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ADENOSINE DURING PROLONGED WAKEFULNESS IN THE RAT BRAIN

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ACADEMIC DISSERTATION

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Sleep that knits up the ravell'd sleave of care,
The death of each day's life, sore labour's bath,
Balm of hurt minds, great nature's second course,
Chief nourisher in life's feast.

William Shakespeare (*Macbeth* 2.2.48-51)

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ABSTRACT

Adenosine is an endogenous substance which, in addition to being a building block for high-energy phosphates, has been associated with a multitude of both physiological and pathological processes including blood pressure, heart rate, pain and inflammation. In the central nervous system, adenosine is a recognized inhibitory neuromodulator, it is a neuroprotective agent against neuronal injury in threatening conditions like ischemia or epileptic seizures. In addition it is known to take part in the regulation of motor activity and sleep, among others.

The sleep inducing effects of adenosine have been well documented, and it is known that this effect is at least partly mediated by adenosine A_1 receptors. It is also known that during prolonged wakefulness adenosine accumulates specifically in the basal forebrain, a key area in the control of wakefulness and cortical activation. There is a transient increase in extracellular adenosine in the cortex, but no accumulation has been measured in any other area, including e.g. the thalamus and the dorsal raphe. As an inhibitory neuromodulator, adenosine inhibits the neuronal excitability in the basal forebrain, thereby decreasing cortical activity and increasing the propensity to sleep.

This study was undertaken to clarify the mechanisms by which adenosine mediates its sleep inducing effects in the basal forebrain, and the possible mechanism(s) of adenosine accumulation at the cellular level in the rat.

The mRNA expression and the receptor binding of the adenosine A_1 and A_{2A} receptors were measured after 3 and 6 hours of sleep deprivation and after 2 hours of recovery sleep. The A_1 mRNA was upregulated during sleep deprivation in the basal forebrain, while the receptor binding remained unchanged. In the olfactory tubercle, the A_{2A} receptor mRNA expression was decreased, while the receptor binding showed a transient decrease to be lower after 3 but not after 6 hours of deprivation. The adenosine A_1 receptor-dependent G-protein activity, which directly measures the G-protein activity, was measured after 1, 2, 3 and 6 hours of deprivation, and after 2 hours of recovery sleep. There was a transient increase of the G-protein activity in the cortex after 2 and 3 hours of sleep deprivation, while the activity remained unaltered in the basal forebrain. On the basis of these data, it is concluded that accumulation of adenosine during prolonged wakefulness induces a fast transient response in the cortex, and a slow, longer lasting and possibly recovery sleep inducing response in the basal forebrain.

The activities of the main physiological regulators of extracellular adenosine concentrations, the enzymes adenosine kinase, ecto- and endo-5'-nucleotidase and the equilibrative nucleoside transporter ENT1, were measured after 3 and 6 hours of sleep deprivation and after 2 hours of recovery sleep. The enzyme activities were unaffected by sleep deprivation in both the cortex and the basal forebrain alike, while there was a decrease in the activity of the equilibrative nucleoside transporter ENT1 in the basal forebrain after 3 hours of sleep deprivation. It is concluded that the decrease of the ENT1 activity during prolonged wakefulness is at least one of the mechanisms causing the increase of extracellular adenosine concentrations during prolonged wakefulness.
The distribution of both equilibrative nucleoside transporters, the ENT1 and the ENT2, in the central nervous system was assessed immunohistochemically by novel antibodies against the transporters, and their localization was demonstrated at the cellular level. Both transporters are expressed in practically all neurons, but the ENT1 is mostly associated with the cellular membrane and the ENT2 has a major intracellular localization. These data suggest that the ENT1 is more directly connected to the transport of adenosine across the cell membrane, while the ENT2 has a more prominent role in the intracellular nucleoside transport in the rat central nervous system.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I. Basheer R, Halldner L, Alanko L, McCarley R.W, Fredholm B, Porkka-Heiskanen T. Opposite changes in adenosine A_1 and A_{2A} receptor mRNA in the rat following sleep deprivation. NeuroReport 2001, 12:1577-80

II. Alanko L, Laitinen J, Stenberg D, Porkka-Heiskanen T. Adenosine A_1 receptor-dependent G-protein activity in the rat brain during prolonged wakefulness. NeuroReport 2004, 15:2133-7

III. Alanko L, Heiskanen S, Stenberg D, Porkka-Heiskanen T. Adenosine kinase and 5'-nucleotidase activity after prolonged wakefulness in the cortex and the basal forebrain of rat. Neurochem Int 2003, 42:449-54

IV. Alanko L, Stenberg D, Porkka-Heiskanen T. Nitrobenzylthioinosine (NBMPR) binding and nucleoside transporter ENT1 mRNA expression after prolonged wakefulness and recovery sleep in the cortex and basal forebrain of rat. J Sleep Res 2003, 12:299-304

V. Alanko L, Porkka-Heiskanen T, Soinila S. Localization of equilibrative nucleoside transporters in the rat brain. Submitted

Original publication I was also included in a thesis entitled "Physiology and Pathophysiology of Central Adenosine A_1 and A_{2A} Receptors" by Linda Halldner in Karolinska Institutet, Sweden.

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ABBREVIATIONS

5’-NT	5’-nucleotidase
ACh	acetylcholine
ADA	adenosine deaminase
ADP	adenosine diphosphate
AK	adenosine kinase
AMP	adenosine monophosphate
ANOVA	analysis of variance
AOPCP	α,β-methylene adenosine diphosphate
ATP	adenosine triphosphate
BF	basal forebrain
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleid acid
CNS	central nervous system
CNT	concentrative nucleoside transporter
CPA	N6-cyclopentyladenosine
CPT	cyclopentyl-1,3-dimethylxanthine
DAB	diaminobenzidine
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenosine
ENT	equilibrative nucleoside transporter
ei	equilibrative insensitive
es	equilibrative sensitive
GABA	γ-amino-butyric acid
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
$[^{35}S]GTP\gamma S$	$[^{35}S]$Guanosine 5'-(γ-thio)triphosphate
IMP	inosine monophosphate
LDT	laterodorsal tegmental nucleus
MPA	medial preoptic area
mRNA	messenger ribonucleic acid
NBMPR	nitrobenzyl-thio-inosine
NF-κB	nuclear factor κB
NO	nitric oxide
PPT	peduculopontine tegmental nucleus
REM	rapid eye movement
RT-PCR	real time polymerase chain reaction
SAH	S-adenosyl homocysteine
SAHH	S-adenosyl homocysteine hydrolase
SCH 58261	5-amino-phenylethylfurylpyrazolo-1,2,4-triazolo-pyrimidine
SD	sleep deprivation
SWS	slow wave sleep

1. REVIEW OF THE LITERATURE

1.1 Introduction

Adenosine is the nucleoside base of adenosine triphosphate (ATP), which is the main high energy compound used in cellular metabolism. In addition to being a metabolic end product and a building block for high-energy phosphates, it is a modulator of several physiological and pathophysiological processes both in the body and in the central nervous system (CNS). It was already noted in 1929 that adenosine has profound effects on the mammalian heart (Drury & Szent-Gyorgyi 1929). In the 1970s the effects of adenosine and adenine nucleotides on cyclic adenosine monophosphate (cAMP) content on cortical slices were reported (Sattin & Rall 1970), followed by the confirmation of the existence of adenosine receptors (van Calker et al 1979). By 1984 it was known that adenosine could affect at least vascular tone, hormone action, neural function, platelet aggregation and lymphocyte differentiation, and as by then substantial evidence had accumulated to show that adenosine levels were at least partly regulated by metabolism of ATP and other nucleotides, Newby introduced the term “retaliatory metabolite” to express the idea that the cell could retaliate against an external stimulus which could cause excessive energy consumption with the metabolic end product itself (Newby 1984). In the CNS adenosine was by then known to inhibit synaptic activity and decrease neuronal excitability (Phillis et al 1979, Dunwiddie & Hoffer 1980, Phillis & Wu 1981) and to modulate cerebral blood flow (Berne et al 1981). Since then adenosine has been acknowledged to take part in the regulation of sleep and arousal and to be a neuroprotective agent in situations like ischemic or seizure-induced injury or trauma, which has led to intense research on its possible clinical use (Rudolphi et al 1992, Fredholm 1997, von Lubitz 1999, Dunwiddie & Masino 2001).

1.2 Adenosine receptors

Adenosine mediates its effects via specific receptors, namely A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al 2001). The first two adenosine receptors were cloned from a canine thyroid library (Libert et al 1989) and they were later shown to be the A_1 and A_{2A} receptors (Maenhaut et al 1990, Libert et al 1991). The A_{2B} and A_3 receptors have been cloned as well (Meyerhof et al 1991, Stehle et al 1992). They are all seven transmembrane domain, G-protein coupled receptors (GPCRs) and

since the original findings they have been cloned from several species (Olah & Stiles 1995). The receptors differ in their affinity to adenosine, their distribution and function. The A_1 and A_{2A} are the high-affinity receptors, as they are activated already at nanomolar adenosine concentrations, while the A_{2B} and A_3 are activated only at micromolar concentrations (Fredholm et al 2000). Functionally the A_1 and A_3 are negatively coupled to adenylate cyclase by $G_{i/o}$ and $G_{q/11}$ proteins and their activation leads to a decrease in the intracellular cAMP levels while the A_{2A} and A_{2B} are positively coupled to adenylate cyclase by G_s, G_{olf}, $G_{q/11}$ and $G_{15/16}$ and consequently their activation has an opposite effect on the cAMP levels (Fredholm et al 2000).

The A_1 receptor is the most abundant and widely expressed of the adenosine receptors in the CNS, being most prominent in the hippocampus, cerebellum and cortex as shown by both mRNA expression studies and immunohistochemical staining (Reppert et al 1991, Rivkees et al 1995). Activation of the A_1 receptors leads to inhibition of adenylate cyclase (van Calker et al 1978), stimulation of potassium conductance (Trussell & Jackson 1985), inhibition of Ca^{2+}-conductance (Dolphin et al 1986) and activation of phospholipase C (Gerwins & Fredholm 1992). The net effect of A_1 activation is a reduction in transmitter release pre-synaptically and post-synaptical hyperpolarization (Haas & Selbach 2000).

The A_{2A} receptor has a more localized expression in the CNS, being concentrated in the striatum, the nucleus accumbens, the olfactory tubercle and the lateral segment of globus pallidus (Jarvis & Williams 1989, Parkinson & Fredholm 1990) with a low expression throughout the rest of the CNS (Dixon et al 1996). The A_{2A} receptor activates the adenylate cyclase pathway and seems to increase neurotransmission, but its role is not only stimulatory (Cunha 2001). The A_{2B} and A_3 have a low expression in the CNS (Dixon et al 1996). They are activated only at pathological concentrations of adenosine and their physiological role is not clear (Haas & Selbach 2000).

1.3 Regulation of extracellular adenosine levels

1.3.1 General

The extracellular levels of adenosine are in the nanomolar range under normal physiological conditions. Originally they were reported in the range of 30-50 nM using cerebral cortical cup technique (Phillis et al 1987). Later, the extracellular concentrations have been shown, using *in vivo* microdialysis, to be 120 nM in the cortex (Pazzagli et al 1994), 40-210 nM in the striatum (Ballarin et al 1991, Pazzagli et al 1993, Pazzagli et al 1995) and 180-270 nM in the basal

forebrain and the thalamus (Porkka-Heiskanen et al 2000). As the K_m values of the A_1 and the A_{2A} receptors are in the nanomolar range (70 nM and 150 nM, respectively) (Dunwiddie & Masino 2001), it has been suggested that they are tonically active in physiological concentrations. This has been verified by recordings after antagonist infusions of both A_1 and A_{2A} receptors (Rainnie et al 1994, Svenningsson et al 1997). Thus a strict control of extracellular adenosine concentration is necessary to maintain the inhibitory tone at an appropriate level.
As it is, the extracellular adenosine levels are regulated by extra- and intracellular metabolism and transport through the cell membrane (Fig. 1). There are two primary sources of extracellular adenosine. Firstly, it can be transported through the cell membrane according to the concentration gradient from the neighboring cells. This efflux of intracellular adenosine (which is often slightly misleadingly called release) occurs when energy expenditure exceeds energy production (i.e. more ATP is being degraded than produced), most prominently in situations like increased neuronal activity, seizures, ischemia or energy depletion (Lloyd et al 1993, Mitchell et al 1993, Brundege & Dunwiddie 1998). Secondly, adenosine can be produced extracellularly from adenosine nucleotides via the nucleotidase pathway (Zimmermann & Braun 1999). The primary source of adenosine for this pathway is ATP, which is known to be co-released with several neurotransmitters i.e. acetylcholine and noradrenaline (White 1977, Fredholm et al 1982, Poelchen et al 2001), and it has been shown that astrocytes can release adenosine nucleotides as well (Queiroz et al 1997).

1.3.2 Extracellular production of adenosine

Extracellularly, adenosine is formed by a chain of nucleotidases which degrade nucleotides into adenosine (Zimmermann & Braun 1999). ATP is hydrolyzed by ecto-ATPase and/or ecto-ATP diphosphorylase (Boeck et al 2002). Adenosine diphosphate (ADP) is dephosphorylated to adenosine monophosphate (AMP) by diphosphorylase as well. AMP is then dephosphorylated into adenosine by ecto-5'-nucleotidase (ecto-5'-NT, EC 3.1.3.5) (Zimmermann 1992). It is a membrane-bound ecto-enzyme with its active site in the extracellular space with a K_m for AMP in the micromolar range, and it is competitively inhibited by ATP, ADP and α,β-methylene adenosine diphosphate (AOPCP) (Zimmermann 1996). In the rat CNS it has been shown to be localized on glial cells with the exception of the mossy fiber terminals in the CA3 region of the hippocampus (Schoen et al 1987, Zimmermann et al 1993, Schoen et al 1999). Yet ecto-5'-NT activity has been noted on neural and glial cells using both biochemical and

immunohistochemical methods (Franco et al 1986, Cunha et al 1992, James & Richardson 1993). This discrepancy has been attributed to possible immunologically distinct isoforms of the enzyme in the CNS (Zimmermann et al 1993, Cunha et al 2000). The degradation of nucleotides into adenosine is fast, extracellular ATP is abolished and metabolized into adenosine in less than 1 second, the dephosprohylation of AMP into adenosine being the rate-limiting step (Dunwiddie et al 1997).

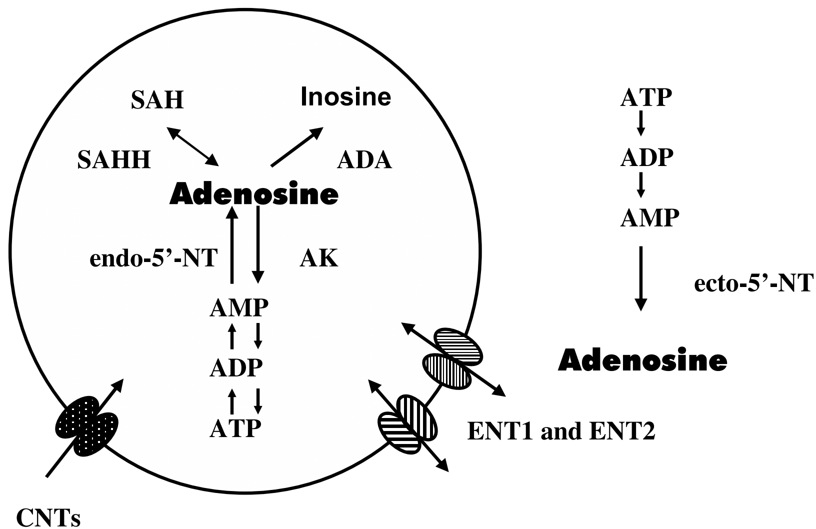

Figure 1. A simplified schema of the main intra- and extracellular pathways of adenosine metabolism and transport. Extracellularly adenosine is formed by a chain of ectonucleotidases, of which the ecto-5'-nucleotidase (ecto-5'-NT) is the rate limiting enzyme. Intracellular formation of adenosine is due to a similar chain of nucleotidases with the endo-5'-nucleotidase (endo-5'-NT) being the rate limiting enzyme. Adenosine is degraded either into adenosine monophosphate (AMP) by adenosine kinase (AK) or into inosine by adenosine deaminase (ADA). A third adenosine metabolizing enzyme is S-adenosylhomocysteine (SAH)-hydrolase (SAHH) which converts SAH into adenosine, or vice versa, but it is thought to have a lesser role. Adenosine in transported through the cell membrane either by the sodium-driven concentrative nucleoside transporters (CNTs) or according to the concentration gradient by equilibrative nucleoside transporters 1 and 2 (ENT1 and ENT2, respectively). ATP= adenosine triphosphate, ADP= adenosine diphosphate.

An alternate source for extracellular adenosine formation is cAMP, as it has been shown that norepinephrine and vasoactive intestinal peptide induce extracellular accumulation of cAMP and adenosine in cortical cultures (Rosenberg & Dichter 1989, Rosenberg & Li 1995a, Rosenberg & Li 1995b). In support of these findings, it has been shown that prolonged superfusion of hippocampal slices with cAMP increases extracellular adenosine formation, but as short periods of increased cAMP levels do not affect adenosine formation, it has been speculated that cAMP would cause only slow responses in extracellular adenosine concentrations (Brundege et al 1997).

1.3.3 Intracellular production of adenosine

Intracellularly, adenosine is formed by at least three different endo-5'-nucleotidases. Two of them have high K_m values, one of which prefers AMP (K_m for AMP 1-14 mM) and the other inosine monophosphate (IMP) as substrate (Zimmermann 1992). The third endo-5'-nucleotidase resembles the ecto-enzyme, it has a low K_m of 14 µM for adenosine and it is likely to have a major role in adenosine production under physiological concentrations (Orford & Saggerson 1996).

Another possible source of intracellular adenosine could the hydrolysis of S-adenosyl homocysteine (SAH) by the SAH-hydrolase (SAHH, EC 3.3.11). In the rat brain there is evidence showing it to be mainly nuclear and present in several brain regions (Patel & Tudball 1986). Furthermore, it has been shown in chick embryos that SAHH has higher activity in the neurons than glia (Ceballos et al 1994). The hydrolysis reaction catalyzed by the SAHH is fully reversible, but as the L-homocysteine levels are very low in the brain, it is thought that the main direction is the hydrolysis of SAH into adenosine and L-homocysteine and not vice versa (Reddington & Pusch 1983).

The SAHH is responsible for approximately one third of the adenosine production in the guinea pig heart, but it is not closely linked to the energy state of the cells (Lloyd et al 1988, Deussen et al 1989). In the rat hippocampal slices, it has been shown that the inhibition of the SAHH does not affect the extracellular adenosine levels under basal conditions (Pak et al 1994), under ischemia-like conditions or during electrical stimulation (Lloyd et al 1993, Latini et al 1995). Thus the SAHH is thought to have a minor role, if any, in the metabolism of adenosine in the rat brain (Brundege & Dunwiddie 1997).

1.3.4 Intracellular metabolism of adenosine

There are two intracellular metabolic pathways which lead to a decrease in adenosine levels: phosphorylation of adenosine into AMP by adenosine kinase (AK, EC 2.7.1.20) or deamination of adenosine into inosine by adenosine deaminase (ADA, EC 3.5.4.4).

AK is a soluble enzyme located in the soma of the cells. It is a low K_m enzyme (2μM) located quite evenly in the rat CNS, whose activity is inhibited with concentrations higher than 0.5 μM (Phillips & Newsholme 1979, Yamada et al 1980). Inhibition of AK has been shown to increase adenosine levels under basal and stimulated conditions (Pak et al 1994, Lloyd & Fredholm 1995, White 1996) and quite recently it was noted that there is an increase in AK expression and a decrease in adenosine concentrations after a kainate induced lesion in the mouse hippocampus (Gouder et al 2004).

ADA, unlike AK, shows a more selective distribution in the rat CNS, being most prominent in the basal hypothalamus as has been shown by both immunoreactivity and enzyme activity measurements (Phillips & Newsholme 1979, Nagy et al 1984). In addition high enzyme activity has been noted in the tuberomamillary nucleus (Mackiewicz et al 2000) and a high mRNA expression in the leptomeninges (Okada et al 2003). The K_m for ADA is approximately 17-45 μM in the rat brain (Phillips & Newsholme 1979), and although it is considered to be an intracellular enzyme, there is evidence of extracellular ADA as well (Franco et al 1986). Selective ADA inhibitors, deoxyformycin and erythro-9-(2-hydroxy-3-nonyl)adenosine (EHNA), have been shown to increase extracellular adenosine levels both under basal conditions and under hypoxia and ischemia *in vivo* (Phillis & O'Regan 1989, Ballarin et al 1991, Phillis et al 1991, Pazzagli et al 1993, Sciotti & Van Wylen 1993, Pazzagli et al 1995). In *in vitro* studies EHNA has been shown to have no effect under basal conditions or after electrical field stimulation (Lloyd & Fredholm 1995, White 1996), but in the same study EHNA did increase adenosine levels after energy depletion (Lloyd & Fredholm 1995). In hippocampal slices the endogenous ADA did not affect extracellular adenosine levels under basal or hypoxic conditions (Pak et al 1994, Zhu & Krnjevic 1994).

As the physiological adenosine concentrations are in the nanomolar range, it is likely that phosphorylation of adenosine by AK is the prominent pathway for metabolizing adenosine under normal physiological conditions and that the deamination by ADA becomes more prominent as the adenosine concentrations increase into the micromolar range, like during seizures and ischemia.

1.3.5 Adenosine transport

In addition to adenosine production and degradation, the extracellular adenosine levels are regulated by adenosine transport through the cell membrane. There are two families of transporters: the concentrative nucleoside transporters (CNT) and the equilibrative nucleoside transporters (ENT) (Thorn & Jarvis 1996). The CNT-type transporters are high-affinity transporters and are, as their name indicates, concentrative, and thus facilitate the influx of adenosine and other nucleosides, driven by the transmembrane sodium gradient. Five different CNTs have been described in man (Cabrita et al 2002), of which CNT1, CNT2 and CNT3 belong to the SLC28 gene family (Gray et al 2004). Two of them, CNT1 and CNT2, have been shown to be expressed in the rat brain (Anderson et al 1996). The role of CNTs in the regulation of the extracellular adenosine concentrations is not known; while it is thought to be less important than that of ENTs, it should be noted that the lack of specific pharmacological tools has hindered research on them (Cabrita et al 2002).

The ENT-type transporters carry nucleosides across the cell membrane in either direction according to the concentration gradient (Thorn & Jarvis 1996). The localization of transporter sites in the rat brain was originally reported using [^{3}H]NBMPR-binding studies (Geiger & Nagy 1984, Bisserbe et al 1985). Shortly thereafter functional studies indicated the presence of two different transporters (Lee & Jarvis 1988), which was later confirmed with *in situ* -hybridization studies (Anderson et al 1999a, Anderson et al 1999b).

The ENTs were originally divided on the basis of their sensitivity to the inhibitor nitrobenzylthioinosine (NBMPR). The equilibrative NBMPR-sensitive (es) transporters are inhibited by nanomolar concentrations and the equilibrative NBMPR-insensitive (ei) transporters are inhibited only by micromolar concentrations of NBMPR. Later, when the transporters were cloned in rat (Yao et al 1997) and humans (Griffiths et al 1997), they were renamed ENT1 and ENT2 (es and ei, respectively). While the ENT2 transporter lacks a specific inhibitor, NBMPR can be used as a relatively specific inhibitor for the ENT1 transporter. NBMPR binds dose-dependently to the ENT1 transporter with high affinity, blocking the adenosine transport. This binding is inhibited by other adenosine transporter inhibitors in a saturable manner, e.g. dipyridamole (Jarvis & Young 1980, Thorn & Jarvis 1996). Due to these properties, the tritium-labelled NBMPR has been used to measure the ENT1 activity since the early studies on nucleoside transporters (Thorn & Jarvis 1996).

Of the two transporters the ENT1 has been thought to be the more important due to its greater activity (Cabrita et al 2002), but it has been suggested that the ENT2 would be the more important transporter in the rat brain due to a higher mRNA expression (Anderson et al 1999a).

1.4 Adenosine and sleep

1.4.1. General

The first report on the hypnogenic properties of adenosine goes back to the middle of the last century, when it was shown that injections of adenosine into the lateral ventricle induced sleep-like behaviour in the cat (Feldberg & Sherwood 1954) and two decades later the same phenomenon was noted in dogs (Haulica et al 1973). Since then adenosine has been in the focus of sleep research and its hypnotic properties have been noted repeatedly as both adenosine and its agonist induce sleep in rats (Dunwiddie & Worth 1982, Virus et al 1983, Radulovacki et al 1984, Radulovacki 1985, Ticho & Radulovacki 1991). Restoration of energy has been suggested to be one function of sleep (Chagoya de Sanchez et al 1993, Benington & Heller 1995), and adenosine has fit well into the theory as it is a metabolic end product; the more energy spent, the more sleep is needed to restore the energy stores. Furthermore, the most widely used pscyhoactive stimulant in the world, caffeine, is an adenosine receptor antagonist (Fredholm et al 1999).

1.4.2 Extracellular adenosine levels during the sleep-wake cycle

Extracellular adenosine concentrations are known to increase during both increased neuronal activity and increased metabolism (Pull & McIlwain 1972, Van Wylen et al 1986, Mitchell et al 1993). On the other hand, wakefulness is known to have greater metabolic activity which has been shown by measuring glucose utilization, cerebral blood flow and oxygen consumption in humans (Madsen et al 1991, Maquet et al 1992, Madsen 1993, Maquet et al 1997) and extracellular glucose and lactate concentrations in rats (Netchiporouk et al 2001, Shram et al 2002).

Thus it could be expected that adenosine concentrations vary during the sleep-wake cycle. It was first shown that the extracellular adenosine levels were lower during the rest phase and higher during the active phase in the hippocampus and neostriatum of rats (Huston et al 1996). The first direct evidence for state-dependency was published in 1997 when it was shown using *in-vivo* microdialysis that the extracellular adenosine levels were higher during slow-wave sleep than

during waking in the cholinergic magnocellular basal forebrain (BF) and the thalamus of cats (Porkka-Heiskanen et al 1997). Later this phenomenon was shown to be present also in the cortex, the dorsal raphe nucleus, the pedunculopontine tegmental area (PPT) and the preoptic hypothalamic area (Porkka-Heiskanen et al 2000). Recently the state-dependency and the circadian variation have been further confirmed in the BF of both young and old rats (Murillo-Rodriguez et al 2004).

1.4.3 The cholinergic basal forebrain and local accumulation of adenosine during prolonged wakefulness

The BF is part of the extrathalamic ventral pathway from the brainstem reticular activating system to the cerebral cortex (Steriade 1996, Jones 2004). It is a heterogenous region in the ventral region of forebrain adjacent to preoptic, supraoptic and rostral infundibular levels of the hypothalamus. During the last decades the BF has been connected with several behavioural functions like attention (Muir et al 1994), cortical arousal (Buzsaki & Gage 1989, Berntson et al 2002), control of sensory processing (Metherate & Ashe 1993, Sarter & Bruno 1997), learning and memory (Bartus et al 1982, Fibiger 1991, McLin, III et al 2002), plasticity (Kilgard & Merzenich 1998) and sleep (Szymusiak 1995).

The BF contains magnocellular cholinergic neurons which innervate widely the limbic system and the cortex providing most of the cholinergic cortical innervation (Jones 2004). The cholinergic cells are located interdispersed in several nuclei, from rostral to caudal: the medial septum, the vertical and horizontal diagonal bands of Broca, the magnocellular preoptic are, the substantia innominata and the nucleus basalis of Meynert (Cullinan & Zaborszky 1991). Even though the BF was originally named according to the cortically projecting magnocellular cholinergic neurons, these cholinergic neurons are a minority and actually outnumbered by 2:1 the rat BF by GABAergic neurons, which have cortical projections as well. In addition, there is a substantial proportion of unidentified cortically projecting neurons (Rye et al 1984, Gritti et al 1993, Gritti et al 1997).

The cholinergic cortically projecting neurons of the BF release acetylcholine (ACh) which excites the cortical neurons and thus stimulates cortical activation (Krnjevic 1967, McCormick & Prince 1986, McCormick 1992). This ACh release has been shown to be highest during waking and REM sleep (Celesia & Jasper 1966, Jasper & Tessier 1971, Marrosu et al 1995). Furthermore, lesions of the BF have been shown to disrupt sleep patterns of of both cats and rats

(Stewart et al 1984)(Szymusiak & McGinty 1986b, Buzsaki et al 1988), and the discharge rates of the supposedly cholinergic neurons have been shown to be highest during waking and REM sleep while being lowest during slow-wave sleep (SWS) in both cats and rats (Detari et al 1984, Szymusiak & McGinty 1986a, Szymusiak & McGinty 1989, Alam et al 1999).

The idea for the connection between adenosine and the cholinergic systems rose when it was shown *in vitro* that A_1 agonists decreased and antagonist increased the neuronal discharge rate in the cholinergic mesopontine laterodorsal and peduculopontine tegmental nuclei (LDT/PPT) by causing hyperpolarization of identified cholinergic cells (Rainnie et al 1994). Later it was shown *in vivo* that infusion of adenosine via microdialysis cannula into the BF and the LDT/PPT of cats (Portas et al 1997) and the BF of rats (Basheer et al 1999) induces sleep.

In addition to being a sleep inducing agent, adenosine was noted to accumulate in the BF of cats and rats during prolonged wakefulness (Porkka-Heiskanen et al 1997, Basheer et al 1999) This effect is site specific as it is present only in the BF and transiently in the cortex (Porkka-Heiskanen et al 2000). Furthermore, it has been shown with combined *in vivo* microdialysis and unit recording that infusion of adenosine into the BF of rats and cats decreases the unit activity of the cortically projecting wake-related neurons (Alam et al 1999, Thakkar et al 2003a).

1.4.4 The roles of the A_1 and A_{2A} receptors during prolonged wakefulness

The A_1 receptors have been associated with the regulation of the sleep-wake cycle since it was noted that A_1 agonist N6-cyclopentyladenosine (CPA) induced sleep when given i.p. or i.c.v. (Benington et al 1995, Schwierin et al 1996). Later it has been shown that unilateral infusion of A_1 receptor antagonist cyclopentyl-1,3-dimethylxanthine (CPT) into the BF of cats and rats increases waking and decreases sleep (Strecker et al 2000). Combined microdialysis and unit recording studies have shown that infusion of A_1 agonist into the BF decrease, and infusion of A_1 antagonist increase single unit activity of wake-active neurons in both cats and rats (Alam et al 1999, Thakkar et al 2003a). Recently it has been shown that bilateral infusion of antisense to the A_1 receptor mRNA decreases non-REM sleep and induces wakefulness (Thakkar et al 2003b). In this method, the antisense binds to the A_1 receptor mRNA preventing the translation of mRNA which is thought to lead to a decreased amount of receptor protein. In addition to decreasing sleep and inducing wakefulness the rats had less non-REM sleep after 6 hours of sleep deprivation (SD) when compared to untreated controls. When considering the evidence presented above, the A_1 receptors have an important role in the homeostatic regulation of sleep in the BF.

There is another line of evidence promoting the importance of A_1 receptors. It has been shown that the activation of the A1 receptors in the BF mobilizes intracellular calcium *in vitro* mainly in the cholinergic cells (Basheer et al 2002) and that a SD induced increase in extracellular adenosine levels increases c-FOS protein levels and NF-κB binding, both known transcription factors, in the BF (Basheer et al 1999). This suggests that adenosine (and SD) has longer lasting, possibly gene transcription affecting and recovery sleep inducing effects in addition to the immediate hyperpolarizing inhibitory effects in the BF (Basheer et al 2001).

The A_{2A} receptors are involved in the regulation of sleep, as it has been shown that infusion of A_{2A} agonists into the subarachnoid space under the rostral basal forebrain induces sleep (Satoh et al 1996, Satoh et al 1998, Satoh et al 1999). Furthermore, it has been shown that activation of prostaglandin D_2 receptors in the rostal basal forebrain leads to release of adenosine, the activation of adenosine A_{2A} receptors and an increase in non-REM sleep (Matsumura et al 1994, Urade & Hayaishi 1999, Mizoguchi et al 2001, Hayaishi 2002). The sleep inducing effects of the prostaglandin D_2 and A_{2A} agonist have been localized in the ventrolateral preoptic area (VLPO) (Scammell et al 1998, Scammell et al 2001), an area which is known to contain GABAergic, sleep-active cells projecting into wake-promoting regions such as the tuberomamillary nucleus (Sherin et al 1996, Sherin et al 1998, Szymusiak et al 1998, Gallopin et al 2000). Quite recently it has been shown that the sleep-active neurons in VLPO are disinhibited by adenosine (Morairty et al 2004). Most of the studies concerning the sleep inducing effects of A_{2A} in the VLPO have been conducted using pharmacological approaches or micromolar adenosine concentrations. Therefore future research is needed to clarify the role of adenosine in controlling the activity of VLPO under normal sleep-wake cycle and prolonged wakefulness.

2. AIMS OF THE STUDY

General aims of the study:

When starting this study, it was well documented that adenosine accumulates during prolonged wakefulness especially in the BF and that it has sleep inducing properties which are at least partially mediated by the A_1 receptors.

The following main questions were set:

1. By which mechanism(s) does the adenosine accumulate in the BF and the cortex during prolonged wakefulness?
2. What is the mechanism(s) by which the recovery sleep inducing effects are mediated in the BF?

Specific aims of the study:

I. To find out whether the prolonged stimulation due to the accumulation of adenosine during SD has effects in the affinity or transcription of the A_1 and the A_{2A} receptors.

II. To clarify the possible changes in the A_1 receptor mediated G-protein activation during prolonged wakefulness.

III. To find out whether the activities of the key adenosine metabolizing enzymes are affected during prolonged wakefulness.

IV. To study possible changes in the activity of the equilibrative nucleoside transporter ENT1, the other possible mechanism affecting the extracellular levels of adenosine.

V. To show the localization of the equilibrative nucleoside transporters ENT1 and ENT2 at the cellular level as differences in the localization can have significance in the regulation of extracellular adenosine concentrations.

3. MATERIALS AND METHODS

As the experimental procedures are described in detail in the original publications included in this thesis, only general information on the experimental techniques is reported here. Unless otherwise stated, the experiments were performed at the Institute of Biomedicine, University of Helsinki.

Experimental animals (I-V)

Adult male Hannover Wistar rats (weight 260-390g), were used in the experiments. In addition, male Long Evans rats (weight 350-450g) were used in RT-PCR experiments which were conducted at Harvard University (I). The Wistar rats were purchased from the Experimental Animal Center of the University of Helsinki and kept in 12/12h light/dark rhythm with free access to standard rat chow and water. All experimental protocols were approved by the respective ethical committees and were in accordance with the laws of Finland and the European Convention.

Sleep deprivation (I-IV)

Rats were sleep-deprived (SD) for 1 to 6 hours by the previously described gentle handling method (Franken et al 1993) starting at lights on (n=6 in all groups) as follows: in all studies involving SD (I-IV) rats were deprived for at least for 3 and 6 hours. In addition, in the studies assessing the adenosine A_1 receptor mediated G-protein activity (II), the activities of the adenosine metabolizing enzymes (III) and the ENT1 transporter activity (IV), an additional group was allowed to sleep for 2 hours after 6 hours of SD. Finally, in the G-protein activity study (II), additional 1 and 2 hour deprivations were carried out.

A time matched control group, which was kept undisturbed in their home cages, was used for each experimental group. At the end of the SD, the rats were decapitated at the same time with their corresponding controls and the brains were rapidly removed. The use of time-matched controls makes it possible to dissociate the circadian and homeostatic components (thus making it possible to assess the effects of sleep deprivation per se). The 1 hour SD group was the only exception, and the values obtained from this group were compared to the 2 hour SD controls, as a time difference of 1 hour is unlikely to cause major differences in G-protein activity under normal resting conditions.

In situ hybridization (I,IV)

The mRNA expression of the rat ENT1 (IV) and of the A_1 and the A_{2A} receptors (I) was assessed as previously described (Johansson et al 1993, Toppila et al 1996).

The brain was cut into 20 µm coronal cryosections. Sections were post fixed in paraformaldehyde and incubated with a ^{35}S labeled oligonucleotide probe. The different probes were complementary to mRNA sequences of the desired proteins; the rat ENT1 probe was complementary to nucleotides 763-810 of the rat ENT1 (Yao et al 1997), the A_1 probe to nucleotides 985-1032 of the rat A_1 receptor (Mahan et al 1991) and the A_{2A} probe to nucleotides 916-959 of the dog A_{2A} receptor (Schiffmann et al 1990). The labeled mRNA was then visualized with emulsion autoradiography and the rat ENT1 mRNA expression was measured by computer densitometry as previously described (Toppila et al 1997). The A_1 and the A_{2A} receptor mRNA expression was assessed in Karolinska Institutet, Stockholm.

RT-PCR (I)

The cDNA was created from extracted total RNA by using reverse transcriptase and specific primers for A_1 receptors and cyclophylin. The cDNAs were then amplified by polymerase chain reaction. The amplified sequences were separated by electrophoresis on polyacrylamide gel and visualized with phosphoimager, and the A1/cyclophylin ratio was calculated. These experiments were performed at the Department of Psychiatry, Harvard Medical School.

A_1 and A_{2A} receptor autoradiography (I)

Receptor autoradiography was assessed as previously described (Parkinson & Fredholm 1992, Johansson et al 1993). Coronal sections were cut and incubated in the presence of 0.25-10 nM tritium labeled 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) or 0.2-10 nM tritium labeled 5-amino-phenylethylfurylpyrazolo-1,2,4-triazolo-pyrimidine (SCH 58261) for detection of A_1 and A_{2A} receptors, respectively. The receptors were visualized autoradiographically. The experiments were performed in Karolinska Institutet.

[^{35}S]Guanosine 5'-(γ-thio)triphosphate autoradiography (II)

The binding assay was performed as previously described (Laitinen 1999). Coronal sections were incubated in the presence of [^{35}S]GTPγS, excess GDP, ADA to abolish the endogenous adenosine

and A_1 receptor agonist 2-chloroadenosine at two concentrations producing near maximal and maximal responses (10^{-5} and 10^{-4} M, respectively). The bound [^{35}S]GTPγS was visualized autoradiographically. The autoradiography assay was done at the Department of Physiology, University of Kuopio.

Measurement of adenosine kinase activity (EC. 2.7.1.20) (III)

The AK activity was analysed radiochemically by monitoring the conversion of [2-^{3}H]-adenosine into [^{3}H]-AMP as previously described (Ward et al 1998). The membranous and soluble fractions were separated by centrifugation and the supernatant was incubated in the presence of EHNA, excess ATP and [^{3}H]-adenosine. The reaction product, [^{3}H]-AMP, was bound to ammonium formate equilibrated DEAE-Sephadex and then eluted with formate buffer. The eluate was taken for scintillation counting.

Measurement of the 5'-nucleotidase activities (EC 3.1.3.5) (III)

Both endo- and ecto-5'-nucleotidases were assayed by monitoring the conversion of [2-^{3}H]-AMP into [^{3}H]-adenosine with a previously described method (Orford et al 1991). The membranous and soluble fractions were separated by centrifugation and the membrane fraction was used for the ecto-5'-NT activity measurements while the endo-5'-nucleotidase activity was measured from the soluble fraction. The samples were incubated in the presence of [2-^{3}H]-AMP and an excess of AMP. The reaction was stopped by desiccation and the activity was measured from the remaining supernatant using scintillation counting.

NBMPR binding (IV)

The NBMPR binding was determined as previously described (Williams et al. 1984) with slight modifications. The membrane fraction was separated from the tissue samples and the NBMPR binding was measured by incubating the membranes in the presence of tritium labeled NBMPR. Radioactivity was measured from the filters using scintillation counting.

Raising the antiserum for rat ENT1 and ENT2 (V)

A 13- and 14-amino acid sequences were synthesized based on published sequences of the rat ENT1 and the rat ENT2, respectively (Yao et al 1997). The synthesized peptides contain amino acid residues 59-77 for the rat ENT1 and 50-63 for the rat ENT2. An additional cystein

residue was attached to the N-terminus and the peptide was coupled to a carrier molecule, either limpet keyhole haemocyanin or thyroglobulin. New Zealand rabbits were immunized with the rat ENT1- or the rat ENT2-haemocyanin conjugate emulsified with Freund´s complete adjuvant. Pre-immune serum samples from all the rabbits were collected before the immunization to be used as control sera. The animals were test bleeded to verify immunization by dot blotting. 10 days after a further booster, the animals were sacrificed and their blood was collected, serum separated and stored at -80 ºC.

Dot-blotting (V)
A dilution series of the ENT1 and the ENT2 -thyroglobulin conjugates (100 µg/ml - 10 ng/ml) were pipetted onto nitrocellulose slips. The slips were incubated with the rat ENT1 or rat ENT2 antisera. The immunocomplexes were visualized with horse radish peroxidase-conjugated secondary antibody and diaminobenzidine (DAB) as a chromogen.

Western blotting (V)
Aliquots of homogenized rat tissue samples containing cortex, hippocampus, hypothalamus, brain stem or cerebellum were weight fractioned on polyacrylamide gel. The separated proteins were transferred onto nitrocellulose filters, which were incubated with the ENT1 or the ENT2 antibody and horse radish peroxidase-conjugated secondary antibody. DAB was used as a chromogen.

Immunohistochemistry (V)
Male Wistar rats were perfused transcardially with 4 % paraformaldehyde under sodium pentobarbital anaesthesia. Sections were incubated with the primary antibody at 4ºC overnight in a humidity chamber. The ENT1 and the ENT2 antisera and the corresponding pre-immune serum were used in dilutions 1:500-1:1 000. After incubation with the primary antibody, the sections were incubated with horse radish peroxidase-conjugated secondary antibody, and DAB was used as a chromogen.

Double staining studies (V)
Sections were equal to the ones used for single antibody stainings. The sections were incubated with the rabbit-raised ENT1 or ENT2 antiserum (1:500), and one of the following

monoclonal antibodies; antibody against neuron-specific antigen NeuN (1:1000), antibody against astrocyte-specific antigen S-100β (1:2000), antibody against oligodendrocyte-specific antigen (1:10000) or antibody against oligodendrocyte-specific antigen OX-42 (1:100). Fluorescein- and rhodamine-conjugated secondary antibodies were incubated consecutively, for 1 h each. The stainings were visualized with fluorescence-microscopy.

Specificity of antibodies (V)

Based on genome bank search, the amino acid sequences used for immunization for ENT1 and ENT2 are not present in any other known peptide. Omitting the primary (ENT1 or ENT2) antiserum removed all staining. In immunofluorescence specimens a faint background autofluorescence was seen. Staining with the pre-immune sera obtained from the same rabbit in which the respective ENT antibody was raised resulted in no staining above the background level. Specificity of the other antibodies used in the study has been described before (for anti-NeuN (Wolf et al 1996); for anti-S-100β (Boyes et al 1986); for anti-oligodendrocyte (Friedman et al 1989); for anti-OX-42 (Ford et al 1995)).

Statistics (I-IV)

The effect of SD on the adenosine A_1 and A_{2A} receptor ligand binding and mRNA expression (I) was evaluated using Student's *t*-test for the RT-PCR experiments, unpaired *t*-test for the *in situ* hybridization experiments and one-way analysis of variance (ANOVA) for the ligand binding experiments.

The effect of SD on adenosine A_1 receptor dependent G-protein activation (II), enzyme activities (III) and ENT1 transporter activity (IV) was evaluated using Student's *t*-test by comparing the values from the sleep deprived animals to the values from the undisturbed control animals decapitated at the same circadian time. The effect of diurnal variation was evaluated from the undisturbed control animals using one-way ANOVA with Student-Newman-Keuls as *post hoc* test (II-IV). The difference between the groups was regarded as significant at $p<0.05$.

4. RESULTS AND DISCUSSION

4.1 The effects of sleep deprivation on adenosine A_1 and A_{2A} receptors

4.1.1 Ligand binding and mRNA expression of the A1 and the A2A receptors during prolonged wakefulness (I)

At the time this study was undertaken, it was known that adenosine accumulates in the BF of both rats and cats during prolonged wakefulness and that infusions of adenosine and adenosine A_1 receptor agonists into the BF induce sleep (Porkka-Heiskanen et al 1997, Portas et al 1997, Basheer et al 1999, Porkka-Heiskanen et al 2000). On the other hand, there was evidence showing that the A_{2A} receptors are involved in the regulation of sleep, as A_{2A} agonists induce sleep in the rostral forebrain (Satoh et al 1996). The possible mechanisms mediating the sleep inducing effects of adenosine in the BF were unknown, as were the possible effects of the sustained agonist stimulation present during prolonged wakefulness. As a first step to study the effects of adenosine on its receptors during prolonged wakefulness, the ligand binding and mRNA expression of the A_1 and A_{2A} receptors were measured after 3 and 6 hours of SD.
The ligand binding of the A_1 receptors was measured from both the BF and the cortex and it remained unaltered in both areas during the SD. In the cortex, the A_1 mRNA was not affected, while in the BF the A_1 mRNA expression was increased. This increase suggests a positive feedback mechanism of A_1 receptors during prolonged wakefulness. The most common response of the GPCRs to prolonged agonist exposure is receptor downregulation (Grady et al 1997) which has been noted in A_1 receptors before (Fredholm et al 2001). On the other hand, there is evidence showing that A_1 receptors are being upregulated during threatening conditions like ischemia in smooth muscle cells (Hammond et al 2004) and hypoglychemia has been shown to induce A1 receptor activation and to participate in hypoglychemia induced neuronal death in cortical neurons (Turner et al 2004). As energy depletion mimicks the effects of sleep deprivation (Kalinchuk et al 2003), it would be tempting to hypothesize that during prolonged wakefulness adenosine induces in the BF changes similar to those induced by hypoglychemia in the cortex. There seems to an upregulation of A_1 receptors, i.e. positive feedback, in situations where the reduction of signalling could be potentially harmful. In such situations, the receptor expression must be upregulated to maintain, and possibly increase, the inhibitory tone until the potentially harmful situation is avoided, in the case of prolonged wakefulness, until sleep is initiated.

The importance of the A_1 receptors was confirmed in our studies in an undirect manner, as the A_{2A} receptor mRNA was not expressed in the BF with either *in situ* hybridization or RT-PCR suggesting the lack of A_{2A} receptors in this region.

The ligand binding and the mRNA expression of the A_{2A} receptors were measured from the striatum, the nucleus accumbens and the olfactory tubercle. It was found that both the ligand binding and the mRNA expression were decreased during sleep deprivation in the olfactory tubercle, but not in the nucleus accumbens or the striatum. The ligand binding was decreased only after 3 but not after 6 hours of SD, while the mRNA expression was significantly decreased after both 3 and 6 hours of SD. The extracellular concentrations of adenosine have not been measured from the olfactory tubercle during normal sleep-wake cycle or prolonged wakefulness. If there is an increase in the extracellular adenosine concentrations, the decreases in ligand binding and mRNA expression are not suprising, as the A_{2A} receptors are known to be rapidly desensitized and downregulated after prolonged exposure to stimuli (Moreau & Huber 1999).

4.1.2. The A_1 receptor mediated signaling is transiently increased in the cortex (II)

It was shown in the previous experiments (I) that the adenosine A_1 receptor mRNA expression increased in the basal forebrain and remained unaltered in the cortex, while the ligand binding remained unaffected. In order to further clarify the activation of the A_1 receptors, it was decided to measure the A_1 receptor dependent G-protein activity, i.e. the A_1 receptor mediated signalling using the $[^{35}S]GTP\gamma S$ autoradiography, a method developed by our collaborator Jarmo Laitinen (Laitinen 1999). With this method, it is possible to assess the G-protein activity directly and *in situ*, which omits the possible pitfalls of ligand binding and mRNA expression assays.

The rats were sleep deprived for 1, 2, 3 or 6 hours and one group was allowed to sleep 2 hours of recovery sleep after 6 hours of SD. The A_1 receptor-stimulated $[^{35}S]GTP\gamma S$ binding was transiently increased in both the frontal and the cingulate cortex after 2 and 3 hours of SD, while a smaller increase was noted in the medial preoptic area (MPA) after 3 hours of SD. In the BF, the $[^{35}S]GTP\gamma S$ binding remained unaltered throughout the deprivation and recovery sleep. The increase in the G-protein activity in both cortical areas was most prominent after 2 hour SD and started to diminish already after the third hour of SD.

The major finding was the rapid and transient increase in A_1 receptor-dependent G protein activity during prolonged wakefulness in the cortex, while A_1 receptor signalling remained

unaltered in the BF. To our best knowledge, this was the first study to assess adenosine A_1 receptor-mediated signalling directly and *in situ* during prolonged wakefulness.

A major feature and advantage of the GPCRs is signal amplification which takes place in several stages; firstly, the activation of one receptor can activate several G-proteins which leads to the dissociation of the α- and $\beta\gamma$-subunits, secondly, both activated subunits affect multiple different effectors, i.e. adenylyl cyclase, protein kinase C etc. and thirdly, the activated effectors have multiple effects further downstream in the signal transducting cascade. In the A_1 receptor, the agonist causes a conformational change leading to the activation of the G-protein. This leads to the binding of the GTP to the α-subunit of the G-protein, which leads to the dissociation of the α-, the $\beta\gamma$-subunit and the receptor. The subunits mediate the effects of GPCRs, which, in the case of A_1 receptors, leads to inhibition of adenylyl cyclase, inhibition of Ca^{2+} conductance, stimulation of K^+ conductance and activation of phospholipase C (Fredholm et al 2001). The subunits are active until GTPase terminates the activity by cleaving the GTP into GDP. The GTPase is an intrinsic part in the Gα-subunit, and it is controlled by proteins known as regulators of G-protein signalling (Neubig & Siderovski 2002). In the [^{35}S]GTPγS binding assays GTP is substituted by [^{35}S]GTPγS which is resistant to hydrolysis by GTPase. This means that if the GTPase activity itself is affected during prolonged wakefulness, the possible changes will remain undetected.

As reviewed previously, there is substantial evidence indicating that adenosine mediates its sleep-inducing effects at least partly through the A_1 receptors in the cortically projecting cholinergic neurons in the BF. Thus it was a surprise that the G-protein activity in the BF remained unaffected despite the known elevated extracellular levels of adenosine during prolonged wakefulness (Porkka-Heiskanen et al 1997, Basheer et al 1999). There are some possible explanations for this lack of effect; Firstly, the A_1 receptor expression is lower in the BF than in the cortex (Rivkees et al 1995, Dixon et al 1996), and only one third of the neurons in the BF are thought to be cholinergic (Gritti et al 1993). Secondly, the extracellular adenosine levels increase slowly within the first two hours of SD up to two-fold (Basheer et al 1999). When these factors are considered, it is possible that the A_1 receptor dependent G-protein activity increases in the cholinergic cells, but that the method used is not sensitive enough to detect it. The other plausible explanation is that the A_1 receptor dependent G-protein activity is regulated differently depending on the brain area, which could explain the slight increase in the G-protein activity noted in the

MPA, which lies in the close vicinity of the BF. When the G-protein activity is compared with the known time scale of the accumation of adenosine in the cortex (Basheer et al 1999), it is noted that the G-protein activation takes place during the first hours of deprivation, which corresponds to the increase phase of the adenosine levels. When the extracellular adenosine levels are at their highest, the G-protein activity has already returned to its normal level, i.e. to the same level with the undeprived control animals. It could be speculated that the A_1 receptor dependent G-protein activity is most strongly affected by the change and not necessarily by the level of the extracellular adenosine.

On the basis of these and previous studies, it is concluded that the accumulation of adenosine during prolonged wakefulness causes a fast transient response in the cortex and a slower, longer lasting and possibly recovery sleep inducing response in the basal forebrain.

4.2 The effects of sleep deprivation on the key adenosine metabolising enzymes and transporter activity

4.2.1 The activities of the adenosine metabolising enzymes are not affected during prolonged wakefulness (III)

As previously discussed, adenosine accumulates in the BF and, to a lesser extent, in the cortex during prolonged wakefulness. The extracellular levels of adenosine are affected by the acitivites of the adenosine metabolizing enzymes and the transport of adenosine between intra- and extracellular spaces.

To gain insight to the possible mechanism(s) which could cause the accumulation of the extracellular adenosine during prolonged wakefulness, the activities of the key adenosine metabolizing enzymes, namely AK and 5'-NTs, were measured after 3 and 6 hours of SD and recovery sleep.

There were no differences in the enzyme activities measured between the sleep deprived and the control animals. These findings have been confirmed, as another report was published shortly after our publication, in which the results were in accordance with ours, with the additional notion of the ADA activity remaining unaffected (Mackiewicz et al 2003). A decreasing diurnal tendency in the enzyme activities was noted, which is in accordance with previous findings and probably is due to the lower energy demand during the rest phase of the animal (Chagoya de Sanchez et al 1993). Despite these findings, small changes in the enzyme activities could take

place during prolonged wakefulness. Firstly, the enzymes were assessed from tissue samples and thus the values obtained represent the mean enzyme activities of a cell population consisting of neurons with different transmitter phenotypes and glia. Consequently, the possible changes between different cell types are not noted. Secondly, all enzyme assays reflect the state of the selected enzymes at the moment the measurement is done, in this case after decapitation and dissection and freezing/melting/thawing of the tissue. Unluckily, the nucleoside metabolism is very rapid and tissue trauma leads to increased adenosine concentrations (Dunwiddie & Masino 2001). Furthermore, it has been shown before that the method for killing the animals affects nucleoside levels (Delaney & Geiger 1996), and thus minor changes in enzyme metabolism might pass unnoticed. And finally, as adenosine is constantly both produced and degraded, a small change in the balance between consumption and production might not affect the enzyme activities sufficiently to be detectable by the enzyme assays. Yet it is relatively safe to assume that no major changes in the enzyme activities of adenosine metabolizing enzymes take place during prolonged wakefulness in the brain areas measured.

4.2.2 The ENT1 ligand binding is decreased during prolonged wakefulness in the basal forebrain (IV)

The possible sources of increased extracellular adenosine concentrations are either increased intracellular adenosine production due to increased activity or increased co-release of ATP, which is further metabolized into adenosine by the ecto-nucleotidases. Yet there is evidence that under basal conditions a significant amount, if not all, of adenosine originates in extracellular nucleotide metabolism (Delaney & Geiger 1998, Barsotti & Ipata 2004). In addition, it has been shown that the inhibition of the ENT1 inhibitor NBMPR into the BF and SD have similar effects on the extracellular adenosine levels which are approximately doubled (Porkka-Heiskanen et al 1997, Basheer et al 1999, Porkka-Heiskanen et al 2000).

As the enzyme activities were found to remain unaffected, the next logical step was to assess the other mechanism known to affect the extracellular adenosine levels, namely, the adenosine transport and especially the equilibrative nucleoside transporters.

The equilibrative nucleoside transporters ENT1 and ENT2 carry nucleosides through the cell membrane according to the concentration gradient. Unfortunetaly there are specific tools only for the ENT1, for which a relatively specific inhibitor, NBMPR, is available (Thorn & Jarvis 1996).

To assess the possible changes in the transporter activity, the NBMPR binding and the mRNA expression of the ENT1 were measured after 3 and 6 hours of sleep deprivation and recovery sleep. The NBMPR binding was decreased in the BF of rats after 6 hours of SD but not in the cortex, suggesting a local decreased activity of the transporter. This would mean that regardles of whether the adenosine is produced intra- or extracellularly, the transport through the cell membrane would be less efficient. The expression of the ENT1 mRNA was not altered during SD when compared to controls, which could be due to the relatively short period of SD.

The measured NBMPR binding describes the combination of the affinity of the ligand to the ENT1 transporter and the amount of transporters. This means that a possible change in the NBMPR binding can result from either a change in the affinity or in the amount of the transporter, and these two factors cannot be separated by this method. Despite this known deficit of the method, there is evidence indicating that the NBMPR binding can be used to assess the transporter activity (Thorn & Jarvis 1996).

On the basis of the previous findings and the noted decrease in equilibrative transport, it is concluded that the accumulation of extracellular adenosine during prolonged wakefulness could be due to the inhibition of uptake of adenosine into the cells, and the continuous co-release of ATP could be due to the higher electrical activity sustained during SD. A subset of supposedly cholinergic neurons in the BF is known to be most active during active waking (Szymusiak et al 2000), and when combined with the decrease of influx of adenosine into the cells it could be sufficient to cause the accumulation of adenosine in the BF during prolonged wakefulness. It is not suggested that this would be the sole mechanism to cause the accumulation of adenosine in the BF during prolonged wakefulness, but that the decreased activity of the ENT1 transporter is included in the mechanism of accumulation. There is substantial evidence showing that one additional mechanism could be nitric oxide (NO), as it has been shown that NO induces adenosine release *in vitro* in hippocampal slices and forebrain neuronal cultures (Fallahi et al 1996, Rosenberg et al 2000). And quite recently it has been shown that NO donors increase nonREM sleep, while NO scavengers prevent recovery sleep after SD (Kalinchuk, personal communication).

In the cortex, however, the extracellular adenosine levels are known to increase transiently during prolonged wakefulness (Porkka-Heiskanen et al 2000), while no change in the transporter activity was noted in this study. One possible mechanism explaining the transient increase could be methodological; the SD by gentle handling is based on introducing novel objects to the rats, thus

keeping them awake. The BF is known to be most active during situations of arousal, like in new surroundings, with the reported biggest difference in single unit activity being between active and quiet wake with a much smaller difference between quiet wake and sleep (Szymusiak et al 2000). Therefore the rise in the extracellular adenosine levels seen in the cortex during the first hours of SD could be due to the increased BF and cortical activity induced by introducing novel objects. It would be of interest to compare the previously noted increases in the extracellular adenosine levels during SD by gentle handling with another method, like the rotating disc method, in which no explorative behaviour is needed.

4.3 The equilibrative transporters are ubiquitously distributed in the rat brain (V)

The equilibrative nucleoside transporters ENT1 and ENT2 have been cloned from rat and human (Yao et al 1997, Griffiths et al 1997) and they have been shown to be expressed in the CNS of both species (Anderson et al 1999a, Anderson et al 1999b, Jennings et al 2001). The ENTs are membrane bound (Cabrita et al 2002) and their distribution has been assessed previously using *in situ* hybridization (ENT1 and ENT2), autoradiographic and membrane binding techniques (ENT1) (Geiger & Nagy 1984, Bisserbe et al 1985, Anderson et al 1999a, Anderson et al 1999b). These methods, while being most useful for general localization studies and activity measurements, give little information concerning the exact localization or amount of the transporter proteins *per se*.

Using novel antibodies against the rat ENT1 and ENT2 raised in our laboratory, we characterized for the first time the distribution of the transporters using immunohistochemical methods. Both transporters were present in almost all neurons throughout the rat brain. The discrepancy between ours and previous results is clear, as Anderson *et al.* previously proposed that the ENT2 would be the pre-dominant transporter in the rat brain, due to higher prevalence of the ENT2 mRNA over the ENT1 mRNA positive neurons (Anderson et al 1999a, Anderson et al 1999b). Their proposition is based on the differences in mRNA expression; the ENT1 mRNA was present in approximately half of the neurons in the parietal cortex and the dorsolateral striatum, while the ENT2 mRNA was noted in over 90% of the neurons. The difference between our findings and those of Andersons is most probably methodological, as mRNA expression does not reveal the amount of protein, only the amount of transcript, while the immunohistochemical methods reveal

the localization of the protein. The protein is not a marker of transporter activity, but it is more accurate than the mRNA in evaluating the possible importance of the transporters.
In the immunofluorescence and double labeling studies a major difference was noted in the distribution of the ENT1 and ENT2 at the cellular level; the ENT1 was located mainly in the cellular membrane, as expected, while the ENT2 showed in addition major somatic staining. Furthermore, some ENT1 and ENT2 positive astrocytes were found with the ENT1 more prominent of the two. In addition, a dense bundle of varicose, intensely ENT1 immunoreactive fibers were consistently seen in the median eminence and, to a lesser extent, in the lateral hypothalamus suggesting that ENT1 is located also in some axons. In accordance with previous studies (Anderson et al 1999a, Anderson et al 1999b), the choroid plexus and ependyme were strongly ENT1 and ENT2 immunoreactive, suggesting that the ENTs are involved in the forming of cerebrospinal fluid.
These findings suggest that the equilibrative transporters may have different roles in the regulation of nucleotide transport, the ENT1 being more important in the nucleoside transport between the extra- and intracellular space, and the ENT2 having a more prominent role in the intracellular nucleoside transport between different intracellular compartments. Furthermore, the ENT1 seems to be located in at least some axons, but the co-localization of the ENT2 cannot be excluded, as the affinity of the antibody and the amount of the ENT2 may be too low to be detected with the methods used in the study.

4.4 Future considerations

The role of the ENT1 and the ENT2 in the regulation of nucleoside transport has yet many interesting phenomena to be investigated. The subcellular localization of both transporters is unknown and of major interest. Furthermore, the specific tools for studying the ENT2 are lacking, and thus the functional role of the transporter is still unclear.
The focus of research shall be pointed at the intracellular localization of both the ENT transporters. The subcellular localization shall be assessed from cell fractions and and later possibly with electron microscopy as well. The CNTs have so far been studied quite sparingly, mostly due to the lack of specific tools, but this seems to be changing. Both the CNT1 and the CNT2 have been cloned from the rat brain, and their functional role reamains unclear (Anderson et al 1996). Moreover it has been shown that the adenosine transporter cloned from the blood

brain barrier is identical to the CNT2 (Li et al 2001). Most recently, evidence has been presented showing that the CNT2 mRNA expression is decreased in the cortex after 24 hours of SD (Duflot et al 2004). It seems that the concentrative nucleoside transporters are finally surfacing and it would be of major interest to find out the functional roles of the CNTs in the CNS and to solve their localization in the cellular level and their distribution between neurons of different transmitter phenotypes and glia.

5. CONCLUSIONS

- The ligand binding of the A_1 receptors remains unaffected during prolonged wakefulness, but the A_1 receptor mRNA expression is increased, suggesting an increased demand for A_1 receptors.
- The A_1 receptor-dependent G-protein activity is transiently increased during prolonged wakefulness in the cortex, but not in the BF.
- The data from these studies suggest that the accumulation of adenosine causes an A_1 receptor mediated fast transient response in the cortex, and a slower, longer lasting, possibly recovery sleep inducing, response in the BF.

- The activity of the key adenosine metabolizing enzymes remains unaffected in the cortex and the BF during prolonged wakefulness and recovery sleep.
- The adenosine transporter ENT1 activity is decreased during prolonged wakefulness in the BF. Combined with the increased cellular activity in the BF during prolonged wakefulness, and thus with the increased release of ATP, this decrease of activity could lead to increased extracellular adenosine concentrations via the ecto-nucleotidase pathway.

- The ENT1 and the ENT2 are widely distributed in the rat brain being present in almost all neurons.
- The ENT1 is located mainly in the cellular membrane, while the ENT2 is located in the soma as well. This could mean that the ENT1 takes directly part in the regulation of extracellular levels of adenosine, and the ENT2 has a more prominent role in intracellular nucleoside transport.

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Every story has an ending, and I end this one with a citation from the very last strip of the cartoon Calvin & Hobbes by Bill Watterson:

It's a magical world, Hobbes ol' buddy, let's go exploring!

Lauri Alanko
Helsinki, May 2005

REFERENCES

Alam MN, Szymusiak R, Gong H, King J, McGinty D. 1999. Adenosinergic modulation of rat basal forebrain neurons during sleep and waking: neuronal recording with microdialysis. *J. Physiol* 521 Pt 3:679-90

Anderson CM, Baldwin SA, Young JD, Cass CE, Parkinson FE. 1999a. Distribution of mRNA encoding a nitrobenzylthioinosine-insensitive nucleoside transporter (ENT2) in rat brain. *Brain Res. Mol. Brain Res.* 70(2):293-7

Anderson CM, Xiong W, Geiger JD, Young JD, Cass CE, Baldwin SA, Parkinson FE. 1999b. Distribution of equilibrative, nitrobenzylthioinosine-sensitive nucleoside transporters (ENT1) in brain. *J. Neurochem.* 73(2):867-73

Anderson CM, Xiong W, Young JD, Cass CE, Parkinson FE. 1996. Demonstration of the existence of mRNAs encoding N1/cif and N2/cit sodium/nucleoside cotransporters in rat brain. *Brain Res. Mol. Brain Res.* 42(2):358-61

Ballarin M, Fredholm BB, Ambrosio S, Mahy N. 1991. Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta Physiol Scand.* 142(1):97-103

Barsotti C, Ipata PL. 2004. Metabolic regulation of ATP breakdown and of adenosine production in rat brain extracts. *Int. J Biochem. Cell Biol.* 36(11):2214-25

Bartus RT, Dean RL, III, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. *Science* 217(4558):408-14

Basheer R, Arrigoni E, Thatte HS, Greene RW, Ambudkar IS, McCarley RW. 2002. Adenosine induces inositol 1,4,5-trisphosphate receptor-mediated mobilization of intracellular calcium stores in basal forebrain cholinergic neurons. *J. Neurosci.* 22(17):7680-6

Basheer R, Porkka-Heiskanen T, Stenberg, D, McCarley RW. 1999. Adenosine and behavioral state control: adenosine increases c-Fos protein and AP1 binding in basal forebrain of rats. *Brain Res. Mol. Brain Res.* 73(1-2):1-10

Basheer R, Rainnie DG, Porkka-Heiskanen T, Ramesh V, McCarley RW. 2001. Adenosine, prolonged wakefulness, and A1-activated NF-kappaB DNA binding in the basal forebrain of the rat. *Neuroscience* 104(3):731-9

Benington JH, Heller HC. 1995. Restoration of brain energy metabolism as the function of sleep. *Prog. Neurobiol.* 45(4):347-60

Benington JH, Kodali SK, Heller HC. 1995. Stimulation of A1 adenosine receptors mimics the electroencephalographic effects of sleep deprivation. *Brain Res.* 692(1-2):79-85

Berne RM, Winn HR, Rubio R. 1981. The local regulation of cerebral blood flow. [Review] [113 refs]. *Progress in Cardiovascular Diseases* 24(3):243-60

Berntson GG, Shafi R, Sarter M. 2002. Specific contributions of the basal forebrain corticopetal cholinergic system to electroencephalographic activity and sleep/waking behaviour. *Eur. J Neurosci.* 16(12):2453-61

Bisserbe JC, Patel J, Marangos PJ. 1985. Autoradiographic localization of adenosine uptake sites in rat brain using [3H]nitrobenzylthioinosine. *J. Neurosci.* 5(2):544-50

Boeck CR, Sarkis JJ, Vendite D. 2002. Kinetic characterization and immunodetection of ecto-ATP diphosphohydrolase (EC 3.6.1.5) in cultured hippocampal neurons. *Neurochem. Int.* 40(5):449-53

Boyes BE, Kim SU, Lee V, Sung SC. 1986. Immunohistochemical co-localization of S-100b and the glial fibrillary acidic protein in rat brain. *Neuroscience* 17(3):857-65

Brundege JM, Diao L, Proctor WR, Dunwiddie TV. 1997. The role of cyclic AMP as a precursor of extracellular adenosine in the rat hippocampus. *Neuropharmacology* 36(9):1201-10

Brundege JM, Dunwiddie TV. 1997. Role of adenosine as a modulator of synaptic activity in the central nervous system. [Review] [200 refs]. *Advances in Pharmacology (New York)* 39:353-91

Brundege JM, Dunwiddie TV. 1998. Metabolic regulation of endogenous adenosine release from single neurons. *Neuroreport* 9(13):3007-11

Buzsaki G, Bickford RG, Ponomareff G, Thal LJ, Mandel R, Gage FH. 1988. Nucleus basalis and thalamic control of neocortical activity in the freely moving rat. *J Neurosci.* 8(11):4007-26

Buzsaki G, Gage FH. 1989. The cholinergic nucleus basalis: a key structure in neocortical arousal. *EXS* 57:159-71

Cabrita MA, Baldwin SA, Young JD, Cass CE. 2002. Molecular biology and regulation of nucleoside and nucleobase transporter proteins in eukaryotes and prokaryotes. *Biochem. Cell Biol.* 80(5):623-38

Ceballos G, Tuttle JB, Rubio R. 1994. Differential distribution of purine metabolizing enzymes between glia and neurons. *J Neurochem* 62(3):1144-53

Celesia GG, Jasper HH. 1966. Acetylcholine released from cerebral cortex in relation to state of activation. *Neurology* 16(11):1053-63

Chagoya de Sanchez V, Hernandez MR, Suarez J, Vidrio S, Yanez L, Diaz MM. 1993. Day-night variations of adenosine and its metabolizing enzymes in the brain cortex of the rat--possible physiological significance for the energetic homeostasis and the sleep-wake cycle. *Brain Res.* 612(1-2):115-21

Cullinan WE, Zaborszky L. 1991. Organization of ascending hypothalamic projections to the rostral forebrain with special reference to the innervation of cholinergic projection neurons. *J Comp Neurol.* 306(4):631-67

Cunha RA. 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem. Int.* 38(2):107-25

Cunha RA, Brendel P, Zimmermann H, Ribeiro JA. 2000. Immunologically distinct isoforms of ecto-5'-nucleotidase in nerve terminals of different areas of the rat hippocampus. *J. Neurochem.* 74(1):334-8

Cunha RA, Sebastiao AM, Ribeiro JA. 1992. Ecto-5'-nucleotidase is associated with cholinergic nerve terminals in the hippocampus but not in the cerebral cortex of the rat. *J Neurochem* 59(2):657-66

Delaney SM, Geiger JD. 1996. Brain regional levels of adenosine and adenosine nucleotides in rats killed by high-energy focused microwave irradiation. *J. Neurosci. Methods* 64(2):151-6

Delaney SM, Geiger JD. 1998. Levels of endogenous adenosine in rat striatum. II. Regulation of basal and N-methyl-D-aspartate-induced levels by inhibitors of adenosine transport and metabolism. *J Pharmacol. Exp. Ther.* 285(2):568-72

Detari L, Juhasz G, Kukorelli T. 1984. Firing properties of cat basal forebrain neurones during sleep-wakefulness cycle. *Electroencephalogr. Clin. Neurophysiol.* 58(4):362-8

Deussen A, Lloyd HG, Schrader J. 1989. Contribution of S-adenosylhomocysteine to cardiac adenosine formation. *J. Mol. Cell Cardiol.* 21(8):773-82

Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, Freeman TC. 1996. Tissue distribution of adenosine receptor mRNAs in the rat. *Br. J. Pharmacol.* 118(6):1461-8

Dolphin AC, Forda SR, Scott RH. 1986. Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. *J Physiol* 373:47-61

Drury AN, Szent-Gyorgyi A. 1929. The physiological activity of adenine compounds with especial reference to their action upon mammalian heart. *J. Physiol* 68:213-37

Duflot S, Riera B, Fernandez-Veledo S, Casado V, Norman RI, Casado FJ, Lluis C, Franco R, Pastor-Anglada M. 2004. ATP-sensitive K(+) channels regulate the concentrative adenosine transporter CNT2 following activation by A(1) adenosine receptors. *Mol. Cell Biol.* 24(7):2710-9

Dunwiddie TV, Diao L, Proctor WR. 1997. Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. *J.* 17(20):7673-82

Dunwiddie TV, Hoffer BJ. 1980. Adenine nucleotides and synaptic transmission in the in vitro rat hippocampus. *British Journal of Pharmacology* 69(1):59-68

Dunwiddie TV, Masino SA. 2001. The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* 24:31-55

Dunwiddie TV, Worth T. 1982. Sedative and anticonvulsant effects of adenosine analogs in mouse and rat. *J. Pharmacol. Exp. Ther.* 220(1):70-6

Fallahi N, Broad RM, Jin S, Fredholm BB. 1996. Release of adenosine from rat hippocampal slices by nitric oxide donors. *J Neurochem* 67(1):186-93

Feldberg W, Sherwood SL. 1954. Infections of drugs into the lateral ventricle of the cat. *J. Physiol* 123(1):148-67

Fibiger HC. 1991. Cholinergic mechanisms in learning, memory and dementia: a review of recent evidence. *Trends Neurosci.* 14(6):220-3

Ford AL, Goodsall AL, Hickey WF, Sedgwick JD. 1995. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. *J. Immunol.* 154(9):4309-21

Franco R, Canela EI, Bozal J. 1986. Heterogeneous localization of some purine enzymes in subcellular fractions of rat brain and cerebellum. *Neurochem Res.* 11(3):423-35

Franken P, Tobler I, Borbély AA. 1993. Effects of 12-h sleep deprivation and of 12-h cold exposure on sleep regulation and cortical temperature in the rat. *Physiol. Behav.* 54:885-94

Fredholm BB. 1997. Adenosine and neuroprotection. *Int. Rev. Neurobiol.* 40:259-80

Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. 2000. Structure and function of adenosine receptors and their genes. *Naunyn Schmiedebergs Arch. Pharmacol.* 362(4-5):364-74

Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* 51(1):83-133

Fredholm BB, Fried G, Hedqvist P. 1982. Origin of adenosine released from rat vas deferens by nerve stimulation. *Eur. J Pharmacol.* 79(3-4):233-43

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. 2001. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 53(4):527-52
Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG. 1989. In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, rip. *Glia* 2(5):380-90
Gallopin T, Fort P, Eggermann E, Cauli B, Luppi PH, Rossier J, Audinat E, Muhlethaler M, Serafin M. 2000. Identification of sleep-promoting neurons in vitro. *Nature* 404(6781):992-5
Geiger JD, Nagy JI. 1984. Heterogeneous distribution of adenosine transport sites labelled by [3H]nitrobenzylthioinosine in rat brain: an autoradiographic and membrane binding study. *Brain Res. Bull.* 13(5):657-66
Gerwins P, Fredholm BB. 1992. ATP and its metabolite adenosine act synergistically to mobilize intracellular calcium via the formation of inositol 1,4,5-trisphosphate in a smooth muscle cell line. *J Biol. Chem.* 267(23):16081-7
Gouder N, Scheurer L, Fritschy JM, Boison D. 2004. Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. *J. Neurosci.* 24(3):692-701
Grady EF, Bohm SK, Bunnett NW. 1997. Turning off the signal: mechanisms that attenuate signaling by G protein-coupled receptors. *Am. J Physiol* 273(3 Pt 1):G586-G601
Gray JH, Owen RP, Giacomini KM. 2004. The concentrative nucleoside transporter family, SLC28. *Pflugers Arch.* 447(5):728-34
Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, Baldwin SA. 1997. Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. *Biochem. J.* 328 (Pt 3):739-43
Gritti I, Mainville L, Jones BE. 1993. Codistribution of GABA- with acetylcholine-synthesizing neurons in the basal forebrain of the rat. *J. Comp Neurol.* 329(4):438-57
Gritti I, Mainville L, Mancia M, Jones BE. 1997. GABAergic and other noncholinergic basal forebrain neurons, together with cholinergic neurons, project to the mesocortex and isocortex in the rat. *J. Comp Neurol.* 383(2):163-77
Haas HL, Selbach O. 2000. Functions of neuronal adenosine receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* 362(4-5):375-81
Hammond LC, Bonnet C, Kemp PJ, Yates MS, Bowmer CJ. 2004. Chronic hypoxia up-regulates expression of adenosine A(1) receptors in DDT(1)-MF2 cells. *Biochem. Pharmacol.* 67(3):421-6
Haulica I, Ababei L, Branisteanu D, Topoliceanu F. 1973. Letter: Preliminary data on the possible hypnogenic role of adenosine. *J Neurochem* 21(4):1019-20
Hayaishi O. 2002. Molecular genetic studies on sleep-wake regulation, with special emphasis on the prostaglandin D(2) system. *J Appl. Physiol* 92(2):863-8
Huston JP, Haas HL, Boix F, Pfister M, Decking U, Schrader J, Schwarting RK. 1996. Extracellular adenosine levels in neostriatum and hippocampus during rest and activity periods of rats. *Neuroscience* 73(1):99-107
James S, Richardson PJ. 1993. Production of adenosine from extracellular ATP at the striatal cholinergic synapse. *J Neurochem* 60(1):219-27
Jarvis MF, Williams M. 1989. Direct autoradiographic localization of adenosine A2 receptors in the rat brain using the A2-selective agonist, [3H]CGS 21680. *Eur. J Pharmacol.* 168(2):243-6
Jarvis SM, Young JD. 1980. Nucleoside transport in human and sheep erythrocytes. Evidence that nitrobenzylthioinosine binds specifically to functional nucleoside-transport sites. *Biochem. J.* 190(2):377-83
Jasper HH, Tessier J. 1971. Acetylcholine liberation from cerebral cortex during paradoxical (REM) sleep. *Science* 172(983):601-2
Jennings LL, Hao C, Cabrita MA, Vickers MF, Baldwin SA, Young JD, Cass CE. 2001. Distinct regional distribution of human equilibrative nucleoside transporter proteins 1 and 2 (hENT1 and hENT2) in the central nervous system. *Neuropharmacology* 40(5):722-31
Johansson B, Ahlberg S, van dP, I, Brene S, Lindefors N, Persson H, Fredholm BB. 1993. Effect of long term caffeine treatment on A1 and A2 adenosine receptor binding and on mRNA levels in rat brain. *Naunyn Schmiedebergs Arch. Pharmacol.* 347(4):407-14
Jones BE. 2004. Activity, modulation and role of basal forebrain cholinergic neurons innervating the cerebral cortex. *Prog. Brain Res.* 145:157-69
Kalinchuk AV, Urrila AS, Alanko L, Heiskanen S, Wigren HK, Suomela M, Stenberg D, Porkka-Heiskanen T. 2003. Local energy depletion in the basal forebrain increases sleep. *Eur. J. Neurosci.* 17(4):863-9
Kilgard MP, Merzenich MM. 1998. Cortical map reorganization enabled by nucleus basalis activity. *Science* 279(5357):1714-8

Krnjevic K. 1967. Chemical transmission and cortical arousal. *Anesthesiology* 28(1):100-5
Laitinen JT. 1999. Selective detection of adenosine A1 receptor-dependent G-protein activity in basal and stimulated conditions of rat brain [35S]guanosine 5'-(gamma-thio)triphosphate autoradiography. *Neuroscience* 90(4):1265-79
Latini S, Corsi C, Pedata F, Pepeu G. 1995. The source of brain adenosine outflow during ischemia and electrical stimulation. *Neurochemistry International* 27(3):239-44
Lee CW, Jarvis SM. 1988. Nucleoside transport in rat cerebral-cortical synaptosomes. Evidence for two types of nucleoside transporters. *Biochem. J.* 249(2):557-64
Li JY, Boado RJ, Pardridge WM. 2001. Cloned blood-brain barrier adenosine transporter is identical to the rat concentrative Na+ nucleoside cotransporter CNT2. *J Cereb. Blood Flow Metab* 21(8):929-36
Libert F, Parmentier M, Lefort A, Dinsart C, Van Sande J, Maenhaut C, Simons MJ, Dumont JE, Vassart G. 1989. Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science* 244(4904):569-72
Libert F, Schiffmann SN, Lefort A, Parmentier M, Gerard C, Dumont JE, Vanderhaeghen JJ, Vassart G. 1991. The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO J* 10(7):1677-82
Lloyd HG, Deussen A, Wuppermann H, Schrader J. 1988. The transmethylation pathway as a source for adenosine in the isolated guinea-pig heart. *Biochem. J.* 252(2):489-94
Lloyd HG, Fredholm BB. 1995. Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem Int.* 26(4):387-95
Lloyd HG, Lindstrom K, Fredholm BB. 1993. Intracellular formation and release of adenosine from rat hippocampal slices evoked by electrical stimulation or energy depletion. *Neurochem. Int.* 23(2):173-85
Mackiewicz M, Nikonova EV, Bell CC, Galante RJ, Zhang L, Geiger JD, Pack AI. 2000. Activity of adenosine deaminase in the sleep regulatory areas of the rat CNS. *Brain Res. Mol. Brain Res.* 80(2):252-5
Mackiewicz M, Nikonova EV, Zimmerman JE, Galante RJ, Zhang L, Cater JR, Geiger JD, Pack AI. 2003. Enzymes of adenosine metabolism in the brain: diurnal rhythm and the effect of sleep deprivation. *J. Neurochem.* 85(2):348-57
Madsen PL. 1993. Blood flow and oxygen uptake in the human brain during various states of sleep and wakefulness. *Acta Neurol. Scand. Suppl* 148:3-27
Madsen PL, Schmidt JF, Wildschiodtz G, Friberg L, Holm S, Vorstrup S, Lassen NA. 1991. Cerebral O2 metabolism and cerebral blood flow in humans during deep and rapid-eye-movement sleep. *J. Appl. Physiol* 70(6):2597-601
Maenhaut C, Van Sande J, Libert F, Abramowicz M, Parmentier M, Vanderhaegen JJ, Dumont JE, Vassart G, Schiffmann S. 1990. RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* 173(3):1169-78
Mahan LC, McVittie LD, Smyk-Randall EM, Nakata H, Monsma FJ, Jr., Gerfen CR, Sibley DR. 1991. Cloning and expression of an A1 adenosine receptor from rat brain. *Mol. Pharmacol.* 40(1):1-7
Maquet P, Degueldre C, Delfiore G, Aerts J, Peters JM, Luxen A, Franck G. 1997. Functional neuroanatomy of human slow wave sleep. *J Neurosci.* 17(8):2807-12
Maquet P, Dive D, Salmon E, Sadzot B, Franco G, Poirrier R, Franck G. 1992. Cerebral glucose utilization during stage 2 sleep in man. *Brain Res.* 571(1):149-53
Marrosu F, Portas C, Mascia MS, Casu MA, Fa M, Giagheddu M, Imperato A, Gessa GL. 1995. Microdialysis measurement of cortical and hippocampal acetylcholine release during sleep-wake cycle in freely moving cats. *Brain Res.* 671(2):329-32
Matsumura H, Nakajima T, Osaka T, Satoh S, Kawase K, Kubo E, Kantha SS, Kasahara K, Hayaishi O. 1994. Prostaglandin D2-sensitive, sleep-promoting zone defined in the ventral surface of the rostral basal forebrain. *Proc. Natl. Acad. Sci. U. S. A* 91(25):11998-2002
McCormick DA. 1992. Neurotransmitter actions in the thalamus and cerebral cortex and their role in neuromodulation of thalamocortical activity. *Prog. Neurobiol.* 39(4):337-88
McCormick DA, Prince DA. 1986. Mechanisms of action of acetylcholine in the guinea-pig cerebral cortex in vitro. *J Physiol* 375:169-94
McLin DE, III, Miasnikov AA, Weinberger NM. 2002. Induction of behavioral associative memory by stimulation of the nucleus basalis. *Proc. Natl. Acad. Sci. U. S. A* 99(6):4002-7
Metherate R, Ashe JH. 1993. Nucleus basalis stimulation facilitates thalamocortical synaptic transmission in the rat auditory cortex. *Synapse* 14(2):132-43
Meyerhof W, Muller-Brechlin R, Richter D. 1991. Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* 284(2):155-60

Mitchell JB, Lupica CR, Dunwiddie TV. 1993. Activity-dependent release of endogenous adenosine modulates synaptic responses in the rat hippocampus. *J Neurosci.* 13(8):3439-47
Mizoguchi A, Eguchi N, Kimura K, Kiyohara Y, Qu WM, Huang ZL, Mochizuki T, Lazarus M, Kobayashi T, Kaneko T, Narumiya S, Urade Y, Hayaishi O. 2001. Dominant localization of prostaglandin D receptors on arachnoid trabecular cells in mouse basal forebrain and their involvement in the regulation of non-rapid eye movement sleep. *Proc. Natl. Acad. Sci. U. S. A* 98(20):11674-9
Morairty S, Rainnie D, McCarley R, Greene R. 2004. Disinhibition of ventrolateral preoptic area sleep-active neurons by adenosine: a new mechanism for sleep promotion. *Neuroscience* 123(2):451-7
Moreau JL, Huber G. 1999. Central adenosine A(2A) receptors: an overview. *Brain Res. Brain Res. Rev.* 31(1):65-82
Muir JL, Everitt BJ, Robbins TW. 1994. AMPA-induced excitotoxic lesions of the basal forebrain: a significant role for the cortical cholinergic system in attentional function. *J Neurosci.* 14(4):2313-26
Murillo-Rodriguez E, Blanco-Centurion C, Gerashchenko D, Salin-Pascual RJ, Shiromani PJ. 2004. The diurnal rhythm of adenosine levels in the basal forebrain of young and old rats. *Neuroscience* 123(2):361-70
Nagy JI, LaBella LA, Buss M, Daddona PE. 1984. Immunohistochemistry of adenosine deaminase: implications for adenosine neurotransmission. *Science* 224(4645):166-8
Netchiporouk L, Shram N, Salvert D, Cespuglio R. 2001. Brain extracellular glucose assessed by voltammetry throughout the rat sleep-wake cycle. *Eur. J Neurosci.* 13(7):1429-34
Neubig RR, Siderovski DP. 2002. Regulators of G-protein signalling as new central nervous system drug targets. *Nat. Rev. Drug Discov.* 1(3):187-97
Newby AC. 1984. Adenosine and the concept of 'retaliatory metabolites'. *Trends in Biochemical Sciences* 9(2):42-4
Okada T, Mochizuki T, Huang ZL, Eguchi N, Sugita Y, Urade Y, Hayaishi O. 2003. Dominant localization of adenosine deaminase in leptomeninges and involvement of the enzyme in sleep. *Biochem. Biophys. Res. Commun.* 312(1):29-34
Olah ME, Stiles GL. 1995. Adenosine receptor subtypes: characterization and therapeutic regulation. *Annu. Rev. Pharmacol. Toxicol.* 35:581-606
Orford M, Mazurkiewicz D, Saggerson D. 1991. Soluble 5'-nucleotidase activities in rat brain. *J Neurochem* 56(1):141-6
Orford MR, Saggerson ED. 1996. A low-Km 5'-nucleotidase from rat brain cytosolic fraction: purification, kinetic properties, and description of regulation by a novel factor that increases sensitivity to inhibition by ATP and ADP. *J. Neurochem.* 67(2):795-804
Pak MA, Haas HL, Decking UK, Schrader J. 1994. Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. *Neuropharmacology* 33(9):1049-53
Parkinson FE, Fredholm BB. 1990. Autoradiographic evidence for G-protein coupled A2-receptors in rat neostriatum using [3H]-CGS 21680 as a ligand. *Naunyn Schmiedebergs Arch. Pharmacol.* 342(1):85-9
Parkinson FE, Fredholm BB. 1992. Magnesium-dependent enhancement of endogenous agonist binding to A1 adenosine receptors: a complicating factor in quantitative autoradiography. *J. Neurochem.* 58(3):941-50
Patel BT, Tudball N. 1986. Localization of S-adenosylhomocysteine hydrolase and adenosine deaminase immunoreactivities in rat brain. *Brain Res.* 370(2):250-64
Pazzagli M, Corsi C, Fratti S, Pedata F, Pepeu G. 1995. Regulation of extracellular adenosine levels in the striatum of aging rats. *Brain Res.* 684(1):103-6
Pazzagli M, Corsi C, Latini S, Pedata F, Pepeu G. 1994. In vivo regulation of extracellular adenosine levels in the cerebral cortex by NMDA and muscarinic receptors. *Eur. J Pharmacol.* 254(3):277-82
Pazzagli M, Pedata F, Pepeu G. 1993. Effect of K+ depolarization, tetrodotoxin, and NMDA receptor inhibition on extracellular adenosine levels in rat striatum. *Eur. J Pharmacol.* 234(1):61-5
Phillips E, Newsholme EA. 1979. Maximum activities, properties and distribution of 5' nucleotidase, adenosine kinase and adenosine deaminase in rat and human brain. *J. Neurochem.* 33(2):553-8
Phillis JW, Edstrom JP, Kostopoulos GK, Kirkpatrick JR. 1979. Effects of adenosine and adenine nucleotides on synaptic transmission in the cerebral cortex. *Can. J Physiol Pharmacol.* 57(11):1289-312
Phillis JW, O'Regan MH. 1989. Deoxycoformycin antagonizes ischemia-induced neuronal degeneration. *Brain Res. Bull.* 22(3):537-40
Phillis JW, Walter GA, O'Regan MH, Stair RE. 1987. Increases in cerebral cortical perfusate adenosine and inosine concentrations during hypoxia and ischemia. *J Cereb. Blood Flow Metab* 7(6):679-86
Phillis JW, Walter GA, Simpson RE. 1991. Brain adenosine and transmitter amino acid release from the ischemic rat cerebral cortex: effects of the adenosine deaminase inhibitor deoxycoformycin. *J Neurochem* 56(2):644-50
Phillis JW, Wu PH. 1981. The role of adenosine and its nucleotides in central synaptic transmission. *Prog. Neurobiol.* 16(3-4):187-239

Poelchen W, Sieler D, Wirkner K, Illes P. 2001. Co-transmitter function of ATP in central catecholaminergic neurons of the rat. *Neuroscience* 102(3):593-602

Porkka-Heiskanen T, Strecker RE, McCarley RW. 2000. Brain site-specificity of extracellular adenosine concentration changes during sleep deprivation and spontaneous sleep: An in vivo microdialysis study. *Neuroscience* 99(3):507-17

Porkka-Heiskanen T, Strecker RE, Thakkar, M, Bjorkum AA, Greene RW, McCarley RW. 1997. Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science* 276(5316):1265-8

Portas CM, Thakkar M, Rainnie DG, Greene RW, McCarley RW. 1997. Role of adenosine in behavioral state modulation: a microdialysis study in the freely moving cat. *Neuroscience* 79(1):225-35

Pull I, McIlwain H. 1972. Metabolism of (14 C)adenine and derivatives by cerebral tissues, superfused and electrically stimulated. *Biochem. J.* 126(4):965-73

Queiroz G, Gebicke-Haerter PJ, Schobert A, Starke K, von K, I. 1997. Release of ATP from cultured rat astrocytes elicited by glutamate receptor activation. *Neuroscience* 78(4):1203-8

Radulovacki M. 1985. Role of adenosine in sleep in rats. *Reviews in Clinical & Basic Pharmacology* 5(3-4):327-39

Radulovacki M, Virus RM, Djuricic-Nedelson M, Green RD. 1984. Adenosine analogs and sleep in rats. *J. Pharmacol. Exp. Ther.* 228(2):268-74

Rainnie DG, Grunze HC, McCarley RW, Greene RW. 1994. Adenosine inhibition of mesopontine cholinergic neurons: implications for EEG arousal. *Science* 263(5147):689-92

Reddington M, Pusch R. 1983. Adenosine metabolism in a rat hippocampal slice preparation: incorporation into S-adenosylhomocysteine. *J Neurochem* 40(1):285-90

Reppert SM, Weaver DR, Stehle JH, Rivkees SA. 1991. Molecular cloning and characterization of a rat A1-adenosine receptor that is widely expressed in brain and spinal cord. *Mol. Endocrinol.* 5(8):1037-48

Rivkees SA, Price SL, Zhou FC. 1995. Immunohistochemical detection of A1 adenosine receptors in rat brain with emphasis on localization in the hippocampal formation, cerebral cortex, cerebellum, and basal ganglia. *Brain Res.* 677(2):193-203

Rosenberg PA, Dichter MA. 1989. Extracellular cAMP accumulation and degradation in rat cerebral cortex in dissociated cell culture. *J. Neurosci.* 9(8):2654-63

Rosenberg PA, Li Y. 1995a. Adenylyl cyclase activation underlies intracellular cyclic AMP accumulation, cyclic AMP transport, and extracellular adenosine accumulation evoked by beta-adrenergic receptor stimulation in mixed cultures of neurons and astrocytes derived from rat cerebral cortex. *Brain Res.* 692(1-2):227-32

Rosenberg PA, Li Y. 1995b. Vasoactive intestinal peptide regulates extracellular adenosine levels in rat cortical cultures. *Neurosci. Lett.* 200(2):93-6

Rosenberg PA, Li Y, Le M, Zhang Y. 2000. Nitric oxide-stimulated increase in extracellular adenosine accumulation in rat forebrain neurons in culture is associated with ATP hydrolysis and inhibition of adenosine kinase activity. *J Neurosci.* 20(16):6294-301

Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB. 1992. Neuroprotective role of adenosine in cerebral ischaemia. *Trends Pharmacol. Sci.* 13(12):439-45

Rye DB, Wainer BH, Mesulam MM, Mufson EJ, Saper CB. 1984. Cortical projections arising from the basal forebrain: a study of cholinergic and noncholinergic components employing combined retrograde tracing and immunohistochemical localization of choline acetyltransferase. *Neuroscience* 13(3):627-43

Sarter M, Bruno JP. 1997. Cognitive functions of cortical acetylcholine: toward a unifying hypothesis. *Brain Res. Brain Res. Rev.* 23(1-2):28-46

Satoh S, Matsumura H, Hayaishi O. 1998. Involvement of adenosine A2A receptor in sleep promotion. *Eur. J Pharmacol.* 351(2):155-62

Satoh S, Matsumura H, Koike N, Tokunaga Y, Maeda T, Hayaishi O. 1999. Region-dependent difference in the sleep-promoting potency of an adenosine A2A receptor agonist. *Eur. J Neurosci.* 11(5):1587-97

Satoh S, Matsumura H, Suzuki F, Hayaishi O. 1996. Promotion of sleep mediated by the A2a-adenosine receptor and possible involvement of this receptor in the sleep induced by prostaglandin D2 in rats. *Proc. Natl. Acad. Sci. U. S. A* 93(12):5980-4

Sattin A, Rall TW. 1970. The effect of adenosine and adenine nucleotides on the cyclic adenosine 3', 5'-phosphate content of guinea pig cerebral cortex slices. *Mol. Pharmacol.* 6(1):13-23

Scammell T, Gerashchenko D, Urade Y, Onoe H, Saper C, Hayaishi O. 1998. Activation of ventrolateral preoptic neurons by the somnogen prostaglandin D2. *Proc. Natl. Acad. Sci. U. S. A* 95(13):7754-9

Scammell TE, Gerashchenko DY, Mochizuki T, McCarthy MT, Estabrooke IV, Sears CA, Saper CB, Urade Y, Hayaishi O. 2001. An adenosine A2a agonist increases sleep and induces Fos in ventrolateral preoptic neurons. *Neuroscience* 107(4):653-63

Schiffmann SN, Libert F, Vassart G, Dumont JE, Vanderhaeghen JJ. 1990. A cloned G protein-coupled protein with a distribution restricted to striatal medium-sized neurons. Possible relationship with D1 dopamine receptor. *Brain Res.* 519(1-2):333-7

Schoen SW, Ebert U, Loscher W. 1999. 5'-Nucleotidase activity of mossy fibers in the dentate gyrus of normal and epileptic rats. *Neuroscience* 93(2):519-26

Schoen SW, Graeber MB, Reddington M, Kreutzberg GW. 1987. Light and electron microscopical immunocytochemistry of 5'-nucleotidase in rat cerebellum. *Histochemistry* 87(2):107-13

Schwierin B, Borbely AA, Tobler I. 1996. Effects of N6-cyclopentyladenosine and caffeine on sleep regulation in the rat. *Eur. J Pharmacol.* 300(3):163-71

Sciotti VM, Van Wylen DG. 1993. Increases in interstitial adenosine and cerebral blood flow with inhibition of adenosine kinase and adenosine deaminase. *J Cereb. Blood Flow Metab* 13(2):201-7

Sherin JE, Elmquist JK, Torrealba F, Saper CB. 1998. Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus of the rat. *J Neurosci.* 18(12):4705-21

Sherin JE, Shiromani PJ, McCarley RW, Saper CB. 1996. Activation of ventrolateral preoptic neurons during sleep. *Science* 271(5246):216-9

Shram N, Netchiporouk L, Cespuglio R. 2002. Lactate in the brain of the freely moving rat: voltammetric monitoring of the changes related to the sleep-wake states. *Eur. J Neurosci.* 16(3):461-6

Stehle JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD, Reppert SM. 1992. Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. *Mol. Endocrinol.* 6(3):384-93

Steriade M. 1996. Arousal: revisiting the reticular activating system. *Science* 272(5259):225-6

Stewart DJ, MacFabe DF, Vanderwolf CH. 1984. Cholinergic activation of the electrocorticogram: role of the substantia innominata and effects of atropine and quinuclidinyl benzilate. *Brain Res.* 322(2):219-32

Strecker RE, Morairty S, Thakkar MM, Porkka-Heiskanen T, Basheer R, Dauphin, LJ, Rainnie DG, Portas CM, Greene RW, McCarley RW. 2000. Adenosinergic modulation of basal forebrain and preoptic/anterior hypothalamic neuronal activity in the control of behavioral state. *Behavioural Brain Research* 115(2):183-204

Svenningsson P, Nomikos GG, Ongini E, Fredholm BB. 1997. Antagonism of adenosine A2A receptors underlies the behavioural activating effect of caffeine and is associated with reduced expression of messenger RNA for NGFI-A and NGFI-B in caudate-putamen and nucleus accumbens. *Neuroscience* 79(3):753-64

Szymusiak R. 1995. Magnocellular nuclei of the basal forebrain: substrates of sleep and arousal regulation. *Sleep* 18(6):478-500

Szymusiak R, Alam N, McGinty D. 2000. Discharge patterns of neurons in cholinergic regions of the basal forebrain during waking and sleep. *Behav. Brain Res.* 115(2):171-82

Szymusiak R, Alam N, Steininger TL, McGinty D. 1998. Sleep-waking discharge patterns of ventrolateral preoptic/anterior hypothalamic neurons in rats. *Brain Res.* 803(1-2):178-88

Szymusiak R, McGinty D. 1986a. Sleep-related neuronal discharge in the basal forebrain of cats. *Brain Res.* 370(1):82-92

Szymusiak R, McGinty D. 1986b. Sleep suppression following kainic acid-induced lesions of the basal forebrain. *Exp. Neurol.* 94(3):598-614

Szymusiak R, McGinty D. 1989. Sleep-waking discharge of basal forebrain projection neurons in cats. *Brain Res. Bull.* 22(2):423-30

Thakkar MM, Delgiacco RA, Strecker RE, McCarley RW. 2003a. Adenosinergic inhibition of basal forebrain wakefulness-active neurons: a simultaneous unit recording and microdialysis study in freely behaving cats. *Neuroscience* 122(4):1107-13

Thakkar MM, Winston S, McCarley RW. 2003b. A1 receptor and adenosinergic homeostatic regulation of sleep-wakefulness: effects of antisense to the A1 receptor in the cholinergic basal forebrain. *J. Neurosci.* 23(10):4278-87

Thorn JA, Jarvis SM. 1996. Adenosine transporters. *Gen. Pharmacol.* 27(4):613-20

Ticho SR, Radulovacki M. 1991. Role of adenosine in sleep and temperature regulation in the preoptic area of rats. *Pharmacology, Biochemistry & Behavior* 40(1):33-40

Toppila J, Alanko L, Asikainen M, Tobler I, Stenberg D, Porkka-Heiskanen T. 1997. Sleep deprivation increases somatostatin and growth hormone-releasing hormone messenger RNA in the rat hypothalamus. *J. Sleep Res.* 6(3):171-8

Toppila J, Asikainen M, Alanko L, Turek FW, Stenberg D, Porkka-Heiskanen T. 1996. The effect of REM sleep deprivation on somatostatin and growth hormone-releasing hormone gene expression in the rat hypothalamus. *J. Sleep Res.* 5(2):115-22

Trussell LO, Jackson MB. 1985. Adenosine-activated potassium conductance in cultured striatal neurons. *Proc. Natl. Acad. Sci. U. S. A* 82(14):4857-61

Turner CP, Blackburn MR, Rivkees SA. 2004. A1 adenosine receptors mediate hypoglycemia-induced neuronal injury. *J. Mol. Endocrinol.* 32(1):129-44

Urade Y, Hayaishi O. 1999. Prostaglandin D2 and sleep regulation. *Biochim. Biophys. Acta* 1436(3):606-15

van Calker D, Muller M, Hamprecht B. 1978. Adenosine inhibits the accumulation of cyclic AMP in cultured brain cells. *Nature* 276(5690):839-41

van Calker D, Muller M, Hamprecht B. 1979. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* 33(5):999-1005

Van Wylen DG, Park TS, Rubio R, Berne RM. 1986. Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia. *J Cereb. Blood Flow Metab* 6(5):522-8

Virus RM, Djuricic-Nedelson M, Radulovacki M, Green RD. 1983. The effects of adenosine and 2'-deoxycoformycin on sleep and wakefulness in rats. *Neuropharmacology* 22(12A):1401-4

von Lubitz DK. 1999. Adenosine and cerebral ischemia: therapeutic future or death of a brave concept? *Eur. J. Pharmacol.* 371(1):85-102

Ward H, Baldwin D, Wang T, Warner H, Seymour K, Marquardt C, McFalls E, Foker JE. 1998. Ion-exchange column chromatographic method for assaying purine metabolic pathway enzymes. *Journal of Chromatography* B, Biomedical Sciences & Applications. 707(1-2):295-300

White TD. 1977. Direct detection of depolarisation-induced release of ATP from a synaptosomal preparation. *Nature* 267(5607):67-8

White TD. 1996. Potentiation of excitatory amino acid-evoked adenosine release from rat cortex by inhibitors of adenosine kinase and adenosine deaminase and by acadesine. *Eur. J Pharmacol.* 303(1-2):27-38

Wolf HK, Buslei R, Schmidt-Kastner R, Schmidt-Kastner PK, Pietsch T, Wiestler OD, Bluhmke I. 1996. NeuN: a useful neuronal marker for diagnostic histopathology. *J. Histochem. Cytochem.* 44(10):1167-71

Yamada Y, Goto H, Ogasawara N. 1980. Purification and properties of adenosine kinase from rat brain. *Biochim. Biophys. Acta* 616(2):199-207

Yao SY, Ng AM, Muzyka WR, Griffiths M, Cass CE, Baldwin SA, Young JD. 1997. Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. *J. Biol. Chem.* 272(45):28423-30

Zhu PJ, Krnjevic K. 1994. Endogenous adenosine deaminase does not modulate synaptic transmission in rat hippocampal slices under normoxic or hypoxic conditions. *Neuroscience* 63(2):489-97

Zimmermann H. 1992. 5'-Nucleotidase: molecular structure and functional aspects. *Biochem. J.* 285 (Pt 2):345-65

Zimmermann H. 1996. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. *Prog. Neurobiol.* 49(6):589-618

Zimmermann H, Braun N. 1999. Ecto-nucleotidases--molecular structures, catalytic properties, and functional roles in the nervous system. *Prog. Brain Res.* 120:371-85

Zimmermann H, Vogel M, Laube U. 1993. Hippocampal localization of 5'-nucleotidase as revealed by immunocytochemistry. *Neuroscience* 55(1):105-12