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REVIEW

Adenosine in the central nervous system: release mechanisms and extracellular concentrations

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Abstract

Adenosine has several functions within the CNS that involve an inhibitory tone of neurotransmission and neuroprotective actions in pathological conditions. The understanding of adenosine production and release in the brain is therefore of fundamental importance and has been extensively studied. Conflicting results are often obtained regarding the cellular source of adenosine, the stimulus that induces release and the mechanism for release, in relation to different experimental approaches used to study adenosine production and release. A neuronal origin of adenosine has been demonstrated through electrophysiological approaches showing that neurones can release significant quantities of adenosine, sufficient to activate adenosine receptors and to modulate synaptic functions. Specific actions of adenosine are mediated by different receptor subtypes (A_1, A_{2A}, A_{2B} and A_3), which are activated by various ranges of adenosine concentrations. Another important issue is the measurement of adenosine concentrations in the extracellular fluid under different conditions in order to know the degree of receptor stimulation and understand adenosine central actions. For this purpose, several experimental approaches have been used both *in vivo* and *in vitro*, which provide an estimation of basal adenosine levels in the range of 50–200 nM. The purpose of this review is to describe pathways of adenosine production and metabolism, and to summarize characteristics of adenosine release in the brain in response to different stimuli. Finally, studies performed to evaluate adenosine concentrations under physiological and hypoxic/ischemic conditions will be described to evaluate the degree of adenosine receptor activation.

Keywords: adenosine release, CNS, extracellular concentrations.

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Over the past 25 years a general consensus has been reached on the crucial role of adenosine in the CNS as a modulator of neurotransmission and a neuroprotective agent against ischemic- and seizure-induced neuronal injury. Adenosine has also been proposed to be a potent regulator of cerebral blood flow (Phillis 1989; Dunwiddie and Fredholm 1997). Besides its more general involvement in cellular metabolism, specific actions of adenosine in the CNS as neuroeffector are believed to be mediated through specific receptors, which have been cloned and classified as A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm *et al*. 1994a). Activation of different receptor subtypes has been reported to mediate different effects of endogenous adenosine. The well-established inhibitory action of adenosine on excitatory neurotransmission is principally ascribed to A_1 receptor activation, whereas excitatory actions on neurones have been observed after A_{2A} receptor stimulation. The A_1-mediated inhibitory effects of adenosine on neurotransmission account for neuroprotective effects that have been demonstrated in a variety of *in vitro* and *in vivo* experimental models, mainly of hypoxia/ischemia and seizures (Rudolphi *et al*. 1992; Von Lubitz 1999). The A_1-mediated neuroprotective effects are attributed to the inhibition of neuronal Ca^{2+} uptake in the presynaptic terminal, which per se is protective and also contributes to the reduction of presynaptic release of different neurotransmitters. Among transmitters, reduction in excitatory amino acids is relevant to the neuroprotective effect of adenosine. Acting on A_1 receptors, adenosine also hyperpolarizes cells, thus reducing neuronal excitability and firing

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Abbreviations used: ADO, adenosine; ADA, adenosine deaminase; AK, adenosine kinase; AOPCP, α,β-methylene adenosine diphosphate; DCF, deoxycoformycin; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenosine; ei, equilibrative-insensitive; es, equilibrative-sensitive; hENT, human equilibrative nucleotide transporter; 5-IT, 5-iodotubercidin; NBMPR, nitrobenzylthioinosine; rENT, rat equilibrative nucleotide transporter; SAH, *S*-adenosylhomocysteine; TTX, tetrodotoxin.

rate (Greene and Hass 1991) with a consequent reduction in cellular metabolism, energy consumption and hypothermia.

In recent years data have been generated showing the neuroprotective actions of compounds acting on A_{2A} receptors (Ongini and Schubert 1998). A_{2A} antagonists have been proved useful in improving motor disabilities in primate models of Parkinson's disease (Kanda *et al*. 1998; Grondin *et al*. 1999), and are considered as potential therapeutic drugs alone or in association with L-DOPA in humans (for a review see Impagnatiello *et al*. 2000). Recently, the finding that brain injury induced by transient focal ischemia is reduced in A_{2A} receptor knockout mice (Chen *et al*. 1999) strengthens the observation that A_{2A} receptor blockade is neuroprotective in different ischemia models (Ongini and Schubert 1998). At the moment, however, the role of the A_{2A} receptor in ischemia is controversial as both A_{2A} agonists and antagonists proved to be neuroprotective in different *in vivo* models of neurotoxicity or ischemia (Ongini and Schubert 1998; Pedata *et al*. 2001). Neuroprotective effects of A_{2A} receptor agonists have been attributed to the activation of non-neuronal A_{2A} receptors located on arterial walls, platelets or neutrophils responsible for vasodilation, inhibition of platelet aggregation and reduction of free radical formation, respectively. On the other hand, neuroprotective effects of A_{2A} antagonists have been attributed to the stimulation of neuronal A_{2A} receptors, thus reducing excitatory amino acid release (for a review see Pedata *et al*. 2001). The possible neuroprotective effect of both the stimulation or the blockade of A_{2A} receptors located on microglial cells, where they regulate the release of inflammation products, is still a matter of debate (Ongini and Schubert 1998; Pedata *et al*. 2001).

Little information is available at the moment on specific effects of A_{2B} receptor stimulation on the CNS, and opposite effects, both protective and injurious, have been ascribed to A_3 adenosine receptor stimulation under ischemic conditions (for a review see Von Lubitz 1999).

As A_1, A_{2A}, A_{2B} and A_3 adenosine receptors may be activated by different ranges of endogenous adenosine concentrations (Fredholm *et al*. 1994a), the levels of endogenous adenosine available to bind to and activate these receptors help control different physiological responses to adenosine. Therefore, the measurement of adenosine concentrations in the extracellular space under different conditions is of great interest in order to understand the degree of receptor stimulation and the central actions of adenosine.

The purpose of this review is to summarize evidence on adenosine release in the brain with particular emphasis on studies performed to evaluate adenosine concentrations in the extracellular space and its modification in response to different stimuli. Pathways of intra- and extracellular metabolism of adenosine will be described, with particular emphasis on adenosine release per se or from released nucleotides.

Pathways of adenosine production, metabolism and transport

Extra- and intracellular formation of adenosine from nucleotides

Adenosine may be formed intracellularly in the CNS from degradation of AMP, and then exit via bi-directional nucleoside transporters, or extracellularly by the metabolism of released nucleotides (Fig. 1). At least four different 5′-nucleotidase activities have been described in mammalian tissues with different biochemical and molecular properties (Zimmermann 1992). In this family of enzymes, the ecto-5′-nucleotidase (e-N), which is responsible for extracellular degradation of AMP, is the best-characterized enzymatic source of adenosine. It exhibits a K_m for AMP in the micromolar range, and is potently inhibited by ATP, ADP and α,β-methylene adenosine diphosphate (AOPCP). Two soluble 5′-nucleotidase with high K_m for AMP (millimolar range) are found in the cytosolic fraction (c-N-I and c-N-II) and another form of 5′-nucleotidase activity (e-Ns), with a low K_m for AMP ($K_m = 15$ μM in the rat brain) and several properties similar to those of ecto-5′-nucleotidase, have been observed in soluble fractions from rat liver, kidney, brain (Piec and Le Hir 1991; Orford and Saggerson 1996) and bovine brain (Montero and Fes 1982).

Extracellular formation

Various studies have been performed to assess biochemical, molecular and pharmacological properties of ecto-5′-nucleotidase, the enzyme responsible for the extracellular source of adenosine (for a review see Zimmermann 1996). Ecto-5′-nucleotidase is a homodimer linked to the plasma membrane through a glycosyl-phosphatidylinositol lipid anchor, with its active site exposed in the extracellular space. Although biochemical and enzyme histochemical analyses have identified ecto-5′-nucleotidase activity in association with both neuronal and glial cells (Franco *et al*. 1986; Cunha *et al*. 1992; James and Richardson 1993), immunocytochemical analysis has revealed that this enzyme is mainly associated with plasma membrane of astrocytes, and oligodendroglial and microglial cells (Schoen *et al*. 1987; Grondal *et al*. 1988). The only examples of a neuronal localization of the enzyme are the mossy fibre terminals in the CA3 region of rat and mouse hippocampus (Zimmermann *et al*. 1993; Schoen *et al*. 1999), and its presence on cultured cerebellar granule cells (Maienschein and Zimmermann 1996). The lack of correlation between the localization of the protein, mainly associated with glial cells, and biochemical evidence for a neuronal enzymatic activity, could be attributed to the existence of immunologically distinct isoforms of the ecto-5′-nucleotidase throughout the CNS (Zimmermann *et al*. 1993; Cunha *et al*. 2000).

Numerous evidence suggest that ATP can be released

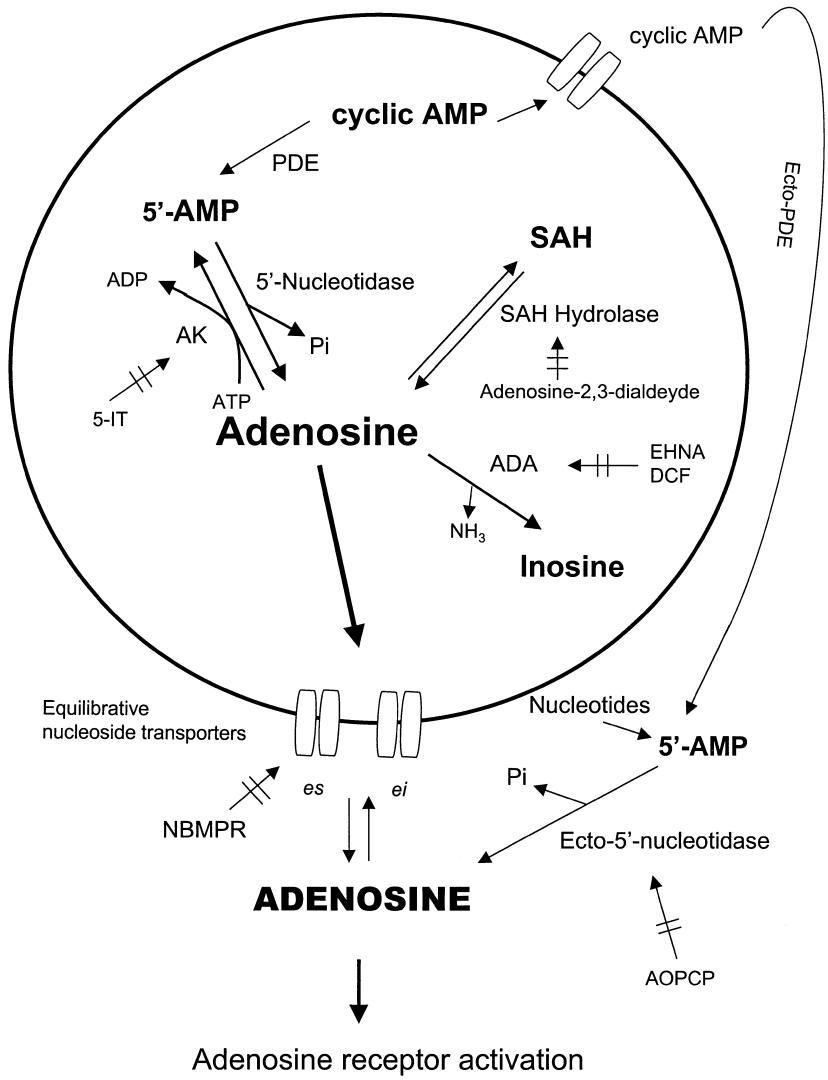

Fig. 1 Pathways of adenosine production, metabolism and transport, with indications of the sites of action of various enzyme inhibitors. Abbreviations are as follows: ADA, adenosine deaminase; AK, adenosine kinase; AOPCP, α,β-methylene ADP; DCF, deoxycoformycin; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenosine; es, equilibrative-*s*ensitive nucleoside transporters; ei, equilibrative-*i*nsensitive nucleoside transporters; 5-IT, 5-iodotubercidin; NBMPR, nitrobenzylthioinosine; PDE, cAMP phosphodiesterase; SAH, *S*-adenosyl homocysteine.

from neurones as a transmitter or as a cotransmitter, thereby generating excitatory responses in several regions of the CNS by acting on specific nucleotide receptors (P_2 receptors; for a review see Zimmermann 1994). Nevertheless, in many systems ATP also elicits inhibitory effects on synaptic functions that are mediated via the activation of adenosine receptors (Dunwiddie and Hoffer 1980; Lee *et al*. 1981). In most of these studies inhibitory responses are blocked by adenosine receptor antagonists and by adenosine deaminase, suggesting that the nucleotide is converted to adenosine before the activation of these receptors. However, it has been shown that ATP also exerts inhibitory actions directly trough stimulation of its receptors and not through conversion to adenosine (Von Kugelgen *et al*. 1992; Bennett and Boarder 2000). Recently it has been shown that inhibitory responses to ATP are partly a result of its conversion to adenosine and partly a result of the activation of P2 receptors, which in turn trigger a release of endogenous adenosine (Nikbakht and Stone 2000).

That ATP-mediated inhibitory effects are caused by a rapid conversion to adenosine has been clearly demonstrated in the hippocampus using electrophysiological approaches (Dunwiddie *et al*. 1997; Cunha *et al*. 1998). The local application of ATP, ADP and AMP near the cell body of CA1 pyramidal neurones, from which inhibitory responses were recorded, results in a rapid conversion to adenosine (within < 1 s), which can activate A_1 receptors and generate an increase in postsynaptic K^+ conductance. From the comparison of the kinetics of adenosine and nucleotide responses, it appears that the transformation of AMP to adenosine represents the rate-limiting step in the catabolism of ATP, caused by the inhibition of 5′-nucleotidase exerted by ADP and ATP (Dunwiddie *et al*. 1997). This observation confirms a previous kinetic characterization of extracellular ATP degradation performed at the striatal cholinergic synapse (James and Richardson 1993), in which authors proposed that in situations of high-frequency release rates, adenosine could not be extracellularly produced until the

end of the stimulation period, when ATP and ADP levels are reduced. In this case an increase in extracellular adenosine concentration during the stimulation period may be primarily caused by a release of adenosine per se. However, after the stimulation period, when ATP and ADP levels decrease below the threshold to inhibit ecto-5-nucleotidase, formation of adenosine from extracellular ATP may occur (James and Richardson 1993).

It appears from literature on this topic that the intra- or extracellular origin of adenosine may also depend on the neuronal preparations used, as well as on the method used to stimulate adenosine release. In the hippocampal slice preparation, for example, only the application of a high-frequency stimulation burst results in adenosine release, which in its later phase is derived from the extracellular catabolism of ATP (Cunha *et al*. 1996). On the other hand, after low-frequency stimulation adenosine is mainly derived per se from cells (Lloyd *et al*. 1993; Pedata *et al*. 1993b; Cunha *et al*. 1996). Using a hippocampal subcellular preparation enriched in mossy fibre synaptosomes, where neuronal localization of ecto-5′-nucleotidase has been demonstrated (Schoen *et al*. 1999), rapid extracellular hydrolysis of ATP with consequent formation of adenosine and inhibition of glutamate release has been observed (Terrian *et al*. 1989). In rat cortical slices and whole brain synaptosomes, the inhibition of ecto-5′-nucleotidase activity consistently decreases basal and NMDA-induced adenosine release, but not the K^+- and glutamate-evoked release (MacDonald and White 1985; Hoehn and White 1990c; Craig and White 1993a). In another study on purified rat striatal cholinergic synaptosomes, the inhibition of ecto-5′-nucleotidase with specific antibodies decreased adenosine formation from released ATP, preventing the inhibition of acetylcholine release by ATP (Richardson *et al*. 1987). *In vivo* estimations of striatal adenosine tissue levels show that a significant quantity of adenosine, both under basal conditions and under NMDA-stimulated conditions, originates from extracellular nucleotide metabolism (Delaney and Geiger 1998).

Under ischemic or hypoxic conditions, most studies demonstrate an intracellular formation of released adenosine (Meghji *et al*. 1989; Lloyd *et al*. 1993), whereas in a recent study conducted in fetal sheep brain, an extracellular origin of adenosine under hypoxia has been observed (Koos *et al*. 1997). An up-regulation of ecto-5′-nucleotidase expression associated with reactive glia has been observed following *in vivo* cerebral ischemia in damaged tissue, with a consequent increase in extracellular adenosine formation (Braun *et al*. 1998). Similar results have also been obtained in PC12 cells submitted to hypoxic conditions (Kobayashi *et al*. 2000). This effect may be involved in the rapid removal of any cytotoxic effect of extracellular ATP (Edwards 1996) and the increase in neuroprotective actions of adenosine (Rudolphi *et al*. 1992).

Another possible extracellular source of adenosine is released cyclic AMP. The specific release of cyclic AMP in the CNS has been reported via a non-specific energy-dependent transporter (Henderson and Strauss 1991). In the extracellular space, cyclic AMP can be converted to 5′-AMP by ecto-phosphodiesterase, and then to adenosine by ecto-5′-nucleotidase (Fig. 1). An extracellular accumulation and degradation of cyclic AMP in response to the application of norepinephrine and VIP has been reported in rat cortical cultures as a possible source of extracellular adenosine (Rosenberg and Dichter 1989; Rosenberg and Li 1995). Alternatively, cyclic AMP may also be converted to 5′-AMP inside the cells, and then the latter may be released, providing an extracellular adenosine source. This possibility has been raised in relation to the NMDA-evoked adenosine release from rat cortical slices (Craig *et al*. 1994). These observations also suggest that several neurotransmitters acting on metabotropic receptors coupled to adenyl cyclase, by increasing the accumulation of cyclic AMP, might regulate extracellular adenosine levels, and thus inhibit the tone in the CNS. In an electrophysiological approach it has been observed that prolonged increases in extracellular cyclic AMP levels induce the formation of adenosine and the production of an adenosine receptor-mediated response in the hippocampus; on the other hand, brief increases in cyclic AMP levels in the proximity of single pyramidal neurones do not induce an adenosine-mediated response (Brundege *et al*. 1997). Thus, changes in intra- or extracellular cyclic AMP and adenosine levels may occur very slowly in response to prolonged changes in neurotransmitter release. In this way cyclic AMP is unable to act as a rapid precursor of extracellular adenosine, and may account only for slow changes in extracellular adenosine concentrations.

Intracellular formation

Two soluble 5′-nucleotidases are found in cytosolic fractions: an IMP-selective cytosolic 5′-nucleotidase, which prefers IMP as substrate (K_m 0.1–1.2 mM) and an AMP-selective cytosolic 5′-nucleotidase, which is 15–20 times more active on AMP than IMP. Because the K_m values for AMP of both cytosolic-5′-nucleotidase activities are much higher (1–14 mM) than the intracellular concentration of AMP under physiological conditions (0.1–0.5 mM), it is possible to envisage that both enzymes respond only to a large increase in AMP concentration during increased metabolic activity. The intracellular concentration of ATP is about 50 times higher than that of AMP, thus only a small variation in ATP catabolism can induce a large increase in AMP concentration. For this reason the intracellular formation of adenosine from catabolism of cytosolic ATP has been proposed to represent a very sensitive signal of increased metabolic rate or metabolic stress. However, more recently it has been shown that substantial adenosine formation by cytosolic 5′-nucleotidase may also occur

under physiological conditions, in the absence of metabolic stress (Sala-Newby *et al*. 1999). Furthermore, under physiological conditions, adenosine may be intracellularly produced by the low-K_m cytosolic 5′-nucleotidase (Orford and Saggerson 1996).

Cytosolic-5′-nucleotidase has been purified and characterized from bovine brain (Montero and Fes 1982), but its presence in the CNS has not been directly demonstrated in immunohistochemical studies. Only recently the enzyme has been cloned from pigeon heart, and its expression has been shown to be abundant in oxidative muscles and brain (Sala-Newby *et al*. 1999). Because specific inhibitors of cytosolic-5′-nucleotidase are not available, the contribution of intracellular adenosine formation to the released adenosine has been mainly demonstrated by indirect experimental approaches. The most important has been the inhibition of ecto-5′-nucleotidase obtained with AOPCP, which allows the correlation between a reduction of adenosine release with the origin of adenosine from released nucleotides. Under basal conditions ecto-5′-nucleotidase inhibition has been found to partially decrease adenosine release from cortical slices and synaptosomes (MacDonald and White 1985; Hoehn and White 1990c), and *in vivo* from cat basal ganglia (Barberis *et al*. 1984). On the other hand, a preferential release of adenosine per se, instead of ATP, has been described in several experimental brain preparations submitted to depolarizing stimuli (K^+, veratridine and electrical stimulation) and glutamate (Pons *et al*. 1980; Barberis *et al*. 1984; MacDonald and White 1985; Hoehn and White 1990c; Lloyd *et al*. 1993; Pedata *et al*. 1993b). An intracellular origin of released adenosine has also been demonstrated from rat hippocampal slices submitted to ischemic conditions (Lloyd *et al*. 1993; Pedata *et al*. 1993b), to transient hyperthermia (Masino and Dunwiddie 1999) and from cultured neurones and/or glia of chick embryo submitted to metabolic poisoning (Meghji *et al*. 1989).

Another experimental approach for determining the origin of released adenosine has been the *in vitro* superfusion of brain tissue with L-homocysteine, which in the presence of intracellular adenosine, induces a reversal of the reaction catalysed by *S*-adenosylhomocysteine (SAH) hydrolase, with the consequent formation of SAH (see Fig. 1). Using this approach, a significant reduction in adenosine release from electrically stimulated cortical slices (McIlwain and Poll 1985), and from hippocampal slices submitted to electrical stimulation and energy depletion (Lloyd *et al*. 1993), has been shown. These results are indicative of an intracellular origin of released adenosine.

In summary, experimental evidence exists of both an intra- and extracellular formation of adenosine in several brain preparations. Under normoxic conditions, extracellular adenosine may derive from presynaptic neurones per se or from released ATP. In this last case formation of adenosine may be directly linked to neurotransmission. Even a release of adenosine per se from the postsynaptic cell has been demonstrated (Smith 1991; Smith and Lu 1991; Brundege and Dunwiddie 1996). On the contrary, under hypoxic/ischemic conditions, extracellular adenosine is mostly dependent on intracellular formation of adenosine, which is linked to the balance between energy supply and demand and thus the formation and degradation of cytoplasmic ATP.

Intracellular formation of adenosine from *S*-adenosylhomocysteine

Another possible intracellular source of adenosine may be the hydrolysis of SAH by SAH hydrolase (Fig. 1). This pathway is not closely linked to the energy state of the cell and provides one-third of adenosine production under normoxic conditions but none under hypoxic conditions in the heart (Lloyd *et al*. 1988; Deussen *et al*. 1989). SAH hydrolase has been localized by immunocytochemistry in the neocortex, hippocampus and cerebellum of the rat (Patel and Tudball 1986). However the selective inhibitor of SAH hydrolase, adenosine-2,3-dyaldehyde, does not significantly influence adenosine release either under normoxic or ischemic conditions, indicating that this pathway does not significantly contribute to adenosine production in the brain (Pak *et al*. 1994; Latini *et al*. 1995).

Adenosine metabolism

Inactivation of extracellular adenosine is principally mediated by uptake across the cell membrane of neurones or neighbouring cells, followed by either phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminase (ADA) (Fig. 1). Several studies investigated the contribution of these enzymes to the regulation of extracellular adenosine concentrations under different conditions.

Adenosine deaminase is a 36-kDa protein, the distribution of which has been extensively studied in the brain. Numerous reports describe a selective expression of ADA in discrete neuronal populations in rat and mouse brain, with the strongest immunoreactivity (Nagy *et al*. 1984, 1986, 1996; Nagy and Daddona 1985; Patel and Tudball 1986) and the highest activity of the enzymes (Phillips and Newsholme 1979; Geiger and Nagy 1986) found in neurones of the basal hypothalamus, which have widespread projections throughout the brain (Staines *et al*. 1987). A correlation has also been found between the regional distribution of ADA-immunoreactive neurones and adenosine uptake sites in the rat brain (Nagy *et al*. 1985), consistent with the need for adenosine to be taken up before it is deaminated.

Although ADA has largely been considered to be a cytosolic enzyme, there have been several reports of an extracellularly located ADA in a variety of tissues, including the brain (Franco *et al*. 1986). Two ADA anchoring proteins have been identified in the brain: CD26 and the A_1 adenosine receptors (for a review see Franco *et al*. 1997). Besides the main role of ectoADA in degrading extracellular

adenosine, more recent evidence points to an extra-enzymatic functional role of the enzyme. Catania *et al*. (1991) first demonstrate an extra-enzymatic effect of ectoADA upon influx of calcium and hydrolysis of polyphosphoinositides in cerebellar neurones. More recently it has been shown that ectoADA associated to A_1 adenosine receptors could act as a positive modulator for adenosine binding and signalling function (Circuela *et al*. 1996; Ruiz *et al*. 2000).

Selective and potent inhibitors of ADA, deoxycoformycin (DCF) and erythro-9-(2-hydroxy-3-nonyl)adenosine (EHNA), are shown to increase brain extracellular concentrations of adenosine measured *in vivo* under basal conditions (Ballarìn *et al*. 1991; Pazzagli *et al*. 1993, 1995; Sciotti and Van Wylen 1993) and under hypoxic or ischemic conditions (Zetterstrom *et al*. 1982; Phillis and O'Regan 1989; Phillis *et al*. 1991). On the other hand, variable effects of ADA inhibitors have been obtained *in vitro* on both basal and evoked adenosine release. The EHNA does not significantly modify either basal (Lloyd and Fredholm 1995; White 1996) or adenosine release evoked by electrical field stimulation (Lloyd and Fredholm 1995), or by application of NMDA (White 1996), although it greatly increases adenosine released after tissue energy depletion (Lloyd and Fredholm 1995). On the other hand, DCF increases both basal and NMDA-evoked adenosine release from cortical slices (White 1996), and NMDA-induced increase in striatal adenosine levels (Delaney and Geiger 1998). Endogenous ADA does not appear to be involved in the modulation of hippocampal synaptic transmission under either normoxic (Pak *et al*. 1994; Zhu and Krnjevic 1994) or hypoxic conditions (Zhu and Krnjevic 1994).

Inside the cell, adenosine could also be phosphorylated to AMP by AK, a cytosolic enzyme of 38–54 kDa. In comparison to ADA, less is known about the distribution of AK in the brain, however no marked differences in AK activity have been found in different rat brain regions (for a review see Geiger *et al*. 1997). The specific inhibitor of AK, 5-iodotubercidin (5-IT), appears to be more effective than ADA inhibitors at increasing extracellular adenosine levels in the brain (Sciotti and Van Wylen 1993; Pazzagli *et al*. 1995). The AK inhibition results in a consistent increase in basal adenosine tissue levels but not in NMDA-increased levels in the striatal region (Delaney and Geiger 1998). On the other hand, a significant increase in extracellular adenosine concentrations has been found *in vitro* both under basal conditions (Lloyd and Fredholm 1995; White 1996), after stimulation of NMDA or non-NMDA receptors (White 1996) and after electrical field stimulation (Lloyd and Fredholm 1995). Inhibition of AK reduces synaptic transmission in the hippocampus, suggesting a significant role of AK in regulating basal extracellular adenosine levels (Pak *et al*. 1994).

Different effects of ADA and AK inhibitors in various experimental conditions could be ascribed to their different affinity values (K_m values) for adenosine. In most preparations, K_m values for AK are between one or two orders of magnitude lower than those calculated for ADA, and in the rat whole brain K_m values are 2 μM for AK and in the range of 17–45 μM for ADA (Phillips and Newsholme 1979). At physiological concentrations of adenosine AK is saturated, and its activity is inhibited when adenosine concentrations rise (> 0.5 μM). Because endogenous adenosine levels are in the nanomolar range under normal physiological conditions, it is likely that the predominant pathway of adenosine metabolism is phosphorylation by AK, and that deamination becomes more important only at higher adenosine concentrations, such as under ischemic conditions.

Recently, the metabolic regulation of adenosine release from single neurones has been investigated with whole-cell recordings from CA1 pyramidal layer of rat hippocampal slices. Contrary to what has been previously obtained with *in vivo* administration or bath superfusion of brain slices with adenosine metabolism inhibitors, the intracellular injection of AK and ADA inhibitors in individual neurones does not modify extracellular adenosine concentrations. The possibility has been raised that multiple neurones must have their adenosine metabolism altered to allow modification of adenosine levels (Brundege and Dunwiddie 1998).

Another possible pathway for adenosine inactivation is the reversible reaction catalysed by SAH hydrolase, forming SAH from adenosine and L-homocysteine. L-Homocysteine availability limits this pathway and, because its levels are very low in the brain, SAH synthesis represents only a minor pathway of adenosine metabolism in brain tissue under basal conditions (Reddington and Pusch 1983).

Adenosine transport

Extracellular adenosine levels are regulated by a bi-directional nucleoside transport process, which may be of further importance as salvage of extracellular nucleosides for intracellular *de novo* synthesis of nucleotides. In the 1980s, functionally distinct nucleoside transporters were characterized and classified into two main categories: (i) equilibrative nucleoside transporters, which carry both purine and pyrimidine nucleosides across cell membranes in either direction and following their concentration gradient; (ii) concentrative nucleoside transporters that mediate the influx of nucleosides coupled under the force of transmembrane sodium gradient. In the CNS the equilibrative type of nuleoside transporter appears to dominate (for a review see Thorn and Jarvis 1996).

Equilibrative transporters are classified based on sensitivity to the selective inhibitor nitrobenzylthioinosine (NBMPR). The equilibrative-sensitive (es) transporter is inhibited by NBMPR at low nanomolar concentrations, whereas the equilibrative-insensitive (ei) transporter should be considered inhibitor resistant, as it is inhibited by

micromolar NBMPR concentrations (Fig. 1). Furthermore the ei nucleoside transporter has a lower affinity for substrates than the es carrier. Another inhibitor of equilibrative transporters is dipyridamole, which in many tissues inhibits the es transporter with greater potency than the ei transporter. However, in rat tissues both nucleoside transporters exhibit equal sensitivity to inhibition by dipyridamole (Thorn and Jarvis 1996). Functional studies indicate the presence of both es and ei transport activity in rat brain (Lee and Jarvis 1988), and recently, both transporters have been cloned from rat (rENT1, rENT2) (Yao *et al*. 1997) and human (hENT1, hENT2) tissues (Griffiths *et al*. 1998). A wide distribution of mRNA throughout the rat brain has been detected for both transporters with a strong hybridization signal in the hippocampus, cerebellum, cortex and striatum. The presence of mRNA for both rENT1 and rENT2 has been found in several cell types, including neurones, astrocytes, vascular smooth cells and choroid plexus epithelial cells (Anderson *et al*. 1999a,b). These studies also indicate that rENT2 (ei) may be the predominant equilibrative transporter in the rat brain. This is in agreement with functional studies finding higher levels of ei transporter activity in rat cortical synaptosomes (Lee and Jarvis 1988).

Five subclasses of concentrative nucleoside transporters have been identified with different properties (for a review see Geiger *et al*. 1997). One of these is selectively inhibited by low nanomolar concentrations of NBMPR, whereas no selective inhibitors have been identified for the other classes. The cDNAs of two of these Na^+-dependent transporters have been cloned and detected in the rat brain, with a specific localization in posterior hypothalamus, superior colliculus, brainstem, striatum, hippocampus, cerebellum and cortex (Anderson *et al*. 1996).

Since equilibrative nucleoside transporters are bi-directional, not only may they facilitate the movement of adenosine into cells when its extracellular concentration exceeds its intracellular concentration, but they should also mediate the efflux of adenosine from cells when its intracellular concentration is increased. In situations in which the Na^+ electrochemical gradient is reversed, the concentrative nucleoside transporters may also provide a means of releasing adenosine from cells. The involvement of bi-directional transporters in the *in vivo* and *in vitro* release of adenosine evoked by several stimuli has been studied utilizing adenosine transport blockers, which can both decrease (Jonzon and Fredholm 1985; Gu *et al*. 1995; Sweeney 1996) and increase (Phillis *et al*. 1989; Andiné *et al*. 1990; Park and Gidday 1990; Ballarìn *et al*. 1991; Pazzagli *et al*. 1993; Fredholm *et al*. 1994b; Gidday *et al*. 1996) extracellular adenosine concentrations, depending on the extent to which they block either uptake or efflux of adenosine from brain tissue. Most studies focus on the role of es nucleoside transporters, because the selective inhibitor NBMPR has been used. On the other hand, the lack of selective inhibitors for ei transporters and Na^+/nucleoside transporters has delayed progress in determining the involvement of these systems in the regulation of extracellular levels and actions of endogenous adenosine.

Characteristics of adenosine release in response to different stimuli

The presence of adenosine in the CNS and the regional distribution of A_1 and A_{2A} adenosine receptors have convinced researchers that adenosine is an intercellular mediator with specific roles in the brain (Impagnatiello *et al*. 2000). In light of these considerations, the study of the release mechanism of adenosine, in comparison with those of classical neurotransmitters, has become very important to the understanding of its cellular origin and roles.

As shown in Tables 1 and 2, adenosine can be released in response to such stimuli as high K^+, electrical stimulation, glutamate receptor agonists, hypoxia, hypoglycemia and ischemia. A wide variety of *in vitro* brain preparations have been used to study the release of adenosine, often leading to different release characteristics and thus to different conclusions about the origin of released adenosine. The use of synaptosomal preparations allows the study of the presynaptic origin of adenosine in response to different stimuli, whereas the brain-slice technique, especially in the hippocampal region, offers the advantage of maintenance of synaptic connections between neurones. The use of highly purified cell cultures permits the study of the release of adenosine from different cell types, i.e. neuronal or glial cells. The main advantage to the use of *in vitro* methods is the possibility of a better-defined application of adenosine-releasing stimuli, including drugs and electrical stimulation. Furthermore, easier modulation of the evoked release can be obtained *in vitro* with the application of inhibitors of adenosine metabolism and transport, or changing composition of the extracellular fluid (i.e. deprivation of Ca^{2+} to study the Ca^{2+}-dependence). As shown by comparing Tables 1 and 2, a more detailed characterization of adenosine release has been obtained with *in vitro* than with *in vivo* approaches. On the other hand, *in vivo* techniques provide the important advantage of having an integrated neuronal system and a measure of extracellular adenosine concentrations (see Estimation of extracellular adenosine concentrations by *in vivo* and *in vitro* methodologies).

Depolarization of neuronal tissue obtained with the application of high KCl concentrations (80–120 mM and 30–60 mM for *in vivo* and *in vitro* studies, respectively) has been shown to release adenosine from various brain regions. An increase of between two- and four-fold in adenosine release has been observed both *in vivo* using the microdialysis technique (Ballarìn *et al*. 1987; Chen *et al*. 1992; Pazzagli *et al*. 1993, 1994), and *in vitro* using brain slices

Table 1 *In vivo* adenosine-releasing stimuli

Stimulus	Brain region*	Methodology	Adenosine increase	Characteristics of evoked adenosine release
KCl	Striatum[1,3,4,5], cortex[2] hippocampus[6], thalamus[7]	Microdialysis	2/4-fold	KCl-evoked release was reduced by TTX (40–50%)[1,2] and D-AP7 (80%)[1,2,3,6].
Electrical stimulation	Cortex[8], striatum[9]	Cortical cup[8] microdialysis[9]	10-fold[8] 1.5–5 fold[9]	
NMDA	Striatum[10,11,12]	Tissue levels/NMDA locally injected[10,11,12]	2/6-fold	NMDA-evoked release is mainly derived from extracellular nucleotide degradation (74%)[11]. Evoked release was increased by the inhibition of adenosine deaminase and adenosine kinase[11], was blocked by MK-801[10,12], does not require the intermediate formation of nitric oxide, but is reduced by free radical scavengers[12].
	Striatum[13], hippocampus[6]	Microdialysis[6,13]	1.5/3.5-fold	NMDA-evoked release is blocked by MK-80113 and 2-AP56.
Kainate	Striatum[12]	Tissue levels/kainate locally injected[12]	3.5/6-fold	Kainate-evoked release was reduced by CNQX (57%) and MK-801 (90%)[12].
	Striatum[9,14], hippocampus[15], thalamus[7]	Microdialysis[7,9,14,15]	2/6-fold	Kainate-evoked release was increased by 5-IT (100%)[14], by 8-SPT (40%)[9], and reduced by TTX (54%), CNQX (92%) and free radical scavengers[15].
Hypoxia	Striatum[5,16], cortex[17] thalamus[17,18]	Microdialysis[5,16,17,18]	2/3-fold[16,17,18] 10-fold[5]	Hypoxia-evoked release is completely derived from extracellular nucleotide degradation[18] and was increased by EHNA16.
	Cortex[19,20,21,22]	Cortical cup[19,20,21,22]	35-fold[19]	Hypoxia-evoked release was suppressed by nifedipine and felodipine[20], and enhanced by DCF21 and dipyridamole[22].
Anoxia	Striatum[5], cortex[19]	Microdialysis[5], cortical cup[19]	15-fold[19] 30-fold[5]	
Global ischemia	Striatum[23,24,25,27], cortex[28,29,30] hippocampus[26,27]	Microdialysis[23–29], cortical cup[30]	10/25-fold[23–26,28–30] 46-fold[27]	Ischemia-evoked release was increased by propentofylline (50%)[26], DCF (350%–1900%)[30], dipyridamole (200%)[28], NBMPR (400%)[29] and SPT (120%)[25], and reduced by D-APV (40%)[23].
Focal ischemia	Striatum[31,32], cortex[33,34]	Microdialysis[31,32,33], cortical cup[34]	8-fold[33] 15/30-fold[31,32,34]	

[1]Pazzagli *et al.* 1993, [2]1994, [3]1995; [4]Ballarìn *et al.* 1987; [5]Van Wylen *et al.* 1986; [6]Chen *et al.* 1992; [7]Dobolyi *et al.* 2000; [8]Sulakhe and Phillis 1975; [9]Sciotti *et al.* 1993; [10]Delaney and Geiger 1995, [11]1998; [12]Delaney *et al.* 1998; [13]Melani *et al.* 1999a; [14]Britton *et al.* 1999; [15]Carswell *et al.* 1997; [16]Zetterstrom *et al.* 1982; [17]Park *et al.* 1987; [18]Koos *et al.* 1997; [19]Phillis *et al.* 1987, [20]1988b,[21]a, [22]1989; [23]Hagberg *et al.* 1986, [24]1987; [25]Sciotti *et al.* 1992; [26]Andiné *et al.* 1990; [27]Dux *et al.* 1990; [28]Park and Gidday 1990; [29]Gidday *et al.* 1996; [30]Phillis *et al.* 1991; [31]Hillered *et al.* 1989; [32]Melani *et al.* 1999b; [33]Matsumoto *et al.* 1992; [34]Phillis *et al.* 1994. *Reported *in vivo* experiments are performed on the rat, except for the following references: 8, cat; 18, sheep; 27, gerbil; 17, 28 and 29, pig.

(Hollins and Stone 1980; Jonzon and Fredholm 1985; Hoehn and White 1989, 1990c) and synaptosomes (Daval and Barberis 1981; MacDonald and White 1985; Cahill *et al*. 1993). A higher increase in adenosine release (eight-fold) has been shown by utilizing cerebellar cell cultures (Philibert and Dutton 1989; Sweeney 1996). Most studies indicate that adenosine is released as such from cells in response to tissue depolarization (Daval and Barberis 1981; Hoehn and White 1990c; Sweeney 1996), with the exception of an *in vitro* study on cortical synaptosomes where KCl-evoked adenosine release is partially (35%) reduced by inhibition of ecto-5′-nucleotidase (MacDonald and White

1985). On the other hand, spontaneous adenosine release both from cortical slices and synaptosomes is consistently derived from extracellular degradation of released nucleotides (MacDonald and White 1985; Hoehn and White 1990c). Although Ca^{2+}-dependence of KCl-evoked adenosine release has been shown with *in vitro* preparations (Hollins and Stone 1980; Jonzon and Fredholm 1985; MacDonald and White 1985; Schousboe *et al*. 1989; Hoehn and White 1990c; Sweeney 1996), the delayed pattern of adenosine efflux after application of KCl suggests a release mechanism different from that of classical neurotransmitters (Hollins and Stone 1980; Daval and Barberis 1981). However, it should be taken into consideration that the velocity of *in vitro* superfusion of brain preparations greatly influences the diffusion of adenosine from the site of release. We have demonstrated that increasing the superfusion rate of hippocampal slices by 10-fold, shorten the pattern of the appearance of adenosine after application of an ischemia-like stimulus, and its release is correlated with the electrophysiological activity of the hippocampal slice (Latini *et al*. 1999). KCl-evoked adenosine release is partially consequent to neuronal propagated electrical activity because a 40–50% reduction in adenosine release has been observed both *in vivo* and *in vitro* in the presence of tetrodotoxin (TTX) (Hoehn and White 1990c; Pazzagli *et al*. 1993, 1994). Increased extracellular adenosine levels may also reflect an increase in the metabolic activity of depolarized cells (not only neurones, but also glial and vascular cells). *In vivo* experiments conducted in striatal, cortical and hippocampal regions have shown that the increase in the extracellular adenosine level brought about by K^+ depolarization, but not the spontaneous adenosine efflux, is mediated primarily by the release of excitatory amino acids and stimulation of NMDA receptors (Chen *et al*. 1992; Pazzagli *et al*. 1993, 1994). On the other hand, only 30% of KCl-evoked adenosine release obtained *in vitro* from cortical slices is reduced in the presence of NMDA receptor antagonists, whereas it is unaffected by non-NMDA receptor antagonists (Hoehn and White 1990c).

The application of electrical field stimulation to brain tissue, which, under controlled conditions of frequency and intensity of stimulation, represents a *quasi-physiological* condition of neuronal activation, increases the efflux of adenosine, both *in vivo* and *in vitro*. A 10-fold increase in the release of tritiated adenosine has been shown after *in vivo* electrical stimulation of cat cerebral cortex, suggesting a presynaptic release of adenosine from nerve terminals (Sulakhe and Phillis 1975). A detailed characterization of the electrically evoked adenosine release from different brain regions has been performed with *in vitro* preparations (Table 2). In response to this *quasi-physiological* stimulus, adenosine is mainly released as such from cells and is not derived from extracellular degradation of nucleotides (Lloyd *et al*. 1993; Pedata *et al*. 1993b; Cunha *et al*. 1996). The cellular source of released adenosine is represented by AMP dephosphorylation, whereas a contribution by SAH hydrolysis to adenosine extracellular accumulation has been excluded (Latini *et al*. 1995). Although nucleotides appear to be the principal source of adenosine, a significant modification in tissue nucleotide levels is not detectable in electrically stimulated hippocampal slices (Latini *et al*. 1995). Under conditions of normal oxygenation of brain tissue, oxidative phosphorylation can rapidly replace the energy compounds utilized for supporting increased neuronal activity. However, an increase in ATP consumption, well above the capacity for rapid ATP resynthesis, may selectively occur in a small compartment of the neuronal cell, such as a dendrite, as a consequence of a limited number of stimuli. The focal increase of intracellular adenosine concentration would then result in localized increased efflux into the extracellular space, via equilibrative transporters.

Release of both radioactive and non-labelled endogenous evoked adenosine from electrically stimulated brain slices is considered to be mainly derived from the activation of presynaptic nerve terminals, as a 70–100% TTX-sensitivity and a partial (50–70%) or complete Ca^{2+} dependence have been described (see Table 2). In particular, direct involvement of both N- and P-type calcium channels in the adenosine release mechanism triggered by electrical stimulation has been demonstrated by the use of selective calcium channels blockers: ω-conotoxin GVIA and ω-agatoxin IVA (Latini *et al*. 1997). Despite these strong observations concerning a neuronal origin of adenosine release, an exocytotic mechanism has not been demonstrated, leaving the nature of the Ca^{2+}-dependent excitation secretion coupling for adenosine unclear. An involvement of adenosine transporters in the electrically evoked adenosine release has been suggested by the reduction of evoked release obtained with NBMPR (Fredholm *et al*. 1994b; Cunha *et al*. 1996). Although nucleoside transporters function in a bi-directional way following adenosine concentration, the increase of electrically evoked adenosine release obtained with propentofylline and dipyridamole, suggests that their effect may be less on efflux than on adenosine uptake (Newby 1986).

In the hippocampal region, electrically evoked adenosine release is partly mediated by the release of excitatory amino acids that act at both NMDA and non-NMDA receptors (Pedata *et al*. 1991). The involvement of amino acid receptor activation on the release of adenosine induced by depolarizing stimuli is also confirmed by observations that receptor stimulation with glutamate, NMDA, kainate and quisqualate directly induces the release of adenosine from several brain regions both *in vivo* and *in vitro*. A two or six-fold increase in adenosine striatal tissue levels has been observed after *in vivo* NMDA and kainate brain microinjection, and a potentiation of this effect has been observed with inhibitors of adenosine metabolism and transport

Table 2 *In vitro* adenosine-releasing stimuli

Stimulus	Brain region	Methodology	Adenosine increase	Characteristics of evoked adenosine release
KCl	Cortex[1,3], hippocampus[2], cerebellum[4]	Brain slices[1,2] synaptosomes[3], cell culture[4] preloaded with [^{3}H]adenine or [^{3}H]adenosine	1.5/2-fold increase in total tritium[1–3] or [^{3}H]adenosine[4] release	Evoked release is partially (50–80%)[1,4] or completely[2] Ca^{2+}-dependent. From synaptosomes adenosine is released as such[3].
	Cortex[5,6,7], cerebellum[8,9], spinal cord[10]	Brain slices[5,6], synaptosomes[7,10], cell culture[8,9]; endogenous adenosine	1.5/4-fold[5,6,7,10] and 7/8-fold[8,9]	From slices and cell culture evoked adenosine is released as such from cells[6,8]; from synaptosomes it originates in part from extracellular nucleotide degradation (35%)[7]. Evoked release is partially (50–70%)[6,8] or completely[7] Ca^{2+}-dependent, increased by Bay K 8644[9,10] and completely inhibited by ω-CgTx[10], partially TTX-sensitive (44%)[6], and partially reduced by APV or MK801 (30%)[6].
Electrical stimulation	Cortex[11,16,17], hippocampus[2,12–15,17–21], striatum[17]	Tissue samples[11], brain slices[2,12–21] Radioactive[2,11–17] and endogenous adenosine[12–14,17–21]	5 min (10 Hz, 30 mA/cm^2, 5 ms): 5/10-fold[12–14,17–21] Radioactive evoked release is higher than endogenous.	Released adenosine is mainly derived from intracellular nucleotides[12,15,19] and not from SAH[26]. Evoked release is increased by propentofylline (150%), dipyridamole (300%)[13] and 5-IT (300%)[14] but not affected by EHNA[14]. Evoked release is TTX-sensitive (70–100%)[11,15–18], mostly (50–70%)[2,15–18] or completely[11] Ca^{2+}-dependent, and reduced by D-AP7 + DNQX (50%)[18]. Bay K 8644 increases (70%) and ω-CgTx or ω-Aga IVA inhibit (78%) evoked release[21]
Glutamate	Cortex[5,6,22,23], hippocampus[18], cerebellum[4]	Brain slices[5,6,18,23], synaptosomes[22], endogenous adenosine. Cell culture preloaded with [^{3}H]adenosine[4]	0.1 mM: 1.2/3-fold[4,22] 5 mM: 5-fold[5,6,18,23]	From cerebellar cell culture evoked release was mainly Ca^{2+}-dependent (80%)[4]. From synaptosomes evoked adenosine originates from extracellular nucleotide degradation, its release is Ca^{2+}-independent, TTX-insensitive, not reduced by MK 801 or DNQX but abolished by inhibition of glutamate uptake[22]. From cortical slices evoked adenosine is mainly released as such[6,23], is increased by dypiridamole (70%)[23], is Ca^{2+}- independent, only partially TTX-sensitive (21%), reduced by APV and MK 801 (50%), and almost completely abolished by MK801 + DNQX[6]. From hippocampal slices evoked release is not reduced either by D-AP7 or DNQX[18].

(Table 1) (Delaney and Geiger 1995, 1998). In accordance with these observations, extracellular adenosine concentrations measured in the striatum and hippocampus by microdialysis are increased after the local application of NMDA and kainate (Table 1). The correlation found between the behavioural effect of motor depression and the increase in extracellular striatal adenosine concentrations obtained after systemic administration of NMDA, provides strong evidence that released adenosine may modify neuronal functions (Melani *et al*. 1999a). The study of *in vitro* adenosine release induced by excitatory amino acid receptor stimulation has pointed out several differences in the characteristics of release in relation to different tissue preparations (Table 2). A Ca^{2+}-dependent release of radiolabelled adenosine has been observed from cultured cerebellar granule cells stimulated with glutamate (Schousboe *et al*. 1989). On the other hand, a Ca^{2+}-independent release of endogenous adenosine from brain slices and synaptosomes is induced by glutamate, NMDA, kainate and quisqualate (Hoehn and White 1990a,b,c). A partial dependence of glutamate-, NMDA- and kainate-evoked adenosine release from neuronal propagated activity has been observed in cortical slices (Hoehn and White 1990b,c), whereas glutamate-evoked release from brain synaptosomes appears to be TTX-insensitive (Hoehn and White 1990a). Adenosine is released as such from cortical slices after the application of glutamate and kainate (Hoehn and White 1990c; Craig and White 1993a), whereas glutamate-induced adenosine release from synaptosomes mainly originates from extracellular degradation of nucleotides (Hoehn and White 1990a). NMDA-induced adenosine release from cortical slices originates from extracellular dephosphorylation of AMP derived from increased intracellular cyclic AMP (Craig and White 1993a; Craig *et al*. 1994). Glutamate-evoked

Table 2. *continued*

Stimulus	Brain region	Methodology	Adenosine increase	Characteristics of evoked adenosine release
NMDA and non-NMDA	Cortex[23–27], hippocampus[18]	Brain slices, endogenous adenosine[18,23–27]	NMDA: 1.8[24]/2.5-fold[18] Kainate: 2.2-fold[24] Quisqualate: 1.5-fold[18,24]	NMDA-evoked adenosine release mainly originates from extracellular dephosphorylation of AMP[23], which derives from intracellular cyclic AMP[25]. Adenosine release does not require the intermediate formation of nitric oxide[27], is partially TTX-sensitive (40%)[24], partially Ca^{2+}-dependent (50%)[23] and completely antagonized by D-AP7[18], APV and MK801[24]. Kainate-evoked adenosine release is released as such from cells[23], is partially TTX-sensitive (20%), Ca^{2+}-independent and reduced by DNQX (30%)[24]. Quisqualate-evoked release is Ca^{2+}-independent[14] and reduced by DNQX (49%)[18,24]. DCF and 5-IT potentiate NMDA and non-NMDA evoked release[26].
Hypoxia	Hippocampus[28]	Brain slices, endogenous adenosine[28]	4-fold	Evoked release is increased by dipyridamole[28].
Ischemia	Hippocampus[12–14,19,20,29]	Brain slices, endogenous adenosine[12–14,19,20,29]	5 min: 6-fold[19,20,29] 10 min: 35-fold[19,29] 35 min: 13-fold[12–14]	Adenosine is released as such from cells[12] and is not derived from SAH[20]. Propentofylline (300%)[13], dipyridamole (40%)[13], 5-IT (120%)[14] and EHNA (140%)[14] increase evoked release. Adenosine outflow is partially TTX-sensitive (42%)[19], Ca^{2+}-independent[19], not significantly modified by D-AP7, D-AP7 + DNQX and D-AP7 + DNQX + DHK[19,29].

[1]Hollins and Stone 1980; [2]Jonzon and Fredholm 1985; [3]Daval and Barberis 1981; [4]Schousboe *et al.* 1989; [5]Hoehn and White 1989, [6]1990c; [7]MacDonald and White 1985; [8]Sweeney 1996; [9]Philibert and Dutton 1989; [10]Cahill *et al.* 1993; [11]Pull and McIlwain 1973; [12]Lloyd *et al.* 1993; [13]Fredholm *et al.* 1994b; [14]Lloyd and Fredholm 1995; [15]Cunha *et al.* 1996; [16]Pedata *et al.* 1988, [17]1990, [18]1991, [19]1993b; [20]Latini *et al.* 1995, [21]1997; [22]Hoehn and White 1990a; [23]Craig and White 1993a; [24]Hoehn and White 1990b; [25]Craig *et al.* 1994; [26]White 1996; [27]Craig and White 1993b; [28]Fowler 1993; [29]Pedata *et al.* 1993a.

release of adenosine from cortical synaptosomes is mediated by intracellular glutamate in that it is inhibited by high-affinity glutamate uptake blockers and not by NMDA and/or non-NMDA receptor activation (Hoehn and White 1990a). Similarly to that described in astrocytes (Magistretti *et al.* 1999), one may consider that in cortical neuronal terminals Na^+-dependent glutamate uptake results in activation of Na^+/K^+ ATPase, with Na^+ extrusion. ATP degradation could lead to adenosine formation and release into the extracellular space. Adenosine release evoked by glutamate in the hippocampus is not reduced in the presence of NMDA and non-NMDA receptor antagonists (Pedata *et al.* 1991), whereas adenosine release evoked by glutamate, NMDA, kainate and quisqualate from cortical slices is reduced or abolished in the presence of selective receptor antagonists (Hoehn and White 1990b,c).

Very recently it has been shown that the increase in the temperature of brain tissue, a situation in which an increased tissue metabolic rate occurs, represents a stimulus to release of adenosine from brain slices. The increase in temperature of the superfusing medium of rat hippocampal slices (from 32°C to 38–40°C) markedly inhibits excitatory synaptic transmission, and induces a temporally related increase in adenosine outflow from slices (1.5-fold) in the absence of significant changes in ATP tissue levels (Masino and Dunwiddie 1999; Masino *et al.* 2001).

Adenosine release occurs in response to an increase in energy demand, such as under conditions of neuronal activation or increased temperature in situations of impairment between energy demand and ATP resynthesis, as verified under hypoxia or ischemia, and a consistent increase in extracellular adenosine concentration is observed both by *in vitro* or *in vivo* approaches. Hypoxic or ischemic conditions can be reproduced *in vitro*, on brain slices or cell cultures by gassing the artificial CSF with nitrogen (95% N2/5% CO_2), and under ischemic conditions by subtracting oxygen and glucose from artificial CSF. Otherwise, a similar effect can be obtained with metabolic inhibitors, such as 2-deoxyglucose and oligomycin, which drastically reduce ATP tissue levels (Meghji *et al.* 1989). The application of

in vitro ischemia-like conditions on hippocampal slices, results in a six- and 30-fold increase after application of 5 or 10 min of ischemia, respectively (Table 2) (Pedata *et al*. 1993a; Latini *et al*. 1995). Several *in vivo* models of hypoxia and ischemia have been used to study adenosine release and to understand mechanisms of protection exerted by adenosine against brain damage. To reproduce *in vivo* hypoxic or anoxic conditions the animal is allowed to breathe a hypoxic gas mixture (8–5% oxygen in nitrogen and 100% nitrogen, respectively). Brain ischemia can be reproduced *in vivo* on the whole brain (global ischemia) or selectively on cortical and striatal regions (focal ischemia). The most common global ischemia models, which reproduce selective neuronal loss in the pyramidal layer of the hippocampus observed in the human brain after a heart attack or coronary artery bypass surgery, are obtained with: (i) bilateral carotid artery occlusion in the gerbil and (ii) four-vessel occlusion, with or without hypotension, in the rat. Animal models of focal ischemia, reproduced with the permanent or transient occlusion of the middle cerebral artery, are believed to be the most pertinent in relation to human stroke. In order to monitor modifications of extracellular adenosine levels under these conditions, both the application of cortical cups on the surface of animal cortex or the insertion of a microdialysis probe in discrete brain regions, have been used. After application of hypoxia, higher increases in extracellular adenosine concentrations are detected with the cortical cup technique (35-fold) (Phillis *et al*. 1987) in comparison with that obtained with microdialysis (3 or 10-fold) (Zetterstrom *et al*. 1982; Van Wylen *et al*. 1986). This difference may largely be a result of the fact that the magnitude of the increases were evaluated on the basis of different basal levels of adenosine detected from the normoxic brain: 1–2 μM by microdialysis and 30–50 nM by the cortical cup technique. Global and focal brain ischemia also induces a very high increase (10- or 30-fold) in the extracellular concentration of adenosine in several brain regions (Table 1).

The cellular source of released adenosine under hypoxic/ischemic conditions is represented by intracellular nucleotide dephosphorylation, with the exclusion of a contribution of SAH hydrolysis (Latini *et al*. 1995). However, in contrast to normoxic conditions, the decrease in tissue oxygen supply results in a consistent modification of tissue nucleotide levels, with a significant decrease in both ATP levels and tissue energy charge and a significant increase in AMP levels (Latini *et al*. 1995). The intracellular increase in adenosine concentration results in an efflux of adenosine into the extracellular space through nucleoside transporters, whereas a significant contribution to increased adenosine by extracellular nucleotide dephosphorylation has been generally excluded (Meghji *et al*. 1989; Lloyd *et al*. 1993) with the exception of fetal sheep brain submitted to hypoxia (Koos *et al*. 1997). The study of the involvement of nucleoside transporters in ischemia-induced adenosine release shows both a decrease (Phillis *et al*. 1988b; Meghji *et al*. 1989) and an increase (Phillis *et al*. 1989; Andiné *et al*. 1990; Park and Gidday 1990; Fredholm *et al*. 1994b; Gidday *et al*. 1996) in extracellular adenosine levels by using nucleoside transporters inhibitors, thus confirming the bi-directional nature of the transporter. Furthermore, transporter inhibitors may have a different effect on different cell types; for example, when dipyridamole is used *in vivo* it does not cross the blood–brain barrier and could therefore have access only to vascular endothelial cells, inhibiting nucleoside efflux or uptake at this level. On the other hand, when it is used *in vitro* on cell cultures or brain slices, it may also inhibit adenosine uptake or efflux from glial and neuronal cells.

Inhibition of intracellular adenosine metabolism with inhibitors of ADA (EHNA and DCF) and AK (5-IT) results in a considerable increase in adenosine release elicited by hypoxia or ischemia, both in *in vitro* and *in vivo* models (Phillis *et al*. 1988a, 1991; Lloyd and Fredholm 1995). Investigations on the relative importance of these enzymes in regulating extracellular adenosine concentrations under *in vitro* ischemic conditions show that ADA plays a much greater role than AK (Lloyd and Fredholm 1995). Several authors, utilizing *in vivo* models of brain ischemia, have demonstrated a protective effect of inhibitors of adenosine metabolism and transport against ischemic brain damage, confirming the important neuroprotective action of adenosine against brain damage (Phillis and O'Regan 1989; Dux *et al*. 1990; Miller *et al*. 1996; Jiang *et al*. 1997).

Adenosine release induced by hypoxic or ischemic stimuli has very different characteristics in respect to adenosine release induced by depolarizing stimuli applied under normoxic conditions. A detailed characterization has been performed by comparing adenosine outflow evoked by *in vitro* ischemia-like conditions applied on hippocampal slices, with adenosine release induced by *quasi-physiological* electrical stimulation (Table 2) (Pedata *et al*. 1988, 1990, 1991, 1993a,b; Latini *et al*. 1995, 1997). *In vitro* ischemia-induced outflow is only partially reduced in the presence of TTX, is completely Ca^{2+} independent and is not mediated by excitatory amino acid receptor stimulation. On the other hand, as described above, adenosine release induced by electrical field stimulation is completely blocked in the presence of TTX, is Ca^{2+} dependent and is thus reduced by 60–70% in the absence of Ca^{2+} or in the presence of calcium channel blockers, and is partly consequent to the activation of NMDA and non-NMDA receptors by endogenous glutamate. On the basis of this characterization, even if the cellular source of adenosine is the same (AMP dephosphorylation), it was speculated that different release mechanisms could be involved in adenosine outflow elicited by the two experimental conditions. Furthermore, under hypoxic/ischemic conditions the increase in extracellular

adenosine levels could derive from different cell types (neurones, glial cells and endothelial cells), thus reflecting a general situation of energy imbalance and not a selective release from neurones. Another important difference is that under ischemic conditions, outflow of adenosine from a neuronal cell may occur anywhere along the cell membrane, and not in specific regions, such as dendrites when they are activated by a depolarizing stimulus.

Does an adenosynergic system exist in the CNS?

As described in the previous paragraphs, the study of the characteristics of adenosine release in relation to different stimuli and in different brain preparations has led to the recognition of different modalities of adenosine production and release under physiological and pathological conditions. At the same time, in the attempt to demonstrate the existence of a purinergic neuronal system, several kinds of investigations have been performed concerning the neuroanatomical origin of adenosine, which interacts with adenosine receptors and the modification of its extracellular concentration in response to different stimuli.

One of the first criteria studied was the presence of adenosine or its synthetic and degradative enzymes in neurones. Immunocytochemical techniques reveal a specific adenosine staining confined to neuronal cells (mainly over the cell body) with a heterogeneous distribution in discrete brain regions: pyramidal layer and dentate gyrus of the hippocampus, thalamic nuclei, cerebral cortex, corpus striatum and cerebellum (Braas *et al*. 1986). The distribution in the brain of specific purinergic markers, such as ADA, adenosine uptake sites, adenosine receptors, etc., and the possible coexpression of these markers in the same brain regions has been investigated by several authors in an attempt to demonstrate a purinergic system. A correlation has been observed in the regional distribution of neurones containing ADA and the bi-directional adenosine transporter, whereas a correlation between adenosine receptors and ADA or transport sites is lacking (for a review see Nagy *et al*. 1990).

Another criterion studied in the purinergic system was the demonstration of regional variations in the release of adenosine, reflecting a non-uniform distribution of neurones from which purine efflux originates. In accordance, it has been shown that specific neurotoxic lesions of the cholinergic and noradrenergic, but not serotoninergic fibers, projecting to the cortex are followed by a marked decrease in the electrically evoked release of radiolabelled purines from rat cortical slices (Pedata *et al*. 1989). Adenosine release from the striatal region is not affected by dopamine denervation (Ballarìn *et al*. 1987), but was strongly reduced by intrastriatal kainic acid injection (Wojcik and Neff 1983). A regional difference in the electrically evoked release of both radioactive and endogenous adenosine has been shown from hippocampal, cortical and striatal slices, with higher levels in the hippocampus and lower in the striatum (Pedata *et al*. 1990).

A specific neuronal origin of adenosine has been shown both by the use of purified neuronal cultures (Philibert and Dutton 1989; Schousboe *et al*. 1989; Sweeney 1996) and with electrophysiological approaches. Strong evidence of an activation-coupled release of adenosine from presynaptic sites in the brain has been provided in a combined *in vivo/in vitro* study, where controlled stimulation of the perforant path in the hippocampal slice of rats injected *in vivo* with [^{3}H]adenosine into the entorhinal cortex results in a significant increase of tritium efflux (Lee *et al*. 1982). Furthermore, in the hippocampal region a release of endogenous adenosine has been more directly demonstrated from GABAergic interneurones, after the activation of NMDA receptors, providing the first evidence that adenosine, selectively released by neuronal cells, can inhibit the release of glutamate from excitatory synapses (Manzoni *et al*. 1994). In a very elegant study, utilizing the whole-cell patch-clamp technique on hippocampal slices, it has been shown for the first time that a single pyramidal neurone is able to release enough adenosine in the extracellular space to inhibit synaptic activity on its own. In this sense adenosine can be considered a retrograde synaptic messenger released from hippocampal pyramidal neurones, which regulates the strength of its excitatory synaptic inputs (Brundege and Dunwiddie 1996).

The evidence reported above does not convincingly prove that adenosine is released from a restricted set of purinergic neurones, and a more widespread presence of adenosine throughout the CNS is now accepted. It is generally assumed that adenosine is released as such from cells, as shown in most studies, and that the mechanism most commonly implicated in adenosine release is the intracellular AMP dephosphorylation and efflux through bi-directional nucleoside transporters. However, evidence described in this and previous sections still does not exclude that an excitation-coupled response of neuronal cells is also responsible for adenosine release. Many studies demonstrate that adenosine is released per se from neuronal terminals, and in many preparations its release is entirely or greatly Ca^{2+}-dependent. The possibility cannot be excluded that adenosine formed intracellularly from AMP is stored in synaptic vesicles and is released under physiological stimuli in an excitation-secretion coupled mechanism from neuronal cells. The presence of adenosine in vesicles, to our knowledge, has not been investigated.

Estimation of extracellular adenosine concentrations by *in vivo* and *in vitro* methodologies

Regardless of which mechanisms result in the presence of adenosine in the synaptic cleft, one of the main issues of

research in the adenosine field is the estimation of adenosine concentrations. Adenosine acts via different receptors, the affinity of which ranges from nM to μM (A_1, 3–30 nM; A_{2A}, 1–20 nM; A_{2B}, 5–20 μM; A_3, >1 μM; Fredholm *et al*. 1994a). Keeping in mind the limitation of extrapolating actual *in vivo* affinity and/or efficacy from values calculated in *in vitro* binding studies, knowledge of actual adenosine concentrations under physiological or pathological conditions may help to suggest which receptor subtypes are preferentially activated. Furthermore, this knowledge should be useful in designing appropriate concentrations of adenosine agonists or antagonists, which might be tested as potential therapeutic drugs in *in vivo* experiments.

Direct measurements of tissue adenosine levels in different brain regions have been performed with several methods, in which precautions have been taken to minimize the post-mortem breakdown of ATP and to rapidly inactivate enzymes responsible for production and metabolism of adenosine. These methods include: immersion of whole animals or decapitated heads into liquid nitrogen (Kluge *et al*. 1977), freezing brains *in situ* using liquid nitrogen (Nordström *et al*. 1977; Rehncrona *et al*. 1978; Schrader *et al*. 1980), the freeze–blow technique (Winn *et al*. 1979, 1981) and high-energy focused microwave irradiation (Delaney and Geiger 1996). In general, *in situ* techniques are considered relatively slow methods of freezing brain tissue in comparison with the freeze–blow technique and microwave irradiation. Similar adenosine levels were found by the different methodologies under control conditions (1–20 nmol/g of tissue). However, although the *in situ* technique did not show an increase of adenosine levels during hypoxia (Rehncrona *et al*. 1978), a consistent adenosine increase was shown by the freeze–blow technique (Winn *et al*. 1981). The more recently developed method of microwave irradiation, besides the very rapid inactivation of enzymes, offers the further advantage of a simultaneous analysis of endogenous adenosine levels in multiple brain regions. By this method it was found that adenosine levels range from 1 nmol/g tissue in the cerebral cortex to 17 nmol/g tissue in cerebellum, whereas similar values were found in striatum (4.5 nmol/g tissue) and hippocampus (4.8 nmol/g tissue) (Delaney and Geiger 1996).

This kind of measurement precludes the precise localization of endogenous adenosine, intracellular or extracellular, and also the understanding of its cellular origin. Therefore, other experimental approaches have been used to determine adenosine concentration in the extracellular brain fluid and also to study their variation in response to physiological or pathological stimuli.

A simple method, which allows the estimation of cerebral adenosine concentrations from unanaesthetized animals, is the collection of cerebrospinal fluid from the cisterna magna by a chronically implanted cannulae. This method, first described by Kiser (1982) and then modified by Barraco *et al*. (1991), made repeated CSF sampling possible with minimal manipulation of the animal and without anaesthesia. This is of particular importance for evaluating the *in vivo* effect of hypoxic conditions on adenosine concentrations, uncomplicated by hypotension associated with anaesthesia. Adenosine concentrations in the range of 40–90 nM were calculated in the CSF by this technique (Park *et al*. 1987; Barraco *et al*. 1991; Dobolyi *et al*. 1998).

Another method used to assay cerebral adenosine concentrations is the cerebral cortical cup technique, originally developed for studies on acetylcholine release from the rat cerebral cortex (Mitchell 1963; Beani *et al*. 1968; Phillis 1968; Pepeu 1973). Because this method damages tissue less than the microdialysis technique, it is likely to give an estimation of adenosine concentrations without being prejudiced by tissue metabolic alterations. By this technique, adenosine concentrations in the range of 30–50 nM were calculated in the perfusate of the rat cerebral cortex under basal conditions (Phillis *et al*. 1987). The main disadvantages of this technique are that it only permits estimation of adenosine concentration in superficial brain regions such as the cerebral cortex and cerebellar cortices, and that these estimations are limited to efflux of adenosine from the uppermost layers of the tissue.

More recently the microdialysis technique (Ungerstedt 1984) has been applied to the measurement of extracellular adenosine levels *in vivo*, allowing determinations in several brain regions (cortex, striatum, hippocampus and thalamus). With this approach, several possible artefacts may prejudice the estimation of adenosine extracellular concentrations: first of all, an incomplete equilibration occurring between the artificial CSF inside the membrane and the surrounding CSF and secondly, the need to introduce a correction factor to calculate the absolute adenosine concentration in the extracellular fluid. Concerning the first possible artefact, if slow rates of perfusion are used, nearly 100% recovery is achieved (Van Wylen *et al*. 1986). Concerning the second possible artefact, the *in vitro* recovery of the fibre is usually used to calculate the actual concentration of adenosine in the dialysis fibre effluent (see Table 3). The percentage of recovery depends on several factors, such as the properties of the dialysis fibre, the rate of perfusion and the composition of the perfusion fluid. A criticism of the use of *in vitro* recovery to calculate adenosine or other transmitter concentrations, is that glial cells can cover the microdialysis membrane *in vivo* (Benveniste 1989). This factor is not considered when calculating the *in vitro* recovery of the fibre. However, the microdialysis operation stimulates a consistent glyosis starting 24 h after the operation. Thus this problem can be circumvented by restricting the experimental time to 24 h.

A further important point in microdialysis studies is that the implantation of the probe induces brain tissue damage and thus the adenosine concentration reflects an alteration in

cellular metabolism. As shown in Table 3, several authors have estimated the adenosine basal concentration using the microdialysis technique. In most experiments, adenosine efflux from the brain was measured in anaesthetized animals 2–4 h after the implantation of the microdialysis probe, when a stable level of adenosine concentration is reached. Adenosine concentrations of 1–2 μM have been found in the rat striatum, 0.85 μM in the hippocampus, 4.8 μM in the thalamus, 0.7 μM in the piglet cortex and 1 μM in the thalamus. When brain dialysate was collected 24 h after the probe implantation, a 20-fold lower level of extracellular adenosine was found in the rat striatum (Ballarìn *et al*. 1991; Pazzagli *et al*. 1993). Determinations made 24 h after probe implantation offer probably more reliable estimations of extracellular adenosine concentrations because they are either less or not related to the alteration of tissue metabolism caused by local tissue damage. Cellular metabolism has recovered from the stress of surgery, as indicated by the recovery of glucose metabolism, which is increased soon after surgery, and by cerebral blood flow normalization (Benveniste *et al*. 1987). Adenosine concentrations measured in the rat 24 h after microdialysis probe insertion and corrected for *in vitro* recovery of the microdialysis membrane are: 40–210 nM in the striatum (Ballarìn *et al*. 1991; Pazzagli *et al*. 1993, 1995; Melani *et al*. 1999b) and 120 nM in the cortex (Pazzagli *et al*. 1994). Adenosine levels in the striatum of aged rats are in the same range as those of young rats (72 nM; Pazzagli *et al*. 1995). These levels are close to those reported by the cortical cup technique. The problem of induction of tissue damage, which can prejudice the estimation of adenosine concentration by the microdialysis technique, can thus be circumvented by assay 24 h after probe implantation.

Another criticism of the microdialysis technique discussed is whether adenosine concentrations estimated by the microdialysis technique reflect adenosine concentrations active on receptors. We must consider that it takes from 5 to 20 min to collect microdialysis samples. These times are needed to reach a sample volume necessary to estimate adenosine concentration with the available and currently used assay methods such as HPLC. Such a long time may allow adenosine to diffuse, be taken-up and then deaminated or phosphorylated. Thus the estimated adenosine concentrations may not reflect the actual adenosine concentration acting on receptors after being released, but represent the equilibrium after diffusion, re-uptake and degradation instead. Consistently, it has been shown that inhibitors of adenosine metabolism, such as inhibitors of AK (5-IT) and ADA (EHNA and DCF), or inhibitors of the nucleoside transporter (dipiridamole, NBMPR and lidoflazine) do increase the extracellular basal adenosine concentration in microdialysis studies (Table 3).

Recently we have used an *in vitro* pharmaco-dynamic approach to calculate the concentration of endogenous adenosine acting at the receptor level (Latini *et al*. 1999). For this purpose, we recorded field excitatory postsynaptic potentials from the CA1 region of rat hippocampal slices (Corradetti *et al*. 1983) both under normoxic conditions and during an ischemia-like episode of 5 min duration. The ischemia-like episode induces a synaptic depression that is reversed by the selective A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). The pharmacological analysis has been performed using a relationship, recently proposed by Barlow (1995), which enables the calculation of concentrations of an agonist of known K_a and $[A_{50}]$ by measuring the effect of an antagonist of known K_b. This *in vitro* methodology enables an adenosine concentration at the receptor level to be measured with a time discrimination of 15 s, which is greater than that achieved with any biochemical approach where the sampling period may allow an estimation of adenosine concentration that likely represents the equilibrium reached in the CSF. Adenosine concentrations calculated by this approach are in the range of 180–240 nM under normoxic conditions, that is the same concentration range (120–200 nM) as that calculated by Dunwiddie and Diao (1994) in the same *in vitro* preparation. As a similar range (30–210 nM) of adenosine concentrations has also been estimated by biochemical approaches of the cortical cup technique or by microdialysis 24 h after probe implantation, it may be assumed that under normoxic conditions, the released adenosine soon equilibrates in the extracellular fluid and therefore adenosine extracellular concentrations estimated by microdialysis are indicative of those that tonically stimulate receptors.

Utilizing this pharmacological approach we have also provided the first calculation of adenosine concentrations acting at the receptor level during an ischemia-like episode in the CNS. In our analysis, modifications of the IC_{50} of the antagonist DPCPX as a function of time were calculated on the basis of synaptic depression observed in the absence and in the presence of DPCPX at different concentrations. IC_{50} values of DPCPX change as a function of time, according to the concentration of adenosine present at each time. By utilizing Barlow's relationship, we estimated that adenosine concentration changes as a function of time, reaching a maximal value of 30 μM 45 s after the reintroduction of oxygen and glucose, and rapidly declining thereafter. The close temporal correlation found between adenosine increase at the receptor level and depression of synaptic responses produced by the *in vitro* ischemic episode, confirms the previous observation that an increase in adenosine efflux from a hippocampal slice closely parallels in time the synaptic depression (Latini *et al*. 1998).

Extracellular adenosine concentrations of 20–40 μM have been measured by microdialysis after the induction of global ischemia in the rat and gerbil (Hagberg *et al*. 1987; Dux *et al*. 1990; Gidday *et al*. 1996). However, these values were

Table 3 Brain adenosine concentrations estimated in the extracellular fluid with microdialysis

Animal	Brain region	Time after probe implantation	Dialysate concentration (μM)	Estimated concentration (μM)	Modifications of basal concentration
Rat	Striatum	1.5 h[1]	0.05[1]	1.9[1]	Increased by EHNA (58%), IT (170%), EHNA + IT (350%) and EHNA + IT + 8-SPT (500%)[4].
		2 h[2,3]	0.4[2]	1–2[2]	
			0.12[3]	0.6[3]	
		0 h[5,6]	3	10	Increased by DCF (40%), DIP (73%) and DIP + NBMPR + lidoflazine (360%)[6].
		2 h[5,6]	0.2	1	
		24 h[6]	0.012	0.040	
		48 h[6]	0.025	0.083	
		0 h[7]	1.2	9.2	Increased by EHNA (260%), DIP (76%), IT (200%) and reduced by TTX (50%)[7,8].
		2 h[7]	0.3	2.31	
		24 h[7]	0.016	0.124	
		24 h[8]	0.015	0.067	
		24 h[9]	0.013	0.210	
	(aged rats)	24 h[8]	0.017	0.072	Increased by IT (200%) and reduced by D-AP7 (54%).
	Cortex	24 h[10]	0.038	0.120	Reduced by TTX (40%).
	Hippocampus	2 h[11,12]	0.12	0.85	
	Thalamus	1 h[13]	0.97	4.85	Reduced by TTX (50%).
Gerbil	Striatum	1.5 h[14]	0.07	0.52	
		8 h[14]	0.025	0.18	
	Hippocampus	1.5 h[14]	0.05	0.51	
		8 h[14]	0.015	0.15	
Piglet	Cortex	1 h[15]	0.14	0.68	Increased by DIP (90%)[16] and NBMPR (140%)[17].
	Thalamus	1 h[15]	0.21	1.03	

[1]Hagberg *et al.* 1987; [2]Zetterstrom *et al.* 1982; [3]Van Wylen *et al.* 1986; [4]Sciotti and Van Wylen 1993; [5]Ballarìn *et al.* 1987, [6]1991; [7]Pazzagli *et al.* 1993, [8]1995; [9]Melani *et al.* 1999b; [10]Pazzagli *et al.* 1994; [11]Chen *et al.* 1992; [12]Carswell *et al.* 1997; [13]Dobolyi *et al.* 2000; [14]Dux *et al.* 1990; [15]Park *et al.* 1987; [16]Park and Gidday 1990; [17]Gidday *et al.* 1996.

measured acutely after microdialysis probe insertion, when the basal adenosine extracellular concentration is 20 times higher than the concentrations found 24 h after surgery (as described above). Actually, lower adenosine concentrations with a peak value of 3 μM were measured 24 h after microdialysis probe implantation in the striatum during brain focal ischemia induced in the rat by medial cerebral artery occlusion (Melani *et al*. 1999b). In agreement with the obtained data, the increase in adenosine concentration under ischemia was lower in microdialysis experiments (14-fold) than in *in vitro* experiments (100–150-fold). From our results, it was therefore inferred that the calculated adenosine concentration of 30 μM during or soon after the ischemic stimulus, represents the concentration of adenosine acting on receptors before diffusion and equilibration in the extracellular fluid. Therefore the degree of receptor stimulation is greater than that extrapolated by the microdialysis adenosine measurement during ischemia.

Very recently another method has been developed to allow on-line measurement of extracellular adenosine in combination with simultaneous electrophysiological recordings. An enzyme-based sensor (Dale 1998) is used to measure adenosine released in the extracellular fluid during hypoxia in the CA1 region of rat hippocampal slices (Dale *et al*. 2000). A progressive increase in the adenosine concentration, closely related in time to a significant depression of synaptic responses, is observed, reaching a peak value of 5.6 μM and 9 μM after 5 or 10 min of hypoxia, respectively. Although this approach, in comparison to biochemical methods, gives a higher temporal resolution of measuring adenosine release during hypoxic conditions, it suffers from various limitations. The wide size of the sensor (500 μm width) precludes its insertion into the tissue without damaging cells, thus it can only be used to measure adenosine release at the slice surface. This could partially invalidate the analysis as superficial layers of slices (50–100 μm) consist of tissue that shows little or no electrophysiological activity and is characterized histologically by a gross disruption of cellular structure (Garthwaite *et al*. 1979). Furthermore, similarly to the

cortical cup or microdialysis technique, this method allows estimation of adenosine concentration after diffusion, uptake and metabolism, and thus does not estimate adenosine levels at the receptors. The exact time course of changes in adenosine concentration at the synapse could also be different from those recorded on the slice surface.

Conclusions

In conclusion, the majority of the evidence reported in this review indicates that adenosine released by physiological stimuli in a first phase originates per se from neurones, whereas later release is attributable to the degradation of extracellularly released nucleotides. Under conditions of increased metabolic activity and of a mismatch between energy supply and demand, such as in hypoxic/ischemic conditions, adenosine mainly derives per se from cells. The mechanism most commonly implicated in adenosine release involves bi-directional nucleoside transporters. However, the evidence of neuronal Ca^{2+}-dependent release of adenosine does not exclude the existence of an excitation-coupled release mechanism. The presence of adenosine in synaptic vesicles, to our knowledge, has not been investigated.

In *in vivo* and *in vitro* studies, adenosine is shown to be present extracellularly at concentrations that can stimulate both the higher affinity A_1 and A_{2A} receptors. Under conditions of hypoxia/ischemia, adenosine rises to concentrations that can also stimulate the lower affinity A_3 and A_{2B} receptors.

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