Adenosine-induced neuroprotection

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Chapter 1

Introduction

The adenosinergic system

General aspects

Adenosine and ATP belong to a group of molecules, called purines, which have similar molecular structure. Purines are important constituents of living cells, for example adenine and guanine are basic components of nucleic acids.

ATP is the universal "currency" of free energy in the cell, acting as a energy donor in most cellular activities. Besides their role in energy transfer, purines also function as important intercellular signaling molecules [65]. When cells use energy, ATP is hydrolyzed into ADP, AMP and finally into adenosine. Under physiological conditions the production and consumption of energy are balanced and the amount of intracellular adenosine is tightly regulated. Since under physiological conditions ATP concentrations in the cell are high (about 3 mM), adenosine concentrations rise sharply if a small amount of ATP is metabolized. Thus, in situations where cells are impaired in their ability to synthesize ATP, the levels of intracellular adenosine will therefore increase rapidly.

Adenosine is transported passively across the cell membrane by facilitated diffusion transporters, which equilibrate the concentration of extra- and intracellular adenosine. Rising intracellular levels of adenosine will thus lead to the release of adenosine, Under basal conditions the concentration of extracellular adenosine in all biological fluids is estimated at 30-300 nM, but as a result of decreased energy supply or increased metabolic activity the concentration can rise to 10 μM or higher [86, 178, 189]. Another source of adenosine is the extracellular breakdown of ATP by ecto-nucleotidases, but this has been suggested to be a minor contributor to the amount of extracellular adenosine [65].

The two main mechanisms responsible for the clearing of adenosine from the extracellular space are transformation into inosine by adenosine deaminase or by reuptake into cells, which occurs by facilitated diffusion or by active transport [43, 65].

The breakdown rate of adenosine in the extracellular space is very high due to the high expression of the appropriate enzymes. Thus the extracellular concentrations of adenosine can rise and decrease rapidly, which makes adenosine an ideal signaling molecule. Figure 1.1 describes pathways of adenosine release, production and degradation and the enzymes involved.

Purinoreceptors

Purinoreceptors have been subdivided into P1 receptors, which bind adenosine as natural ligand and P2 receptors which can bind ATP, ADP, adenine dinucleotides but also pyrimidines like UTP and UDP [32]. P1 or adenosine receptors have

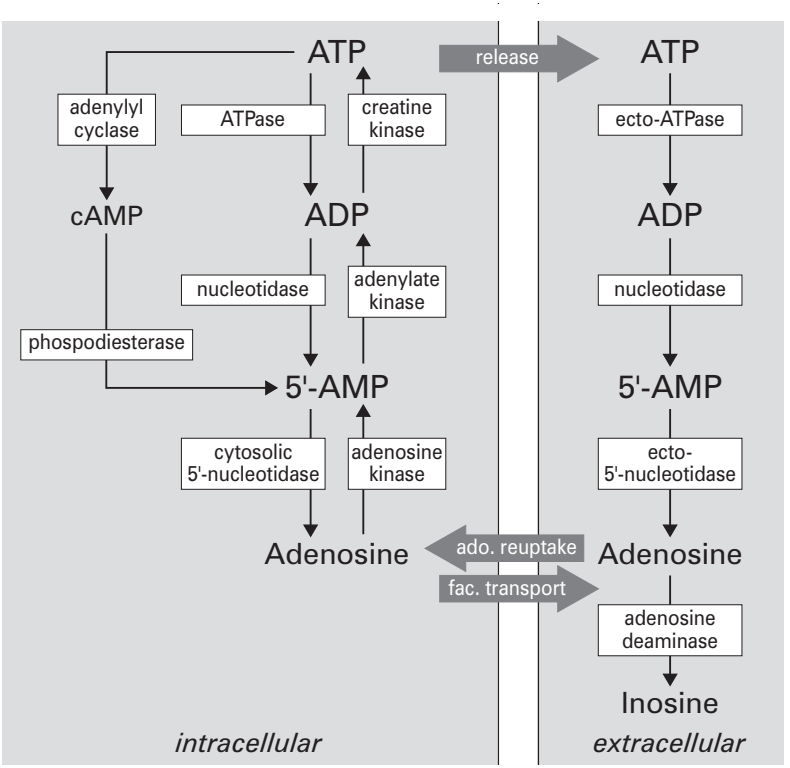

Figure 1.1. Main pathways for the formation of adenosine. ATP is being released from damaged cells, where it can be metabolized into adenosine by ectonucleotidases. Intracellularly, ATP can also be metabolized into adenosine, which is then being released by facilitated transport. Adenosine can be cleared from the extracellular space by reuptake into the cell or by degradation into inosine. (Derived from refs 65, 237).

initially been divided in two subtypes. This classification between A_1 and A_2 was based on their effect on cAMP [219]. Currently, the adenosine receptor family contains the four subtypes, A_1, A_{2A}, A_{2B} and A_3, which all couple to G-proteins and have the typical seven-transmembrane structure as shown in figure 1.2 [85]. Originally it was reported that adenosine A_1 and A_3 interact primarily with G_i-proteins and induce inhibition of adenylyl cyclase whereas adenosine A_{2A} and A_{2B} receptors couple preferentially to G_s-proteins and thus stimulate adenylyl cyclase and increase cAMP levels [86, 152, 218]. Adenosine receptors, however, have also been reported to interact with other G-proteins and signal through various other pathways, independent of adenylyl cyclase, as reviewed recently [189]. In table 1.1 an overview of the second messenger pathways induced by adenosine receptors has been provided. Several reviews addressing the structure,

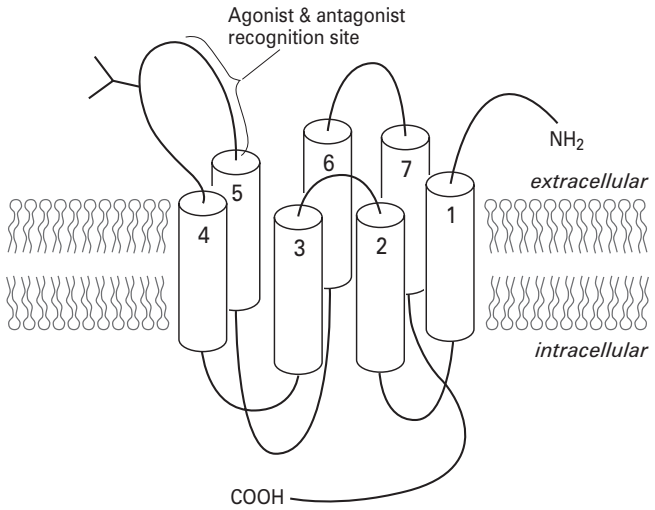

Figure 1.2. Structure of the adenosine A_1 receptor. As other G-protein coupled receptors, the adenosine A_1 receptor has 7 transmembrane domains (1-7) which have an α-helix structure. The most important region for agonist and antagonist binding is indicated. (Adapted from ref 170).

classification and pharmacology of adenosine receptors have been published [2, 86, 152, 153, 157, 201].
P2 receptors are divided in a family of ligand gated ion channels, P2X receptors and G-protein coupled receptors termed P2Y receptors. Several subtypes of both families have been cloned and characterized [170].

Physiological functions of adenosine in the periphery

In situations of increased energy use or a decreased energy supply the consumption of ATP overrides its'generation. As a result the balance is shifted towards higher levels of adenosine, which is rapidly released from the cell. The resulting increased extracellular levels of adenosine and the subsequent stimulation of cell-surface adenosine receptors will generally result in an inhibition of the cell metabolism (Figure 1.3). Thus adenosine-based phosphate metabolism provides a very basic feedback system linking energy demand to energy supply, which controls the metabolic rate in order to prevent energy depletion and subsequent cellular damage [58, 65, 98, 157].
Already since 1929, when adenosine was described to be involved in cardiovascular regulation [61], extensive research on the many physiological functions of adenosine has been performed [22, 63, 111, 202, 230].

Table 1.1. Overview of G-protein coupling and second messenger signaling of adenosine receptors. (Adapted from refs 86, 189).

adenosine receptor subtype	G-protein	effects of G-protein coupling	MAPK subtype	signaling pathway
A_1	$G_{i1/2/3}$	↓ cAMP ↑ IP_3/DAG (PLC) ↑ Arachidonate (PLA_2) ↑ choline, DAG (PLD) ↑ K^+ channels ↓ Q,P, N type Ca^{2+} channels	ERK1/2	$G_{i/0}$ > βγ > Tyr kinase[a] > P13K> MEK1
	G_0			
A_{2A}	G_s	↑ cAMP	ERK1/2	Gs > cAMP > PKA > Rap1[b] > B-Raf > MEK1
	G_{olf}	↑ cAMP		
	$G_{15/16}$	↑ IP_3	ERK1/2	$G_{\alpha s}$ > cAMP > PKA > Src > Ras
A_{2B}	G_s	↑ cAMP	ERK1/2	G_s > cAMP > P13K> MEK1
	$G_{q/11}$	↑ IP_3/DAG (PLC)	p38	G_s > cAMP > PKA
A_3	$G_{i2/3}$	↓ cAMP ↑ IP_3/DAG (PLC) ↑ choline, DAG (PLD) ↑ K^+ -ATP channels ↑ Cl- channels	ERK1/2	$G_{i/0}$ > βγ > P13K> Ras > MEK1
	$G_{q/11}$	↑ IP_3/DAG (PLC)		

In keeping with the already mentioned role of adenosine, coupling energy consumption to energy demand, adenosine receptors are distributed in almost all biological tissues and in many different species [147, 172, 173]. Although adenosine may have different actions depending on cell type, the ultimate result of the action is the control of metabolic rate. Thus, adenosine has several actions, which directly modulate energy supply. Adenosine induces relaxation of vascular smooth muscle cells causing vasodilatation, thereby increasing blood flow. In the kidney, adenosine causes vasoconstriction thus reducing renal blood flow and indirectly regulating blood pressure [100, 231]. Adenosine plays a role in the modulation of cardiac and respiratory function as well [144, 198, 208]. For example, adenosine is involved in hypoxia-induced angiogenesis, thereby counteracting the effects of a reduced energy supply [135]. In addition, adenosine

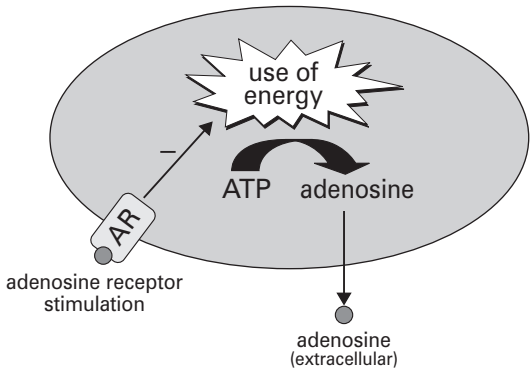

Figure 1.3. The adenosinergic system forms a negative feedback loop to regulate cell metabolism. Adenosine levels rise in cases of excessive energy use. Adenosine is transported out of the cell, binding to adenosine receptors on the surface. Activation of adenosine receptors slows down energy usage, which will eventually result in reduced formation of adenosine.

Table 1.2. Overview of physiological systems/pathological conditions in which adenosine plays a role.

physiological system/pathology	references
central nervous system	[65, 175, 202]
sleep	[17, 168, 169]
circadian rhythm	[69]
anxiety	[82, 110]
drugs of abuse induced actions	[64, 203]
pain modulation	[110, 166, 184, 185]
cardiac system	[14, 144, 154, 208]
blood flow	[58, 164, 208]
angiogenesis	[62, 94, 135, 136]
platelet aggregation	[126, 183]
respiratory system	[95, 198]
mast cell degranulation, asthma	[83, 125]
immune system	[47, 126]
kidney	[42, 100]
gastrointestinal tract	[177]
lipolysis	[103, 186, 212]
cell growth, proliferation	[33]
apoptosis	[4, 151]
embryogenesis	[119]

is involved in several other physiological functions, which are not directly linked to energy control. Adenosine is known to mediate anti-inflammatory effects, which could protect tissues from damage [77, 126, 204]. Furthermore, adenosine is involved in platelet aggregation, gastrointestinal mobility, mast cell degranulation, pain modulation, induction of sleep, cell growth, proliferation and apoptosis. In table 1.2 an overview of all the different functions in which adenosine plays a role has been provided.

Physiological functions of adenosine in the nervous system

Generally the brain consumes approximately 20% of our total energy. Consequently, the brain is very vulnerable to fluctuations in energy supply. In this respect adenosine plays an important role by coupling energy use to energy demand. Large amounts of adenosine are produced and released during conditions of increased energy use such as high neuronal activity during seizures, or under conditions of reduced energy supply like in ischemia or hypoglycemia. [180]. Stimulation of cell surface adenosine receptors in the brain, that are mainly of the A_1 subtype, protects neurons by retaining neuronal firing and inhibiting the release of excitatory neurotransmitters, including glutamate [202]. These actions are primarily described for neurons in the brain but adenosine seems to exert similar effects in the spinal cord [56].
Whereas under pathological conditions adenosine is neuroprotective, under physiological conditions adenosine acts as a neuromodulator by regulating a general inhibitory tone in the brain. This neuromodulatory role at the synapse level is mediated by stimulation of inhibitory A_1 receptors and facilitatory A_{2A} receptors [48, 231].
Besides neuromodulatory and neuroprotective effects, adenosine also induces trophic effects in neurons and glia cells. Adenosine stimulates neurite outgrowth [36], increases glia cell proliferation and promotes myelination [200]. These actions are more extensively reviewed in **chapter 2**.

Adenosine is also involved in other functions in the central nervous system. For instance adenosine is known to induce sleep [168], which explains the activating properties of the unspecific adenosine antagonist caffeine [87]. There is some evidence that adenosine may play a role in the effects of drug abuse, since opiates, benzodiazepines as well as ethanol inhibit adenosine reuptake [65]. Since adenosine is known to have analgesic effects [66, 110], it has been suggested that analgesic effects of for example morphine are actually caused by its effect on adenosine [63, 64, 203].

Functions of different adenosine receptors in the nervous system

Although adenosine receptors are found throughout the brain, their expression varies in specific brain regions [55, 175] (Figure 1.4). The specific expression pattern of the different receptor subtypes is related to their specific functions. The functions of adenosine receptors have been analyzed by using selective adenosine receptor antagonists and by the generation of mouse strains with targeted deletions of adenosine A_1, A_{2A} and A_3 receptor subtypes [110, 124, 149, 235].

Adenosine A_1 receptors

Adenosine A_1 receptors are clearly involved in neuroprotection. These receptors are found throughout the brain, but show especially high expression in vulnerable areas like for example the hippocampus. Adenosine A_1 receptors are found both pre- and postsynaptically in neurons, where they play an important role in inhibiting the release of excitatory neurotransmitters and inducing hyperpolarization respectively. Thus presynaptically, adenosine inhibits the release of the excitotoxic neurotransmitters glutamate, probably through the inhibition of Ca^{2+}-influx [63, 65, 70]. Postsynaptically, adenosine counteracts depolarization by stabilization of the Mg^{2+} blockade of NMDA receptors [178, 181]. Adenosine also actively reduces postsynaptic Ca^{2+}-influx, probably by an inhibition of N-type voltage-dependent Ca^{2+}-channels [60, 129, 148, 159, 195, 210]. Moreover, stimulation of adenosine A_1 receptors causes hyperpolarization of the postsynaptic resting membrane potential via G-protein-dependent activation of inwardly

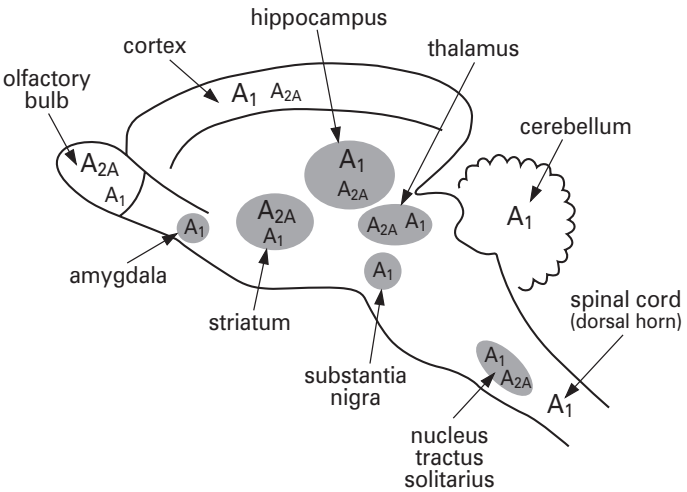

Figure 1.4. Distribution of adenosine A_1 and A_{2A} receptors in the brain. Bigger fonts indicate high levels of expression. (Adapted from ref 175).

rectifying K^+-channels (GIRKs) [160, 214]. In addition, adenosine A_1 receptor stimulation enhances a calcium-dependent potassium current, in much the same way as $GABA_B$ receptor stimulation, although different G-proteins might be involved [90, 98].

Adenosine A_{2A} receptors

In general adenosine A_{2A} receptors are involved in the facilitation of neuronal firing. In close interaction with adenosine A_1 receptors they modulate synapse function. Although A_{2A} receptors have been found in all brain regions they are particularly expressed in the nucleus accumbens, olfactory tubercle and striatum, where they are co-localized with dopamine D_2 receptors [88, 122, 143, 155, 170]. It has been shown that antagonistic interactions between adenosine A_{2A} and dopamine D_2 receptors and also between adenosine A_1 and dopamine D_1 receptors are partly responsible for the motor stimulant effects of adenosine receptor antagonists like caffeine [88]. Furthermore, involvement of adenosine A_{2A} receptors in locomotion, anxiety, aggression, motivation and reward in drug addiction and psychotic-like behavior have been suggested [37, 39,124,143]. Most of these functions have been revealed using adenosine A_{2A} receptor knock out mice. [37, 38, 52, 143].

Adenosine A_{2A} receptors are also involved in the control of cerebral blood flow [58, 170].

Adenosine A_{2B} receptors

Adenosine A_{2B} receptors have been found in most tissues but are generally expressed at low levels. Low expression levels were also found throughout the brain [59]. Since selective ligands for the adenosine A_{2B} subtype are lacking, and no A_{2B} knock mouse has been generated yet, less is known on its physiological role. It has been suggested that A_{2B} receptors play a role in vascularization and control of cerebral blood flow [93, 170, 193]. Stimulation of A_{2B} receptors has been shown to induce release of vascular endothelial growth factor in both peripheral and cerebral endothelial cells [73, 78, 93, 94]. There are indications that A_{2B} receptors are involved in neuroexcitatory actions and that stimulation of these receptors would aggravate tissue injury [72]. A_{2B} receptors are also expressed in glia cells where they have been shown to induce release of interleukin-6 [76, 191].

Adenosine A_3 receptors

Adenosine A_3 receptors are widely distributed in the brain, but its physiological role is largely unknown [170]. Adenosine A_3 receptors mediate inhibition of synaptic transmission in neurons in concert with A_1 receptors [31] and they play a role in modulating synaptic plasticity [46]. More extensive research on the role

of the A_3 receptor in the brain has been done by the group of Von Lubitz [224, 226-228]. Von Lubitz and colleagues showed that stimulation of A_3 receptors induces apoptosis of brain tissue and they therefore suggested that the A_3 receptor acts as a "death receptor". Inducing apoptosis of badly damaged neurons in stroke would be beneficial since it would limit neuroinflammation and infarct size [228].
In contrast, stimulation of the A_3 receptor with low concentrations of adenosine seems to induce neuroprotective effects [3, 4, 71, 106]. These seemingly conflicting actions do have physiological significance. High levels of adenosine in the core area of an ischemic insult would induce apoptosis through action of A_3 receptors, while in the surrounding brain tissue lower adenosine levels exert neuroprotective effects mediated by the same receptors. Furthermore, several reports indicate that adenosine A_3 receptor stimulation in glia cells leads to neuroprotection by inducing cytoskeleton rearrangement [1, 5].

The four different adenosine receptor subtypes have a different affinity for adenosine. Whereas A_1 and A_{2A} receptors have relatively high (nanomolar range) affinities for adenosine, A_{2B} and A_3 receptors have a much lower affinity and are only activated at micromolar concentrations [65]. These differences in affinity may reflect functional significance. Thus different receptors with different functional responses are activated by varying extracellular concentrations of adenosine. Moreover, adenosine at varying concentrations not only activates different receptor subtypes, but also induces multiple, sometimes even opposite effects by activation of the same receptor subtype. These observations show that the adenosinergic system regulates a complex interplay of biological activities.

Adenosine in pathology and therapy

Adenosine-based treatment of disorders

Since adenosine is involved in many physiological functions, drugs that interact with the adenosinergic system (so called "adenosine-based drugs") could be developed to treat a variety of pathological conditions. But at the same time these adenosine-based drugs cause serious side effects because adenosine receptors are so widely distributed. This explains why presently only very few adenosine-based drugs are used in the clinic, even though extensive research on the physiological roles of adenosine has been done since 1929 [61].
Currently, adenosine is only therapeutically used as intravenous application to treat patients suffering from supraventricular tachycardias (see [176] and *www.adenocard.com*). Adenosine-based treatment of other disorders is still at an early stage of investigation.

High levels of extracellular adenosine have been associated with the pathophysiology of lung diseases [24]. Adenosine A_{2B} and A_3 receptors play a role in adenosine-induced mast cell degranulation and bronchoconstriction and have therefore been associated with the pathophysiology of asthma [74, 171, 185, 236]. In order to block adenosine-induced bronchoconstriction asthmatic patients use theophylline, a non-specific adenosine antagonist [72]. High doses of theophylline, however, can lead to seizure activity, so more specific adenosine receptor antagonists, which show fewer side effects are preferable. Since specific antagonists for A_{2B} receptors are not available, currently only adenosine A_3 antagonists are under investigation as possible anti-asthmatic drugs [75, 83, 197].

It has been reported that adenosine and adenosine analogues induce apoptosis in various types of tumor cells [13, 35, 115, 139, 188]. Furthermore, it has been reported that particularly adenosine A_3 receptors are beneficial in the treatment of cancer [79, 140, 151]. In addition to inducing apoptosis in tumor cells, stimulation of adenosine A_3 receptors protects tissue from damage by chemotherapy and induces the release of granulocyte colony-stimulating factor (G-CSF) which stimulates the proliferation of bone marrow cells [16, 80]. Currently, 2-chlorodeoxyadenosine is tested in clinical trials for the treatment of glioma (see *www.clinicaltrials.gov*).

Adenosine is also known to mediate anti-inflammatory effects, like suppression of phagocytosis and reduction of free radical generation, which could protect tissues from damage [77, 101, 126, 204].

Adenosine and the treatment of neurological diseases

It is well established that adenosine induces neuroprotective activity in the brain [53, 111, 178, 202]. These effects of adenosine might have significant therapeutic potential in acute brain injuries like brain trauma and stroke, but also in a wide range of chronic neurological diseases including seizures, Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis [10, 27, 65, 175, 179, 216, 230].

Before considering possible pharmacological tools to manipulate the adenosinergic system it is important to realize that prolonged stimulation of adenosine receptors leads to receptor desensitization. This process involves uncoupling of the activated receptor from its G-protein by receptor phosphorylation mediated by G-protein kinases (GRK's). Internalization of receptors into intracellular compartments may also occur [28]. Ligand stimulation for hours to days causes receptor down regulation. In this case degradation of receptors leads to a decrease in actual receptor number.

Desensitization of adenosine A_1 receptors in several tissues including brain, requires exposure to agonist for at least 15 minutes to hours or even days, while

adenosine A_3 receptors in astrocytes undergo significant desensitization already after several minutes after stimulation [44, 170, 213]. Long-term stimulation of adenosine receptors with antagonists generally leads to an increase in receptor number [170].
Stimulation of adenosine A_1 receptors as well as inhibition of adenosine A_{2A} receptors reduces neuronal damage when administered acutely [51]. Accordingly, mice lacking adenosine A_{2A} receptors show less neuronal damage in ischaemia models [30, 38]. Surprisingly, it has also been reported that chronic stimulation of adenosine A_1 receptors or chronic inhibition of adenosine A_{2A} receptors, aggravates neuronal damage [53, 107]. These contradictory results, a phenomenon called "effect inversion", may be caused by desensitization and up-regulation of adenosine receptors due to the chronic agonists and antagonists treatment, respectively [53, 107].

Possible pharmacological approaches to increase the neuroprotective effects of adenosine

It is clear that the adenosinergic system is important to maintain a healthy nervous system. It may thus be attractive to evaluate therapeutic approaches to several neurological diseases based on the adenosinergic system. Several pharmacological approaches to manipulate the adenosinergic system are available and can be divided in two categories: synthetic adenosine derivatives that directly stimulate adenosine receptors or factors that indirectly increase the effectiveness of endogenous adenosine. Table 1.3 shows a summary of neuroprotective effects of different pharmacological approaches in various models of brain pathology.

STIMULATION OF ADENOSINE RECEPTORS

Stable synthetic adenosine derivatives that can cross the blood-brain barrier make much better candidates for clinical use than adenosine, which is instantly degraded. For clinical use it is also mandatory that these compounds are effective even when administered hours after the pathological event, e.g. stroke. The adenosine A_1 agonists CHA and R-PIA and the adenosine-amine congener ADAC showed neuroprotective effects 30 minutes to several hours after cerebral ischaemia [108, 130, 225].
However, due to the widespread distribution of adenosine receptors throughout the body, especially the A_1 subtype, peripheral side effects often occur when using adenosine A_1 receptor agonists [202]. This could be prevented by using adenosine A_{2A} receptor antagonists, which have less effect on heart rate and blood pressure than adenosine A_1 receptor agonists [202].

Table 1.3. Neuroprotective effects of adenosine by using different therapeutic approaches.

Experimental model	Drug	Mechanism	Effect	refs
Kainic acid injection in hippocampus	adenosine	agonist	protection	[133]
Kainic acid induced toxicity	2-CA	agonist	protection	[11]
rat hippocampal cell culture cell injury	CPA	A_1 agonist	protection	[142]
vessel occlusion in rats (forebrain ischaemia)/ KA induced seizures	R-PIA	A_1 agonist	protection	[26]
carotid artery occlusion in gerbils hypoxia, ischaemia	ADAC	A_1 agonist	protection	[223]
carotid artery ligation in newborn rats hypoxia, ischaemia	PD 81,273	allosteric enhancer of A_1 receptor binding	protection	[99]
hyperglycemic cerebral ischaemia	PD 81,273	allosteric enhancer of A_1 receptor binding	protection	[138]
preconditioning MCA occlusion followed by longer MCA occlusion hypoxia, ischaemia	DPCPX	A_1 antagonist	reduction of protective effect of preconditioning	[146]
quinolic acid injection# combined with free radicals (xanthine) in hippocampus	SCH 58261 ZM 241358	A_{2A} antagonist	protection	[19]
β-amyloid toxicity in cultured rat neurons	caffeine	antagonist	protection	[51]
	ZM 241358	A_{2A} antagonist		
carotid artery occlusion in newborn rats hypoxia, ischaemia	theofylline	antagonist	protection	[30]
	SCH 58261	A_{2A} antagonist		
quinolic acid induced neurotoxicity#	SCH 58261	A_{2A} antagonist	protection	[167]
MPTP induced neurotoxicity*	SCH 58261	A_{2A} antagonist	protection	[40]
MPTP induced neurotoxicity*	KW-6002	A_{2A} antagonist	protection	[104]
MCA occlusion in rats hypoxia, ischaemia	GP683	adenosine kinase inhibitor	protection	[211]
MCA occlusion in rats hypoxia, ischaemia	5'd-5IT	adenosine kinase inhibitor	protection	[109]
cell death by stimulation with macrophage/microglial products	propentofylline	adenosine uptake inhibitor	protection	[81]
carotid artery occlusion hypoxia, ischaemia	propentofylline	adenosine uptake inhibitor	increased cerebral blood flow	[217]
subclavian and brachiocephalic artery occlusion. ischaemia	NBTI	adenosine uptake inhibitor	protection against reperfusion injury	[91]
bilateral artery occlusion in gerbils ischaemia	deoxyco-formycin	adenosine deaminase inhibitor	protection	[165]

animal model for Huntington's disease, * animal model for Parkinson's disease. 2-CA = 2-chloroadenosine, CPA= N^6- cyclopentyladenosine, R-PIA = R- N^6-phenylisopropyladenosine, ADAC = adenosine amine congener, PD 81,273 = a 2-amino-3-benzylthiophene(no details given in paper), DPCPX = 8-cyclopentyl-1,3-dipropylxanthine, SCH 58261 = 7-(2-phenylethyl)-5-amino-2-(2-furyl)pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine, ZM 241358 = 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol, KW-6002 = (E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione, GP 683 = 4-(N-phenylamino)-5-phenyl-7-(5'-deoxy β- D-ribofurasonyl)pyrrolo[2,3-d]pyrimidine, 5'd-5IT = 5'-deoxy-5-iodotubercidin, NBTI = nitrobenzylthioinosine, KA = kainic acid.

Increasing the effectiveness of endogenous adenosine

Compounds that increase the effectiveness of endogenous adenosine will only enhance effects at sites of high extracellular adenosine levels. Presumably, this approach would induce tissue/region-specific effects without much peripheral side effects. Inhibition of enzymes that metabolize adenosine, like adenosine deaminase or adenosine kinase have been shown to increase the neuroprotective effects of adenosine [53, 109, 165]. The extracellular concentration of adenosine can also be increased by inhibition of adenosine reuptake [81]. Yet another approach to increase the effectiveness of adenosine is the use of factors, so-called allosteric enhancers, that do not activate the adenosine receptor itself but enhance the binding of endogenous adenosine to the receptor [99].

Alzheimer's disease

It has been suggested that chronic neurological diseases as well as acute brain injuries could be treated with trophic factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF). However, poor penetration of the blood-brain barrier and the occurrence of side effects limits the use of exogenous application of these factors. Therefore stimulating the local production of trophic factors seems a more attractive approach [43].

Two synthetic purine derivatives, propentofylline and AIT-082, are currently under evaluation in clinical trials for the treatment of Alzheimer's disease. Both compounds have been shown *in vivo* to increase the mRNA expression for NGF, neurotrophin-3 and basic fibroblast growth factor (bFGF) *in vivo*. As NGF is considered to protect cholinergic neurons, which degenerate in Alzheimer's disease, it has been suggested that propentofylline and AIT-082 might have a neuroprotective effect in Alzheimer's disease [43, 57, 96, 118].

Propentofylline acts as an adenosine uptake inhibitor thereby maintaining high concentrations of adenosine in the extracellular space. Furthermore, it has been demonstrated that propentofylline stimulates the production of NGF in cultured mouse astrocytes [194]. In addition, the capacity of propentofylline to improve cerebral blood flow presumably also contributes to its' neuroprotective effect [43, 217]. It is not yet known whether propentofylline has been successful in clinical trials since the pharmaceutical company involved is reluctant to publish the results. A meta-analysis of clinical trial results published so far, did not show any beneficial effect of propentofylline in patients with Alzheimer's disease [84].

AIT-082 is under evaluation in clinical trials as a memory-enhancing agent since it increases NGF release from glia cells, it enhances NGF induced neurite outgrowth in a neuron-like cell line and it protects neuronal tissue from damage *in vivo* [57, 141, 174]. The mechanism of AIT-082-induced protection remains to be elucidated, but it has been suggested that AIT-082 increases the release of adenosine

from astrocytes. First clinical trials have been performed with AIT-082, now renamed as Neotrofin^{TM}, to investigate pharmacokinetics and tolerability [96].

Parkinson's disease

Parkinson's disease is caused by degeneration of dopaminergic neurons in the substantia nigra that innervate the striatum. The subsequent decreased levels of dopamine in the striatum lead to a disturbed regulation of motor behavior causing the symptoms typically observed in Parkinson's disease. In the striatum dopamine D_2 receptors are co-localized with adenosine A_{2A} receptors whereas dopamine D_1 receptors are in close proximity of adenosine A_1 receptors. Through a system of receptor cross talk, adenosine counteracts the actions of the neuro-transmitter dopamine. Whereas stimulation with dopamine or other dopamine D_2 receptor agonists enhances motor activity, stimulation of adenosine A_{2A} receptors reduces this effect [88]. Likewise, stimulation with adenosine A_1 receptor agonists counteracts the enhancing effect of dopamine D_1 receptor agonists on motor behavior. These interactions are probably responsible for the motor stimulant effects of adenosine receptor antagonists like caffeine. Furthermore, adenosine A_{2A} receptor antagonists have been reported to attenuate the neurotoxicity observed in a mouse model of Parkinson's disease [40, 104]. In addition, adenosine A_{2A} receptor antagonists were found not only to diminish the symptoms of Parkinson's disease but also to potentiate the effect of L-DOPA [229]. L-DOPA, a dopamine precursor, which is currently used to treat Parkinson's disease, shows significant side effects like dyskinesia that are observed especially in patients that receive high dosages of L-DOPA. If adenosine A_{2A} receptor antagonists indeed increase the efficacy of L-DOPA, lower doses of L-DOPA could be used and less side effects would occur [25]. All these findings suggest that adenosine A_{2A} receptor antagonists could be useful in the treatment of Parkinson's disease [192]. Recently, phase II clinical trials of the adenosine A_{2A} receptor antagonist, KW-6002 (Istradefylline^{R}) have been performed and showed relief of Parkinson's disease motor symptoms without side effects [102, 114, 120]. Phase III clinical trials will start shortly (Schwarzschild, personal communication).

Adenosine A_1 receptor expression in pathological events

During seizure activity, cerebral hypoxia and ischemia elevated extracellular concentrations of adenosine have been found in brain tissue [65, 67]. It has been assumed that these elevated levels of extracellular adenosine cause endogenous anticonvulsant activity as well as neuroprotection [66, 178]. Since the main protective actions of adenosine are mediated via the A_1 receptor, it is likely that the expression level of this receptor has a significant influence on the efficiency of neuroprotection by adenosine. The study of adenosine A_1 receptor expression in disease is therefore of particular interest.

Several conflicting reports addressing the level of adenosine A_1 receptor expression in epilepsy have been published. Some reports show that adenosine A_1 receptors are chronically reduced in epilepsy [65, 92, 150]. Others have found an upregulation of these receptors [9, 221]. It is thus not clear whether changes in adenosine A_1 receptor expression might be a causal factor in the pathophysiology of epilepsy.
Several reports describe modification of adenosine A_1 receptor activity that is related to the aging process [41, 128]. This modification is most likely due to a decrease in adenosine A_1 receptor expression, which has been observed in aged rats and mice [49, 50, 68, 156, 196].
A reduction of adenosine A_1 receptors has also been observed in autopsy and post-mortem samples of patients with Alzheimer's disease [112] as well as dementia with sclerosis type pathology [54].

Glia cells

Most of the research on adenosine receptors in the brain has been focussed on neurons. Mechanisms of neuroprotection induced through neuronal adenosine receptors have been well described. In addition glia cells form a considerable component of the nervous system and play an important role in for example brain development, nerve tissue maintenance, modulation of synaptic transmission, formation of the blood-brain barrier and brain immune function [113]. This thesis will partly focus on the role of glia cells in adenosine-induced neuroprotection. I therefore briefly describe here the properties and functions of glia cells.

Microglia

Unlike neurons and astrocytes, microglia are derived from bone-marrow and migrate to the brain during development. Microglia are the primary immunocompetent cells of the brain. Under physiological conditions microglia are resting ramified cells, with many large processes to monitor their surroundings. If damage in the CNS occurs, microglia become activated, retract their processes and adapt a round macrophage-like morphology [199]. Microglia can orchestrate neuroinflammation by producing cytokines and by presenting antigens. Furthermore microglia can phagocytose both cells and cell debris [7]. Since microglia can produce neurotoxic substances, activation of microglia, so-called reactive microgliosis has been linked to the pathophysiology of many types of brain pathology [12, 15, 205]. In addition to their detrimental activity, microglia can also assume a neurosupportive role by producing neurotrophins [145, 205, 206]. Whether microglia mediate detrimental or beneficial effects probably depends on a variety of factors. Indeed it has been shown that adenosine receptors are involved in the regulation of microglia activation [105].

Astrocytes

Astrocytes are the most abundant cells in the brain; by estimation the number of neurons in the human brain is outnumbered ten times by astrocytes. During early brain development astrocytes form growth tracts to guide the migration of neurons. In this development phase astrocytes also produce trophic factors thus supporting neurons in their development. In the adult brain, astrocytes play an essential role in the maintenance of the nervous system. Astrocytes that surround glutamatergic synapses regulate the extracellular glutamate concentration by actively taking up glutamate (Figure 1.5). This mechanism prevents the occurrence of glutamate excitotoxicity [8]. Since the active uptake of glutamate requires a large amount of energy, it is clear that a balanced cell metabolism in astrocytes surrounding the synaptic cleft is of the utmost importance. It is likely that besides their involvement in inhibiting neurotransmitter release from the presynaptic neuron, adenosine receptors also play an essential role in regulating cell metabolism in astrocytes surrounding the synaptic cleft.

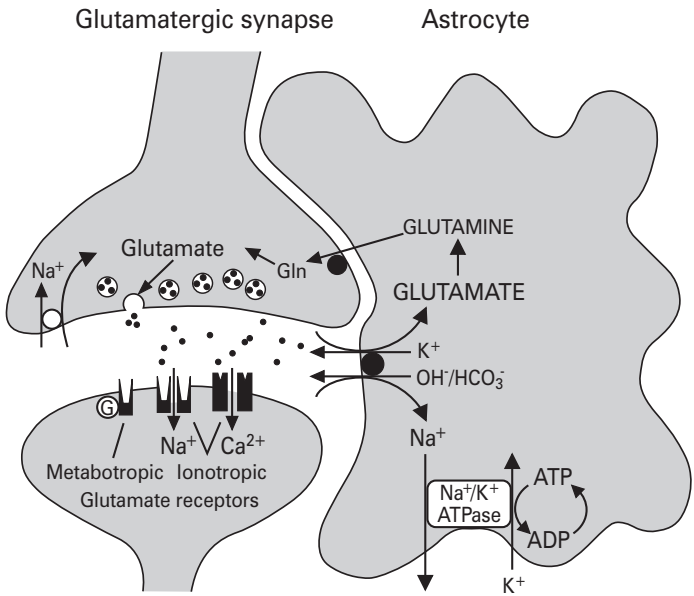

Figure 1.5. Regulation of glutamate concentration in the synaptic cleft by astrocytes. Glutamate released presynaptically stimulates glutamate receptors on the postsynaptic neuron leading to depolarization. Excessive stimulation of glutamate receptors can lead to an increase of the intracellular Ca^{2+} concentration, resulting in neuronal damage. Astrocytes control the action of glutamate by actively taking up glutamate from the synaptic cleft. Glutamate is cotransported with Na^+, leading to an increase of the Na^+ concentration in astrocytes. Na^+ concentrations in astrocytes are regulated by the energy consuming Na^+/ K^+ - ATPase (Adapted from ref 215).

During physiological conditions but also in brain pathology, astrocytes are known to produce and release neuroprotective substances. Furthermore, astrocytes constitute an important component of the blood-brain barrier, a structure that prevents antigens from entering the brain.

Both astrocytes and microglia fulfill various important roles in regulation of neuronal function. It is likely that adenosine is widely involved in the molecular mechanisms underlying the multiple functions of these glia cells [43]. Therefore part of this thesis is focussed on glial adenosine receptors and their involvement in glial function is being reviewed in **chapter 2**.

Interleukin-6 in the brain

The cytokine interleukin-6 (IL-6), like adenosine is released during neuropathological conditions and has been shown to mediate neuroprotective effects. IL-6 belongs to the family of neuropoietic cytokines, which consists of ciliary neurotrophic factor (CNTF), leukemia inhibiting factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), IL-6 and IL-11 [209].
This family of cytokines is involved in several biological functions including immune responsivity and hematopoiesis [117]. IL-6 does not only elicit its functions in the peripheral immune system. Since IL-6 is also produced by neurons, astrocytes and microglia, various additional functions of IL-6 in the brain have been proposed [21, 97, 182, 187, 220, 233]. Under physiological conditions IL-6 levels in the brain are very low or undetectable, but during pathological events like neuroinflammation, ischaemia and seizures, IL-6 levels rise dramatically [97, 127, 134, 161, 222]. IL-6 mediates contrasting effects in the brain [89]. IL-6 is involved in the neuroimmune response, causing neuronal degeneration. Therefore, IL-6 has been associated with the pathophysiology of neurodegenerative disorders like Alzheimer's disease [18, 158, 234]. On the other hand, IL-6 plays a role in neuronal and glial differentiation and survival [89, 97]. Numerous reports show neuroprotective effects of IL-6. *In vitro*, IL-6 protects neurons during ischemia or induced excitotoxicity [123, 137, 232]. *In vivo*, IL-6 shows neuroprotective effects in a number of different animal models [6, 20, 127, 132]. IL-6 deficient mice showed increased neuronal death in animal models like experimentally induced brain-injury, the MPTP (Parkinson's disease) model and after kainic acid-induced seizures [29, 162, 163, 207].
IL-6 elicits effects in CNS cells by binding to the IL-6 receptor, which is associated with the transmembrane transduction peptide gp130. Two forms of the IL-6 receptor have been identified; an extracellular soluble form and a membrane bound form. Under both conditions the receptor can induce signal transduction

after binding to gp130 [116]. The gp130-IL6 receptor complex can activate second messenger pathways involving JAK kinases and homodimerization of STAT3, which then act on the IL-6 response element to activate gene transcription leading to protein synthesis [190]. An alternative pathway involves the RAS/MAPK cascade and the activation of the nuclear factor NF-IL-6 [97].
Although the signaling pathway involved in IL-6 action has been described, the complete signaling cascade leading to the neuroprotective effects of IL-6 is still largely unknown. IL-6 might have neuroprotective properties due to induction of other neuroprotective substances since IL-6 has been found to induce the expression of vascular growth factor, a factor involved in angiogenesis [45]. Furthermore, IL-6 was found to induce the release of NGF from astrocytes [34, 121, 131].

Aim of the thesis

Adenosine is released during pathological conditions and has significant neuroprotective effects mainly by stimulating adenosine A_1 receptors in neurons. These neuroprotective effects are increased following upregulation of adenosine A_1 receptors. Much research has been performed to enhance the neuroprotective effects of adenosine experimentally. Since direct interference with the adenosinergic system causes side effects, it would be preferable to find ways to increase the neuroprotective effects of adenosine indirectly, for example by finding factors that increase adenosine A_1 receptor expression.

Like adenosine, the proinflammatory cytokine interleukin-6 (IL-6) is released during pathological conditions and IL-6 is also known to reduce neuronal damage and mortality. In contrast to adenosine, however, little is known so far regarding the mechanism of IL-6 mediated neuroprotection. Recent findings *in vitro* have shown that IL-6 is being released by cultured astrocytes after adenosine receptor stimulation [76, 191]. Furthermore, it has been shown that stimulation with IL-6 increases the expression of adenosine A_1 receptors in nervous tissue, which implies that the neuroprotective effect of IL-6 might partially be due to upregulation of adenosine A_1 receptors [23]. From these findings we propose a model for interactions between the adenosinergic system and IL-6 (Figure 1.6).

The aim of the current thesis is to further investigate the interactions between the adenosinergic system and IL-6 and to check whether IL-6 has an effect on adenosine A_1 receptor expression in pathological conditions.

While the mechanisms of direct adenosine-induced neuroprotection in neurons are well understood, less is known on putative neuroprotective effects of

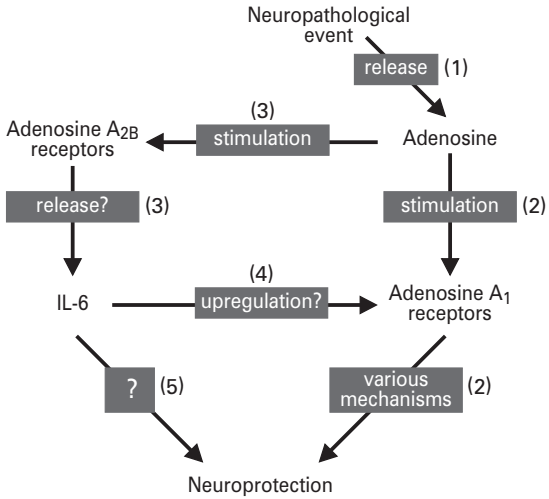

Figure 1.6. Proposed model of interactions between adenosine and IL-6.
Neuropathological events lead to an increase of extracellular adenosine concentration (1). Adenosine A_1 receptors in neurons are stimulated, resulting in membrane hyperpolarization and inhibition of neurotransmitter release, which will protect neurons (2). Stimulation of adenosine A_{2B} receptors on glial cells leads to a release of interleukin-6 (3), which will subsequently result in adenosine A_1 receptor upregulation (in neurons) (4) and thereby increasing adenosine's neuroprotective effects. Question marks (3,4) indicate that it is not yet known if these interactions exist *in vivo*. IL-6 has neuroprotective effects but the mechanisms are unknown (5).

adenosine that are mediated by glia cells. Therefore part of this thesis focuses on the role of glia cells in adenosine-induced neuroprotection.
In **chapter 2** we have reviewed current knowledge on the neuroprotective substances that are released by glia cells after adenosine receptor stimulation. In addition, in **chapter 3** we show that the chemokine CCL2 is another factor with "presumed" neuroprotective effects that is released after stimulation of glial adenosine receptors.
Most research on receptor pharmacology has been performed on rat and human adenosine receptors while mouse adenosine receptors have not been fully characterized. In order to study the adenosinergic system in knock out models in mice, it was necessary to investigate pharmacological properties of mouse adenosine receptors. In **chapter 4** we therefore present a pharmacological characterization of the mouse adenosine A_1 receptor using functional studies and radioligand binding assays.
In **chapter 5** we have investigated the effect of IL-6 on the regulation of adenosine A_1 receptor expression during seizures. Finally, the results have been summarized and discussed in **chapter 6**.

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