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Plasticity of purine release during cerebral ischemia: clinical implications?

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Abstract

Adenosine is a powerful modulator of neuronal function in the mammalian central nervous system. During a variety of insults to the brain, adenosine is released in large quantities and exerts a neuroprotective influence largely via the A_1 receptor, which inhibits glutamate release and neuronal activity. Using novel enzyme-based adenosine sensors, which allow high spatial and temporal resolution recordings of adenosine release in real time, we have investigated the release of adenosine during hypoxia/ischemia in the *in vitro* hippocampus. Our data reveal that during the early stages of hypoxia adenosine is likely released *per se* and not as a precursor such as cAMP or an adenine nucleotide. In addition, repeated hypoxia results in reduced production of extracellular adenosine and this may underlie the increased vulnerability of the mammalian brain to repetitive or secondary hypoxia/ischemia.

Keywords: adenosine - inosine - glutamate - hypoxia - ischemia - seizure - epilepsy - TBI - purines - purinoceptor - ATP - nucleotides - nucleosides

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Adenosine as an ubiquitous modulator of neuronal function

The purine nucleoside adenosine plays an important modulatory role in a wide variety of physiological processes such as the regulation of sleep [1] and pain [2], control of transmitter release [3], cardiac function [4] and spinal motor patterns [5].

Adenosine exerts its actions via four G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3) all of which have been cloned from a variety of mammalian species [6, 7]. The A_1 receptor has the widest tissue distribution and via G_i/G_o exerts the profound inhibitory influence of adenosine on neuronal activity. A_{2A} receptors have their highest expression in striatum with lower levels of expression elsewhere in the brain. A_{2A} and A_{2B} receptors (the mRNA of the latter are found at low levels in brain) are coupled to G_s (G_{olf} in striatum) and results in stimulation of cAMP production in a variety of tissues although stimulation of IP_3 production has also been reported [7]. The distribution of A_3 receptor mRNA is described as intermediate in brain, being found in the hippocampus and cerebellum, and the receptor can couple to both G_i and G_q [6, 7].

Adenosine is the primary full agonist at all four adenosine receptor subtypes. In CHO cells transfected with human adenosine receptors, the rank order of potency (EC_{50} value) for the effects of adenosine against each of the receptors was 54 nM (A_1), 56 nM (A_3), 960 nM (A_{2A}) and 11.3 μM (A_{2B}) [8], although the situation at native receptors *in vivo* may differ from that in recombinant systems. However, inosine, the metabolite of adenosine formed by the action of adenosine deaminase (EC 3.5.4.4; Fig. 1), is an agonist at A_3 receptors with an EC_{50} for rat mast cell degranulation of around 2 μM [9]. At recombinant human adenosine receptors, inosine was a much more potent agonist at A_3 receptors (EC_{50} = 81 nM) versus A_1 receptors (EC_{50} = 6.7 μM), whilst being ineffective against A_2 receptors [8]. That inosine is a potent agonist at A_3 receptors has implications for pathological conditions and will be discussed later.

Adenosine in CNS disease and injury

In addition to a housekeeping role for adenosine, adenosine and adenosine receptors have also been implicated in several chronic neurodegenerative disorders [10]. In Alzheimer's Disease, the consumption of caffeine (a broad spectrum adenosine receptor antagonist) may lessen the occurrence of Alzheimer's Disease [11]. In Parkinson's Disease, adenosine A_{2A} receptor antagonists such as KW-6002 (Istradefylline) reduce Parkinsonian symptoms in humans [12, 13] and adenosine A_1 receptor agonists and A_{2A} receptor antagonists produce beneficial effects in animal models of Huntington's disease [14].

Over and above this role for adenosine and adenosine receptors in chronic conditions, adenosine is released in large quantities during many acute insults to the mammalian, but more pertinently human, central nervous system (CNS) such as ischemia [15, 16], trauma [17, 18] and seizure activity [19]. Under these conditions adenosine is believed to exert an important protective influence. During pathologies which involve reduced cerebral blood flow, the release of adenosine is initiated by smaller reductions in blood flow than that required for the release of the potentially excitotoxic glutamate [20]. Thus adenosine initiates its retaliatory action ahead of the release of glutamate.

Interactions between the adenosine receptors

Adenosine exerts its protective actions largely via A_1 receptors through a combination of pre and postsynaptic effects on neuronal function. These actions, reduction of glutamate release (Fig. 2) via inhibition of presynaptic calcium channels, direct interference with the vesicle release machinery and potentially activation of presynaptic K^+ channels [3] coupled with activation of postsynaptic hyperpolarizing currents [21] and perhaps direct inhibition of the NMDA receptor [22] effectively shuts down neuronal activity, preventing spread of excitation throughout the brain and reducing metabolic demands. Manipulations which increase adenosine A_1 receptor activation (agonists, uptake or metabolism inhibitors) generally reduce injury to brain tissue whereas reduction in the activation of A_1 receptors (antagonists, promotion of metabolism, A_1 knockouts) worsen neuronal damage [23–25].

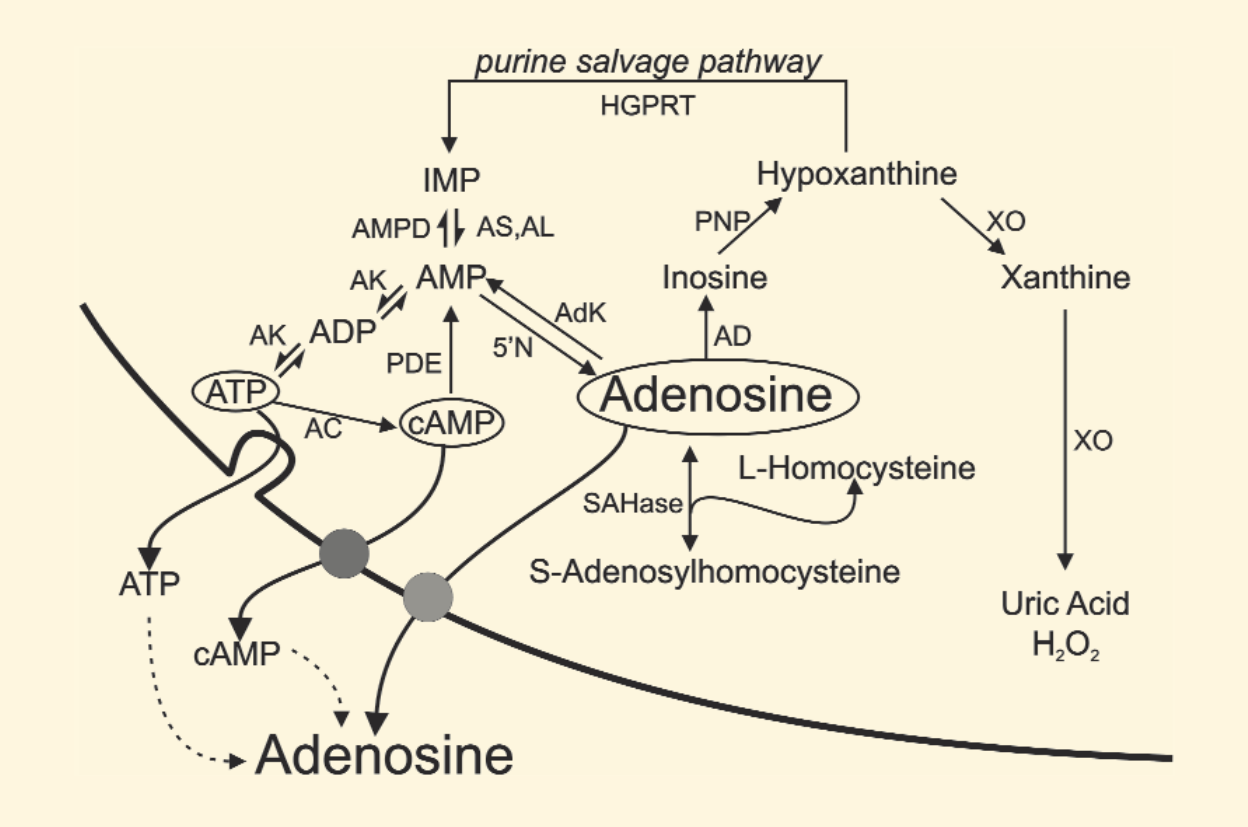

Fig. 1 Schematic representation of the production, release and metabolism of adenosine. Molecules circled can be released directly and, in the case of ATP and cAMP, converted via ectonucleotidases to adenosine [39]. Notice that the formation of xanthine, as well as transport of adenosine across the blood brain barrier into the systemic circulation, represent sources of adenosine loss from the central nervous system, whilst S-adenosyl-L-homocysteine represents an intracellular "sink" for adenosine. AD, adenosine deaminase (EC 3.5.4.4); PNP, purine nucleoside phosphorylase (EC 2.4.2.1); HGPRT, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); XO, xanthine oxidase (EC 1.1.3.22); SAHase, S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1); AdK, adenosine kinase (EC 2.7.1.20) 5'N, 5'-nucleotidase (EC 3.1.3.5); AK, adenylate kinase (EC 2.7.4.3); AC, adenylate cyclase (EC 4.6.1.1); PDE cAMP phosphodiesterases (EC 3.1.4.17); AMPD, AMP deaminase (EC 3.5.4.6); AS, adenylosuccinate synthase (EC 6.3.4.4); AL, adenylosuccinate lyase (EC 4.3.2.2); IMP, inosine monophosphate.

However, despite the beneficial effects of adenosine release, there can be deleterious consequences through activation of A_{2A} and A_3 receptors. This complex issue revolves around whether agonists or antagonists of these receptors (including those for A_1 receptors; [26]) are given acutely or chronically. Generally, antagonists of A_{2A} and A_3 receptors are protective when given acutely, whereas agonists are harmful, but the situation reverses with chronic pre-treatment of animals [27, 28]. Indeed, A_3 receptor knockout mice showed enhanced neurodegeneration and cognitive dysfunction in response to chronic intermittent CO-induced hypoxia, and both of these outcomes could be mimicked in wild type mice by repeated application of MRS 1523, an A_3 receptor antagonist [29]. Generation of A_{2A} knockout mice has added another level of complexity to this story: in adult A_{2A} knockouts, focal cerebral ischemia results in reduced cerebral infarction and improved behavioural outcome [30], whereas in neonatal mice subjected to hypoxia/ischemia, A_{2A} knockout resulted in increased brain injury and subsequent impaired locomotor activity in adulthood [31].

At least part of the complex interactions between the various adenosine receptors can be

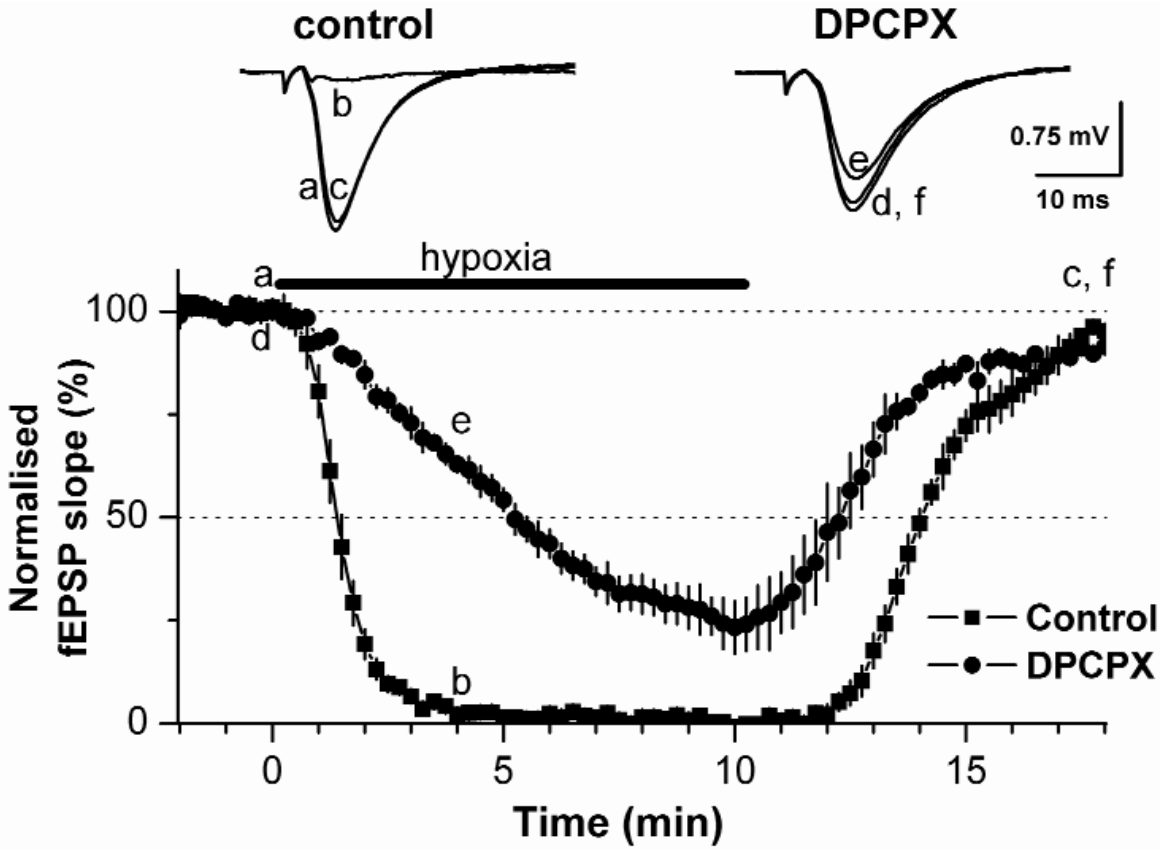

Fig. 2 Dominant role played by an accumulation of extracellular adenosine and activation of adenosine A_1 receptors in the hypoxic depression of excitatory synaptic transmission in area CA1 of the rat hippocampus. Under control conditions (squares; n = 3), excitatory synaptic transmission is rapidly depressed by hypoxia (black bar). In the presence of the A_1 antagonist DPCPX (circles, n = 3 additional slices; 100 nM) the hypoxic depression of the field excitatory postsynaptic potential (fEPSP) is greatly attenuated. Inset field excitatory postsynaptic potentials (fEPSPs) are taken at the times indicated before (a, d), during (b, e) and after (c, f) the period of hypoxia in the absence (a,b,c) or presence of DPCPX (d,e,f).

explained by findings that both A_{2A} and A_3 receptors can reduce the inhibitory actions of A_1 receptors [32, 33], and at least for the A_{2A} receptor, an inhibitory influence which increases with development and with increased density of A_{2A} receptors [34]. Furthermore, the potential for A_3 receptor activation by inosine, which also rises dramatically during hypoxia/ischemia, strengthens the likelihood that some form of desensitisation of A_1 receptors might occur during or after hypoxia/ischemia. Such desensitisation would promote increased glutamate release and neuronal activity, factors that would predispose to greater neuropathology. We have proposed [35] that this could potentially explain the dramatic recovery of excitatory synaptic transmission observed in the face of large post-hypoxic concentrations of extracellular adenosine (and inosine) (Fig. 3). The interactions between the adenosine receptors contributes, at least in part, to the "fine-tuning" role of adenosine in the mammalian CNS [6, 36].

Source of extracellular adenosine

In order for adenosine to exert its modulatory/protective role and for these various interactions to occur requires the release of adenosine into the extracellular space. It will come as a surprise that

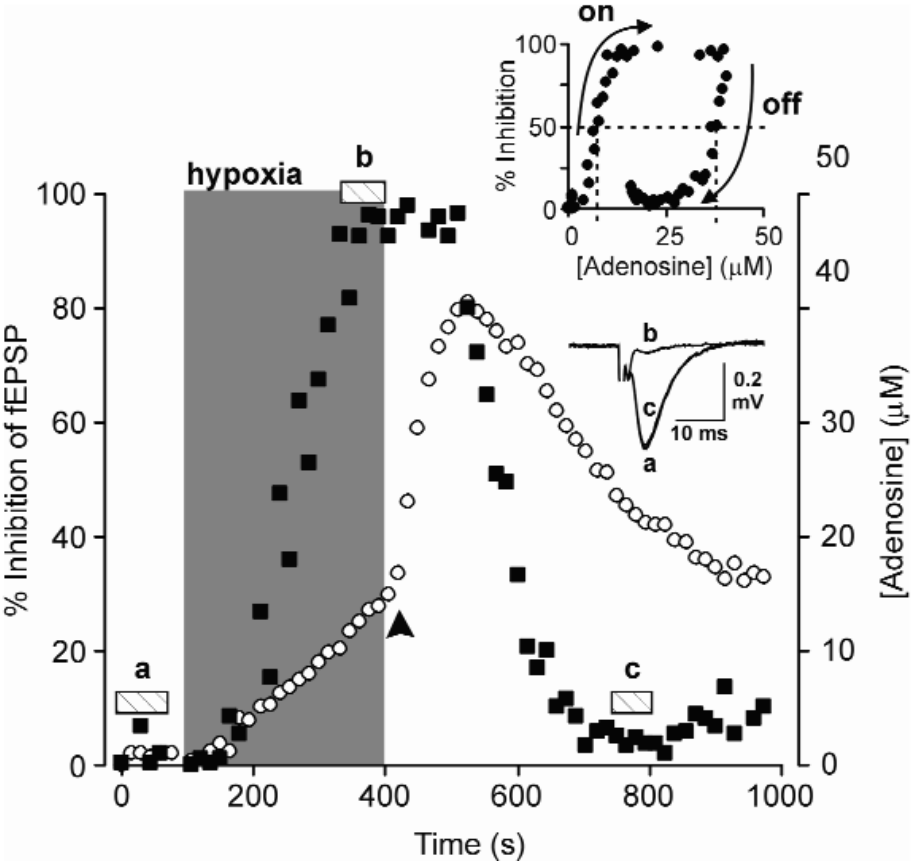

Fig. 3 The relationship between extracellular adenosine and the depression of excitatory synaptic transmission is not symmetrical. Experiment in which inhibition of synaptic transmission (squares) during hypoxia (grey box) and post-hypoxic recovery is compared with adenosine production measured from within area CA1 of the hippocampal slice using the MK-II adenosine sensor inserted into stratum radiatum (circles). Note full depression of fEPSP (inset fEPSP, b) at low concentrations of extracellular adenosine (e.g. 10 μM), whereas full recovery (inset fEPSP, c) is observed in the face of higher adenosine concentrations (e.g. 20 - 30 μM). The top inset plots inhibition of fEPSP versus adenosine concentration. Arrows depict direction of time during the experiment. Note shift in IC_{50} (dotted lines) from 7 μM during the depression phase to 38 μM during recovery. The lower inset shows the average of four consecutive fEPSPs taken at the three times indicated (a, b, c), at different levels of extracellular adenosine before, during and after the period of hypoxia, respectively. Arrowhead indicates the occurrence of the post-hypoxic purine efflux during which time the fEPSP paradoxically recovers. Taken from [35] with permission from Blackwell Publishing.

the mechanism by which extracellular adenosine levels are increased during pathologies is still unclear. The scheme in Fig. 1 alludes to the complexity of adenosine production, metabolism and release, but belies the fact that adenosine can be released by a whole host of brain cells including neurones, interneurones, glial and endothelial cells. Dunwiddie and Masino [37] explain that the release of adenosine reflects the brain's inability to synthesise enough ATP to meet demands imposed upon it, either because the substrates are absent (eg during hypoxia/ischemia) or because energy requirements have increased (eg during seizures). Thus, the potential cellular sources of adenosine and the ways in which ATP can be depleted imply that the mechanism by which adenosine is released may depend upon the nature of the stimulus.

Given that ATP can be released directly as a neurotransmitter [38], extracellular adenosine can also be formed via the actions of ectonucletotidases [39]. Indeed activity-dependent changes in synaptic responses attributable to adenosine A_1 receptor

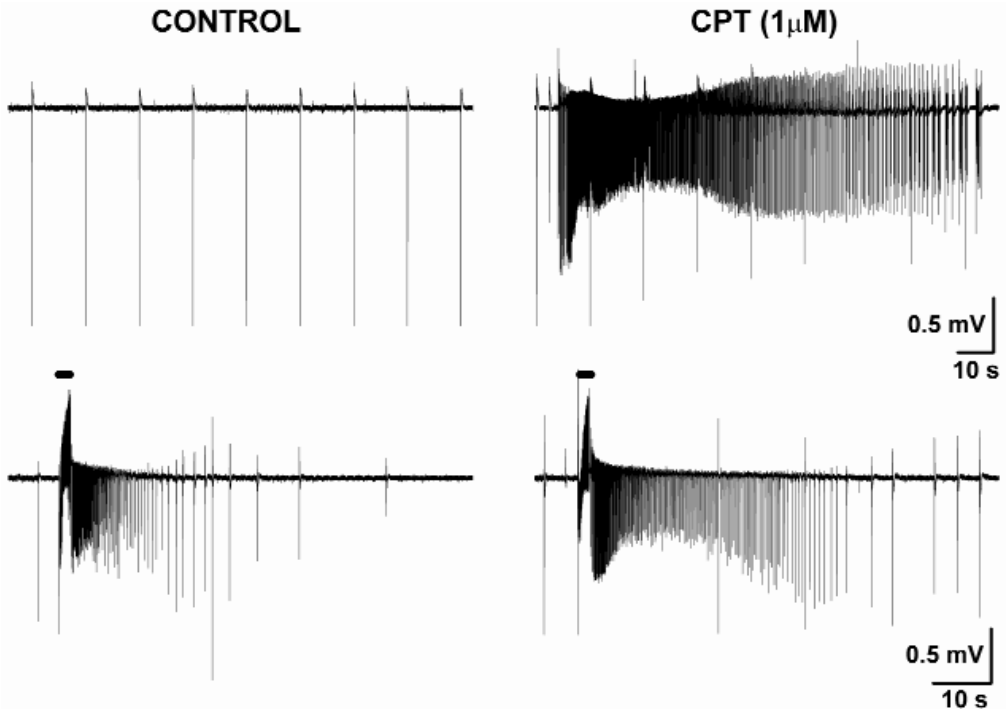

Fig. 4 Endogenous adenosine exerts a profound inhibitory influence on seizure activity in area CA1. Experiments performed in nominally Mg^{2+}-free aCSF in which the top panels represent basal electrical activity and the lower panels depict stimulus-evoked epileptiform activity (2 s, 60 Hz stimulation denoted by black bar). Periodic deflections, most clearly observed in the top left panel, reflect fEPSPs evoked at 15 s intervals to monitor basal synaptic transmission. Spontaneous seizure activity was seldom observed (20 % of slices) in Mg^{2+}-free aCSF (top left), but was greatly increased in frequency (> 70 % of slices) and intensity in slices perfused with the adenosine A_1 antagonist CPT (top right). Similarly, in a different slice, CPT caused a dramatic enhancement in the duration and intensity of evoked seizure (bottom right) compared to in the absence of CPT (bottom left).

activation have been observed in hippocampus [40–42], nucleus accumbens [43] and dorsal horn neurones that release ATP as a co-transmitter with GABA [44]. This might be especially pronounced during seizure activity, which in many models is exaggerated by adenosine A_1 receptor antagonism [45] (Fig. 4). However, we, as others, have little evidence that the extracellular release and metabolism of ATP contributes appreciably to the accumulation of extracellular adenosine, at least in the early stages of hypoxia *in vitro* (Fig. 5) or *in vivo* [46]. Indeed the release of adenosine during hypoxia is distinctly not Ca^{2+}-dependent as removal of extracellular Ca^{2+} actually increases ischemic [47] or hypoxic [48] adenosine release, obviating a need for Ca^{2+}-dependent transmitter release as a trigger for adenosine release. Furthermore, hypoxic/ischemic adenosine release is not affected by ionotropic glutamate receptor antagonism [35, 47] arguing against glutamate receptor activation as being a necessary stimulus for adenosine release. Similarly, blockers of volume-regulated anion channels do not affect the adenosine-dependent depression of excitatory synaptic transmission [49], suggesting that they too are not recruited during the early stages of hypoxia/ischemia, if at all, whereas they clearly act as a conduit for glutamate and aspartate release during metabolic stress [50–54].

More likely the breakdown of intracellular ATP will give rise initially to the release of adenosine *per se* via equilibrative nucleoside transporters. Nonetheless, under more severe conditions, ATP release and adenosine derived from ATP release has been observed *in vitro* [55, 56] and *in vivo* [57]. Indeed, an increase in the expression and activity of

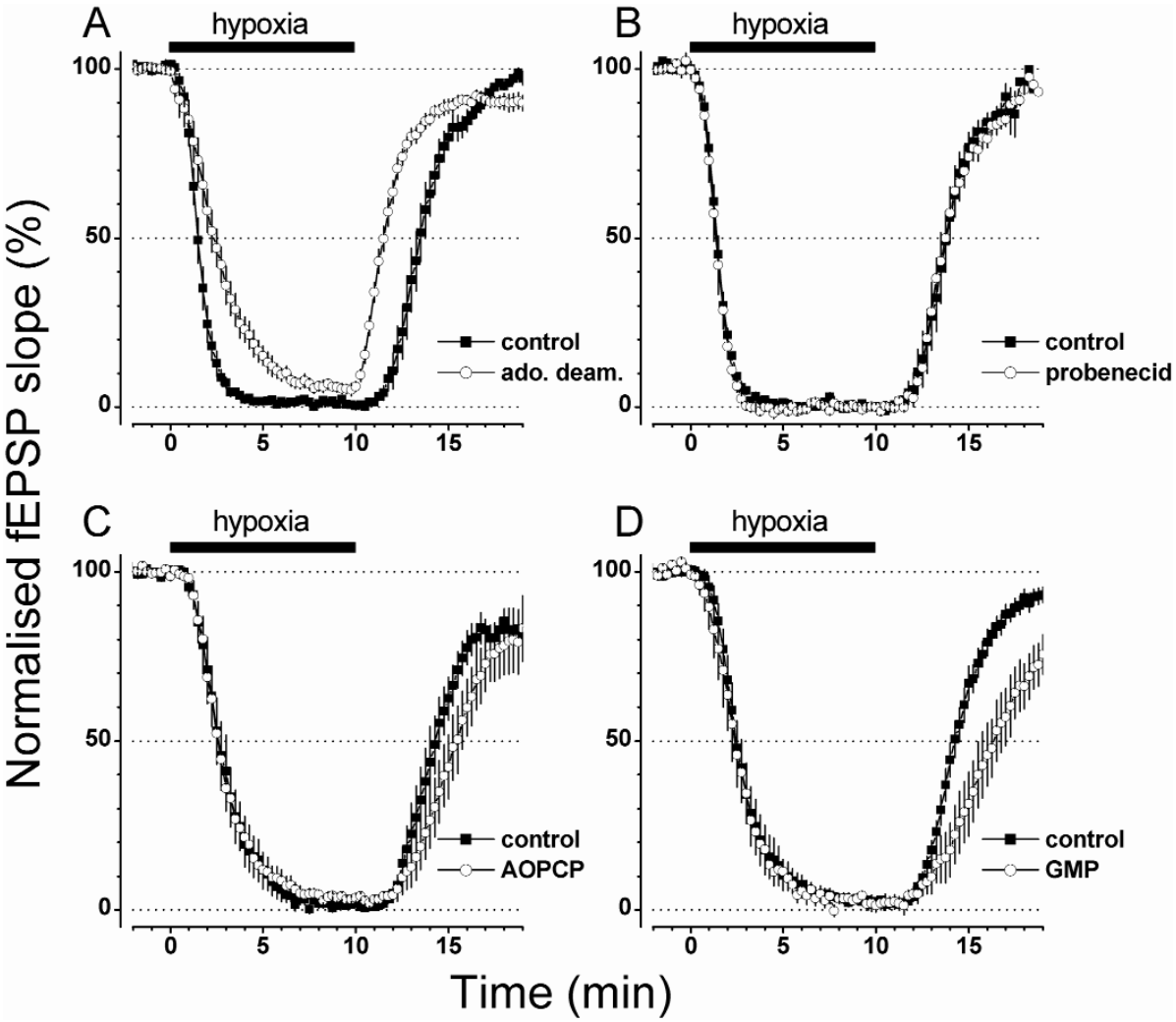

Fig. 5 Extracellular metabolism of adenine nucleotides does not appreciably contribute to the hypoxic depression of excitatory synaptic transmission in area CA1 of the rat hippocampus. In contrast to adenosine deaminase (A; circles; 2 U/ml; n = 6), which metabolises extracellular adenosine, slows the rate at which hypoxia depresses the fEPSP and allows faster recovery of the fEPSP compared to separate interleaved control experiments (squares; n = 6), neither the cAMP transport inhibitor probenecid (B; circles; 1 mM; n = 5) nor the ecto-5'-nucleotidase inhibitors α,β-methyleneadenosine 5'-diphosphate (C; AOPCP; circles; 300 μM; n = 11) or GMP (D; circles; 2 mM; n = 8) had any appreciable effect on the depression of the fEPSP induced by hypoxia when compared to interleaved controls (squares; n = 6, 7 and 11 for probenecid, AOPCP and GMP, respectively). Although probenecid had no effect on the post-hypoxic recovery of the fEPSP, GMP, and to a lesser extent AOPCP, did seem to retard recovery. At present we have no explanation for this observation although it is unlikely to be related to their ability to block ecto-5'-nucleotidase. GMP, AOPCP and probenecid depressed the basal fEPSP (25, 15 and 20 %, respectively). For GMP this may be related to the antagonism of glutamate receptors (eg [91]). A depression caused by AOPCP has been observed previously [92] and may be related to inhibition of A_{2A} receptor-mediated excitation [93]. The depression caused by probenecid, although not observed in another study in CA1 [94], could potentially be due to probenecid-stimulated increase in kynurenic acid, an endogenous glutamate receptor antagonist [95, 96]. A caveat to the use of ecto-nucleotidase inhibitors should be added: they may be less effective against very high concentrations of released nucleotides [97]. Small differences between A,B and C,D in the rate of depression of the fEPSP by hypoxia are due to different set ups being used for the experiments.

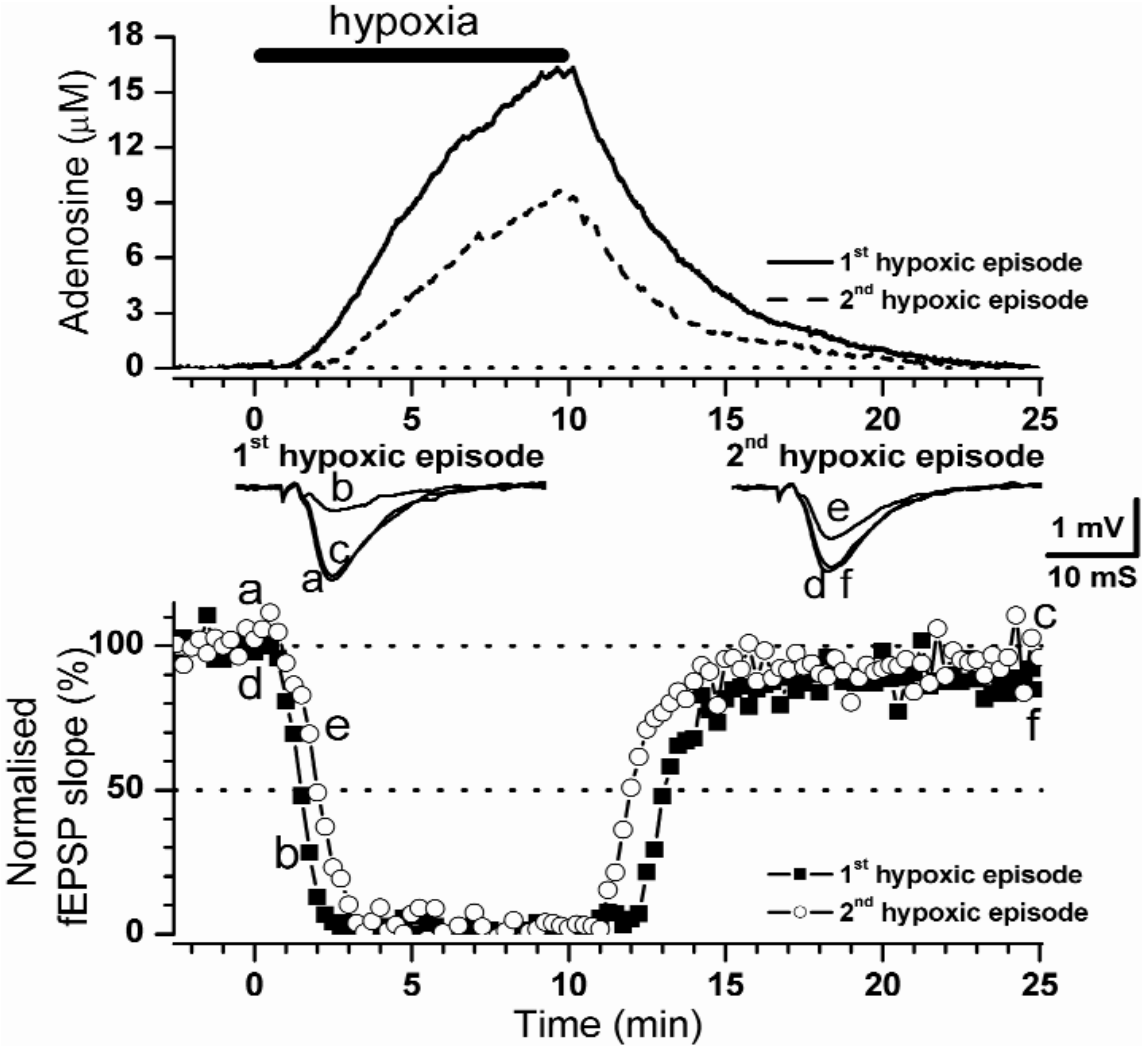

Fig. 6 Adenosine depletion caused by repeated hypoxia. Experiment in which the Mk-1 adenosine sensor [48, 98], placed on the surface of the slice, was used to measure extracellular adenosine during two sequential 10 min periods of hypoxia from the CA1 region of the hippocampal slice. Top panel shows the adenosine signal associated with the first (solid line) and second (broken line) periods of hypoxia. Notice the rapid release of adenosine and consequent depression of the fEPSP (bottom panel; squares) during the first period of hypoxia. During the second period of hypoxia, adenosine release is reduced (top panel, broken line) and the fEPSP takes longer to depress and recovers quicker (bottom panel, circles). Inset fEPSPs are taken at the times indicated and show the reduced depressant effects of hypoxia during the second (d, e) compared to the first (a, b,) periods of hypoxia. See also [35, 69].

several ectonucleotidases after cerebral ischemia *in vivo* [58] suggest that ATP may be released directly during severe ischemia and that its conversion to adenosine is an attempt to ameliorate the potentially damaging consequences of ATP (P2) receptor activation [59, 60].

Availability of adenosine during repeated or prolonged hypoxia/ischemia

Given the importance of the release of adenosine during hypoxia/ischemia, evidence is accumulating that the release of adenosine in response to physiological and pathological stimuli is not fixed, but labile and depends upon prior release of adenosine.

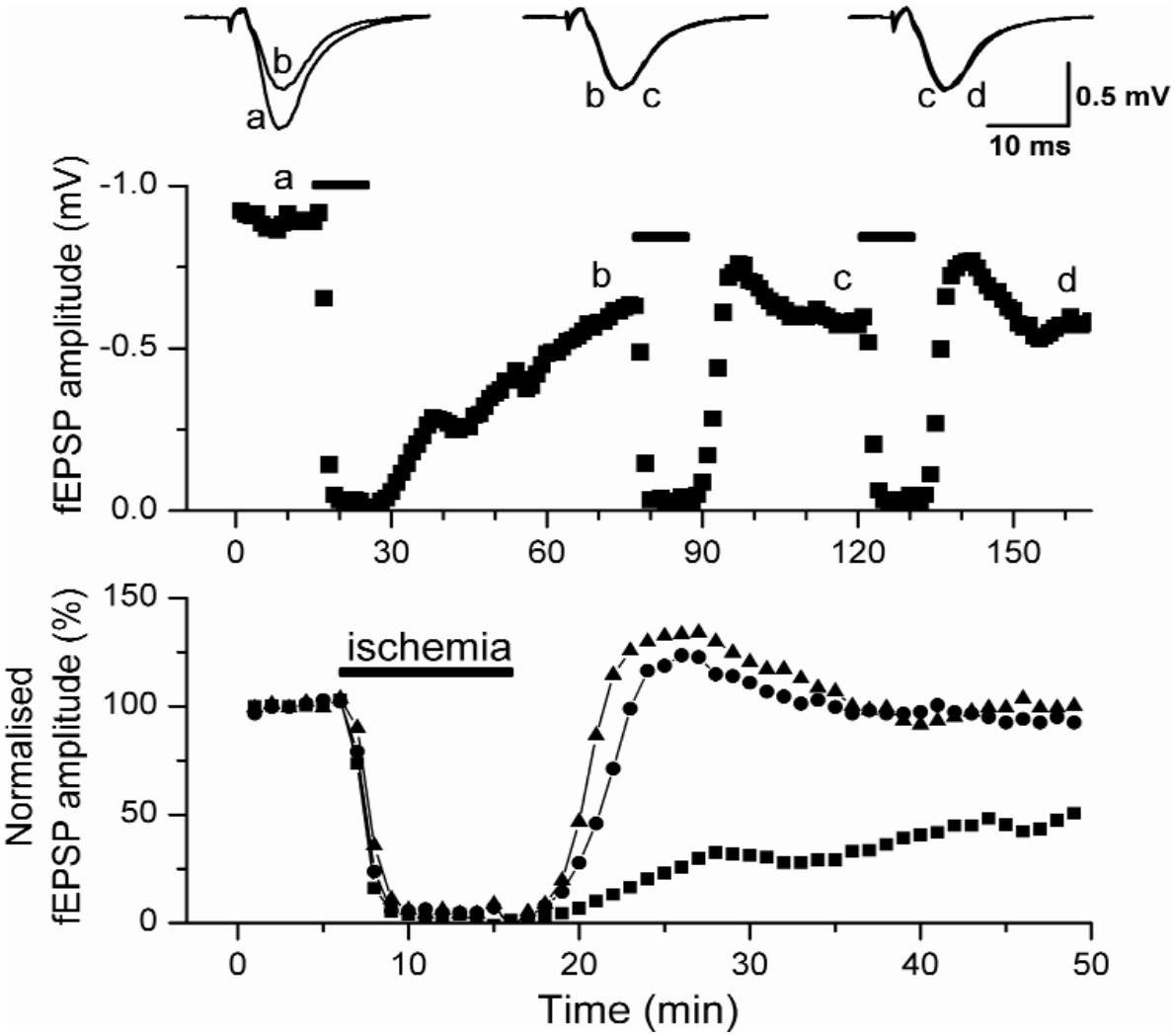

Fig. 7 Ischemic preconditioning or adenosine depletion? Sequential periods of ischemia (aCSF devoid of glucose and oxygen; [99]) result in dramatically faster post-ischemic recovery of the fEPSP in area CA1. The top panel shows the entire time-course of an experiment in which 3 ischemic episodes (black bars) of identical duration were delivered to the hippocampal slice. The first resulted in a protracted recovery period (compare inset fEPSPs a and b), whereas the second and third ischemic episodes resulted in very rapid recoveries of the fEPSP (compare inset fEPSPs b & c and c & d). The lower panel shows only the 50 minutes of the experiment around each of the three ischemic episodes shown above. Notice how the initial depressions become slower and the recoveries accelerated between the first (squares) second (circles) and third (triangles) ischemic episodes. Such accelerated recoveries of the fEPSP after repeated *in vitro* ischemia has been interpreted as the *in vitro* equivalent of the ischemic preconditioning phenomenon. However an alternative interpretation is a reduction in the release of extracellular adenosine.

This is most apparent during pathological conditions where levels of adenosine rise dramatically. For example, in gerbil striatum and hippocampus, reduced adenosine release was observed during the second of two 5 min *in vivo* global ischemic episodes given 30 min apart [61]. This led to the perceptive comment that *"...these findings suggest that the release of adenosine by an ischemia stimulus is reduced by a prior major release of purines"* [61]. More recently, reduced adenosine release was observed in rat cerebral cortex during the second of two 10 min periods of global ischemia delivered 2 hours apart [62] and less adenosine-mediated hypoxic cerebral vasodilatation was observed fol-

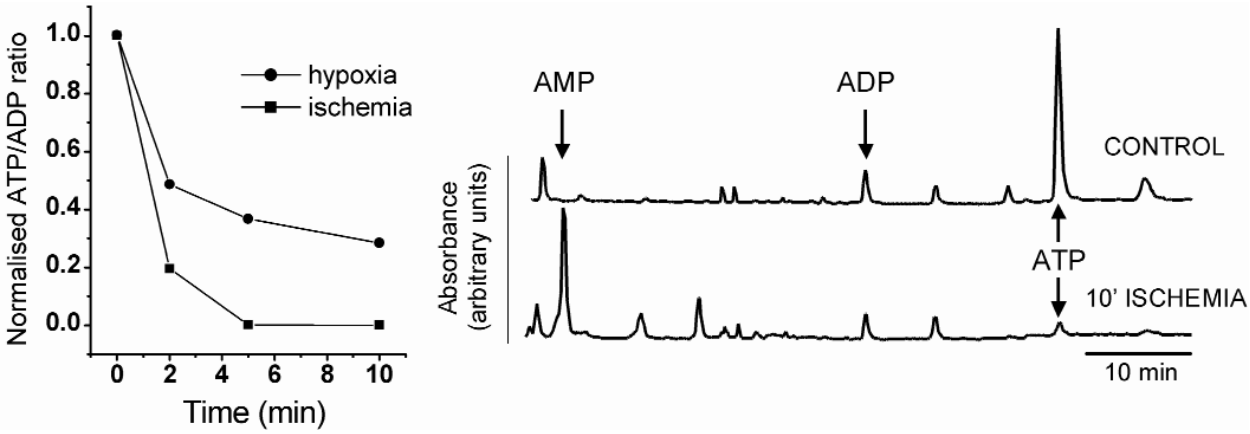

Fig. 8 Depletion of ATP during ischemia. Left panel, normalised ATP/ADP ratio for cerebrocortical brain slices exposed to hypoxia (circles) or ischemia (squares). Note dramatic decline in ratio during ischemia. Right panel, HPLC chromatograms from two separate sister slices treated in parallel in control and after 10 mins of ischemia. Notice the dramatic decrease in ATP and consequent increase in AMP. Modified from [100].

lowing a period of seizure activity in neonatal piglets [63]. Furthermore, in a murine model of asphyxia, hippocampal adenosine production did not scale with the number of hypoxic episodes [64]. In addition, it would seem that during prolonged ischemia extracellular adenosine levels are not maintained, but decline over time [20, 65–67]. Adenosine depletion has also been observed during physiological activity, as opposed to being exclusively associated with insults to the mammalian brain. In the rat nucleus tractus solitarus *in vivo*, two stimulations of the hypothalamic defense area given 5 minutes apart resulted in reduced adenosine release during the second stimulation, which was associated with a shorter period of apnoea [68]. Thus, the labile nature of adenosine availability has important implications for many physiological and pathological processes in the mammalian brain.

Implications of adenosine depletion for neuronal function

On the basis of our observations of adenosine depletion [35, 69] (and Fig. 6) in which sequential hypoxic episodes result in reduced production of adenosine, we have suggested that reduced availability of adenosine may have deleterious consequences for the mammalian brain exposed to repeated or secondary insults. Since adenosine exerts such a powerful inhibitory influence of glutamate release during hypoxia [49, 70, 71] (and Fig. 2), any diminution in adenosine availability will result in increased glutamatergic excitation. Certainly this can been seen *in vitro* where repeated hypoxic episodes are associated with slower depressions and more rapid post-hypoxic recoveries of excitatory synaptic transmission [69]. Similarly, observations of more rapid recovery of glutamatergic transmission on repeated ischemia (Fig. 7) can be interpreted as being due to reduced adenosine availability.

Depletion of adenosine release will thus predispose to increased glutamate release, increased postsynaptic depolarization via AMPA and NMDA receptor activation, increased Ca^{2+}-loading, increased neuronal excitability and increased metabolic demands, with potentially pathological consequences. This hypothesis is a plausible explanation, at least in part, for the 1 - 4 hour period of increased vulnerability of the mammalian brain after an initial injury observed in several *in vivo* models of ischemia (e.g. [72, 73]) and trauma (e.g. [74]).

Potential basis for adenosine depletion

The mammalian brain uses purine salvage (Fig. 1) as opposed to purine synthesis to restore nucleotide

levels [75, 76]. This reliance on salvage makes the brain, as in heart [77] vulnerable to ATP-depleting insults. Depletion of adenine nucleotides after hypoxia/ischemia has been known for many years [66, 78–82] (Fig. 8). The production of xanthine, which is not a substrate for purine salvage (Fig. 1), during cerebral metabolic stress in humans [83, 84] and animals [62, 85–88] as well as loss of adenosine *per se* from the brain [15, 16], likely contribute to reduced post-insult levels of adenine nucleotides. Indeed inhibition of xanthine oxidase has been shown to improve post-ischemic levels of adenine nucleotides [89] as has inhibition of adenosine transport, perhaps by reducing the loss of adenosine to the periphery [90]. Since adenine nucleotides are a primary source of adenosine, hypoxia/ischemia- or even activity-induced adenine nucleotide depletion may underlie the subsequent reduced production of adenosine. It might therefore be expected that a reduction in cellular ATP would have downstream consequences for adenosine availability.

Conclusions

Adenosine is a powerful and ubiquitous modulator of neuronal function in the mammalian CNS. The release of adenosine during insults to the brain exerts an important neuroprotective influence, which can be disrupted by antagonism of adenosine A_1 receptors or by experimental manipulations designed to reduce the availability of extracellular adenosine. Surprisingly, reduced extracellular adenosine is observed during repeated hypoxic/ischemic episodes *in vivo* and *in vitro* and it is possible that this period of reduced adenosine availability may underlie the increased vulnerability of the mammalian brain to repetitive or secondary hypoxia/ischemia. Greater understanding of adenosine availability with a view to maintaining or improving post-ischemic adenosine levels may thus be of benefit in a range of acute human neurological conditions.

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