From Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

Adenosine Receptors and Stress

- studies using methylmercury, caffeine and hypoxia

Olga Björklund

Karolinska Institutet

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Picture of the brain was kindly provided by Ana Gruber.

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None has yet drank a honey´d draught
Unmixed with cup of bitter gall,
And cup of gall for honey equally doth call,
That so, the mixture one may easier drink.

The Mountain Wreath
P.P. Nyegosh, Bishop of Montenegro

To the memory of my beloved mother Ljiljana

Abstract

Brain development is a precisely organized process that can be disturbed by various stress factors present in the diet (e.g. exposure to xenobiotics) as well as insults such as decreased oxygen supply. The consequent adverse changes in nervous system function may not necessarily be apparent until a critical age when neurodevelopmental defects may be unmasked by a subsequent challenge. Adenosine and its receptors (AR) (A_1, A_{2A}, A_{2B} and A_3) which participate in the brain stress response, are potential targets for neuroprotective drugs. The aim of work presented in this thesis was to investigate a possible role of AR in modulating the effects of developmental alterations caused by exposure to three stressors, the fish pollutant methylmercury (MeHg), the adenosine antagonist caffeine or decreased oxygen supply, before and/or after birth. To address these questions we used both AR ligands and genetically engineered mice with altered expression of AR.

MeHg bioaccumulates in fish, shellfish and sea mammals and is neurotoxic especially to the developing brain since it readily crosses the placenta, blood brain barrier and reaches the breast milk. Exposure of dams to very low levels of MeHg (0.2 mg/l in the drinking water) from gestational day 7 to lactational day 7 caused behavioral alterations in offspring that were sex and age-dependent. A decreased response to psychostimulant amphetamine in 2-month-old males perinatally-treated with MeHg pointed to disturbances in dopaminergic functions. Presence of caffeine, that partially blocks A_1R and $A_{2A}R$, attenuated the locomotor alterations induced by MeHg. The basal and amphetamine-stimulated locomotor activity of A_1R knock-out (KO) and $A_{2A}R$ KO mice was relatively unaffected by treatment with MeHg, suggesting that these receptors are involved in the protective effect of perinatal caffeine against MeHg toxicity during gestation and lactation.

Although the health consequences of ordinary caffeine consumption are probably minor, there are lingering concerns about caffeine intake during pregnancy and lactation. Modest maternal caffeine intake (0.3 g/l in the drinking water) in our studies induced long-lasting changes in the mouse offspring, evidenced by an increased motor activity and an amplified response to psychostimulants (cocaine and amphetamine) in adult age, irrespective of sex. Similar alterations were observed in A_1R KO mice, as well as mice with partial deletion of A_1R gene (A_1R heterozygote mice) suggesting that adenosine A_1R are involved in the alterations triggered by caffeine exposure during development. Furthermore, if the mother partially lacked A_1R the offspring displayed more hyperactivity and responded more strongly to cocaine stimulation as adults than did mice of a WT mother, regardless of their genotype. Our results suggest that perinatal caffeine, by acting on A_1R in the mother, is responsible for long-lasting behavioral changes in the offspring.

Characterization of the A_3R KO mice with regard to motor activity, response to psychostimulants, and susceptibility to neurotoxic challenge suggested that genetic elimination of this receptor has broad effects in early development. Despite sparse distribution of A_3R in neurons, life-long deletion of the A_3R caused increased basal motor activity in A_3R KO mice, decreased response to drugs that do not primarily act via A_3R such as caffeine and cocaine, reduced activity upon natural stimuli (darkness), and greater susceptibility of female mice to MeHg toxicity.

Our in vitro studies focused on identifying the intracellular pathways activated by oxygen deprivation and cobalt chloride ($CoCl_2$) exposure in primary cultures of mouse astrocytes. These cells are particularly important in hypoxia because they immediately activate an array of protective mechanisms that can decrease neuronal injury. Cobalt caused numerous toxic effects in primary glial cells reminiscent of events caused by oxygen deprivation such as activation of HIF-1 pathway with increased expression of HIF-1α regulated genes, decreased ATP levels, mitochondrial damage and cell death (apoptosis and necrosis). Thus $CoCl_2$ appears to be a useful tool for mimicking some aspects of oxygen deprivation in astrocytes.

Our preliminary in vitro data suggest that endogenous adenosine and adenosine analogues acting on A_1R and A_3R exert cytoprotective effects against oxygen deprivation and $CoCl_2$ induced toxicity in primary mouse astrocytes. These observations were supported by our in vivo study using Rice-Vannucci hypoxic ischemia model, where genetic elimination of A_1R, $A_{2A}R$ and A_3R caused aggravated brain damage in different maturational stages.

Thus, adenosine receptors seem to be important during development. Depending on the subtype they can influence the effect of xenobiotics and protect against ischemic conditions.

Key words: adenosine, adenosine receptors, knock-out mice, methylmercury, caffeine, hypoxia, cobalt chloride

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List of Publications

1. Björklund O, Kahlström J, Salmi P, Ögren SO, Vahter M, Chen JF, Fredholm BB, Daré E.
The effects of methylmercury on motor activity are sex- and age-dependent, and modulated by genetic deletion of adenosine receptors and caffeine administration.
Toxicology 241 (2007), 119-133

2. Björklund O, Kahlström J, Salmi P, Fredholm BB.
Perinatal caffeine, acting on maternal adenosine A_1 receptors, causes long-lasting behavioral changes in mouse offspring.
Submitted to European Neuropsychopharmacology

3. Björklund O, Halldner-Henriksson L, Yang JN, Eriksson MT, Jacobson MA, Daré E, Fredholm BB.
Decreased response to caffeine and other psychostimulants in A_3 adenosine receptor knock-out mice
Submitted to Physiology & Behavior

4. Karovic O, Tonazzini I, Rebola N, Edström E, Lövdahl C, Fredholm BB, Daré E.
Toxic effects of cobalt in primary cultures of mouse astrocytes. Similarities with hypoxia and role of HIF-1α.
Biochemical Pharmacology 73 (2007), 694-708

Additional relevant publications

Daré E, Schulte G, Karovic O, Hammarberg C, Fredholm BB.
Modulation of glia cell functions by adenosine receptors.
Physiology & Behavior 92 (2007), 15-20

Björklund O, Shang M, Tonazzini I, Daré E, Fredholm BB.
Adenosine A_1 and A_3 receptors protect astrocytes from hypoxic damage.
Submitted to Biochemical Pharmacology

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Abbreviations

AC	adenylyl cyclase
AR	adenosine receptors
AIF	apoptosis inducing factor
BBB	blood brain barrier
$[Ca^{2+}]_i$	intracellular calcium
cAMP	cyclic adenosine3´,5´- monophosphate
Cl-IB-MECA	chloro-N^6-(3-iodobenzyl)-5´-N-methyl-carboxamidoadenosine
$CoCl_2$	cobalt chloride
DNA	deoxy-ribonucleic acid
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
E	embryonal day
ERK 1/2	signal-regulated protein kinase-1/2
G protein	guanosine triphosphate-binding protein
HIF-1	hypoxia-inducible factor 1
HO-1	heme oxygenase 1
HSP	heat shock protein
iNOS	inducible nitric oxide synthase
KO	knock-out
LDH	lactate dehydrogenase
LTP	long term potentiation
MCAO	middle cerebral artery occlusion
MeHg	methylmercury
MPTP	mitochondrial permeability transition pore
NMDA	N-methyl-D-aspartic acid
NECA	5´-N-ethylcarboxamidoadenosine
mRNA	messenger ribonucleic acid
$\Delta\Psi_m$	mitochondrial membrane potential
PND	postnatal day
PARP	poly(ADP-ribose) polymerase
ROS	reactive oxygen species
SCH 58261	5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
TNFα	tumor necrosis factor alpha
TUNEL	terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling
TLC	thin layer chromatography
WT	wild type

1 Introduction

Brain development occurs in different phases and each developmental stage is reached according to a tightly regulated program involving cell proliferation, migration, differentiation, maturation and apoptosis. To function properly, the brain requires a precise number of cells with the correct characteristics in the right place at the right time (Rodier 1994). An insult that interferes with any of these mechanisms can have adverse effects on the development and subsequent function of the nervous system. The consequences of developmental damage may not become apparent until later in life, when a neurodevelopmental defect may be unmasked by a challenge (Reuhl 1991). By far the most important factor is nutrition: the availability and utilization of food are prime determinants of growth and development. The mother's consumption of different substances can influence the outcome for the infant (Rodier 1994). Exposure to xenobiotics, such as drugs and pollutants, and other stress inducers, e.g. decreased oxygen supply, before and after birth has been identified as one of the key risk factors for neurodevelopmental disorders, including cerebral palsy, epilepsy, learning disabilities, autism, dyslexia, attention-deficit hyperactivity disorder, decreased intelligence and mental retardation (Grandjean and Landrigan 2006; Volpe 2001).

1.1 Brain cells

The brain and spinal cord are made up of different types of cells, including neurons, glial and ependymal cells. Neurons process and transmit information through the nervous system. Glia consists of astrocytes, microglia, oligodendrocytes, Schwann cells and satellite cells. Glial cells make up some 90 percent of the brain cells and are responsible for a wide variety of functions, such as neuronal support and maintenance, myelin production and regulation of synaptic transmission by release of glutamate, D-serine and adenosine (Haydon and Carmignoto 2006).

Astrocytes are the predominant glial cell type and play an important role in physiological and pathological conditions. They take up transmitters and maintain extracellular ion levels, actively control synaptogenesis, synapse number, synapse function and plasticity. Oligodendrocytes and Schwann cells provide neurons with insulation and trophic support, especially during development, and they determine the structure and electrical properties of

axons (Barres 2003). Microglia are specialized macrophages acting as the first and main form of active immune defense in the CNS. Microglia are constantly on the move, scanning the CNS for damaged neurons, plaques, and infectious agents and are extremely sensitive even to small pathological changes. They react to chemical and structural changes in their environment by altering their morphological and biochemical properties, changing from resting to activated phagocytosing phenotype (Daré et al., 2007).

The period when the brain grows most rapidly, known as the brain growth spurt, is characterized by a proliferation of astrocytes and oligodendrocytes. This stage of development has been shown to be very sensitive to toxic insults, suggesting that glial cells also are a target for neurotoxicants (Aschner and Allen 2000). In response to the brain injury, astrocytes undergo morphological changes that are characterized by hypertrophy and an increase in proteins such as glial fibrillary acidic protein and adenosine kinase (Boison 2006). Because of their close proximity to the capillaries, changes in astrocytes are among the earliest events following ischemia and reperfusion (Panickar and Norenberg 2005). Astrocytes immediately activate an array of mechanisms that decrease neuronal injury: they produce trophic factors, regulate transmitter and ion concentrations and remove excess glutamate from the extracellular milieu (Aschner et al., 2002). Thus, astrocytes have a direct impact on neuronal survival and synaptic function, as well as neurogenesis and neural repair (Aschner et al., 2002; Swanson et al., 2004).

1.2 Cell stress and damage

Cellular homeostasis in tissues is maintained by three mechanisms: proliferation, differentiation and death. Stressful stimuli activate a number of auto-protective mechanisms including the production of heat shock proteins, anti-inflammatory cytokines, growth factors and endogenous antioxidants (Leker and Shohami 2002). However, if the insult is persistent and severe the cell will eventually make a “decision” to die by either necrosis or apoptosis. Apoptosis is an energy demanding, regulated process characterized by activation of signaling pathways leading to specific cleavage of proteins and DNA, condensation of the nucleus, cell shrinkage, phosphatidylserine flips from the inner to the outer plasma membrane and fragmentation into apoptotic bodies that can be phagocytosed (Orrenius et al., 2003). Unlike the apoptotic process, during which the plasma membrane is intact, necrosis involves swelling

of the cell and its organelles resulting in cell lysis and risk of tissue inflammation due to leakage of the intracellular content (Orrenius et al., 2003).

Signaling pathways that lead to apoptosis are divided into extrinsic and mitochondrial pathways (Fig. 1). The latter are characterized by mitochondrial alterations followed by release of mitochondrial factors such as cytochrome c, apoptosis inducible factor (AIF), endonuclease G (EndoG) etc. The extrinsic pathway is instead triggered by ligand binding to members of the death receptor family (Gupta 2001).

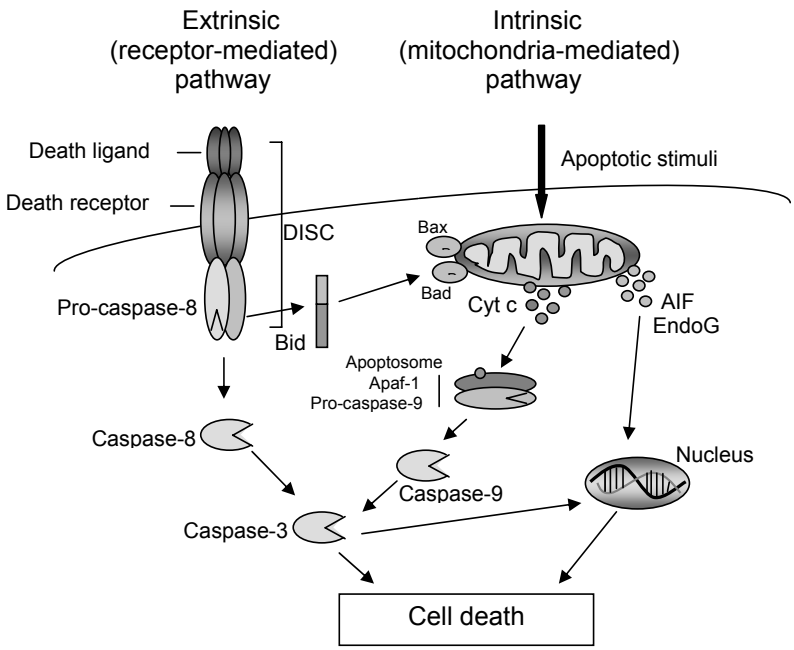

Fig. 1. Signaling pathways leading to apoptosis

The extrinsic pathway is induced by the activation of cell-surface death receptors into the death-inducible signalling complex (DISC) resulting in the activation of pro-caspase-8. Depending on the cell type, this caspase can next activate caspase-3, which cleaves target proteins and leads to apoptosis, or stimulate a pro-apoptotic Bcl2-family member, Bid, to translocate and oligomerize with Bax and/or Bak present on mitochondria. This is followed by the release of factors, e.g. cytochrome c, which forms a cytosolic apoptosome complex with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 which activates caspase-3.
The intrinsic pathway is activated by signals acting directly or indirectly on the mitochondria, resulting in either caspase-mediated cell death via formation of apoptosome complex, or in caspase-independent apoptosis involving the release from mitochondria and translocation to the nucleus of proteins such as AIF and EndoG.
Modified from Orrenius et al., 2003.

1.3 Neurotoxic challenge

1.3.1 Methylmercury (MeHg)

Methylmercury is a neurotoxic pollutant. Emission of mercury occurs naturally mainly from the earth's crust, but also from anthropogenic sources, including mining, chloroalkali manufacturing and combustion of fossil fuels. MeHg is formed by microbial methylation of inorganic mercury in sediments and soil, and is bioaccumulated and biomagnified in the aquatic food chain. Contaminated fish, shellfish and sea mammals represent the dominant source of human exposure.

Dietary MeHg is almost totally absorbed in gastrointestinal tract and rapidly enters the bloodstream; it easily crosses the blood-brain barrier (BBB) making the brain a primary target organ (Clarkson 1997). Two of the most well-known catastrophes involving mercury occurred in Minamata, Japan, and Iraq, where thousands of people were exposed to high levels of MeHg (Bakir et al., 1973; Takeuchi et al., 1959). The studies on these populations showed adverse effects on CNS both in adults and infants. However, the fetus and the infant appeared to be particularly susceptible to MeHg neurotoxicity, as the compound readily crosses the placenta and is excreted in milk (Clarkson 1997). Consequently, recommendations that pregnant women limit their intake of fish have been introduced in several countries (JECFA 2003).

Neurological alterations in humans can occur even after exposure to fairly low levels of MeHg (Grandjean et al., 1997). It exerts adverse effects on human cognition and behavior (Clarkson and Magos 2006). Epidemiological studies have shown that MeHg levels were inversely associated with children's scores on neuropsychological tests in some populations with high fish consumption in New Zealand and Faeroe Islands (Davidson et al., 2004; Grandjean et al., 1997). The children examined displayed deficits in motor skills, attention and verbal tests at the age of 14 years, indicating that the damage induced by methylmercury is probably permanent (Debes et al., 2006). Conversely, cohort studies from the Seychelles showed no clear correlation between the MeHg exposure and adverse effects, although some authors have suggested that adverse effects may become evident in higher cognitive functions that develop with age (Davidson et al., 2004; Debes et al., 2006; Landrigan et al., 2005). Despite major efforts to reduce the use and emission of mercury in the environment, MeHg contamination remains a persistent problem. Since fish is an important source of nutrients and

crucial part of many people's diet, it is important to keep controlling mercury levels. Mean total mercury content in fish and seafood in European countries was estimated to 109 ± 845 µg/kg (EFSA 2004) with the variability in the contents depending on the region and type of fish consumed. However, the mean consumption in different countries across Europe differs greatly and exposures can vary by a factor 10. The mean weekly intake for a 60 kg adult varies from 0.1 µg/kg (the Netherlands) to 1 µg/kg body weight (Norway) (EFSA 2004) and is below the provisional tolerably weekly intake for methylmercury set by the Joint Food and Agriculture Organization of the United Nations/World Heath Organization Expert Committee on Food Additives (JECFA) (1.6 µg/kg body weight per week) (JECFA 2003). An evaluation by the (U.S.) National Research Council (NRC) established an intake limit of 0.7 µg/kg body weight per week (NRC 2000).

Experimental studies in rats and mice on the effects of low maternal exposure to MeHg report that the offspring show alterations in locomotor activity, increased sensitivity to behavioral effects of amphetamine such as hyperactivity, stereotypic and self-injurious behavior (Daré et al., 2003; Gímenez-Llort et al., 2001; Rasmussen and Newland 2001). A predisposition to depression-like behavior, learning disabilities, impairment of reference and working memory were also observed in rodent model, supporting the epidemiological data on humans (Clarkson and Magos 2006; Kakita et al., 2000; Onishchenko et al., 2007).

The in vitro and in vivo studies point to several mechanisms in selected brain regions (e.g. cerebellum) as specific targets for MeHg. Methylmercury is one of the agents that could lead to neuronal migration and synaptogenesis failure after perinatal exposure (Komuro and Rakic 1998; Rodier 1994). MeHg can alter dopamine release or modify the expression of dopamine receptors, e.g. reduce D_2 receptor binding in caudate putamen (Daré et al., 2003; Faro et al., 2002; Gímenez-Llort et al., 2001). A decrease in monoamine neurotransmitter release as well as an increase in glutamate efflux have been observed after MeHg treatment (Aschner et al., 2000; Castoldi et al., 2006). Increased formation of reactive oxygen species (ROS) as well as reduced oxidative defense (decreased levels of glutathione, which is involved in free radical scavenging), uncontrolled release of intracellular calcium from damaged mitochondria and cytoskeletal alterations caused by microtubule dysfunction (MeHg binds to tubulin via SH groups) are the prime mechanisms by which MeHg exposure can lead to cell stress and death (for ref see Johansson et al., 2007a). Very low levels of MeHg can also inhibit spontaneous neuronal differentiation of neural stem cells (Tamm et al., 2006).

It has been emphasized that neonatal exposure to low doses of toxic agents and dietary components (such as selenium which is known to protect from MeHg toxicity) can influence how the adult animal reacts to the administration of xenobiotics, implying that the differences in adult susceptibility to environmental pollutants need not be only an inherited condition (Fischer et al., 2007; Reed et al., 2008).

1.3.2 Ischemia

Cerebral ischemia may result from a variety of causes that impair cerebral blood flow and deprive the brain of both oxygen and glucose. When persistent, such impairment in blood flow eventually leads to cell death (Leker and Shohami 2002). Interestingly, the most deleterious consequences of ischemia are observed after the restoration of oxygen supply, in the so called reperfusion phase. During this period injury to the BBB, a surge of free radical production and an inflammatory response (increased release of pro-inflammatory cytokines) occur. There is a vast literature on the mechanisms of neuronal damage following experimental ischemia and reperfusion (Leker and Shohami 2002). The experimental studies have highlighted several therapeutic possibilities, but frustratingly, few of these have been successful in clinical trials (Green 1997).

1.3.2.1 Oxygen deprivation

Hypoxia is a common occurrence in everyday life, arising both during normal activities such as breath holding, climbing to altitude and birth and in pathological states like sleep apnea, some types of congenital heart disease, or pulmonary disease, myocardial infarction and stroke. The transcriptional regulator hypoxia-inducible factor 1 (HIF-1), consisting of two subunits (HIF-1α and HIF-1β), is an essential mediator of oxygen homeostasis in the cell. During normoxic conditions HIF-1α is degraded whereas in hypoxia the α-subunit is stabilized by blocked ubiquitination and proteasomal degradation (Fig. 2). Increased HIF-1α protein stability and activity of HIF-1 complex, regulates transcription of a set of hypoxia-responsive genes (e.g. glycolytic enzymes, erythropoietin and heat shock proteins) that promote cell survival and recovery after hypoxia (Semenza 2000). However, there are also reports showing involvement of HIF-1 in transcription of some pro-apoptotic factors that can lead to cell death (Bruick 2000). Moreover, the lack of oxygen inhibits mitochondrial

metabolism and activates the inefficient anaerobic metabolism of glucose, causing a local rise in lactate production and fall in pH with consequent acidosis (Barber et al., 2003).

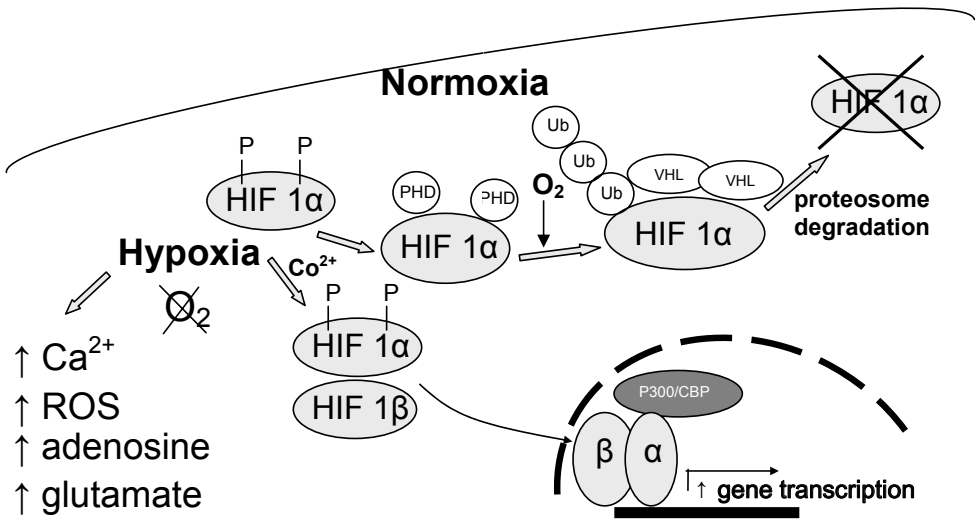

Fig. 2. Stabilization of HIF-1α and other effects of hypoxia

Under normoxic conditions, prolyl hydroxylation of HIF-1α by prolyl-hydroxylase-domain-containing proteins (PHD) leads to binding of von-Hippel-Lindau tumor suppressor protein (VHL) and recruitment of ubiquitin-ligase complex (Ub). This results in the ubiquitination and degradation of HIF-1α by the 26S proteosome. Hydroxylase activity is inhibited when the concentration of oxygen is low. During hypoxia PHDs are also targeted for proteasome-mediated degradation by the ubiquitin-ligase complex. In the absence of PHD activity HIF-1α is stabilized and can activate expression of its target genes involved in glycolysis, angiogenesis, erythropoiesis and cell survival. Hypoxia also leads to increase of intracellular calcium, ROS formation, adenosine and glutamate levels in many cell types.

The energy-dependent functions of cell membranes to maintain ion homeostasis become progressively impaired during hypoxia: K^+ leaks out of the cell, Na^+ and water as well as Ca^{2+} enter the cell, where Ca^{2+} impairs mitochondrial function (Barber et al., 2003). All these changes in ion homeostasis lead to self-promoting cascade of pathophysiological events such as excitotoxicity and energy depletion, free radicals formation, membrane depolarisation, inflammation and apoptosis (Endres and Dirnagl 2002).

1.3.2.2 The hypoxia-mimic cobalt

Some of the characteristic effects of cobalt are thought to be mediated by interaction with the cellular oxygen-sensing machinery. Like low oxygen tension, cobalt at normoxic

conditions is able to stabilize the α-subunit of HIF-1, Fig. 2 (Epstein et al., 2001). By increasing HIF-1α levels in the brain cobalt can protect the brain against hypoxic ischemia 24 h later and for this reason it can be used as a preconditioning agent (Jones and Bergeron 2001; Ran et al., 2005). Cobalt can cause DNA fragmentation (Zou et al., 2001), activation of caspases (Zou et al., 2002), increased production of reactive oxygen species (ROS) (Araya et al., 2002; Zou et al., 2001), augmented phosphorylation of mitogen activated protein (MAP) kinases (Yang et al., 2004; Zou et al., 2002) and elevated levels of p53 (Chandel et al., 2000). Because of the similarities with some of the cell death pathways induced by hypoxia cobalt is used for in vitro studies as a mimic of hypoxia. The data available in the literature indicate that cobalt is cytotoxic to many cell types, including neural cells (Olivieri et al., 2001; Wang et al., 2000; Yang et al., 2004) and can induce cell death by apoptosis and necrosis (Huk et al., 2004).

Cobalt is an essential element for humans, being necessary for the formation of vitamin B12 (hydroxycobalamin). The human body contains about 1–2 mg of cobalt (Elinder et al., 1986); cobalt deficiency causes anemia and increases the risk of developmental abnormalities and growth failure in infants (Stabler and Allen 2004). However, excessive levels of cobalt can be detrimental to the organism. The route of exposure is frequently dermal or via inhalation (Jensen and Tuchsen 1990) that occurs mostly in industrial refining. Cobalt toxicity includes cardiomyopathy (Kesteloot et al., 1968), adverse pulmonary effects (Wehner et al., 1977) and carcinogenicity (Heath 1956). This heavy metal is also suspected to cause neurotoxic effects and lead to memory deficit (Jordan et al., 1997).

1.4 Metabolism and functions of adenosine

Adenosine is an endogenous substance present in all cells and body fluids. Concentrations of adenosine reflect the metabolic state and regulate biological processes throughout the body. Adenosine controls many brain functions in physiological and pathophysiological conditions and has potent neuroprotective properties (Fredholm et al., 2005a). This neuromodulator plays a role in myelination, sleep, ischemia, epilepsy, pain, Alzheimer's disease, Parkinson desease, Huntington's disease and adddiction (Fig. 3).

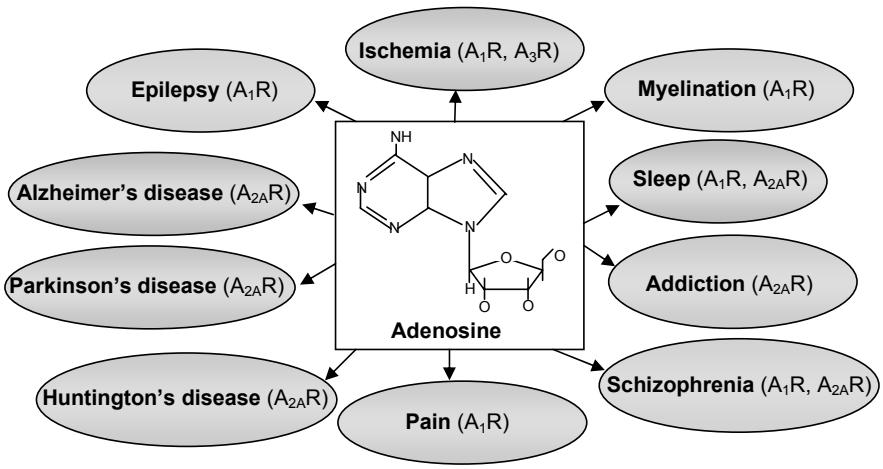

Fig. 3. Involvement of the adenosine system in brain. Modified from Boison D 2006.

Under physiological conditions generation of adenosine occurs both intracellularly and extracellularly. The intracellular and extracellular pools of adenosine are tightly regulated by bidirectional equilibrative and concentrative nucleoside transporters (Fredholm et al., 2005a). Adenosine is produced intracellularly by either dephosphorylation of adenosine 5'phosphates (ATP, ADP, AMP) by 5´-nucleotidase or by hydrolysis of S-adenosylhomocysteine (Fredholm et al., 2005b). Extracellular adenosine can be formed by rapid hydrolysis of nucleotides such as ATP via a cascade of ectonucleotidases (e.g. CD39 and CD73) (Cunha 2001; Zimmermann 2000). Resting adenosine concentrations in the brain are normally kept in the range of 25-250 nM (Dunwiddie and Masino 2001) primarily by phosphorylation to AMP catalyzed by intracellularly located adenosine kinase or degradation to inosine by adenosine deaminase (which is present both intra- and extracellularly), a process with several fold lower affinity (Fig. 4) (Lloyd and Fredholm 1995). Inosine is further degraded to the stable end-product uric acid and both metabolites have potent anti-inflammatory and neuroprotective effects (Hasko et al., 2005).

During metabolic stress the levels of extracellular adenosine can increase 30-100 times (to 10–50 μM) in part due to the down-regulation of adenosine kinase in astrocytes, but also by astrocytic activity-dependent release of ATP and its extracellular degradation to adenosine: this leads to heterosynaptic depression (Boison 2006). Thus, whenever intracellular levels of adenine nucleotides fall as a result of excessive energy use, the intracellular levels of adenosine will rise dramatically (Rudolphi et al., 1992). In addition, regulated release of ATP,

e.g. from astrocytes, can cause high local concentrations of adenosine (Boison 2006). Variations in adenosine release may play a role in controlling blood flow, matching it to the metabolic needs of the tissues. The protective effect of adenosine is based on its ability to inhibit cell functions under stressful conditions (e.g. hypoxia/ischemia, inflammation, trauma, excessive neuronal firing) and thus minimize the metabolic requirements of cells (Fredholm et al., 1999). That is why adenosine has been termed a "retaliatory metabolite" (Newby 1984).

Several mechanisms have been proposed to explain why adenosine protects nervous tissue against hypoxia and ischemia. These include adenosine's ability to dilate blood vessels and thereby increase blood flow, to reduce the release of neurotransmitters, to inhibit NMDA-receptor-mediated glutamate responses, to inhibit neutrophil accumulation in vessels after ischemia, to exert an antioxidant effect, or to induce hypothermia which is a neuroprotective factor in itself (Fredholm 1996).

1.5 Adenosine receptors

Adenosine is known to regulate normal functions (sleep and arousal, locomotion, anxiety, cognition, memory), but it is also active under pathophysiological conditions when it can depress neuronal activity, modulate pain, and reduce epilepsy. These effects appear to be mediated by adenosine receptors (AR) (A_1, A_{2A}, A_{2B} and A_3) which belong to the superfamily of G protein-coupled receptors and contain the typical seven transmembrane domains with an extracellular N-terminus and an intracellular C-terminus. AR were defined in the 1970's, based on their effects on adenylyl cyclase (AC), antagonism by methylxanthines, different affinity for adenosine and the potency of selective agonists and antagonists (Fredholm et al., 1994). AR are targets for the most widely used psychostimulant, caffeine, which produces effects by antagonizing those of adenosine at A_1 and A_{2A} receptors (Fredholm et al., 1999).

AR interact with either inhibitory G_i proteins (A_1R, A_3R) or stimulatory G_s/G_{olf} proteins ($A_{2A}R$, $A_{2B}R$). G_i and G_s mediate signal transduction via AC by decreasing or increasing, respectively, intracellular levels of cyclic AMP (cAMP) (Fredholm et al., 2005b). Downstream events appear to be dependent on the AR subtype and on the cell type. Major signal transduction routes are the activation of protein kinase A by cAMP, the stimulation of

the mitogen-activated protein kinase (MAPK), elevation of intracellular Ca^{2+} concentration and the activation of phospholipase C (Fredholm et al., 2000).

1.5.1 Adenosine receptors in the brain

All of the four AR subtypes are found in the brain, although they are differentially expressed in different types of cells (for ref see Fredholm et al., 2001a). A_1R show high expression throughout the brain. The $A_{2A}R$, on the other hand, are very abundant in basal ganglia, but are expressed only at low level in other brain regions. The distribution of $A_{2B}R$ and A_3R is less well characterized. They appear to be widespread in the brain, although at low density (Fig. 4).

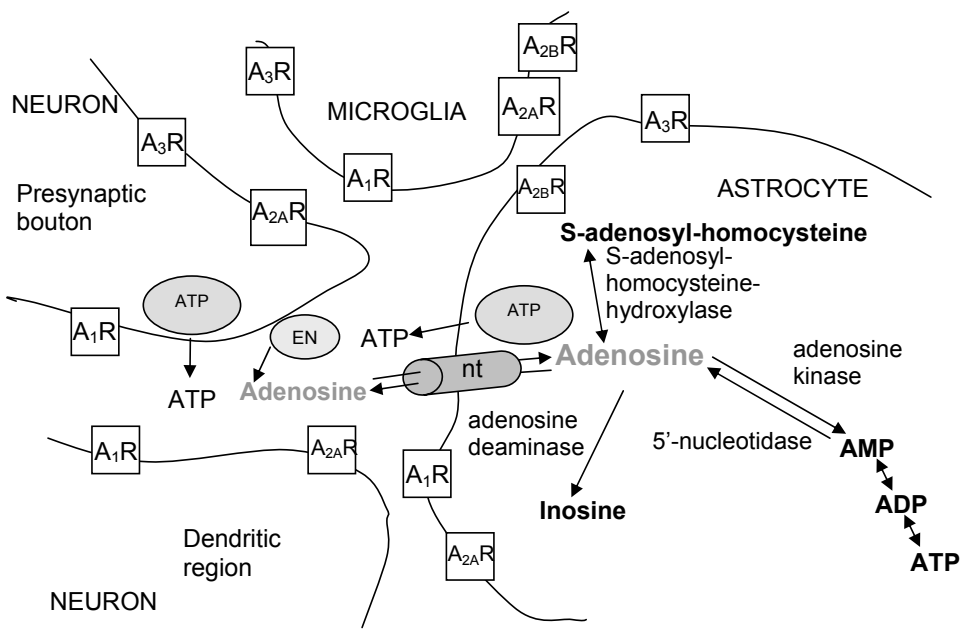

Fig. 4. Pathways of adenosine metabolism in brain.

Under physiological conditions adenosine concentrations depend on adenosine kinase and 5'nucleotidase actions. Alternative metabolic routes of adenosine involve either the reversible hydrolysis of S-adenosylhomocysteine by S-adenosyl-homocysteine hydroxylase or the deamination of adenosine to inosine by adenosine deaminase. Intra and extracellular concentrations of adenosine equilibrate rapidly via nucleoside transporters expressed ubiquitously (nt, shown only in astrocytes in the figure). ATP that is released from either neurons or astrocytes is cleaved extracellularly by ectonucleotidases (EN), and this contributes to the formation of adenosine. Adenosine exerts its actions by binding to A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. Modified from Boison 2006.

Despite having neuroprotective properties adenosine may under some circumstances contribute to neurotoxicity (de Mendonca et al., 2000). These opposite phenomena might be

due to the activation of various subtypes of adenosine receptors which exert different actions depending on the cellular location (Fredholm et al., 2001a).

Genetically engineered mice with altered expression of adenosine receptors are important tools to study a potential dysfunction of the adenosine system in normal physiology and disease. Although resulting phenotype can represent a developmental effect and there is a lack of tissue specificity, these limitations can be overcome by combining the studies with adenosine receptor ligands as well as by creating conditional knock-outs (Fredholm et al., 2005b).

1.5.1.1 A_1R

A_1R are abundant in most areas of the brain with the highest levels in the hippocampus, cerebellum and cerebral cortex (for ref see Fredholm et al., 2005a). They are mainly pre-synaptically located though some can be found in the nerve-cell bodies (Fredholm et al., 2005a). A_1R are also found on astrocytes, microglia and oligodendrocytes (Fredholm et al., 2005a). A_1R mRNA is detected from embryonal day (E14) and receptor protein continues to increase postnatally as does the coupling of the receptor to G proteins (postnatal day 5-15) (Åden et al., 2000; Fredholm et al., 2005a; Weaver 1996).

Physiological concentrations of adenosine can activate A_1 receptors (Dunwiddie and Masino 2001). Therefore, small increases in the ambient concentrations of adenosine can enhance inhibitory A_1R mediated functions (stimulation of K^+ conductance, inhibition of NMDA action, reduced Ca^{2+} entry and phospholipase C activation) (for ref see Fredholm et al., 2005a). As a consequence, the release of various neurotransmitters, in particular glutamate, is inhibited by presynaptic A_1R while the activation of A_1R at postsynaptic sites leads to membrane hyperpolarization with subsequent inhibition of neuronal firing (Fredholm et al., 2005a). Adenosine acting on A_1R can increase production of NGF, TGF-β1, IL-6 and S100β, postulated neuroprotective substances (Ciccarelli et al., 1999; Schwaninger et al., 1997). This could contribute to neuroprotection after ischemic insult, attenuated neuroinflammation and prevention of epileptic seizures (for ref see Fredholm et al., 2005a).

Functional data indicate interactions between dopamine D_1 and A_1R, which are colocalized in medium-sized striatal neurons, where they regulate motor activity (Ferre et al., 1994). A_1R agonists induce sleep and sleep like EEG whereas antagonists reduce sleep (Fredholm et al., 2005a).

A_1R KO mice

Studies on A_1R knock-out mice have helped in elucidating many of the functions of A_1R. Two lines of A_1R KO mice have been generated, one 129/OlaHsd × C57BL/6 and the other 129/SvJ × C57BL/6 (for ref see Fredholm et al., 2005b). These mice develop normally, have a normal or slightly higher body temperature, though reduced survival rates and muscle strength as well as increased body weight after five months of age (Gimenez-Llort et al., 2002; Johansson et al., 2007b; Yang et al., 2007). A_1R KO mice have higher heart rate, blood pressure, Na^+ and fluid excretion than the corresponding wild type mice (Brown et al., 2006; Yang et al., 2007).

Mice lacking A_1R do not respond to the analgesic effects of adenosine analogues and show increased nociceptive response compared to the wild types (Fredholm et al., 2005b). They also exhibit increased anxiety and aggressiveness, decreased habituation to the new environment, and no differences in spatial reference and working memory. They represent an important tool for revealing the relative importance of A_1R in the behavioral actions of caffeine (Fredholm et al., 2005b). Studies on A_1R knock-out mice revealed that A_1R facilitates caffeine effects on motor activity, although they do not play a crucial role in the motor depressant effects of high doses of caffeine (Halldner et al., 2004). Long term potentiation (LTP) was greatly reduced in the mossy fiber pathways in hippocampal slices from A_1R KO mice providing evidence that adenosine via A_1R augments LTP (Moore et al., 2003). Studies on locomotor activity in A_1R KO mice report everything from no difference, to lower or higher activity and these observations seem to depend on the strain and sex of mice, methodology and the phase of the light-dark cycle (Gimenez-Llort et al., 2002; Johansson et al., 2001; Yang et al., 2007). Even though A_1R are implicated in sleep regulation, sleep was unaltered in A_1R KO mice and it appeared that A_1R are not important in caffeine's ability to induce wakefulness (Fredholm et al., 2005a; Huang et al., 2005).

As stated earlier, adenosine levels are rapidly elevated following acute insult, providing tonic adenosine-mediated inhibition, seizure control and neuroprotection in WT, but not in A_1R KO animals (Fedele et al., 2006; Johansson et al., 2001). Although hippocampal slices taken acutely from adult mice do show reduced functional recovery from hypoxic insult in A_1R KO mice (Johansson et al., 2001), no significant difference could be observed in the damage resulting from ischemia in vivo in adult A_1R KO compared to WT mice (Olsson et al., 2004). However, effects of adenosine acting on A_1R may differ depending on the maturity of the organism, since immature animals genetically lacking the A_1R showed decreased loss of white matter after hypoxia (Turner et al., 2003). This is consistent with the protective effects of caffeine found after hypoxic ischemia in 7 day-old rat (Bona et al., 1995). The reason for these opposing effects might be explained by the different outcome of NMDA receptor activation and consequent changes in intracellular Ca^{2+} levels in neonates versus adults (Turner et al., 2004).

1.5.1.2 $A_{2A}R$

$A_{2A}R$ are very abundant in basal ganglia where they have a predominantly post-synaptic localization, but they are also expressed pre-synaptically in nerve terminals as well as in other brain cells and blood vessels throughout the brain (Fredholm et al., 2005a; Jarvis et al., 1989). Low levels of $A_{2A}R$ mRNA were first detected on E14, when they had a diffuse distribution, but later concentrate in striatum around PND 3-14 (Åden et al., 2000; Weaver 1993). In the striatum, $A_{2A}R$ are selectively expressed in the GABAergic striatopallidal neurons where they are coupled to G_{olf} proteins and colocalize with postsynaptic dopamine D_2 receptors, while in other regions they bind to G_s (Fredholm et al., 2005b; Svenningsson et al., 1999).

$A_{2A}R$ are involved in many important functions of the CNS such as protection against kainate-induced excitotoxicity, facilitation of synaptic transmission, modulation of the dynamic neuronal plasticity, increase of glutamate efflux and regulation of reactive astrocytes formation (for ref see Fredholm et al., 2005a). $A_{2A}R$ play a significant role in the control of inflammatory reactions; $A_{2A}R$ activation leads to vasodilatation, inhibition of pro-inflammatory cytokines, increases in release of anti-inflammatory cytokines (IL-10) and stimulation of $A_{2A}R$ on platelets inhibits aggregation (Fredholm et al., 2005a; Hasko et al., 1996). The $A_{2A}R$ expression is upregulated by hypoxia (Arslan et al., 2002) and by cytokines

(Trincavelli et al., 2002). Activation of $A_{2A}R$ promotes proliferation of cultured astrocytes (Brambilla et al., 2003). $A_{2A}R$ activation may exert detrimental effects in early phase of injury (e.g. by facilitating glutamate efflux), while in the later inflammatory phase activation of these receptors could be protective (Trincavelli et al., 2002).

Deficiency of $A_{2A}R$ results in alterations of various neuronal responses, including a functional hypodopaminergic state (Dassesse et al., 2001) and a partial reduction in the catalepsy caused by manipulating the dopaminergic and cholinergic systems (Svenningsson et al., 1999). Due to these modulating actions $A_{2A}R$ antagonists appear to be good targets for treating Parkinson's disease and depression (Svenningsson et al., 1999).

Besides the ability to form heterodimers with D_2 receptors, $A_{2A}R$ can also form homodimers ($A_{2A}R$-$A_{2A}R$ interactions) or heterodimers with A_1R that can alter their sensitivity to adenosine and to adenosine's antagonist, caffeine. These dimers have been implicated in tolerance to motor effects after chronic treatment with caffeine (Ciruela et al., 2006). Adenosine is a modulator of nociceptive pathways, both at peripheral and spinal levels (Sawynok and Yaksh 1993) and direct stimulation of nociceptive nerve terminals is regulated through the activation of $A_{2A}R$ (McQueen and Ribeiro 1986). Adenosine $A_{2A}R$ antagonists enhance the activity of mice in the forced swim and tail suspension test by prolonging the escape-directed behavior, pointing to an important role in depression (El Yacoubi et al., 2001).

$A_{2A}R$ KO mice

All three strains of $A_{2A}R$ KO mice that have been generated so far, on CD1, C57BL/6 or Sv-129 background, were found to be viable and breed normally. The $A_{2A}R$ KO strain of mice on CD1 background had significantly higher body weight than the WT mice of both sexes, while no differences were found in two other strains (for ref see Fredholm et al., 2005b). Blood pressure, heart rate, and platelet aggregation in these mice were found to be increased in the line bred onto CD1 background (Ledent et al., 1997), whereas this did not appear to be the case in two other lines (Fredholm et al., 2005b).

Exploratory behavior of $A_{2A}R$ KO mice was increased at 4 weeks of age (Åden et al., 2003) but in adulthood was reported to be reduced, maybe due to the increased anxiety (Chen

et al., 2000; Ledent et al., 1997). $A_{2A}R$ KO appeared to be less immobile in the depression tests and more aggressive towards intruders than the WT in resident intruder test (Chen et al., 2000; El Yacoubi et al., 2001). They respond more slowly to painful (thermal) stimuli (Ledent et al., 1997) and have reduced startle habituation, startle amplitude and prepulse inhibition (Wang et al., 2003). Exploratory behavior is normally increased by caffeine, cocaine and amphetamine, but the increase is smaller in $A_{2A}R$ KO mice (Chen et al., 2000; Ledent et al., 1997). Dopamine D_1, D_2, NMDA and AMPA receptor binding was found to be normal in these mice, though extracellular concentration of dopamine was decreased in striatum and extracellular glutamate was enhanced (Chen et al., 2000; Dassesse et al., 2001). This might explain why the locomotor response to dopamine agonists is reduced in mice with a genetic deletion of $A_{2A}R$ (Chen et al., 2000). In the study by Huang and colleagues, the significance of $A_{2A}R$ activation on caffeine induced wakefulness was confirmed, since no effect of caffeine was observed in $A_{2A}R$ KO mice (Huang et al., 2005).

Brain damage after focal ischemia has been reported to be attenuated in adult $A_{2A}R$ KO mice compared to the WT (Chen et al., 1999). Selective $A_{2A}R$ inactivation by transplantation of bone marrow cells from $A_{2A}R$ KO mice into WT mice reduced MCAO-induced focal infarction in the brain (Yu et al., 2004). On the other hand, aggravated brain damage is observed after hypoxic ischemia in $A_{2A}R$ KO neonates (Åden et al., 2003).

1.5.1.3 $A_{2B}R$

The characterization of $A_{2B}R$ functions is still at an early stage, because pharmacological tools and knock-out mice have only recently become available. These receptors have been shown to be protective in inflammation by inducing IL-6 production, and by counteracting excessive vascular adhesion (Fiebich et al., 1996; Fredholm and Altiok 1994; Yang et al., 2006). $A_{2B}R$ have cardioprotective properties and are involved in inflammation (Eckle et al., 2007; Hua et al., 2007; Yang et al., 2006), while blockade of the $A_{2B}R$ in the lungs by enprofylline, an adenosine antagonist, exerts beneficial effects in the lungs (Holgate 2005).

The results obtained so far show that $A_{2B}R$ are widespread in the brain, although at low density (Fredholm et al., 2005a). $A_{2B}R$ activation promotes glycogen synthesis in astrocytes through modulation of gene expression (Allaman et al., 2003). Hypoxia can directly increase

the expression of $A_{2B}R$ because of a canonical HIF-1α binding site in the promoter (Kong et al., 2006).

$A_{2B}R$ KO mice

$A_{2B}R$ KO mice develop normally, achieve normal body weights, have regular blood pressure and survive more than a year (Hua et al., 2007; Yang et al., 2006). Mast cell activation is enhanced in mice deficient in the $A_{2B}R$ and they exhibit increased susceptibility to antigen induced anaphylaxis (Hua et al., 2007). Interestingly, $A_{2B}R$ KO mice also show markedly increased endothelial permeability, and are resistant to preconditioning (Eckle et al., 2007; Yang et al., 2006).

1.5.1.4 A_3R

Adenosine A_3 receptors are the most recently discovered of the adenosine receptors. They are present at low density in neurons (nerve terminals of cortical and hippocampal neurons), and are found in astrocytes, microglial cells and blood vessels in the brain (for ref see Fredholm et al., 2005a).

Lack of high affinity A_3R specific radioligands and other pharmacological tools has impeded thorough characterization of A_3R activity. It has been speculated that these receptors react only to high concentrations of adenosine and that they therefore may play a role mostly during pathological conditions (Jacobson et al., 1995). However, in humans adenosine is as potent at A_3R as it is at A_1 and A_{2A} receptors (Fredholm et al., 2001b; Schulte and Fredholm 2000).

Some in vitro studies have shown that the A_3R mediate the toxic effects of adenosine in brain cells (Abbracchio et al., 1997; Di Iorio et al., 2002). However, protective effects of A_3R against brain pathology have recently been reported in both in vitro and in vivo studies. Pre-exposure to the A_3R agonist Cl-IB-MECA counteracted a hypoxia-mediated decrease in viability of rat cortical cultures as well as reduced cerebral infarction and increased locomotor activity in rats after middle cerebral artery occlusion (Chen et al., 2006). In fetal astrocytes and microglia cells A_3R activate ERK1/2 phosphorylation, which is a known survival signal

(Hammarberg et al., 2004; Neary et al., 1996). Stimulation of A_3R in astrocytes increases the release of protective cytokines and synthesis of matrix metalloproteinases (Dorf et al., 2000). Adenosine acting through A_3R is a potentially important inhibitor of the release of tumor necrosis factor (TNFα), a key cytokine in the inflammatory cascade (Levy et al., 2006). A_3R have a protective function in the cardiovascular system (Parsons et al., 2000), influence immune function and mast cell degranulation (Fedorova et al., 2003).

A_3R KO mice

These mice appear to develop in an essentially normal manner, exhibit a normal morphology and have no major fertility problems (Fedorova et al., 2003). A_3R KO mice have decreased intraocular pressure (Avila et al., 2002).

Mice with genetic deletion of A_3R exhibit increased locomotion in the open field test, the elevated plus maze and light/dark box, whereas the deletion appears to have a depressant effect in depression predictive tests (Fedorova et al., 2003). This genotype shows decreased reaction to some painful stimuli and deficits in generating the localized inflammatory response to carrageenan (Wu et al., 2002).

Consistent with previous reports of the neuroprotective actions of A_3R agonists, A_3R KO mice show an increase in neurodegeneration in response to repeated episodes of hypoxia (Fedorova et al., 2003). In addition, after MCA occlusion, a more extensive cerebral infarction was found in the A_3R KO mice compared to the WT controls, suggesting that the A_3R are tonically activated during ischemia (Chen et al., 2006).

1.6 Caffeine

The alkaloid caffeine (1,3,7-trimethylxanthine) is the most widely consumed psychostimulant substance, present in numerous dietary products (coffee, tea, cocoa beverages and chocolate bars). It was isolated and its structure identified in 1875 (Arnaud 1987). Due to its widespread use and low abuse potential, caffeine is considered an atypical drug of abuse (Fredholm et al., 1999).

Caffeine is absorbed rapidly and almost completely when given by the oral route (Arnaud 1987) with peak plasma concentration after 30–60 min and plasma $T_{1/2}$ of 3–5 h in humans (Fredholm et al., 1999). Systemic clearance is essentially equal to metabolic elimination. Caffeine is metabolized in the liver by cytochrome P450IA2 to paraxanthine (84%), theobromine (12%) and theophylline (4%). Paraxanthine is further transformed to 1,7-dimethylurate by cytochrome P450 or to 1-methylurate by cytochrome P450 and N-acetyltransferase. Because of its lipophilicity and limited protein binding, caffeine passes the blood-brain barrier and the placenta and is excreted in milk. Significant levels are observed in brain within 5 min, whereas peak levels appear within 30 min after oral administration in humans (Bonati et al., 1984). Many factors influence the absorption and kinetics of caffeine such as species, age (in neonates caffeine clearance is 20 times slower than in adults because of the undeveloped oxidase systems), genetics (fast and slow acetylating rates because of N-acetyltransferase polymorphism), exercise (moderate exercise increases the peak plasma concentration and reduces the $T_{1/2}$), pregnancy (2.5–7 times prolongation of $T_{1/2}$ occurs during late pregnancy), disease (caffeine is metabolized primarily by liver enzymes and liver diseases decrease the rate of caffeine demethylation), smoking/enzyme inducers (stimulation of the hepatic enzyme P450IA2 causes reduction of caffeine's $T_{1/2}$ to one-half), and drugs (coadministration can lead to impairment of caffeine elimination because of competition at the enzymatic level) (Sawynok and Yaksh 1993).

The total worldwide consumption of caffeine (irrespective of source) has been estimated to approximately 70 to 76 mg/person/day. Interestingly, the levels of caffeine intake in countries such as Sweden and Finland reach more than 400 mg/person/day (Fredholm et al., 1999). In humans, the reinforcing effects of caffeine are limited to the intake of low to moderate doses (Fredholm et al., 1999). Some individuals experience anxiety and distress in response to caffeine and this has been correlated with $A_{2A}R$ polymorphism (Alsene et al., 2003).

Caffeine exerts biphasic effects on behavior depending on the administered dose both in humans (Nehlig and Boyet 2000) and in laboratory animals (Daly and Fredholm 1998). At low doses caffeine stimulates locomotor activity, inhibits sleep, and sharpens alertness (Fredholm et al., 2001a). Subjective effects induced by caffeine at higher doses are characterized by negative feelings such as anxiety, nervousness and insomnia, a condition referred to as "caffeinism" (Mumford and Holtzman 1991; Nehlig and Boyet 2000). In low

doses, which are the most relevant to human use, effects elicited by caffeine administration are mainly achieved by blocking adenosine A_1 and A_2 receptors resulting in modulation of dopamine receptor activation in the mesolimbic dopamine system (Ferre et al., 2001; Fredholm et al., 1999). More than 10 times higher concentrations are needed to afford significant inhibition of A_3R (Fredholm et al., 2001b). Stimulatory effects of caffeine have been assigned to the activation of A_{2A}R (El Yacoubi et al., 2001), though some evidence for the involvement of A_1R has also been reported (Ferre et al., 2008; Halldner et al., 2004; Kuzmin et al., 2006).

Although the health consequences of ordinary caffeine consumption are probably minor there are concerns about caffeine intake during pregnancy and lactation. It is notable that in contrast to alcohol and tobacco consumption during pregnancy, approximately 70% of expectant mothers continue to drink beverages containing caffeine at normal or near normal rate (Bracken et al., 2003; Olsen et al., 1991). As stated earlier, newborns can accumulate pharmacologically active doses of caffeine because of their slow caffeine metabolism (Fredholm et al., 1999). However, several studies in humans have shown that consumption of moderate amounts of caffeine during pregnancy has no measurable consequences on the fetus and newborn infant (Castellanos and Rapoport 2002; Nehlig and Debry 1994). On the other hand, chronic maternal consumption of large quantities of coffee (more than 7 cups/day) during gestation and lactation correlated with an increased risk of intrauterine growth retardation (Fenster et al., 1991), lower birth weight and decreased head circumference (Nehlig and Debry 1994) and represents a risk for adverse pregnancy outcomes (Cnattingius et al., 2000). Recent evidence obtained from animal studies suggests that chronic consumption of caffeine may modify the psychomotor effects of other drugs of abuse, such as nicotine, cocaine and amphetamine (Cauli and Morelli 2005). Caffeine, through A_{2A}R, exerts neuroprotective effects on dopaminergic neuron degeneration (Chen et al., 2001). In this regard, epidemiological studies have shown an inverse correlation between Parkinson's disease and the consumption of caffeine (Ascherio et al., 2001).

2 Aims

1. Investigate the behavioral outcomes of perinatal exposure to MeHg in mice where adenosine receptors (A_1R and $A_{2A}R$) were partially blocked by caffeine treatment or eliminated by genetic modification.

2. Analyze the effects of perinatal exposure to caffeine on motor activity, focusing on the role of A_1R.

3. Characterize the A_3R KO mice with regard to motor activity, response to psychostimulants and susceptibility to neurotoxic challenge.

4. Identify the intracellular pathways activated by oxygen deprivation and cobalt exposure in primary cultures of mouse astrocytes.

5. Evaluate possible neuroprotective effects of adenosine receptor subtypes under hypoxia-like conditions in astrocytes.

6. Study the role of A_1R, $A_{2A}R$ and A_3R under hypoxic ischemia in immature and mature mice.

3 Materials and methods

The materials and techniques used in present thesis are listed in the Table 1 and were thoroughly described in manuscripts included in this thesis.

Table 1. Techniques used in this thesis

	Methods	**Paper**
1.	Genetically modified mice	I, II, III, prel. res.
2.	Breeding and genotyping mice	I, II, III, IV, prel. res.
3.	Exposure of animals to chemicals	I, II, III
4.	Rice-Vannucci hypoxic ischemia model	prel. res.
5.	Histopathology score	prel. res.
6.	Open field model	I, II, III, prel. res.
7.	Telemetry system	III
8.	Rotarod	I, II, III
9.	Beam walking	prel. res.
10.	Tissue preparation	I, II, III, prel. res.
11.	RNA extraction and cDNA synthesis	III, IV
12.	Reverse transcription polymerase chain reaction (RT-PCR)	III, IV
13.	Real-time RT-PCR	III, IV
14.	In situ hybridization	II, III
15.	Autoradiography	II, III
16.	Determination of total mercury in brain	I
17.	Primary astrocyte cultures	IV, prel. res.
18.	Astrocytoma D384 cell line	prel. res.
19.	Exposure of cells to hypoxic environment	IV, prel. res.
20.	Exposure of cells to chemicals	IV, prel. res
21.	Luminescence ATP detection assay	IV, prel. res.
22.	Trypan blue exclusion test	IV, prel. res.
23.	LDH assay	IV, prel. res.
24.	Chromatin condensation analysis by propidum iodide staining	IV, prel. res.
25.	TUNEL assay	IV
26.	Vital triple staining with Annexin V-FITC, Hoechst 33358 and PI	IV
27.	Immunoblotting	IV
28.	Immunocytochemistry	IV
29.	Reactive oxygen species (ROS) measurement	IV
30.	ELISA for HIF-1α detection	IV
31.	Intracellular calcium $[Ca^{2+}]_i$ measurement	IV
32.	Mitochondrial membrane potential ($\Delta\Psi m$) measurement	IV
33.	Bromodeoxyuridine staining	IV
34.	Detection of apoptosis by flow cytometry	prel. res
35.	Thin layer chromatography (TLC)	prel. res.

3.1 Animals

Four types of mice were used in this thesis: *adenosine A_1 receptor knock-out* (Johansson et al., 2001), *adenosine A_{2A} receptor knock-out* (Chen et al., 1999), *adenosine A_3 receptor deficient mice* generated by Merck Research Laboratories (Salvatore et al., 2000) and *C57BL/6 mice* (wild type, WT). To generate the A_1R KO mice, the second coding exon of the mouse adenosine A_1R gene was inactivated in mouse E14.1 embryonic stem cells and 129/OlaHsd/C57BL/6 hybrid mice were generated. A_{2A}R KO mice were made on mixed 129-Steel × C57BL/6 and the A_3R KO mice on 129 × C57BL/6 genetic background. All AR KO mice were backcrossed for more than 10 generations with C57BL/6 to achieve essentially pure congenic lines. Animals were bred at the Department of Physiology and Pharmacology, Karolinska Institutet. They were housed at a constant room temperature (22°C; 12 h light/dark cycle, lights on at 6 a.m.) with *ad libitum* access to food and water and were routinely genotyped by PCR. The experimental procedures regarding the handling of the animals were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Ethics Committee of Northern Stockholm.

3.2 Exposure

Dams were administered doses of 1 μM MeHg and/or 0.3 g/l caffeine in the drinking water for either 3 weeks (gestational day 7 to day 7 of lactation, paper I and III) or 6 weeks (gestational day 1 to day 21 of lactation, paper I and II). Determination of total mercury in brains was done at PND21 (paper I) by alkaline solubilization/reduction and cold vapor atomic fluorescence spectrophotometry (Berglund et al., 2005).

3.3 Behavioral studies

In papers I, II, III and preliminary results behavioral analysis was done primarily using the **Open field model**. This system allows investigating basal activity and effects of acute stimulation on motor functions of an individual mouse by recording the locomotor activity in a solid and sound-attenuating square box equipped with light beams and photocells. Breaking of the light beams (horizontal and vertical) is recorded as counts by the computer. We have analyzed the following variables: horizontal activity (total number of beam breakings),

locomotion (interruptions of photocells in the lower rows when the animal breaks two adjacent beams, that is, when an actual transfer is made) and rearing activity (all interruptions of photocells in the upper rows). This equipment does not allow the recording of small movements, e.g. tremor, reflexes and tail movements. In paper III additional motor activity measurements were made by **telemetry** (Data Sciences, St. Paul, MN, USA). After implantation of the transmitter into the peritoneal cavity, the mouse was monitored for several days in a row in its home cage where it is housed alone or in group. Telemetry is, however, less sensitive than the Open field model since it does not distinguish between vertical and horizontal movements, and generally speaking, larger movements are required in order to be counted.

Rotarod performance was used as coordination and balance test following exposure to neurotoxicants or damage caused by diminished oxygen delivery. The animal's task is to keep from falling off a rotating drum and its performance is measured as the latency time, i.e. how long until it falls off. Preliminary results were also obtained with the **beam walking test** for evaluation of balance and coordination after ischemic insult. This test has previously been shown to be more sensitive for detection of motor disturbances after ischemia than the Rotarod. The mouse is placed at one end of a thin beam and is allowed to traverse the distance 3 times while the number of foot faults for each of the hind limbs is counted separately.

3.4 Neurochemistry

We measured the expression of immediate early genes, tyrosine hydroxylase and proenkephalin by **in situ hybridization** and dopamine (D_1 and D_2) receptor binding by **autoradiography** (paper II and III). Expression of A_1R and $A_{2A}R$ mRNA was measured by in situ hybridization (paper III).

3.5 Assessment of cell damage in cultured astrocytes

In paper IV and preliminary results we have employed **primary cultures of mouse astrocytes** as well as human **astrocytoma D384 cells**. Cells were exposed to **oxygen deprivation** in a CO_2 incubator (Queue, LABEQUIPTM, Ontario, Canada) connected to a nitrogen source (1% O_2, 5% CO_2, 94% N_2) or exposed to **cobalt chloride ($CoCl_2$)**, a mimic of

hypoxia. The damage caused by these conditions after different exposure times was assessed using various methods such as: measurement of total **ATP levels** as cytotoxicity index, **trypan blue staining** (cell membrane impermeable dye) for distinguishing cells with intact cell membrane (healthy and apoptotic) from cells with permeable cell membrane (necrotic cells) and for counting the total cell number. We also measured the release of **cytoplasmic LDH** into the medium to evaluate cell membrane integrity. Assessment of nuclear morphology was done by staining fixed cells with **propidium iodide** (PI) where a small nucleus and intense chromatin staining indicates apoptotic cells. **Vital staining** with cell-permeable dye **Hoechst 33358,** cell-impermeable **PI** and **Annexin V-FITC** was used to further evaluate and distinguish occurrence of apoptosis (condensed chromatin and exposure of phosphatidyl serine on the membrane surface) vs necrosis (PI-positive cells with damaged cell membrane). Detection of **DNA fragmentation** (apoptosis) was done by terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (**TUNEL**) assay. **Flow cytometry** (**FACS**) was used for analyzing the cell cycle and apoptosis in fixed cells stained with PI. By scoring nuclei labeled by **bromodeoxyuridine (BrdU)** we evaluated cell proliferation. Detection of polypeptides was performed with **immunocytochemistry**, which enables detection of proteins at the single cell level, and standard Western blotting (**immunoblotting)** for detection of proteins in cell extracts, e.g. caspase cleavage products generated during the apoptotic process. HIF-1α levels were determined by **ELISA** and evaluation of gene expression was performed by **real-time RT PCR**. **Formation of oxygen radicals** (**ROS**) was assessed by staining with carboxy-H2DCFDA dye, which becomes fluorescent after deacetylation and oxidation in living cells. Changes in $\mathbf{[Ca^{2+}]_i}$ induced by stimulation with ATP were measured using the fluorescent Ca^{2+} sensitive dye Fluo-3. The voltage sensitive probe tetramethylrhodamine ethylester (TMRE) was used for evaluation of the **mitochondrial membrane potential** ($\mathbf{\Delta\Psi_m}$). **Purine release measurements and TLC** were performed to assess the level of released adenosine and its metabolites in the medium after exposure to various insults**.**

3.6 Experimental model of hypoxic ischemic injury in vivo

Rice-Vannucci hypoxic ischemia model: In the study described in the preliminary results, consistent focal ischemic damage was induced in the mouse by unilateral ligation of the common carotid artery and timed exposure to hypoxia. This method works both in

immature (10 day old) and mature (2 months old) mice. Animals were anesthetized with isoflurane (1% for 2 minutes), the local anesthetic marcain was administered s.c. prior to incision and the left common carotid artery was electrocoagulated. After 1 h of recovery, the pups/adult mice were exposed to hypoxia (10% O_2/balance N_2) for 1 h. At the end of hypoxia, pups/adult mice were kept in the incubator in air with temperature maintained at 36°C for 30 minutes. The damage was evaluated after 14 days using behavioral and morphological methods.

This model is well characterized and is considered to share important features with birth asphyxia in the human neonate (10 day-old mouse corresponds to the stage of maturity of a human newborn) as well as with stroke in adults, with regard to blood flow changes and cellular metabolic derangements. Gradual decrease in cerebral blood flow after hypoxia causes damage in the brain areas adjacent to the middle cerebral artery and producing a histopathological injury in ipsilateral cerebral cortex, hippocampus and striatum. Because animals subjected to this model generally survive the acute phase, it allows evaluation of long term consequences, such as functional and sensory motor impairment. The functional changes related to the brain damage are evaluated by **histopathology** (cresyl violet) and **immunohistochemistry** (microtubule associated protein MAP-2, 1:500 (Blomgren et al., 1995)) using a scoring system on a 0-24 point scale total for each brain (Sheldon et al., 1998). To derive the total score, eight brain regions most affected by hypoxic ischemia are scored on a 0-3 scale and the scores are summed (cortex, striatum, hippocampus). Motor impairments are assessed by monitoring locomotor activity in the **open field** and evaluating balance and coordination using the **beam walking test**. However the model has some shortcomings such as the unilateral distribution of brain injury, unilateral changes in cerebral blood flow and lack of multi-organ dysfunction, all of which differ from what is seen in the clinical setting.

4 Results and discussion

4.1 Effects of perinatal methylmercury and caffeine exposure on mouse offspring. Role of adenosine receptors (Paper I)

In this study, we investigated a possible role of adenosine receptors in modulating the effects of developmental neurotoxicity caused by the fish pollutant methylmercury (MeHg). Presence of other factors included in the diet (e.g. caffeine) could either potentiate the damage to the nervous system caused by the neurotoxicants or activate protective pathways. Behavioral outcomes of low perinatal MeHg exposure were studied in mice where the A_1 and A_{2A} adenosine receptors were either partially blocked by caffeine treatment or eliminated by genetic modification (A_1R and A_{2A}R knock-out mice). Dams were given doses that mimic human intake, i.e. 1 μM MeHg (0.2 mg/l) and/or 0.3 g/l caffeine (correlating to about 3-5 cups of coffee per day) in the drinking water for 3 weeks (gestational day 7 to day 7 of lactation).

Exposure to these very low levels of methylmercury caused behavioral alterations that were sex and age-dependent. MeHg caused no cerebellar alterations severe enough to be detected by rotarod. Male wild type mice exposed to MeHg displayed a decrease in motor activity at PND21 as compared to untreated controls, whereas the opposite was observed at the age of 2 months. This could either be due to the maturation differences between adolescent and adult males caused by the rapid decline of brain mercury concentration and consequent adaptive changes affecting locomotion (Stern et al., 2001) or due to the fact that the dopaminergic system is still under development at PND21 (Gerfen 1996). As discussed in earlier studies, MeHg can alter dopamine release or modify the expression of dopamine receptors (Daré et al., 2003; Faro et al., 2002). This could explain the decreased locomotor response after amphetamine stimulation in 2 month-old pre-treated WT males. We found no clear effects in treated WT female mice of either age, in keeping with the results previously reported in both rats and humans (Johansson et al., 2007a; Vahter et al., 2007). The gender effect might be due to the faster body clearance of mercury in females than in males, although we detected equal brain Hg levels in both sexes at PND21, or to the reported higher susceptibility of males to oxidative stress - a likely consequence of MeHg exposure (Vahter et al., 2007).

Maternal caffeine intake caused lower body weight in 3-week-old mice but no behavioral changes as assessed by open-field analysis or rotarod. However, at adult age an increased spontaneous motor activity and an amplified response to psychostimulants (cocaine and amphetamine) were found, irrespective of sex. Increased locomotion and spontaneous activity were reported in rodents subjected to either moderate or high doses of caffeine during pregnancy/lactation (Cauli and Morelli 2005). Perinatal exposure to caffeine might in some way influence the dopaminergic system during brain development (Cauli and Morelli 2005) causing changes which could persist and be important for the increase of the rewarding effects of drugs classically abused in humans. This line of thinking is further supported by the finding that chronic consumption of caffeine prevents the extinction of cocaine self-administration in rodents and induces place preference conditioning, probably through A_1R (Fredholm et al., 2005a).

In the male offspring co-exposed to MeHg and caffeine, the behavioral changes induced by MeHg were attenuated by caffeine. Thus, young co-exposed males displayed spontaneous activity similar to the untreated controls. When the mice reached adulthood, the overall activity in the co-exposed mice appeared to be similar to that in the group treated with caffeine alone. There was no difference in the response to amphetamine between the co-exposed and untreated males. At PND21 combined exposure did not affect the behavior of female mice, though at adult age their spontaneous as well as psychostimulant-induced activity followed the same pattern as in the group exposed to caffeine only.

Spontaneous motor activity measured in the open field paradigm was similar in adolescent A_1R KO and WT mice, but when A_1R KO mice reached adulthood, spontaneous activity was clearly increased in both sexes. However, males but not females, responded less to amphetamine, which could be due to the higher baseline activity prior to amphetamine injection. The spontaneous behavior of A_1R KO mice showed remarkable similarities with that of mice perinatally treated with caffeine, pointing to an involvement of A_1R in the changes induced by exposure to caffeine during development.

Parameters of motor activity were increased in adolescent $A_{2A}R$ KO males as observed in the previous study by Ådén and colleagues (Åden et al., 2003). At 2 months of age the motor activity of $A_{2A}R$ KO and WT mice was alike, but $A_{2A}R$ KO males had an impaired response to different doses of amphetamine (2.5 and 10 mg/kg). Impaired responses to

psychostimulants in $A_{2A}R$ KO mice have also been reported earlier (Fredholm et al., 2005a). The reason for discrepancies between psychostimulant-induced behavioral responses obtained by a genetic approach (deletion of $A_{2A}R$) versus a pharmacological approach (blockade of $A_{2A}R$, i.e. by caffeine) lies in the fact that the $A_{2A}R$ KO animals have a 45% lower extracellular concentrations of dopamine (Dassesse et al., 2001).

The open field behavior of A_1R KO and $A_{2A}R$ KO mice was essentially unaffected by the perinatal treatment with MeHg, perhaps indicating that these genotypes are less vulnerable to MeHg than WT. Since genetic deletion of adenosine A_1R and $A_{2A}R$ had ameliorating effects, these receptors are likely to be involved in the protective effect of perinatal caffeine in attenuating MeHg damage.

There are also some general conclusions that can be drawn. 1) In future studies of the long-term consequences of environmental agents it will be relevant to relate outcome not only to exposure levels, but also to the genotype of the affected individual. Differences in AR genes and genes influencing adenosine levels are only one of several potentially important variables. 2) The exposure of individuals to other agents, including caffeine, nicotine and ethanol, must be taken into account when assessing the impact of environmental toxic agents.

4.2 Consequences of perinatal caffeine exposure on mouse offspring (Paper II)

This study was designed to further investigate the perinatal exposure to caffeine, since the data on the caffeine consumption effects among pregnant women is inconclusive and somewhat contradictory, and a majority of women continue to drink caffeine beverages during pregnancy. One of the main concerns with perinatal exposure to caffeine was a potential to influence subsequent susceptibility to addictive drugs. Here we administrated 0.3 g/l in the drinking water throughout pregnancy and lactation to mimic ordinary human consumption patterns. We have assessed the offspring's behavioral status, including motor functions and psychomotor activation, by employing another type of psychostimulant, i.e. cocaine (10 mg/kg).

Perinatally caffeine-treated WT offspring exhibited increased locomotor activity in an open field as adults, irrespective of sex. The finding agreed with our previous study (paper I)

where the caffeine exposure window was shorter, to match the MeHg treatment. It was also consistent with findings obtained in humans showing that exposure to low or modest doses of caffeine correlates with hyperactivity in late adolescence or adulthood (Linnet et al., 2003). The perinatally treated offspring also responded to cocaine challenge with greater locomotor activity than mice not perinatally exposed to caffeine. This observation was in agreement with our previous report on the higher effect of amphetamine stimulation in female offspring treated perinatally with caffeine for 3 weeks (paper I). Thus, even a narrower time window of caffeine exposure during development might be sufficient to have life-long consequences.

Since low doses of caffeine antagonize brain adenosine A_1R and $A_{2A}R$ we also tested the response of A_1R KO and $A_{2A}R$ KO mice to cocaine. The response to this psychostimulant was increased in mice with genetic deletion of A_1R, correlating with the results obtained after chronic treatment with caffeine, while the opposite was true for $A_{2A}R$ KO mice. Based on these results we decided to further examine the role of A_1R in the effects of caffeine treatment.

Some effects of perinatal exposure to caffeine were mimicked in mice heterozygous (Hz) for adenosine A_1R, which have half the normal number of such receptors. This is relevant because regular human consumption of caffeine leads to the blockade of about half of the body's A_1R (Johansson et al., 2001). Adult A_1R Hz offspring showed a behavioral profile of hyperactivity quite similar to WT groups exposed perinatally to caffeine, and their response to cocaine was also enhanced. That A_1R Hz mice have higher basal motor activity has been reported earlier (Gimenez-Llort et al., 2002). Furthermore, it appeared that the mother's genotype, not the offspring's, was critical for behavioral changes in adult offspring. The fact that the drug phenotype could be mirrored by a phenotype of genetic modification made it possible to discriminate between maternal and filial actions. Thus, if the mother partially lacked A_1R the offspring displayed more hyperactivity and responded more strongly to cocaine stimulation as adults than did the adult offspring of a WT mother, regardless of their genotype. In addition, WT offspring from A_1R Hz mother also showed altered response to psychostimulant cocaine. The effect of the mother's genotype was further supported by the finding that WT offspring from WT mother but A_1R Hz grandmother preserved higher locomotor response to cocaine compared to WT mice whose mother and grandmother were both wild types.

Early exposure to psychostimulant drugs may lead to a phenomenon called "neuronal imprinting" where a drug may have effects that are not necessarily immediate but manifest later in life (Andersen 2005). In view of the fact that the rewarding properties of all psychostimulants, including to some extent caffeine (Daly and Fredholm 1998), are a result of actions of the drugs on the mesolimbic dopamine system (Feldman et al., 1997), it could be hypothesized that a potential to generalize between different types of psychoactive drugs may develop after perinatal exposure to caffeine.

It has been reported that repeated administration of low doses of caffeine can induce cross-sensitization to the motor stimulant effects of amphetamine, cocaine and dopamine D_1 and D_2 receptor agonists (Cauli and Morelli 2005). This might be linked to a preferential interaction between A_1R and D_1 receptors in ventral striatum (Cauli and Morelli 2005). Our examination of various neurochemical parameters did not provide any direct evidence that a major disruption of the dopaminergic pathways was involved in psychostimulation in caffeine exposed mice. However, we did find an elevation of preproenkephalinA mRNA levels in dopaminergic neurons in caudate-putamen of offspring born to A_1R Hz mothers.

Thus, we conclude that perinatal caffeine, by acting on A_1R in the mother, appears to be responsible for long-lasting behavioral changes in the offspring. The genetic approach has allowed us to completely differentiate between maternal and fetal actions. This would not be feasible using drugs, since it is impossible to administer psychoactive drugs only to the dam and not to the fetus, and vice versa. However, we have insufficient evidence to conclude whether the long-lasting behavioral changes after perinatal caffeine treatment that we have observed are detrimental or beneficial.

4.3 Characterization of A_3R KO mice: motor activity, response to caffeine challenge and vulnerability to methylmercury damage (Paper III)

In this study we wanted to thoroughly investigate the behavioral consequences of genetic deletion of the adenosine A_3R in mice. The knock-out approach was chosen to address a possible role of these receptors during development: the effect of A_3R stimulation or blockade in the brain is hard to determine pharmacologically because of the lack of specific ligands, especially for rodent receptors. We have evaluated open field behavior in adenosine A_3R KO mice, both under basal conditions and after stimulation with psychostimulants.

Overall locomotor activity of 21-day-old and 2-month-old A_3R KO males was higher compared to male WT mice. This was in agreement with the observation of an increased locomotor activity in adult A_3R KO males reported by Fedorova and colleagues (Fedorova et al., 2003). Rearing activity was also higher in young A_3R KO females compared to the WT, but no differences were observed in spontaneous locomotion in adult age. No differences were found in body weight or in rotarod performance in males, whereas in females the body weight was higher in A_3R KO than in WT. Thus, despite low levels of A_3R expression in central nervous system (Fredholm et al., 2005a), A_3R appear to play some role in modulating motor activity. Furthermore, A_3R KO mice had a decreased response to stimulation with different doses of caffeine (7.5-75 mg/kg), irrespective of sex. This was a surprising observation considering that more than 10 times higher concentrations of caffeine are needed to inhibit A_3R than those needed for A_1R and A_{2A}R (Fredholm et al., 2001b). Effects of another psychostimulant, amphetamine, were also reduced in male and female A_3R KO mice compared to the WT groups, implying changes in motor regulation influenced by the dopaminergic system. A_3R have been shown to play a role in the regulation of synaptic plasticity after chronic cocaine treatment in rats: chronic cocaine increased dopamine levels and, through a cascade of events, elevated PKC activity resulting in impairment of group II mGluR (Huang et al., 2007). Telemetric recordings done throughout the light-dark cycle revealed less excitation of overall activity in A_3R KO females compared to the WT mice in darkness, which is a natural arousal stimulus in mice. To explore the possibility that attenuated signaling via dopaminergic pathways or changes in the adenosine receptor expression might be responsible for the observed phenomena, we examined the binding capacity of D_1 and D_2 receptors as well as the levels of A_1R, A_{2A}R and A_{2B}R mRNA in A_3R KO brains. No differences were found between A_3R KO and WT brains.

The possibility that A_3R function plays a role in modulating the brain response to perinatal challenge with the environmental pollutant MeHg was also examined. This developmental neurotoxicant is known to interfere with the neural migration process (Rodier 1994) and to target the dopaminergic system (Johansson et al., 2007a). As reported in paper I, exposure to MeHg (1 μM in drinking water) during pregnancy and lactation produced behavioral alterations in adolescent and adult WT males, whereas WT females were unaffected. In contrast, both adolescent and adult A_3R KO females exposed to methylmercury displayed an increase in locomotion, indicating a higher susceptibility to deleterious effects of organometal

exposure and probable neuroprotective functions that the A_3R may exert. A_3R deletion in development was reported earlier to aggravate CO-mediated hypoxia (Fedorova et al., 2003).

Consequences of A_3R stimulation could be related to indirect effects on other molecular systems or to activation of various cell types other than neurons during development. Functional A_3R are located on the surface of astrocytes and microglia cells (Fredholm et al., 2005a) and deletion of A_3R may have an impact on neuronal damage through altered reactivity of glia cells which respond to extracellular adenosine (Rebola et al., 2005). Excessive A_3R activation on astrocytes may also cause desensitization of A_1R-mediated inhibition of glutamate transmission (O'Shea 2002). The importance of functional microglia has been particularly emphasized for keeping the balance between synaptic regulation and neuronal death of e.g. dopaminergic neurons during development (Bessis et al., 2007; Cullheim and Thams 2007). Activated microglia penetrate the areas of human cerebral cortex and white matter involved in motor, sensory and associative systems where they can during the first trimester prevent axon extension into the cortical plate (Judas et al., 2005; Monier et al., 2006; Salvatore et al., 2000).

In conclusion, we have found that life-long deletion of the A_3R leads to behavioral adaptation, including an altered response to drugs that do not primarily act via A_3R. Thus, results from the literature and our own data suggest that the role of A_3R may be quite indirect and perhaps excited during development. The observed decreased in the response of mice with genetic deletion of A_3R to stimulation with caffeine was a truly surprising finding that opens new possibilities for the effects of methylxanthines.

4.4 Effects of $CoCl_2$ and hypoxia in primary cultures of mouse astrocytes (Paper IV)

The purpose of the study was to compare the pathways induced by oxygen deprivation and cobalt chloride ($CoCl_2$) in primary cultures of mouse astrocytes. We investigated the possible involvement of e.g. HIF-1α pathway, oxidative stress and calcium signaling after the exposure to cobalt that has been shown to mimic some aspects of hypoxia in vivo (Jones and Bergeron 2001) as well as in vitro (Zou et al., 2001).

Information on the mechanism of astroglial injury and death during the insult caused by oxygen deprivation is scarce. It is believed that astrocytes are less susceptible than neurons to hypoxia because they have the ability to compensate the lack of oxygen by switching from oxidative phosphorylation to the glycolytic pathway, whereas neurons do not use anaerobic metabolism as main source of energy (Almeida et al., 2001). Reports show that exposure to hypoxia or simulated ischemia decreases ATP levels and induces apoptosis and necrosis in rat astrocytes (Gregory et al., 1990; Yu et al., 2001). The failure of energy metabolism leads to cellular dysfunction and swelling due to the influx and accumulation of Na^+, Ca^{2+}, and water (Gregory et al., 1990). The pathways of cell death caused by ischemia seem to be many, including activation of NMDA receptors, increased production of ROS, loss of trophic factors, and an increase in the ratio of proapototic/antiapoptotic Bcl-2 family proteins. These changes in the environment consequently lead to depression of mitochondrial respiration, Ca^{2+} accumulation and swelling, mitochondrial permeability transition, and release of proapototic proteins, resulting in caspase dependent and caspase independent cell death (Hagberg 2004).

In our study, hypoxia induced stabilization of HIF-1α already after 3 h followed by increased stabilization of HIF-1 regulated genes such as heme-oxygenase (HO-1). HO-1 is known to exert protective effects by controlling the redox state of the cell (Maines 1988). In accordance with the literature, a short period of (6 h) hypoxia caused ATP depletion and increase in apoptosis, whereas prolonged exposure (24 h) led to the onset of necrotic death.

Very little data on the effects of cobalt on astrocytes is available in the literature. It is known that cobalt can activate extracellular signal-regulated protein kinase1/2 (ERK 1/2) and cause cell death by apoptosis in C6 glioma cells (Yang et al., 2004). $CoCl_2$ (0.2-0.8 mM) caused dose-dependent ATP depletion, apoptosis (cell shrinkage, phosphatidylserine externalization and chromatin rearrangements) and secondary necrosis (alteration of cell membrane permeability with leakage of cytoplasmic enzymes into the culture medium) in primary mouse astrocytes. In our setting, stabilization of HIF-1α was also an early event after oxygen deprivation. This was followed by an increased expression of HIF-1 regulated genes, e.g. stress protein HO-1, pro-apoptotic factor Nip3 and inducible nitric oxide synthase (iNOS). Cobalt increased the generation of oxygen radicals, but antioxidants and iNOS inhibitors did not prevent toxicity, implying that ROS and NO are not likely to trigger Co^{2+} toxicity in astrocytes. We also found an increase in the phosphorylation of ERK1/2 and p38,

but MEK1 inhibitors could not reduce cytotoxicity. Caspase activation contributed modestly to damage, as shown by the low levels of cleaved PARP and the lack of protective effect of the caspase inhibitor Z-VAD-FMK. Cobalt is a well known blocker of voltage gated calcium channels (MacVicar 1987) and we have observed inhibition of the ATP response by cobalt in glial cells, probably due to the reduced Ca^{2+} influx through P2X ligand-gated calcium channels.

As reported in studies on hypoxia models, the mitochondria appeared to be a main target of cobalt toxicity as well. This was shown by the early loss of mitochondrial membrane potential ($\Delta\Psi_m$) and release of apoptosis inducing factor (AIF). Pre-incubation of astrocytes with bongkrekic acid, an inhibitor of the mitochondrial permeability transition pore (MPT), reduced ATP depletion significantly. Our data suggest that the apoptotic process caused by Co^{2+} involves the opening of the MPT pore, possibly due to the increased level of Nip3.

Thus, several aspects of hypoxia, such as HIF-1α stabilization and deterioration of mitochondria, were mimicked by $CoCl_2$ in the primary astrocyte cultures. One can conclude that some of the features caused by oxygen deprivation can be mimicked by exposure to cobalt, which could represent an easy and reliable tool in studies on the mechanisms underlying ischemic infarction. However, we are not sure that the toxic effects of cobalt are entirely explained by those effects that partially mimic aspects of hypoxia.

4.5 Preliminary results

4.5.1 Role of adenosine receptor subtypes in astrocytes during hypoxic stress

In the present study we wanted to evaluate the role of adenosine receptors in glial cells during stress conditions such as hypoxia or exposure to cobalt chloride. So far, the majority of the studies addressing the role AR in modulating astrocyte functions during hypoxia have employed pharmacological tools, i.e. agonists and antagonists that are sometimes unspecific, especially in the case of the A_3R. Here we have used an alternative approach to ablate the function of specific AR, i.e. their genetic deletion. We have investigated the cytotoxic effects of hypoxia-like conditions in primary astrocytes prepared from specific AR KO mice. Human astrocytoma D384 cells were also used in order to investigate the effect of hypoxia on purine

release. The conditions of toxic challenge were selected based on the findings obtained in paper IV.

As stated earlier, adenosine is important as a modulator of glial function in emergency situations (Boison 2006; Daré et al., 2007). The concentration of adenosine is known to quickly rise during lack of oxygen to reset the energy balance in the cells (Jacobson and Gao 2006). Some recent reports refer to astrocytes as the main source of adenosine production during early stages of hypoxia via the direct release from the cytosol (Martin et al., 2007), but excellent evidence has also been provided on adenosine production via extracellular adenine nucleotides during hypoxia (Parkinson and Xiong 2004). Several studies have demonstrated that all four AR subtypes are present in astrocytes (Daré et al., 2007). Our results suggest that a major part of the decrease in intracellular ATP induced by hypoxia can be related to ATP breakdown and consequent release of purines (adenosine, inosine, hypoxanthine etc) even at early time points, whereas this is not the case for $CoCl_2$ (Fig. 5A). Pre-treatment with NECA, an unselective adenosine receptor agonist, protected WT primary astrocytes and D384 cells from apoptotic death following hypoxia or $CoCl_2$ exposure (Fig. 5B).

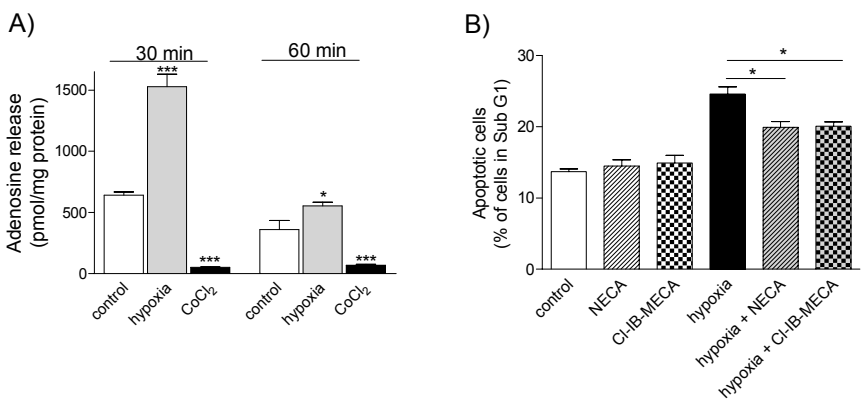

Fig. 5. (A) Purine release from D384 cells incubated with [^{3}H]adenine for 30 min at 37°C. Release of [^{3}H]purines was evoked by buffer, hypoxia and 0.8 mM $CoCl_2$. Released [^{3}H]adenosine was isolated and quantified by TLC. Values are mean ± S.E.M. (*$p < 0.05$, ***$p < 0.001$, vs. buffer control, One way ANOVA followed by Tukey's post-hoc test). (B) Analysis of apoptotic nuclei by flow cytometry in human astrocytoma D384 cells after pre-treatment with the unspecific adenosine agonist NECA (1 μM) or the A_3R agonist Cl-IB-MECA (0.8 μM) for 24 h. Lack of oxygen induced apoptosis that was reduced by the two pre-treatments. Values are expressed as mean ± S.E.M, n = 3 and analyzed by One way ANOVA (*$p < 0.05$).

Our data support the general view that adenosine, by acting on A_1R located on neural cells, ameliorates ischemic damage, since cytotoxicity was more pronounced in A_1R KO astrocytes (Fig. 6A) and tended to be higher in WT cells in the presence of the A_1R antagonist

DPCPX. Indeed, the A_1R can inhibit reactive gliosis that occurs late in ischemia and is linked to production of toxic factors from astrocytes (Ciccarelli et al., 1994; Neary et al., 1996). Also other effects mediated by A_1R activation are likely to reduce ischemic injury, e.g. changes in excitatory synaptic transmission linked to reduced presynaptic transmitter release and post-synaptic membrane hyperpolarization with inhibition of neuronal firing (Martin et al., 2007; Pascual et al., 2005).

A comparable degree of susceptibility to cobalt and hypoxia induced toxicity was observed in $A_{2A}R$ KO and WT astrocytes indicating that $A_{2A}R$ are relatively unimportant. Some signs of a minor protective effect were found in $A_{2A}R$ KO astrocytes (Fig. 6A) as well as in human D384 cells pre-treated with the $A_{2A}R$ antagonist SCH58261. It is known that glial $A_{2A}R$ can regulate the formation of reactive astrocytes in vitro and may also indirectly control the levels of extracellular glutamate by regulating the function of glutamate transporters, i.e. enhancing glutamate efflux from cultured astrocytes (Brambilla et al., 2003; Li et al., 2001). Our finding is of particular interest considering the possible use of $A_{2A}R$ antagonists for treating stroke (Chen et al., 1999).

A_3R KO glial cells were more affected by the lack of oxygen than WT cells (Fig. 6A). In accordance, the A_3R agonist Cl-IB-MECA reduced ATP depletion caused by hypoxia in primary astrocytes. It also reduced apoptosis in human astroglioma D384 cells after oxygen deprivation (Fig. 5B). A_3R KO cells showed more pronounced necrosis after exposure to 24 h hypoxia and $CoCl_2$ (Fig. 6B). Low concentrations of A_3R agonists (Cl-IB-MECA and the less specific IB-MECA) have been previously reported to be neuroprotective in other astrocyte models, probably via increased ERK1/2 phosphorylation (Abbracchio et al., 1998). Interestingly, the protective role of A_3R in the brain has emerged in two in vivo studies where a higher degree of neuronal damage was seen in A_3R KO mice in comparison with WT mice following CO-mediated hypoxia or after middle cerebral artery occlusion (MCAO) (Chen et al., 2006; Fedorova et al., 2003). It is also worth noting that stimulation of A_3R in astrocytes in vitro was shown to induce the release of the neuroprotective cytokine CCl2 (Wittendorp et al., 2004).

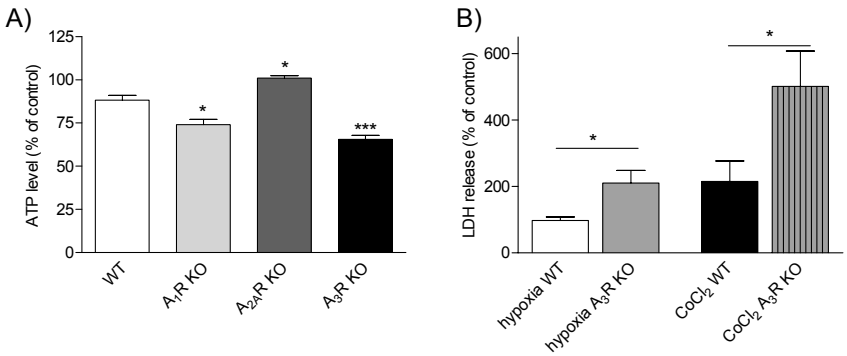

Fig. 6. (A) Effects on ATP levels after 24 h exposure to hypoxic environment in primary astrocytes prepared from different adenosine receptor knock-out and WT mice. Values are expressed as percentage of the ATP content in untreated cells of the same genotype, means ± S.E.M., n = 3-4. Statistical analysis was performed with One-way ANOVA followed by Tukey's post hoc test (*p < 0.05, **p < 0.01; ***p < 0.001, vs.WT). (B) Hypoxia and $CoCl_2$ (0.8 mM) resulted in an increase in LDH release after 24 h exposure. This effect was more pronounced in A_3R KO astrocytes (hypoxia: p = 0.028, $CoCl_2$: p = 0.045, Student's t-test) as compared to the WT cells. Values are expressed as means ± S.E.M. (n = 3-4).

In conclusion, breakdown of ATP into adenosine occurs during the early stages of hypoxia, whereas cobalt has no such effect. Endogenously produced adenosine, as well adenosine analogues acting on A_1R and A_3R appear to protect primary mouse astrocytes from the hypoxic insult. Further understanding of the mechanisms that reduce astrocytic death after hypoxia could be relevant for the development of pharmacological treatments for stroke and birth asphyxia.

4.5.2 Characterizing the role of AR during hypoxic ischemia in immature and mature mice

Because of the inconsistencies in the literature regarding the role of the different adenosine receptors in hypoxic ischemia and during various stages of maturation (see Introduction) this project was undertaken to elucidate their role using a single model in mice of different ages. The AR knock-out mice used in this study were all on the same genetic background. We chose the Rice-Vannucci hypoxic ischemia model which is established in immature animals as well as in adult mice. It allows long term survival that enables evaluation of functional impairment and assessment of sensorimotor functions.

Our preliminary data show a significantly aggravated injury 2 weeks after hypoxic ischemia in both immature A_1R KO mice and mature A_1R KO mice (Table 2). This protective effect of A_1R is in agreement with the results obtained by Johansson and colleagues, where

A_1R deletion impaired recovery from in vitro hypoxia in hippocampal slices compared to slices from WT, probably by lowering the excitatory amino acid release and postsynaptic depolarization (Johansson et al., 2001). However, in a study using a global ischemia model, no differences between the damage in A_1R KO and WT adult mice were detected, while significant aggravation of the damage was found in WT mice injected with A_1R antagonist prior to the insult (Olsson et al., 2004). The discrepancies might be due to the differences in the model of hypoxia employed as well as to the use of mice with mixed genetic background in the cited study.

A significantly increased morphological damage was found in $A_{2A}R$ KO pups (Table 2). We did not observe differences in behavioral test results of adult mice of the two genotypes two weeks after the insult, but there was a tendency to increased locomotion and increased number of mistakes in the beam walking test in $A_{2A}R$ KO pups compared to the WT. The present data are in agreement with results obtained using the same model of ischemia in 7-day-old $A_{2A}R$ KO mice on CD1 background, where aggravated damage was observed in cortex, striatum and hippocampus 5 and 21 days after hypoxic ischemia (Åden et al., 2003). In this study behavioral examination after 3 weeks and 3 months showed worsening of the sensorimotor abilities in the mice with genetic deletion of $A_{2A}R$. Thus, the results show that despite the different strains of $A_{2A}R$ KO mice used in the two studies, the differences in age when pups were subjected to the insult (in our study PND10 and in Ådén's model PND 7), as well as the variation in the evaluation date, the $A_{2A}R$ seem to have a protective role in the neonatal model of hypoxic ischemia in vivo. $A_{2A}R$ stimulation mediates vasodilatation and inhibition of neutrophil activation which could explain the present phenomena (Åden et al., 2003).

$A_{2A}R$ did not appear to play a major role in Rice-Vannucci model of ischemia in adult mice. This was in agreement with studies performed on adult animals on the same genetic background as ours (C57BL/6×129 Steel) where $A_{2A}R$ inactivation resulted in reduced brain damage and improved neurological score evaluated 5 days after focal ischemia in $A_{2A}R$ KO (Chen et al., 1999). The potential detrimental effects of $A_{2A}R$ activation may be due to the facilitation of glutamate efflux. Since the same results were obtained in animals of the same age in different models the discrepancies between effects of $A_{2A}R$ in adult and immature mice are likely to be explained by age related factors (and not by differencies in models of ischemia).

We found significantly increased susceptibility to hypoxic ischemia damage in both A_3R KO pups and adult mice compared to the corresponding WT operated groups (Table 2). Significantly higher score in beam walking (indicating greater motor impairment) as well as reduced horizontal activity and rearing were found in young operated A_3R KO mice when compared to the operated WT pups, showing behavioral alterations in A_3R KO mice. This implies a protective role of the A_3R in hypoxic ischemia. One possible mechanism is that A_3R can diminish the release of TNF alpha, as reported in a study with adult A_3R KO mice as well as in rats injected with A_3R agonist Cl-IB-MECA in the Rice-Vannucci hypoxic ischemia model (Chen et al., 2006).

Table 2. Histopathology score (0-24) 2 weeks after hypoxic ischemia. Mann-Whitney *U* test, comparison to WT mice (*p<0.05, **p<0.01, ***p<0.001).

Mice	WT	A_1R KO	$A_{2A}R$ KO	A_3R KO
Immature	median 11	median 16***	median 15.5*	median 17**
	range 0-23.5, n = 41	range 0-22, n = 23	range 0-21.5, n = 13	range: 0-24, n = 27
Mature	median 1.5	median 7.25**	median 0.5	median 4*
	range 0-21.5, n = 74	range 0-22, n = 34	range 0-21.5, n = 39	range 0-22, n = 49

In summary, preliminary data from our in vivo studies show a protective role of A_1R, $A_{2A}R$ and A_3R in different maturational stages. Whereas the effect of $A_{2A}R$ has not been straightforward, the results on A_1R and A_3R are in concordance with our in vitro data obtained in primary mouse astrocytes after hypoxic insult (see 4.5.1.).

5 Conclusions

1. Exposure to very low doses of MeHg during the perinatal period causes altered behavior in male mice.

2. Perinatal caffeine attenuates MeHg-induced behavioral changes in mouse offspring by blocking A_1R and $A_{2A}R$, as evidenced by reduced behavioral consequences of MeHg exposure in A_1R KO and $A_{2A}R$ KO mice.

3. Exposure of the dams to caffeine leads to increased locomotor activity and a stronger response to psychostimulants in mouse offspring.

4. A_1R KO mice have a higher basal locomotor activity than WT mice and respond to a psychostimulant in the same manner as caffeine pre-treated mice.

5. It seems that perinatal caffeine, by acting on adenosine A_1R in the mother, can induce long-lasting behavioral changes in the offspring.

6. Young $A_{2A}R$ KO mice show higher basal activity than WT mice, but this is not observed in adult age. Adult $A_{2A}R$ KO mice respond less to the psychostimulant activation.

7. A_3R KO mice show increased basal activity in comparison with WT mice and respond less to caffeine, cocaine and natural stimuli (darkness). Female A_3R KO mice show greater susceptibility to MeHg toxicity. A_3R may play a role in brain development.

8. A_1R and A_3R appear to show cytoprotective effects against hypoxic conditions, i.e. oxygen deprivation and $CoCl_2$ induced toxicity, in mouse astrocytes.

9. Our preliminary in vivo study tentatively shows that the brain damaging effects of hypoxic ischemia are aggravated in both immature and mature animals with genetic deletion of either A_1R or A_3R, as well in young $A_{2A}R$ KO mice.

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7 References

Abbracchio, M.P., Ceruti, S., Brambilla, R., Franceschi, C., Malorni, W., Jacobson, K.A., von Lubitz, D.K., Cattabeni, F., 1997. Modulation of apoptosis by adenosine in the central nervous system: a possible role for the A3 receptor. Pathophysiological significance and therapeutic implications for neurodegenerative disorders. Ann. N. Y. Acad . Sci. 825, 11-22.

Abbracchio, M.P., Ceruti, S., Brambilla, R., Barbieri, D., Camurri, A., Franceschi, C., Giammarioli, A.M., Jacobson, K.A., Cattabeni, F., Malorni, W., 1998. Adenosine A_3 receptors and viability in astrocytes. Drug. Develop. Res. 45, 379-86.

Åden, U., Herlenius, E., Tang, L.Q., Fredholm, B.B., 2000. Maternal caffeine intake has minor effects on adenosine receptor ontogeny in the rat brain. Pediatr Res 48, 177-83.

Åden, U., Halldner, L., Lagercrantz, H., Dalmau, I., Ledent, C., Fredholm, B.B., 2003. Aggravated brain damage after hypoxic ischemia in immature adenosine A2A knockout mice. Stroke 34, 739-44.

Allaman, I., Lengacher, S., Magistretti, P.J., Pellerin, L., 2003. A_{2B} receptor activation promotes glycogen synthesis in astrocytes through modulation of gene expression. Am .J. Physiol. Cell Physiol. 284, C696-704.

Almeida, A., Almeida, J., Bolanos, J.P., Moncada, S., 2001. Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. Proc Natl Acad Sci U S A 98, 15294-9.

Alsene, K., Deckert, J., Sand, P., de Wit, H., 2003. Association between A2a receptor gene polymorphisms and caffeine-induced anxiety. Neuropsychopharmacology 28, 1694-702.

Andersen, S.L., 2005. Stimulants and the developing brain. Trends Pharmacol Sci 26, 237-43.

Araya, J., Maruyama, M., Inoue, A., Fujita, T., Kawahara, J., Sassa, K., Hayashi, R., Kawagishi, Y., Yamashita, N., Sugiyama, E., Kobayashi, M., 2002. Inhibition of proteasome activity is involved in cobalt-induced apoptosis of human alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 283, L849-58.

Arnaud, M.J., 1987. The pharmacology of caffeine. Prog Drug Res 31, 273-313.

Arslan, G., Kull, B., Fredholm, B.B., 2002. Anoxia redistributes adenosine A(2A) receptors in PC12 cells and increases receptor-mediated formation of cAMP. N-S Arch Pharmacol 365, 150-7.

Ascherio, A., Zhang, S.M., Hernan, M.A., Kawachi, I., Colditz, G.A., Speizer, F.E., Willett, W.C., 2001. Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. Ann Neurol 50, 56-63.

Aschner, M., Allen, J.W., 2000. Astrocytes in methylmercury, ammonia, methionine sulfoximine and alcohol-induced neurotoxicity. Neurotoxicology 21, 573-9.

Aschner, M., Yao, C.P., Allen, J.W., Tan, K.H., 2000. Methylmercury alters glutamate transport in astrocytes. Neurochem. Int. 37, 199-206.

Aschner, M., Sonnewald, U., Tan, K.H., 2002. Astrocyte modulation of neurotoxic injury. Brain Pathol 12, 475-81.

Avila, M.Y., Stone, R.A., Civan, M.M., 2002. Knockout of A3 adenosine receptors reduces mouse intraocular pressure. Invest Ophthalmol Vis Sci 43, 3021-6.

Bakir, F., Damluji, S.F., Amin-Zaki, L., Murtadha, M., Khalidi, A., al-Rawi, N.Y., Tikriti, S., Dahahir, H.I., Clarkson, T.W., Smith, J.C., Doherty, R.A., 1973. Methylmercury poisoning in Iraq. Science 181, 230-41.

Barber, P.A., Demchuk, A.M., Hirt, L., Buchan, A.M., 2003. Biochemistry of ischemic stroke. Adv Neurol. 92, 151-164.

Barres, B.A., 2003. What is a glial cell? Glia 43, 4-5.

Berglund, M., Lind, B., Bjornberg, K.A., Palm, B., Einarsson, O., Vahter, M., 2005. Inter-individual variations of human mercury exposure biomarkers: a cross-sectional assessment. Environ Health 4, 20.

Bessis, A., Bechade, C., Bernard, D., Roumier, A., 2007. Microglial control of neuronal death and synaptic properties. Glia 55, 233-8.

Blomgren, K., McRae, A., Bona, E., Saido, T.C., Karlsson, J.O., Hagberg, H., 1995. Degradation of fodrin and MAP 2 after neonatal cerebral hypoxic-ischemia. Brain Res 684, 136-42.

Boison, D., 2006. Adenosine kinase, epilepsy and stroke: mechanisms and therapies. Trends Pharmacol Sci 27, 652-8.

Bona, E., Aden, U., Fredholm, B.B., Hagberg, H., 1995. The effect of long term caffeine treatment on hypoxic-ischemic brain damage in the neonate. Pediatr Res 38, 312-8.

Bonati, M., Latini, R., Tognoni, G., Young, J.F., Garattini, S., 1984. Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. Drug Metab Rev 15, 1355-83.

Bracken, M.B., Triche, E.W., Belanger, K., Hellenbrand, K., Leaderer, B.P., 2003. Association of maternal caffeine consumption with decrements in fetal growth. Am J Epidemiol 157, 456-66.

Brambilla, R., Cottini, L., Fumagalli, M., Ceruti, S., Abbracchio, M.P., 2003. Blockade of A_{2A} adenosine receptors prevents basic fibroblast growth factor-induced reactive astrogliosis in rat striatal primary astrocytes. Glia 43, 190-194.

Brown, R.D., Thoren, P., Steege, A., Mrowka, R., Sallstrom, J., Skott, O., Fredholm, B.B., Persson, A.E., 2006. Influence of the adenosine A1 receptor on blood pressure regulation and renin release. Am J Physiol Regul Integr Comp Physiol 290, R1324-9.

Bruick, R.K., 2000. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A 97, 9082-7.

Castellanos, F.X., Rapoport, J.L., 2002. Effects of caffeine on development and behavior in infancy and childhood: a review of the published literature. Food Chem Toxicol 40, 1235-42.

Castoldi, A.F., Blandini, F., Randine, G., Samuele, A., Manzo, L., Coccini, T., 2006. Brain monoaminergic neurotransmission parameters in weanling rats after perinatal exposure to methylmercury and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153). Brain Res 1112, 91-8.

Cauli, O., Morelli, M., 2005. Caffeine and the dopaminergic system. Behav Pharmacol 16, 63-77.

Chandel, N.S., Vander Heiden, M.G., Thompson, C.B., Schumacker, P.T., 2000. Redox regulation of p53 during hypoxia. Oncogene 19, 3840-3848.

Chen, G.J., Harvey, B.K., Shen, H., Chou, J., Victor, A., Wang, Y., 2006. Activation of adenosine A3 receptors reduces ischemic brain injury in rodents. J. Neurosci. Res. 84, 1848-55.

Chen, J.F., Huang, Z., Ma, J., Zhu, J., Moratalla, R., Standaert, D., Moskowitz, M.A., Fink, J.S., Schwarzschild, M.A., 1999. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci 19, 9192-200.

Chen, J.F., Beilstein, M., Xu, Y.H., Turner, T.J., Moratalla, R., Standaert, D.G., Aloyo, V.J., Fink, J.S., Schwarzschild, M.A., 2000. Selective attenuation of psychostimulant-induced behavioral responses in mice lacking A(2A) adenosine receptors. Neuroscience 97, 195-204.

Chen, J.F., Xu, K., Petzer, J.P., Staal, R., Xu, Y.H., Beilstein, M., Sonsalla, P.K., Castagnoli, K., Castagnoli, N., Jr., Schwarzschild, M.A., 2001. Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. J Neurosci 21, RC143.

Ciccarelli, R., Di Iorio, P., Ballerini, P., Ambrosini, G., Giuliani, P., Tiboni, G.M., Caciagli, F., 1994. Effects of exogenous ATP and related analogues on the proliferation rate of dissociated primary cultures of rat astrocytes. J. Neurosci. Res. 39, 556-566.

Ciccarelli, R., Di Iorio, P., Bruno, V., Battaglia, G., D'Alimonte, I., D'Onofrio, M., Nicoletti, F., Caciagli, F., 1999. Activation of A_1 adenosine or mGlu3 metabotropic glutamate receptors enhances the release of nerve growth factor and S-100β protein from cultured astrocytes. Glia 27, 275-281.

Ciruela, F., Casado, V., Rodrigues, R.J., Lujan, R., Burgueno, J., Canals, M., Borycz, J., Rebola, N., Goldberg, S.R., Mallol, J., Cortes, A., Canela, E.I., Lopez-Gimenez, J.F., Milligan, G., Lluis, C., Cunha, R.A., Ferre, S., Franco, R., 2006. Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. J Neurosci 26, 2080-7.

Clarkson, T.W., 1997. The toxicology of mercury. Crit Rev Clin Lab Sci 34, 369-403.

Clarkson, T.W., Magos, L., 2006. The toxicology of mercury and its chemical compounds. Crit Rev Toxicol 36, 609-62.

Cnattingius, S., Signorello, L.B., Anneren, G., Clausson, B., Ekbom, A., Ljunger, E., Blot, W.J., McLaughlin, J.K., Petersson, G., Rane, A., Granath, F., 2000. Caffeine intake and the risk of first-trimester spontaneous abortion. N Engl J Med 343, 1839-45.

Cullheim, S., Thams, S., 2007. The microglial networks of the brain and their role in neuronal network plasticity after lesion. Brain Res Rev.

Cunha, R.A., 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem. Int. 38, 107-25.

Daly, J.W., Fredholm, B.B., 1998. Caffeine--an atypical drug of dependence. Drug Alcohol Depend 51, 199-206.

Daré, E., Fetissov, S., Hökfelt, T., Hall, H., Ögren, S.O., Ceccatelli, S., 2003. Effects of prenatal exposure to methylmercury on dopamine-mediated locomotor activity and dopamine D2 receptor binding. N-S Arch Pharmacol 367, 500-8. Epub 2003 Apr 9.

Daré, E., Schulte, G., Karovic, O., Hammarberg, C., Fredholm, B.B., 2007. Modulation of glial cell functions by adenosine receptors. Physiol Behav 92, 15-20.

Dassesse, D., Massie, A., Ferrari, R., Ledent, C., Parmentier, M., Arckens, L., Zoli, M., Schiffmann, S.N., 2001. Functional striatal hypodopaminergic activity in mice lacking adenosine A(2A) receptors. J. Neurochem. 78, 183-98.

Davidson, P.W., Myers, G.J., Shamlaye, C., Cox, C., Wilding, G.E., 2004. Prenatal exposure to methylmercury and child development: influence of social factors. Neurotoxicol Teratol 26, 553-9.

de Mendonca, A., Sebastiao, A.M., Ribeiro, J.A., 2000. Adenosine: does it have a neuroprotective role after all? Brain Res Brain Res Rev 33, 258-74.

Debes, F., Budtz-Jorgensen, E., Weihe, P., White, R.F., Grandjean, P., 2006. Impact of prenatal methylmercury exposure on neurobehavioral function at age 14 years. Neurotoxicol Teratol 28, 363-75.

Di Iorio, P., Kleywegt, S., Ciccarelli, R., Traversa, U., Andrew, C.M., Crocker, C.E., Werstiuk, E.S., Rathbone, M.P., 2002. Mechanisms of apoptosis induced by purine nucleosides in astrocytes. Glia 38, 179-190.

Dorf, M.E., Berman, M.A., Tanabe, S., Heesen, M., Luo, Y., 2000. Astrocytes express functional chemokine receptors. J Neuroimmunol 111, 109-21.

Dunwiddie, T.V., Masino, S.A., 2001. The role and regulation of adenosine in the central nervous system. Annu. Rev. Neurosci. 24, 31-55.

Eckle, T., Krahn, T., Grenz, A., Kohler, D., Mittelbronn, M., Ledent, C., Jacobson, M.A., Osswald, H., Thompson, L.F., Unertl, K., Eltzschig, H.K., 2007. Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. Circulation 115, 1581-90.

EFSA: Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to mercury and methylmercury in food. European Food Safety Authority Journal, 2004, pp. 1-14.

El Yacoubi, M., Ledent, C., Parmentier, M., Bertorelli, R., Ongini, E., Costentin, J., Vaugeois, J.M., 2001. Adenosine A2A receptor antagonists are potential antidepressants: evidence based on pharmacology and A2A receptor knockout mice. Br J Pharmacol 134, 68-77.

Elinder, C.G., Friberg, L., Lind, B., Nilsson, B., Svartengren, M., Overmark, I., 1986. Decreased blood lead levels in residents of Stockholm for the period 1980-1984. Scand J Work Environ Health 12, 114-20.

Endres, M., Dirnagl, U., 2002. Ischemia and stroke. Adv. Exp. Med. Biol. 513, 455-73.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J., Ratcliffe, P.J., 2001. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107, 43-54.

Faro, L.R., do Nascimento, J.L., Alfonso, M., Duran, R., 2002. Mechanism of action of methylmercury on in vivo striatal dopamine release. Possible involvement of dopamine transporter. Neurochem. Int. 40, 455-65.

Fedele, D.E., Li, T., Lan, J.Q., Fredholm, B.B., Boison, D., 2006. Adenosine A1 receptors are crucial in keeping an epileptic focus localized. Exp Neurol 200, 184-90.

Fedorova, I.M., Jacobson, M.A., Basile, A., Jacobson, K.A., 2003. Behavioral characterization of mice lacking the A3 adenosine receptor: sensitivity to hypoxic neurodegeneration. Cell Mol Neurobiol 23, 431-47.

Feldman, R.S., Meyer, J.S., Quenzer, L.F., 1997. Principles of Neuropharmacology. Principles of Neuropharmacology, 612.

Fenster, L., Eskenazi, B., Windham, G.C., Swan, S.H., 1991. Caffeine consumption during pregnancy and spontaneous abortion. Epidemiology 2, 168-74.

Ferre, S., Popoli, P., Gimenez-Llort, L., Finnman, U.B., Martinez, E., Scotti de Carolis, A., Fuxe, K., 1994. Postsynaptic antagonistic interaction between adenosine A1 and dopamine D1 receptors. Neuroreport 6, 73-6.

Ferre, S., Popoli, P., Gimenez-Llort, L., Rimondini, R., Muller, C.E., Stromberg, I., Ogren, S.O., Fuxe, K., 2001. Adenosine/dopamine interaction: implications for the treatment of Parkinson's disease. Parkinsonism Relat Disord 7, 235-241.

Ferre, S., Ciruela, F., Borycz, J., Solinas, M., Quarta, D., Antoniou, K., Quiroz, C., Justinova, Z., Lluis, C., Franco, R., Goldberg, S.R., 2008. Adenosine A1-A2A receptor heteromers: new targets for caffeine in the brain. Front Biosci 13, 2391-9.

Fiebich, B.L., Biber, K., Lieb, K., van Calker, D., Berger, M., Bauer, J., Gebicke-Haerter, P.J., 1996. Cyclooxygenase-2 expression in rat microglia is induced by adenosine A_{2a}-receptors. Glia 18, 152-160.

Fischer, C., Fredriksson, A., Eriksson, P., 2007. Neonatal co-exposure to low doses of an ortho-PCB (PCB 153) and methyl mercury exacerbate defective developmental neurobehavior in mice. Toxicology.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., Williams, M., 1994. Nomenclature and classification of purinoceptors. Pharmacol Rev 46, 143-56.

Fredholm, B.B., Altiok, N., 1994. Adenosine A_{2B} receptor signalling is altered by stimulation of bradykinin or interleukin receptors in astroglioma cells. Neurochem. Int. 25, 99-102.

Fredholm, B.B.: Adenosine and neuroprotection. In: *Neuroprotective agents and cerebral ischemia*. Eds.: A. R. Green and A. J. Cross. Academic Press, London, 1996, pp. 259-280.

Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A., Zvartau, E.E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 51, 83-133.

Fredholm, B.B., Arslan, G., Halldner, L., Kull, B., Schulte, G., Wasserman, W., 2000. Structure and function of adenosine receptors and their genes. N-S Arch Pharmacol 362, 364-74.

Fredholm, B.B., AP, I., Jacobson, K.A., Klotz, K.N., Linden, J., 2001a. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53, 527-552.

Fredholm, B.B., Irenius, E., Kull, B., Schulte, G., 2001b. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. Biochem. Pharmacol. 61, 443-8.

Fredholm, B.B., Chen, J.F., Cunha, R.A., Svenningsson, P., Vaugeois, J.M., 2005a. Adenosine and brain function. Int. Rev. Neurobiol. 63, 191-270.

Fredholm, B.B., Chen, J.F., Masino, S.A., Vaugeois, J.M., 2005b. Actions of Adenosine at Its Receptors in the CNS: Insights from Knockouts and Drugs. Annu. Rev. Pharmacol. Toxicol. 45, 385-412.

Gerfen, C., Wilson, CJ: The basal ganglia. In: *Handbook in Chemical Neuroanatomy*. Ed.: L. Swanson, Björklund, A, Hökefelt, T (eds.). Elsevier, Amsterdam, 1996, pp. 371-468.

Gimenez-Llort, L., Fernandez-Teruel, A., Escorihuela, R.M., Fredholm, B.B., Tobena, A., Pekny, M., Johansson, B., 2002. Mice lacking the adenosine A1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate. Eur J Neurosci 16, 547-50.

Gímenez-Llort, L., Ahlbom, E., Dare, E., Vahter, M., Ogren, S., Ceccatelli, S., 2001. Prenatal exposure to methylmercury changes dopamine-modulated motor activity during early ontogeny: age and gender-dependent effects. Environ Toxicol Pharmacol 9, 61-70.

Grandjean, P., Weihe, P., White, R.F., Debes, F., Araki, S., Yokoyama, K., Murata, K., Sorensen, N., Dahl, R., Jorgensen, P.J., 1997. Cognitive deficit in 7-year-old

children with prenatal exposure to methylmercury. Neurotoxicol Teratol 19, 417-28.

Grandjean, P., Landrigan, P.J., 2006. Developmental neurotoxicity of industrial chemicals. Lancet 368, 2167-78.

Green, R.A., Cross, J.A.: Neuropritective agents and cerebral ischaemia. In: *International Review of Neurobiology*, 1997.

Gregory, G.A., Welsh, F.A., Yu, A.C., Chan, P.H., 1990. Fructose-1,6-bisphosphate reduces ATP loss from hypoxic astrocytes. Brain Res 516, 310-2.

Gupta, S., 2001. Molecular steps of death receptor and mitochondrial pathways of apoptosis. Life Sci 69, 2957-64.

Hagberg, H., 2004. Mitochondrial impairment in the developing brain after hypoxia-ischemia. J Bioenerg Biomembr 36, 369-73.

Halldner, L., Aden, U., Dahlberg, V., Johansson, B., Ledent, C., Fredholm, B.B., 2004. The adenosine A1 receptor contributes to the stimulatory, but not the inhibitory effect of caffeine on locomotion: a study in mice lacking adenosine A1 and/or A2A receptors. Neuropharmacology 46, 1008-17.

Hammarberg, C., Fredholm, B.B., Schulte, G., 2004. Adenosine A_3 receptor-mediated regulation of p38 and extracellular-regulated kinase ERK1/2 via phosphatidylinositol-3'-kinase. Biochem. Pharmacol. 67, 129-134.

Hasko, G., Szabo, C., Nemeth, Z.H., Kvetan, V., Pastores, S.M., Vizi, E.S., 1996. Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. J. Immunol. 157, 4634-40.

Hasko, G., Pacher, P., Vizi, E.S., Illes, P., 2005. Adenosine receptor signaling in the brain immune system. Trends Pharmacol Sci 26, 511-6.

Haydon, P.G., Carmignoto, G., 2006. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev 86, 1009-31.

Heath, J.C., 1956. The production of malignant tumours by cobalt in the rat. Br J Cancer 10, 668-73.

Holgate, S.T., 2005. The Quintiles Prize Lecture 2004. The identification of the adenosine A2B receptor as a novel therapeutic target in asthma. Br J Pharmacol 145, 1009-15.

Hua, X., Kovarova, M., Chason, K.D., Nguyen, M., Koller, B.H., Tilley, S.L., 2007. Enhanced mast cell activation in mice deficient in the A2b adenosine receptor. J Exp Med 204, 117-28.

Huang, C.C., Yang, P.C., Lin, H.J., Hsu, K.S., 2007. Repeated cocaine administration impairs group II metabotropic glutamate receptor-mediated long-term depression in rat medial prefrontal cortex. J Neurosci 27, 2958-68.

Huang, Z.L., Qu, W.M., Eguchi, N., Chen, J.F., Schwarzschild, M.A., Fredholm, B.B., Urade, Y., Hayaishi, O., 2005. Adenosine A2A, but not A1, receptors mediate the arousal effect of caffeine. Nat Neurosci 8, 858-9.

Huk, O.L., Catelas, I., Mwale, F., Antoniou, J., Zukor, D.J., Petit, A., 2004. Induction of apoptosis and necrosis by metal ions in vitro. J Arthroplasty 19, 84-87.

Jacobson, K.A., Siddiqi, S.M., Olah, M.E., Ji, X.D., Melman, N., Bellamkonda, K., Meshulam, Y., Stiles, G.L., Kim, H.O., 1995. Structure-activity relationships of 9-alkyladenine and ribose-modified adenosine derivatives at rat A3 adenosine receptors. J. Med. Chem. 38, 1720-35.

Jacobson, K.A., Gao, Z.G., 2006. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 5, 247-64.

Jarvis, M.F., Jackson, R.H., Williams, M., 1989. Autoradiographic characterization of high-affinity adenosine A2 receptors in the rat brain. Brain Res 484, 111-8.

JECFA: Summary and Conclussions of the Sixty-first Meeting of the Joint FAO/WHO Expert Committee on Food Additives, 2003.

Jensen, A.A., Tuchsen, F., 1990. Cobalt exposure and cancer risk. Crit Rev Toxicol. 20, 427-437.

Johansson, B., Halldner, L., Dunwiddie, T.V., Masino, S.A., Poelchen, W., Gimenez-Llort, L., Escorihuela, R.M., Fernandez-Teruel, A., Wiesenfeld-Hallin, Z., Xu, X.J., Hardemark, A., Betsholtz, C., Herlenius, E., Fredholm, B.B., 2001. Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. Proc Natl Acad Sci U S A 98, 9407-12. Epub 2001 Jul 24.

Johansson, C., Castoldi, A.F., Onishchenko, N., Manzo, L., Vahter, M., Ceccatelli, S., 2007a. Neurobehavioural and molecular changes induced by methylmercury exposure during development. Neurotox Res 11, 241-60.

Johansson, S.M., Yang, J.N., Lindgren, E., Fredholm, B.B., 2007b. Eliminating the antilipolytic adenosine A) receptor does not lead to compensatory changes in the antilipolytic actions of PGE2 and nicotinic acid. Acta Physiol. (Oxf) 190, 87-96.

Jones, N.M., Bergeron, M., 2001. Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain. J Cereb Blood Flow Metab 21, 1105-1114.

Jordan, C.M., Whitman, R.D., Harbut, M., 1997. Memory deficits and industrial toxicant exposure: a comparative study of hard metal, solvent and asbestos workers. Int J Neurosci 90, 113-128.

Judas, M., Rados, M., Jovanov-Milosevic, N., Hrabac, P., Stern-Padovan, R., Kostovic, I., 2005. Structural, immunocytochemical, and mr imaging properties of periventricular crossroads of growing cortical pathways in preterm infants. AJNR Am J Neuroradiol 26, 2671-84.

Kakita, A., Wakabayashi, K., Su, M., Yoneoka, Y., Sakamoto, M., Ikuta, F., Takahashi, H., 2000. Intrauterine methylmercury intoxication. Consequence of the inherent brain lesions and cognitive dysfunction in maturity. Brain Res 877, 322-30.

Kesteloot, H., Roelandt, J., Willems, J., Claes, J.H., Joossens, J.V., 1968. An enquiry into the role of cobalt in the heart disease of chronic beer drinkers. Circulation 37, 854-64.

Komuro, H., Rakic, P., 1998. Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca2+ fluctuations. J Neurobiol 37, 110-30.

Kong, T., Westerman, K.A., Faigle, M., Eltzschig, H.K., Colgan, S.P., 2006. HIF-dependent induction of adenosine A2B receptor in hypoxia. FASEB J. 20, 2242-50.

Kuzmin, A., Johansson, B., Gimenez, L., Ogren, S.O., Fredholm, B.B., 2006. Combination of adenosine A1 and A2A receptor blocking agents induces caffeine-like locomotor stimulation in mice. Eur Neuropsychopharmacol 16, 129-36.

Landrigan, P.J., Sonawane, B., Butler, R.N., Trasande, L., Callan, R., Droller, D., 2005. Early environmental origins of neurodegenerative disease in later life. Environ Health Perspect 113, 1230-3.

Ledent, C., Vaugeois, J.M., Schiffmann, S.N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J.J., Costentin, J., Heath, J.K., Vassart, G., Parmentier, M., 1997. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. Nature 388, 674-8.

Leker, R.R., Shohami, E., 2002. Cerebral ischemia and trauma-different etiologies yet similar mechanisms: neuroprotective opportunities. Brain Res Brain Res Rev 39, 55-73.

Levy, O., Coughlin, M., Cronstein, B.N., Roy, R.M., Desai, A., Wessels, M.R., 2006. The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. J. Immunol. 177, 1956-66.

Li, X.X., Nomura, T., Aihara, H., Nishizaki, T., 2001. Adenosine enhances glial glutamate efflux via A_{2a} adenosine receptors. Life Sci 68, 1343-1350.

Linnet, K.M., Dalsgaard, S., Obel, C., Wisborg, K., Henriksen, T.B., Rodriguez, A., Kotimaa, A., Moilanen, I., Thomsen, P.H., Olsen, J., Jarvelin, M.R., 2003. Maternal lifestyle factors in pregnancy risk of attention deficit hyperactivity disorder and associated behaviors: review of the current evidence. Am J Psychiatry 160, 1028-40.

Lloyd, H.G., Fredholm, B.B., 1995. Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. Neurochem. Int. 26, 387-95.

MacVicar, B.A., 1987. Morphological differentiation of cultured astrocytes is blocked by cadmium or cobalt. Brain Res 420, 175-7.

Maines, M.D., 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J. 2, 2557-68.

Martin, E.D., Fernandez, M., Perea, G., Pascual, O., Haydon, P.G., Araque, A., Cena, V., 2007. Adenosine released by astrocytes contributes to hypoxia-induced modulation of synaptic transmission. Glia 55, 36-45.

McQueen, D.S., Ribeiro, J.A., 1986. Pharmacological characterization of the receptor involved in chemoexcitation induced by adenosine. Br J Pharmacol 88, 615-20.

Monier, A., Evrard, P., Gressens, P., Verney, C., 2006. Distribution and differentiation of microglia in the human encephalon during the first two trimesters of gestation. J. Comp. Neurol. 499, 565-82.

Moore, K.A., Nicoll, R.A., Schmitz, D., 2003. Adenosine gates synaptic plasticity at hippocampal mossy fiber synapses. Proc Natl Acad Sci U S A 100, 14397-402.

Mumford, G.K., Holtzman, S.G., 1991. Qualitative differences in the discriminative stimulus effects of low and high doses of caffeine in the rat. J Pharmacol Exp Ther 258, 857-65.

Neary, J.T., Rathbone, M.P., Cattabeni, F., Abbracchio, M.P., Burnstock, G., 1996. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci. 19, 13-18.

Nehlig, A., Debry, G., 1994. Potential teratogenic and neurodevelopmental consequences of coffee and caffeine exposure: a review on human and animal data. Neurotoxicol Teratol 16, 531-43.

Nehlig, A., Boyet, S., 2000. Dose-response study of caffeine effects on cerebral functional activity with a specific focus on dependence. Brain Res 858, 71-7.

Newby, A.C., 1984. Adenosine and the concept of "retaliatory metabolites". Trends Biochem. 9, 42-44.

NRC: Toxicological effects of methylmercury. *Committee on the Toxicological Effects of methylmercury, US National Research Council.* Washington, DC, 2000.

O'Shea, R.D., 2002. Roles and regulation of glutamate transporters in the central nervous system. Clin Exp Pharmacol Physiol 29, 1018-23.

Olivieri, G., Hess, C., Savaskan, E., Ly, C., Meier, F., Baysang, G., Brockhaus, M., Muller-Spahn, F., 2001. Melatonin protects SHSY5Y neuroblastoma cells from

cobalt-induced oxidative stress, neurotoxicity and increased beta-amyloid secretion. J Pineal Res 31, 320-325.

Olsen, J., Overvad, K., Frische, G., 1991. Coffee consumption, birthweight, and reproductive failures. Epidemiology 2, 370-4.

Olsson, T., Cronberg, T., Rytter, A., Asztely, F., Fredholm, B.B., Smith, M.L., Wieloch, T., 2004. Deletion of the adenosine A1 receptor gene does not alter neuronal damage following ischaemia in vivo or in vitro. Eur J Neurosci 20, 1197-204.

Onishchenko, N., Tamm, C., Vahter, M., Hokfelt, T., Johnson, J.A., Johnson, D.A., Ceccatelli, S., 2007. Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice. Toxicol Sci 97, 428-37.

Orrenius, S., Zhivotovsky, B., Nicotera, P., 2003. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 4, 552-65.

Panickar, K.S., Norenberg, M.D., 2005. Astrocytes in cerebral ischemic injury: morphological and general considerations. Glia 50, 287-98.

Parkinson, F.E., Xiong, W., 2004. Stimulus- and cell-type-specific release of purines in cultured rat forebrain astrocytes and neurons. J. Neurochem. 88, 1305-12.

Parsons, M., Young, L., Lee, J.E., Jacobson, K.A., Liang, B.T., 2000. Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D. FASEB J. 14, 1423-31.

Pascual, O., Casper, K.B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J.Y., Takano, H., Moss, S.J., McCarthy, K., Haydon, P.G., 2005. Astrocytic purinergic signaling coordinates synaptic networks. Science 310, 113-6.

Ran, R., Xu, H., Lu, A., Bernaudin, M., Sharp, F.R., 2005. Hypoxia preconditioning in the brain. Dev Neurosci 27, 87-92.

Rasmussen, E.B., Newland, M.C., 2001. Developmental exposure to methylmercury alters behavioral sensitivity to D-amphetamine and pentobarbital in adult rats. Neurotoxicol Teratol 23, 45-55.

Rebola, N., Rodrigues, R.J., Oliveira, C.R., Cunha, R.A., 2005. Different roles of adenosine A1, A2A and A3 receptors in controlling kainate-induced toxicity in cortical cultured neurons. Neurochem. Int. 47, 317-25.

Reed, M.N., Banna, K.M., Donlin, W.D., Newland, M.C., 2008. Effects of gestational exposure to methylmercury and dietary selenium on reinforcement efficacy in adulthood. Neurotoxicol Teratol 30, 29-37.

Reuhl, K.R., 1991. Delayed expression of neurotoxicity: the problem of silent damage. Neurotoxicology 12, 341-6.

Rodier, P.M., 1994. Vulnerable periods and processes during central nervous system development. Environ Health Perspect 102 Suppl 2, 121-4.

Rudolphi, K.A., Schubert, P., Parkinson, F.E., Fredholm, B.B., 1992. Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol Sci 13, 439-445.

Salvatore, C.A., Tilley, S.L., Latour, A.M., Fletcher, D.S., Koller, B.H., Jacobson, M.A., 2000. Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. J. Biol. Chem. 275, 4429-34.

Sawynok, J., Yaksh, T.L., 1993. Caffeine as an analgesic adjuvant: a review of pharmacology and mechanisms of action. Pharmacol Rev 45, 43-85.

Schulte, G., Fredholm, B.B., 2000. Human adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. Mol. Pharmacol. 58, 477-482.

Schwaninger, M., Neher, M., Viegas, E., Schneider, A., Spranger, M., 1997. Stimulation of interleukin-6 secretion and gene transcription in primary astrocytes by adenosine. J. Neurochem. 69, 1145-1150.

Semenza, G.L., 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88, 1474-80.

Sheldon, R.A., Sedik, C., Ferriero, D.M., 1998. Strain-related brain injury in neonatal mice subjected to hypoxia-ischemia. Brain Res 810, 114-22.

Stabler, S.P., Allen, R.H., 2004. Vitamin B12 deficiency as a worldwide problem. Annu Rev Nutr 24, 299-326.

Stern, S., Cox, C., Cernichiari, E., Balys, M., Weiss, B., 2001. Perinatal and lifetime exposure to methylmercury in the mouse: blood and brain concentrations of mercury to 26 months of age. Neurotoxicology 22, 467-77.

Svenningsson, P., Le Moine, C., Fisone, G., Fredholm, B.B., 1999. Distribution, biochemistry and function of striatal adenosine A2A receptors. Prog. Neurobiol. 59, 355-96.

Swanson, R.A., Ying, W., Kauppinen, T.M., 2004. Astrocyte influences on ischemic neuronal death. Curr Mol Med 4, 193-205.

Takeuchi, T., Kambara, T., Morikawa, N., Matsumoto, H., Shiraishi, Y., Ito, H., 1959. Pathologic observations of the Minamata disease. Acta Pathol Jpn 9(Suppl), 769-83.

Tamm, C., Duckworth, J., Hermanson, O., Ceccatelli, S., 2006. High susceptibility of neural stem cells to methylmercury toxicity: effects on cell survival and neuronal differentiation. J. Neurochem. 97, 69-78.

Trincavelli, M.L., Costa, B., Tuscano, D., Lucacchini, A., Martini, C., 2002. Up-regulation of A(2A) adenosine receptors by proinflammatory cytokines in rat PC12 cells. Biochem. Pharmacol. 64, 625-31.

Turner, C.P., Seli, M., Ment, L., Stewart, W., Yan, H., Johansson, B., Fredholm, B.B., Blackburn, M., Rivkees, S.A., 2003. A1 adenosine receptors mediate hypoxia-induced ventriculomegaly. Proc Natl Acad Sci U S A 100, 11718-11722.

Turner, C.P., Blackburn, M.R., Rivkees, S.A., 2004. A1 adenosine receptors mediate hypoglycemia-induced neuronal injury. J Mol Endocrinol 32, 129-44.

Vahter, M., Akesson, A., Liden, C., Ceccatelli, S., Berglund, M., 2007. Gender differences in the disposition and toxicity of metals. Environ Res 104, 85-95.

Volpe, J.J.: Neurobiology of the newborn, 4th edn. W.B. Saunders Company, Philadelphia, 2001, pp. 1-912.

Wang, G., Hazra, T.K., Mitra, S., Lee, H.M., Englander, E.W., 2000. Mitochondrial DNA damage and a hypoxic response are induced by CoCl(2) in rat neuronal PC12 cells. Nucleic Acids Res 28, 2135-2140.

Wang, J.H., Short, J., Ledent, C., Lawrence, A.J., van den Buuse, M., 2003. Reduced startle habituation and prepulse inhibition in mice lacking the adenosine A2A receptor. Behav Brain Res 143, 201-7.

Weaver, D.R., 1993. A2a adenosine receptor gene expression in developing rat brain. Brain Res Mol Brain Res 20, 313-27.

Weaver, D.R., 1996. A1-adenosine receptor gene expression in fetal rat brain. Brain Res Dev Brain Res 94, 205-23.

Wehner, A.P., Busch, R.H., Olson, R.J., Craig, D.K., 1977. Chronic inhalation of cobalt oxide and cigarette smoke by hamsters. Am Ind Hyg Assoc J 38, 338-46.

Wittendorp, M.C., Boddeke, H.W., Biber, K., 2004. Adenosine A_3 receptor-induced CCL2 synthesis in cultured mouse astrocytes. Glia 46, 410-418.

Wu, W.P., Hao, J.X., Halldner-Henriksson, L., Xu, X.J., Jacobson, M.A., Wiesenfeld-Hallin, Z., Fredholm, B.B., 2002. Decreased inflammatory pain due to reduced carrageenan-induced inflammation in mice lacking adenosine A3 receptors. Neuroscience 114, 523-7.

Yang, D., Zhang, Y., Nguyen, H.G., Koupenova, M., Chauhan, A.K., Makitalo, M., Jones, M.R., St Hilaire, C., Seldin, D.C., Toselli, P., Lamperti, E., Schreiber, B.M., Gavras, H., Wagner, D.D., Ravid, K., 2006. The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. J Clin Invest 116, 1913-23.

Yang, J.N., Tiselius, C., Dare, E., Johansson, B., Valen, G., Fredholm, B.B., 2007. Sex differences in mouse heart rate and body temperature and in their regulation by adenosine A1 receptors. Acta Physiol. (Oxf) 190, 63-75.

Yang, S.J., Pyen, J., Lee, I., Lee, H., Kim, Y., Kim, T., 2004. Cobalt chloride-induced apoptosis and extracellular signal-regulated protein kinase 1/2 activation in rat C6 glioma cells. J Biochem Mol Biol 37, 480-486.

Yu, A.C., Wong, H.K., Yung, H.W., Lau, L.T., 2001. Ischemia-induced apoptosis in primary cultures of astrocytes. Glia 35, 121-30.

Yu, L., Huang, Z., Mariani, J., Wang, Y., Moskowitz, M., Chen, J.F., 2004. Selective inactivation or reconstitution of adenosine A2A receptors in bone marrow cells reveals their significant contribution to the development of ischemic brain injury. Nat Med 10, 1081-7.

Zimmermann, H., 2000. Extracellular metabolism of ATP and other nucleotides. N-S Arch Pharmacol 362, 299-309.

Zou, W., Yan, M., Xu, W., Huo, H., Sun, L., Zheng, Z., Liu, X., 2001. Cobalt chloride induces PC12 cells apoptosis through reactive oxygen species and accompanied by AP-1 activation. J. Neurosci. Res. 64, 646-53.

Zou, W., Zeng, J., Zhuo, M., Xu, W., Sun, L., Wang, J., Liu, X., 2002. Involvement of caspase-3 and p38 mitogen-activated protein kinase in cobalt chloride-induced apoptosis in PC12 cells. J. Neurosci. Res. 67, 837-843.