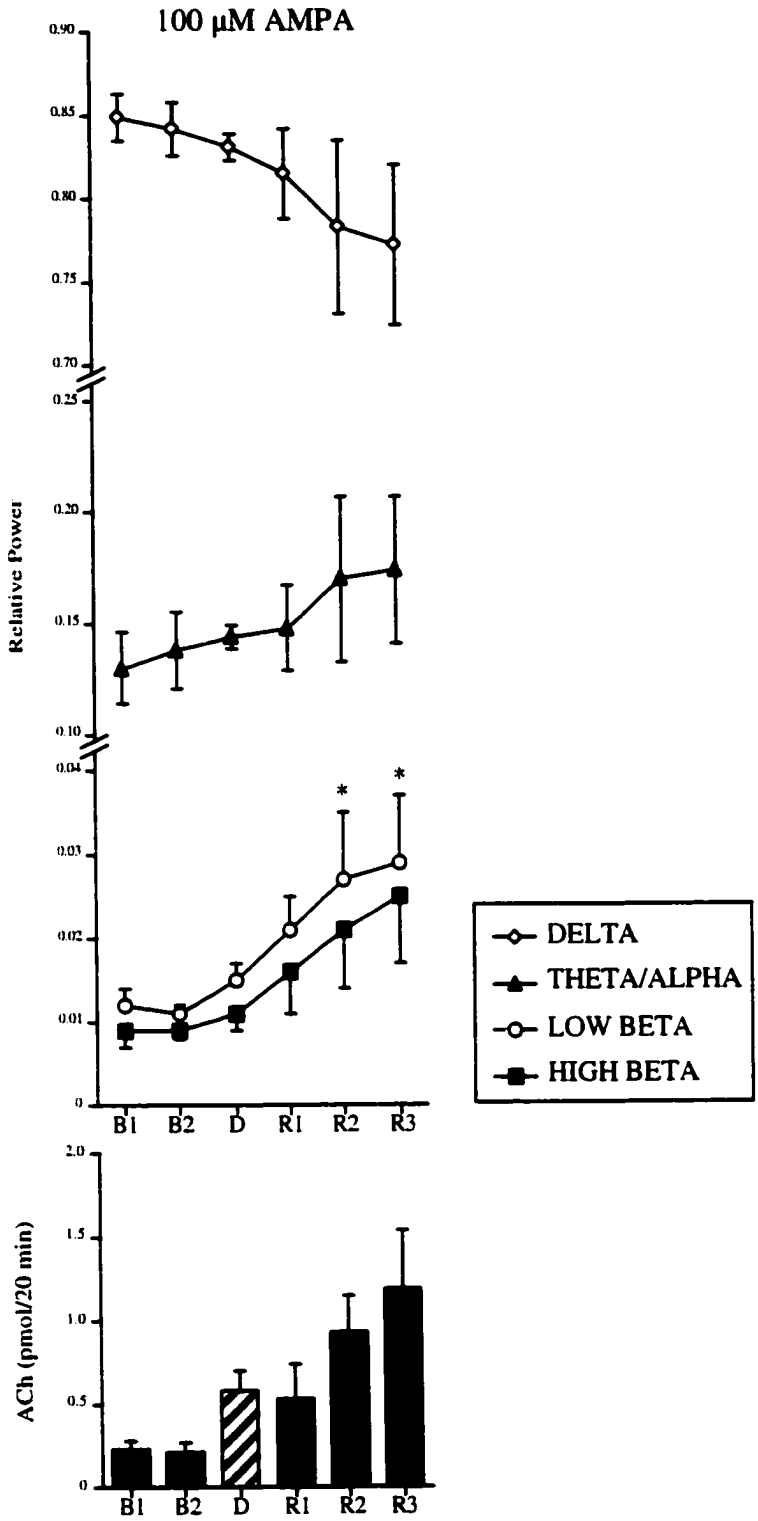

100 µM AMPA
Relative Power
0.90
0.85
0.80
0.75
0.70
0.25
0.20
0.15
0.10
0.04
0.03
0.02
0.01
0
*
*
B1
B2
D
R1
R2
R3
DELTA
THETA/ALPHA
LOW BETA
HIGH BETA
ACh (pmol/20 min)
2.0
1.5
1.0
0.5
0
B1
B2
D
R1
R2
R3

Figure 26

Figure 27. The effects of 0.1 mM NMDA into the basal forebrain on cortical ACh release and the relative power of delta, theta/alpha, low beta and high beta activity (n = 5). Mean ± S.E.M. Abbreviations: B1, first 20-min baseline; B2, second 20-min baseline; D, drug infusion; R1, R2, and R3, the first, second, and third 20-min recovery samples, respectively (no drug present).

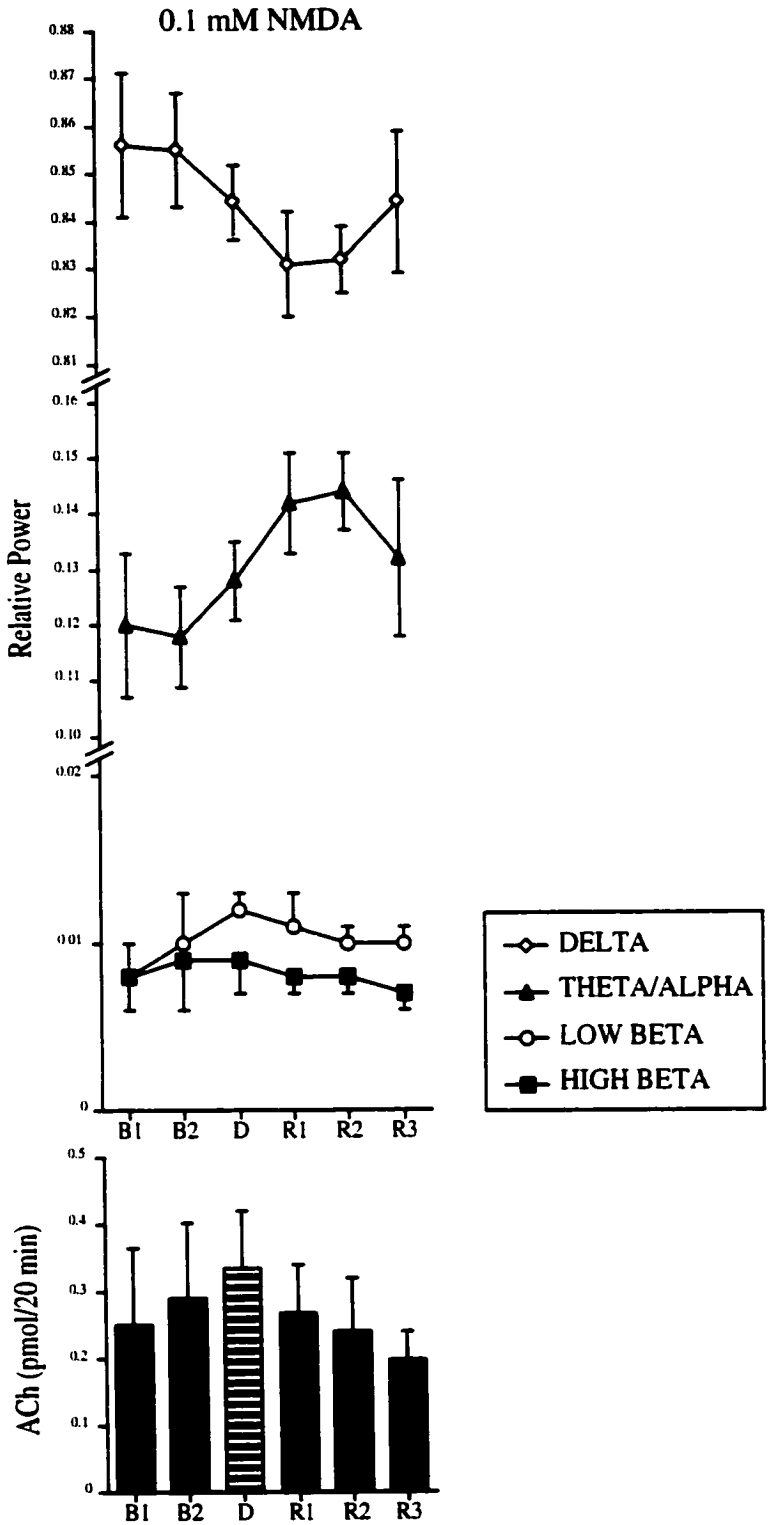

0.1 mM NMDA
Relative Power
0.88
0.87
0.86
0.85
0.84
0.83
0.82
0.81
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.02
0.01
0
B1
B2
D
R1
R2
R3
DELTA
THETA/ALPHA
LOW BETA
HIGH BETA
ACh (pmol/20 min)
0.5
0.4
0.3
0.2
0.1
0
B1
B2
D
R1
R2
R3

Figure 27

Figure 28. The effects of 1 mM NMDA applied to the basal forebrain on cortical ACh release and the relative power of delta, theta/alpha, low beta and high beta activity (n = 5). Mean ± S.E.M. Abbreviations: B1, first 20-min baseline; B2, second 20-min baseline; D, drug infusion; R1, R2, and R3, the first, second, and third 20-min recovery samples, respectively (no drug present).

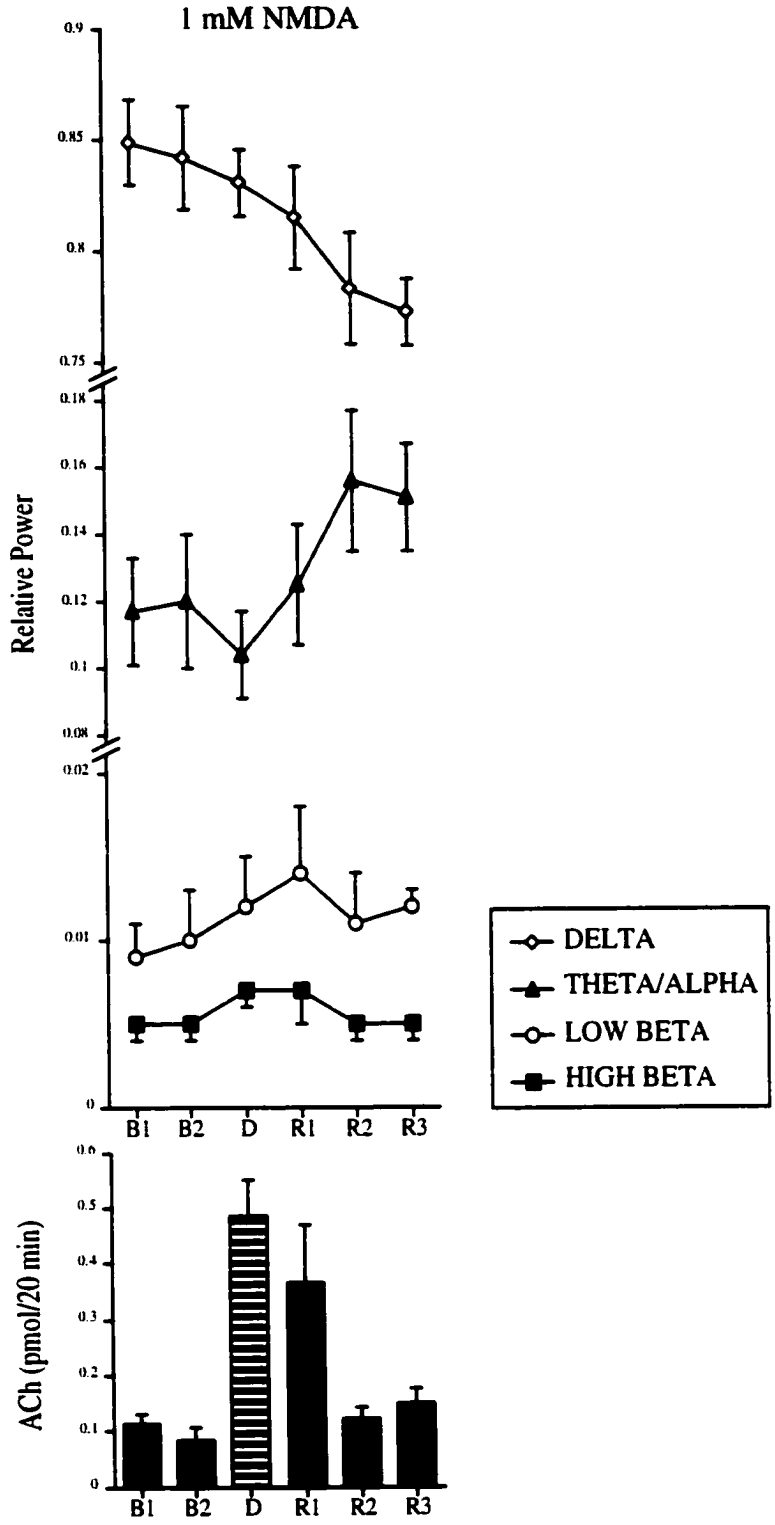
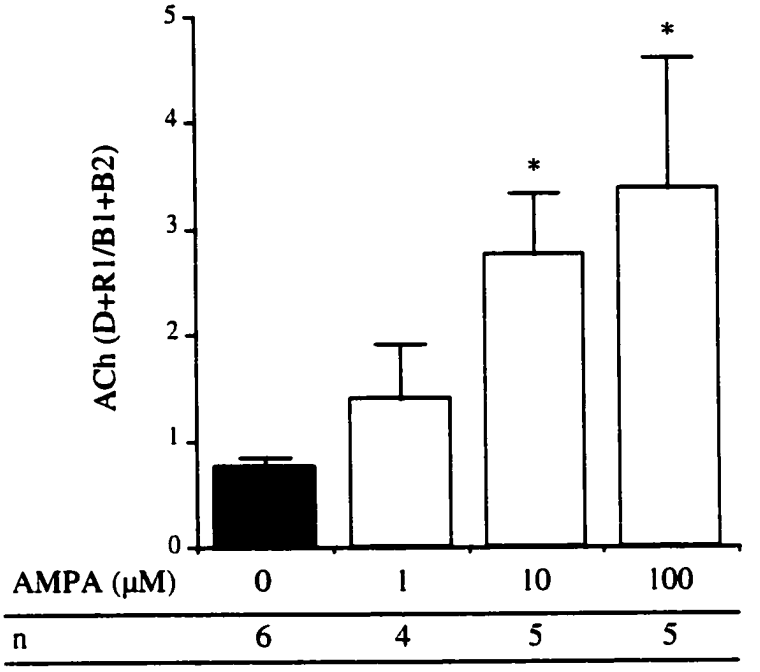

1 mM NMDA
Relative Power
0.9
0.85
0.8
0.75
0.18
0.16
0.14
0.12
0.1
0.08
0.02
0.01
0
B1
B2
D
R1
R2
R3
DELTA
THETA/ALPHA
LOW BETA
HIGH BETA
ACh (pmol/20 min)
0.6
0.5
0.4
0.3
0.2
0.1
0
B1
B2
D
R1
R2
R3

Figure 28

Figure 29. Summary of the effects of infusions of different concentrations of the ionotropic glutamate receptor agonists (A) AMPA and (B) NMDA into the basal forebrain on cortical ACh outflow. Comparisons were made between the average amount of ACh released during the third (drug) and fourth (first recovery) samples versus the average of the two baseline samples. Mean ± S.E.M.; $*P < 0.05$ vs control (black bar).

A


ACh (D+R1/B1+B2)
0
1
2
3
4
5
*
*
AMPA (μM) 0 1 10 100
n 6 4 5 5

B

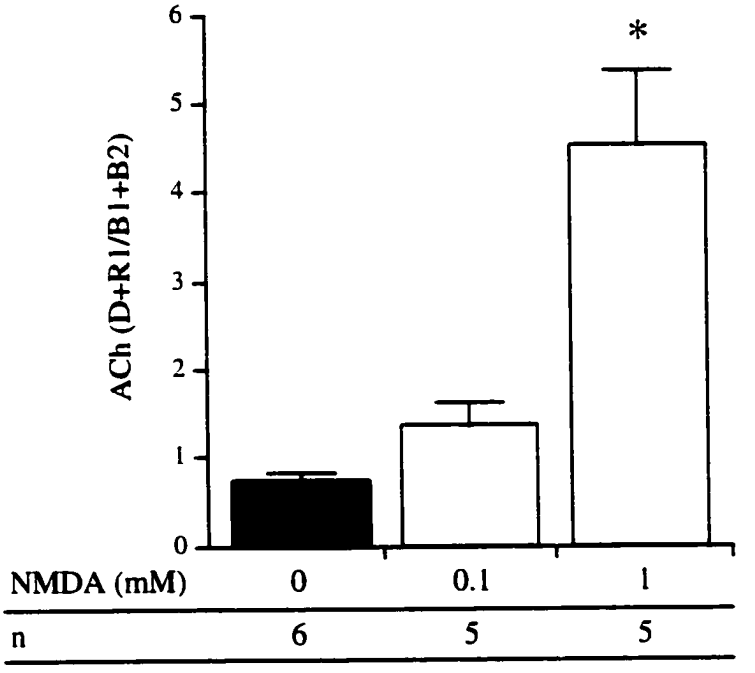

ACh (D+R1/B1+B2)
0
1
2
3
4
5
6
*
NMDA (mM) 0 0.1 1
n 6 5 5

Figure 29

Figure 30. Summary of the effects of different concentrations of AMPA on cortical EEG. Numbers represent the ratios of the third and fourth sample to the first and second sample. Mean ± S.E.M.

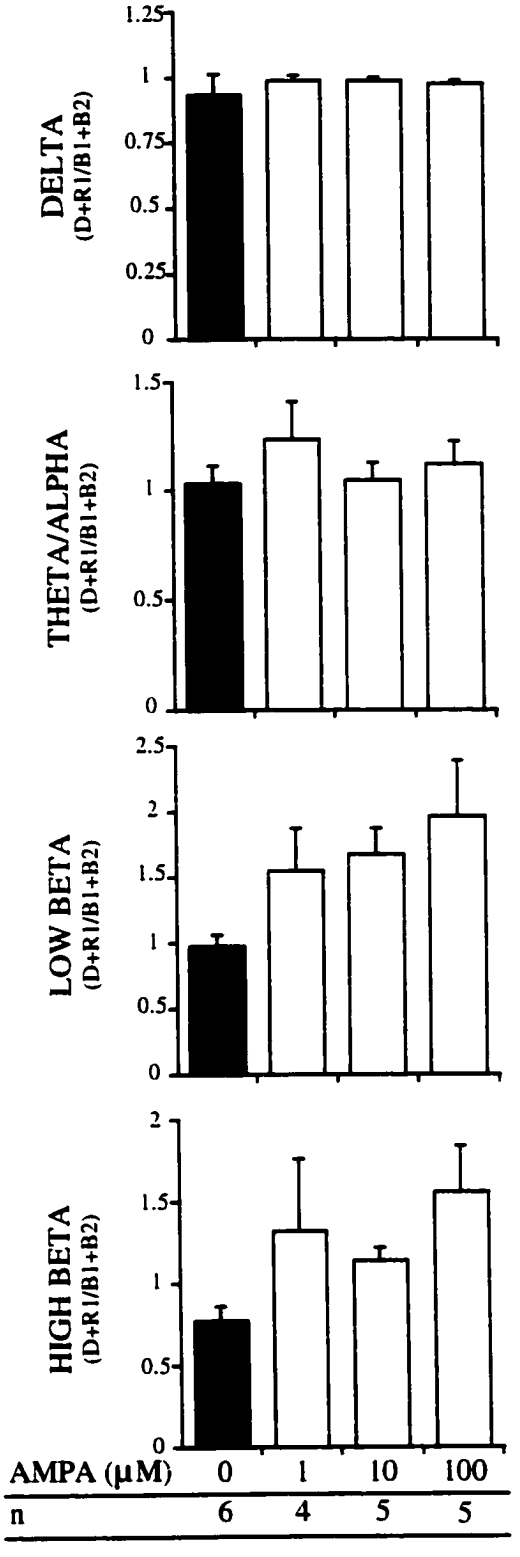

DELTA
(D+R1/B1+B2)
1.25
1
0.75
0.5
0.25
0
THETA/ALPHA
(D+R1/B1+B2)
1.5
1
0.5
0
LOW BETA
(D+R1/B1+B2)
2.5
2
1.5
1
0.5
0
HIGH BETA
(D+R1/B1+B2)
2
1.5
1
0.5
0
AMPA (μM) 0 1 10 100
n 6 4 5 5

Figure 30

Figure 31. Summary of the effects of different concentrations of NMDA on cortical EEG. Numbers represent the ratios of the third and fourth sample to the first and second sample. Mean ± S.E.M.

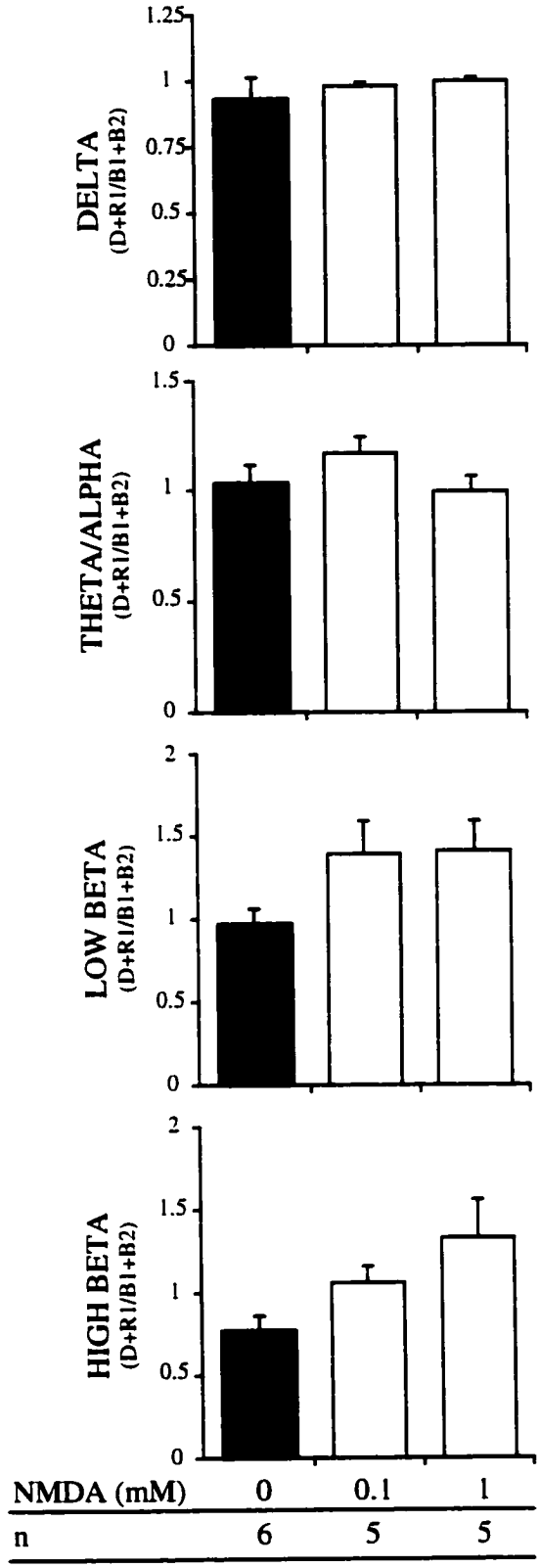

DELTA
(D+R1/B1+B2)
1.25
1
0.75
0.5
0.25
0
THETA/ALPHA
(D+R1/B1+B2)
1.5
1
0.5
0
LOW BETA
(D+R1/B1+B2)
2
1.5
1
0.5
0
HIGH BETA
(D+R1/B1+B2)
2
1.5
1
0.5
0
NMDA (mM) 0 0.1 1
n 6 5 5

Figure 31

Figure 32. The effect of various basal forebrain treatments on cortical ACh release and EEG activity. Each point represents a single animal. The line of best fit is shown through these points.

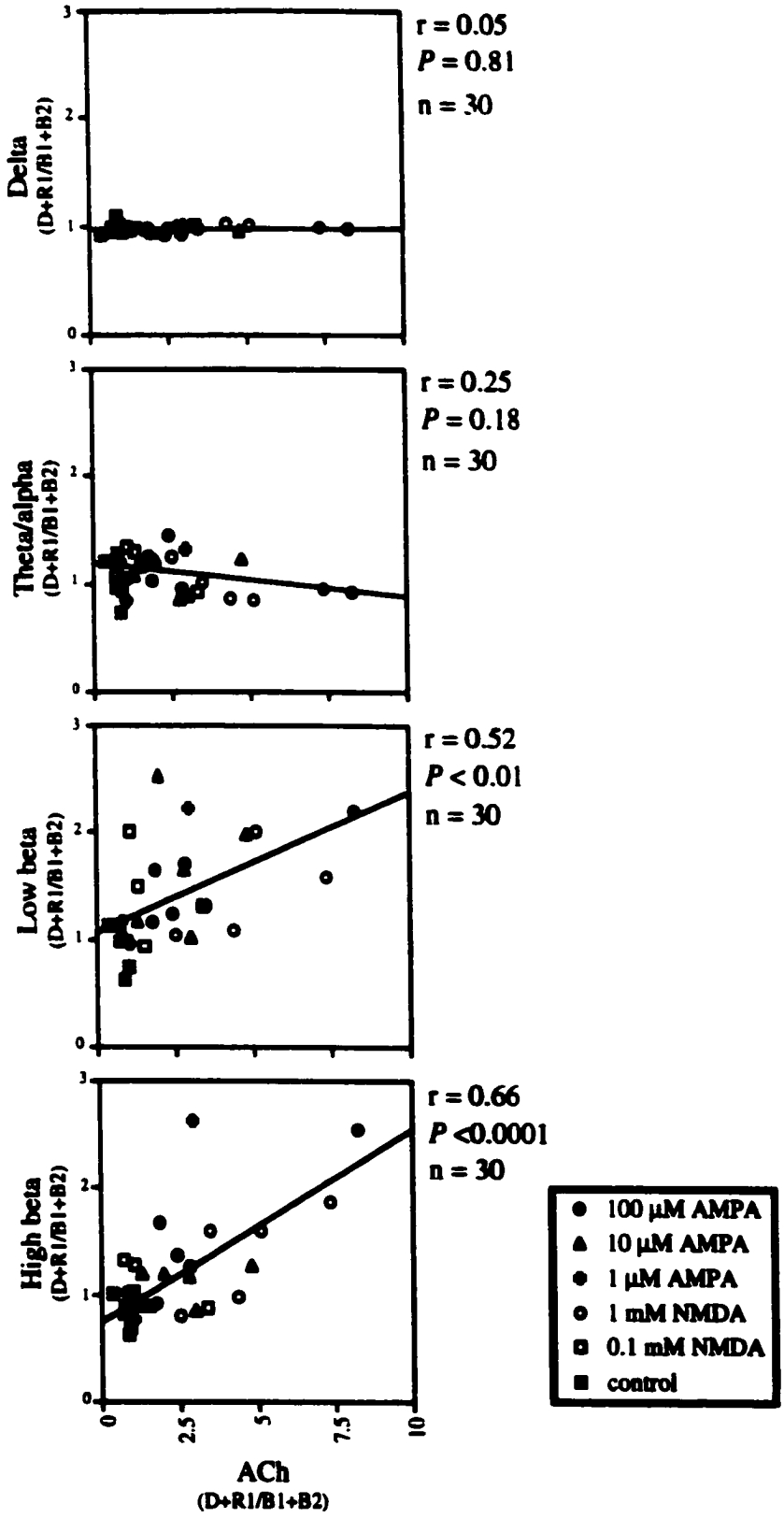
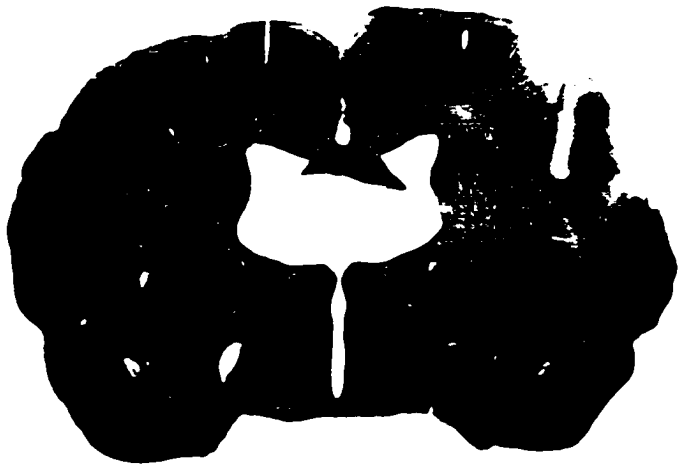

Delta
(D+R1/B1+B2)
r = 0.05
P = 0.81
n = 30
Theta/alpha
(D+R1/B1+B2)
r = 0.25
P = 0.18
n = 30
Low beta
(D+R1/B1+B2)
r = 0.52
P < 0.01
n = 30
High beta
(D+R1/B1+B2)
r = 0.66
P <0.0001
n = 30
100 µM AMPA
10 µM AMPA
1 µM AMPA
1 mM NMDA
0.1 mM NMDA
control
0
2.5
5
7.5
10
ACh
(D+R1/B1+B2)

Figure 32

Figure 33. Photomicrographs of cresyl violet-stained coronal sections through (A) the somatosensory cortex to confirm the position of the bipolar recording electrode and (B) the basal forebrain to confirm the location of the microdialysis probe (arrows). The track for the cortical microdialysis probe can also be observed. Some cortical damage due to the initial surgery is evident in A. Magnification = 5.2x.

A



B

Figure 33

Discussion

The present study demonstrated that infusion of AMPA and NMDA into the basal forebrain by reverse dialysis in urethane-anesthetized rats significantly enhanced cortical ACh release. Despite the substantial increase in ACh efflux, only slight changes to cortical EEG activity were observed. Although not statistically significant, the observed increase in high-frequency EEG activity as a result of activation of ionotropic glutamate receptors in the basal forebrain is consistent with earlier reports (Cape and Jones, 2000). A significant correlation between cortical ACh release and the occurrence of high frequency (>12 Hz) activity was demonstrated in the present study.

Technical considerations

The advantages and limitations to microdialysis have been discussed in the preceding two chapters. The effects of drug administration in this study are interpreted as reflecting physiological responses of healthy neurons. While the possibility that AMPA or NMDA induced cell death due to excitotoxicity cannot be totally excluded, this is unlikely for the following reasons. Abnormally intense or prolonged activation of ionotropic glutamate receptors may be lethal to healthy neurons (Kerchner et al., 1999; Koh et al., 1990). In the present experiment, drugs were administered via reverse dialysis at 1 μM to 1 mM concentrations in the probe for only 20-minutes. Since the drugs were delivered by reverse dialysis, the tissue was not exposed to the full concentration of agonist present within the perfusate since

the dialysis membrane acted as a partial barrier to the diffusion of drugs into the surrounding tissue (Shippenberg and Thompson, 1997).

Application of NMDA or AMPA to the basal forebrain increases cortical ACh release

Activation of ionotropic glutamate receptors by NMDA or AMPA enhanced cortical ACh efflux in urethane-anesthetized rats in a concentration dependent manner. This suggests that enhanced cortical ACh release due to application of glutamate to the basal forebrain (Kurosawa et al., 1989) involves, at least in part, activation of NMDA and AMPA receptors. It has been shown previously that delivery of an NMDA receptor antagonist to the NBM decreased ACh outflow from the cortex in freely moving rats (Giovannini et al., 1997). It was also shown that brainstem stimulation-induced cortical ACh release could be partially blocked by infusion of an NMDA receptor antagonist into the basal forebrain but application of an AMPA receptor antagonist was less effective (Rasmusson et al., 1996). Rasmusson et al. (1996) also noted that simultaneous application of the NMDA receptor antagonist and the AMPA receptor antagonist to the basal forebrain nearly abolished synaptically evoked cortical ACh efflux. Thus, cortical ACh outflow appears to be regulated by both NMDA and AMPA receptors in the basal forebrain.

The activity of basal forebrain neurons has been shown to increase following exposure to ionotropic glutamate receptor agonists. Application of NMDA to basal forebrain slices *in vitro* has been shown to induce burst firing in hyperpolarized cholinergic neurons (Khateb et al., 1995). Burst firing may increase the probability of neurotransmitter release compared to single spikes (Lisman, 1997).

It is possible that infusion of ionotropic glutamate receptor agonists in the present study directly induced rhythmic bursting in basal forebrain cholinergic neurons that, in turn, increased cortical ACh efflux.

Ionotropic glutamate receptor activation has relatively minor effects on cortical EEG activity

The relatively minor effect of ionotropic glutamate receptor agonist application to the basal forebrain on cortical EEG in the present study was surprising. It had previously been reported that brainstem stimulation-induced activation of cortical EEG could be inhibited by infusion of ionotropic glutamate receptor antagonists into the basal forebrain (Rasmusson et al., 1994, 1996). Cape and Jones (2000) have demonstrated that microinjections of NMDA or AMPA increased high-frequency cortical EEG activity in freely moving rats. The large differences in EEG responses to drug application between animals and between groups of animals in the present study have no doubt influenced the statistical analysis.

The lack of significant changes, despite consistent trends, in most of the EEG samples examined may have been due to effects of the anesthetic which may have had a depressant effect on the activity of cortical neurons (Angel, 1993). Due to the physical constraints of the experimental setup, EEG was only recorded from one cortical site. Cape and Jones (2000) noted that changes in cortical EEG due to NMDA and AMPA microinjections into the basal forebrain were not entirely consistent across the four cortical areas sampled. For example, it was shown that the

retrosplenial cortex displayed a more pronounced increase in theta activity compared to other cortical areas.

It is possible that the effects observed in the present study may have been due to thalamocortical projections, cortically projecting GABAergic neurons, or other neurotransmitter systems. Changes in the activity and firing pattern of thalamocortical neurons are correlated with changes in cortical EEG activation (Steriade et al., 1993). Complete removal of the thalamus in cats results in cortical EEG dominated by high-amplitude slow-waves for up to 25 days (Villablanca, 1974). It is possible that cholinergic basal forebrain neurons regulate cortical EEG activation primarily via inputs to the thalamus. Both cholinergic and non-cholinergic projections to thalamus, specifically the reticular thalamic nucleus, have been shown to originate from the basal forebrain (Parent et al., 1988; Asanuma and Porter, 1990). Modulation of the activity of neurons within the reticular thalamic nucleus would in turn influence the activity of thalamocortical neurons (McCormick, 1992). Since the results presented in the present study demonstrated little change in cortical EEG, the activity of neurons within the reticular thalamic nucleus may not have been affected. Thus, activation of thalamocortical neurons may not have been significantly altered by intra-basal forebrain infusion of ionotropic glutamate receptor agonists.

In addition to thalamic control of cortical EEG, cortically projecting GABAergic neurons within the basal forebrain may also contribute to EEG activation. The GABAergic neurons in the basal forebrain have been shown project to cortical GABAergic interneurons (Freund and Meskenaite, 1992). Activation of basal forebrain GABAergic neurons that project to the cortex could therefore induce

cortical activation via disinhibition. In the present study, infusions of AMPA or NMDA may not have sufficiently affected cortically projecting GABAergic neurons. Thus, changes in cortical EEG would not have occurred.

Finally, cortical EEG activation may reflect the activity of several neurotransmitter systems. Dringenberg and Vanderwolf (1998) suggest that cortical EEG activation is maintained by multiple systems working in parallel. In particular, both cholinergic and serotonergic systems working in concert have been implicated in EEG arousal (Vanderwolf, 1988; Dringenberg and Vanderwolf, 1998). Infusion of NMDA or AMPA into the basal forebrain is not likely to influence cortical serotonergic levels.

Cortical ACh outflow and high frequency EEG activity are correlated

While the changes in cortical EEG within groups were modest, when the data were collapsed across groups a significant correlation between the amounts of ACh released in the cortex and the presence of high frequency (>12 Hz) activity was shown. Recently, the correlation between the activity of identified cholinergic basal forebrain neurons and cortical EEG activity has been described. Manns et al. (2000) demonstrated that cortical EEG activation induced by tail-pinch in urethane-anesthetized rats was strongly correlated with increased firing rate of identified cholinergic neurons within the basal forebrain. The firing pattern of the basal forebrain cholinergic neurons changed from a tonic-firing mode to rhythmical bursting during sensory stimulation-induced cortical EEG arousal (Manns et al., 2000).

High-frequency cortical EEG activity was elicited by infusion of glutamate into the basal forebrain of anesthetized rats (Metherate et al., 1992). This effect was blocked by atropine, a muscarinic antagonist (Metherate et al., 1992). In light of the present results and those reported by Cape and Jones (2000), it is likely that changes in high-frequency activity induced by intra-basal forebrain infusions of glutamate were due to activation of ionotropic glutamate receptors and the subsequent increase in cortical ACh release.

Functional implications

Glutamatergic inputs to the basal forebrain have been shown to originate from various regions including the prefrontal and piriform cortices (Zaborszky et al., 1997), amygdala (Zaborszky et al., 1984), thalamus, and hypothalamus (Carnes et al., 1990). Regulation of the activity of basal forebrain neurons could have important behavioural consequences. Infusion of either NMDA or AMPA into the basal forebrain of freely moving animals has been shown to increase wakefulness and reduce slow-wave sleep (Manfridi et al., 1999). In addition to this effect, Manfridi et al. (1999) also noted that AMPA, but not NMDA, decreased rapid-eye-movement sleep. These alterations in behavioural state may be due, at least in part, to modulation of the activity level of cholinergic neurons and the subsequent changes in cortical ACh release. In addition to sleep and wakefulness, cortical ACh has been implicated in synaptic plasticity and cortical reorganization (Kilgard and Merzenich, 1998) as well as attention (Sarter and Bruno, 2000). It has been shown that blocking NMDA receptors within the basal forebrain disrupts the performance of animals

performing attentional tasks (Turchi and Sarter, 1998). Thus glutamatergic modulation of cholinergic basal forebrain neurons may regulate many cognitive processes.

General Discussion

Study 4.

Original hypotheses:

A. Application of the ionotropic glutamate receptor agonist NMDA to the basal forebrain will enhance ACh release in the cortex with little or no change in cortical EEG activation.

B. Application of the ionotropic glutamate receptor agonist AMPA to the basal forebrain will result in enhanced cortical ACh release and increased cortical EEG activation.

The results described in this chapter demonstrate that intra-basal forebrain infusion of either NMDA or AMPA induces ACh release from the cortex of urethane-anesthetized rats. These results by and large support my original hypotheses. However, only modest changes in cortical EEG activity were observed with either ionotropic glutamate receptor agonist.

The relatively small changes in cortical EEG arousal following intra-basal forebrain infusions of the glutamate receptor agonists NMDA and AMPA were surprising given the profound effect each of these drugs had on cortical ACh release. This implies that ACh release alone is not sufficient for cortical EEG activation.

The correlation between cortical ACh release and the occurrence of high-frequency activity may reflect the excitatory effects of ACh on cortical neurons. Application of ACh onto cortical neurons has been shown to depolarize cortical

neurons (Krnjevic and Phillis, 1963; Spehlmann, 1963; see Chapter I). ACh induced depolarization of neurons would elicit excitatory postsynaptic potentials, increase the probability of action potential generation, and, ultimately, affect the EEG.

CHAPTER V. GENERAL CONCLUSIONS

The work presented in this thesis was an examination of factors that regulate cortical ACh outflow and EEG activation. Specifically, the effects of intracortical adenosine on synaptically evoked cortical ACh outflow and the distribution of the mRNA for the A_1 and A_{2A} adenosine receptors were examined. The effects of local infusion of glutamate on cortical ACh release evoked by brainstem stimulation were also reported. Finally, the effects of ionotropic glutamate receptor agonists applied to the basal forebrain on cortical ACh outflow and EEG activation were described. From the studies presented in this thesis, several conclusions can be drawn.

Firstly, adenosine inhibits evoked cortical ACh release by activating A_1 adenosine receptors within the cortex. As described in Chapter II, local infusion of adenosine reduced cortical ACh efflux evoked by electrical stimulation of the PPT. A selective A1 adenosine receptor agonist mimicked this effect. While the limitations of the microdialysis technique make it impossible to determine if these receptors are expressed on cholinergic terminals, previous studies strongly suggest that this is the case. The most conclusive evidence that A_1 adenosine receptors are located on presynaptic cholinergic terminals is derived from studies examining the effects of adenosine on synaptosomes. Pedata et al. (1986) demonstrated that adenosine inhibits potassium evoked ACh release from cortical synaptosomes in a concentration dependent manner. It is therefore likely that adenosine applied to the cortex by reverse dialysis activates presynaptic adenosinergic heteroreceptors on cholinergic terminals.

The results of the *in situ* hybridization study suggest that mRNA for the A_1 adenosine receptor is expressed by neurons within the basal forebrain. The original purpose of the second study in Chapter II was to colocalize mRNA for the A_1 adenosine receptor using in situ hybridization with the enzyme ChAT using immunocytochemistry. Unfortunately, the technical difficulties encountered could not be overcome. However, the results of physiological studies suggest that cholinergic neurons express adenosine receptors. Rainnie et al. (1994) demonstrated that adenosine inhibited the firing rate of all neurons tested in the diagonal band. Superfusion of an adenosine receptor antagonist increased the activity of basal forebrain neurons (Rainnie et al., 1994). Presumably, some of the cells tested would have been cholinergic.

Intracortical application of glutamate in the third study had the unexpected result of reducing synaptically evoked cortical ACh release. This effect was shown to be due to activation of intracortical ionotropic glutamate receptors. Further testing demonstrated that GABA mediated the effects of glutamate on ACh efflux. This suggests that activation of intracortical glutamate receptors evoked cortical GABA release that, in turn, decreased ACh efflux. Inhibition of cortical ACh release by local infusion of GABA has been demonstrated recently (Giorgetti et al., 2000). Inhibition of cortical ACh release by serotonin (Ramírez et al., 1996), norepinephrine (Beani et al., 1986), and histamine (Giorgetti et al., 1997) has been shown to involve GABA. This type of modulation of ACh release may ultimately result in the outflow of ACh within very concise regions of the cortex. Ultimately, this may result in the selective enhancement of cortical responses to specific inputs.

The final study presented in this thesis demonstrated that activation of ionotropic glutamate receptors in the basal forebrain enhances cortical ACh outflow. In addition to this effect, there was a slight increase in cortical EEG activation. This suggests that the inhibitory glutamatergic-GABAergic circuit present within the cortex does not exist within the basal forebrain or plays only a secondary role in regulating cortical ACh release. The relatively minor changes in cortical EEG arousal were surprising given the amount of ACh that was released in response to activation of NMDA and AMPA receptors in the basal forebrain. It has been reported recently that extensive loss of ChAT activity in the cortex (up to 84%) due to immunotoxic lesions of cholinergic basal forebrain neurons had only modest effects on all frequency bands of the cortical EEG (Holschneider et al., 1999). This strongly suggests that noncholinergic inputs play a significant role in modulating cortical EEG activation.

These data suggest that cortical ACh release can be regulated by several neurotransmitter and neuromodulatory systems at both the level of the axon terminal and the cell body. Given the importance of ACh in regulating attentional processes, behavioural state, and learning and memory, determining how ACh release may be modified could further our understanding of these cognitive processes.

APPENDIX A

EXPERIMENTAL SOLUTIONS

I. Perfusion Solutions.

0.9% saline

9.0 g	sodium chloride

Bring volume to 1 liter with distilled water.

0.2 M monobasic phosphate buffer

46.9 g	NaH_2PO_4

Bring volume to 1.7 liters with distilled water

0.2 M dibasic phosphate buffer

85.2 g	Na_2HPO_4
650 ml	0.2 M monobasic phosphate buffer

Bring volume to 3.65 liters with distilled water.

20% paraformaldehyde

200 g	paraformaldehyde

Bring volume to 1 liter with distilled water. Heat, but do not boil, while stirring for 45 minutes. Add 12 drops of 10 N NaOH. Stir for an additional 5 minutes. Remove from heat, cool to room temperature, and filter.

4% paraformaldehyde in 0.1 M phosphate buffer

200 ml	0.2 M dibasic phosphate buffer
120 ml	distilled water
80 ml	20% paraformaldehyde

II. In Situ Hybridization.

DEPC water

Add 1 ml of diethylpyrocarbonate (Sigma) for every liter of deionized water. Stir overnight in the fume hood at room temperature and then autoclave.

Hybridization Buffer

In a 50 ml sterile Falcon polypropylene tube:

25 ml	100% deionized formamide (Sigma)
10 ml	20X SSC
2.5 ml	0.5 M sodium phosphate
0.5 ml	50X Denhardt's solution (Sigma)
1.0 ml	10 mg/ml sonicated hydrolyzed salmon sperm DNA (Gibco)
1.0 ml	5 mg/ml polyadenylic acid (Sigma)
5.0 g	dextran sulphate (Sigma)
1.25 ml	10 mg/ml yeast tRNA (Sigma)

Bring volume to 50 ml with DEPC water. Store in -20°C freezer.

20X SSC

175.3 g	sodium chloride
88.2 g	sodium citrate (Sigma)

Dissolve in 800 ml with DEPC water. Adjust the pH to 7.0 with 10N NaOH. Bring volume to 1 liter.

III. Microdialysis.

HPLC mobile phase

14.2 g	Na_2HPO_4
0.077 g	EDTA
10 ml	ProClin (Bioanalytical Systems, Inc.)

Bring volume to 2 liters with deionized water. Adjust pH with orthophosphoric acid. Filter and degas mixture.

0.13 M CaCl

3.607 g	CaCl

Bring volume to 250 ml with deionized water.

0.3 M KCl

5.592 g	KCl

Bring volume to 250 ml with deionized water.

0.1 M $MgSO_4$

3.9 g	$MgSO_4$

Bring volume to 250 ml with deionized water.

Artificial cerebral spinal fluid

1 ml	0.13 M CaCl
1 ml	0.3 M KCl
1 ml	0.1 M $MgSO_4$
730.5 mg	NaCl

Add to 90 ml of deionized water and adjust pH to 7.0-7.2 with 0.2 N NaOH. Bring volume to 100 ml with deionized water. Filter with 0.22 μm filter paper.

10 mM Neostigmine

0.334 g	neostigmine sulphate

Bring volume to 10 ml with deionized water.

10 mM Atropine

0.677 g	atropine sulphate

Bring volume to 10 ml with deionized water.

Microdialysis perfusion solution

10 μl	10 mM neostigmine
10 μl	10 mM atropine

Bring volume to 10 ml with artificial cerebral spinal fluid.

0.1% NaH_2PO_4

0.1 g	NaH_2PO_4

Bring volume to 100 ml with deionized water.

10 mM Acetylcholine

150 mg	acetylcholine chloride

Bring volume to 82.6 ml with 0.1% NaH_2PO_4.

Acetylcholine standard solutions

Dilute 10 µl of 10 mM acetylcholine in 50 ml artificial cerebral spinal fluid and mix well. Place 1 ml of artificial cerebral spinal fluid into marked eppendorf tubes and add the following amounts of the diluted acetylcholine solution to obtain the desired standards:

1 pmol	add 25.6 µl diluted acetylcholine
2 pmol	add 52.6 µl diluted acetylcholine
4 pmol	add 112 µl diluted acetylcholine

Mix well.

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