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Caffeine and the biological role of adenosine receptors

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RESUMEN

Cafeína y función biológica de los receptores de adenosina

Hace aproximadamente 30 años se descubrió que la cafeína, la más empleada de todas las substancias psico-estimulantes, es capaz de antagonizar los efectos de la adenosina a concentraciones que se alcanzan durante el consumo normal de café y otros alimentos ó bebidas. Este descubrimiento tiene varias consecuencias importantes: 1) existen receptores para los cuales la adenosina es el agonista y la cafeína el antagonista; 2) Ya que el antagonista es biológicamente activo, los receptores deben de estar activados por la adenosina endógena. Esta breve visión de conjunto, la cual refleja el contenido del Discurso de Entrada en la Real Academia de Farmacia del Profesor B. Fredholm, presentará los datos que avalan las conclusiones previamente enunciadas.

El metabolismo de la adenosina y sus niveles en condiciones normales y fisiopatológicas es conocido con detalle. Actualmente han sido clonados y caracterizado farmacologicamente cuatro receptores de adenosina. Todos ellos están acoplados a proteínas G y tienen cascadas de señalización y distribuciones diversas en el organismo. Es probable que los efectos biológicos de la cafeína se realicen mayoritariamente a través de receptores A_1 y A_{2A}. Los datos obtenidos con ratones genéticamente modificados, anulando estos genes confirma su función biológica.

Palabras clave: Cafeína.— Enfermedad de Parkinson.— Factores CREB.— Nucleo estriado— Neuronas GABAergicas.— Proteinas G.— Receptores de adenosina.— Receptores de dopamina.

SUMMARY

Caffeine and the biological role of adenosine receptors

About thirty years ago it was realized that caffeine, the most widely used of all psychoactive drugs, is able to antagonize the effects of adenosine at concentrations achieved during normal human consumption. This finding had several important implications: 1) there are receptors at which adenosine is the agonist and caffeine the antagonist; 2) since the antagonist is biologically active it should mean that the receptors are activated by endogenous adenosine. This brief overview, which reflects the contents of my introductory lecture at the Academy, will present some data vindicating these conclusions.

The metabolism of adenosine and its levels under normal and pathophysiological conditions has been elucidated. Now four different adenosine receptors have been cloned and pharmacologically characterized. They are all G protein coupled. Their different signalling characteristics are briefly summarized as is their distribution. Based on these data t is concluded that caffeine probably exerts its effects by blocking adenosine A_1 and A_{2A} receptors. The biological role(s) of these receptors is finally presented using data from knock-out mice.

Key words: Cafeine.— Parkinson's disease.— CREB factors.— Striatum.— GABAergic neurons.— G proteins.— Adenosine receptors.— Dopamine receptors.

About thirty years ago it was realized, from the work of i.a. Ted Rall and John Daly, that caffeine, the most widely used of all psychoactive drugs, is able to antagonize the effects of adenosine at concentrations achieved during normal human consumption (see Fredholm; 1980). This finding had several important implications: 1) there are receptors at which adenosine is the agonist and caffeine the antagonist; 2) since the antagonist is biologically active it should mean that the receptors are activated by endogenous adenosine. This brief overview, which reflects the contents of my introductory lecture at the Academy, will present some of the data from my own laboratory vindicating these conclusions. The interested reader is referred to other more compendious reviews (Ferré, Fredholm, Morelli, Popoli and Fuxe; 1997, Fredholm, Bättig, Holmén, Nehlig and Zvartau; 1999, Svenningsson, Le Moine, Fisone and Fredholm; 1999, Fredholm, IJzerman, Jacobson, Klotz and Linden; 2001, Fredholm; 2002, Svenningsson and Fredholm; 2002, Fredholm and Svenningsson; 2002 Submitted, Fredholm, Cunha and Svenningsson; 2003). I also need to

point out that the extremely important contributions by other scientists will not be covered here.

Regulation of the levels of adenosine

It should be clearly understood that adenosine does not act as a classical hormone or neurotransmitter: it is not stored in vesicles, it is not released by exocytosis, it does not appear to transfer information from the pre- to the postsynaptic components and it does not act only or predominantly in synapses. Instead, adenosine fulfils a double role (see Cunha; 2001a), acting both as a homeostatic trans-cellular messenger, and as a modulator, controlling i.a. neurotransmitter release and neuronal excitability.

Adenosine can appear in the extracellular milieu through three different mechanisms: 1) the release of adenosine as such through nucleoside transporters upon increase of the intracellular levels of adenosine; 2) the extracellular formation of adenosine through the ecto-nucleotidase pathway on release of adenine nucleotides, and 3) the extracellular formation of adenosine on release of cAMP (reviewed in Dunwiddie and Masino; 2001). The third pathway has been found to be of minor importance in more integrated preparations when physiological parameters affected by adenosine are being studied (Brundege, Diao, Proctor and Dunwiddie; 1997).

The idea that adenosine is mostly released as such through the different classes of nucleoside transporters is probably the most popular hypothesis. It was formulated essentially by analogy with the way extracellular adenosine is generated upon cytotoxic insults, like hypoxia or metabolic poisoning (for references, see Cunha; 2001a, Dunwiddie and Masino; 2001, Latini and Pedata; 2001). In stressful situations, there is an imbalance between energy supply and demand, leading to a net hydrolysis of ATP. Via a series of steps, this ATP is converted to adenosine, leading to a dramatic increase in the intracellular concentration of adenosine, which, at rest, is around 50 nM. The presence of non-concentrative bi-directional adenosine transporters will then force extracellular adenosine to rise in parallel with intracellular adenosine. Given that intracellular

ATP levels are some 100,000 times higher, it is obvious that substantial changes in adenosine levels can occur without any major changes in ATP levels. Indeed, extracellular adenosine can be formed without any measurable change in the energy status of CNS preparations (Mitchell, Lupica and Dunwiddie; 1993, Doolette; 1997). Given that the energy demanded – and hence the utilization of ATP – is substantial in localized neuronal compartments as a consequence of work needed to maintain ion balance, it is clear that substantial local adenosine formation could occur via this mechanism. Nevertheless, it is difficult to explain the time course of extracellular adenosine build-up, which may become sufficient to modulate synaptic transmission in less than 20 ms (Mitchell et al.; 1993), a time course much lower than the K_{cat} of most enzymes and adenosine transporters. Also, this release of adenosine through nucleoside transporters is at odds with the ability of inhibitors of nucleoside transport to potentiate the effects of endogenous adenosine (for references, see Cunha; 2001a, Latini and Pedata; 2001), which indicates that the role of nucleoside transporters is mostly to clear up rather than to mediate adenosine release, at least in non-stressful situations. Nevertheless, and as discussed previously, this finding may be explained if in any compartment there are cells that produce adenosine, and others that release itsee (Fredholm, Lindström and Wallman-Johansson; 1994).

The last hypothesis concerning the origin of extracellular adenosine is based on the extracellular catabolism of released adenine nucleotides, mainly of released ATP, through the ecto-nucleotidase pathway (for reviews, see Zimmermann; 2000, Cunha; 2001b). In fact, in contrast to adenosine, ATP is present in synaptic vesicles and the vesicular release of ATP on stimulation of nerve terminals is well documented (see references in Cunha; 2001a). However, only a few studies have so far gathered direct evidence for the role of ecto-nucleotidases in forming extracellular adenosine (see references in Cunha; 2001a-b). This might be due to the difficulty to block completely an enzyme system that is extremely efficient and where the spatial relationship between enzyme and effector system is very close (Dunwiddie, Diao and Proctor; 1997, Cunha, Sebastião and Ribeiro; 1998, Cunha; 2001b). It should also be pointed out that neurochemical studies have concluded that the extracellular adenosine, which is relevant

for modulation of synaptic transmission, might originate not only from nerve terminals but also from the activated postsynaptic component (see references in Cunha; 2001a, Dunwiddie and Masino; 2001, Latini and Pedata; 2001) as well as from surrounding non-neuronal cells. Both astrocytes and dendritic compartments are apparently devoid of a fast exocytotic apparatus and yet they are also able to release ATP (Caciagli, Ciccarelli, Di Iorio, Ballerini and Tacconelli; 1988) by a mechanism largely unknown (Bodas, Aleu, Pujol, Martin-Satué, Marsal and Solsona; 2000, Bodin and Burnstock; 2001).

Thus, there are several mechanisms that contribute to the formation of extracellular adenosine. And most importantly, it should be stressed that to understand how adenosine is released, one needs to keep in mind that different stimuli will trigger different ways of generating extracellular adenosine. Figure 1 schematically represents the possible contributions of the different compartments in the CNS to control the extracellular levels of purines.

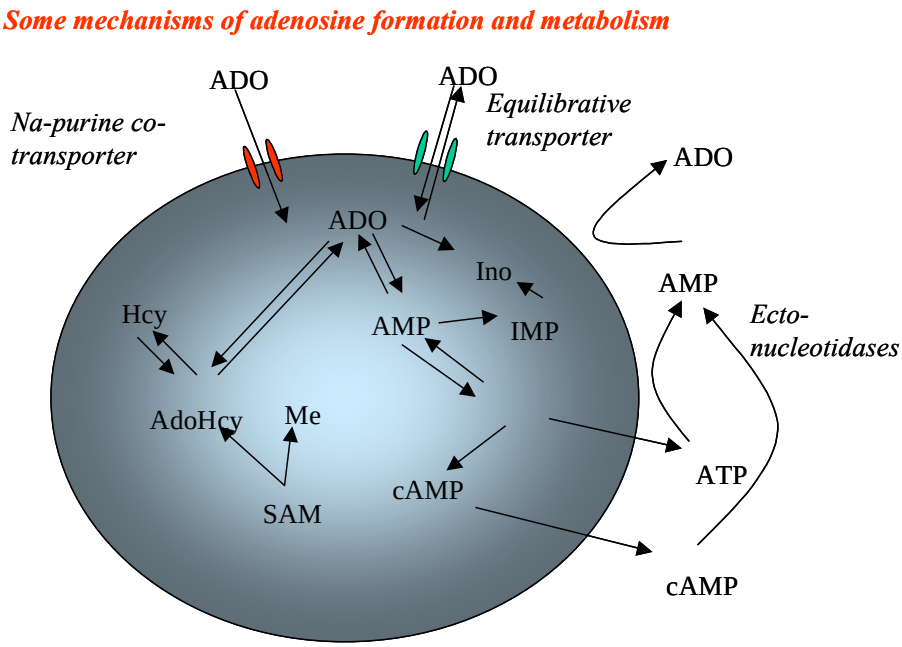

FIGURE 1. Schematic representation of the mechanisms by which adenosine levels are regulated.

In contrast, the clearance of extracellular adenosine is better understood. It mostly occurs through the action of the non-concentrative adenosine transporters, and the effectiveness of the uptake system is guaranteed by the efficient intracellular metabolisation of adenosine. Indeed, adenosine can either be converted into inosine through adenosine deaminase or be phosphorylated into AMP via adenosine kinase. The comparison of the kinetic parameters of each of the enzymes lead to the proposal that adenosine kinase was the initial pathway to salvage intracellular adenosine, with adenosine deaminase only coming to play when large amounts of adenosine have to be cleared. This is confirmed by the general greater effectiveness of adenosine kinase inhibitors to increase the extracellular levels of adenosine under physiological situations and the increased effectiveness of adenosine deaminase inhibitors to increase the extracellular levels of adenosine with increased neurotoxic insults.

This probably reflects the relative importance of neuronal versus non-neuronal uptake of adenosine since the two enzymatic activities have apparently different cellular locations with adenosine kinase being enriched in neurons and adenosine deaminase being more abundant in astrocytes It should also be mentioned that adenosine can also be extracellularly deaminated into its inactive metabolite inosine through an ecto-adenosine deaminase (Franco, Casadó, Ciruela, Saura, Mallol, Canela and Lluis; 1997), although the relevance of this pathway for adenosine clearance in the CNS remains to be established (Cunha; 2001b).

An alternative pathway for intracellular metabolization of adenosine would be via the S-adenosylhomocysteine pathway. But although this pathway is of relevance for the control of intra- and extracellular levels of adenosine in cardiomyocytes for instance, it appears to be of limited importance in the nervous system.

The balance between the effectiveness of releasing and clearing up adenosine will give raise to a transient extracellular build up of adenosine. However, the presumably different location of release sites and uptake sites makes it difficult to estimate an extracellular concentration of adenosine since one should instead discuss the amplitude of extracellular adenosine gradient. Pharmacological manipulation of the activity of adenosine transporters and adenosine receptor responses have lead to an estimate of the transient concentration of adenosine facing A_1 receptors between 200-400 nM in the rat brain (Dunwiddie and Diao; 1994). This estimate of an effective concentration of adenosine is similar to those found in microdialysis studies without stimulating or insulting the CNS (Hagberg, Andersson, Lacarewicz, Jacobson, Butcher and Sandberg; 1987). Upon stimulation (During and Spencer; 1992) or upon stressful conditions (Hagberg et al.; 1987) the extracellular amounts of adenosine can build up to tenths of micromolar.

Adenosine receptors

Despite the fact that adenosine receptors were well characterized and partially purified the two first adenosine receptors cloned, A_1 and A_{2A}, came from a library of orphan receptors from the dog thyroid (Maenhaut, Van Sande, Libert, Abramowicz, Parmentier, Vanderhaegen,

Dumont, Vassart and Schiffmann; 1990, Libert, Schiffmann, Lefort, Parmentier, Gerard, Dumont, Vanderhaeghen and Vassart; 1991). Soon the same receptors were cloned from rat and humans (Mahan, McVittie, Smyk-Randall, Nakata, Monsma Jr, Gerfen and Sibley; 1991, Furlong, Pierce, Selbie and Shine; 1992), and a related receptor, the A_{2B} receptor, was cloned from rat brain (Stehle, Rivkees, Lee, Weaver, Deeds and Reppert; 1992). These receptors had all been predicted from extensive pharmacological studies. The fourth receptor, A_3, was more unexpected. By now these four adenosine receptors have been cloned from several mammalian and non-mammalian species. A_1, A_{2A} and A_{2B} receptors are well conserved among mammals, but A_3 receptors show considerable structural variability.

For all four adenosine receptors the coding region is split up by an intron in a region corresponding to the second intracellular loop (Fredholm, Arslan, Halldner, Kull, Schulte and Wasserman; 2000). Already when the structure of the A_1 receptor was first reported the presence of two major transcripts was noted. Transcripts containing three exons, called exons 4, 5 and 6 were found in all tissues expressing the receptor, whereas transcripts containing exons 3, 5 and 6 are in addition found in tissues such as brain, testis and kidney, that express high levels of the receptor (Ren and Stiles; 1994, 1995). There are two promoters, a proximal one denoted promoter A, and a distal one denoted promoter B, which are about 600 bp apart. The 5'-untranslated region of the adenosine A_{2A} receptor gene also displays two alternative promoters, although it lacks the diversity of exons found in the A_1 receptor gene (Chu, Tu, Lee, Kuo, Lai and Chern; 1996, Lee, Chang, Su, Lin, Sun, Lai and Chern; 1999). The A_{2A} receptor shows one hybridizing transcript in most tissues examined (Stehle et al.; 1992, Ren and Stiles; 1994, 1995). The rat A_{2B} receptor shows two hybridizing transcripts of 1.8 and 2.2 kb, where the latter is the dominant one (Stehle et al.; 1992). This could, in analogy with the above, suggest the presence of multiple promoters. The human A_3 receptor shows two transcripts: the most abundant is approximately 2 kb in size, and the less abundant one about 5 kb (Atkinson, Townsend-Nicholson, Nicholl, Sutherland and Schofield; 1997), perhaps indicating similarities with the A_1 receptor gene.

The distribution of receptors tells us where agonists and antagonists given to the intact organism can act. Furthermore, the rather low levels of endogenous adenosine present under basal physiological conditions have the potential of activating receptors where they are abundant, but not where they are sparse (Svenningsson, Nomikos and Fredholm; 1999).

There is much information on the distribution of the A_1 and A_{2A} receptors from several different species, because good pharmacological tools including radioligands (see below) are available. There are also several studies that have used antibodies to localize adenosine A_1 and A_{2A} receptors receptors in brain (Rosin, Robeva, Woodard, Guyenet and Linden; 1998, Hettinger, Lee, Linden and Rosin; 2001). In the case of the A_{2B} and A_3 receptors the data are less impressive. Here one tends to rely on data on the expression of the corresponding mRNA. Some of this information is summarized in Table 1.

Adenosine A_1 receptor mRNA is widespread in the brain, with the highest levels in cell bodies in hippocampus, cerebellum and cerebral cortex (Mahan et al.; 1991, Reppert, Weaver, Stehle and Rivkees; 1991). Studies using immunohistochemistry and ligand autoradiography have shown that adenosine A_1 receptor protein and the corresponding mRNA do not exactly match in several regions of the central nervous system (Johansson, Ahlberg, van der Ploeg, Brené, Lindefors, Persson and Fredholm; 1993, Swanson et al.; 1995). Much of the differential distribution can probably be explained by the fact that a substantial number of adenosine A_1 receptors are present at nerve terminals. For example, a careful examination of the comparative distribution of adenosine A_1 receptor mRNA and protein in hippocampus showed that this mRNA is enriched in cell bodies in the granular layer of dentate gyrus and the pyramidal layers of CA1 and CA3, whereas [^{3}H]DPCPX binding and A_1 receptor immunoreactivity is predominantly found in the dentate hilus stratum moleculare, stratum lacunosum, stratum radiatum and stratum oriens (Swanson et al.; 1995). Double immunofluorescence experiments showed that A_1 receptor protein co-localize with SMI-31 that labels axons, but to a lesser extent with MAP2a, b, which labels cell bodies and dendrites, or with synaptophysin, which labels synapses.

Interestingly, the levels of adenosine A_1 receptor mRNA and protein are differently regulated by, e.g., long term antagonist treatment (Johansson, Ahlberg, van der Ploeg, Brené, Lindefors, Persson and Fredholm; 1993) and during development (Ådén, Herlenius, Tang and Fredholm; 2000). The general distribution of adenosine A_1 receptors is similar between rodents and humans (Fastbom, Pazos and Palacios; 1987, Svenningsson, Hall, Sedvall and Fredholm; 1997, Schindler, Harris, Hayes, Papotti and Humphrey; 2001). Initial attempts to localize adenosine A_1 receptors in the living brain using [^{11}C]KF15372 and [^{11}C]MPDX and positron emission tomography (PET) have been made (Noguchi, Ishiwata, Furuta, Simada, Kiyosawa, Ishii, Endo, Suzuki and Senda; 1997, Shimada, Ishiwata, Kiyosawa, Nariai, Oda, Toyama, Suzuki, Ono and Senda; 2002).

Adenosine A_{2A} receptor mRNA is highly enriched in the striatum (Schiffmann, Libert, Vassart and Vanderhaeghen; 1991, Fink, Weaver, Rivkees, Peterfreund, Pollack, Adler and Reppert; 1992, Svenningsson, Hall, Sedvall and Fredholm; 1997). Lower levels are also found in extrastriatal areas, such as lateral septum, cerebellum, cortex and hippocampus (Dixon, Gubitz, Sirinathsinghji, Richardson and Freeman; 1996, Svenningsson, Hall, Sedvall and Fredholm; 1997). Most striatal neurons (95%) are GABAergic projection neurons. These neurons can be divided into two major subtypes based on their target areas and neuropeptide contents. One sub-population projects to globus pallidus and contain enkephalin. Another sub-population projects to substantia nigra pars reticulata/the entopeduncular nucleus and contains substance P and dynorphin. Interestingly, adenosine A_{2A} receptors are selectively expressed in the enkephalin-containing striatopallidal neurons (Schiffmann, Libert, Vassart and Vanderhaeghen; 1991, Fink et al.; 1992, Augood and Emson; 1994, Svenningsson, Hall, Sedvall and Fredholm; 1997). In addition to the GABAergic projection neurons, there are also cholinergic and GABAergic interneurons in striatum. It is still controversial whether these interneurons contain adenosine A_{2A} receptors. Studies using in situ hybridization have been unable to detect adenosine A_{2A} receptor mRNA in interneurons (Schiffmann, Libert, Vassart and Vanderhaeghen; 1991, Augood and Emson; 1994, Svenningsson, Hall, Sedvall and Fredholm; 1997). However, a single cell PCR study detected

adenosine A_{2A} receptor mRNA in cholinergic interneurons (Richardson, Dixon, Lee, Bell, Cox, Williams, Pinnock and Freeman; 2000).

Studies using immunohistochemistry and ligand autoradiography show high levels of adenosine A_{2A} receptors in all sub-regions of striatum (Jarvis and Williams; 1988, Parkinson and Fredholm; 1990, Rosin et al.; 1998). In addition, high levels of adenosine A_{2A} receptors have also been found in globus pallidus. These receptors are located on nerve terminals from the striatal projection neurons which innervate globus pallidus (Rosin et al.; 1998). Using A_{2A} receptor-selective antibodies and immunohistochemistry at the light- and electron-microscopic levels, Rosin and her colleagues (Rosin et al.; 1998, Hettinger et al.; 2001) have shown that striatal adenosine A_{2A} receptors are found in most neuronal compartments, i.e. dendrites, terminals of axon collaterals and in soma. However, the highest levels are found in dendrites and dendritic spines that form asymmetric synapses. These synapses receive input from glutamatergic terminals and are of excitatory nature. This postsynaptic localization of A_{2A} receptors implies that A_{2A} receptors may play an important role in the regulation of synaptic plasticity. Indeed, a functional correlate to this anatomical finding has recently been demonstrated, namely that NMDA receptor-dependent long-term potentiation in the nucleus accumbens is significantly attenuated by selective A_{2A} receptor antagonists or in A_{2A} receptor KO mice (d'Alcantara, Ledent, Swillens and Schiffmann; 2001).

The distribution of adenosine A_{2A} receptors is similar in rodents and humans (Martinez-Mir, Probst and Palacios; 1991, Schiffmann, Libert, Vassart and Vanderhaeghen; 1991). However, the levels of extrastriatal adenosine A_{2A} receptors appear to be higher in humans than in rodents. Since there is accumulating evidence for a critical role of adenosine A_{2A} receptors in the pathophysiology of several neurological and psychiatric disorders, most notably Parkinson's disease and schizophrenia, it will be of great interest to be able to monitor the levels of adenosine A_{2A} receptors in the living brain using PET. There are several reports about various ligands, including [^{11}C]KW-6002, [^{11}C]IS-DMPX, [^{11}C]KF 18446 and [^{11}C]KF 17837 (Stone-Elander, Thorell, Eriksson, Fredholm and Ingvar; 1997, Ishiwata, Noguchi, Wakabayashi,

Shimada, Ogi, Nariai, Tanaka, Endo, Suzuki and Senda; 2000, Hirani, Gillies, Karasawa, Shimada, Kase, Opacka-Juffry, Osman, Luthra, Hume and Brooks; 2001). However, these PET ligands do not appear ideal since non-specific, extrastriatal binding is high.

Biochemical studies have demonstrated low levels of adenosine A_{2B} receptors on most neurons and glia cells and in situ hybrization studies specifically demonstrated the presence of adenosine A_{2B} receptor mRNA in the hypophyseal pars tuberalis (Stehle et al.; 1992). The levels of A_3 receptors in the brain are also low, but there appear to be species differences, with the levels being higher sheep and humans than in rodents (Salvatore, Jacobson, Taylor, Linden and Johnson; 1993).

Signaling via Adenosine Receptors

The adenosine A_1 and A_2 receptors were initially subdivided on the basis of their ability to inhibit and stimulate adenylyl cyclase, respectively (Londos, Cooper and Wolff; 1980). Indeed, A_1 and A_2 receptors are coupled to members of the G_i group and G_s group of G proteins, respectively (Table 1).

	Adenosine A_1 receptor	**Adenosine A_{2A} receptor**	**Adenosine A_{2B} receptor**	**Adenosine A_3 receptor**
Previous/Alternative names	R_i ; AA1R	A_{2a}, R_s; AA2AR	A_{2b}, R_s; AA2BR	AA3A
Selective agonists	CPA, CCPA, CHA	CGS 21680, HE-NECA, CV-1808, CV-1674, ATL146e	None	Cl-IB-MECA
Selective antagonists	DPCPX[a] 8-cyclopentyl-theophylline, WRC0571	selective: SCH 58261[f,g] moderately selective: ZM241385[a], KF 17387, CSC	MRS1754[j], enprofylline	MRS 1220, MRE 3008-F20, MRS 1191; MRS 1523
Radioligands	[^{3}H]-DPCPX; [^{3}H]-CHA	[^{3}H]-CGS 21680; [^{3}H]-SCH 58261; [^{3}H]-ZM-241385	([^{3}H]-ZM-241385; [^{3}H]-DPCPX) [^{3}H]-MRS1754	[^{3}H]- MRE 3008-F20
Antibodies	Several commercial and non-commercial peptide antibodies	Several commercial and non-commercial peptide antibodies;	Some commercial and non-commercial peptide antibodies	Some commercial and non-commercial peptide antibodies
Structural information (Accession numbers)	Human 326 aa, P30542, , rat 326 aa, P25099,	Human 410 aa, P29274, rat 409 aa, P30543, mouse 409 aa, UO5672	Human 328 aa, P29275, rat 332 aa, P29276 mouse 332 aa, UO5673	Human 318 aa, P33765, rat 320 aa P28647(Alternative splicing in rat can yield product with 337 aa) Mouse 320 aa, AF069778
G protein coupling	G_i, G_o	G_s, G_{olf}	Gs, G_q	G_i
Expression pattern in nervous system.	High expression: Brain (cortex, cerebellum, hippocampus). Dorsal horn of spinal cord. Eye, adrenal gland Intermediate levels: Other brain regions.	High expression: Striatopallidal GABAergic neurons (in caudate-putamen, nucleus accumbens, tuberculum olfactorium), olfactory bulb Low levels:Rest of brain	Intermediate levels: Blood vessels, eye, median eminence, *mast cells (human?)* Low levels: Adrenal gland, pituitary gland	Intermediate levels: *Cerebellum (human?), hippocampus (human?)* Low levels: Most of brain (rat, mouse).
Gene name/chromosomal location	chr 1q32.1	chr 22g11.2	chr 17p11.2-12;	chr 1p21-13,
Main physiological function(s)	Bradycardia; inhibition of lipolysis; reduced glomerular filtration; tubero-glomerular feedback, antinociception;	Regulation of sensorimotor integration in basal ganglia; inhibition of platelet aggregation and	Relaxation of smooth muscle in vasculature and intestine; inhibition of monocyte and macrophage function,	Enhancement of mediator release from mast cells (some species). Preconditioning (some species).

	reduction of sympathetic and parasympathetic activity; presynaptic inhibition; neuronal hyperpolarization; ischemic preconditioning.	polymorphonuclear leukocytes; vasodilatation, protection against ischemic damage, stimulation of sensory nerve activity.	stimulation of mast cell mediator release (some species).	
Knockout phenotype	Anxiety, hyperalgesia, decreased tolerance to hypoxia, loss of tubero-glomerular feedback	Anxiety, hypoalgesia, hypertension, increased tolerance to ischemia, altered sensitivity to motor stimulant drugs, decreased platelet aggregation.		Altered inflammatory reactions, decreased edema, altered release of inflammatory mediators.

The A_3 receptor is also G_i coupled. In addition, there is some evidence from transfection experiments that the adenosine receptors may signal via other G proteins, but it is not known if such coupling is physiologically important. Recently, evidence was presented that whereas the A_{2A} receptor is coupled to G_s in most peripheral tissues it is coupled to G_{olf} in striatum (Kull, Svenningsson and Fredholm; 2000). Endogenous A_{2B} receptors of HEK 293 cells, human HMC-1 mast cells and canine BR mast cells may be dually coupled to G_s and G_q

After activation of the G proteins, enzymes and ion channels are affected as can be predicted from what is known about G protein signaling. Thus, A_1 receptors mediate inhibition of adenylyl cyclase, activation of several types of K^+-channels (probably via β,γ-subunits), inactivation of N, P and Q-type Ca^{2+} channels, activation of phospholipase Cβ etc. The same appears to be true for A_3 receptors. In CHO cells transfected with the human adenosine A_3 receptor both adenylyl cyclase inhibition and a Ca^{2+} signal are mediated via a $G_{i/o}$-dependent pathway. Given that many of the steps in the signaling cascade involve signal amplification it is not surprising that the position of the dose-response curve for agonists will depend on which particular effect is measured Both A_{2A} and A_{2B} receptors stimulate the formation of cAMP, but other actions, including mobilization of intracellular calcium (e.g. Mirabet, Mallol, Lluis and Franco; 1997), have also been described. Actions of adenosine A_{2A} receptors on neutrophil leukocytes are due in part to cAMP, but cyclic AMP-independent effects of A_{2A} receptor activation in these cells have also been suggested.

Activation of A_1 receptors can dose and time dependently activate ERK1/2 via β,γ-subunits released from pertussis toxin-sensitive $G_{i/o}$ proteins and phosphoinositol-3-kinase.

Activation of A_{2A} receptors also increases MAPK activity (Sexl, Mancusi, Holler, Gloria-Maercker, Schutz and Freissmuth; 1997) but the signaling pathways used by the A_{2A} receptor seem to vary with the cellular background and the signaling machinery that the cell possesses (Seidel, Klinger, Freissmuth and Holler; 1999). A_{2A} receptor activation may not only stimulate, but also inhibit ERK phosphorylation (Hirano, Aoki, Ogasawara, Kodama, Waga, Sakanaka, Shimizu and Nakamura;

1996, Arslan and Fredholm; 2000), probably via PKA-dependent phosphorylation of Raf-1. The adenosine A_{2B} receptor is the only subtype that has so far been shown to activate not only ERK1/2, but also JNK and p38 perhaps via activation of $G_{q/11}$, PLC, genistein-insensitive tyrosine kinases, ras, B-raf and MEK1/2 (Gao, Chen, Weber and Linden; 1999). Studies in transfected cells show a nearly 100-fold higher potency of both NECA and adenosine in inducing ERK1/2 phosphorylation than in inducing cAMP production. The EC_{50} value for ERK1/2 phosphorylation in transfected CHO lies in the nanomolar range, whereas cAMP production is half-maximally activated around 1-5 μM NECA. This emphasizes that G protein-coupled adenosine receptors can have substantially different potencies on different signaling pathways in the same cellular system. The adenosine A_3 receptor activates ERK1/2 in human fetal astrocytes and in transfected CHO cells (Schulte and Fredholm; 2000).

Adenosine A_{2A} receptors in striatum interact with dopaminergic mechyanisms

In 1974 Kjell Fuxe and Urban Ungerstedt showed that theophylline could by itself induce the same type of rotation-behavior that was induced by drugs that directly or indirectly stimulated dopamine receptors and that it could markedly enhance dopamine-mediated effects (Fuxe and Ungerstedt; 1974). In that study the effect was interpreted as secondary to blockade of phosphodiesterase (PDE) and was therefore taken as evidence for an important role of cyclic AMP as a mediator of dopamine actions. However, in a follow-up study where I (BF) was involved several different PDE inhibitors were examined and it was found that the potency of the drugs fitted much better with their potency as adenosine antagonists (or enhancers) than their potency as PDE inhibitors (Fredholm, Fuxe and Agnati; 1976). Together these studies showed that methylxanthines, probably by blocking adenosine receptors, could potentially be used as treatment in PD.

Studies, in two laboratories, of dopamine stimulated adenylyl cyclase in brain also showed that methylxanthines could lower “basal” enzyme activity and that adenosine could stimulate it (Fredholm; 1977, Premont, Perez and Bockaert; 1977). This was observed in dopamine-rich areas of

the brain including caudate-putamen and tuberculum olfactorium, but not in other brain areas. This suggested that these parts of the brain might have a different set of adenosine receptors than other brain areas.

This contention received support during the following decade as methods to study receptors using binding techniques were developed. The first studies used relatively non-selective radioligands but pharmacological means to discriminate between multiple binding sites Lee). Later studies used a rather selective ligand for A_{2A} receptors, including CGS 21680, (Alexander and Reddington; 1989, Jarvis, Jackson and Williams; 1989, Jarvis, Schulz, Hutchison, Do, Sills and Williams; 1989, Parkinson and Fredholm; 1990). Altogether these studies vindicated the belief that a special form of adenosine receptors, the A_{2A} receptor, is enriched in dopamine rich areas of the brain and that this offers a rationale for examining the role of adenosine in mediating or modulating behaviours and traits traditionally associated with dopamine.

The availability of more selective adenosine receptor agonists and antagonists also enforced the idea that behavioral consequences of adenosine-A_2 and dopamine-receptor mediated effects tended to be opposite (Fredholm, Herrera-Marschitz, Jonzon, Lindström and Ungerstedt; 1983, Heffner, Wiley, Williams, Bruns, Coughenour and Downs; 1989, Brown, Gill, Evenden, Iversen and Richardson; 1991).

The interactions between adenosine and dopamine receptors in striatum continued to be studied at the biochemical level. The A_{2A} receptor being coupled to a member of the G_s family of G proteins, and the D_2 receptor being coupled to a G_i protein would interact negatively at the level of second messengers and beyond. It was demonstrated that there were interactions between adenosine A_{2A} receptors at several levels. Using binding it was found that high affinity binding of D_2 agonists could be reduced by stimulation of adenosine A_{2A} receptors (Ferré, von Euler, Johansson, Fredholm and Fuxe; 1991). This finding suggested that there were interactions directly between the receptors (Figure 2), an issue that has been forcefully pursued by Kjell Fuxe and his colleagues.

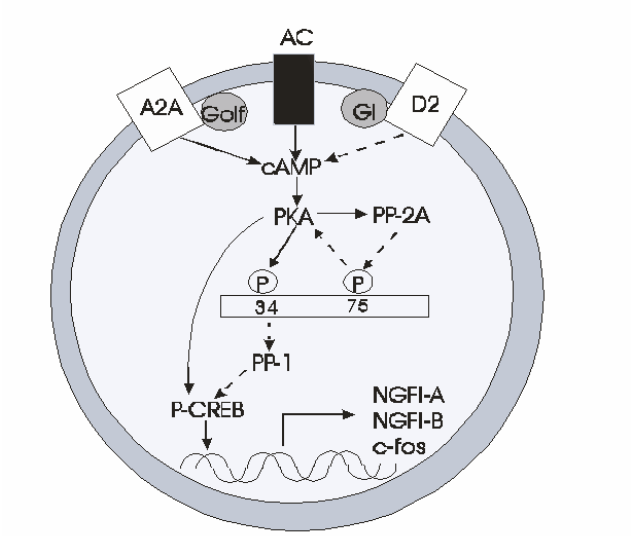

FIGURE 2. Schematic illustration of the interactions between adenosine and dopamine

The next major conceptual advance was the cloning of several adenosine receptors, as described above. These findings not only conclusively proved that there are two distinct adenosine A_2 receptors, but also provided a set of novel tools that proved very useful. In situ hybridisation was used to pinpoint the cells that express A_{2A} receptors in the brain. Using increasingly sophisticated methods it was proven that the bulk of A_{2A} expression is confined to one set of neurones in the striatum, namely those GABAergic output neurons that constitute the so-called indirect pathway (Schiffmann, Jacobs and Vanderhaeghen; 1991, Schiffmann, Libert, Vassart and Vanderhaeghen; 1991, Fink et al.; 1992, Johansson, Ahlberg, van der Ploeg, Brené, Lindefors, Persson and Fredholm; 1993, Pollack, Harrison, Wooten and Fink; 1993, Johansson, Georgiev and Fredholm; 1997, Svenningsson, Le Moine, Kull, Sunahara, Bloch and Fredholm; 1997). These cells also express the bulk of the dopamine D_2 receptors. Hence the link between A_{2A} and dopamine D_2 receptors was further strengthened.

Techniques with a cellular resolution were also used to try to determine the roles of adenosine A_{2A} receptors in the intact striatum. This was based

on early findings showing that expression of immediate early genes could be used to pinpoint changes in neuronal activity and/or signal transduction (Sheng and Greenberg; 1990). We observed that stimulatory doses of caffeine and selective A_{2A} receptor antagonists caused a decrease in the expression of IEGs, known to be regulated by the cAMP/CREB cascade, in striatopallidal neurons (Svenningsson, Nomikos and Fredholm; 1995, Svenningsson, Nomikos, Ongini and Fredholm; 1997). These and subsequent studies (Pinna, Wardas, Cozzolino and Morelli; 1999, Chen, Moratalla, Impagnatiello, Grandy, Cuellar, Rubinstein, Beilstein, Hackett, Fink, Low, Ongini and Schwarzschild; 2001) provide strong evidence that adenosine, via A2A receptors, exert a robust tonic activation of the cAMP/CREB/IEG cascade in striatopallidal neurons. Moreover, this result also provided evidence that multiple D2 receptor-mediated effects by dopamine can be attributed to an antagonism of this adenosine-mediated activation of striatopallidal neurons.

In order to increase our understanding of the interactions of adenosine and dopamine at the signal transduction level, we proposed a collaborative project with Paul Greengard to study the effects of adenosine A_{2A} selective compounds and caffeine on the phosphorylation of dopamine and cAMP phosphoprotein of 32 kDa (DARPP-32). DARPP-32 is highly enriched in all striatal GABAergic medium-sized projection neurons and is an important mediator of dopaminergic signaling (Greengard; 2001). Its function is determined by its relative phosphorylation state at several different threonine/serine residues, of which the most studied is a PKA-site Thr34. When this residue is phosphorylated it converts DARPP-32 into an inhibitor of protein phosphatase-1, which, in turn, regulates the activity of multiple transcription factors, including CREB, ion channels and ionotropic receptors (Fig 2). In initial studies conducted in brain slices prepared from striatum, it was found that CGS 21680 potently increases phosphorylation at Thr34 (Svenningsson, Lindskog, Rognoni, Fredholm, Greengard and Fisone; 1998). This effect was additive to that of SKF81297, a selective D_1 agonist, and could be counteracted by quinpirole, a selective D2 agonist (Lindskog, Svenningsson, Fredholm, Greengard and Fisone; 1999). This result identified adenosine, via A2A receptors, as a key regulator of the phosphorylation state of DARPP-32 in

striatopallidal neurons. Subsequently, we developed a method to reliably detect DARPP-32 phosphorylation in vivo and could demonstrate that the A_{2A} antagonist used, SCH 58261, significantly counteracted the increase in DARPP-32 phosphorylation that was observed following treatment with selective D_2 receptor antagonists (Svenningsson, Lindskog, Ledent, Parmentier, Greengard, Fredholm and Fisone; 2000). Likewise, the ability of D_2 antagonists to increase DARPP-32 phosphorylation was dramatically reduced in A_{2A} receptor KO mice. These data therefore provided further support for the notion that adenosine acting on A_{2A} receptors is an important mediator of establishing a basal cyclic AMP level, which is necessary for many effects of dopamine's action via D_2 receptors. In order to address the involvement of DARPP-32 in the behavioural actions of caffeine and selective adenosine A2A receptor compounds, we administred such compounds to DARPP-32 KO and studied effects on locomotor behaviour. As expected from the biochemical data, it was found that the ability of CGS 21680 to induce hypolocomotion was attenuated in DARPP-32 KO mice (Lindskog, Svenningsson, Pozzi, Kim, Fienberg, Bibb, Fredholm, Nairn, Greengard and Fisone; 2002). Similarly, the ability of caffeine and SCH 58261 to induce hyperlocomotion was attenuated in DARPP-32 KO mice. In this paper, an additional effect of A_{2A} receptors on DARPP-32 phosphorylation was shown, namely that A_{2A} agonism via cAMP-dependent mechanisms increases the phosphorylation of Thr34-DARPP-32, but decreases the phosphorylation at Thr75-DARPP-32. Conversely, caffeine and SCH 58261 increase phosphorylation at Thr75-DARPP-32. This site has recently been shown to be phosphorylated by Cdk5 and when this happens DARPP-32 is converted into an inhibitor of PKA. Thus, by increasing the phosphorylation of Thr75-DARRP-32, caffeine and selective A2A receptor antagonists will further increase the inhibition of PKA. This feed-forward mechanism, which is also utilized by D2 receptor agonists, will therefore potentiate the inhibitory influence of adenosine on the cAMP/PKA/CREB/IEG signaling pathway in striatopallidal neurons (Fig 1d).

A_{2A} receptor antagonists and Parkinson´s disease

In parallell with the development of an increasingly clear understanding of the biochemical and molecular underpinning of the adenosine-dopamine interactions there has been extensive work on the effectiveness of adenosine A_{2A} antagonists in various experimental models of PD. It will carry too far to try to present these results. However, a recent study showed that A_{2A} receptor antagonism could reduce not only symptoms of PD, but also the loss of dopamine neurons induced by MPTP (Chen, Xu, Petzer, Staal, Xu, Beilstein, Sonsalla, Castagnoli, Castagnoli Jr and Schwarzschild; 2001). Furthermore, it was shown that in fact persistent L-DOPA effects require A_{2A} receptors (Fredduzzi, Moratalla, Monopoli, Cuellar, Xu, Ongini, Impagnatiello, Schwarzschild and Chen; 2002).

Thus, over the years the concept that A_{2A} and D_2 receptors interact in such a way that A_{2A} antagonists could prove to be useful in PD has developed strongly. There are however concerns. One potential concern is related to tolerance. It is very well known that some actions of caffeine develop rapid tolerance (Fredholm, Bättig, Holmén, Nehlig and Zvartau; 1999, Svenningsson, Nomikos and Fredholm; 1999). However, caffeine effects in PD models do not (Xu, Xu, Chen and Schwarzschild; 2002), and there is also no tolerance to selective A_{2A} antagonsts in models that show tolerance to caffeine (Halldner, Lozza, Lindström and Fredholm; 2000).

Another, and perhaps more serious, concern is related to the fact that A_{2A} receptors regulate other things than activity in striatopallidal neurons. It has long been known that adenosine regulates platelet activation (Haslam and Cusack; 1981), and now we know that A_{2A} receptors are responsible for this (Ledent, Vaugeois, Schiffmann, Pedrazzini, El Yacoubi, Vanderhaeghen, Costentin, Heath, Vassart and Parmentier; 1997).

Similarly A_{2A} receptors are critically important in regulating neuttrophil leucocyte activity (Cronstein; 1994), and activity of macrophages. Even more importantly A_{2A} receptors do regulate inflammatory reactions in general (Ohta and Sitkovsky; 2001). Therefore, long-term blockade of adenosine A_{2A} receptors might cause undesirable peripheral morbidity.

A potential way to attack this problem was afforded when it was discovered that A_{2A} receptors in striatum are coupled to G_{olf} proteins (Kull et al.; 2000), whereas on platelets, neutrophils and lymphocytes G_s

mediates the A_{2A} effects. If it proves possible to find agents that selectively affects A_{2A}-G_{olf} more selective drugs may be found.

Physiology of adenosine A_1 receptors as determined from studies using knock out mice

Even though quite selective agonists and antagonists are available for adenosine A_1 receptors (Fredholm, IJzerman, Jacobson, Klotz and Linden; 2001), and consequently much is known about their role in physiology and pathophysiology we embarked on a classical gene knock out strategy. The second coding exon was targeted as described (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm; 2001) and experiments were conducted on littermates from matings of heterozygote animals (A1R +/-) with a mixed C57Bl6/129OlaHsd background.

The offspring showed the expected frequency (1/4 A1R +/+; 2/4 A1R +/- and 1/4 A1R -/-). There were no clear differences in the growth and maturation over the first five months between genotypes (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm; 2001), but subsequently +/- and, especially, -/- mice tended to die faster than their +/+ littermates (Giménez-Llort, Fernández-Teruel, Escorihuela, Fredholm, Tobena, Pekny and Johansson; 2002). So far we have not observed any major differences in fertility between genotypes, even though there is evidence that the A_1 receptor is involved in sperm capacitation.

A1R +/- and -/- mice showed no gross behavioural abnormalities. However, their grip strength, as judged by the so called wire hanger test, was reduced (Giménez-Llort et al.; 2002). This was somewhat surprising given the evidence that methylxanthines in doses that predominantly affect adenosine receptors appear, if anything, to increase muscle strength.

Although overall locomotor activity was unaltered in A1R -/- mice (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm; 2001), we have found that the

increase in motor activity that accompanies transition from light to dark is reduced in the knockout mice (Giménez-Llort et al.; 2002). Surprisingly, A1R +/- mice were in this regard indistinguishable from wild-type animals. However, exploratory activity (assayed in the open field test and the hole-board) tended to be increased in the heterozygotes (Giménez-Llort et al.; 2002). This would tally with the known stimulatory effect of methylxanthines, including caffeine (Fredholm, Bättig, Holmén, Nehlig and Zvartau; 1999).

The A1R -/- showed increased anxiety using two commonly used tests, the elevated plus maze and the dark-light box (Giménez-Llort et al.; 2002). This is interesting because hyperanxiety was also observed in A2A -/- mice (Ledent et al.; 1997). Future experiments will tell if the traits in the two strains is synergistic. It is also interesting to compare with the fact that high doses of caffeine that acts on A_1 and A_{2A} receptors produces anxiety in animals and man (Fredholm, Bättig, Holmén, Nehlig and Zvartau; 1999). Using the resident intruder test A1R -/- mice also exhibited increased aggressiveness (Giménez-Llort et al.; 2002), again a trait also observed in A2A -/- mice (Ledent et al.; 1997). By contrast, no effect was observed when examining memory functions with the Morris water maze.

Using the hippocampal slice preparation the inhibitory adenosine effects were shown to be eliminated in A1R -/- mice (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm; 2001). This is an important finding since it shows that there are no important effects mediated via any of the other receptors in this preparation, despite the fact that important effects of both A_{2A} (Sebastiao and Ribeiro; 1996) and A_3 receptors (Dunwiddie, Diao, Kim, Jiang and Jacobson; 1997) has been reported. Another important finding was that the dose-response curve for adenosine was shifted significantly (two-fold) to the left in A1R +/- mice, which possess almost exactly half the normal number of receptors. This emphasises the important general point that potency of adenosine analogues is strongly dependent on the number of receptors. There were no adaptive changes in responses to $GABA_B$ receptor activation or any adaptive changes in A_{2A} receptors.

Ever since the pioneering work of Berne and Gerlach a role for adenosine in mediating responses to hypoxia and ischemia has been postulated. We have so far examined the role of A_1 receptors in two such responses. In the hippocampal slice preparation responses to hypoxia include a fast decrease in responses to excitatory stimulation. We found that virtually all of this decrease was lost in slices from A1R -/- mice (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm; 2001). Furthermore, A1R -/- slices did not fully recover after hypoxia, as did A1R +/+ slices. These findings clearly demonstrate that adenosine acting on A_1 receptors is critically important in regulating the responsiveness of neurons to decreased supply of metabolizable energy, enabling the neurons to survive such energy shortage.

A somewhat similar effect was observed when activity of respiratory neurons in the immature brainstem was studied. The normal depression of rate of spontaneous firing induced by hypoxia was lost or markedly reduced in A1R -/- mice. Also in this preparation the recovery seen in A1R +/+ mice was less complete in A1R -/- brainstem neurons (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm; 2001).

Body temperature was similar in all genotypes, but the temperature lowering effect of adenosine analogues was less pronounced in A1R +/-, and, particularly, A1R -/- mice (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm; 2001).

It has long been known that adenosine can control pain (Sawynok and Sweeney; 1989) and adenosine receptors, particularly A_1 receptors, have been known to be present in spinal cord (Geiger et al.; 1984, Fastbom, Post and Fredholm; 1990). It was therefore no major surprise when we found that the analgesic effects of intrathecal adenosine analogues were essentially lost in A1R -/- mice (Johansson, Halldner, Dunwiddie, Masino, Poelchen, Giménez-Llort, Escorihuela, Fernández-Teruel, Wiesenfeld-Hallin, Xu, Hårdemark, Betsholtz, Herlenius and Fredholm;

2001). Somewhat more surprising was the finding that there was a clear hyperalgesia in these animals. This clearly suggests that adenosine acting at A_1 receptors constitute a significant endogenous analgesic mechanism.

The actions of adenosine are however mediated also by other adenosine receptors. Thus adenosine A_{2A} rerceptors, presumably located at sensory nerve endings, medate hyperalgesia (Ledent et al.; 1997). A_3 receptors are – at least in mice – important as promoters of peripheral inflammation that leads to pain.

It has long been known that adenosine can decrease cardiac performance and reduce heart rate (Drury and Szent-György; 1929), and this effect, which is clinically relevant, is linked to A_1 receptors. The depressant effect of exogenous adenosine analogues was virtually eliminated in A1R -/- mice. It was therefore surprising that heart rate in A1R -/- mice proved to be similar to that in their wild type littermates.

Blood pressure in anaesthetised mice was elevated in the A1R -/- genotype (12 mm Hg), and there was even a tendency towards an elevated blood pressure in A1R +/- mice (5 mm Hg) (Brown, Ollerstam, Johansson, Skött, Fredholm and Persson; 2001). Since A1R -/- mice also had an elevated level of plasma renin a possible explanation is that the blood pressure elevation is due to angiotensin. However, an antagonist did not differ in its blood pressure lowering effect between genotypes (Brown, Ollerstam, Johansson, Skött, Fredholm and Persson; 2001). There were no major differences in blood vessel reactivity between genotypes, so we are still looking for the cause of the blood pressure increase. Given that the A2AR -/- genotype is also hypertensive (Ledent et al.; 1997) it will be interesting to know if the double knock-out shows a marked blood pressure elevation.

In preliminary studies we have found that isolated hearts from A1R -/- mice do not differ in their response to acute ischemia from hearts from their wild type littermates. However, we do see a markedly reduced protective effect of remote preconditioning. Ongoing studies aims at demonstrating the underlying mechanisms.

Sodium excretion was markedly (two-fold) elevated in both A1R +/- and -/- mouse kidneys (Brown, Ollerstam, Johansson, Skött, Fredholm and

Persson; 2001). This agrees with the known effects of caffeine and other adenosine receptor antagonists. By contrast, potassium excretion and glomerular filtration rate was unaltered. Most importantly, tubuloglomerular feedback is completely eliminated in A1R -/- mice (Brown, Ollerstam, Johansson, Skött, Fredholm and Persson; 2001). These results emphasize that renal A_1 receptors constitute important drug targets.

Knock-out mice can be used not only to determine the role(s) of a particular gene product in physiology and pathophysiology, but also to determine the selectivity of drugs. So far we have finished a study that demonstrates that at least some of the effects of ATP (and other adenine nucleotides) in the hippocampus are in fact mediated by A_1 receptors, since the responses are completely eliminated in A1R -/- mouse hippocampi. It will be important in the future to perform similar studies in other tissues, since adenine nucleotides are so very rapidly broken down at cell membranes generating high local concentrations of adenosine

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