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Astrocytic adenosine kinase regulates basal synaptic adenosine levels and seizure activity but not activity-dependent adenosine release in the hippocampus

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ABSTRACT

Adenosine is an endogenous inhibitor of excitatory synaptic transmission with potent anticonvulsant properties in the mammalian brain. Given adenosine's important role in modulating synaptic transmission, several mechanisms exist to regulate its extracellular availability. One of these is the intracellular enzyme adenosine kinase (ADK), which phosphorylates adenosine to AMP. We have investigated the role that ADK plays in regulating the presence and effects of extracellular adenosine in area CA1 of rat hippocampal slices. Inhibition of ADK activity with 5′-iodotubercidin (IODO; 5 μM) raised extracellular adenosine, as measured with adenosine biosensors, and potently inhibited field excitatory post-synaptic potentials (fEPSPs) in an adenosine A_1R-dependent manner. In nominally Mg^{2+}-free aCSF, which facilitated the induction of electrically-evoked epileptiform activity, adenosine biosensor recordings revealed that seizures were accompanied by the transient release of adenosine. Under these conditions, IODO also inhibited the fEPSP and greatly suppressed epileptiform activity evoked by brief, high-frequency stimulation. During spontaneous seizures evoked by the A_1R antagonist CPT, adenosine release was unaffected by IODO. This suggests that ADK activity does not limit activity-dependent adenosine release. On the basis of strong ADK immunoreactivity in GFAP-positive cells, astrocytes are likely to play a key role in regulating basal adenosine levels. It is this action of ADK on the basal adenosine tone that is permissive to seizure activity, and, by extension, other forms of activity-dependent neuronal activity such as synaptic plasticity.

1. Introduction

The purine nucleoside adenosine regulates many physiological and pathological processes via four G-protein coupled receptors (A_1, A_{2A}, A_{2B}, A_3) (Fredholm et al., 2005). These physiological processes range from regulation of transmitter release, in particular that of the major excitatory neurotransmitter, glutamate, to the induction of sleep following the gradual accumulation of adenosine during wakefulness (Basheer et al., 2004). In addition, the release of adenosine during a host of pathological events in the mammalian brain is believed to exert a strong neuroprotective influence via inhibition of glutamate release and neuronal and network activity, thereby reducing nutrient demand, as well as acting as a vasodilator during such insults as stroke, epileptic seizures and head injury (Cunha, 2005).

Given the powerful and ubiquitous nature of adenosine action within the CNS, basal levels of extracellular adenosine are carefully regulated and are estimated to be in the region of 30–300 nM (Fredholm et al., 2001). The two main pathways for the control of extracellular adenosine involve phosphorylation of cytosolic adenosine to AMP, which is mediated by adenosine kinase (ADK), or deamination by adenosine deaminase (ADA) resulting in the formation of inosine. In rat brain, K_m values are ~2 μM for ADK and ~17 μM for ADA (Phillips and Newsholme, 1979). This suggests that under normal physiological conditions adenosine is metabolised primarily via the action of ADK, with the contribution of ADA increasing as the extracellular adenosine concentration is elevated, for example, during metabolic stress (Lloyd and Fredholm, 1995; Latini and Pedata, 2001). These K_m values also suggest that adenosine kinase is the primary regulator of intracellular, and, most likely by virtue of the ubiquitous equilibrative nucleoside transporters (King et al., 2006), basal extracellular adenosine concentration (Boison, 2006).

Accordingly, inhibition of ADK, but not ADA, in hippocampal slices increased extracellular levels of adenosine and depressed

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excitatory synaptic transmission in an A_1R-dependent manner (Pak et al., 1994; Lloyd and Fredholm, 1995). More recently, hippocampal grafts of ADK-deficient myoblasts, fibroblasts or embryonic stem cells raised extracellular adenosine levels *in vivo* and were able to retard kindling-induced seizure activity in rats (Boison, 2006, 2007).

Thus, ADK is a key regulator of the concentration, and hence effects, of extracellular adenosine. In this study we have examined the role of ADK in an *in vitro* model of electrically-evoked epileptiform activity. We show that inhibition of ADK, which is located primarily in GFAP-positive astrocytes, elevates synaptic adenosine and greatly suppresses glutamatergic excitatory synaptic transmission in both control and nominally Mg^{2+}-free aCSF in an A_1R-dependent manner. In addition, seizure activity evoked by brief, high-frequency stimulation was greatly attenuated. These data suggest that not only do astrocytes and ADK play a pivotal role in the regulation of synaptic adenosine under both physiological and pathological conditions, they also imply that basal ADK activity is permissive to seizure activity. Thus, factors influencing the activity or levels of ADK are therefore likely to have important consequences for activity-dependent neuronal function, such as synaptic plasticity, whilst targeting ADK may provide novel therapies for epilepsy and other neurological disorders.

2. Materials and methods

2.1. Slice preparation

Hippocampal slices were obtained from 16 to 22 day-old Sprague–Dawley rats of either sex. The animals were killed by cervical dislocation in accordance with Schedule 1 of the UK Government Animals (Scientific Procedures) Act 1986 and with the approval of the local Ethical Review Committee. The animals were then decapitated and the whole brain rapidly dissected and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing 11 mM Mg^{2+}. Slices were cut using a Vibratome (Intracel, Royston, Herts, UK), using techniques described previously (Dale et al., 2000; Pearson et al., 2001; Etherington and Frenguelli, 2004) in which sagittal sections were cut from each bisected hemisphere to yield 600 μm transverse hippocampal slices. Such thicker slices are necessary to support high-frequency stimulation-induced seizures (Etherington and Frenguelli, 2004). Slices were initially maintained, submerged, in an incubation chamber filled with circulating oxygenated (95% O_2/5% CO_2) standard aCSF (1 mM Mg^{2+}) at room temperature for at least 1 h before use. The composition of the standard aCSF solution was (in mM): NaCl, 124; $CaCl_2$, 2; $NaHCO_3$, 26; NaH_2PO_4, 1.25; D-glucose, 10; $MgSO_4$, 1; pH 7.4 with 95% O_2/5% CO_2.

2.2. Extracellular recording

A single slice was transferred to a submersion-type recording chamber, placed on a suspended nylon mesh (to ensure all round perfusion) and continuously perfused at 6 ml/min with aCSF. The perfusion medium was gassed with 95% O_2/5% CO_2 and maintained at 30–31 °C. To monitor basal synaptic transmission during the experiment, the afferent Schaffer collateral–commissural pathway from area CA3 to the CA1 region was stimulated at 15 s intervals (100 μs pulses) using a twisted Teflon-coated tungsten bipolar stimulating electrode (~100 μm in diameter) placed in stratum radiatum. Field excitatory post-synaptic potentials (fEPSPs) were recorded from stratum radiatum of area CA1 using an aCSF-filled glass microelectrode (<5 MΩ). Stimulus parameters and acquisition and analysis of fEPSPs were under the control of LTP software (courtesy of Dr. Bill Anderson and Professor Graham Collingridge, Bristol University, UK; http://www.ltp-program.com/indexLtp24.htm) (Anderson and Collingridge, 2001). Continuous electrophysiological recordings (1 Hz–3 kHz) were made simultaneously using Spike 2 software (sampling rate 10 kHz) and a CED micro 1401 interface (Cambridge Electronic Design, Cambridge, UK) or with custom software. The ability to evoke and record fEPSPs with a separate program (LTP) facilitated the identification of fEPSPs among ongoing seizure activity.

2.3. Adenosine biosensor measurements

To detect the release of adenosine and inosine we inserted 50 μm diameter miniature microelectrode biosensors (Sarissa Biomedical Ltd; Coventry UK; http://www.sarissa-biomedical.com) into the stratum radiatum region of 600 μm hippocampal slices, essentially as described previously (Frenguelli et al., 2003, 2007). In brief, these sensors consisted of a 50 μm platinum wire which was coated with a matrix containing enzymes resulting in the metabolism of adenosine and inosine to hydrogen peroxide, which was detected as an oxidation current on the polarized (+500 mV) platinum wire. A third microelectrode lacking the enzymes served as a null control for non-specific electro-active interferents. A screening layer effectively prevented electroactive substances such as 5-HT, noradrenaline, dopamine, urate and ascorbate from contributing to the sensor signal. The patency of this layer was tested with 10 μM 5-HT at the end of the experiment, whilst 10 μM adenosine and inosine were applied to calibrate the sensors. Output from the sensors was fed to a PC running custom software via a potentiostat (Duostat ME200$^+$; Sycopel International Ltd; Jarrow, UK). Where possible we have subtracted the contribution of inosine to the adenosine/inosine signal and thus present adenosine release in terms of absolute concentration (μM). However, this was not possible in all cases, likely due to the fact that seizure activity may not be uniform across stratum radiatum, which may result in inaccurate differential recordings of net adenosine release. In these cases we present values for adenosine/inosine release as μM′, to indicate that the contribution of inosine (approximately 50% of the sensor signal during hypoxia/ischemia) has not been subtracted (Frenguelli et al., 2003, 2007).

2.4. Seizure protocol

After ensuring a stable baseline recording in standard aCSF the medium was changed to a nominally Mg^{2+}-free aCSF, to facilitate the induction of seizure activity, and allowed to stabilize for 30 min. Following this, seizures, recorded with the same electrode in area CA1, were induced at approximately 10 min intervals by means of a 2 s, 60 Hz stimulus delivered at test intensity (high-frequency stimulation; HFS). Comparisons of seizure duration were made between seizures evoked under control conditions and seizures elicited in the presence of the adenosine kinase inhibitor 5′-iodotubercidin (IODO; 5 μM) and the A_1R antagonist 8-cyclopentyltheophylline (CPT; 1 μM). Further seizures were evoked while washing out the adenosine kinase inhibitor to observe any reversibility in its effects. Seizure duration was measured as the time from the initiation of seizure activity to the last ictal discharge. Often the seizure immediately followed on from the HFS with no interruption between the two. In these cases, the start of the seizure was taken as being at the end of the 2 s HFS train. The application of CPT often resulted in recurrent spontaneous seizure activity in either the presence or absence of IODO. The evoked and spontaneous seizures were considered to have ended if one or more seconds elapsed between two successive ictal discharges. We noted that evoked seizure activity was somewhat reduced in slices in which multiple sensors had been inserted. This may be due to severing of the afferent fibres.

2.5. Immunohistochemistry

Hippocampal slices (600 μm) were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature (20–22 °C) then immersed in 30% sucrose overnight at 4 °C. Thin sections (40 μm) were cut from these fixed slices on a cryostat and incubated overnight at 4 °C in a Tris–Triton solution (pH 7.4) containing antibodies to ADK (1:5000, rabbit; Boison et al., 2002) and GFAP (1:5000, mouse; Chemicon, MAB360), 2% donkey serum and 0.2% Triton X-100. Sections were then given 4 × 5 min washes in TBS then incubated at room temperature for 1 h in Tris-Triton solution (pH 7.4) containing secondary antibody conjugated to: Cy3 (donkey anti-rabbit; Jackson ImmunoResearch Labs) plus Alexa 488 (donkey anti-mouse; Molecular Probes), 2% donkey serum and 0.2% Triton X-100, *or* Cy5 (donkey anti-rabbit) plus Alexa 488 (donkey anti-mouse), 2% donkey serum and 0.2% Triton X-100. All secondary antibodies were used at 1:500 dilution. Following 4 × 5 min washes in TBS, sections were mounted on slides with Hydromount® and coverslipped. Fluorescence imaging of ADK and GFAP dual labeling was performed with a Zeiss LSM 510 confocal microscope using a 40× water immersion objective.

2.6. Chemicals

Chemicals used in the aCSF were supplied by VWR (Lutterworth, Leics, UK). 4-Amino 5-iodo-7(b-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (5-iodotubercidin; IODO), dipyridamole (DIPY) and *S*-(4-nitrobenzyl)-6-thioinosine (NBTI) were obtained from Sigma–Aldrich (Poole, Dorset, UK) and 8-cyclopentyltheophylline (CPT) from RBI (Poole, Dorset, UK). 5′-iodotubercidin, dipyridamole and *S*-(4-nitrobenzyl)-6-thioinosine were dissolved in dimethyl-sulphoxide (DMSO, 0.05, 0.01 and 0.01% final concentration of DMSO, respectively).

2.7. Data analysis

Data on average seizure duration (2–3 seizures) from within slices were pooled into groups (drug and control) and compared by means of paired or independent *t*-tests as indicated in the text. Significance was noted at the level of $P < 0.05$. Data are presented as mean ± SEM and n = number of slices. No more than two slices per animal were used for any condition and a minimum of three animals were used per manipulation.

3. Results

3.1. ADK powerfully regulates extracellular adenosine levels

Treatment of 600 μm rat hippocampal slices with the ADK inhibitor 5′-iodotubercidin (IODO; 5 μM) resulted in a rapid and profound depression of the fEPSP slope to 6.1 ± 2.2% of control ($n = 5$; Fig. 1). To establish whether this depression of glutamatergic transmission was dependent on an increase in extracellular adenosine and activation of A_1Rs, we applied the A_1R antagonist CPT (1 μM). Under basal conditions CPT enhanced the fEPSP by 9.3 ± 2.5% ($n = 5$; data not shown) indicative of an adenosine tone similar to that from 400 μm thick hippocampal slices (Gadalla et al., 2004). This indicates that 600 μm slices do not experience any elevation in basal adenosine tone attributable to hypoxia (Pearson et al., 2006). However, CPT was able to completely reverse the inhibition of the fEPSP induced by IODO (144.3 ± 26.5% of control; $n = 3$; Fig. 1). This suggests that inhibition of ADK results in an increase in extracellular adenosine (see below) and subsequent activation of A_1Rs.

3.2. Profile of epileptiform activity in nominally Mg^{2+}-free aCSF

A commonly used model of seizure activity involves the removal of extracellular Mg^{2+}, which facilitates activation of the NMDA receptor and thus predisposes hippocampal slices to seizure activity. Perfusion of hippocampal slices with nominally Mg^{2+}-free aCSF resulted in a rapid and dramatic increase in excitatory synaptic transmission, which stabilized at 180 ± 1% of baseline ($n = 15$) after 30 min (Fig. 2). However, this alone was only infrequently (~20% of slices) capable of inducing spontaneous seizure activity, defined as a sustained period of activity capable of depressing basal synaptic transmission. In slices which did not exhibit spontaneous seizure activity, seizures were elicited by means of a 2 s, 60 Hz burst of high-frequency stimulation (HFS) delivered to the Schaffer collateral–commissural pathway (Fig. 2A and B).

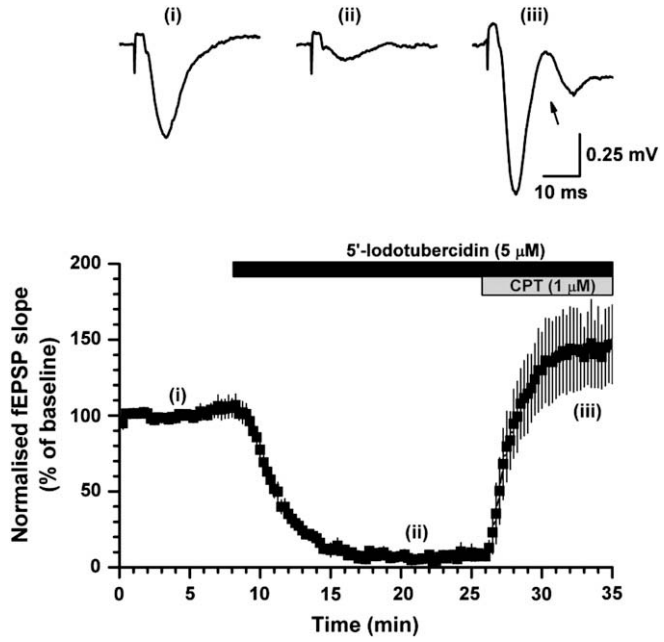

Fig. 1. Regulation of extracellular adenosine by ADK. Inhibition of ADK by IODO (5 μM; black bar; pooled data from 3 of 5 experiments in which IODO was applied) caused a profound depression of the fEPSP slope that was reversible upon application of the A_1R antagonist CPT (1 μM; grey bar). Inset are fEPSPs taken (i) before and (ii) during IODO, and (iii) after application of CPT. Note increased excitability (appearance of the population spike, arrow) after application of CPT.

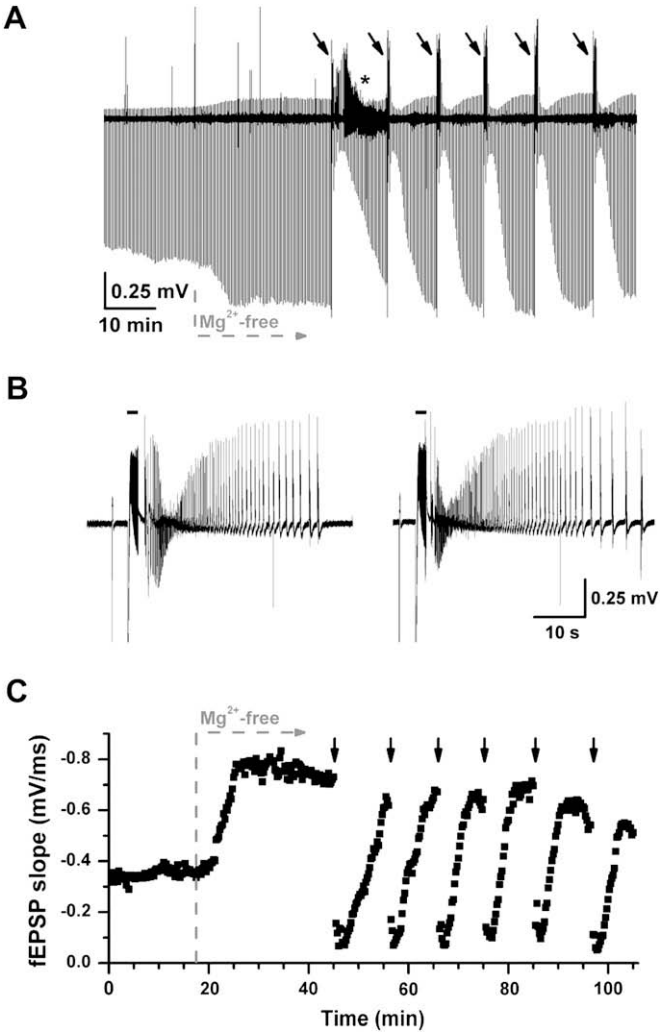

Fig. 2. Profile of seizure activity in nominally Mg^{2+}-free aCSF. (A) Full time-course of typical seizure experiment. Periodic downward deflections, in this and subsequent figures, reflect fEPSPs evoked at 15 s intervals. Nominally Mg^{2+}-free aCSF (Mg^{2+}-free) was applied as indicated by broken grey line and resulted in an increase in synaptic transmission. High-frequency stimulation (60 Hz, 2 s; arrows) at 10 min intervals evoked reproducible epileptiform activity and reversible depressions of the fEPSP. Asterisk denotes period of increased interictal (but not seizure) activity. (B) Two examples of seizures from the experiment depicted in (A) in response to the 3rd (left) and 4th (right) periods of HFS (black bar). (C) Time-course of fEPSP slope measurements in experiment depicted in (A). Nominally Mg^{2+}-free aCSF was applied as indicated. Arrows indicate timing of HFS.

Evoked seizure activity generally displayed a tonic-clonic profile, with a period of high-frequency smaller-amplitude spikes followed by lower-frequency large-amplitude spikes and lasted for 22.6 ± 2.2 s ($n = 7$; Fig. 2B). Capturing individual fEPSPs before, during and after HFS-induced seizures allowed us to observe a profound (93.1 ± 4.0% of baseline), but temporary reduction in synaptic transmission, which recovered to baseline within approximately 6 min, but greatly outlasted the observed seizure (Etherington and Frenguelli, 2004) (Fig. 2A and C).

Seizure activity was under the control of endogenous adenosine (Etherington and Frenguelli, 2004) (Fig. 3). In 11 consecutive slices, seizure activity evoked by HFS in nominally Mg^{2+}-free aCSF was elicited from 5 slices and measured 17.1 ± 3.1 s (Fig. 3A and B). Subsequent application of the A_1R antagonist CPT (1 μM) resulted in spontaneous seizures in all 11 slices (mean duration 67.2 ± 4.8 s), lowered the threshold for HFS-induced seizure generation and greatly increased the duration of evoked seizures (34.1 ± 5.3 s;

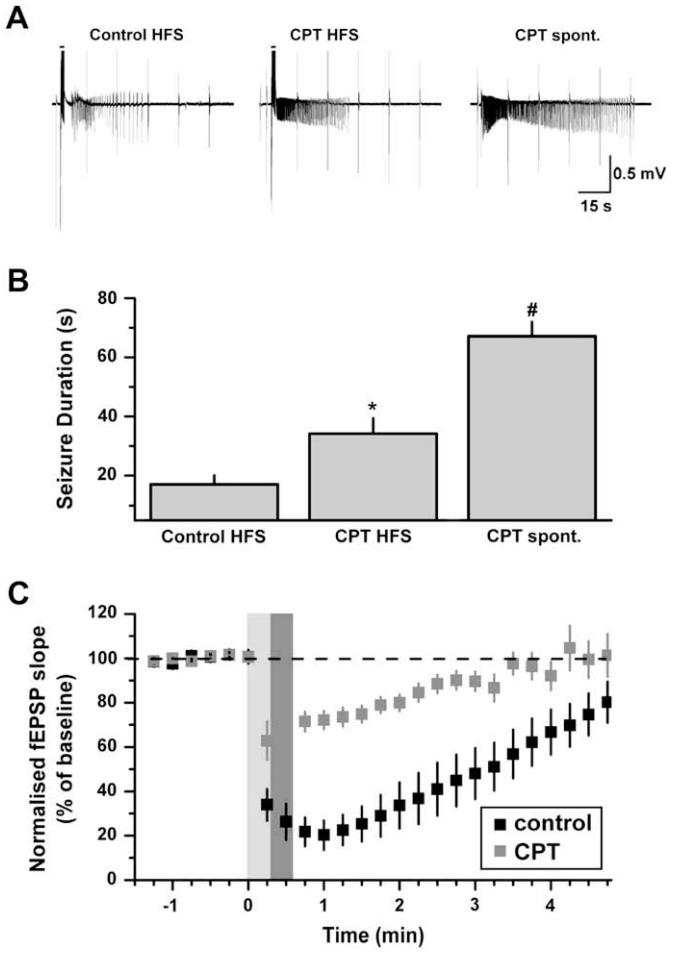

Fig. 3. Endogenous adenosine limits seizure activity. (A) Profile of HFS-induced seizure activity (small black bar) in the absence (control HFS) and presence (CPT HFS) of the adenosine A_1 receptor antagonist CPT. Rightmost trace (CPT spont.) depicts spontaneous seizure recorded in the presence of CPT. (B) Pooled data (5–11 experiments) demonstrating the increase in HFS-evoked seizure duration in the presence of CPT (CPT HFS) and the long duration of spontaneous seizures in CPT (CPT spont.). Asterisk denotes significantly different from control whilst hash sign indicates further significant increase over CPT HFS seizures. (C) Seizure-induced depression of the fEPSP is greatly attenuated by CPT. Filled squares: depression of fEPSP caused by HFS-evoked seizures under control conditions. Light grey bar depicts average control seizure duration (~17 s). Grey squares show reduced depression of the fEPSP in the presence of CPT, despite longer seizure (additional dark grey bar; ~34 s).

$n = 8$; $P < 0.05$, independent t-test compared to controls; Fig. 3A and B). Furthermore, the seizure-induced peak depression of the fEPSP (80.5 ± 5.9% depression; Fig. 3C) was greatly attenuated by CPT (43.8 ± 7.6% depression; independent t-test, $P = 0.006$) despite the HFS-induced seizures being twice as long.

3.3. Role of adenosine kinase in regulating epileptiform activity

Given the importance of endogenous adenosine in this model of seizure activity, we tested whether the primary route of adenosine metabolism, ADK, played a role in regulating the availability of extracellular adenosine and hence activation of inhibitory A_1Rs.

Similar to the situation in 1 mM Mg^{2+}, treating slices with IODO (5 μM) caused a significant reduction (to 9.1 ± 0.9% of control; paired t-test; $P < 0.05$; $n = 4$) in baseline synaptic transmission (Fig. 4). This depression was fully reversed on application of the A_1R antagonist CPT (102.8 ± 8.9%; $n = 4$; Fig. 4), and resulted in spontaneous seizure activity in the majority of slices (see below).

As a consequence of the profound effect on basal synaptic transmission, epileptiform activity was significantly attenuated:

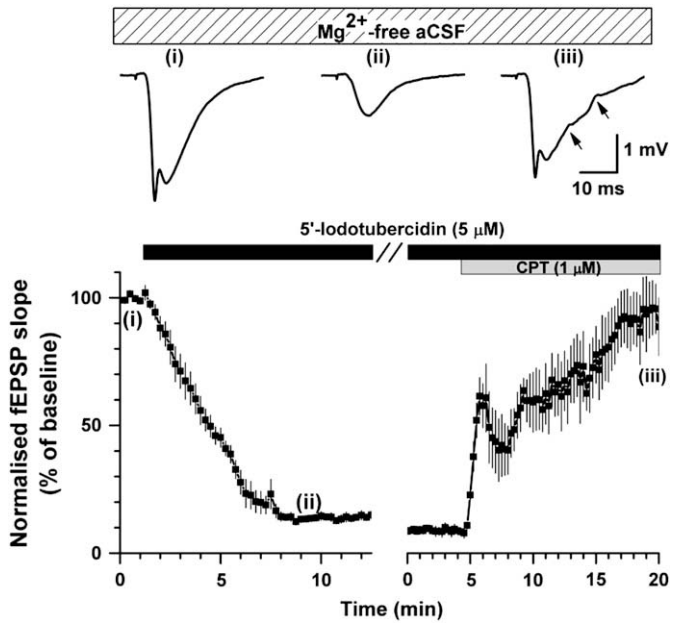

Fig. 4. ADK regulates extracellular adenosine in nominally Mg^{2+}-free aCSF. (*Left panel*). Inhibition of ADK by IODO (5 μM; black bar; $n = 4$) caused a strong inhibition of excitatory synaptic transmission. This depression was reversed by the A_1R antagonist CPT (1 μM; grey bar; *right panel*). Break in graph reflects 30–60 min range over which IODO was applied to the slice. Data are normalized to previous control baseline (*left panel*) and immediately prior to CPT application (*right panel*). Inset are fEPSPs taken (i) before and (ii) during IODO, and (iii) after application of CPT. Note appearance of multiple population spikes (arrows) after application of CPT.

the duration of evoked seizures was reduced from 18.5 ± 3.6 s under control conditions of nominally Mg^{2+}-free medium to only 4.1 ± 1.1 s in the presence of the adenosine kinase inhibitor (paired t-test; $P = 0.002$, $n = 12$; Fig. 5A). In two of three slices in which it was tested, the effect on seizure duration was partially reversed upon washout of the adenosine kinase inhibitor (mean duration 13.5 s).

The dramatic reduction of HFS-induced epileptiform activity in the presence of IODO resulted in a large attenuation of the seizure-induced depression of synaptic transmission (25.8 ± 3.7% depression) compared with corresponding controls (76.4 ± 6.0% depression, paired t-test; $P < 0.05$, $n = 12$, Fig. 5B). The HFS-induced depression of the fEPSP was often (10/12 slices) preceded by a brief period of excitation. This may reflect the enhancement of residual glutamatergic transmission by greater activation of excitatory A_{2A} receptors through the elevated extracellular tone of adenosine (Cunha et al., 1994; Cunha, 2008).

The reduction of the HFS-induced depression of the fEPSP by IODO was partially reversed upon washout of the adenosine kinase inhibitor (67.0 ± 12.8% depression, $n = 3$; not shown). To counteract the effects of IODO we applied CPT (1 μM) in the continued presence of IODO. This resulted in the recovery of synaptic transmission (Fig. 4) and the appearance of spontaneous seizure activity in 6 of 9 slices (Fig. 5C). This spontaneous activity restricted the delivery of HFS. In three slices where it was possible to deliver HFS, the seizures evoked measured 26.1 ± 8.2 s, effectively reversing the seizure suppression induced by IODO (Fig. 5D), and caused a 51.1 ± 9.5% depression of the fEPSP (not shown).

3.4. Direct determination of extracellular adenosine release

The previous experiments using the ADK inhibitor IODO and the A_1R antagonist CPT indirectly imply that both seizure activity and inhibition of ADK cause elevations of extracellular adenosine. We tested these hypotheses directly by using enzyme-based

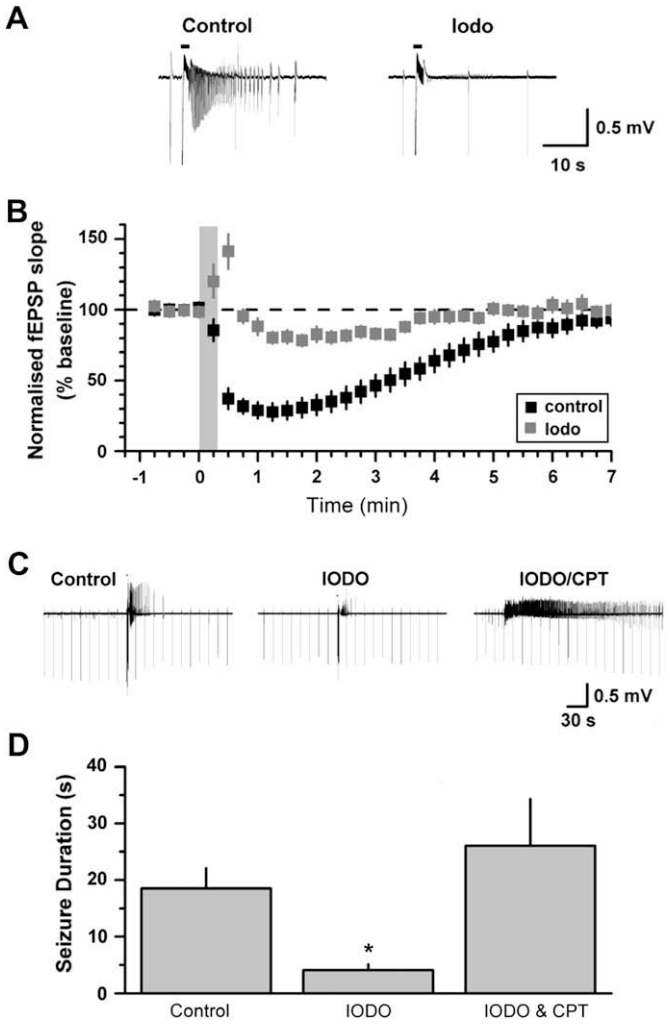

Fig. 5. Seizure activity is greatly attenuated following inhibition of ADK. (A) Epileptiform activity induced by HFS (small black bar) in nominally Mg^{2+}-free aCSF was greatly attenuated by IODO (5 μM; *right panel*) compared to control conditions in the absence of IODO (control; *left panel*). (B) The reversible depression of synaptic transmission induced by seizures under control conditions (filled squares) was also greatly attenuated in the presence of IODO (grey squares). Average duration of seizures (~18 s) in control conditions is indicated by grey bar, but too short to depict (~4 s) in the presence of IODO. (C) The IODO-induced depression of seizure activity (*middle panel*; IODO) is reversed by the A_1 receptor antagonist CPT (1 μM; IODO/CPT; *right panel*). Note seizure in IODO/CPT is a spontaneous seizure, seen in 6/9 slices exposed to this combination of drugs. (D) Pooled data showing the inhibition of HFS-evoked seizure duration by IODO ($n = 9$) and its reversal by CPT in the 3 slices not showing spontaneous seizure activity.

microelectrode biosensors for adenosine and its primary metabolite inosine.

In nominally Mg^{2+}-free aCSF, HFS resulted in adenosine/inosine release measuring 1.0 ± 0.3 μM′ ($n = 13$), associated with seizures of 8.9 ± 1.2 s in duration. In a subset of slices it was possible to observe a net adenosine signal which measured 1.0 ± 0.3 μM ($n = 5$), an example of which can be seen in Fig. 6A. Null sensors recorded no signal other than a transient response associated with the HFS stimulus (not shown). In ten slices, adenosine sensors were used to measure the change in extracellular adenosine associated with the application of IODO. In all cases IODO caused an increase in the purine tone (2.7 ± 0.6 μM′), which was associated with a depression of the fEPSP to 12.3 ± 2.6% ($n = 10$) of the previous control. We were able to measure a net increase in basal adenosine concentration of 1.0 ± 0.2 μM in 6 of these slices (Fig. 6B). In order to test whether the adenosine efflux induced by IODO was mediated by adenosine equilibrative nucleoside transporters (ENT), we applied IODO in the presence of a combination of the ENT1 and ENT2 inhibitors dipyridamole (DIPY; 10 μM) and *S*-(4-nitrobenzyl)-6-thioinosine (NBTI; 5 μM; $n = 9$) (Dunwiddie and Diao, 1994; Pearson et al., 2001; Gadalla et al., 2004; Frenguelli et al., 2007). DIPY/NBTI caused a net elevation of the purine tone in 7 of 9 slices (Fig. 6C), which on average measured 0.6 ± 0.2 μM′ ($n = 7$), and a depression of the fEPSP to 20.5 ± 4.8% of control ($n = 9$; not shown). Subsequent application of IODO (5 μM) resulted in a further elevation of extracellular adenosine/inosine by 1.5 ± 0.3 μM′ ($n = 8$; Fig. 6C) and an additional inhibition of the fEPSP to 4.9 ± 1.3% of control ($n = 9$; not shown). This elevation in extracellular adenosine/inosine is lower (but not significantly) than that caused by IODO alone suggesting that the combination of ENT inhibitors had reduced, but not completely prevented the efflux of adenosine caused by ADK inhibition. Thus, either DIPY/NBTI is ineffective in completely blocking ENT1 and ENT2, or additional mechanisms exist by which adenosine exits the cell under these conditions.

Application of IODO reduced the release of adenosine/inosine during HFS-induced seizures from 1.2 ± 0.3 μM′ adenosine/inosine to 0.7 ± 0.2 μM′ adenosine/inosine ($n = 8$; Fig. 6D). This counter-intuitive result likely reflects the fact that IODO reduced the duration of the seizures (from 10.1 ± 1.3 s to 7.8 ± 1.8 s; $n = 8$; Fig. 6D). In these slices, subsequent application of CPT restored excitatory synaptic transmission (to 139.3 ± 18.8% of baseline; $n = 7$), HFS-induced seizure duration (29.0 ± 10.6 s; $n = 6$) and adenosine/inosine release (1.2 ± 0.5 μM′; $n = 6$).

Assessing the role of ADK on seizure-induced adenosine release is complicated by the fact that inhibition of ADK elevates basal adenosine and hence reduces the duration of seizures. We therefore examined whether ADK regulates activity-dependent release of adenosine by performing these experiments in the presence of the A_1R antagonist CPT to obviate the inhibitory effects of adenosine. We found that application of CPT (1 μM; $n = 8$) evoked spontaneous seizures of 54.5 ± 9.2 s duration, which caused the release of 3.2 ± 0.5 μM′ adenosine/inosine (Fig. 6E). In 4 of these slices the net adenosine release measured 2.1 ± 0.5 μM. In separate slices we applied IODO prior to the application of CPT to test whether this could alter the subsequent adenosine release during the CPT-evoked seizure. Application of CPT after IODO induced spontaneous seizures lasting 65.8 ± 4.7 s (not significantly different from CPT alone). The seizures in the presence of IODO caused the release of 3.2 ± 0.9 μM′ adenosine/inosine ($n = 7$; Fig. 6F). Thus, the amount of adenosine released during spontaneous seizures in IODO/CPT was no different from that in CPT alone. This suggests that the release of adenosine during seizures is not limited by the action of ADK and instead may arise from other sources such as the extracellular breakdown of ATP.

3.5. Predominant astrocytic localization of ADK

To demonstrate whether synaptic adenosine could be regulated via a non-neuronal mechanism, we investigated the cellular localization of ADK by performing immunohistochemical staining against ADK in thin (40 μm) cryosections of hippocampal slices from 16 to 22 day-old animals, typical of those used for electrophysiological recordings. This age range reflects a period during which the developmental shift of ADK expression from pyramidal neurons to astrocytes is already completed, while the developmental shift of ADK expression from granular neurons to astrocytes is still ongoing (Studer et al., 2006). Consistent with this previously described developmental shift in ADK expression in the murine hippocampus, we observed co-localization between ADK and GFAP-positive astrocytes in area CA1, but a lack of ADK expression in pyramidal neurons (Fig. 7A). However, in dentate gyrus, in

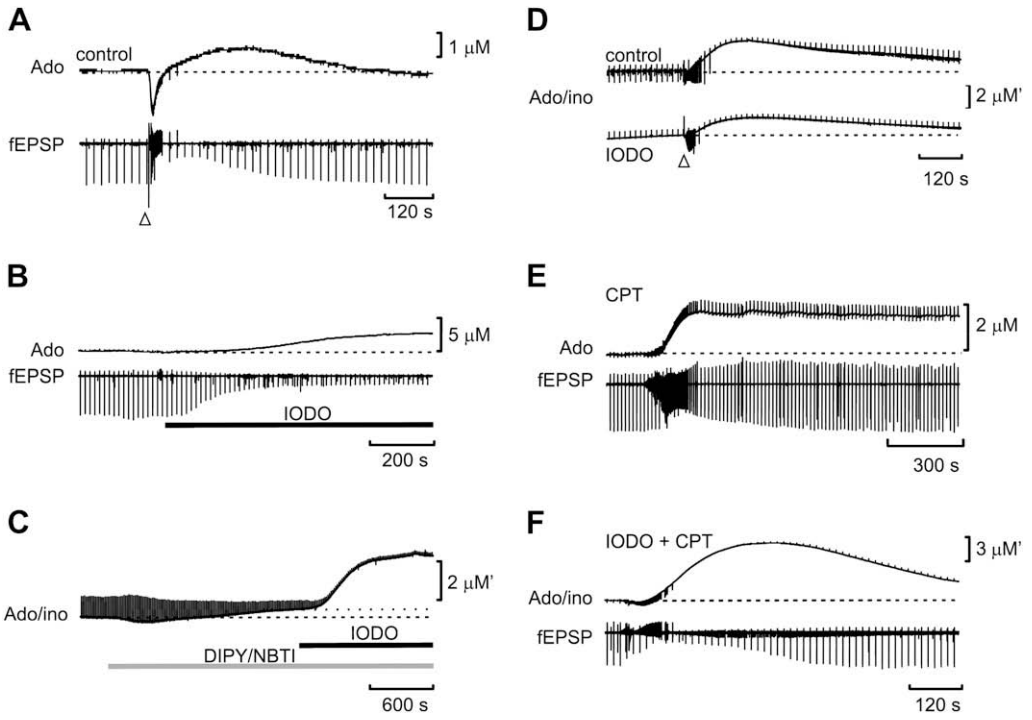

Fig. 6. Direct measurement of adenosine release during seizures and ADK inhibition. (A) Seizure, transient depression of the fEPSP and adenosine release evoked by HFS (arrowhead) under control conditions in nominally Mg^{2+}-free aCSF. (B) Application of the ADK inhibitor iodotuberocidin (IODO; 5 μM) resulted in an increase in extracellular adenosine and a subsequent inhibition of the fEPSP. (C) Pretreatment of hippocampal slices with the adenosine equilibrative nucleoside transport inhibitors DIPY (10 μM) and NBTI (5 μM; grey bar) elevated extracellular adenosine and depressed the fEPSP (periodic upward deflections on the sensor trace), but did not prevent a further elevation in extracellular adenosine caused by IODO (black bar). (D) Comparison of seizure activity and adenosine release in the absence (control; upper trace) and presence of IODO (lower trace). Note in the presence of IODO: (a) reduced amplitude of fEPSP (periodic deflections) before HFS (open triangle); (b) reduced seizure activity immediately after HFS and (c) reduced ado/ino release. (E) Application of the A_1R antagonist CPT (1 μM) elicits a spontaneous seizure and considerable adenosine release, which due to A_1 receptor antagonism by CPT, does not depress the fEPSP. The subsequent interictal activity may have contributed to the continued elevation of extracellular adenosine. (F) Similarly, application CPT, in the continued presence of IODO, evoked a spontaneous seizure and ado/ino release. A subsequent spontaneous seizure (not shown) interrupted the return of the ado/ino signal to baseline.

addition to astrocytes, appreciable staining of granule cells was observed being consistent with the remnant expression of ADK in granular neurons over this developmental time span (Fig. 7B).

4. Discussion

4.1. Adenosine as an endogenous anticonvulsant

Extracellular adenosine increases in the mammalian, including human, brain during seizure activity and both terminates ictal activity and mediates post-ictal refractoriness (During and Spencer, 1992). In the present study we provide the first continuous, on-line determinations of adenosine release during both evoked and spontaneous seizure activity. The former, typically of ~10–20 s duration, elevates extracellular adenosine to approximately 1 μM above basal for several minutes, which is sufficient to both transiently suppress glutamatergic excitation and terminate seizure activity. In contrast, spontaneous seizures occurring in the presence of the adenosine A_1R antagonist CPT last in excess of 50 s and are associated with sustained ~2–3 μM increases in extracellular adenosine. This additional adenosine likely stems from the increased seizure activity, but we cannot exclude the possibility, as described in cerebellum (Wall and Dale, 2007), that the A_1 antagonist is alleviating an autoinhibition of adenosine release by A_1Rs. The persistence of adenosine after seizure activity, as per hypoxia/ischemia (Pearson et al., 2006), likely contributes to post-insult suppression of glutamatergic excitation and may confer a neuroprotective influence. These findings confirm the important role of adenosine and adenosine A_1Rs as powerful inhibitors of epileptiform activity in the mammalian hippocampus (Etherington and Frenguelli, 2004).

We have additionally investigated the primary mechanism regulating the availability of adenosine – ADK – to determine its role in controlling adenosine release and action during seizure activity. We find that inhibition of ADK results in an elevation of extracellular adenosine as measured by adenosine biosensors. However, inhibition of the adenosine equilibrative nucleoside transporters (ENT1 and ENT2) (King et al., 2006) with DIPY and NBTI, which also elevated extracellular adenosine, reduced, but did not fully prevent the IODO-induced rise in adenosine or a subsequent inhibition of the fEPSP caused by this additional extracellular adenosine. These observations indicate that a component, potentially around 40–50%, of the efflux caused by ADK inhibition is via ENTs. The remainder may reflect limited sensitivity of ENT1 and ENT2 to these inhibitors, or may indicate alternative mechanisms for adenosine efflux.

The functional implications of this rise in extracellular adenosine induced by ADK inhibition are a profound A_1R-dependent depression of the fEPSP and a dramatic attenuation of electrically-evoked seizures. These observations thus provide a mechanistic explanation for the anticonvulsant actions of ADK inhibition and support the contention that ADK is a prime target in human epilepsy by virtue of its ability to regulate the extracellular levels of the potent endogenous anticonvulsant adenosine (Boison, 2006). We have also shown that spontaneous seizures evoked by the A_1R antagonist CPT do not elicit greater adenosine release when ADK is inhibited, as might be expected if ADK acted as a "sink" for adenosine under these conditions. This implies that seizure-evoked adenosine release may arise from a distinct, but parallel source not directly influenced by ADK. One such source might be the activity-dependent release of ATP and its subsequent extracellular metabolism to adenosine. Nonetheless, these results imply that basal ADK

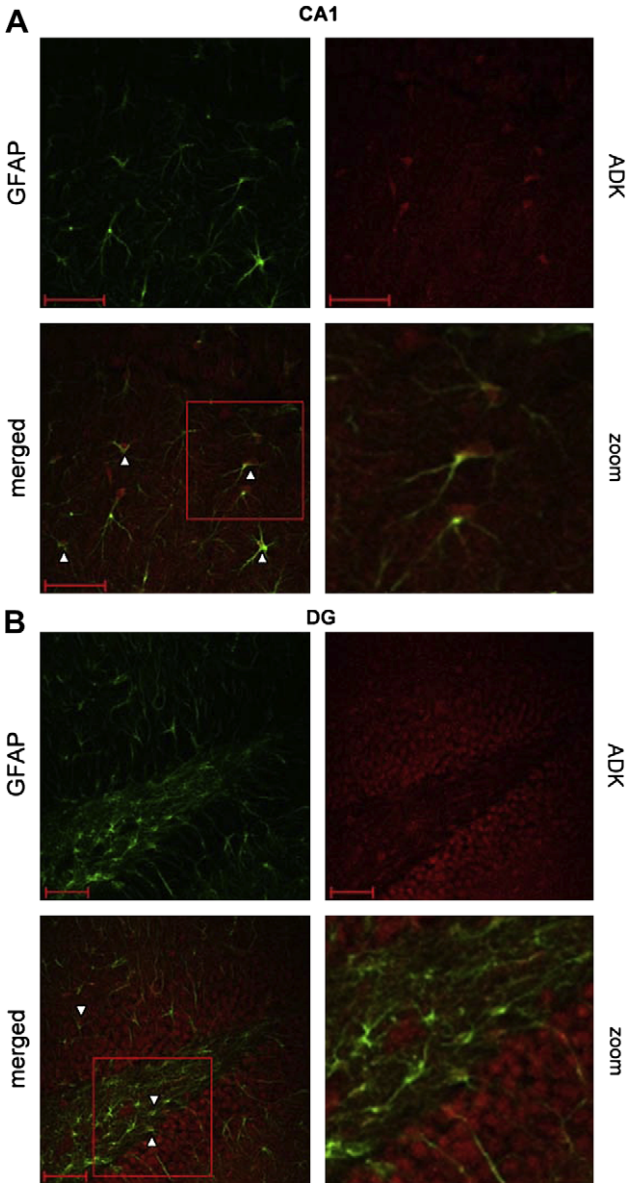

Fig. 7. Immunohistochemical localization of ADK in area CA1 and dentate gyrus. (A) ADK immunoreactivity (red) in area CA1 was primarily located in GFAP-positive astrocytes (green). The merged image shows this co-localization with a number of GFAP/ADK-positive astrocytes (white arrowheads). Boxed region is enlarged in the bottom right panel (*zoom*). In contrast, at this stage of development, dentate gyrus granule neurons remain ADK-positive (red), with some GFAP-positive astrocytes (green) in the hilar region showing ADK immunoreactivity (red). The merged image shows this co-localization (white arrowheads). Boxed region is enlarged in the bottom right panel (*zoom*). Scale bar measures 50 μm.

activity, via maintaining an inward gradient for extracellular adenosine uptake, is permissive not only to seizure activity, but also to other forms of neuronal signaling requiring high-frequency, activity-dependent activation of neurons, such as synaptic plasticity.

4.2. ADK and the control of glutamate release in normal and pathological conditions

Inhibition of ADK, but not ADA, raised basal and electrically-evoked adenosine levels in rat hippocampal (Lloyd and Fredholm, 1995) and spinal cord slices (Golembiowska et al., 1996). Inhibition of ADK has also been shown to inhibit excitatory synaptic transmission, population spikes and cause post-synaptic hyperpolarisation of CA1 neurones, actions likely due to activation of pre- and post-synaptic adenosine A_1Rs (Pak et al., 1994). ADK also plays a role in regulating levels of extracellular adenosine in brain regions other than the hippocampus. For example, 1–2 μM IODO inhibited cerebellar parallel fibre fEPSPs by 50% (Wall et al., 2007), whilst 10 μM IODO inhibited EPSCs in laterodorsal tegmentum by a similar amount (Arrigoni et al., 2001). These studies implicate the intracellular phosphorylation of adenosine to AMP by ADK as an important mechanism by which to limit the release of adenosine into the extracellular space. Our present study extends this work into the influence of ADK, not only for basal glutamatergic synaptic transmission, but also for conditions characterized by excessive or uncontrolled synaptic excitation – i.e., during epileptic seizures.

Previously, the systemic or intracerebral injection of ADK inhibitors has been shown to be anticonvulsant in bicuculline or maximal electroshock seizures in rats (Zhang et al., 1993; Wiesner et al., 1999). More recent approaches have centered on delivering encapsulated myoblasts, fibroblasts or embryonic stem cells lacking ADK into the cerebral ventricles of rats (Huber et al., 2001; Guttinger et al., 2005a,b). Animals that received such grafts were greatly protected from kindling-induced seizures for the duration of graft viability (2–8 weeks). Thus, the potent anticonvulsant action of adenosine kinase inhibitors has led to their candidacy as potential therapeutic agents in the treatment of epilepsy (Kowaluk and Jarvis, 2000; McGaraughty et al., 2005), a candidacy that would be strengthened by greater insight into cellular mechanisms of seizure suppression by ADK inhibition.

Accordingly, we have shown that ADK inhibition results in a profound suppression of excitatory synaptic transmission and electrically-evoked seizures in rat hippocampal slices. These effects of ADK inhibition were reversed by the antagonism of A_1Rs, which reaffirms the importance of ADK in both regulating the concentration of extracellular adenosine and, in turn, the importance of extracellular adenosine and A_1Rs in controlling glutamatergic excitation under normal and pathological conditions. These conclusions are strengthened by direct and real-time observation of elevation of extracellular adenosine during inhibition of ADK. These studies thus provide the mechanistic basis for ADK-targeted antiepileptic therapy.

4.3. Regulation of ADK expression and activity

To what extent do ADK levels, activity or localization change that might have a bearing on the extracellular concentration of adenosine, the control of excitatory synaptic transmission and the propensity for seizure activity? Recent work has clearly shown that ADK displays an intriguing pattern of developmental expression in the mouse (Studer et al., 2006), which we have confirmed here in the rat. Initially, ADK expression is predominately neuronal, but in area CA1, migrates to an exclusively astrocytic distribution by postnatal day 14. This may have acute physiological effects reflected by the lower adenosine tone in younger rather than older animals (Studer et al., 2006), which could underlie the vulnerability of immature and young animals to seizures, and the resistance of older animals to seizures (Wong, 2005). Furthermore, astrocytes contribute to this adenosine tone via a parallel pathway involving the SNARE-dependent release of ATP and its subsequent metabolism to adenosine (Pascual et al., 2005). Thus, astrocytes mediate both the release (via ATP) and sequestration (via ADK) of extracellular adenosine and may accordingly be the primary regulators of the presence and effects of adenosine.

However, this important role is not without risk: astrogliosis, a characteristic of epileptic foci (Khurgel and Ivy, 1996), results in an increase in ADK expression and an exacerbation of the epileptic state (Gouder et al., 2004). That it is the high levels of ADK and not astrogliosis *per se* that engenders seizure activity is indicated by the

increased seizure propensity observed in mice overexpressing ADK (Fedele et al., 2005). Thus, a vicious cycle could ensue whereby seizure activity instigates astrogliosis and ADK overexpression, which in turn results in less ambient adenosine and hence a lower threshold for subsequent seizures (Boison, 2008).

ADK activity and expression are also under more acute regulatory control. For example, shortly after kainic acid treatment there is a transient downregulation of ADK levels, which may exert a protective, anticonvulsant role (Gouder et al., 2004). In addition, hypoxia/ischemia is known to cause inhibition (Decking et al., 1997; Lynch et al., 1998; Kobayashi et al., 2000) and downregulation of ADK (Pignataro et al., 2008) which may contribute, at least in part, to the rapid increase in extracellular adenosine seen under these conditions (Frenguelli et al., 2003, 2007; Pearson et al., 2006), whilst ADP-induced aggregation of ADK exerts another level of control (Sen et al., 2006). Thus, the dynamic regulation of ADK expression and activity is likely to have an important bearing on the availability and role of extracellular adenosine.

4.4. Implications of ADK inhibition for the treatment of pathological conditions

As phosphorylation of adenosine to AMP by ADK is normally the predominant route of adenosine metabolism, inhibition of ADK represents a potential mechanism by which adenosine release may be amplified. This is made all the more attractive since this amplification will be exaggerated at sites with a high turnover of adenine nucleotides – i.e., those experiencing seizures or hypoxia/ischemia – and which would benefit most from increased adenosine levels. However, the role of ADK in a range of physiological functions, including purine salvage, and the fact that ADK knockout is lethal shortly after birth due to liver toxicity (Boison et al., 2002) augurs caution in the use of chronic systemic ADK inhibitors. Nonetheless, considerable medicinal chemistry has been performed on ADK inhibitors and recent compounds show efficacy in pain and epilepsy models with fewer side effects compared with adenosine agonists (McGaraughty et al., 2005). An alternative approach is the local delivery of adenosine-releasing cells, for example those lacking ADK, into an epileptic focus (Boison, 2006, 2007). This would have the advantage of circumventing side effects associated with administration of adenosine receptor agonists, including reduced blood pressure and heart rate, hypothermia and motor depression. Although the attempts at this have been successful in preventing seizures, the short-term viability of these implants does not favour their use in chronic therapy. However, with current advances in stem cell technology, it may not be long before an injection of adenosine-releasing stem cells, capable of integration into the neuropil of the epileptic focus, is offered in preference to removal of temporal lobe structures.

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