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AN *IN VIVO* MICRODIALYSIS STUDY OF NEUROTRANSMITTER EVOKED ADENOSINE RELEASE IN THE RAT CEREBRAL CORTEX

by

Hilary J. Bennett

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

at

Dalhousie University
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October 27, 2000

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

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by Hilary Jane Bennett

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

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ABSTRACT

Adenosine is an inhibitory neuromodulator in the central nervous system. For extracellular adenosine to play a physiological role in the brain, it must be present at effective concentrations. The purpose of these studies was to identify the neurotransmitters that evoke an increase in extracellular adenosine in the cerebral cortex and to identify the receptor subtypes that mediate these effects using *in vivo* microdialysis.

Activation of ionotropic receptors by glutamate has been shown to increase adenosine in cortical slice. However, the possible effects of metabotropic glutamate receptor activation on the concentration of extracellular adenosine has not been examined. We tested whether activation of metabotropic and ionotropic glutamate receptors increases the extracellular concentration of adenosine *in vivo*. The group I/II metabotropic glutamate receptor agonist *trans*-(±)-1-amino-(1S,3R)-cyclopenyanedicarboxylic acid increased extracellular adenosine as did the specific group I agonist (S)-3,5-dihydroxyphenylglycine. Activation of the group II or III metabotropic receptors did not affect extracellular adenosine levels. NMDA, AMPA and kainic acid also significantly increased the concentration of extracellular adenosine.

Previous experiments using cortical slice and *in vivo* preparations have shown that activation of muscarinic receptors alone has no effect on basal adenosine release but enhanced the evoked release of adenosine. Given the role of nicotinic receptors in the modulation of neurotransmitter release, we tested whether activation of nicotinic receptors increases extracellular adenosine levels *in vivo*. Acetylcholine and the acetylcholinesterase inhibitor neostigmine increased extracellular adenosine levels, and the effects of neostigmine were blocked by the nicotinic receptor antagonist mecamylamine. Activation of muscarinic receptors using the nonselective agonist oxotremorine and a selective M1 receptor agonist also increased extracellular adenosine levels. Both nicotine and the nicotinic receptor agonist epibatidine increased the concentration of extracellular adenosine.

Given the role of noradrenaline in modulating the activity of cortical neurons, it is important to determine whether noradrenaline evokes the release of adenosine in the cerebral cortex *in vivo*. Noradrenaline and the noradrenergic reuptake inhibitor desipramine increased extracellular adenosine levels. The α_1-adrenergic receptor agonist phenylephrine and the β-adrenergic agonist isoproterenol increased extracellular adenosine levels, whereas the α_2-adrenergic receptor agonist clonidine did not have an effect.

Taken together, these studies suggest that glutamatergic, cholinergic and noradrenergic transmission may contribute to the regulation of extracellular adenosine levels *in vivo* and may represent a mechanism for controlling neuronal excitability in the cerebral cortex.

LIST OF ABBREVIATIONS AND SYMBOLS

ACPD	*trans*-(±)-1-amino-(1S,3R)-cyclopenyanedicarboxylic acid
AD	adenosine deaminase
AK	adenosine kinase
AMP	adenosine 5'-monophosphate
AMPA	(α-amino-3-hydroxy-5methyl-4-isoxazoleproprionic acid)
ATP	adenosine 5'-triphosphate
cAMP	adenosine 3',5'-cyclic monophosphate
CGS 21680	(2-[p-(2-carboxyethyl)-phenyiethylamino]-5'-*N*-ethylcarboxamidoadenosine)
CHA	cyclohexyladenosine
ChAT	choline acetyltransferase
CNS	central nervous system
(±)-CPP	[(±)-3-(2-Carboxypiperazin-4-yi)-propyl-1-phosphonic acid]
CPA	N^6-cyclopentyladenosine
CPT	8-cyclopentyltheophylline
DAG	diacylglycerol
DHPG	(S)-3,5-dihydroxyphenylglycine
DNQX	6,7-dinitroquinoxaline-2,3-dione
EEG	electroencephalogram
G protein	guanosine 5'-triphosphate binding protein
GABA	γ-aminobutyric acid
HPLC	high performance liquid chromatography

IB-MECA	(1-deoxy-1-[6-[(3-iodophenyl)-methyl]amino)-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide)
IP_3	inositol-1,4,5-triphosphate
L-DOPA	dihydroxyphenylalanine
NBTI	nitrobenzylthioinosine
NECA	5'-*N*-ethyl-carboxamidoadenosine (NECA)
NMDA	N-methyl-D-aspartate
NREM	non-rapid eye movement
PKC	protein kinase C
PPT	pedunculopontine tegmental nucleus
SAH	S-adenosylhomocysteine

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CHAPTER I. INTRODUCTION

The physiological actions of adenosine, including bradycardia, vasodilation of coronary vessels and induction of sleep, were first described by Drury and Szent-Györgyi in 1929. More recently, it has been shown that adenosine is a major inhibitory neuromodulator in the central nervous system (CNS). Presynaptically, adenosine inhibits the release of glutamate, acetylcholine, noradrenaline, serotonin and dopamine (Fredholm and Dunwiddie, 1988). Postsynaptic inhibitory actions of adenosine have also been shown in many structures including the locus coeruleus. The goal of this thesis was to identify the neurotransmitter receptors that are involved in an increase in extracellular adenosine in the freely moving animal.

1. ADENOSINE

1.1. Production

Adenosine is a nucleoside consisting of a ribose sugar linked to the purine base adenine (Fig. 1). For extracellular adenosine to play a physiological role in the CNS, it must be present at effective concentrations. The extracellular concentration of adenosine depends upon several factors including the rates of intracellular adenosine production and release, uptake into the cell, and the presence of extracellular enzymes that metabolize adenosine. Neurons and glia are both potential cellular sources of extracellular adenosine in the brain (Meghji and Newby, 1990). Adenosine is released from these cells via nucleoside transporters or nucleotide transporters followed by enzymatic conversion to adenosine. Although it has been suggested that

Fig. 1. The chemical structure of adenosine. Adenosine is a purine consisting of a ribose sugar linked to adenine.

Adenosine

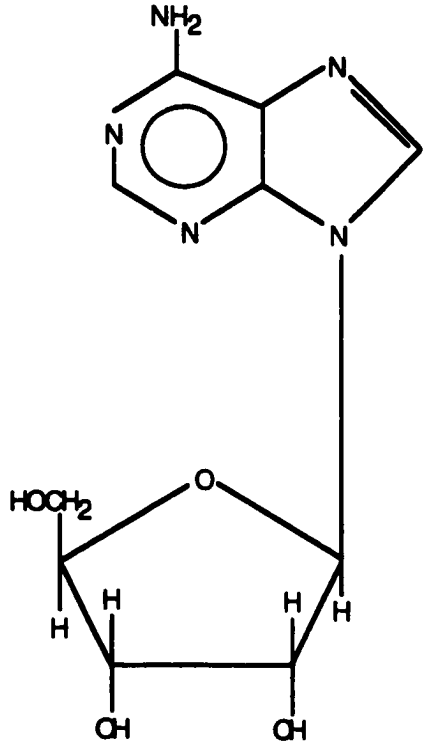


Fig. 1

adenosine is released from presynaptic terminals (Sulakhe and Phillis, 1975), there is currently no evidence to confirm this.

There are several pathways for the production of adenosine within the cell (Fig. 2). The dephosphorylation of adenosine 5'-monophosphate (AMP) originating from either adenosine 5'-triphosphate (ATP) or adenosine 3',5'-cyclic monophosphate (cAMP) is one source of intracellular adenosine (Senba, 1998). Within the cell ATP is degraded to AMP and subsequently to adenosine by 5'-nucleotidase. The conversion of ATP to adenosine is rapid and occurs within 200 msec in hippocampal neurons (Dunwiddie et al., 1997). The conversion of cAMP to adenosine occurs in two steps: cAMP is converted to AMP by cAMP phosphodiesterases (Schwartz and Kandel, 1995), and then to adenosine by 5'-nucleotidase. Another source of intracellular adenosine is S-adenosylmethionine (SAM). The enzyme SAH-hydrolase converts SAM to S-adenosylhomocysteine (SAH) and then converts SAH to adenosine (Arch and Nesholme, 1978; Dunwiddie, 1985; Schläfer et al., 1994; Abraham, 1995). SAH-hydrolase has been immunocytochemically localized mainly in neurons of the cortex, hippocampus and cerebellum (Patel and Tudball, 1986) and is more active in neuronal than glial cultures (Ceballos et al., 1994). Once adenosine is produced within the cell it can be released directly into the extracellular space through a bidirectional nucleoside transporter.

The conversion of ATP and cAMP also occurs in the extracellular space. The ATP that is released to the extracellular space by synaptic vesicles or through a nucleotide transporter is broken down by a series of ectoenzymes to

Fig. 2. The production and metabolism of adenosine (ADN). Adenosine 5'-triphosphate (ATP) within the cell is degraded to adenosine 5'-diphosphate (ADP) and then to adenosine 5'-monophosphate (AMP) by a series of enzymes. AMP is converted to ADN by 5'-nucleotidase (5'N). The conversion of S-adenosylhomocysteine (SAH) by SAH-hydrolase is a second source of intracellular adenosine. Within the cell, cyclic AMP (cAMP) is degraded to AMP by cAMP-phosphodiesterase (PDE) and then to ADN by 5'N. Intracellular adenosine can be released to the extracellular space via bidirectional nucleoside transporters. Extracellular ATP can also be degraded by ecto-ATPase to ADP which is subsequently degraded to AMP by ecto-ADPase and then to ADN by ecto-5'N. Extracellular cAMP can also be degraded to ADN by ecto-PDE and ecto-5'N. Extracellular adenosine is degraded by ecto-adenosine deaminase (ecto-AD) to inosine (IN). Adenosine is also removed from the extracellular space by uptake into the cell via the nucleoside transporter and subsequent conversion to inosine by cytosolic AD or to AMP by adenosine kinase (AK; Phillis and Newsholme, 1979; Phillis and Wu, 1981; Linden, 1999). Additional abbreviations: hypoxanthine-guanine phosphoribosyl transferase (HGPRT); inosine monophosphate (IMP); nucleoside phosphorylase (NP); uric acid (UR); xanthine (XA); xanthine oxidase (XO).

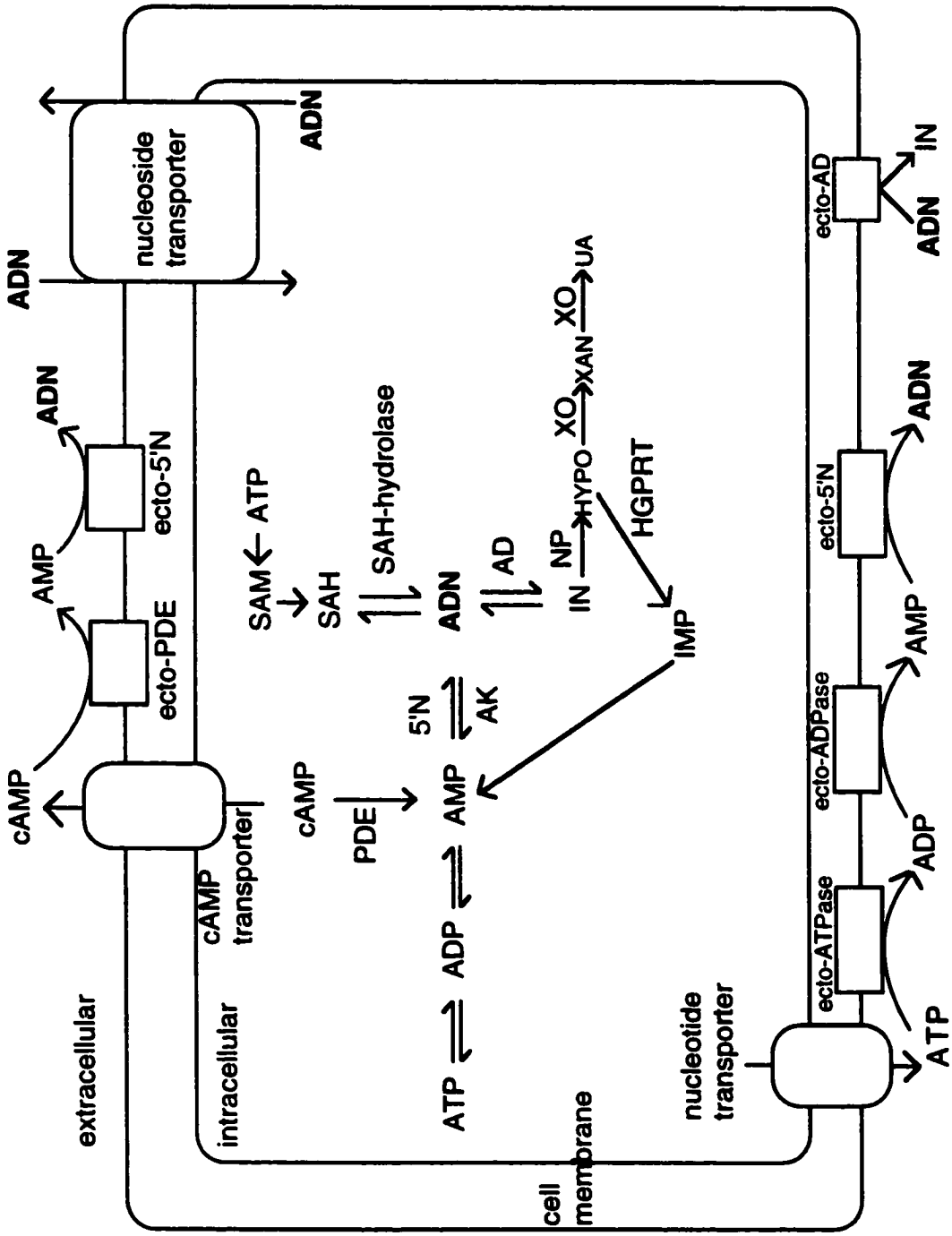

extracellular
intracellular
cell membrane
cAMP
AMP
ADN
ecto-PDE
ecto-5'N
nucleoside transporter
cAMP transporter
nucleotide transporter
ecto-AD
ecto-ADPase
ecto-ATPase
ATP
ADP
AMP
ADN
IN
cAMP
PDE
5'N
AK
SAM
ATP
SAH
SAH-hydrolase
ADN
AD
NP
IN
HYPO
XO
XAN
XO
UA
HGPRT
IMP

Fig. 2

AMP and then by ecto-5'-nucleotidase to adenosine. Immunological isoforms of ecto-5'-nucleotidase have been found in rat nerve terminals (Cunha et al., 2000). Extracellular cAMP that was released from the cell via a cAMP transporter is metabolized by ecto-cAMP-phosphodiesterase to AMP and then by ecto-5'-nucleotidase to adenosine (Linden, 1999). Increases in extracellular cAMP levels may lead to slower increases in extracellular adenosine levels compared to increases in adenosine levels following the release of nucleotides (Brundege et al., 1997).

1.2. Metabolism

Adenosine is rapidly metabolized in plasma and brain. The half-life of adenosine in human plasma is only 0.6-1.5 sec (Möser et al., 1989). After a 15 sec perfusion of [^{3}H] adenosine into the internal carotid artery, only 10% of the radioactivity remained as adenosine in the brain, the rest was incorporated into metabolites of adenosine (Pardridge et al., 1994).

Adenosine is removed from the extracellular space by uptake into neurons or glia through the nucleoside transporter. Intracellularly adenosine is degraded and subsequently converted to inosine by adenosine deaminase (Dunwiddie, 1985). Adenosine deaminase is also found as an ectoenzyme, which converts adenosine to inosine in the extracellular space (Franco et al., 1997). In the cell adenosine is converted to AMP by adenosine kinase (Dunwiddie, 1985; Ijzerman and Van der Wenden, 1997). Of these two enzymes, adenosine kinase plays a greater role in regulating extracellular

adenosine concentrations under basal conditions, whereas adenosine deaminase plays a greater role in regulating extracellular adenosine levels when adenosine formation is increased by energy depletion (Lloyd and Fredholm, 1995).

1.3. Nucleoside transporters

There are two major classes of nucleoside transporters: equilibrative and concentrative. The equilibrative transporters can transport nucleosides in either direction, depending on the direction of the concentration gradient for facilitated diffusion (Griffith and Jarvis, 1996). Two subtypes of equilibrative transporters have been identified: the NBTI-sensitive (es), which is inhibited by low concentrations of nitrobenzylthioinosine (NBTI; 1-10 nM), and NBTI-insensitive (ei) transporters (Jarvis, 1988). The es and ei transporters show similar distributions in the brain and are present in the cortex (Geiger and Nagy, 1984; Anderson et al., 1999a). The mRNA for the es and ei transporters have been found in both neurons and astrocytes (Anderson et al., 1999a,b). Currently, little information is available regarding intracellular localizations of the transporters.

The concentrative nucleoside transporters transport nucleosides into the cell against their concentration gradients by coupling transport to the Na^+-gradient generated by the Na^+-K^+ pump (Wang et al., 1997). To date, five major subtypes (N1-N5) of these transporters have been characterized (Wang et al., 1997). Under basal conditions the net flux of adenosine is into the cell (Arch and Nesholme, 1978).

1.4. Adenosine receptors

Adenosine exerts its effects through activation of cell surface receptors, which are classified into A_1, A_{2A}, A_{2B}, and A_3 subtypes (Table 1). Nanomolar concentrations of adenosine are required for activation of the rat A_1 (10 nM) and A_2 (30 nM) receptors whereas micromolar concentrations (1 μM) are required for activation of the A_3 receptor (Jacobson et al., 1995; Latini et al., 1996; Senba, 1998). Although two adenosine reactive sites are located on adenylate cyclase, an excitatory "R" and an inhibitory "P" site (Londos and Wolff, 1977), the physiological significance of these sites remain unknown.

1.4.1. A_1 receptors

The adenosine A_1 receptor has been cloned from tissues from a variety of species including rat, bovine, rabbit and human (Mahan et al., 1991; Reppert et al., 1991; Olah et al., 1992; Palmer and Stiles, 1995; Olah and Stiles, 1995). Adenosine A_1 receptors, through their coupling to $G_{i/o}$ proteins (G protein; guanosine 5'-triphosphate binding protein), inhibit adenylate cyclase (Mahan et al., 1991; Reppert et al., 1991; Zhou et al, 1992; Salvatore et al., 1993; Foster et al., 1995; Stiles, 1997). Adenosine acting at A_1 receptors also opens K^+ and closes Ca^{2+} channels (Trussell and Jackson, 1985; Dolphin et al., 1986) and, depending on the tissue, activates or inhibits phospholipase C (Linden, 1991).

Adenosine A_1 receptors are distributed widely throughout the rat brain, including the cerebral cortex, hippocampus and thalamus (Goodman and Snyder, 1982; Mahan et al., 1991; Reppert et al., 1991). Within the cerebral

Table 1. Classification and properties of adenosine receptor subtypes.

	A_1	A_{2A}	A_{2B}	A_3
transducer	$G_{i/o}$	G_s	$G_{s/q}$	$G_{i/o}$
primary effector	AC	AC	AC	AC
second messenger	↓cAMP	↑cAMP	↑cAMP	↓cAMP
secondary effectors	↑K^+ ↓Ca^{2+} ↑↓PLC		↑Ca^{2+} ↑PLC	
agonists	ADN NECA L-PIA CHA CPA	ADN NECA L-PIA CGS 21680	ADN NECA L-PIA	ADN NECA IB-MECA
antagonists	caffeine theophylline DPCPX	caffeine theophylline CGS 15943	caffeine theophylline	
location	widespread	basal ganglia, cortex	pars tuberalis	low levels in cortex, striatum

Abbreviations: adenylate cyclase (AC); adenosine (ADN); 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo[1,5-c]quinazolin-5-imine (CGS 15943); (2-*p*-carboxyethyl)-phenylamino-5'-*N*-carboxyamidoadenosine (CGS 21680); N^6-cyclohexyladenosine (CHA); N^6-cyclopentyladenosine (CPA); 8-cyclopentyl-1,3-dipropylxanthine (DPCPX); 1-deoxy-1-[6-[(3-iodophenyl)-methyl]amino)-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA);*N*-ethylcarboxamidoadenosine (NECA); N^6-phenylisopropyladenosine (PIA).

References: Daval et al., 1991; Linden, 1991; Stehle et al., 1992; Zhou et al., 1992; Fredholm et al., 1994; Brundege and Dunwiddie, 1997; Stiles, 1997; Linden et al., 1998; Rosin et al., 1998

cortex the highest density of autoradiographic labeling was found in layers I, IV, and VI with lower levels detected in layers II, III, and V (Goodman and Snyder,1982). Using monoclonal antibodies against A_1 receptors, immunoreactivity in the cortex was found in the cell bodies, dendrites and axonal initial segments of pyramidal neurons and also in some interneurons (Rivkees et al., 1995; Ochishi et al., 1999). Electron microscopic studies also revealed A_1 receptor immunoreactivity in both presynaptic and postsynaptic structures, as well as in glial elements in the cortex (Ochishi et al., 1999). The expression of adenosine A_1 receptor mRNA in microglia in the rat brain has also been shown (Fiebich et al., 1996).

Adenosine acting at presynaptic A_1 receptors inhibits the release of glutamate, acetylcholine, noradrenaline, serotonin and dopamine (Fredholm and Dunwiddie, 1988; Shen and Johnson, 1997). Adenosine also reduces release of γ-aminobutyric acid (GABA) in the substantia nigra pars compacta and ventral tegmental area (Wu et al., 1995). Activation of presynaptic A_1 receptors leads to the inhibition of neurotransmitter release, either by activating K^+ efflux and hyperpolarizing the presynaptic terminal or by inhibition of Ca^{2+} influx.

Postsynaptic inhibitory effects of adenosine are mediated by activation of outward K^+ current. For example, adenosine acting at A_1 receptors leads to the activation of a voltage- and Ca^{2+}-insensitive K^+ conductance and also enhances a voltage-insensitive Ca^{2+}-dependent K^+ conductance to produce a hyperpolarization of the membrane potential (Greene and Haas, 1991).

Postsynaptic inhibitory actions of adenosine have been shown in structures involved in arousal including cholinergic neurons in the mesopontine tegmentum and also in the locus coeruleus (Shefner and Chiu, 1986; Rainnie et al., 1994).

1.4.2. A_2 receptors

The A_{2A} receptor has been cloned from tissues of a variety of species including rat, canine, and human (Olah and Stiles, 1995). A_{2A} receptors, through coupling to G_s proteins, activate adenylate cyclase (Greene and Haas, 1991; Stehle et al., 1992; Stiles, 1997). Activation of these receptors also leads to the release of neurotransmitters, including acetylcholine, glutamate and noradrenaline (Cunha et al., 1995; Sebastião and Ribeiro, 1996). A_{2A} receptors also modulate the release of GABA from rat hippocampal nerve terminals (Cuhna and Ribeiro, 2000). Adenosine A_{2A} receptors were found to be located primarily in the basal ganglia, but are also expressed in microglia in the rat brain (Fiebich et al., 1996). Dense A_{2A} receptor-like immunoreactivity was found in the striatum and nucleus accumbens whereas less dense labeling was found in other areas of the brain including layers V and VI of the cerebral cortex (Rosin et al., 1998). Consistent with these results, binding of the A_{2A} receptor ligand [^{3}H]CGS 21680 (2-[p-(2-carboxyethyl)-phenylethylamino]-5'-*N*-ethylcarboxamido-adenosine) was found in the striatum, nucleus accumbens, globus pallidus and olfactory tubercle as well as in the cerebral cortex, hippocampus, thalamus and cerebellum (Johansson and Fredholm, 1995). However, subsequent studies found that the high affinity [^{3}H]CGS 21680

binding site in the striatum had different binding characteristics from the binding site in the cerebral cortex and hippocampus (Cuhna et al., 1996).

Compared with the A_{2A} receptor, the A_{2B} receptor has a higher threshold for activation by adenosine (Hide et al., 1992). The A_{2B} receptor has been cloned from both rat and human libraries and is expressed in a variety of cells (Stehle et al., 1992; Olah and Stiles, 1995). Through coupling to G_s protein, the A_{2B} receptor activates adenylate cyclase (Greene and Haas, 1991; Stehle et al., 1992; Stiles, 1997). This receptor also activates phospholipase C and mobilizes Ca^{2+} by signalling through G_q proteins (Linden et al., 1998). A_{2B} receptor mRNA has been localized to the hypophyseal pars tuberalis in the brain (Stehle et al., 1992). A_{2B} receptor mRNA is expressed by astrocytes in the rat brain (Fiebich et al., 1996) and activation of these receptors mediates cAMP increases in these cells (Peakman and Hill, 1994). A_{2B} receptors have been implicated in diverse functions such as mast cell activation, vasodilation, and cell growth regulation (Feoktistov and Biaggioni, 1997).

1.4.3. A_3 receptors

The adenosine A_3 receptor has been cloned from rat, sheep and human tissues (Zhou et al., 1992; Salvatore et al., 1993; Linden, 1994). The A_3 receptor, through coupling to $G_{i/o}$ protein, inhibits adenylate cyclase (Mahan et al., 1991; Reppert et al., 1991; Zhou et al, 1992; Salvatore et al., 1993; Foster et al., 1995; Stiles, 1997). A low level of expression of A_3 receptor mRNA has been detected in the cortex, striatum and olfactory bulb (Zhou et al., 1992). A_3

receptor mRNA is also expressed in microglia in the rat brain (Fiebich et al., 1996).

1.5. Physiological functions

For adenosine to exert a physiological function, it must be present at sufficient concentrations in the extracellular space. Although the exact mechanisms by which a stimulus results in the release of adenosine are unknown, several conditions will evoke the release of adenosine in the brain.

1.5.1. Ischemia and hypoxia

The rate of adenosine production is enhanced when energy demand is greater than the rate of energy supply, such as during cerebral hypoxia and ischemia (Rudolphi et al., 1992). For example, the extracellular concentration of adenosine in the striatum increases after middle cerebral artery occlusion in the awake, freely moving rat (Melani et al., 1999). Under ischemic conditions the main source of adenosine in hippocampal slice is shown to be dephosphorylation of AMP by 5'-nucleotidase (Latini et al., 1995). A 5 min period of ischemia in hippocampal slices produced an efflux of adenosine, which in turn depressed electrical activity in the slices, probably via activation of A_1 receptors (Pedata et al., 1993). Adenosine may protect against some of the pathological effects of hypoxia and ischemia by inhibiting the release of excitatory amino acids, inhibiting the influx of Ca^{2+}, and by dilating blood vessels in the brain (Rudolphi et al., 1992; von Lubitz et al., 1995a). Further

studies are required to determine the therapeutic potential of adenosine as a protective agent in hypoxia and ischemia.

1.5.2. Epilepsy

Adenosine is considered by some investigators to be an endogenous anticonvulsant. Indeed, the release of adenosine is increased in rat brain within 10 sec of the onset of seizures (Winn et al., 1980). Furthermore, adenosine analogues have potent anticonvulsant actions in a variety of seizure models (Dragunow, 1988; Chin, 1989). Acute administration of A_1 receptor agonists reduces or prevents seizure activity elicited by chemical and electrical stimuli (Jacobson et al., 1996). Although it is unclear whether acute stimulation of A_{2A} or A_3 receptors antagonizes seizure generation, chronic administration of the A_3 receptor agonist IB-MECA (1-deoxy-1-[6-[(3-iodophenyl)-methyl]amino)-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide) provided protection against N-methyl-D-aspartate (NMDA)-induced seizures (von Lubitz et al., 1995b). An increase in K^+ conductance induced by adenosine acting at A_1 receptors may account for some of the antiepileptic effects of adenosine (Dragonow, 1988). Further studies are required to determine the full therapeutic potential of adenosine as a treatment for epilepsy.

1.5.3. Analgesia

Increase in endogenous adenosine or activation of adenosine receptors has produced antinociceptive effects in rat. Local administration of adenosine

kinase inhibitors in combination with an adenosine deaminase inhibitor, which together would have increased extracellular adenosine levels, produced antinociceptive effects in the rat formalin test (Sawynok et al., 1998). Formalin also induced the release of adenosine in the rat hindpaw, and this release was augmented by local administration of an adenosine kinase inhibitor (Liu and Sawynok, 1999). Adenosine has also been shown to exert antinociceptive effects mediated by A_1 receptors in the spinal cord (Sawynok et al., 1986; Lee and Yaksh, 1996). Adenosine is thought to exert its analgesic effects through a decrease in the release of pain-inducing peptides and excitatory amino acids (Sawynok, 1999). The manipulation of extracellular adenosine may provide new methods for the control of pain.

1.5.4. Control of behavioral state

Adenosine is thought to be a mild sleep-promoting factor in the brain. In the rat cortex, adenosine, inosine and hypoxanthine levels were found to be high between 08:00 and 20:00 h with a peak around 12 noon and a second peak from 02:00-04:00 h (Chagoya de Sánchez et al., 1993). Adenosine concentrations were higher during spontaneous wakefulness than during slow wave sleep in the cortex, basal forebrain, thalamus and dorsal raphe nucleus in the cat (Strecker et al., 1999). In both rat and cat, the extracellular concentration of adenosine increased in the basal forebrain during sleep deprivation (Basheer et al., 1999; Strecker et al., 1999).

Adenosine may exert its somnogenic effects by acting at A_1 receptors in

the basal forebrain to inhibit the activity of cholinergic neurons that modulate arousal (Portas et al., 1997). Administration of nitrobenzylthioinosine (NBTI) via microdialysis in the basal forebrain and ventroanterior/ventrolateral thalamus increased the local concentration of adenosine (Porkka-Heiskanen et al., 1997). In addition, perfusion of NBTI in the basal forebrain, but not in the thalamus, decreased wakefulness, suggesting that the effects of adenosine on arousal may be specific to regions containing cholinergic neurons known to modulate arousal (Porkka-Heiskanen et al., 1997).

1.6. Physiological and endogenous factors that release adenosine

Several physiological factors have been shown to increase the extracellular concentration of endogenous adenosine in the brain, including an increase in temperature (Masino and Dunwiddie, 1999), high K^+, glutamate (McIlwain and Poll, 1986), histamine (Pull and McIlwain, 1975), interleukin-1 (Luk et al., 1999) and vasoactive intestinal polypeptide (Rosenberg and Li, 1995a). Serotonin was found to release adenosine from primary afferent nerve terminals in spinal cord synaptosomes (Sweeney et al., 1988). GABA did not have an effect on the release of [^{14}C]adenine derivatives in cortical slice (Pull and McIlwain, 1975).

An increase in extracellular K^+ concentration releases adenosine from the cerebral cortex and hippocampus (van Wylen et al., 1986; Hoehn and White, 1989; Chen et al., 1992). In cortical and hippocampal slices, K^+ evoked adenosine release was reduced by NMDA receptor antagonists (Hoehn and

White, 1990; Chen et al., 1992). Similarly, under K^+-depolarizing conditions, adenosine efflux was reduced by antagonism of NMDA receptors in the cortex and striatum *in vivo* (Pazzagli et al., 1993, 1994). These studies indicate that the release of adenosine by K^+ may be due to the release of glutamate acting at NMDA receptors.

Electrical stimulation of the cat sensorimotor cortex increased [^{3}H]adenosine release in a study using the cortical cup technique (Sulakhe and Phillis, 1975). Electrical stimulation of cortical, hippocampal or striatal slice increased release of adenosine and adenine derivatives (McIlwain and Poll, 1985; Pedata et al., 1988, 1990; Pazzagli et al., 1990; Sciotti et al., 1993). The electrically evoked release of adenosine was greater in hippocampal slice compared to cortical and striatal slices (Pedata et al., 1990). Electrical stimulation also increased extracellular adenosine in the caudate nucleus of anesthetized rat (Sciotti et al., 1993).

The increase in extracellular adenosine evoked by electrical stimulation was from the degradation of AMP in hippocampal slice (Latini et al., 1995) and rabbit nerve fibers (Maire et al., 1984). Electrical stimulation also increased adenosine, inosine and hypoxanthine content and diminished ATP, ADP and AMP content in cortical tissue, whereas S-adenosylhomocysteine content was unchanged (McIlwain and Poll, 1985). These studies indicate that electrical stimulation evoked the release of a nucleotide that was degraded by ectoenzymes to adenosine.

Furthermore, the electrically evoked increase in ^{3}H-purine release in

hippocampal slice was partly dependent upon extracellular Ca^{2+} (Jonzon and Fredholm, 1985). Also, P- and N-type voltage dependent Ca^{2+} channels have been shown to be involved in the coupling between electrical stimulation and adenosine release (Latini et al., 1997). The electrically evoked release of adenosine was also abolished by tetrodotoxin and reduced by the removal of Ca^{2+} and by an NMDA receptor antagonist either alone or in the presence of DNQX (6,7-dinitroquinoxaline-2,3-dione; Pedata et al., 1991). Taken together, these results suggest that electrical stimulation evoked the release of excitatory amino acids, which acted at NMDA and nonNMDA receptors to promote the release of a nucleotide that was subsequently broken down to adenosine extracellularly.

2. NEUROTRANSMITTERS THAT AFFECT THE RELEASE OF ADENOSINE

Glutamate, acetylcholine and noradrenaline have all been shown to affect the concentration of extracellular adenosine (Hoehn and White, 1990a; Rosenberg et al., 1994; Semba and White, 1997). Therefore, background information about these neurotransmitters will be provided, followed by a review of their effects on adenosine release, as is currently understood.

2.1. Glutamate

Glutamate is the main excitatory neurotransmitter in the brain (Fonnum, 1984; Orrego and Villaneuva, 1993). Within the mitochondria of the nerve

terminal glutamine is converted to glutamate by glutaminase, and in the cytoplasm glutamate is packaged within synaptic vesicles. Depolarization of the axon terminal results in the release of glutamate from the vesicles into the synaptic cleft in a process called exocytosis. The action of glutamate in the synaptic cleft is terminated when it is taken up into glial cells by the glutamate transporters GLT-1 (glutamate transporter-1, also called EAAT2 or excitatory amino acid transporter 2) and GLAST (L-glutamate/L-aspartate transporter, also called EAAT1) and converted into glutamine by glutamine synthase. Glutamine is then cycled back to the nerve terminal (Martinez-Hernandez et al., 1977; Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Feldman et al., 1997; Robinson and Dowd, 1997; Satoh, 1998; Dingledine and McBain, 1999; Hertz et al., 1999). Glutamate transporters are also present in neuronal membranes. However, it is the activity of the glial transporter, not the neuronal transporter, that maintains low extracellular levels of glutamate and thereby prevents glutamate neurotoxicity (Rothstein et al., 1996). Glutamate is also a precursor in the biosynthesis of GABA (Dingledine and McBain, 1999).

Glutamate is the main transmitter in a variety of pathways in the CNS including those involved in corticocortical, corticofugal and sensory systems (Peinado and Mora, 1986; Cotman et al., 1987). The anatomical sources of glutamate in the cerebral cortex are cortical projection neurons, a subclass of cortical interneurons, and terminals of axons from thalamocortical projection neurons. In the rat somatosensory cortex a large population of neurons were found to be immunoreactive for glutamate, and most of this population were

pyramidal cells in layers II, III, V and VI (Conti et al., 1987). These pyramidal cells can release glutamate from terminals in their target structures. Pyramidal cells in layers II and III send association fibers to other cortical areas of the same hemisphere, as well as commissural fibers to the other hemisphere through the corpus callosum. The intracortical excitatory pathways are from layers II and III to V, from layer IV to layers II and III and from layer V to VI. Pyramidal cells in layers V and VI send subcortical projections to structures in the brainstem (Tsumoto, 1990). Stellate cells in layer IV are the only type of excitatory interneuron in the cortex and use glutamate as their transmitter (Barr and Kiernan, 1993). Finally, the cortex receives major glutamatergic projections from the thalamus (Ottersen et al., 1983).

2.1.1. Glutamate receptors

There are two types of glutamate receptor: ionotropic and metabotropic. The ionotropic glutamate receptors are classified into the NMDA, AMPA and kainate receptors (Table 2). The metabotropic receptors are classified into group I, II and III receptors (Table 3).

2.1.1.1. NMDA receptors

The NMDA receptor incorporates a channel that is characterized by Ca^{2+} permeability, a requirement for glycine as a co-agonist, and a voltage-dependent Mg^{2+} block (Seeburg, 1993). The Na^{+}/Ca^{2+} influx through the channel is blocked by extracellular Mg^{2+} ions at a site within the channel at

Table 2. Classification and properties of ionotropic glutamate receptors.

	NMDA	AMPA	Kainate
receptor subunits	NR1 NR2A NR2B NR2C NR2D	GluR1 GluR2 GluR3 GluR4	GluR5 GluR6 GluR7 KA1 KA2
channel permeability	Na^+, K^+, Ca^{2+}	Na^+, K^+ (Ca^{2+} for receptors lacking GluR2)	Na^+, K^+
agonists	glutamate aspartate NMDA	glutamate aspartate AMPA kainic acid quisqualic acid	glutamate aspartate kainic acid
antagonists	CPP D-APV	CNQX DNQX	CNQX DNQX

References: Seeburg, 1993; Hammond, 1996; Lerma, 1998; Ozawa et al., 1998.

Abbreviations: 3-(2-carboxypiperazin-4-yl)-propyl-1-phosponic acid (CPP); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); 2-amino-5-phosphonopentanoic acid (D-APV); 6,7-dinitroquinoxaline-2,3-dione (DNQX).

Table 3. Classification and properties of metabotropic glutamate receptors.

	Group I	Group II	Group III
receptors	mGluR1 mGluR5	mGluR2 mGluR3	mGluR4 mGluR6 mGluR7 mGluR8
transducer	G_s	G_i	G_i
primary effector	PLC	AC	AC
second messengers	IP_3/DAG	cAMP	cAMP
agonists	glutamate ACPD DHPG	glutamate ACPD LCCG-1	glutamate L-AP4
antagonists	MCPG	MCPG	

Reference: Gereau and Conn, 1996.

Abbreviations: adenylate cyclase (AC);*trans*-(1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD); diacylglycerol (DAG); (S)-3,5-dihydroxyphenylglycine (DHPG); inositol triphosphate (IP_3); L-AP4; L(+)-2-amino-4-phosphonobutyric acid (L-AP4); (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (LCCG-1); α-methyl-carboxyphenyl-glycine (MCPG); phospholipase C (PLC);

voltages close to the resting membrane potential of the cell. Therefore, depolarization of the membrane is required to remove the block of the channel caused by Mg^{2+} ions (Hammond, 1996). The channel is also permeable to K^{+} and Cs^{+} ions (Ozawa et al., 1998). The NMDA receptor also contains distinct binding sites for Zn^{2+} and other molecules including polyamines, ethanol and tricyclic dizoclipine (MK-801; Harris, 1995). Two molecules, an agonist (either glutamate or NMDA) and glycine are required to open the channel. Glycine is an amino acid that also acts as an inhibitory neurotransmitter and is present in sufficient concentrations in the cerebrospinal fluid to produce its effect on the NMDA receptor (Hammond, 1996).

There is a variety of neuronal ionotropic glutamate receptor subunit proteins. NMDA receptors are comprised of proteins from the NR1 and NR2 gene families. The NR2 proteins are further divided into four subunits named NR2A to D. NMDA receptors can be reconstituted *in vitro* as a heteromer consisting of an NR1 subunit and one of the four NR2 subunits (Seeburg, 1993). Each of the ionotropic glutamate receptor subunits have in common a large extracellular N-terminus and four hydrophobic membrane segments named M1-M4 (Ozawa et al., 1998).

All of the NMDA receptor subunits have been observed in the cerebral cortex. The NR1 protein was found by using immunocytochemistry in all layers of the parietal cortex. The most intense staining was found in layers II, III and V, and staining was localized primarily in postsynaptic densities (Brose et al., 1993; Petralia et al., 1994a). The pyramidal cells in layers III and V and their

apical dendrites were moderately stained with an NR2A/B antibody (Petralia et al., 1994b). The NMDA receptor subunits NR2A, 2B, 2C and 2D were also located in the cerebral cortex (Wenzel et al., 1995).

2.1.1.2. AMPA receptors

The agonists for the AMPA receptor include glutamate, aspartate, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) and quisqualic acid. This receptor was originally named the quisqualate receptor; however, quisqualate was found also to activate metabotropic glutamate receptors, in addition to ionotropic glutamate receptors, so the receptor was renamed. Four subunits, GluR1-4 have been identified. Functional AMPA receptors can be reconstituted *in vitro* by expressing one or co-expressing any two of the GluR1-4 subunits (GluR; glutamate receptor; Seeburg, 1993). The diversity of AMPA receptors results not only from variations in subunit stoichiometry but also from alternative splicing which is responsible for the subunits existing in "flip and flop" molecular states, with the flip state allowing more current entry into the cell than the flop state (Hammond, 1996). The inward AMPA receptor current results from the flow of Na^+ ions and the channel is also permeable to K^+ and Cs^+ ions (Hammond, 1996). The channel has a negligible permeability to Ca^{2+} ions when the GluR2 subunit is present, whereas AMPA receptors lacking the GluR2 subunit are highly permeable to Ca^{2+} (Hollmann et al., 1991; Burnashev et al., 1992).

Immunocytochemical studies have shown differences in the pattern of

distribution among individual GluR subunits. Neurons in all cortical layers were labeled for GluR1 (Conti et al., 1994) and pyramidal neurons in layer V were intensely stained and immunoreactive dendrites were present throughout the cortex (Martin et al., 1993). GluR2/3 immunoreactive neurons were found in all cortical layers except layer I (Conti et al., 1994) and immunoreactivity for GluR2/3/4c was most intense in pyramidal neurons in layers III and upper V and in the neuropil in layers I-III. GluR4 immunoreactive neurons were present in all cortical layers (Conti et al., 1994) and in cortical astrocytes (Martin et al., 1993). In rat parietal cortex, neuropilar staining for GluR2/3 and GluR4 was equally dense in layers II-VI (Petralia and Wenthold, 1992). The large pyramidal neurons in layer V were densely stained with antibodies to GluR1 and to GluR2/3 in the rat cortex (Petralia and Wenthold, 1992). Cortical neurons, therefore, exhibit a degree of heterogeneity with regard to the distribution of AMPA receptor subunits.

2.1.1.3. Kainate receptors

Although kainic acid or kainate activates AMPA receptors, it also activates its own class of receptor named the kainate receptor (Ozawa et al., 1998). Glutamate and aspartate are endogenous agonists for this receptor, which incorporates a channel that is permeable to Na^+ and K^+ ions. Kainate receptors are encoded by two gene families, and five subunits of kainate receptors have been cloned. Subunits GluR5 to 7 form functional homomeric receptors, whereas subunits KA1 and KA2 only form functional channels when

they are coexpressed with one or more of GluR5 to 7 (Lerma, 1998). Kainate is a potent convulsant agent when administered *in vivo* (Ozawa et al., 1998).

In the rat cortex immunocytochemical staining for KA2 was found to be the densest in pyramidal neurons of layer V and neurons of deep layer VI (Petralia et al., 1994c). GluR6/7 immunoreactivity was dense in layers IV, V and VI (Petralia et al., 1994c). For both of these antibodies, staining of postsynaptic densities, membranes and cytoplasm was observed throughout the depth of the cerebral cortex, but definitive presynaptic staining was not detected.

2.1.1.4. Metabotropic receptors

The glutamate metabotropic receptors are coupled to G proteins and divided into three groups based on sequence homology and pharmacology (Table 3). Group I receptors include mGluR1 and mGluR5 and are positively coupled to G_o/G_q to stimulate phospholipase C which activates the inositol trisphosphate and diacylglycerol signal transduction pathways. Group II receptors include mGluR2 and mGluR3, whereas Group III receptors include mGluR4, mGluR6, mGluR7 and mGluR8. The Group II and III receptors through their coupling to G_i inhibit adenylate cyclase and decrease the production of cAMP (Gereau and Conn, 1996).

The mRNA for the metabotropic glutamate receptor mGluR2 was located in the cerebral cortex where labeling of neuronal cell bodies of pyramidal and nonpyramidal cells was distributed densely in layers IV and V, and sparsely in layers I-III and VI (Ohishi et al., 1993). Diffuse immunostaining for mGluR2 in

the rat cerebral cortex was intense or moderate in layers I-IV and moderate in layers V and VI (Ohishi et al., 1998). In the somatosensory cortex, staining was less intense in the barrels than in the surrounding neuropil or septa (Ohishi et al., 1998).

In the rat cerebral cortex, mGluR1α and mGluR1β mRNA expression was detected in some neurons in layers IV and V (Berthele et al., 1998). mGluR3 mRNA was also expressed in the rat cerebral cortex (Tanabe et al., 1993). Immunoreactivity for mGluR4 was found in layers IV and V of the rat cortex (Phillips et al., 1997). Intense immunoreactivity for mGluR5 was located in the rat cerebral cortex (Shigemoto et al., 1993). Labeling was most intense across layer II (Romano et al., 1995). In the rat cerebral cortex immunoreactivity for mGluR7a was localized in layers II and III with weak labeling detected in layer V (Bradley et al., 1998). GluR8 mRNA has also been detected in the rat cerebral cortex (Saugstad et al., 1997). The metabotropic glutamate receptors, therefore, exhibit a degree of heterogeneity in their patterns of distribution in the cerebral cortex.

2.1.2. Glutamate evoked adenosine release

Administration of glutamate releases adenosine from rat cortical synaptosomes (Hoehn and White, 1990a). However, this release was not mediated by receptors, but by the transport of glutamate into presynaptic terminals, and the subsequent release of a nucleotide and its extracellular conversion to adenosine (Hoehn and White, 1990a). Glutamate also releases

adenosine from slices of rat parietal cortex (Hoehn and White, 1989; Hoehn and White, 1990b). Unlike in cortical synaptosomes, the release of adenosine in cortical slice by glutamate involves both NMDA and non-NMDA receptors (Hoehn and White, 1990b,c). Activation of nonNMDA receptors releases adenosine per se in a Ca^{2+}-independent manner, whereas activation of NMDA receptors releases a nucleotide which is converted extracellularly to adenosine (Craig and White, 1993). cAMP, protein kinase C or nitric oxide formation does not appear to be involved in NMDA evoked adenosine release in cortical slice (Craig and White, 1993; Craig et al., 1994; Wang and White, 1998). Administration of glutamate, NMDA and quisqualate evokes the release of adenosine in hippocampal slice (Pedata et al., 1991; Manzoni et al., 1994). NMDA and kainic acid also evoke the release of adenosine from the hippocampus using in vivo microdialysis in the anesthetized rat (Chen et al., 1992; Carswell et al., 1997). Finally, kainic acid produced an increase in extracellular adenosine in the caudate nucleus of anesthetized rat (Sciotti et al., 1993). These results indicate that activation of NMDA and nonNMDA receptors evokes the release of adenosine in several structures of the brain.

2.3. Acetylcholine

Acetylcholine plays a role in a variety of functions including learning and memory, cortical arousal, attention, and sleep (Semba et al., 1991; Heimer, 1995). The cerebral cortex receives cortical projections from the cholinergic nuclei in the basal forebrain. The parietal cortex receives projections from

cholinergic neurons in the ventromedial globus pallidus and the substantia innominata (Bigl et al., 1982: McKinney et al., 1983). In the rat, the choline acetyltransferase (ChAT) immunoreactive fiber distribution varies from one area of the parietal cortex to another (Lysakowski et al., 1989). In the primary somatosensory area, the distribution of fibers is most dense at the border between layers IV and V, with layers I and V being moderately dense and layers II, III and VI of lighter density. In the secondary somatosensory area, fiber density is greatest in layers I and V, with moderate staining in layers II and III. In addition to the cholinergic innervation from the basal forebrain, the rodent cerebral cortex possesses intrinsic neurons that are ChAT positive (Levey et al., 1984). Acetylcholine release is higher during periods of activity or arousal in rat somatosensory as well as visual and motor cortices (Jiménez-Capdeville and Dykes, 1996).

Acetylcholine is synthesized from acetyl-CoA and choline and this reaction is catalyzed by ChAT. Acetylcholine is mainly synthesized in the nerve terminal, although some synthesis also occurs in the cell body. At the terminal, acetylcholine is transported into synaptic vesicles and released into the synaptic cleft following depolarization of the nerve terminal. The released acetylcholine binds to its receptors and the action of acetylcholine in the synapse is terminated by its conversion to acetate and choline by acetylcholinesterase (Taylor and Brown, 1999; Tohyama, 1998). Acetylcholine receptors were originally classified into two types based on the pharmacological actions of nicotine and muscarine. It is now known that

multiple subtypes exist within each of these two receptor types.

2.3.1. Nicotinic receptors

Nicotinic receptors have been shown to play roles in neuronal development, learning and memory and reward (Jones et al., 1999), and have been implicated in a variety of pathological conditions such as Alzheimer's disease and epilepsy (Lindstrom, 1997). Activation of presynaptic nicotinic acetylcholine receptors enhances the release of noradrenaline, dopamine, GABA, serotonin and acetylcholine (Role and Berg, 1996).

The nicotinic receptors belong to the superfamily of ionotropic receptors that also includes $GABA_A$, $5HT_3$ and glycine receptors (Sargent, 1993). Nicotinic receptors are permeable to Na^{2+} and Ca^{2+} ions and mediate fast inward current (Sargent, 1993). Neuronal nicotinic receptors are composed of α (α2-9) and β (β2-4) subunits. Blocking by α-bungarotoxin identifies the presence of the homomeric α7, α8 or α9 receptors (Colquhoun and Patrick, 1997). Neuronal nicotinic receptors that are not blocked by α-bungarotoxin are comprised of α (α2-6) and β (β2-4) subunits (Lindstrom et al., 1995). Functional complexes with the compositions of $(\alpha4)_2(\beta2)_3$ and $(\alpha7)_5$ have been obtained by heterologous expression of these subunits (Anand et al., 1991; Cooper et al., 1991). In mammals, α4β2 receptors represent 90% of the brain nicotinic receptors with high affinity for nicotine (Whiting and Lindstrom 1988; Flores et al., 1992). Many α4β2 receptors are thought to be located presynaptically, and these particular receptors may be responsible for modulation of transmitter release (Lindstrom et al., 1995). Although other heteromeric and homomeric

species of receptor exist in the brain, they appear less abundant (Sargent, 1993).

In the rat cortex α2 subunit mRNA was localized to neurons in layers V and VI, whereas neurons expressing β2 subunit mRNA were found in all layers (Wada et al., 1989). Immunoreactivity for the α4 subunit was found most prominently in the cell bodies and apical dendrites of pyramidal cells in layer V and was also found through layers II to VI (Nakayama et al., 1995). Binding of [^{125}I]α-bungarotoxin was also high in the cerebral cortex, especially in layers I and IV (Clarke et al., 1985). Lesion studies suggest that cortical [^{3}H]nicotine binding sites, particularly within layer IV in the parietal cortex, are located on noncholinergic afferents arising from the thalamus (Lavine et al., 1997).

2.3.2. Muscarinic receptors

Muscarinic receptors belong to a superfamily of G protein-coupled receptors consisting of seven transmembrane spanning domains. Of the five muscarinic receptors that have been cloned, expressed and defined pharmacologically, M1, M3 and M5 couple to G_q and G_{11} and activate phospholipase C, whereas M2 and M4 couple to G_i and G_0 to inhibit cAMP, inhibit Ca^{2+} channels and activate K^+ channels (Table 4; Kashihara et al., 1992; McKinney, 1993; Löffelholz, 1996; Yan and Surmeier, 1996; Taylor and Brown, 1999; M refers to the receptor protein, whereas m refers to the gene for the receptor).

In the rat cortex, m_1, m_2 and m_4 receptor immunoreactivities are

Table 4. Classification and properties of muscarinic receptors.

	M1	M2	M3	M4	M5
transducer	G_q	$G_{i/0}$	G_q	$G_{i/0}$	G_q
primary effector	PLC	AC	PLC	AC	PLC
second messengers	IP_3/DAG	cAMP	IP_3/DAG	cAMP	IP_3/DAG
secondary effectors		$\downarrow Ca^{2+}$ $\uparrow K^+$		$\downarrow Ca^{2+}$ $\uparrow K^+$	
agonists	ACh muscarine oxotrem CDD 0097	ACh muscarine oxotrem	ACh muscarine oxotrem 4-DAMP	ACh muscarine oxotrem	ACh muscarine oxotrem
antagonists	atropine pirenzepine	atropine	atropine	atropine	atropine
location	cortex hippocampus	cortex hippoampus thalamus	cortex hippocampus	cortex hippocampus	

References: Levey et al., 1991; Smith et al., 1991; Kashihara et al., 1992; McKinney, 1993; Schliebs and Robner, 1995; Löffelholz, 1996; Yan and Surmeier, 1996; Taylor and Brown, 1999.

Abbreviations: adenylate cyclase (AC); acetylcholine (ACh), diacylglycerol (DAG), 4-diphenylacetoxy-N-methylpiperidine (4-DAMP); inositol triphosphate (IP_3), oxotremorine (oxotrem); phospholipase C (PLC).

differentially localized. Immunoreactivity for the m_1 protein is present in most cortical neurons and dense in neuropil of layers II, III, and IV. The m_2 protein is dense in layer IV and the border of layers V/VI and appears to be located presynaptically since it was associated mostly with fibers and presumptive terminals and only occasionally with perikarya. Immunoreactivity for the m_4 protein is less dense and localized in the neuropil of layers II and III (Levey et al., 1991). M3 receptor densities are highest in layers I and II and lowest in layer VI (Smith et al., 1991).

The M1 receptor appears to be the most abundant subtype in the cerebral cortex (McKinney, 1993). Application of acetylcholine to cortical pyramidal cells *in vivo* or *in vitro* results in a slow excitatory response associated with an increase in neuronal excitability, that is mediated by an inhibition of two K^+ current, I_{AHP} and I_M (McCormick and Williamson, 1989; McCormick, 1993). This increase in neuronal excitability may contribute to the cortical desynchronization observed during wakefulness and rapid eye movement sleep (McCormick and Williamson, 1989; McCormick, 1990; McCormick, 1993).

2.3.3. Acetylcholine and adenosine release

Lesioning of the cholinergic fibers projecting to the cortex from the nucleus basalis resulted in a decrease in electrically stimulated [^{3}H]purine release in the cortex (Pedata et al., 1989), suggesting that cholinergic transmission may contribute to cortical adenosine release *in vivo*. However,

acetylcholine did not increase the efflux of [^{14}C]adenine derivatives from cortical tissue (Pull and McIlwain, 1975). Carbachol alone did not affect the basal release of adenosine, but did enhance NMDA evoked adenosine release in cortical slice (Semba and White, 1997). In this study, the facilitory effects of carbachol on NMDA evoked adenosine release were blocked by atropine and by a muscarinic M3 receptor antagonist. Carbachol had no effect on adenosine release evoked by AMPA. In electrically stimulated cortical slices, the muscarinic agonist oxotremorine enhanced [^{3}H]-labeled purine release (Pedata et al., 1988). These findings suggest that activation of muscarinic receptors alone had no effect on basal adenosine levels but did enhance the evoked release of adenosine.

2.4. Noradrenaline

Noradrenaline is a neurotransmitter in the brain and has been shown to play a role in a wide variety of functions in the central nervous system including pain, depression, vigilance and hunger (Aston-Jones et al., 1991; Feldman et al., 1997; Kandel et al., 2000). The noradrenergic neurons of the locus coeruleus are the sole source of noradrenergic innervation to the cerebral cortex and the subcortical projections of these neurons are targeted to primary sensory and association nuclei (Séguéla et al., 1990; Guyenet, 1991; Aston-Jones et al., 1995). Enhancement of locus coeruleus neuronal activity increases encephalographic (EEG) measures of arousal in the cortex, and the activity of locus coeruleus neurons varies with the sleep-wake cycle, indicating

that the locus coeruleus has a role in behavioral state control (Aston-Jones and Bloom, 1981; Berridge and Foote, 1994).

The first step in the synthesis of noradrenaline, and catecholamine synthesis in general, is the conversion of tyrosine to dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase within the cytosol. L-DOPA is converted into dopamine by aromatic L-amino acid decarboxylase. Dopamine is then taken up into vesicles and subsequently converted into noradrenaline by dopamine β-hydroxylase. Noradrenaline is released by exocytosis into the synaptic cleft upon depolarization of the axon in a Ca^{2+}-dependent manner. The action of noradrenaline at the synapse is terminated by reuptake into presynaptic nerve terminals by cell membrane transporters and its intracellular degradation by monoamine oxidase and catechol-O-methyltransferase (Feldman et al., 1997; Kuhar et al., 1999). Tricyclic antidepressants inhibit noradrenaline uptake. Drugs such as amitriptyline and imipramine also inhibit serotonin uptake, whereas desipramine selectively blocks noradrenaline uptake (Richelson and Pfenning, 1984). The uptake of noradrenaline by the neuronal membrane transporter is dependent upon the Na^+ gradient across the membrane and is also Cl^- dependent (Kuhar et al., 1999).

The receptors for noradrenaline are adrenergic receptors and these receptors belong to the G protein coupled receptor superfamily. The adrenergic receptors can be divided into two broad categories, α and β, and the α category can be divided further into α_1- and α_2-receptors. Through their interaction with G_q protein, activation of the α_1-receptor releases inositol trisphosphate and

diacylglycerol intracellularly, which leads to an increase in intracellular Ca^{2+}. The α_1-receptors are further divided into α_{1A}-, α_{1B}-, and α_{1D}-receptors (Schwinn et al., 1991; Wu et al., 1992; Perez et al., 1993; Bylund et al, 1994; Table 5). The α_2-adrenergic receptors, α_{2A}, α_{2B} and α_{2C}, inhibit adenylate cyclase through coupling to G_i protein (Kurose et al., 1991; Coupry et al., 1992; Duzic et al., 1992; Table 6). Three β-adrenergic receptors, β_1, β_2 and β_3, activate adenylate cyclase through coupling to G_s (Table 7). A fourth receptor, β_4, has been identified pharmacologically but has a low affinity for noradrenaline (Kauman 1997; Galitzky et al., 1997; Sarsero et al., 1998).

The adrenergic receptors that have been found to be localized in the cortex include the α_1-, α_{2A}-, β_1- and β_2-receptors (Bylund et al., 1994; Pieribone et al., 1994; Hieble et al., 1995; King et al., 1995; Furuyama et al., 1998). The application of noradrenaline to cortical neurons recorded extracellularly *in vivo* has revealed a wide variety of postsynaptic responses (reviewed by McCormick et al., 1991). These responses include weak inhibition mediated by β-receptors, slow excitatory responses mediated by α_1-receptors and modulatory responses mediated by both α- and β-receptors. These modulatory responses to noradrenaline produce an increase in the ratio between responses that are specific to the stimulus compared to the baseline firing rate (ie., increases the signal-to-noise ratio).

2.4.1. Noradrenaline and adenosine release

Previous reports on the effect of noradrenaline on the release of

Table 5. Classification and properties of α_1-adrenergic receptors.

	α_{1A} (previously α_{1C})	α_{1B}	α_{1D}
transducer	G_q	G_q	G_q
primary effector	PLC	PLC	PLC
second messenger	IP_3/DAG	IP_3/DAG	IP_3/DAG
secondary effector	$\uparrow Ca^{2+}$	$\uparrow Ca^{2+}$	$\uparrow Ca^{2+}$
agonists	NA phenylephrine	NA phenylephrine	NA phenylephrine
antagonists	prazosin	prazosin	prazosin
location	cortex cerebellum	cortex thalamus	cortex hippocampus

Abbreviations: diacylglycerol (DAG); inositol triphosphate (IP_3); noradrenaline (NA); phospholipase C (PLC).

References: Schwinn et al., 1991; Wu et al., 1992; Perez et al., 1993; Bylund et al., 1994.

Table 6. Classification and properties of α_2-adrenergic receptors.

	α_{2A}	α_{2B}	α_{2C}
transducer	G_i	G_i	G_i
primary effector	AC	AC	AC
second messenger	↓cAMP	↓cAMP	↓cAMP
secondary effector	↓Ca^{2+} ↑K^+	↓Ca^{2+} ↑K^+	↓Ca^{2+} ↑K^+
agonists	NA clonidine	NA clonidine	NA clonidine
antagonists	yohimbine	yohimbine	yohimbine
location	cortex hippocampus locus coeruleus amygdala	thalamus cerebellum	hippocampus amygdala

Abbreviations: adenylate cyclase (AC); noradrenaline (NA).

References: Kurose et al., 1991; Coupry et al., 1992; Duzic et al., 1992.

Table 7. Classification and properties of β-adrenergic receptors.

	β_1	β_2	β_3	β_4
transducer	G_s	G_s	G_s	G_s
primary effector	AC	AC	AC	AC
second messenger	↑cAMP	↑cAMP	↑cAMP	↑cAMP
agonists	NA isoproterenol	adrenaline < NA isoproterenol	NA isoproterenol	NA (low affinity)
antagonists	propranolol	propranolol	propranolol	bupranolol
location	cortex striatum hippocampus	cortex	adipose tissue	cardiac tissue

Abbreviations: adenylate cyclase (AC); noradrenaline (NA).

References: Kauman 1997; Galitzky et al., 1997; Sarsero et al., 1998.

adenosine are discrepant. Given that activation of adrenergic receptors increases intracellular levels of cAMP, it was thought that administration of noradrenaline would increase endogenous adenosine. However, noradrenaline at 5-50 μM did not evoke an increase in [^{14}C]adenine derivatives in cortical tissue, although it did increase cAMP levels (Pull and McIlwain, 1975). Higher concentrations of noradrenaline (0.1 or 1 mM) also did not evoke the release of [^{3}H]adenosine from cortical slice (Stone et al., 1981). Unlike in cortical slices, in cortical cultures after 10 min exposure to noradrenaline, the intracellular cAMP content was increased (Rosenberg and Dichter, 1989). In response to β-adrenergic stimulation, extracellular cAMP was secreted from cultured astrocytes and degraded to AMP and adenosine (Rosenberg et al., 1994). Furthermore, in cortical cultures containing both neurons and astrocytes, noradrenaline acting at β-adrenergic receptors significantly stimulated intracellular cAMP accumulation, cAMP transport, and extracellular adenosine accumulation (Rosenberg and Yi, 1995b). In contrast, dopamine, serotonin and histamine did not have an effect on the cAMP or adenosine content of these cultures. Forskolin and vasoactive intestinal polypeptide also stimulated extracellular adenosine accumulation in cortical culture, suggesting that activation of adenylate cyclase may be a common mechanism regulating extracellular adenosine levels (Rosenberg and Yi, 1995a, 1996). In spinal cord synaptosomes, noradrenaline produced a Ca^{2+}-dependent release of a nucleotide which was subsequently degraded extracellularly to adenosine by ecto-5'-nucleotidase (Sweeney et al., 1987).

Finally, neurotoxic lesion of the noradrenergic pathway innervating the cortex resulted in a decrease in cortical noradrenaline content which was associated with a decrease in ^{3}H-labeled purine release consisting mostly of adenosine, inosine and hypoxanthine in cortical slice (Pedata et al., 1989). Although this study may be interpreted to suggest that noradrenergic transmission contributes to extracellular adenosine levels *in vivo*, the effects of noradrenaline on extracellular adenosine concentration in the intact cortex have not been examined.

3. OBJECTIVES AND HYPOTHESES

The cerebral cortex has a role in many "high-level" functions including the processing of sensory information, speech production and comprehension. Adenosine has a potent depressant action on cerebral cortical neurons, inhibiting the activity of spontaneously firing corticospinal cells (Phillis et al., 1974; Stone and Taylor, 1978; Stone, 1982). Inhibitors of adenosine uptake potentiate the depressant actions of adenosine on cortical neurons (Phillis and Kostopoulos, 1975). It was suggested that adenosine is released from presynaptic nerve terminals in a Ca^{2+}-independent manner and may function as an inhibitory neuromodulator in the cerebral cortex (Sulakhe and Phillis, 1975). Therefore, manipulation of extracellular adenosine levels may be an effective mechanism for controlling cortical excitability.

Given the proposed role of adenosine as an inhibitory neuromodulator in the cerebral cortex, it would be important to identify the physiological factors

leading to an increase in extracellular adenosine levels *in vivo*. Activation of ionotropic receptors by glutamate has been shown to increase adenosine release in cortical slice (Hoehn and White, 1991). However, the possible effects of metabotropic glutamate receptor activation on extracellular adenosine levels have not been examined. Therefore, it was hypothesized that:

I. Activation of both ionotropic and metabotropic glutamate receptors increases extracellular adenosine concentration in the cerebral cortex of the unanesthetized rat.

Previous experiments using cortical slice and *in vivo* preparations have shown that activation of muscarinic receptors alone had no effect on basal adenosine levels but enhanced the evoked release of adenosine (Pedata et al., 1988; Pazzagli et al., 1994; Semba and White, 1997). Given the general role of nicotinic receptors in the modulation of the release of several neurotransmitters and the role of acetylcholine in modulating the activity of cortical neurons, the effect of activation of nicotinic receptors on the concentration of extracellular adenosine was tested *in vivo*. It was hypothesized that:

II. Activation of nicotinic and muscarinic receptors increases the concentration of extracellular adenosine in the cortex of unanesthetized rat.

In light of previous experiments demonstrating the release of adenosine following β-adrenergic receptor stimulation in cortical culture (Rosenberg et al.,

1994) and the role of noradrenaline in modulating the activity of cortical neurons, it is important to determine whether noradrenaline evokes the release of adenosine in the cerebral cortex *in vivo* and to determine whether activation of α-adrenergic receptors might also increase the concentration of extracellular adenosine. It was hypothesized that:

III. Activation of α- and β-adrenergic receptors increases the concentration of extracellular adenosine in the cortex *in vivo*.

CHAPTER II. METHODS

Microdialysis is defined as the use of a semipermeable membrane to allow the passage of selected solutes along a concentration gradient (DiChiara et al., 1996). An artificial gradient is created between the dialysis membrane and the extracellular space by the continuous perfusion of the microdialysis probe with a perfusion fluid lacking the solute to be recovered (Gerin and Privat, 1996). The solute will then enter the microdialysis membrane by simple diffusion since its concentration is greater outside the membrane than inside. Due to the size of the membrane, microdialysis does not sample transmitters directly in the synaptic cleft, but in the extracellular fluid surrounding the dialysis membrane (Di Chiara et al., 1996).

The advantages of the microdialysis technique to sample extracellular fluid are clearly numerous (Collin, 1988; Ungerstedt, 1991; Obrenovich et al., 1993; Westerink, 1995; Shippenberg and Thompson, 1997). The area of tissue displaced by the probe is smaller than that for cortical cup or push-pull perfusion. The damage of neural tissue is also greatly reduced since the flow of the perfusion fluid is contained within the probe. Microdialysis can be performed in an awake and freely moving animal and can sample fluid from almost any tissue and organ of the body. Finally, enzymes that may degrade the neurochemical are generally excluded from entering the probe due to the molecular weight cutoff of the membrane.

Despite the many advantages of microdialysis, a number of limitations do exist. Microdialysis has a limited time resolution which is largely determined by the smallest amount of a given neurochemical that can be detected. The

dialysis process also creates an area around the probe in which solutes permeable to the membrane are depleted (Shippenberg and Thompson, 1997). The technique can also influence the experimental or pathological conditions under study by buffering transient changes in the extracellular fluid concentration (Obrenovitch et al., 1995). For example, the failure to provide appropriate concentrations of Ca^{2+} ions in the perfusate may alter the levels of the analyate of interest (Moghaddam et al., 1989; De Boer et al., 1990; Osborne et al., 1991). Although morphologically normal synapses were seen even within 100 μm from the probe edge (Clapp-Lilly et al., 1999), the membrane would also sample extracellular fluid from the area of damage immediately surrounding the probe. Finally, although the concentration of neurotransmitters can be estimated based on the recovery rate of the dialysis membrane, it is unclear as to how the concentration in the extracellular space relates to that at the synaptic cleft (Westerink, 1995).

High performance liquid chromatography (HPLC) is commonly used to determine the concentration of the transmitter of interest in the sample recovered from the microdialysis probe. Liquid chromatography is a technique to partition a sample or solute between a moving or mobile phase (liquid buffer, into which the sample is injected) and a stationary phase (chromatographic column on which the separation takes place) according to the size, polarity or ionic charge of the molecules in the sample. During HPLC, the mobile phase is forced through a short narrow-bore (2-5 mm) column by a high pressure pump.

Reverse phase liquid chromatography was used in the studies described

in this thesis. In reverse phase chromatography, the molecules contained in the sample are separated by passage through a chromatographic column containing the chemically bonded phase, which is more hydrophobic or more nonpolar than the buffer in which the sample was dissolved. With this method the molecules are eluted in the order of increasing hydrophobicity (ie, the more polar a solute the faster it will be eluted from the column).

1. Microdialysis probe construction

Microdialysis probes constructed in the laboratory were used in all of the studies in this thesis. Probe construction (Fig. 3) began with the insertion of silica inlet (35 mm) and outlet (25 mm) tubes (outer diameter 150 μm, inner diameter 75 μm; Polymicro Technologies Inc, Phoenix, AZ) into a cylindrical microdialysis membrane (AN69, outer diameter 340 μm when wet, inner diameter 240 μm when wet, wall thickness 50 μm when wet, mean pore diameter 29 Å, maximum pore diameter 55 Å, 10 kDa molecular weight cutoff; Hospal Gambro Inc, St-Léonard, PQ). The end of the membrane through which the inlet and outlet tubes were inserted was sealed with glue (2 Ton Clear Epoxy; Devcon Corporation, Danvers, MA). This tube assembly was placed inside a stainless 22 Ga steel cannula (10 mm; Small Parts Inc, Miami Lakes, FL) and secured in place with glue to form the shaft portion of the probe. The portions of the inlet and outlet tubes that emerged from the shaft were each placed inside a 23 Ga cannula for protection and glued into place. The free end of the membrane was cut so that 2 mm was left free of glue to be used as the

Fig. 3. Schematic diagram of a microdialysis probe constructed in the laboratory.

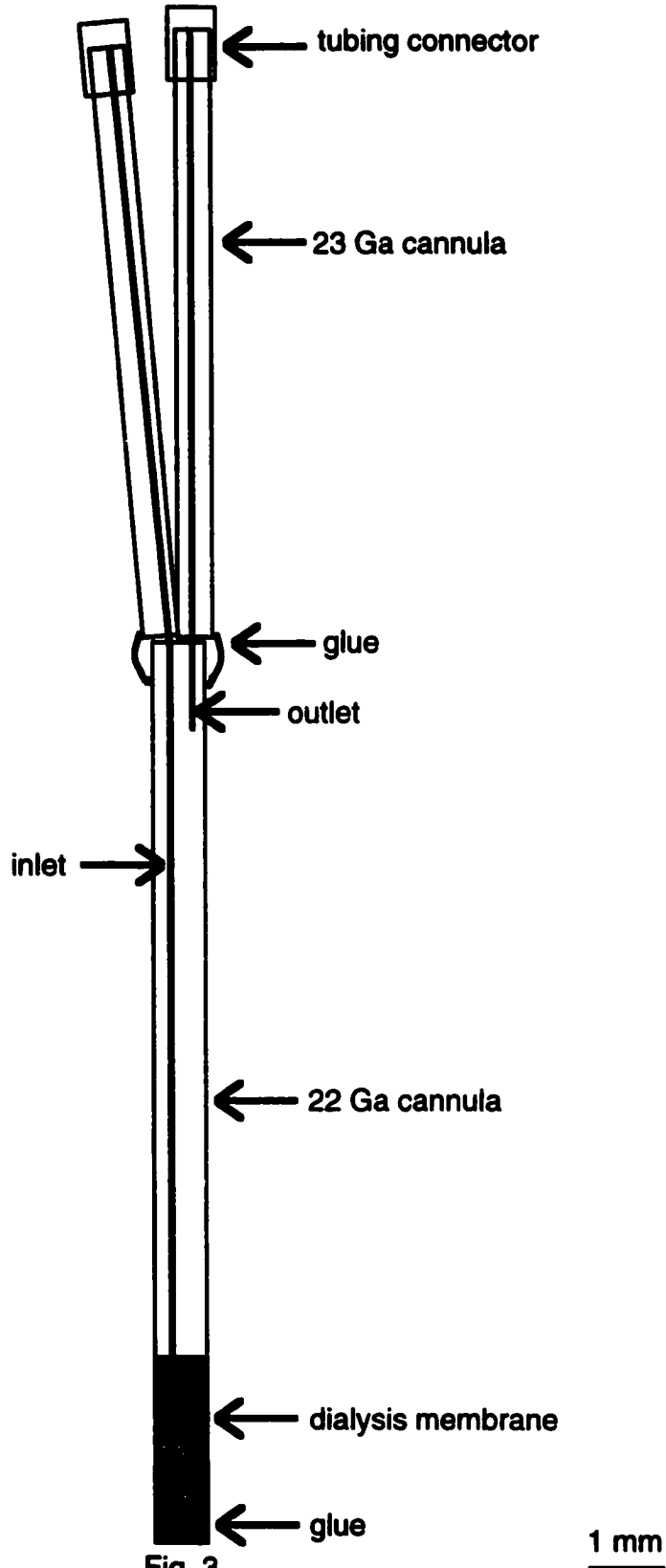

tubing connector
23 Ga cannula
glue
outlet
inlet
22 Ga cannula
dialysis membrane
glue
1 mm

Fig. 3

dialysis portion of the probe. The end of the probe (0.5 mm) was then sealed with glue. Finally, tubing connectors (Bioanalytical Systems (BAS), Lafayette, IN) were inserted onto the ends of the inlet and outlet tubes.

Immediately before implantation, the dialysis membrane of the probe was dipped in the antibacterial reagent Proclin (BAS) and the probe was flushed with artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 1.3 mM CaCl, 3 mM KCl and 1 mM $MgSO_4$ in milliQ water, pH 7.2). In order to determine the percentage of adenosine recovered by the 2 mm microdialysis membrane, ACSF was flushed through 2 probes that were placed in known concentrations of adenosine for 20 min. The first probe was tested in 117.70 and 58.85 pmol of adenosine and the second probe was tested three times in 117.70 pmol of adenosine. The average recovery of adenosine for these five tests was 11% (range: 5-19%). This recovery is comparable to another study in which microdialysis probes constructed with a dialysis membrane (AN69) 3.5 mm in length and perfused at 2 µl/min had a recovery of 13% adenosine (Pazzagli et al., 1993).

2. Microdialysis probe implantation

Two hundred and ninety-one adult male Wistar rats (250-300 g; Charles River, St. Constant, PQ) were housed under a 12 h light-dark cycle (lights on from 7 am to 7 pm) and were provided water and rat chow *ad libitum*. The experimental protocol with animals met the guidelines of the Canadian Council of Animal Care (Olfert et al., 1993) and was approved by the Dalhousie

University Committee of Laboratory Animals. Animals were weighed and given an injection of a mixed anesthetic solution (3 ml ketamine, 0.8 ml xylazine, 0.3 ml acepromazine maleate and 0.9 ml saline; 0.1 ml/100 g, i.m.). This solution was chosen to produce surgical anesthesia and a smooth recovery (Olfert et al., 1993). The skin over the skull was shaved and the rat placed in a stereotaxic frame. The scalp was swabbed with 70% ethanol, a midline incision made, the periosteum was removed and the skull was cleaned with 0.3% hydrogen peroxide. To aid in the adhesion of dental cement to the skull, 2 stainless steel bone screws (Small Parts Inc., Miami Lakes, FL) were screwed into the skull. Using bregma as the reference point, a hole was drilled for the vertical implantation of the microdialysis probe in the somatosensory cerebral cortex (located in the parietal cortex; Paxinos and Watson, 1998) using the following coordinates with the incisor bar positioned 3.3 mm below the interaural line: AP+1.4, right or left 5.0, and DV -2.7 mm (Paxinos and Watson, 1998). The probe was then cemented to the skull using a two-part dental cement (orthodontic resin: L.D. Caulk Company, Milford, DE). Finally, to protect the probes from any damage a polyurethane container intended for embedding electron microscopy specimens (Marivac, Halifax, NS), from which the closed end had been removed, was placed around each probe and cemented to the skull.

Two probes were implanted, one in each cortex, in the majority of cases in order to reduce the number of animals required for the studies. The baseline levels of adenosine recovered from a probe on one side were not affected by

the addition of any drug in the contralateral probe; examples of the stability of the baseline adenosine levels are presented in Figs. 8A and 14A. Two hours elapsed from the completion of the experiment using the first probe to the delivery of drug in the second probe. Finally, the probes did not receive the same concentration of the same drug in any animal.

3. Experimental procedure

Experiments were conducted between 7:30 am and 7:30 pm. After approximately 24 h recovery from surgery, PEEK tubing (BAS) was inserted into the connectors of the probe. The rat was then placed in a large clear plastic observation bowl (Fig. 4). The tubing from the inlet of the probe was inserted into a liquid valve, also called a liquid switch (Uniswitch; BAS), and the tubing from the outlet was inserted into a collection tube that was placed in a fraction collector (FRAC-100; Pharmacia Biotech Inc, Baie d'Urfé, PQ). The liquid switch was connected to a syringe pump (Baby Bee Syringe Pump with a 3-syringe bracket and a Worker Bee Controller; BAS). Therefore, the flow of ACSF was from the syringe through the switch, to the intake of the probe, through the probe, out the probe and to the collector.

The probes were infused with ACSF at a rate of 2 µl/min for the duration of the experiment. Samples were collected both during and after drug perfusion in 20 min intervals (Table 8). Each 40 µl sample was collected into 20 µl of 0.15 M $ZnSO_4$ and then 20 µl saturated 0.15 M $Ba(OH)_2$ was immediately added. Each sample was mixed and then frozen at -20 °C for less

Fig. 4. The experimental setup for in vivo microdialysis in the freely moving rat. A. A rat is placed inside a plastic U-shaped bowl under a table that housed the microdialysis equipment including a syringe pump, a liquid switch and a fraction collector. B. A close-up of a rat inside the observation chamber. Two capsules that were used to protect the microdialysis probes from damage are visible. Dental cement is the material visible around the base of the capsules. Microdialysis probes inside the capsules are also visible. The tubings exit the probes and are connected to the fraction collector shown in (A).

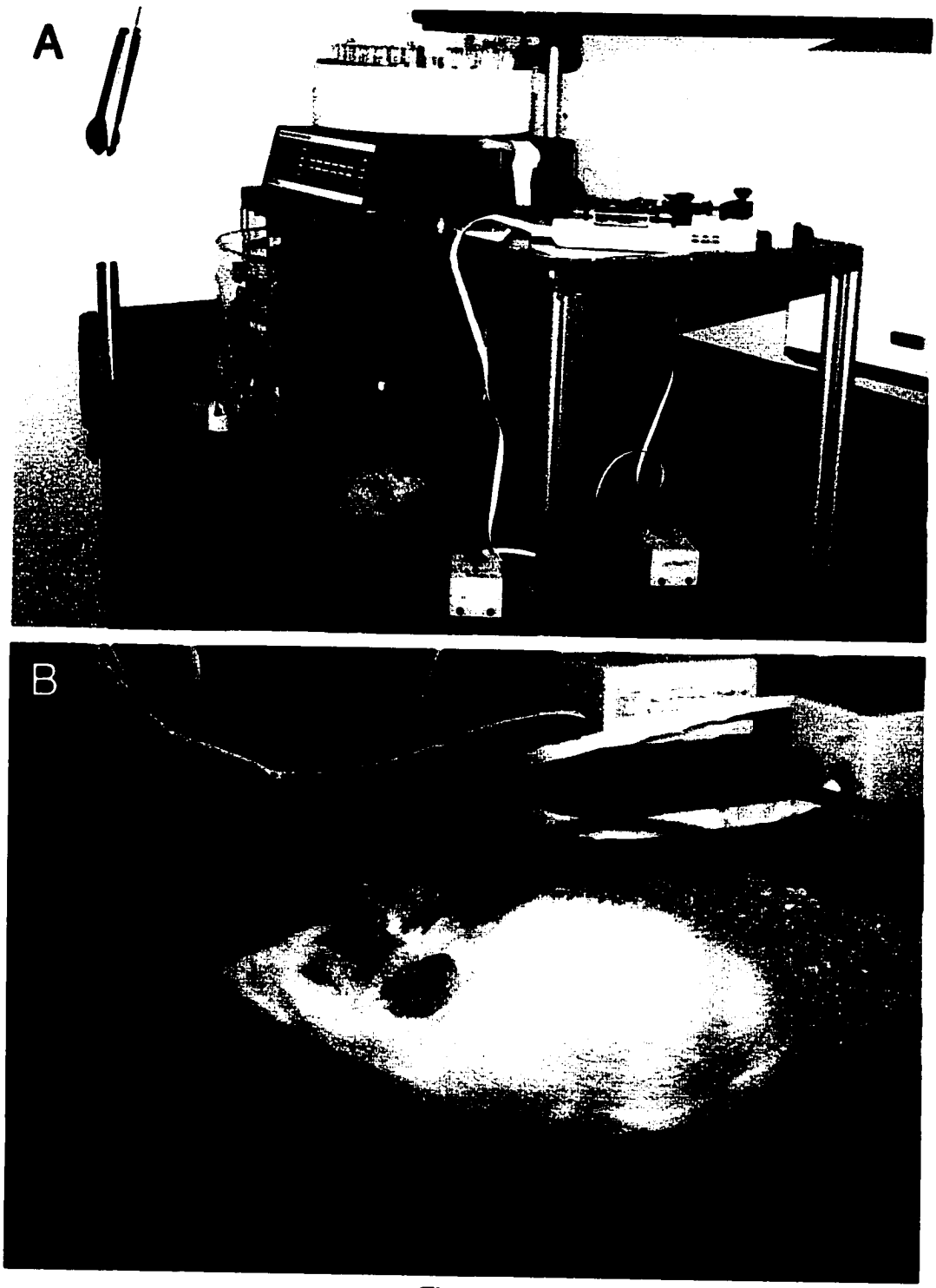


Fig. 4

Table 8. Time course for the collection of samples in each animal.

Probe 1			Probe 2		
sample	time (min)	condition	sample	time (min)	condition
1	60	ACSF	1	60	ACSF
2	60	ACSF	2	60	ACSF
3	20	drug	3	20	ACSF
4	20	ACSF	3	20	ACSF
5	20	ACSF	3	20	ACSF
6	60	ACSF	4	60	ACSF
7	60	ACSF	5	60	ACSF
8	20	ACSF	6	20	drug
8	20	ACSF	7	20	ACSF
8	20	ACSF	8	20	ACSF

than 1 week (Craig and White, 1993). The samples were incubated in $ZnSO_4/Ba(OH)_2$ in order to cause the precipitation of ATP and AMP (Wojcik et al., 1981). A one hour collection time was necessary to detect basal levels of adenosine. Antagonists were added 20 min prior to and during perfusion of the drug under investigation (Table 9).

At the conclusion of the experiment, the rat was given an overdose injection of sodium pentobarbitol (>100 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, ON) and perfused via the ascending aorta with 100 ml of saline followed by 400 ml of a 4% paraformaldehyde (TAAB Laboratories Equipment Ltd, Reading, UK) solution in 0.1 M phosphate buffer (pH 7.4; BDH Inc., Toronto, ON) at a rate of 20 ml/min. The brain was removed and placed in the same fixative overnight at 4 °C, then transferred to a 30% sucrose solution in phosphate buffer until sectioning was done.

4. Detection of adenosine

The adenosine content in the samples was determined by modification of the method of Wojcik and Neff (1982) essentially as described previously by Craig and White (1993). The procedure for the quantification of adenosine used in this thesis involved incubation of the sample with chloroacetylaldehyde to form the fluorescing adenosine derivative 1-*N*6-ethenoadenosine. With this method, 95% of adenosine is converted to its etheno-derivative (Jacobson et al., 1983). The ethenoadenosine was separated by HPLC and quantified by fluorometry.

Table 9. Time course for the collection of samples when an antagonist is delivered prior to and during an agonist.

Probe 1			Probe 2		
sample	time (min)	condition	sample	time (min)	condition
1	60	ACSF	1	60	ACSF
2	60	ACSF	2	60	ACSF
3	20	antagonist	3	20	ACSF
4	20	antagonist + agonist	4	20	ACSF
5	20	ACSF	4	20	ACSF
6	20	ACSF	4	20	ACSF
7	60	ACSF	5	60	ACSF
8	60	ACSF	6	60	ACSF
9	20	ACSF	7	20	antagonist
10	20	ACSF	8	20	antagonist + agonist
10	20	ACSF	9	20	ACSF
10	20	ACSF	10	20	ACSF

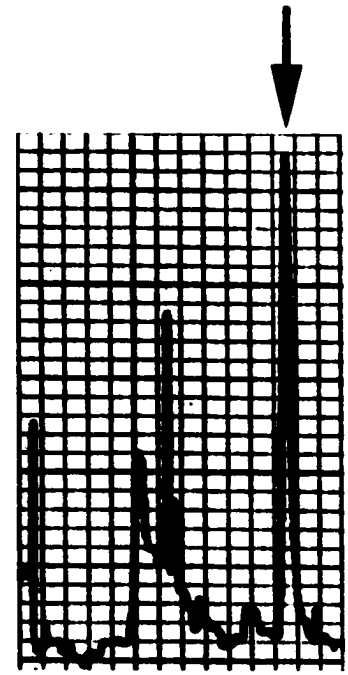
The microdialysis samples were thawed and centrifuged at 11,600 g for 6 min. The supernatant was collected and, following the addition of 4.5% chloroacetylaldehyde was boiled for 20 min (Craig & White, 1993). The etheno-derivative of adenosine was assayed using HPLC with fluorescence detection (Craig & White, 1993). Briefly, 200 ml of milliQ water was degassed and pumped through the HPLC for 25 min at a rate of 1.0 ml/min via a Waters 510 HPLC pump (Waters; Mississauga, ONT) to condition the HPLC column (Radial-Pak C_{18} liquid chromatography cartridge with a 5 mm ID, Waters). The mobile phase consisted of a 50 mM acetate buffer (pH 4.5) containing 10% (v/v) acetonitrile and 2.4 mM octanesulfonic acid and was pumped through the HPLC for 20 min at a speed of 1.0 ml/min, then run for 30 min at a speed of 0.7 ml/min. Each standard or sample was injected into the HPLC using a Waters 712 WISP autoinjector and the amount of adenosine was recorded using a Waters 470 scanning fluorescence detector. The limit of detection was 60 nmol of adenosine.

Adenosine standards in ACSF were treated identically to the samples and peak heights of adenosine standards exhibited a linear relationship with respect to the amount of adenosine. The standards were used to calculate a regression line and from the equation for the slope of this line the amount of adenosine in each sample was calculated (Fig. 5). To determine the concentration of adenosine in each sample, the absolute number of pmoles of adenosine was divided by the volume of the sample.

The statistical significance of the effects of agonists on the concentration

Fig. 5. Examples of a chromatogram of an adenosine standard and of a microdialysis sample from the cerebral cortex. The chromatograms of an adenosine standard (14.71 pmol adenosine; A) and a microdialysis sample (calculated to be 7.94 pmol adenosine; B) both show adenosine peaks (arrows) at 6 min retention time.

A



B

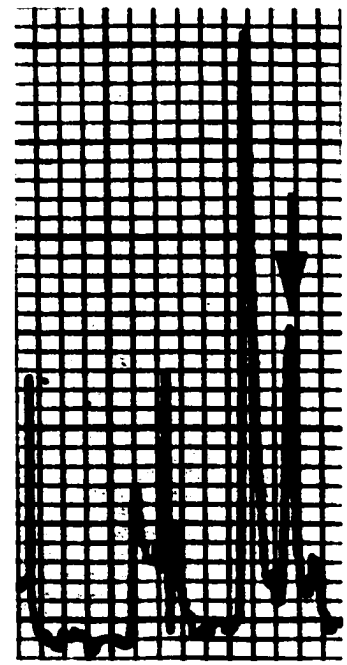


Fig. 5

of extracellular adenosine was determined by ANOVA with repeated measures. In the cases where an agonist was found to have a significant effect on the concentration of extracellular adenosine, the difference between concentration of adenosine during delivery of the agonist and the baseline concentration was examined using Fisher's protected least significant difference test (LSDT; SuperANOVA, Abacus Concepts). The evoked release of adenosine was determined by subtracting the concentration of adenosine in the preceding sample from the concentration of adenosine during drug perfusion, and was used to compare the effects of antagonists using Student's two-tailed t-test (Statview 5.0, Abacus Concepts). Values of $p \leq 0.05$ were considered to be significant.

The concentrations of adenosine reported in this thesis were not corrected for the 11% mean recovery of adenosine by the microdialysis probes, for the following reasons. First, the recovery rate varies among microdialysis probes. Given that the probes were implanted in the brain semichronically and that the rat was perfused with paraformaldehyde prior to removal of the brain, it was not possible to test the recovery rate of each probe before and after the experiment. Furthermore, due to a number of limitations associated with the microdialysis technique and the detection of adenosine, it is not straightforward to determine an overall error factor in the calculation of the concentration of adenosine. Microdialysis measures recovered adenosine, which is usually < 100% of the adenosine molecules present in the extracellular space adjacent to the probe because of the semipermeable nature of the dialysis membrane.

The conversion of adenosine to its etheno-derivative also results in an approximately 5% loss of adenosine (Jacobson et al., 1983). Based on these factors, it is most likely that the concentrations of adenosine reported in this thesis are an underestimation of the actual concentration of adenosine in the extracellular space.

5. Histology

Sections that contained the probe tracts through the cerebral cortex were mounted on chrome-gel slides and air dried overnight. Next, slides were dehydrated through an ascending series of ethanol to xylene, then rehydrated in a descending series of ethanol, and finally placed in 70% ethanol for 10 min. The slides were quickly rinsed in water to remove excess alcohol and then placed in a solution of 0.1% cresyl violet for 5 min. Next, the slides were placed in a differentiating solution containing 95% ethanol, chloroform and glacial acetic acid until the corpus callosum turned white. The slides were then dehydrated in an ascending series of ethanol to xylene and coverslipped.

Data from an additional 29 rats was excluded from statistical analysis because the microdialysis probes were not confined within the parietal cortex.

6. Drugs

The drugs used are listed in Table 10. All drugs, with the exception of AIDA and prazosin hydrochloride, were dissolved in ACSF and administered though the microdialysis probe. AIDA at a concentration of 100 mM was made

Table 10. List of drugs administered via the microdialysis probe.

Drug	Type	Supplier
Glutamatergics		
trans-(±)-ACPD; *trans*-(±)-1-Amino-(1S,3R)-cyclopentanedicarboxylic acid	group I/II metabotropic receptor agonist	RBI
AIDA; (RS)-1-aminoindan-1,5,-dicarboxylic acid	selective group I metabotropic receptor antagonist	Tocris
AMPA; α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid		Tocris
DHPG; (S)-3,5-dihydroxyphenylglycine	selective group I metabotropic receptor agonist	Tocris
glutamate (L-glutamic acid)		Sigma
kainic acid		RBI
L-AP4; L(+)-2-Amino-4-phosphonobutyric acid	group II metabotropic receptor agonist	RBI
LCCG-1; (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine	group II metabotropic receptor agonist	RBI
NMDA; N-methyl-D-aspartic acid		RBI
Cholinergics		
acetylcholine chloride		Sigma
atropine sulfate salt	nonselective muscarinic receptor antagonist	Sigma
carbachol (carbamylcholine chloride)	nicotinic and muscarinic receptor agonist	Sigma

Table 10. continued.

Drug	Type	Supplier
CDD-0097 hydrochloride	selective muscarinic M_1 receptor agonist	RBI
epibatidine dihydrochloride	nicotinic receptor agonist	RBI
mecamylamine hydrochloride	nicotinic receptor antagonist	Sigma
neostigmine methylsulfate	acetylcholinesterase inhibitor	Sigma
(-)-nicotine	nicotinic receptor agonist	Sigma
oxotremorine methiodide	nonselective muscarinic receptor agonist	Sigma
Noradrenergics		
clonidine hydrochloride;	α_2-adrenergic receptor agonist	Sigma
desipramine hydrochloride	noradrenaline reuptake inhibitor	RBI
isoproterenol hydrochloride	nonselective β-adrenergic receptor agonist	Sigma
(-)noradrenaline		Sigma
L-phenylephrine hydrochloride	α_1-adrenergic receptor agonist	Sigma
prazosin hydrochloride	α_1-adrenergic receptor antagonist	Tocris
propranolol hydrochloride	nonselective β-adrenergic receptor antagonist	Tocris

in 0.1 M NaOH then diluted to 1 mM with ACSF. The final concentration of NaOH in the perfusion fluid was 1 mM. Prazosin was dissolved to a concentration of 100 mM in 100% ethanol then diluted with ACSF. Ethanol was diluted to 0.01% in the perfusion fluid. Neither NaOH nor ethanol had an effect on the concentration of extracellular adenosine. The concentrations of drugs used were approximately 10 times greater than those reported in previous *in vitro* studies. The use of higher concentrations of drugs in microdialysis are necessary due to the incomplete permeability of the microdialysis membrane (Shippenberg and Thompson, 1997).

CHAPTER III.

EFFECTS OF IONOTROPIC AND METABOTROPIC GLUTAMATE RECEPTOR ACTIVATION ON THE CONCENTRATION OF EXTRACELLULAR ADENOSINE.

Glutamate, the main excitatory neurotransmitter in the brain, evokes the release of adenosine in cortical slice (Hoehn and White, 1990b). There is ample evidence from studies using cortical slice (Hoehn and White, 1991) and some evidence from *in vivo* studies (Pazzagli et al., 1994) that implicates ionotropic glutamate receptors in the increase in adenosine evoked by glutamate. However, few studies have explored the possibility that activation of metabotropic glutamate receptors also mediates increases in extracellular adenosine. Given that metabotropic glutamate receptors modulate transmitter release (Nakanishi, 1994), it was hypothesized that activation of metabotropic as well as ionotropic receptors increases the concentration of extracellular adenosine *in vivo*. In order to identify the mechanisms that would lead to increased levels of adenosine in the brain, metabotropic and ionotropic glutamate receptor agonists were tested for their ability to increase extracellular adenosine levels in the cortex of unanesthetized rat using *in vivo* microdialysis.

METHODS

The methods are outlined in Chapter II.

RESULTS

Figure 6 is an example of a Nissl stained section of the brain showing the location of microdialysis probes in the left and right parietal cortices. The histology of other animals included in the statistical analyses was similar to this section.

Fig. 6. A Nissl stained coronal section of a rat brain. The holes indicate the locations of the microdialysis probes that were implanted in the right and left parietal cortices. Scale bar = 0.5 mm.

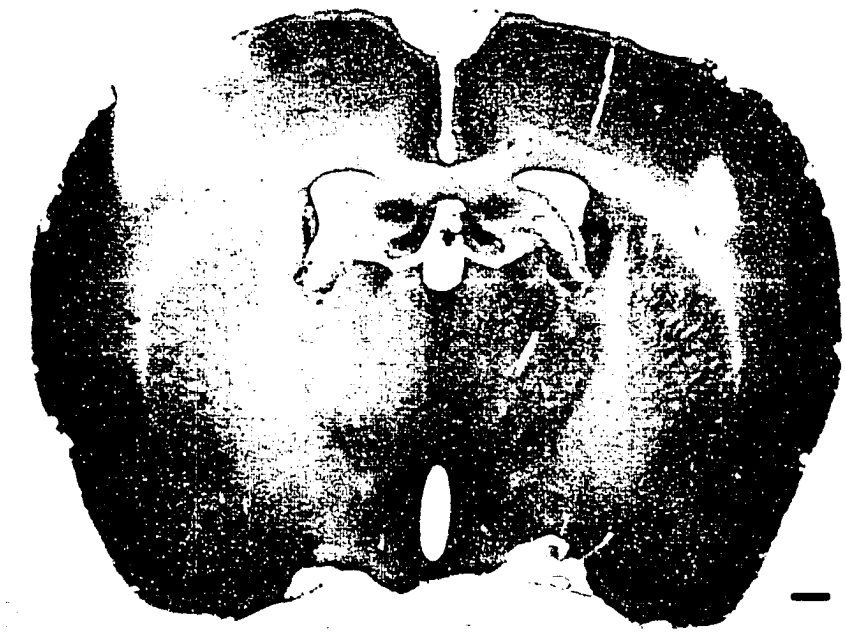


Fig. 6

During these experiments, rats spent most of their time resting or sleeping with eyes closed, but occasional head movements, grooming and locomotion were also seen. No gross behavioral changes were observed in the animals during the perfusion of any drug.

The mean concentration of extracellular adenosine over a 120 min period was 50 ± 5 nM (± standard error of the mean; n=159).

As shown in Figure 7, the delivery of glutamate through the microdialysis probe increased the extracellular adenosine levels in a concentration dependent manner (treatment effect, $F(3,25) = 4.78$, $p < 0.01$). The time course of the effect was similar for 5 and 10 mM glutamate. Delivery of either 5 or 10 mM glutamate significantly increased the concentration of extracellular adenosine during drug delivery (140 min) and at 160 min compared to the baseline concentration at 120 min (5 mM glutamate: $F(4,32) = 8.30$, $p < 0.001$; Fisher's PLSD test $p < 0.001$ and 0.05, respectively; 10 mM glutamate: $F(4,24) = 5.30$, $p < 0.01$; $p < 0.001$ and $p < 0.05$, respectively).

NMDA increased extracellular adenosine levels, and this increase was concentration-dependent (interaction between treatment and time, $F(12,100) = 2.96$, $p < 0.01$; Fig. 8A). A concentration of 200 μM NMDA significantly increased adenosine levels during the drug delivery compared to the baseline levels at 120 min ($F(4,28) = 8.41$, $p < 0.001$; $p < 0.001$). At a higher concentration of 300 μM, NMDA increased adenosine levels during drug delivery and at 160 min ($F(4,16) = 14.38$, $p < 0.001$; $p < 0.01$ and 0.001, respectively). The administration of 200 μM NMDA in the ipsilateral probe did

not affect the concentration of adenosine in the contralateral probe (control; also see Chapter II).

The administration of AMPA also increased extracellular adenosine levels in a concentration dependent manner (effect of treatment $F(2,21) = 16.16$, $p < 0.001$; Fig. 8B). AMPA at concentrations of 25 and 50 μM significantly increased the concentration of extracellular adenosine during drug delivery and in the next sample (25 μM: $F(4,28) = 4.32$, $p < 0.01$; $p < 0.05$ for 140 and 160 min; 50 μM: $F(4,28) = 7.79$, $p < 0.001$; $p < 0.001$ for 140 min and $p < 0.05$ for 160 min).

Kainic acid had a significant effect on the concentration of extracellular adenosine (effect of time, $F(4,88) = 14.90$, $p < 0.001$; Fig. 8C). At a concentration of 50 μM, kainic acid significantly increased adenosine concentration during drug delivery and the concentration of adenosine remained elevated for the duration of the experiment ($F(4,28) = 17.40$, $p < 0.001$; $p < 0.001$ for 140, 160 and 180 min). Two higher concentrations of kainic acid, 200 and 400 μM, also increased adenosine levels during the drug delivery compared to the baseline at 120 min ($F(4,24) = 3.84$, $p < 0.05$; $p < 0.01$; and $F(4,32) = 5.95$, $p < 0.01$; $p < 0.01$, respectively).

The group I/II metabotropic receptor agonist ACPD had a significant effect on extracellular adenosine levels (effect of treatment, $F(2,21) = 11.27$, $p < 0.001$; Fig 9A). ACPD at a concentration of 750 μM increased adenosine levels during drug perfusion and adenosine levels remained elevated for the duration of the collection period ($F(4,28) = 14.12$, $p < 0.001$, $p < 0.001$ for 140, 160 and

180 min). A higher concentration, 1 mM, also increased adenosine levels during drug delivery and adenosine levels again remained elevated for the remainder of the collection period ($F(4,28) = 5.36$, $p < 0.01$; $p < 0.01$ and 0.05 for both, respectively).

The selective group I metabotropic receptor agonist DHPG also had a significant effect on the concentration of extracellular adenosine (effect of time $F(4,56) = 9.05$, $p < 0.001$; Fig. 9B). DHPG at 100 μM increased extracellular adenosine levels during the drug delivery ($F(4,20) = 3.41$, $p < 0.05$; $p < 0.01$). A higher concentration of DHPG, 1 mM, also increased extracellular adenosine levels during delivery and at 160 min compared to the baseline at 120 min ($F(4,16) = 6.87$, $p < 0.01$; $p < 0.05$ for both time points). Neither the group II metabotropic receptor agonist LCCG-1 nor the group III metabotropic receptor agonist L-AP4 affected extracellular adenosine levels (Fig. 9C).

The evoked release of adenosine by 1 mM DHPG was blocked by the group I metabotropic receptor antagonist AIDA (1 mM; $p < 0.01$, Student's t-test; Fig. 10A). The evoked release of adenosine by 5 mM glutamate was not affected by the addition of 1 mM AIDA to the perfusate (Fig. 10B). AIDA alone did not have an effect on extracellular adenosine levels.

Fig. 7. Effects of glutamate on extracellular adenosine in vivo. A 20 min infusion of either 5 or 10 mM glutamate evoked a significant increase in the concentration of extracellular adenosine during drug delivery (indicated by a horizontal bar) and at 160 min compared to the baseline concentration of adenosine at 120 min (*$p < 0.05$, ***$p < 0.001$). Lower concentrations of glutamate, 1 and 2 mM, did not have an effect. The number in brackets indicates the number of experiments.

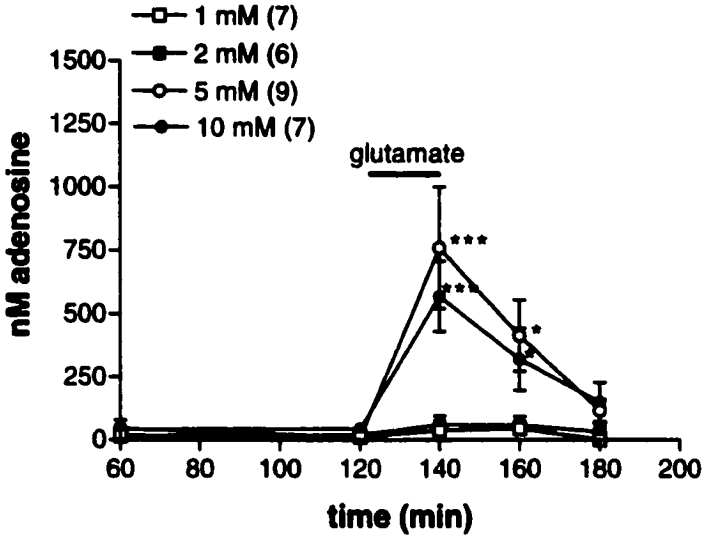

1 mM (7)
2 mM (6)
5 mM (9)
10 mM (7)
glutamate
nM adenosine
1500
1250
1000
750
500
250
0
60
80
100
120
140
160
180
200
time (min)
***
**
*
*

Fig. 7

Fig. 8. Effects of ionotropic glutamate receptor agonists on extracellular adenosine. A. NMDA at concentrations of 200 and 300 μM evoked a significant increase in extracellular adenosine during drug delivery compared to the baseline concentration of adenosine at 120 min. The concentration of adenosine in the contralateral probe was not affected by the delivery of 200 μM adenosine in the ipsilateral probe (control). Neither 50 nor 100 μM NMDA affected the concentration of extracellular adenosine. B. AMPA at 25 and 50 μM evoked a significant increase in extracellular adenosine levels compared to baseline, whereas a lower concentration of 10 μM did not have an effect. C. Kainic acid at all concentrations tested (50, 200 and 400 μM) evoked a significant increase in extracellular adenosine compared to baseline. The number in brackets indicates the number of experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the baseline concentration of adenosine at 120 min.

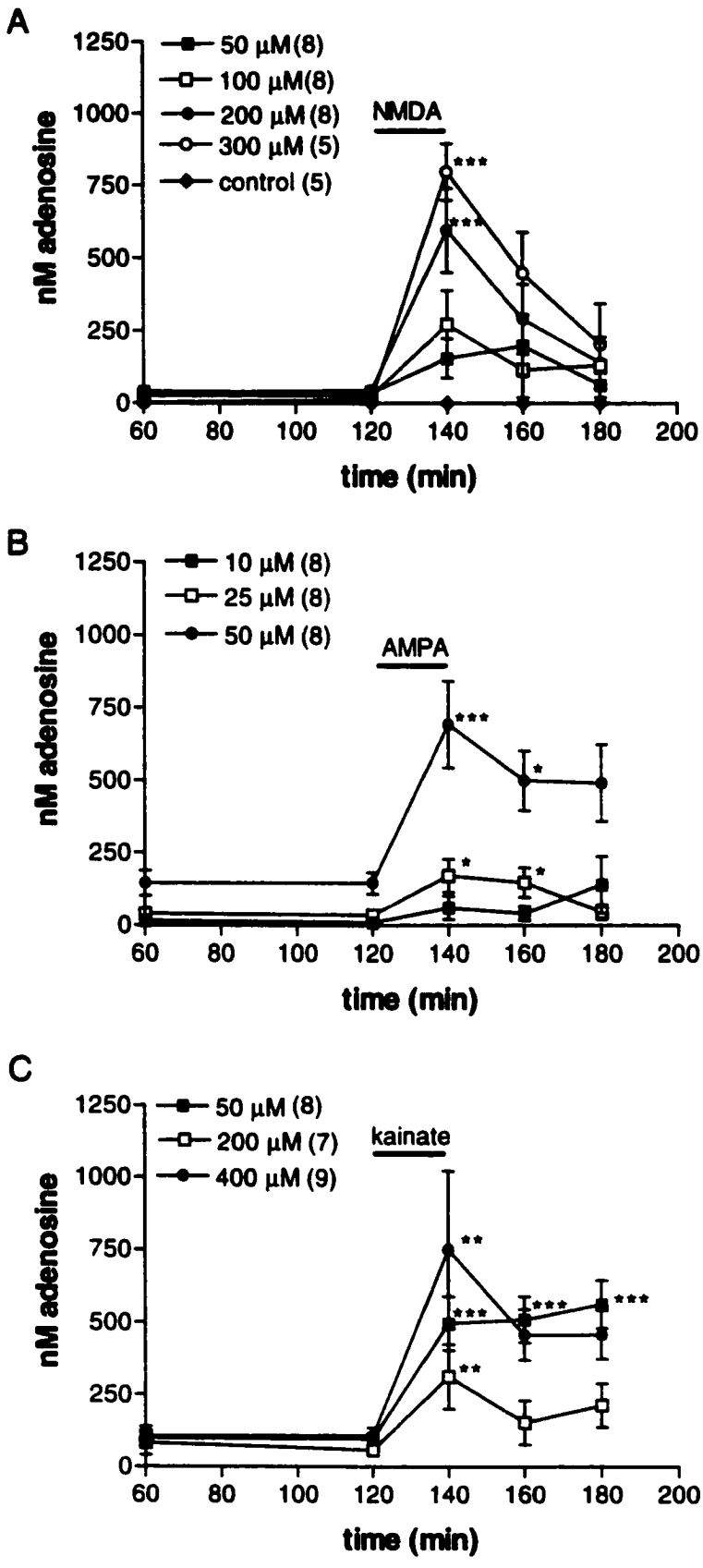

A
50 µM(8)
100 µM(8)
200 µM(8)
300 µM (5)
control (5)
NMDA
nM adenosine
time (min)
B
10 µM (8)
25 µM (8)
50 µM (8)
AMPA
nM adenosine
time (min)
C
50 µM (8)
200 µM (7)
400 µM (9)
kainate
nM adenosine
time (min)

Fig. 8

Fig. 9. Effects of metabotropic glutamate receptor agonists on extracellular adenosine levels. A. The group I and II metabotropic glutamate receptor agonist *trans*-(±)-ACPD at concentrations of 750 μM and 1 mM evoked a significant increase in extracellular adenosine concentration. A lower concentration of 500 μM did not have an effect. B. The group I metabotropic agonist (S)-3,5-DHPG at concentrations of 0.1 and 1 mM also evoked a significant increase in adenosine concentration compared to baseline at 120 min. C. Neither the group II agonist LCCG-1 (1 mM) nor the group III agonist L-AP4 (1 mM) had an effect. The number in brackets indicates the number of experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to baseline concentration at 120 min.

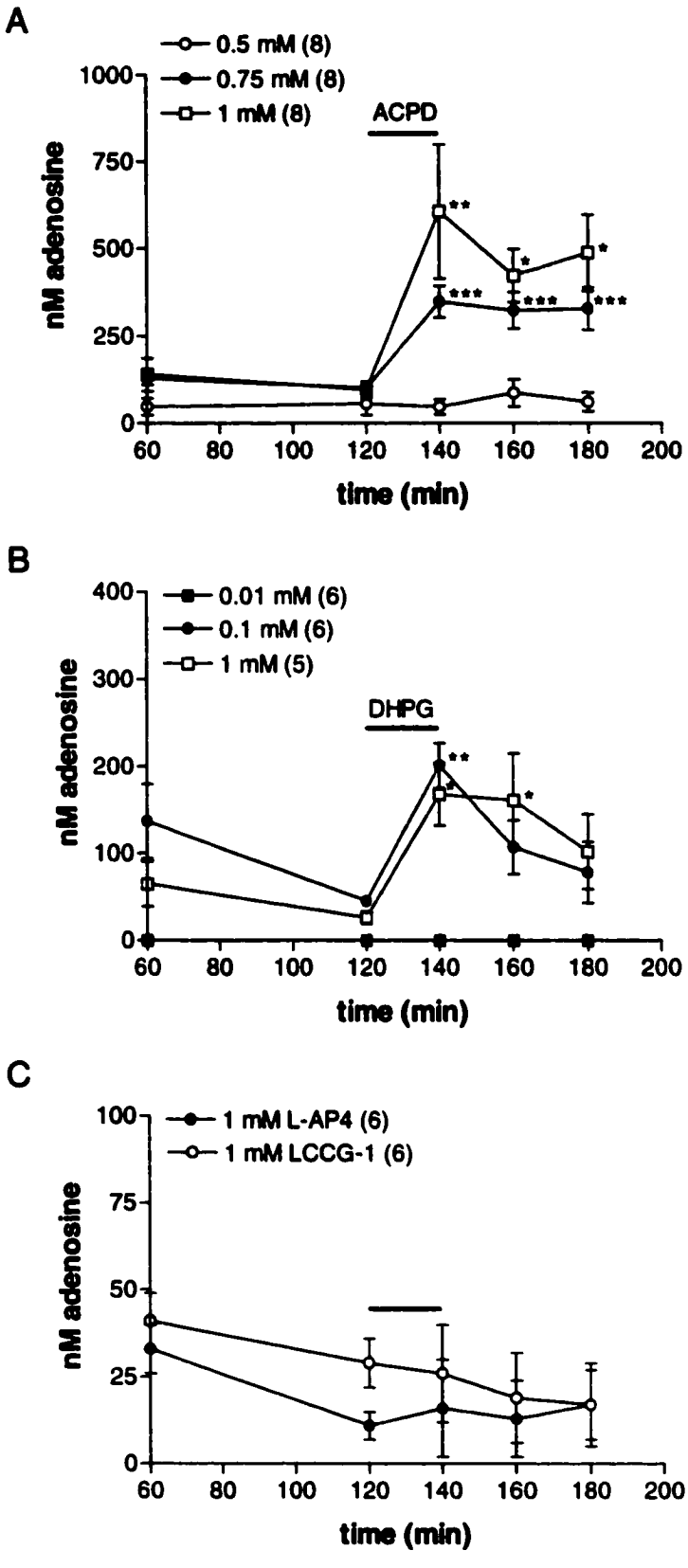

A
0.5 mM (8)
0.75 mM (8)
1 mM (8)
ACPD
nM adenosine
1000
750
500
250
0
60
80
100
120
140
160
180
200
time (min)
B
0.01 mM (6)
0.1 mM (6)
1 mM (5)
DHPG
nM adenosine
400
300
200
100
0
time (min)
C
1 mM L-AP4 (6)
1 mM LCCG-1 (6)
nM adenosine
100
75
50
25
0
time (min)

Fig. 9

Fig. 10. Effect of the group I metabotropic receptor antagonist AIDA on the extracellular concentration of adenosine evoked by DHPG and glutamate. A. AIDA at 1 mM significantly reduced DHPG evoked adenosine release (**$p < 0.01$). B. The release of adenosine by 5 mM glutamate (glu) was reduced, although not significantly, by AIDA. The number in brackets indicates the number of experiments.

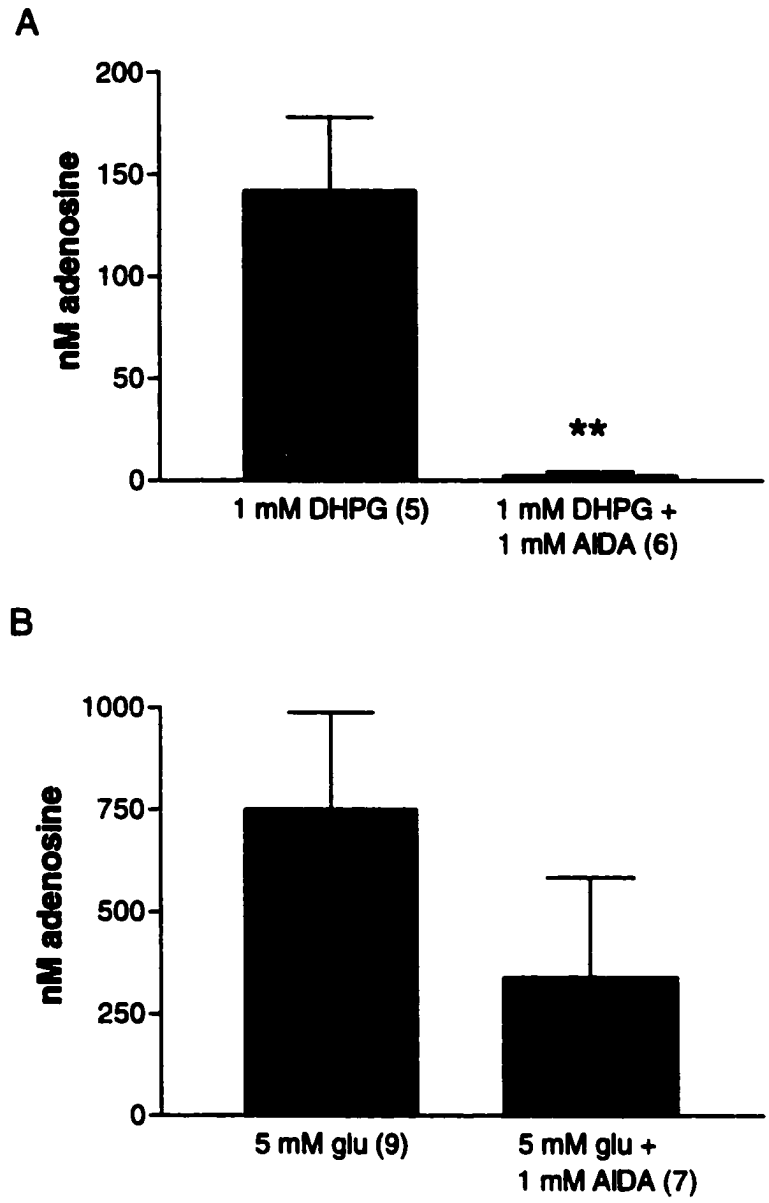

A
200
150
100
50
0
nM adenosine
**
1 mM DHPG (5)
1 mM DHPG +
1 mM AIDA (6)
B
1000
750
500
250
0
nM adenosine
5 mM glu (9)
5 mM glu +
1 mM AIDA (7)

Fig. 10

DISCUSSION

Application of exogenous glutamate resulted in an increase in the concentration of extracellular adenosine in the cerebral cortex *in vivo*. Activation of metabotropic as well as ionotropic glutamate receptors also evoked an increase in the concentration of extracellular adenosine. The present study is the first to demonstrate that activation of group I metabotropic glutamate receptors increases the concentration of extracellular adenosine in the cerebral cortex.

The experiments did not begin until 24 h after probe implantation to allow adenosine levels produced by injury to return to baseline levels (Pazzagli et al., 1993). The 1 h collection time was necessary for the recovery of basal adenosine levels, following the example set in another study using *in vivo* microdialysis in the unanesthetized rat (Pazzagli et al., 1993).

The mean basal level of adenosine obtained in this study was 50 nM. These values are comparable to previously reported values of 5 to 100 nM, which have not been corrected for the percent recovery of the probe, using *in vivo* microdialysis in the cerebral cortex, striatum, caudate nucleus and hippocampus of anesthetized or unanesthetized rats (Zetterström et al., 1982; Ballarin et al., 1991; Pazzagli et al., 1993; Kaku et al., 1994; Pazzagli et al., 1994, 1995; Huston et al., 1996; Carswell et al., 1997; Porkka-Heiskanen et al., 1997).

In experiments using a cortical slice preparation (Hoehn and White, 1990b,c; Craig and White, 1993; Semba and White, 1997), glutamate, NMDA,

AMPA and kainate all evoked a significant increase in adenosine release. Activation of ionotropic receptors has also been found to release adenosine in the striatum (Delany et al., 1998) and hippocampus (Manzoni et al., 1994; Carswell et al., 1997). The present study extends these previous findings to include activation of AMPA and kainate receptors as a mechanism of adenosine release *in vivo*.

Activation of metabotropic glutamate receptors using the group I/II agonist *trans*-ACPD or the group I agonist DHPG evoked a significant increase in extracellular adenosine *in vivo*. *Trans*-ACPD is a racemic mixture of the stereoisomers 1S,3R-ACPD and 1R,3S-ACPD with 1S,3R-ACPD being more potent than 1R,3S-ACPD (Schoepp et al., 1991). DHPG is a highly selective agonist at group I metabotropic receptors, but not at group II or III receptors (Schoepp et al., 1994). It is likely, therefore, that the increase in extracellular adenosine during perfusion of *trans*-ACPD in the present study was due to the activation of group I metabotropic receptors, since, as discussed below, the group II agonist LCCG-1 did not have an effect.

Selective activation of the inhibitory group II and III metabotropic receptors using LCCG-1 and L-AP4, respectively, did not affect extracellular adenosine levels. Similarly, a previous study using hippocampal slice activation of the group II metabotropic receptor also did not affect basal adenosine release (Di Iorio et al., 1996). This is not surprising, since these receptors inhibit adenylate cyclase activity (Nakanishi, 1994). Together, the results of the present study and those of earlier studies suggest that the group I,

but not group II or III, metabotropic glutamate receptors can mediate increases in the concentration of extracellular adenosine in the parietal cortex *in vivo*.

It is possible that some of the increase in extracellular adenosine concentration induced by *trans*-ACPD and DHPG in the present study might have resulted from increased levels of endogenous glutamate since 1,S,3,*R*-ACPD and DHPG both induced glutamate release in the parietal cortex *in vivo* by activation of presynaptic group I metabotropic receptors (Moroni et al., 1998). Also, coadministration of AMPA and ACPD has been reported to release glutamate synergistically from cortical astrocytes (Bezzi et al., 1998). It remains to be determined whether glutamate released by these presynaptic and astrocytic mechanisms evokes the release of adenosine by acting at ionotropic and/or metabotropic glutamate receptors.

The extracellular levels of adenosine achieved by glutamate perfusion were in the range of 600 nM in the dialysate. When the concentration of adenosine in the dialysate is corrected for the 11% recovery of the microdialysis probe, glutamate evoked the release of approximately 6 μM adenosine. This value is comparable to the concentration of adenosine evoked by high K^+ (Pazzagli et al., 1993) or by an ischemic or hypoxic episode (Zetterström et al., 1982; Latini et al., 1999), and is 10-fold greater than the estimated EC_{50} for adenosine to depress field excitatory postsynaptic potentials in hippocampal slice (Dunwiddie and Diao, 1994). It is possible therefore that the extracellular adenosine evoked by glutamate in the present study had an effect on the activity of cortical neurons. This finding also suggests that extracellular

glutamate may contribute to the high levels of extracellular adenosine that are present in the brain following an ischemic or hypoxic insult, although this possibility remains to be tested.

In cortical slice, glutamate evoked adenosine release was diminished by 50% by NMDA receptor antagonists (Hoehn and White, 1990b). In the present study the group I metabotropic receptor antagonist AIDA reduced glutamate evoked adenosine levels, although this was not statistically significant. These observations indicate that activation of metabotropic glutamate receptors contributes less than that of ionotropic receptors to increased extracellular adenosine concentration by endogenous glutamate *in vivo*.

In conclusion, the present results indicate that glutamate acting at all subtypes of the ionotropic receptor and at group I metabotropic receptors increases the concentration of extracellular adenosine in the parietal cortex *in vivo*. Glutamatergic neurotransmission therefore contributes to the level of endogenous extracellular adenosine in the cerebral cortex by activation of ionotropic, and to a lesser degree, metabotropic receptors.

CHAPTER IV.

EFFECTS OF NICOTINIC AND MUSCARINIC RECEPTOR ACTIVATION ON THE CONCENTRATION OF EXTRACELLULAR ADENOSINE.

Acetylcholine plays a role in a variety of functions including cortical arousal (Semba, 1991). The cerebral cortex receives cholinergic innervation from a population of cholinergic neurons in the basal forebrain (Saper, 1984; Semba and Fibiger, 1989). Activation of muscarinic receptors alone does not affect basal adenosine release, but has been shown to modulate evoked adenosine release in the cortex both *in vitro* and *in vivo* (Pedata et al., 1988; Pazzagli et al., 1994; Semba and White, 1997).

The effects of nicotinic receptor activation on adenosine release has not been examined. Nicotine is a central nervous system stimulant (Volle and Koelle, 1975) and lesion studies suggest that cortical [^{3}H]nicotine binding sites in the parietal cortex are located on noncholinergic afferents arising from the thalamus (Lavine et al., 1997). Given that one of the presynaptic effects of nicotinic receptor activation is to modulate transmitter release (Wonnacott, 1997; MacDermott et al., 1999), it was hypothesized in this thesis that activation of nicotinic receptors can increase extracellular adenosine levels in the cortex *in vivo*. Therefore, both nicotinic and muscarinic receptor agonists were tested for their ability to affect extracellular adenosine levels in the cortex using *in vivo* microdialysis in unanesthetized rat.

METHODS

The methods are outlined in Chapter II.

RESULTS

The probe locations in the parietal cortex in this study were similar to those shown in Figure. 6. During these experiments, rats spent most of their time resting or sleeping with eyes closed, but occasional head movements, grooming and locomotion were also seen. No gross behavioral changes were observed in the animals during the administration of any drug.

The mean concentration of extracellular adenosine in the dialysate over a 120 min baseline period was 39 ± 7 nM (n=87).

Figure 11A shows that 100 μM acetylcholine significantly increased the concentration of extracellular adenosine during drug delivery (140 min) compared to the baseline concentration at 120 min (ANOVA, $F(4,40) = 3.23$, $p < 0.05$; Fisher's PLSD, $p < 0.01$). The acetylcholinesterase inhibitor neostigmine at a concentration of 100 μM also increased extracellular adenosine levels during drug delivery ($F(4,20) = 4.53$, $p < 0.01$; $p < 0.01$; Fig. 11A). The evoked release of adenosine by neostigmine was blocked by the nicotinic antagonist mecamylamine (50 μM; Student's t-test, $p < 0.05$; Fig. 11B). Mecamylamine alone did not affect baseline levels of adenosine.

Nicotine at a concentration of 100 μM significantly increased extracellular adenosine levels during drug delivery and at 160 min ($F(4,20) = 9.84$, $p < 0.001$; $p < 0.001$ for both; Fig. 12). The potent nicotinic receptor agonist epibatidine also had a significant effect on extracellular adenosine levels (effect of time, $F(4,52) = 18.57$, $p < 0.001$; Fig. 12). Epibatidine at 1 μM increased extracellular adenosine concentration during drug delivery and the concentration of

adenosine remained elevated ($F(4,24) = 7.30$, $p < 0.001$; $p < 0.01$, $p < 0.05$ and $p < 0.01$ for 140, 160 and 180 min, respectively). A higher concentration of epibatidine (10 μM) also increased adenosine concentration during drug delivery and the concentration of adenosine remained elevated for the duration of the experiment ($F(4,28) = 11.93$, $p < 0.001$; $p < 0.001$, 0.05 and 0.01).

The nonselective cholinergic receptor agonist carbachol at concentrations of 200 μM or 1 mM did not affect extracellular adenosine levels (Fig. 13A). The nonselective muscarinic receptor agonist oxotremorine at 100 μM significantly increased extracellular adenosine concentration during drug delivery compared to the baseline concentration at 120 min ($F(4,28) = 4.06$, $p < 0.05$; $p < 0.05$). The specific M1 receptor agonist CDD 0097 (500 μM) also significantly increased extracellular adenosine concentration; this effect was long lasting and reached significance at 160 and 180 min compared to the baseline concentration at 120 min ($F(4,28) = 6.22$, $p < 0.01$; $p < 0.05$ for both). In the presence of the M1 receptor antagonist pirenzepine (1 μM), CDD 0097 did not affect the extracellular concentration of adenosine (Fig. 13B). Pirenzepine alone did not affect extracellular adenosine levels.

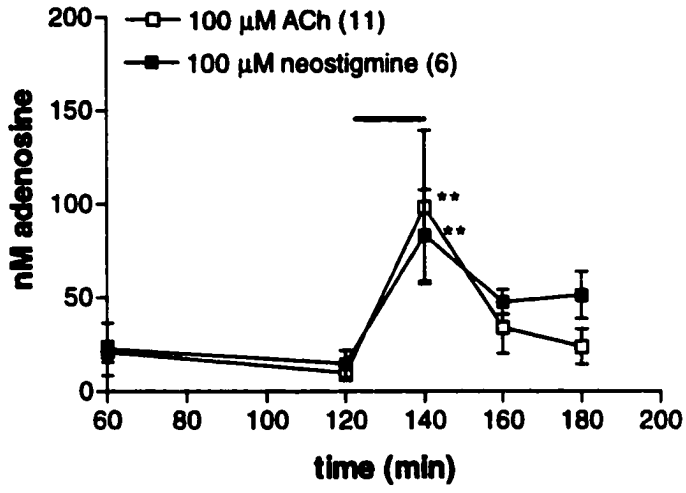
In an attempt to block the effects of muscarinic receptor activation, the muscarinic receptor antagonist atropine was perfused through the microdialysis probe. Unexpectedly, atropine alone affected the concentration of extracellular adenosine (effect of time, ($F(4,120) = 7.42$, $p < 0.0001$; Fig. 14A). Atropine at 10 μM significantly increased extracellular adenosine levels during drug delivery ($F(4,40) = 5.78$, $p < 0.001$; $p < 0.01$). A higher concentration of atropine

(100 μM) significantly increased extracellular adenosine levels during drug delivery and also at 160 min ($F(4,48) = 5.24$, $p < 0.01$; $p < 0.01$ and 0.05, respectively). A lower concentration at 1 μM did not affect extracellular adenosine levels. The increase in adenosine evoked by 10 μM atropine was blocked by the nicotinic receptor antagonist mecamylamine (50 μM; $p < 0.05$; Fig. 14B). The administration of 10 μM atropine in the ipsilateral probe did not affect the concentration of adenosine in the contralateral probe (control; see Chapter II).

The evoked release of adenosine by 5 mM glutamate was not affected by the addition of either 0.2 or 1 mM carbachol (Fig. 15A; 5 mM glutamate from Fig. 7). The evoked increase in extracellular adenosine by 200 μM NMDA was also not affected by the addition of 1 mM carbachol (Fig. 15B; 200 μM NMDA from Fig. 8A).

Fig. 11. Effects of acetylcholine and the acetylcholinesterase inhibitor neostigmine on the concentration of extracellular adenosine. A. A 20 min delivery (horizontal bar) of either acetylcholine (100 μM) or neostigmine (100 μM) through the microdialysis probe increased the concentration of extracellular adenosine compared to the baseline concentration at 120 min. B. The evoked release of adenosine by neostigmine (100 μM) was blocked by the nicotinic receptor antagonist mecamylamine (50 μM). The number in brackets indicates the number of experiments. * $p < 0.05$, ** $p < 0.01$

A


100 μM ACh (11)
100 μM neostigmine (6)
nM adenosine
200
150
100
50
0
60
80
100
120
140
160
180
200
time (min)
**
**

B

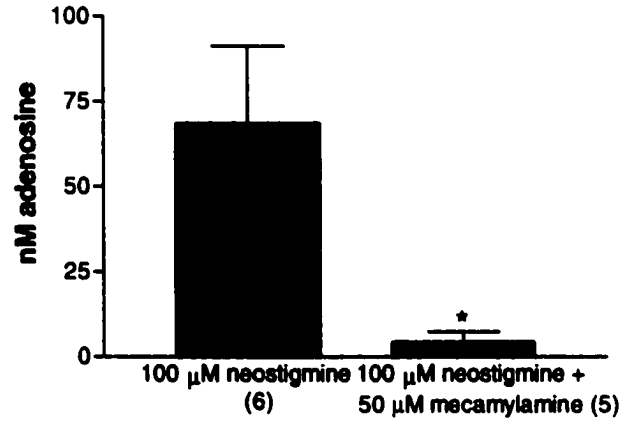

nM adenosine
100
75
50
25
0
100 μM neostigmine (6)
100 μM neostigmine + 50 μM mecamylamine (5)
*

Fig. 11

Fig. 12. Effects of nicotinic receptor activation on the concentration of extracellular adenosine. A 20 min perfusion of nicotine (100 μM) significantly increased extracellular adenosine during drug delivery and at 160 min compared to the baseline concentration at 120 min. The nicotinic receptor agonist epibatidine at both concentrations tested (1 and 10 μM) increased extracellular adenosine compared to the baseline level at 120 min. The number of experiments is indicated in the brackets. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

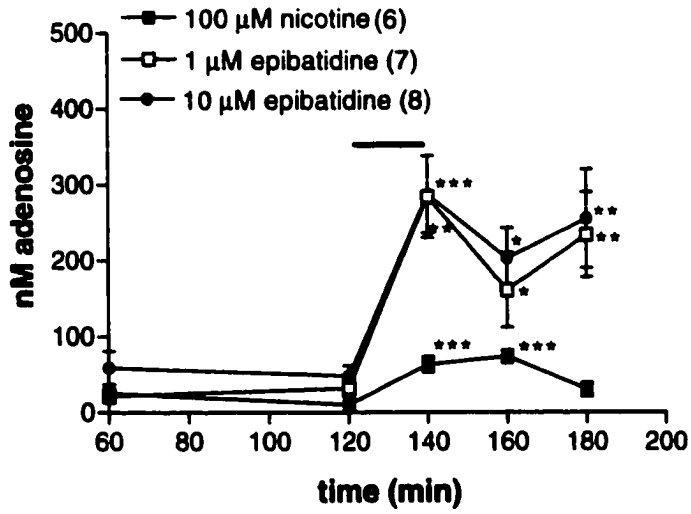
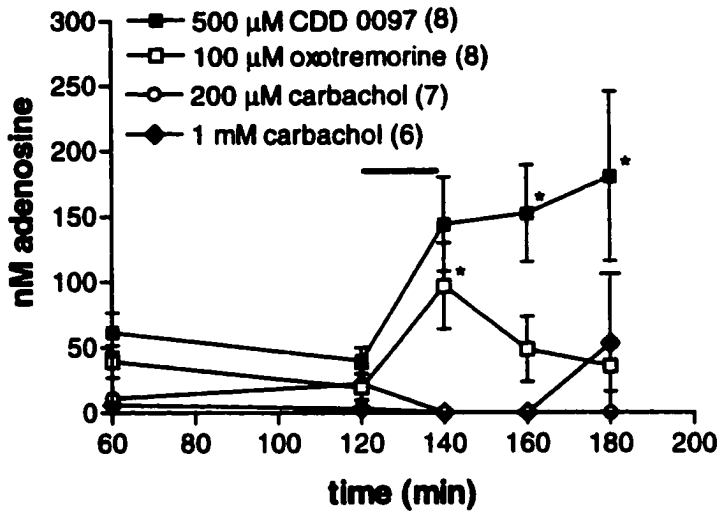

100 µM nicotine (6)
1 µM epibatidine (7)
10 µM epibatidine (8)
nM adenosine
500
400
300
200
100
0
60
80
100
120
140
160
180
200
time (min)

Fig. 12

Fig. 13. Effects of muscarinic receptor activation on adenosine release. A. The muscarinic and nicotinic receptor agonist carbachol at both concentrations tested (0.2 mM and 1 mM) did not affect extracellular adenosine. The nonselective muscarinic receptor agonist oxotremorine (100 μM) significantly increased extracellular adenosine compared to the baseline concentration at 120 min. The selective M1 receptor agonist CDD 0097 (500 μM) significantly increased extracellular adenosine at 160 and 180 min compared to the baseline at 120 min. B. Addition of the M1 receptor antagonist pirenzepine (1 μM) to the perfusate prevented the increase in adenosine evoked by CDD 0097. The number in brackets indicates the number of experiments. *$p < 0.05$

A


500 µM CDD 0097 (8)
100 µM oxotremorine (8)
200 µM carbachol (7)
1 mM carbachol (6)
nM adenosine
300
250
200
150
100
50
0
60
80
100
120
140
160
180
200
time (min)

B

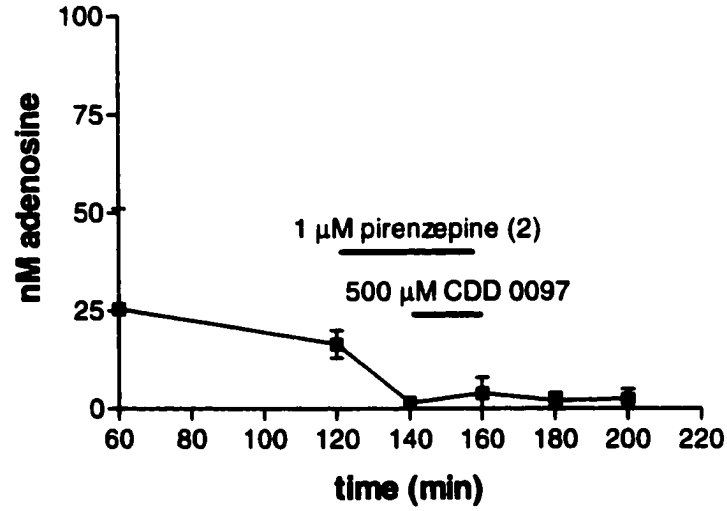

nM adenosine
100
75
50
25
0
1 µM pirenzepine (2)
500 µM CDD 0097
60
80
100
120
140
160
180
200
220
time (min)

Fig. 13

Fig. 14. Effects of the nonselective muscarinic receptor antagonist atropine on the concentration of extracellular adenosine. A. Atropine increased extracellular adenosine at the higher concentrations tested (10 and 100 μM; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the baseline concentration at 120 min). The administration of 10 μM atropine in the probe did not affect the concentration of adenosine in the contralateral probe (control). B. The evoked release of adenosine by atropine was blocked by the nicotinic receptor antagonist mecamylamine ($^{*}p < 0.05$). The number of experiments are indicated in the brackets.

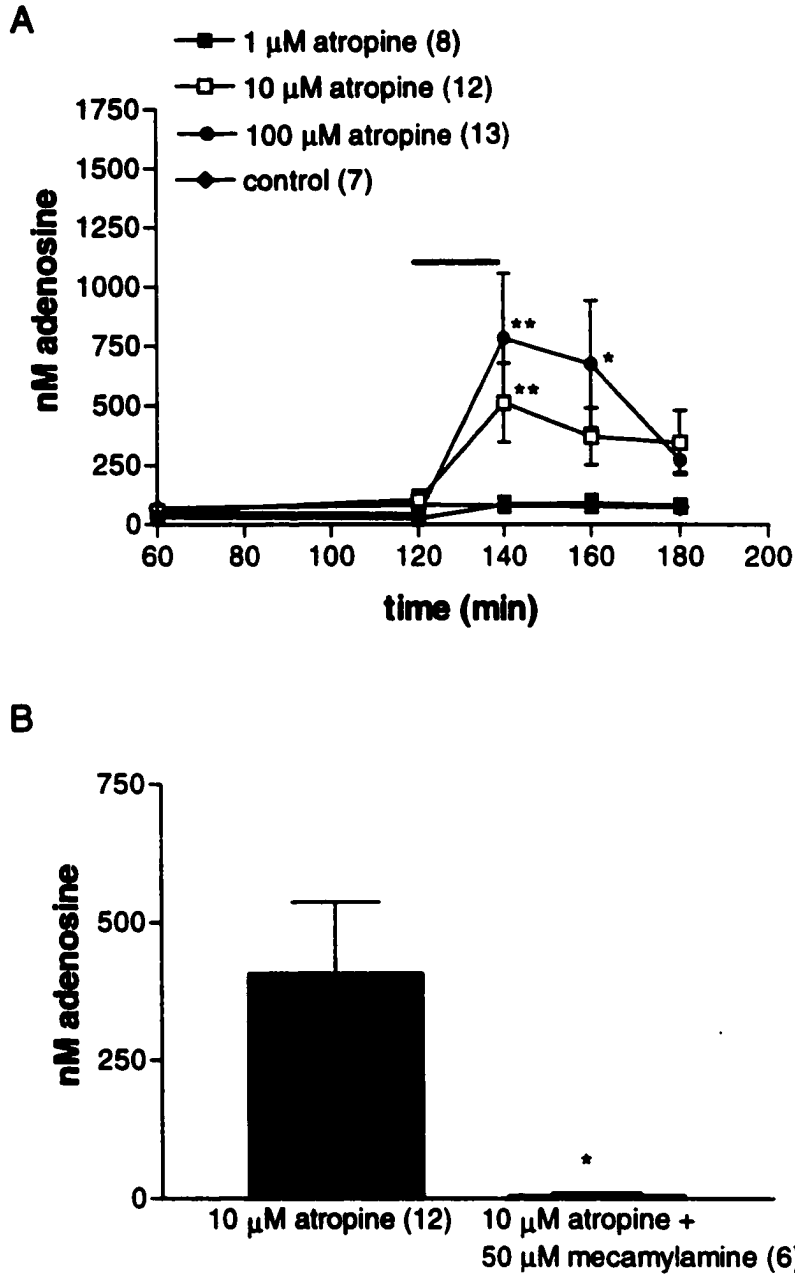
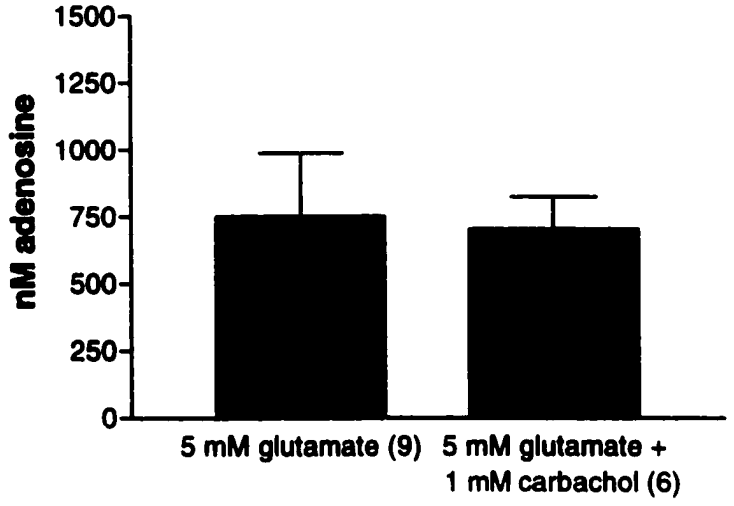

A
1 μM atropine (8)
10 μM atropine (12)
100 μM atropine (13)
control (7)
nM adenosine
1750
1500
1250
1000
750
500
250
0
60
80
100
120
140
160
180
200
time (min)
**
**
*
B
nM adenosine
750
500
250
0
*
10 μM atropine (12)
10 μM atropine +
50 μM mecamylamine (6)

Fig. 14

Fig. 15. The effect of carbachol on glutamate and NMDA evoked adenosine release. A. Carbachol did not affect glutamate (A) or NMDA (B) evoked adenosine release. The number in brackets is the number of experiments.

A


1500
1250
1000
750
500
250
0
nM adenosine
5 mM glutamate (9)
5 mM glutamate +
1 mM carbachol (6)

B

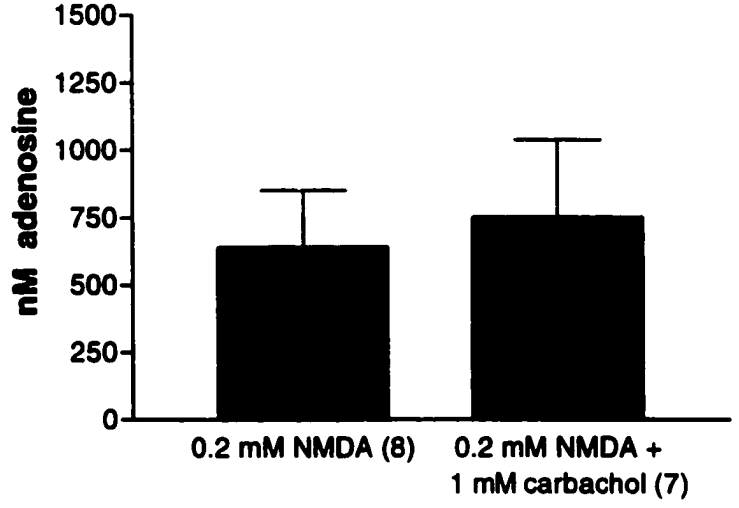

1500
1250
1000
750
500
250
0
nM adenosine
0.2 mM NMDA (8)
0.2 mM NMDA +
1 mM carbachol (7)

Fig. 15

DISCUSSION

Application of exogenous acetylcholine resulted in an increase in the concentration of extracellular adenosine in the cerebral cortex *in vivo*. This was mimicked by an acetylcholinesterase inhibitor which presumably increased endogenous acetylcholine levels. In cortical slice, the cholinesterase inhibitor physostigmine enhanced electrically stimulated [^{3}H]-labeled purine release consisting mostly of adenosine, inosine and hypoxanthine (Pedata et al., 1988). These findings suggest that increases in endogenous acetylcholine may increase the concentration of extracellular adenosine.

Acetylcholine acts at both nicotinic and muscarinic receptors. Given that nicotinic receptors modulate neurotransmitter release (Wonnacott, 1997; MacDermott et al., 1999), it was examined whether nicotinic receptor activation has an effect on extracellular adenosine levels *in vivo*. Activation of nicotinic receptors by the agonist nicotine or by the potent and nonselective nicotinic receptor agonist epibatidine (Gerzanich et al., 1995) increased the concentration of extracellular adenosine. Semba and White (1997) showed that epibatidine did not affect basal adenosine levels *in vitro*, but this is not surprising since slices are deafferented tissue (Reid et al., 1988) and would have a lower level of neuronal activity. To the best of our knowledge this is the first study to demonstrate an effect of nicotinic receptor activation on extracellular adenosine levels.

Unexpectedly, delivery of 10 μM of the nonselective muscarinic receptor antagonist atropine significantly increased the concentration of extracellular

adenosine. Atropine has been shown to increase acetylcholine levels in the cortex (Dudar & Szerb 1969; Quirion et al., 1994) most likely via blockade of the presynaptic, and presumably M2, autoreceptor which functions to inhibit acetylcholine release (Meyer and Otero, 1985; Lapchak et al., 1989; Hoss et al., 1990; Quiron et al., 1994). It is postulated that in the present study atropine increased the level of endogenous acetylcholine, which in turn increased adenosine release via activation of nicotinic receptors. Since the muscarinic receptors were presumably blocked by atropine in these experiments, and the effect of atropine was blocked by the nicotinic receptor antagonist mecamylamine, this suggests that activation of nicotinic receptors mediated the increase in adenosine produced by atropine.

A previous study using *in vivo* microdialysis in the unanesthetized rat reported that atropine did not have any effect on basal adenosine levels or on K^+ evoked adenosine release (Pazzagli et al., 1994). This study used a concentration of 1.5 μM atropine which may not have been high enough to see an excitatory effect on adenosine release, since in the present study, 1 μM atropine had no effect. Interestingly, this increase in adenosine by atropine occurred at a dose (10 μM) commonly used in studies on recovery of acetylcholine by microdialysis, to block the presynaptic muscarinic autoreceptors (Dudar & Szerb 1969; Rasmusson et al., 1994), suggesting that this concentration of atropine enhanced extracellular acetylcholine levels. Taken together, these results suggest that activation of nicotinic receptors by endogenous acetylcholine may be one mechanism by which the concentration

of extracellular adenosine is elevated *in vivo*.

Mecamylamine, a noncompetitive antagonist at the nicotinic receptor ion channel (Banerjee et al., 1990), blocked increases in extracellular adenosine levels produced by neostigmine and atropine. Although mecamylamine has been shown to reduce NMDA-induced currents (O'Dell and Christensen, 1988), most studies used systemic injections of a high dose (1 mg/kg) of mecamylamine, which would attain a concentration of 10 μM in the body if evenly distributed (Clarke et al., 1994). It is likely that the concentration of mecamylamine used in the present study (50 μM) would be reduced upon diffusion from the probe, to approximately 5 μM in the vicinity of the probe. Therefore, although the possibility cannot be ruled out, it is unlikely that the concentration of mecamylamine used in the present study antagonized NMDA receptors.

In the present study the nonspecific muscarinic agonist oxotremorine increased the concentration of extracellular adenosine. Oxotremorine has been found to enhance K^+ evoked adenosine release *in vivo* (Pazzagli et al., 1994) and to enhance [^{3}H]-labeled purine release in electrically stimulated rat cortical slices (Pedata et al., 1988). Activation of M1 muscarinic receptors by the specific agonist CDD-0097 also increased extracellular adenosine levels in the present study. Previously, it was found that the M1 selective antagonist pirenzepine did not affect the facilitation by carbachol of NMDA evoked adenosine release in cortical slice (Semba & White, 1997). However, since in this previous study the effects of agonists specific to muscarinic receptor

subtypes on adenosine release were not examined, the possibility remains that activation of M1 receptors may evoke the release of adenosine *in vitro*.

A previous study using cortical slice showed that the muscarinic and nicotinic agonist carbachol alone did not affect basal adenosine levels, but enhanced NMDA evoked adenosine release and that this enhancement was reduced by the M3 muscarinic receptor antagonist 4-DAMP (Semba and White, 1997). However, in the parietal cortex of the unanesthetized rat, carbachol did not affect basal, glutamate or NMDA evoked adenosine release. The failure of carbachol *in vivo* to enhance glutamate or NMDA evoked adenosine release was unexpected. It had been assumed, as in the case of cortical slice, that carbachol would decrease the voltage-sensitive Mg^{2+} block of the NMDA receptor, thereby enhancing NMDA evoked adenosine release (Semba and White, 1997). In the case of freely moving rats it is possible that the neurons releasing adenosine were already partially depolarized and, therefore, the addition of carbachol was not necessary to remove the Mg^{2+} block of the NMDA receptor. In light of the present finding that activation of both muscarinic and nicotinic receptors increased extracellular adenosine, the lack of an effect of carbachol alone or in combination with other agonists is difficult to interpret.

Adenosine has been postulated to act as a feedback modulator of synaptic transmission (Dunwiddie, 1985). In the cerebral cortex, extracellular levels of acetylcholine are under the tonic inhibitory control of adenosine via the A_1 receptor (Phillis et al., 1993; Kurokawa et al., 1996; Materi et al., 2000). Adenosine and A_1 receptor agonists reduced the electrically stimulated release

of acetylcholine *in vitro* (Pedata et al., 1983; Spignoli et al., 1984) and inhibited synaptically evoked cortical acetylcholine release via the A_1 receptor *in vivo* (Materi et al., 2000).

An increase in extracellular levels of adenosine by acetylcholine, acting at nicotinic receptors *in vivo*, may serve to inhibit further release of acetylcholine in the cortex. Acetylcholine inhibits specific K^+ currents in cortical pyramidal cells thereby increasing neuronal excitability and contributing to the cortical desynchronization observed during wakefulness and rapid eye movement sleep (McCormick and Williamson, 1989; McCormick, 1990; McCormick, 1993). Therefore, a reduction of cortical acetylcholine levels by adenosine may serve to decrease neuronal excitability and may regulate cortical arousal and behavioral states.

CHAPTER V.

EFFECTS OF ADRENERGIC RECEPTOR ACTIVATION ON THE CONCENTRATION OF EXTRACELLULAR ADENOSINE.

The cerebral cortex receives innervation from noradrenergic neurons in the locus coeruleus (Séguéla et al., 1990; Guyenet, 1991; Aston-Jones et al., 1995). Given that the activation of β-adrenergic receptors increases extracellular adenosine levels in cultures of cortical tissue (Rosenberg et al., 1994, Rosenberg and Yi, 1995b), it was hypothesized that activation of β-adrenergic receptors would increase extracellular adenosine levels *in vivo* as well. The possible role of α-adrenergic receptors mediating adenosine release has not been explored. Therefore, to identify the mechanisms that would lead to increased levels of the inhibitory neuromodulator adenosine in the brain, the ability of α- and β-adrenergic receptor agonists to increase extracellular adenosine were tested in the cortex using *in vivo* microdialysis of unanesthetized rat.

METHODS

The methods are outlined in Chapter II.

RESULTS

The probe locations in the parietal cortex were similar to those shown in Figure. 6. During these experiments, rats spent most of their time resting or sleeping with eyes closed, but occasional head movements, grooming and locomotion were also seen. No gross behavioral changes were observed in the animals during the administration of any drug.

The mean extracellular concentration of adenosine over a 120 min baseline period was 20 ± 4 nM (n=51).

As shown in Figure 16A, noradrenaline increased the level of extracellular adenosine in a concentration dependent manner (effect of treatment, $F(3,21) = 37.91$, $p < 0.001$). Noradrenaline at a concentration of 200 μM significantly increased extracellular adenosine concentration at 160 and 180 min compared to the baseline concentration at 120 min ($F(4,16) = 3.56$, $p < 0.05$; $p < 0.05$ for both time points). A higher concentration of noradrenaline (500 μM) also significantly increased extracellular adenosine concentration during drug delivery and the concentration of adenosine remained elevated for the duration of the experiment ($F(4,28) = 11.97$, $p < 0.001$; $p < 0.001$ for 140, 160 and 180 min). The noradrenaline reuptake inhibitor desipramine also increased extracellular adenosine concentration during drug delivery ($F(4,24) = 4.74$, $p < 0.01$, $p < 0.05$; Fig. 16B).

To identify the receptor subtypes that mediated the effects of noradrenaline, selective agonists were used. The α_1-adrenergic receptor agonist phenylephrine at a concentration of 100 μM significantly increased extracellular adenosine concentration during drug delivery and at 160 and 180 min ($F(4,20) = 6.39$, $p < 0.01$; $p < 0.05$ for all; Fig. 17A). Phenylephrine (100 μM) administered in the presence of the α_1-adrenergic receptor antagonist prazosin (10 μM) did not affect extracellular adenosine concentration (Fig. 17B). Prazosin alone did not affect the extracellular concentration of adenosine. Unlike phenylephrine, the α_2-adrenergic receptor agonist clonidine at a concentration of 100 μM did not affect extracellular adenosine levels (Fig. 17A).

The nonselective β-adrenergic receptor agonist isoproterenol at a

concentration of 100 μM significantly increased extracellular adenosine levels during drug perfusion ($F(4,24) = 3.51$, $p < 0.05$; $p < 0.05$; Fig. 17A). Isoproterenol (100 μM) perfused in the presence of the nonselective β-adrenergic receptor antagonist propranolol (100 μM) did not affect adenosine levels (Fig. 17B). Propranolol alone did not affect the extracellular concentration of adenosine.

Fig. 16. Effects of exogenous and endogenous noradrenaline (NA) on the concentration of extracellular adenosine. A. Noradrenaline at the higher concentrations tested (200 and 500 μM) significantly increased extracellular adenosine compared to the baseline at 120 min. B. The noradrenergic reuptake inhibitor desipramine (10 μM) also increased extracellular adenosine compared to the baseline at 120 min. The number in brackets indicates the number of experiments. *$p < 0.05$, ***$p < 0.001$

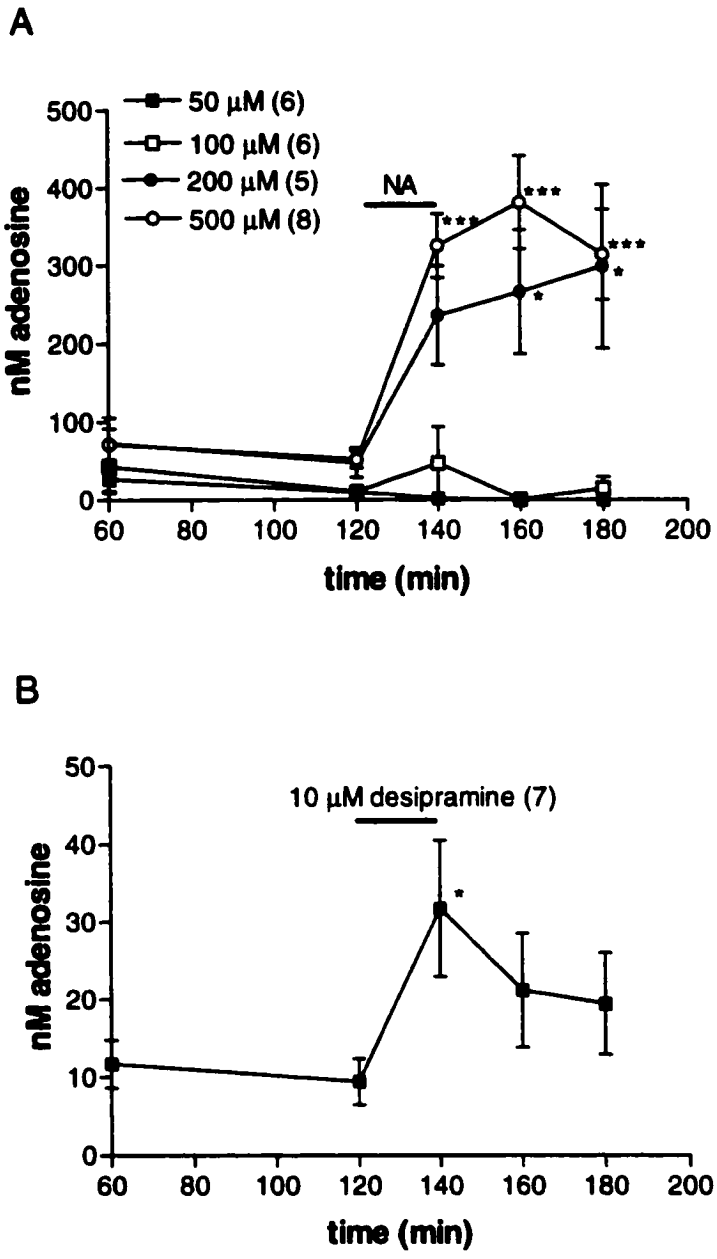
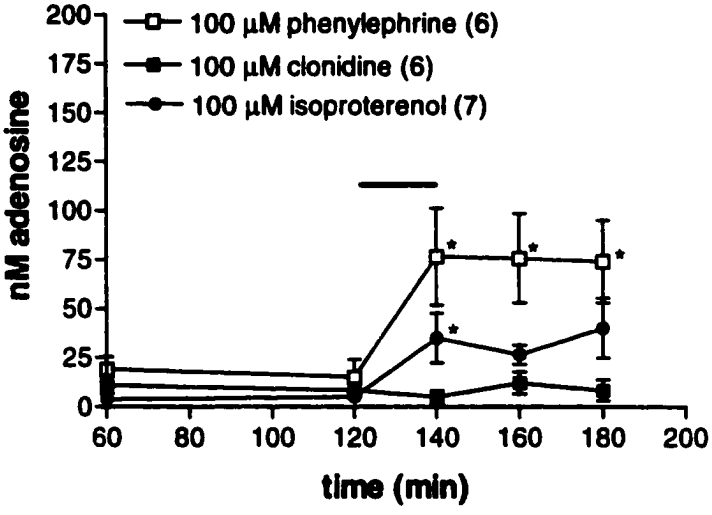

A
50 µM (6)
100 µM (6)
200 µM (5)
500 µM (8)
NA
nM adenosine
time (min)
B
10 µM desipramine (7)
nM adenosine
time (min)

Fig. 16

Fig. 17. Effects of adrenergic receptor agonists on extracellular adenosine in the absence or presence of selective receptor antagonists *in vivo*. A. The α_1-adrenergic receptor agonist phenylephrine (100 μM) and the β-adrenergic receptor agonist isoproterenol (100 μM) significantly increased the concentration of extracellular adenosine compared to baseline concentration at 120 min. Activation of α_2-adrenergic receptors by clonidine (100 μM) did not have an effect. B. Pretreatment with the α_1-adrenergic receptor antagonist prazosin and the β-adrenergic receptor antagonist propranolol before administration of phenylephrine and isoproterenol, respectively, prevented the increase in adenosine concentration. The number in brackets indicates the number of experiments. $^*p < 0.05$

A


100 µM phenylephrine (6)
100 µM clonidine (6)
100 µM isoproterenol (7)
nM adenosine
200
175
150
125
100
75
50
25
0
60
80
100
120
140
160
180
200
time (min)

B

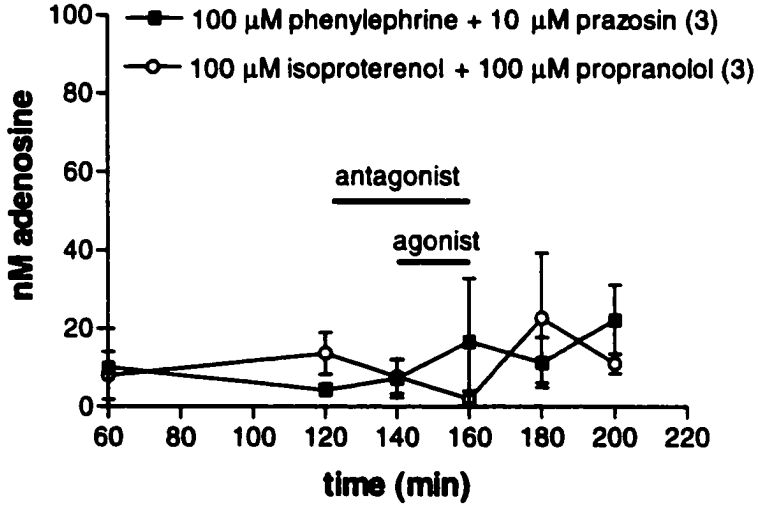

100 µM phenylephrine + 10 µM prazosin (3)
100 µM isoproterenol + 100 µM propranolol (3)
nM adenosine
100
80
60
40
20
0
antagonist
agonist
60
80
100
120
140
160
180
200
220
time (min)

Fig. 17

DISCUSSION

Local application of exogenous noradrenaline elevated the concentration of extracellular adenosine in the cerebral cortex of unanesthetized rat *in vivo*. Similarly, desipramine which, by blocking the noradrenaline reuptake transporter increases extracellular noradrenaline concentrations (Martin et al., 1976), mimicked the effects of exogenous noradrenaline on the concentration of extracellular adenosine. Taken together, these studies suggest that noradrenergic transmission may contribute to the regulation of extracellular adenosine levels *in vivo*.

Although the α_1 receptor is coupled to inositol trisphosphate release through interaction with G_q protein, there is also evidence that cAMP accumulates in response to α_1 receptor activation (Morgan et al., 1984; Cotecchia et al., 1990; Perez et al., 1996; Chen et al., 1999). This cAMP increase results from direct activation of G_s, and possibly via indirect pathways secondary to phosphoinositide turnover (Horie et al., 1995). Therefore, it is possible that activation of α_1- adrenergic receptor leads to an increase in cAMP, which is subsequently broken down by cAMP phosphodiesterase to AMP and then by 5'-nucleotidase to adenosine. This is the first study to demonstrate an effect of α_1-adrenergic receptor activation on the concentration of extracellular adenosine in the brain.

Activation of β-adrenergic receptors by the nonselective agonist isoproterenol also increased the concentration of extracellular adenosine in the parietal cortex of unanesthetized rat. Rosenberg and colleagues (1995b) found

that both noradrenaline and isoproterenol stimulated intracellular cAMP accumulation, cAMP transport and extracellular adenosine accumulation in cortical culture. Forskolin and vasoactive intestinal polypeptide also increased extracellular levels of adenosine, and this adenosine was derived from intracellular cAMP (Rosenberg and Li, 1995a, 1996). Therefore, intracellular cAMP accumulation, release of cAMP and its extracellular degradation to adenosine may be a common pathway for the regulation of extracellular adenosine levels by transmitters whose receptors are coupled to adenylate cyclase.

One consequence of increased extracellular adenosine might be to inhibit further noradrenaline release. Adenosine inhibits electrically stimulated [^{3}H]-noradrenaline release in rabbit cortical slice by acting at presynaptic adenosine A_1 receptors (von Kügelgen et al., 1992a,b). Alternatively, the action of increased adenosine may be to potentiate the effects of noradrenaline. Adenosine and AMP enhance the depression of neuronal firing rate by noradrenaline in rat parietal cortex *in vivo* (Stone and Taylor, 1978). Given that adenosine A_2 receptors are coupled to G_s proteins and activate adenylate cyclase (Greene and Haas, 1991; Stehle et al., 1992; Stiles, 1997), it is probable that stimulatory effects of adenosine on cAMP levels are mediated by this receptor.

Although these two possibilities may appear contradictory to each other, a study by Taylor and Stone (1980) suggests that both types of modulation may occur in the brain. The iontophoretic application of adenosine or AMP reduced

the inhibition of cortical neurons evoked by stimulation of the locus coeruleus if applied before the stimulation. If applied after the stimulation, adenosine enhanced the evoked inhibition of cortical neurons, suggesting that adenosine may act presynaptically to inhibit, and postsynaptically to enhance the effects of the activity of noradrenergic neurons. One explanation for these effects may be that adenosine A_1 receptors are located presynaptically on noradrenergic terminals whereas A_{2A} receptors are located postsynaptically on cortical neurons, although anatomical studies are necessary to verify this possibility. Release of adenosine by noradrenaline, therefore, may serve to modify synaptic transmission in a complex manner in the cerebral cortex.

Application of noradrenaline produces a block of a Ca^{2+}-activated K^+ current I_{AHP} resulting in an increase in the excitability of cortical neurons, and this response is mediated by β-adrenergic receptors (McCormick and Williamson, 1989). A reduction of cortical noradrenaline levels by adenosine, therefore, may serve to decrease neuronal excitability in the cortex.

CHAPTER VI. GENERAL DISCUSSION

The purpose of these studies was to identify the neurotransmitters that evoke an increase in extracellular adenosine in the cerebral cortex *in vivo* and to identify the receptor subtypes that mediate these effects. It was found that activation of ionotropic and group I metabotropic glutamate receptors, nicotinic and M1 muscarinic receptors and α_1- and β-adrenergic receptors increase extracellular adenosine levels (Fig. 18). Activation of group II and III metabotropic glutamate receptors and α_2-adrenergic receptors did not affect extracellular adenosine levels. Based on the findings of these studies, the following conclusion can be drawn.

Glutamatergic, cholinergic and noradrenergic transmission may contribute to the regulation of the extracellular concentration of adenosine in the cerebral cortex *in vivo*.

1. Receptor activation and adenosine release

The intracellular intermediary steps linking receptor activation to the release of adenosine by neurons were beyond the scope of the present studies. However, one likely mechanism producing increases in extracellular adenosine levels is the activation of adenylate cyclase; this produces an increase in intracellular cAMP, then transport of cAMP out of the cell and subsequent degradation to AMP by cAMP phosphodiesterase and then to adenosine by ecto-5'-nucleotidase. This mechanism may be responsible for the increase in adenosine levels evoked by β-adrenergic receptor activation *in vivo*, since

Fig. 18. A diagram summarizing the effects of activation of glutamatergic, cholinergic and noradrenergic receptors on adenosine release *in vivo*. Activation of NMDA, AMPA, kainate (KA) and group I metabotropic glutamate receptors increased extracellular adenosine levels in the cortex of the unanesthetized rat. Activation of muscarinic M1 and nicotinic receptors also increased extracellular adenosine levels. Atropine increased extracellular adenosine levels presumably by blocking muscarinic M2 autoreceptors on cholinergic terminals to increase extracellular acetylcholine (ACh), which then acted on nicotinic receptors. Activation of α_1- and β-adrenergic receptors also increased extracellular adenosine. Activation of group II and III metabotropic glutamate receptors and α_2-adrenergic receptors had no effect on the concentration of extracellular adenosine in the cortex. The location of all receptors on one cell is not implied by this diagram.

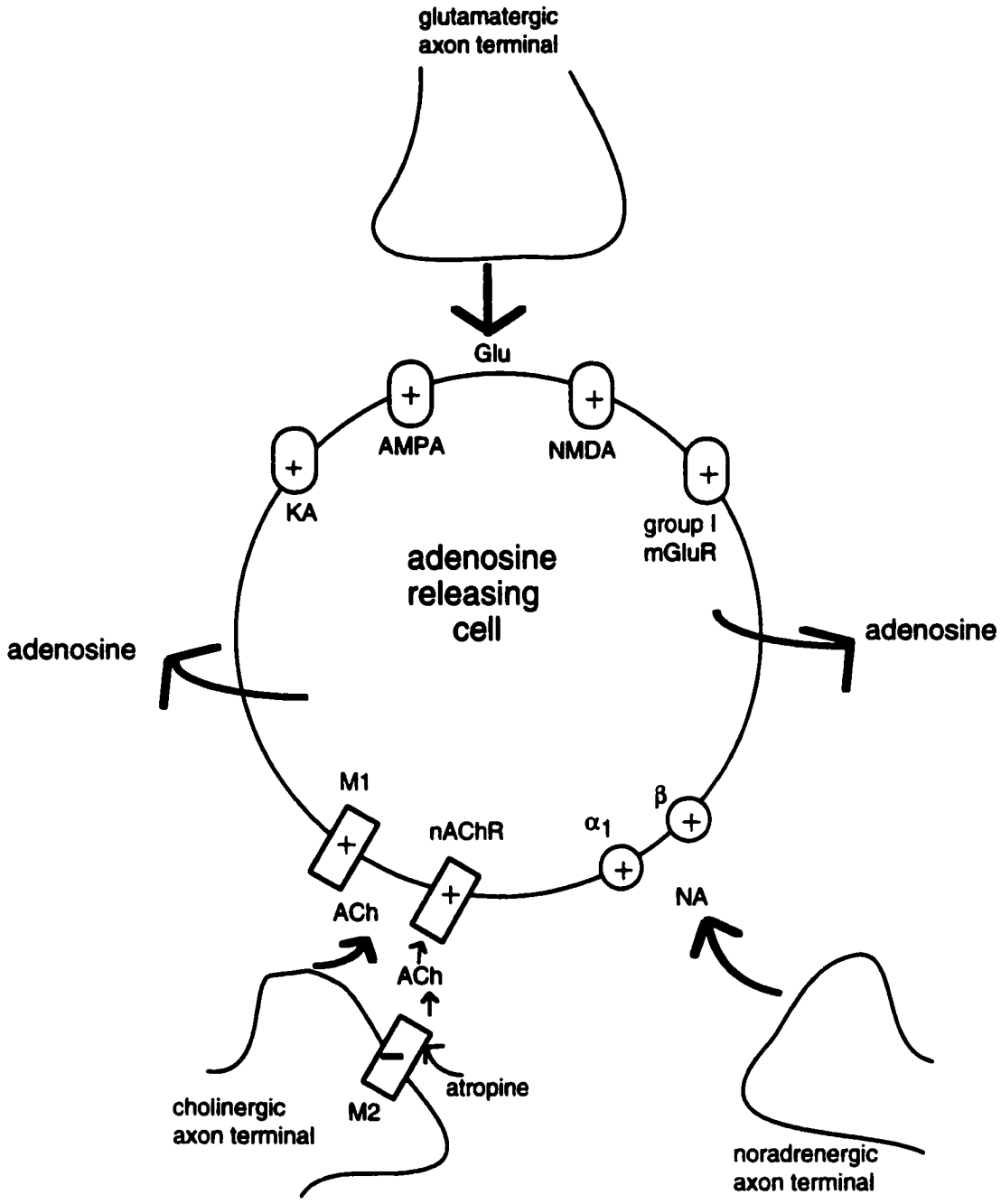

glutamatergic
axon terminal
Glu
AMPA
NMDA
KA
group I
mGluR
adenosine
releasing
cell
adenosine
adenosine
M1
nAChR
β
α1
ACh
ACh
NA
atropine
M2
cholinergic
axon terminal
noradrenergic
axon terminal

Fig. 18

activation of these receptors is linked to an increase in intracellular cAMP. To confirm this idea, a cAMP phosphodiesterase inhibitor such as rolipram (Thompson, 1991) could be delivered through the probe prior to and during the delivery of isoproterenol.

Although attempts have been made previously to determine how activation of NMDA receptors leads to adenosine release, these have not yielded definitive conclusions. In slices of rat parietal cortex activation of NMDA receptors releases a nucleotide that is converted to extracellular adenosine and this release is Ca^{2+}-dependent, whereas non-NMDA receptor activation releases adenosine in a Ca^{2+}-independent manner (Craig and White, 1993). It is not known whether nicotinic receptor activation releases adenosine in a Ca^{2+}-dependent or independent manner. In hippocampal slice P- and N-type voltage-sensitive Ca^{2+} channel blockers inhibited electrically evoked adenosine release (Latini et al., 1997). Therefore, both P- and N-type Ca^{2+} channels play a direct role in the calcium entry involved in the coupling process between electrical stimulation and adenosine release (Latini et al., 1997). It remains to be determined whether Ca^{2+}-dependent release of adenosine evoked by NMDA, and possibly by nicotinic receptor activation, occurs *in vivo*. It is also possible that depolarization of the neuronal membrane by activation of NMDA and nicotinic receptors and subsequent increase in metabolism resulted in an increase in extracellular adenosine through the breakdown of intracellular ATP.

How the activation of G-protein linked receptors leads to adenosine release from the cell is not known. Although it was beyond the scope of this

thesis to answer this question, it is possible to speculate on potential mechanisms. Through their coupling with G_q proteins, activation of mGluR1 and mGluR5 as well as muscarinic M1, M3 and M5 and α_1-adrenergic receptors leads to the activation of protein kinase C (PKC), which in turn liberates the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 is immediately hydrolysed to inositol-1,4-bisphosphate which requires the breakdown of ATP to ADP (Darnell et al., 1990). Although speculative at this time, it is possible that ADP, produced as a byproduct of IP_3 hydrolysis may be degraded to AMP and then to adenosine. However, given the lack of involvement of PKC in adenosine release from cortical slice (Wang and White, 1998), the activation of PKC may not be a likely explanation for the release of adenosine following G protein activation. It is possible that the effects of mGluR1 and mGluR5, muscarinic M1, M3 and M5 and α_1-adrenergic receptor activation are mediated through interaction with other second messengers or unknown G proteins.

In summary, the extracellular concentration of adenosine can be elevated by neuronal depolarization, activation of ion channels or activation of G proteins, although the exact mechanisms by which this occurs are not currently known.

2. Neurotransmitter evoked adenosine release

We found that delivery of glutamate, acetylcholine and noradrenaline increase the concentration of extracellular adenosine in the cortex of

unanesthetized rat, suggesting that these neurotransmitters may contribute to increases in extracellular adenosine in the cortex under normal physiological conditions. In order to determine which neurotransmitter had the greatest effect, the EC_{50} would need to be calculated, although this may be difficult to accomplish in the freely moving, unanesthetized rat due to the potential for seizures and other undesirable effects of drugs administered at high doses.

One previous study suggests that several neurotransmitters may act together to evoke adenosine release. A bilateral excitotoxic lesion of the nucleus basalis magnocellularis by quisqualic acid reduced cortical choline acetyltransferase activity, but did not alter the adenosine release from the cortex as measured by microdialysis (Pazzagli et al., 1994). Although it may be concluded that acetylcholine does not have an effect on cortical adenosine release, the finding of the present thesis that delivery of exogenous acetylcholine evoked the release of adenosine, suggests otherwise. A possible explanation for the apparent discrepancy between the previous and present studies is that the decrease in cholinergic activity in the previous study may have been compensated for by other neurotransmitter systems. From the present studies we can speculate that glutamatergic and noradrenergic systems may have been responsible for maintaining the level of adenosine and thus compensating for the loss of acetylcholine. Under normal physiological conditions glutamatergic, cholinergic and noradrenergic transmission may contribute concurrently to increases in the concentration of extracellular adenosine *in vivo*.

3. Future experiments

3.1. Indirect release of adenosine

Few investigators have examined the possibility that the activation of two or three neurotransmitter systems together would synergistically enhance adenosine release *in vivo*. Because the present experiments were conducted in the freely moving, unanesthetized rat it, is possible that some release of adenosine was secondary to the release of adenosine by another neurotransmitter.

Immunolesion by 192IgG-saporin of cholinergic basal forebrain neurons resulted in a significant reduction of α_2- and β-adrenergic receptor binding in the parietal cortex, suggesting that these receptors are located on presynaptic cholinergic terminals (Heider et al., 1997). A systemic injection of the β-adrenergic receptor antagonist propranolol reduced the basal release of acetylcholine in the cerebral cortex of anesthetized rats (Paul, 1987). Thus, it is possible that the release of adenosine evoked by isoproterenol may have been mediated by the release of acetylcholine due to stimulation of β-adrenergic receptors on cholinergic terminals in the cortex (Fig. 19). Mecamylamine could be administered prior to and during isoproterenol exposure in order to determine if the effects of isoproterenol are mediated by acetylcholine acting at nicotinic receptors. Clonidine and other α_2-adrenergic receptor agonists decrease acetylcholine release in the cortex of conscious rat and guinea pig (Moroni et al., 1983; Tellez et al., 1997) and are therefore unlikely to mediate the indirect release of adenosine.

Fig. 19. Potential indirect mechanisms for release of adenosine. Acetylcholine (ACh), nicotine or epibatidine acting at presynaptic nicotinic receptors (nAChR) on glutamatergic terminals may increase extracellular glutamate, which in turn may act at ionotropic or group I metabotropic receptors to increase the concentration of extracellular adenosine. Noradrenaline acting at presynaptic β-adrenergic receptors on cholinergic terminals may increase extracellular acetylcholine, which may act at nicotinic or muscarinic M1 receptors to increase the levels of endogenous adenosine.

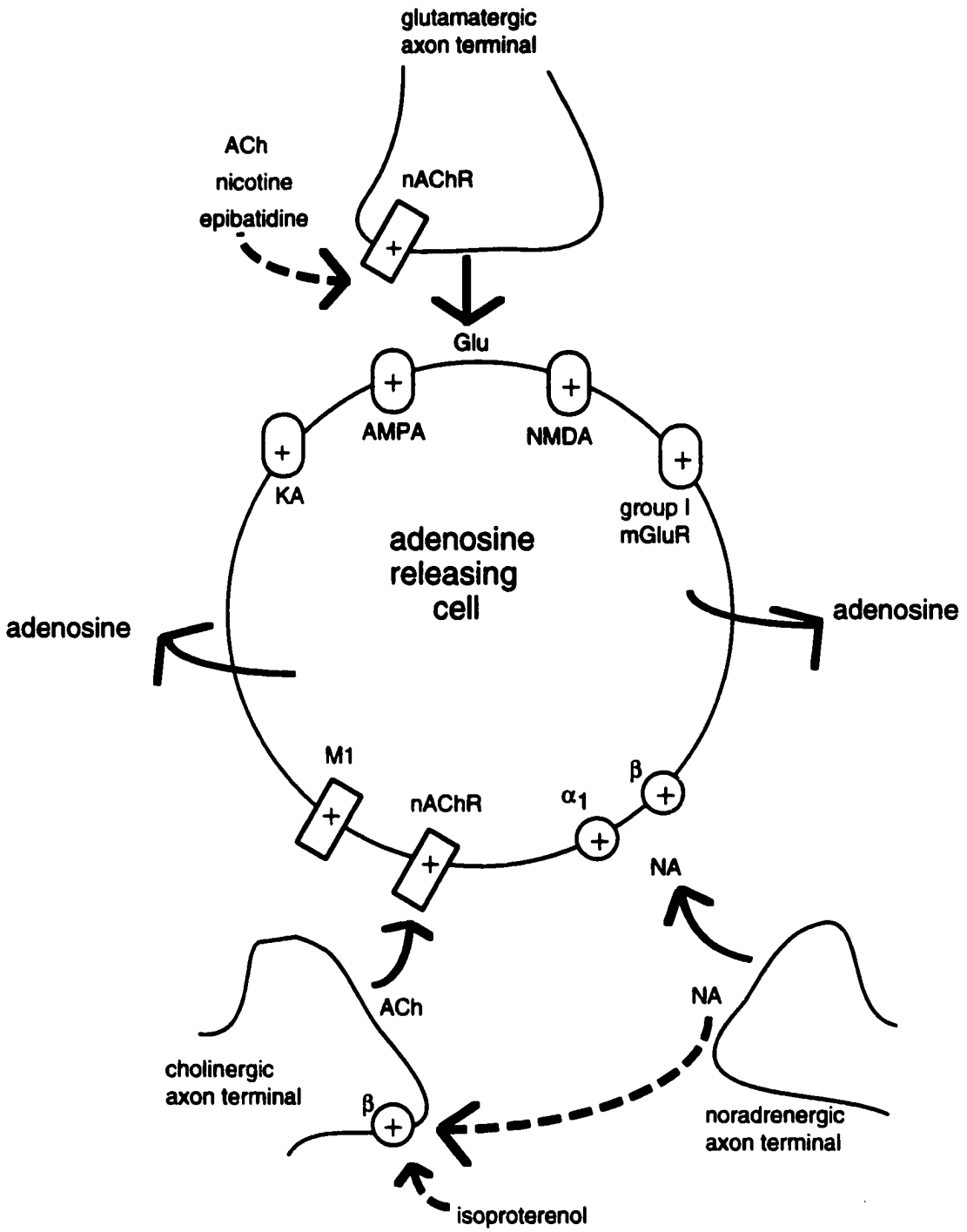

glutamatergic
axon terminal
ACh
nicotine
epibatidine
nAChR
Glu
AMPA
NMDA
KA
group I
mGluR
adenosine
releasing
cell
adenosine
adenosine
M1
nAChR
α1
β
NA
ACh
NA
cholinergic
axon terminal
β
noradrenergic
axon terminal
isoproterenol

Fig. 19

Electrolytic and chemical lesioning of thalamocortical fibers reduced cortical labeling of [^{3}H]nicotine, suggesting that a subset of nicotinic receptors are located presynaptically on thalamocortical terminals (Sahin et al., 1992; Gioanni et al., 1999). Direct application of nicotine via a microdialysis probe in the prefrontal cortex increased extracellular glutamate (Gioanni et al., 1999). Therefore, it is possible that nicotine and epibatidine may have increased extracellular adenosine indirectly through the release of glutamate (Fig. 19). NMDA, AMPA, kainic acid or group I metabotropic receptor antagonists could be perfused prior to and during exposure to epibatidine or nicotine in order to determine whether release of glutamate mediates the effects of nicotinic receptor activation on extracellular adenosine levels.

3.2. Effects of other muscarinic receptor agonists

Carbachol did not affect basal, glutamate or NMDA evoked adenosine release. One possible explanation for the lack of effects of carbachol is that activation of all four muscarinic receptor subtypes together, or in combination, may inhibit the accumulation of extracellular adenosine. Although the M1 receptor agonist CDD 0097 increased the extracellular concentration of adenosine, only a high concentration of this agonist was tested. It is possible that a high concentration was required in order to overcome a possible inhibitory effect by activation of M2 and M4 muscarinic receptors on adenosine release. This may also explain the lack of effect of carbachol, i.e., activation of nicotinic receptors increased extracellular adenosine levels, whereas co-

activation of muscarinic and nicotinic receptors by carbachol did not. Although carbachol acting at nicotinic receptors may have increased the concentration of extracellular adenosine, it may have also reduced extracellular adenosine levels at the same time by acting at the M2 and M4 muscarinic receptors. The net effect, therefore, would be no detectable change in the concentration of extracellular adenosine. To test this hypothesis, a decrease in extracellular adenosine by activation of M2 and/or M4 receptors would first need to be shown. Then, an M2 or M4 muscarinic receptor agonist could be perfused through the microdialysis probe together with epibatidine in order to mimic the actions of carbachol.

4. Lack of an effect of antagonists on basal adenosine release

With the exception of atropine, none of the antagonists tested had an effect on the basal levels of adenosine. Given that these experiments were conducted in the cortex of conscious rats, in which neurotransmission would be ongoing, this is somewhat surprising. Few studies, however, have demonstrated decreases in the basal release of adenosine by an antagonist. The delivery of an agonist would presumably activate all of its receptors, producing a net and detectable increase in extracellular adenosine. An antagonist may produce a decrease in adenosine too small to be detected. Alternatively, the effects of an antagonist on adenosine release may be localized to a particular synapse, and since microdialysis does not sample from the synapse, would be too discrete to be detected. It is possible, therefore, that

the limitations of microdialysis may be responsible for the observed lack of effects of antagonists on the basal release of adenosine.

Alternatively, the lack of effect of antagonists, with the exception of atropine, on the extracellular concentration of adenosine levels suggests that glutamatergic, cholinergic and noradrenergic transmission does not contribute to the basal concentration of adenosine in the present experimental conditions. It was necessary to add exogenous glutamate, acetylcholine and noradrenaline or to increase the endogenous concentrations of acetylcholine and noradrenaline to increase adenosine levels. This suggests that glutamate, acetylcholine and noradrenaline may not influence adenosine levels under physiological conditions, but may only contribute to adenosine levels under pathological conditions or conditions in which the extracellular concentrations of these neurotransmitters is increased beyond normal ranges.

5. Functions of adenosine in the cerebral cortex

One function of adenosine is to act as a retaliatory metabolite or feedback modulator of synaptic activity (Newby, 1984; Brundege and Dunwiddie, 1997). Elevation of the adenosine concentration in a single CA1 pyramidal neuron induced the release of adenosine into the extracellular space and inhibited the excitatory inputs to that cell via presynaptic adenosine receptors (Brundege and Dunwiddie, 1996). Therefore, the concentration of extracellular adenosine in the vicinity of presynaptic nerve terminals may change rapidly to modulate synaptic activity.

Benington and Heller (1995) postulate that release of adenosine in the cortex may be increased by accumulated sleep need. However, electrical stimulation of the pedunculopontine tegmental nucleus, which would mimic prolonged wakefulness, did not affect the concentration of extracellular adenosine in the cortex (see Appendix). Furthermore, a 6 h sleep deprivation produced a nonsignificant increase in adenosine in the cortex (Strecker et al., 1999). In contrast, 6 h sleep deprivation increased the extracellular concentration of adenosine in the basal forebrain (Porrka-Heiskanen et al., 1997; Basheer et al., 1999). The question remains as to whether the primary sleep-promoting actions of adenosine occur in the cerebral cortex.

6. Conclusions

Many of the effects of adenosine result in the reduction of neuronal activity, such as inhibition of neuronal firing and inhibition of neurotransmitter release. Adenosine can therefore act as an inhibitory modulator of synaptic activity and neuronal function in the CNS. Activation of ionotropic and metabotropic glutamate receptors, nicotinic and M1 muscarinic receptors and α_1- and β-adrenergic receptors increased the concentration of extracellular adenosine in the parietal cortex of unanesthetized rat. It is therefore probable that glutamatergic, cholinergic and noradrenergic transmission contribute to increased extracellular adenosine levels *in vivo*. Such neurotransmitter mediated regulation of adenosine levels may thus represent a major mechanism for controlling the excitability of cortical neurons.

APPENDIX

DOES STIMULATION OF THE PEDUNCULOPONTINE TEGMENTAL NUCLEUS EVOKE THE RELEASE OF ADENOSINE IN THE BASAL FOREBRAIN IN VIVO?

Adenosine is thought to be a mild sleep-promoting factor in the brain. The effects of adenosine on arousal may be specific to regions containing cholinergic neurons, such as the basal forebrain. Perfusion of the adenosine uptake inhibitor NBTI in the basal forebrain, but not in the thalamus, decreased wakefulness in the cat (Porkka-Heiskanen et al., 1997). Furthermore, the extracellular concentrations of adenosine in the basal forebrain of rat and cat were increased during sleep deprivation (Basheer et al., 1999; Strecker et al., 1999). The mechanisms of these increases in adenosine concentration are not known.

The purpose of this study was to determine whether the concentration of adenosine in the basal forebrain could be increased synaptically by stimulation of the pedunculopontine tegmental nucleus (PPT). The PPT projects to the basal forebrain and plays a role in wakefulness (Semba, 1999). The major excitatory input to the cholinergic neurons of the basal forebrain from the PPT is mediated via glutamatergic synapses in the basal forebrain (Rasmusson et al., 1994). Measurements of acetylcholine release in the cerebral cortex using microdialysis have demonstrated that the ability of PPT stimulation to desynchronize the cortical electroencephalogram (EEG) can be modulated within the basal forebrain (Rasmusson et al., 1996). It was hypothesized that sustained activation of basal forebrain neurons, through electrical stimulation of

the PPT to mimic prolonged wakefulness, would result in an increase in the concentration of extracellular adenosine in the basal forebrain of the rat.

METHODS

1. Surgery

Microdialysis probes with a dialysis membrane 1.5 mm in length were constructed as outlined in Chapter II. This length of membrane was chosen in order to collect samples from within the dorsal-ventral axis of the basal forebrain. Bipolar stimulating electrodes were constructed by soldering a gold-plate pin (Newark Electronics, Mount Royal, PQ) to each end of a 4 cm piece of insulated stainless steel wire. The wire was then folded in half and the two halves twisted together. The twisted wires were cut at an angle to ensure that the ends were not in contact.

Under anesthesia (ketamine anesthetic solution; 3 ml ketamine, 0.8 ml xylazine, 0.3 ml acepromazine maleate and 0.9 ml saline; 0.1 ml/100 g, i.m.) a microdialysis probe was implanted in the basal forebrain using the following coordinates: AP -1.2, ML -2.8 and DV -8.6 from dura (Paxinos and Watson, 1998). The stimulating electrodes were implanted in the PPT at the following coordinates: AP -8.0, ML -1.6 and DV -6.2 from dura (Paxinos and Watson, 1998). The incisor bar was set at 3.3 mm below the interaural line.

2. Experimental procedure

After 24 h recovery from surgery, the rat was anesthetized with 1.4 g/kg

(i.p.) urethane. The body temperature was maintained within one degree of 37.2 °C using a rectal thermometer connected to a feedback-controlled heating pad (Shurite, New Haven, Connecticut). The inlet and outlet of the probe were connected via tubing to the liquid switch and fraction collector, respectively. The probes were infused with ACSF at a rate of 2 µl/min for the duration of the experiment. Samples were collected in 20 min intervals. Each 40 µl sample was collected into 20 µl of 0.15 M $ZnSO_4$ to which 20 µl saturated 0.15 M $Ba(OH)_2$ was immediately added. The sample was mixed and then frozen at -20 °C (Craig and White, 1993). The procedure for the detection of adenosine was as described in Chapter II.

At the conclusion of the experiment, the rat was given an overdose injection of sodium pentobarbitol (> 100 mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, ON) and perfused via the aorta with 100 ml of saline followed by 400 ml of a 4% paraformaldehyde (TAAB Laboratories Equipment Limited, Reading, UK) solution in 0.1 M phosphate buffer (pH 7.4; BDH Inc., Toronto, ON) at a rate of 20 ml/min. The brain was placed in the same fixative overnight at 4 °C placed in 30% sucrose solution in phosphate buffer until it was sectioned.

3. Histology

To confirm the sites of probes and stimulating electrodes in the basal forebrain and PPT, respectively, the brains were cut on a microtome into coronal sections of 60 µm thickness and collected serially in 0.05 M TBS. The

sections containing the basal forebrain were processed for ChAT immunohistochemistry. All incubations were conducted on a shaker at room temperature except for incubation in the primary antibody which was carried out at 4 °C. The sections were rinsed in 0.05 M Tris-buffered saline (TBS; Tris base from Boehringer Mannheim Co, Indianapolis, IN), treated in 0.3% hydrogen peroxide in 0.05 M TBS for 30 min, rinsed in 0.05 M TBS, and blocked in 10% normal goat serum (Sigma) for 1 h. The sections were then incubated for 24 h in a monoclonal anti-ChAT antibody (Boehringer Mannheim) at a dilution of 1:50. This and all other antibody dilutions were made with 0.05 M TBS containing 0.01% sodium azide, 2% normal goat serum, and 0.3% Triton X-100. The sections were then rinsed and incubated for 2 h in a solution containing a biotinylated goat anti-mouse IgG antibody at a dilution of 1:500 (Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.05 M TBS to which goat serum and Triton were added. Next, the sections were rinsed and placed in avidin-biotin complex (ABC) solution (Vector Laboratories, Burlingame, CA) for 2 h. The sections were rinsed, and incubated in a 3,3' diaminobenzidine (DAB) solution (0.025% DAB and 1 mM imidazole in 0.05 M Tris buffer) for 10 min. Hydrogen peroxide was then added for a final concentration of 0.0075%, and the reaction continued until a brown reaction product appeared. Finally, the sections were rinsed, mounted on chrome gel coated slides, air dried overnight and dehydrated in an ascending series of ethanol to xylene and coverslipped. Control for staining specificity were performed by omitting the primary antibody, and this yielded no specific staining.

Sections through the PPT were processed for NADPH-diaphorase histochemistry using a modification of the method of Vincent and Kimura (1992). The sections were placed in a solution of 1 mg/ml ß-NADPH (tetrasodium salt, reduced form; Sigma) and 0.1 mg/ml nitroblue tetrazolium (Sigma) in 0.05M tris-buffered saline (TBS; Tris base from Boehringer Mannheim) at pH 8.0 containing 0.3% Triton X-100 (Sigma). The sections were incubated until the tissue turned a blue-purple colour, after which they were rinsed in 0.05M TBS. The sections were mounted on slides, air dried overnight, then dipped in xylene and coverslipped.

RESULTS

1. Stimulation parameters

We began with the stimulation parameters used by Rasmusson and colleagues (1994, 1996) of 100 Hz, 400 µA, 0.2 msec pulses and 1 sec stimulus train/30 sec for 1 h (n=5) and then tried the following stimulus parameters:

1. 100 Hz, 400 µA, 0.2 msec pulses, 1 sec train/15 sec (n=4)
2. 100 Hz, 400 µA, 0.2 msec pulses, 2 sec train/15 sec (n=3)
3. 100 Hz, 400 µA, 0.2 msec pulses, 2 sec train/6 sec (n=2)
4. 100 Hz, 600 µA, 0.2 msec pulses, 1 sec train/30 sec (n=1)
5. 100 Hz, 600 µA, 0.2 msec pulses, 1 sec train/1 sec (n=1)
6. 100 Hz, 600 µA, 0.2 msec pulses, 1 sec train/10 sec (n=1)
7. 100 Hz, 600 µA, 0.2 msec pulses, 2 sec train/6 sec (n=1)
8. 100 Hz, 800 µA, 0.2 msec pulses, 2 sec train/6 sec (n=1)

Stimulation of the PPT using these parameters did not increase adenosine concentrations in the basal forebrain. We also used total stimulation durations of 2 h (n=2), 3 h (n=1) and 6 h (n=1) with stimulus parameters of 100 Hz, 400 μA, 0.2 msec pulses, 2 sec train/6 sec. Varying the stimulus parameters or total stimulation duration time did not affect the concentration of extracellular adenosine in the basal forebrain of urethane anesthetized rats.

2. Halothane anesthesia

Since it was thought that the use of urethane during the experiments might have suppressed adenosine release, experiments were conducted under halothane anesthesia, with the animal anesthetized initially with 4% halothane and then maintained on 1.25-2% (1 h stimulation; 100 Hz, 400 μA, 0.2 msec pulses, 2 sec train/6 sec; n=2). These experiments did not show a PPT-stimulated increase in adenosine concentration in the basal forebrain.

3. Stimulation and/or microdialysis in other brain areas

The posterior hypothalamus is a forebrain structure with a major role in regulating arousal (Marrocco et al., 1994). Electrical stimulation of the posterior hypothalamus did not increase extracellular adenosine concentration in the basal forebrain above baseline values (2 h stimulation; 100 Hz, 400 μA, 0.2 msec pulses, 2 sec train/6 sec; n=1). Electrical stimulation of the thalamus also had no effect on the concentration of extracellular adenosine in the cerebral cortex (2 h stimulation; 100 Hz, 400 μA, 0.2 msec pulses, 2 sec train/6 sec; n=1).

4. Prevention of adenosine breakdown or reuptake into the cell

It was thought that extracellular adenosine levels may have been increased due to PPT stimulation, and that the adenosine may be quickly broken down or taken up into cells and therefore not recovered by the microdialysis probe. The addition of the adenosine kinase inhibitors EHNA (100 μM and 1 mM) and iodotubercidin (5 μM) to the perfusion medium did not affect extracellular adenosine before or during PPT stimulation (Fig. 20; 100 Hz, 600 μA, 0.2 msec pulse, 2 sec train/6 sec). The use of the nucleoside transporter blocker dipyridamole alone (100 and 500 μM) or in combination with NBTI (4 μM) in the perfusion medium did not affect adenosine concentration during PPT stimulation (100 Hz, 600 μA, 0.2 msec pulse, 2 sec train/6 sec). The effects of these inhibitors on the basal concentration of adenosine are unclear.

Finally, it was thought that an inhibitory tone may already exist in the brain and prevent the release of adenosine. Therefore, the $GABA_A$ receptor antagonist bicuculline (333 μM) was added to the perfusion medium. This did not affect extracellular adenosine in the basal forebrain (100 Hz, 600 μA, 0.2 msec pulse, 2 sec train/6 sec).

5. EEG recordings

In order to confirm that stimulation of the PPT produced low-voltage fast activity in the cortical EEG, two recording electrodes and one ground electrode were implanted into the cerebral cortex of the rat and the cortical EEG was amplified using a Grass wideband AC EEG pre-amplifier and DC driver

Fig. 20 Effects of adenosine kinase and adenosine reuptake inhibitors. The adenosine kinase inhibitors EHNA or iodotubercidin did not affect the concentration of extracellular adenosine in the basal forebrain. The nucleoside transport inhibitor dipyridamole alone or in combination with the transport inhibitor NBTI also did not affect extracellular adenosine.

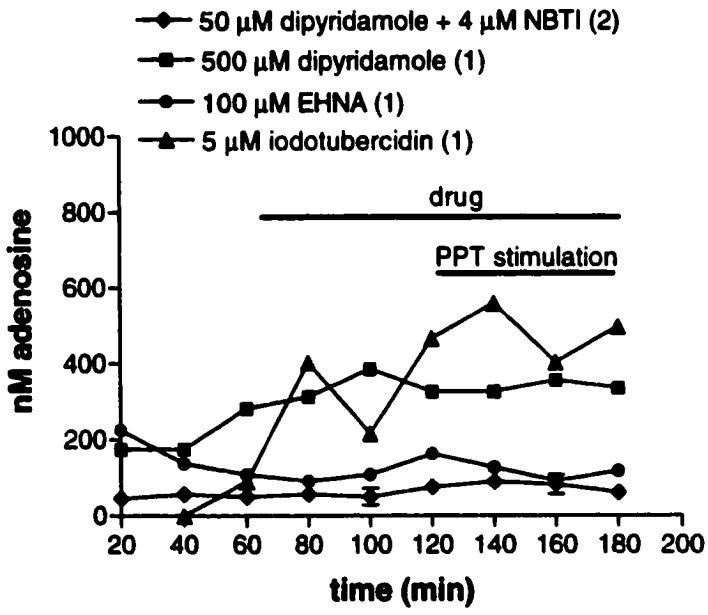

50 μM dipyridamole + 4 μM NBTI (2)
500 μM dipyridamole (1)
100 μM EHNA (1)
5 μM iodotubercidin (1)
drug
PPT stimulation
nM adenosine
1000
800
600
400
200
0
20
40
60
80
100
120
140
160
180
200
time (min)

Fig. 20

amplifier and recorded using a Grass polygraph. Stimulation of the PPT (100 Hz, 600 μA, 0.2 msec pulses, 2 sec train/6 sec; n=3) with 100 μM dipyridamole in the perfusion solution resulted in low-voltage fast activity in the cortical EEG.

DISCUSSION

Based on the lack of positive results in the present series of experiments, it may be concluded that stimulation of the PPT does not increase the concentration of extracellular adenosine in the basal forebrain of anesthetized rat. The possibility remains that PPT stimulation may increase extracellular adenosine in the basal forebrain of unanesthetized rat. A second possibility is that stimulation of the PPT produced an increase in extracellular adenosine that was too small to be detected. One way to test this possibility would be to use a microbore instead of a normal bore column. In a normal bore column, which was used in the present experiments, the stationary phase is packed into columns whose internal diameter is > 1.0 mm. In a microbore column, the stationary phase is packed into columns whose internal diameter is 1.0 mm or less. The benefit of such a column is an enhanced detection sensitivity (Kilpatrick, 1997). Indeed, the use of a microbore column provided a limit of detection in the fmol range (10^{-15}; Porkka-Heiskanen et al., 1997), whereas the use of a normal bore column in the present experiments provided a limit of detection in the pmol range (10^{-12}). Another possibility is that the release of adenosine was too short-lasting to be detected with microdialysis. Finally, it is

possible that adenosine was released, although not in the vicinity of the probe. Further experiments are required either in unanesthetized rat or with the use of a microbore column to determine whether electrical stimulation evokes the release of adenosine *in vivo*.

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