J Physiol 587.18 (2009) pp 4497–4508

The Journal of Physiology

Adenosine signalling at immature parallel fibre–Purkinje cell synapses in rat cerebellum

Alison Atterbury and Mark J. Wall

Neuroscience Group, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

The purine adenosine is an extracellular signalling molecule involved in a large number of physiological and pathological conditions throughout the mammalian brain. However little is known about how adenosine release and its subsequent clearance change during brain development. We have combined electrophysiology and microelectrode biosensor measurements to investigate the properties of adenosine signalling at early stages of cerebellar development, when parallel fibre–Purkinje cell synapses have recently been formed (postnatal days 9–12). At this stage of development, we could detect little or no inhibitory A_1 receptor tone in basal conditions and during trains of stimuli. Addition of pharmacological agents, to inhibit adenosine clearance, had only minor effects on synaptic transmission suggesting that under basal conditions, the concentration of adenosine moving in and out of the extracellular space is small. Active adenosine release was stimulated with hypoxia and trains of electrical stimuli. Although hypoxia released significant concentrations of adenosine, the release was delayed and slow. No adenosine release could be detected following electrical stimulation in the molecular layer. In conclusion, at this stage of development, although adenosine receptors and the mechanisms of adenosine clearance are present there is very little adenosine release.

(Received 4 June 2009; accepted after revision 30 July 2009; first published online 3 August 2009)
Corresponding author M. J. Wall: Neuroscience Group, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK. Email: mark.wall@warwick.ac.uk

Abbreviations AD, adenosine deaminase; ADO biosensor, adenosine biosensor/INO inosine biosensor; CPA, N^6-cyclopentyladenosine; 8-CPT, 8-cyclopentyltheophylline; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; PF, parallel fibre; PNP, purine nucleoside phosphorylase; XO, xanthine oxidase.

The purine adenosine is involved in many diverse CNS processes including sleep, locomotion and respiration and provides neuroprotection during hypoxia and ischaemia. (for review see Boison, 2007). The mechanism of how adenosine is released into the extracellular space is still, in many cases, unclear (for review see Wall & Dale, 2008) but could occur by extracellular ATP metabolism (ATP released by exocytosis, Edwards *et al.* 1992; Dale, 1998; or through gap junction hemi channels, Pearson *et al.* 2005), adenosine transport from the cell cytoplasm (Craig & White, 1993; Sweeney, 1996) or possibly by the exocytosis of adenosine itself (Wall & Dale, 2007). Once released, the actions of adenosine are terminated by a combination of extracellular metabolism to inosine (by the enzyme adenosine deaminase) and transport into neuronal or glial cytoplasm by equilibrative transporters such as ENT1 and ENT2 or concentrative transporters (for review see Noji *et al.* 2004; Baldwin *et al.* 2004). Once internalised adenosine is phosphorylated (by adenosine kinase) to form AMP, maintaining low levels of intracellular adenosine (for review see Boison, 2007). Because the levels of adenosine are determined by the interplay of enzymes and transporters, a number of studies have investigated which components play a major role in determining extracellular adenosine concentration. In many studies blocking adenosine kinase has a major effect on adenosine concentration where extracellular metabolism (via adenosine deaminase) appears less important (Pak *et al.* 1994; Lloyd & Fredholm, 1995; Wall *et al.* 2007).

Although adenosine release probably occurs in most brain areas, relatively little is known about how adenosine release, receptor signalling and clearance change during brain development. At the calyx of Held in mice, A_1 receptor expression declines during the second postnatal week (Kimura *et al.* 2003). In the hippocampus, there is a developmental increase in the A_1 receptor tone at CA1 synapses, with no change in receptor properties (Psarropoulou *et al.* 1990). There is also a shift of adenosine kinase expression from neurones to astrocytes (Studer *et al.*

DOI: 10.1113/jphysiol.2009.176420

2006). Adenosine appears a more potent anticonvulsant in hippocampal slices from adult rats compared to immature rats (Descombes *et al.* 1998). This is supported by the observation that pair pulse facilitation shows greater enhancement in young adult slices (P28–35) compared to juvenile slices (P15–21) even though the amount of synaptic depression was similar (Dumas & Foster, 1998).

Thus the aim of this study is to combine biosensor measurements and electrophysiology to investigate the properties of adenosine signalling at a period early in cerebellar development, soon after synapse formation. The glutamatergic parallel fibre–Purkinje cell synapse is formed around postnatal day 5 in the rat (Altman, 1972) and we have investigated the properties of adenosine signalling between postnatal days 9 and 12 (the earliest period when extracellular field parallel fibre–Purkinje cell EPSPs can reliably be measured). Previous studies have shown that adenosine A_1 receptors are present at the synapses at postnatal days 10–15 (Takahashi *et al.* 1995), with A_1 receptors expressed in the cerebellum before birth (Weaver, 1996). In previous studies (from P21–28) rats we have shown that there is a tone of extracellular adenosine which tonically inhibits parallel fibre–Purkinje cell synapses. The concentration of this adenosine is controlled, in the main, by the level of adenosine kinase activity (Wall *et al.* 2007). The source of this extracellular adenosine is currently unclear but it does not appear to arise from action potential-dependent release (Wall & Dale, 2007).

Methods

Preparation of cerebellar slices

Transverse slices of cerebellar vermis (400 μm) were prepared from male Wistar rats, at postnatal days 9–28 (P9–28). As described previously (Wall *et al.* 2007) and in accordance with the UK Animals (Scientific Procedures) Act 1986, male rats were killed by cervical dislocation and decapitated. The cerebellum was rapidly removed and slices were cut on a Microm HM 650V microslicer in cold (2–4°C) high Mg^{2+}, low Ca^{2+} artificial cerebrospinal fluid (aCSF; composed of, mM: 127 NaCl, 1.9 KCl, 7–8 $MgCl_2$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 10 D-glucose (pH 7.4 when bubbled with 95% O_2–5% CO_2, 300 mosmol l^{-1}). Slices were stored in normal aCSF (1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$) at room temperature for 1–6 h before recording.

Extracellular recording

An individual slice was transferred to a recording chamber, submerged in aCSF and perfused at 6 ml min^{-1} (30–32°C). The slice was placed upon a suspended grid to allow perfusion from above and below, and this reduce the likelihood of hypoxia. Furthermore, all solutions were vigorously bubbled (95% O_2–5% CO_2) and all tubing had low gas permeability (Tygon). For the stimulation of parallel fibres, square voltage pulses (2–5 V, 200 μs duration) were delivered by an isolated pulse stimulator (model 2100, AM systems, Everett, WA, USA) via a concentric bipolar metal stimulating electrode (FHC Inc., Bowdoin, ME, USA) placed on the surface of the molecular layer. The recording electrode (an aCSF filled microelectrode) was placed on the same track along which the parallel fibres travel ('on-beam', Yuan & Atchison, 1999). A typical extracellular field potential consisted of a presynaptic fibre volley followed by a component which could be blocked by 1 μM TTX and greatly reduced by 5 mM kynurenate. This component is probably produced by parallel fibre mediated glutamatergic excitatory synaptic currents in Purkinje cells and interneurones (Clark & Barbour, 1997). Parallel fibre EPSP amplitude was estimated from the kynurenate sensitive potential, which was measured by subtracting what remained in kynurenate from control potentials. Confirmation of PF EPSP identity was achieved by evoking pairs of EPSPs (interval 50 ms) and observing facilitation (20–30%), and by examining the pharmacological profile (inhibition by A_1, $GABA_B$ or mGlu4R receptor agonists). Extracellular recordings were made using an ISO-DAM extracellular amplifier (WPI, Sarasota, FL, USA), filtered at 1 kHz and digitised on-line (10 kHz) with a Micro 1401-2 (Mark 2) interface controlled by Spike software (v. 6.1) (Cambridge Electronic Design, Cambridge, UK).

Purine biosensors

Biosensors were obtained from Sarissa Biomedical Ltd (Coventry, UK). In brief the biosensors consisted of entrapped enzymes (adenosine deaminase, AD; purine nucleoside phosphorylase, PNP; and xanthine oxidase, XO) within a matrix that was deposited around a Pt or Pt/Ir (90/10) wire etched to ~50 μm (Llaudet *et al.* 2003). The biosensor had an exposed length of ~500 μm that was coated with enzymes and thus capable of detecting purines. Biosensors were screened, which greatly reduced the responses to electro-active interferents (such as 5-HT, noradrenaline, dopamine and ascorbate). Three types of purine biosensor were used in this study to identify the released substances. Firstly, a screened null sensor, possessing the matrix but no enzymes, was used to control for the release of any non-specific electro-active interferents. Secondly, screened biosensors containing PNP and XO (responsive to inosine and hypoxanthine) and NP, XO and AD ((INO) responsive to inosine, hypoxanthine and adenosine (ADO)) were used. The difference signal between these two types of biosensors gave the specific adenosine and adenosine metabolite (inosine and

hypoxanthine) signals. A full description of the properties of the biosensors has already been published but they show a linear response to increasing concentration of analyte, are fast to respond, and have a rise time of less than 10 s (Llaudet *et al.* 2003).

The biosensors were positioned just above the surface of the molecular layer. Biosensors were bent so their longitudinal surface was parallel to the slice surface, thus increasing the detection area (Wall & Dale, 2007). Biosensors were calibrated with known concentrations (10 μM) of adenosine and inosine. The screening of biosensors was checked by applying 10 μM of 5-HT. Calibration was performed before the slice was present in the perfusion chamber and after the slice had been removed; this allowed measurement of any reduction in sensitivity during the experiment. Biosensor signals were acquired at 1 kHz with a Micro 1401-2 (Mark 2) interface using Spike (v. 6.1) software.

Drugs and substances

All drugs were made up as 10–100 mM stock solutions, stored frozen and then thawed and diluted with aCSF on the day of use. Adenosine, inosine, *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), 8-cyclopentyltheophylline (8-CPT), and dipyridamole were purchased from Sigma. Iodotubercidin was purchased from Sigma and Molekula (Fine Chemicals, Gillingham, Dorset, UK). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was purchased from Tocris-Cookson (Bristol, UK). Kynurenic acid and CNQX were purchased from Ascent Scientific (Bristol, UK). Hypoxia was induced by changing the inflow from oxygenated aCSF to aCSF bubbled with N_2 (95%)–CO_2 (5%).

Results

The tonic inhibition of parallel fibre–Purkinje cell synapse by endogenous adenosine is greatly reduced or absent in slices from P9–12 rats

Previous studies have shown that A_1 adenosine receptors at parallel fibre–Purkinje cell synapses tonically inhibit synaptic transmission, suggesting the continual presence of extracellular adenosine (Takahashi *et al.* 1995; Wall *et al.* 2007). We have investigated whether such tonic A_1 receptor-mediated inhibition is present at a developmental period soon after the formation of parallel fibre–Purkinje cell synapses (postnatal days 9–12). Application of the A_1 receptor antagonist 8-CPT (1–2 μM) had no effect on the amplitude of PF EPSPs or the paired pulse ratio in 9 out of 20 slices (45%, Fig. 1*A* and *B*). In the remaining 11 slices, 8-CPT increased PF EPSP amplitude by 13.4 ± 2.6%. Overall the block of A_1 receptors only increased EPSP amplitude by 7.4 ± 5.6% ($n = 20$), which is significantly less ($P < 0.001$) than that observed in interleaved experiments on slices from P21–28 rats (45.6 ± 7% increase, 8 slices Fig. 1*B* and see Wall *et al.* 2007). There was no correlation between the age of rat (between P9 and 12) and the presence or absence of an adenosine receptor tone.

A reduced adenosine A_1 receptor tone, early in development, could result from low receptor expression or the expression of low affinity A_1 receptors. This was tested by applying adenosine (100 μM) to inhibit PF EPSPs ($n = 54$ slices) and then following wash, applying 8-CPT (1–2 μM) to measure the A_1 receptor tone. There were some synapses where adenosine (100 μM) had little or no effect on synaptic transmission. These synapses were not investigated further as presumably they would have no A_1 receptor tone. In the remaining synapses (where 100 μM adenosine produced at least a 50% reduction in PF EPSP amplitude), adenosine produced the same reduction in PF EPSP amplitude whether or not an inhibitory adenosine receptor tone was present (no tone, 70.5 ± 5.4% *vs.* 68.5 ± 4.7% with mean tone of 14.3 ± 2.8%; Fig. 1*A* and *C*). The inhibition produced by adenosine (100 μM) was not different from that observed in slices from P21–28 rats (61.5 ± 2.1%, $n = 47$ slices; and see Wall *et al.* 2007). There was also no significant change in the adenosine-mediated increase in paired pulse facilitation between young (increased from 1.38 ± 0.02 to 1.85 ± 0.04, $n = 54$) and adult slices (increased from 1.45 ± 0.02 to 1.92 ± 0.06, $n = 47$). To check the properties of the A_1 receptors more carefully, dose–response curves were constructed for the non-hydrolysable A_1 receptor agonist N^6-cyclopentyladenosine (CPA). The curves constructed from P9–12 and P21–28 could be superimposed (Fig. 1*D*). Thus the lack of adenosine tone at immature synapses does not appear to result from changes in A_1 receptor properties or expression. In all experiments, the sensitivity of synapses was first tested (with 100 μM adenosine) and synapses were rejected if adenosine did not produce at least a 50% reduction in EPSP amplitude.

The presence or absence of tonic A_1 receptor activation has marked effects on trains of activity

Numerous studies have demonstrated that presynaptic inhibition has important effects on trains of activity, with a reduction in initial transmitter release allowing synaptic transmission to persist for longer during a train of stimuli (for example see Kreitzer & Regehr, 2000; Kimura *et al.* 2003; Wong *et al.* 2006). We have investigated what effect the adenosine tone has on trains of EPSPs (100 EPSPs, 20 Hz) in young and mature slices. During trains of stimuli, transmission in slices from P9–12 rats was first facilitated and then depressed (Fig. 2*A*). PF EPSP amplitude was increased to a maximum of 157 ± 10.9% of

the first EPSP amplitude ($n = 6$) at around the 10th EPSP and then decreased to an amplitude less than the first EPSP by around the 70th EPSP. Blocking of A_1 receptors (with 2 μM 8-CPT) had no significant effect on the amplitude of PF EPSPs during the train (Fig. 2*A*). This was expected since there is little or no tonic activation of A_1 receptors, but it also suggests that there is little or no adenosine release during the train (see Wall & Dale, 2007). Addition of (100 μM) adenosine produced a marked change in the trains of PF EPSPs, with significantly ($P < 0.01$) greater facilitation (maximum 215 ± 27%) and the EPSP amplitude remaining elevated above the amplitude of the first EPSP for the duration of the train (Fig. 2*A*).

Trains of EPSPs were also evoked at parallel fibre–Purkinje cell synapses in slices from P21–28 rats where there is a much larger inhibitory A_1 receptor tone (Fig. 2*B*). Like immature synapses, PF EPSP amplitude was facilitated (146 ± 8.1%) and then depressed, although this depression happened much earlier in the train (EPSP amplitude less than first EPSP amplitude by around the 30th EPSP, Fig. 2*B*). Addition of adenosine (100 μM) caused a marked increase in the degree

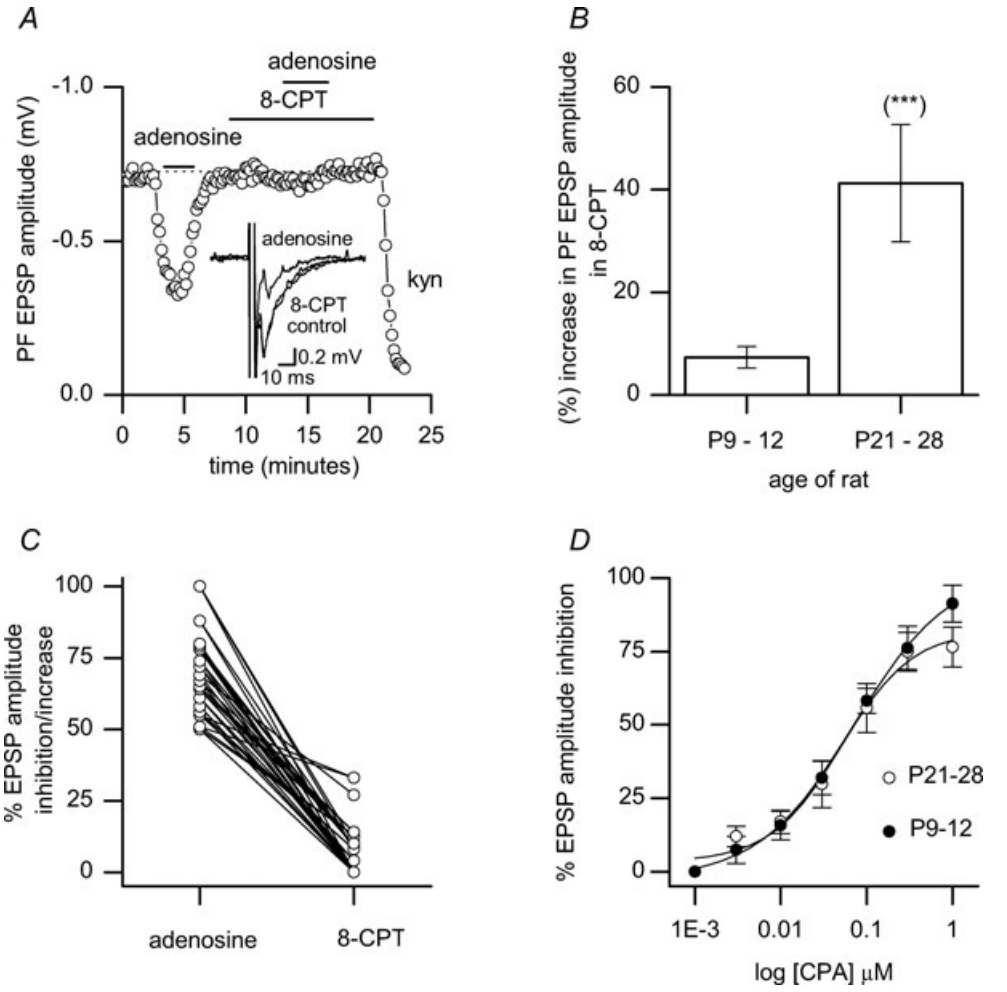

Figure 1. Little or no tonic activation of presynaptic A_1 receptors at immature parallel fibre to Purkinje cell synapses

A, plot of the amplitude of individual parallel fibre (PF) EPSPs (field) against time in a slice from a P10 rat. Although application of adenosine (100 μM) produced a decrease in EPSP amplitude of ∼55%, block of A_1 receptors with 8-CPT (2 μM) had no effect on EPSP amplitude. Addition of adenosine (100 μM) in the presence of 8-CPT had no effect on EPSP amplitude showing A_1 receptors were blocked. Synaptic transmission was blocked at the end of the experiment with 5 mM kynurenate (Kyn). Inset, superimposed average EPSPs from *A* in control, adenosine (100 μM) and 8-CPT (2 μM). *B*, summary of the effects of 8-CPT on the amplitude of PF EPSPs recorded from 20 slices from P9–12 rats and 8 slices from P21–28 rats. *C*, plot of the reduction in PF EPSP amplitude following application of adenosine (100 μM) against the increase in PF EPSP amplitude following block of A_1 receptors with 8-CPT (2 μM). Adenosine was applied first, then washed and then 8-CPT was applied (as in *A*). *D*, concentration–response curves constructed for the reduction in PF EPSP amplitude by the A_1 receptor agonist CPA in P9–12 ($n = 5$) and P21–28 ($n = 4$) slices.

of facilitation ($255 \pm 32\%$), which was maintained at ~200% throughout the train (Fig. 2*B*). Addition of 8-CPT (1–2 μM) caused a small but significant ($P < 0.05$) reduction in the degree of facilitation (from $146 \pm 8.1\%$ to $129 \pm 32\%$) followed by almost complete depression of transmission (11% of first EPSP amplitude) of EPSP amplitude and then recovery (to ~50% of the first EPSP, Fig. 2*B*). The actions of 8-CPT appeared larger later in the train suggesting that adenosine release occurred during the train (see Wall & Dale, 2007).

There is a low concentration of extracellular adenosine metabolites in immature slices

In a previous study (Wall *et al.* 2007), although A_1 receptors were tonically activated in cerebellar slices from P21–28 rats, biosensors placed in or above the slices could not detect adenosine but instead detected the adenosine metabolites inosine and hypoxanthine. It can be predicted that the concentration of these metabolites would be reduced in immature slices since there is little or no A_1 receptor tone. The extracellular purine tone was measured by moving purine (ADO and INO) and null sensors close to the slice surface. Sensors were bent so they were parallel to the slice surface, thus increasing the amount of sensing surface in close proximity to the slice (this will increase the measured purine tone compared to Wall *et al.* (2007), where the sensors were not bent). The advantage of this experiment is that the slice is not damaged by sensor insertion and no resultant purine release occurs (see Wall *et al.* 2007). As predicted, there was only a small increase in baseline current when ADO or INO sensors were moved close to the slice surface (mean change in current: 43.4 ± 4.1 pA, ADO sensors; 61.7 ± 18 pA, INO sensors; $n =$ 15 slices; Fig. 3*A*). There was little or no change in baseline current when a null sensor was moved close to or away from the slice surface (Fig. 3*A*). After correcting for calibration, there was no significant difference between the current measured on the ADO and INO sensors, and thus the current is not produced by adenosine but is produced by the metabolites (inosine and hypoxanthine, see Wall *et al.* 2007). If we assume that all of the purine detected is inosine then the net concentration of inosine measured on the surface of the slice was 0.25 ± 0.03 μM ($n = 15$ slices, Fig. 3*B*). Interleaved experiments with slices from P21–28 rats showed an almost 5-fold larger increase in current when purine sensors were moved close to the slice surface (224.3 ± 35.9 pA, Fig. 3*A*, $n = 8$ slices, equivalent to 1.6 ± 0.3 μM inosine also see Wall *et al.* 2007). The low extracellular concentration of adenosine metabolites suggests that the lack of adenosine A_1 receptor activation does not result from the metabolism of significant concentrations of extracellular adenosine (Fig. 3*B*).

Interrupting adenosine clearance has only small effects on the inhibitory A_1 receptor tone

The lack of adenosine tone could stem from either the rapid removal of adenosine or reduced extracellular production. Adenosine is removed from the extracellular space by a combination of extracellular metabolism (adenosine deaminase) and uptake (equilibrative and

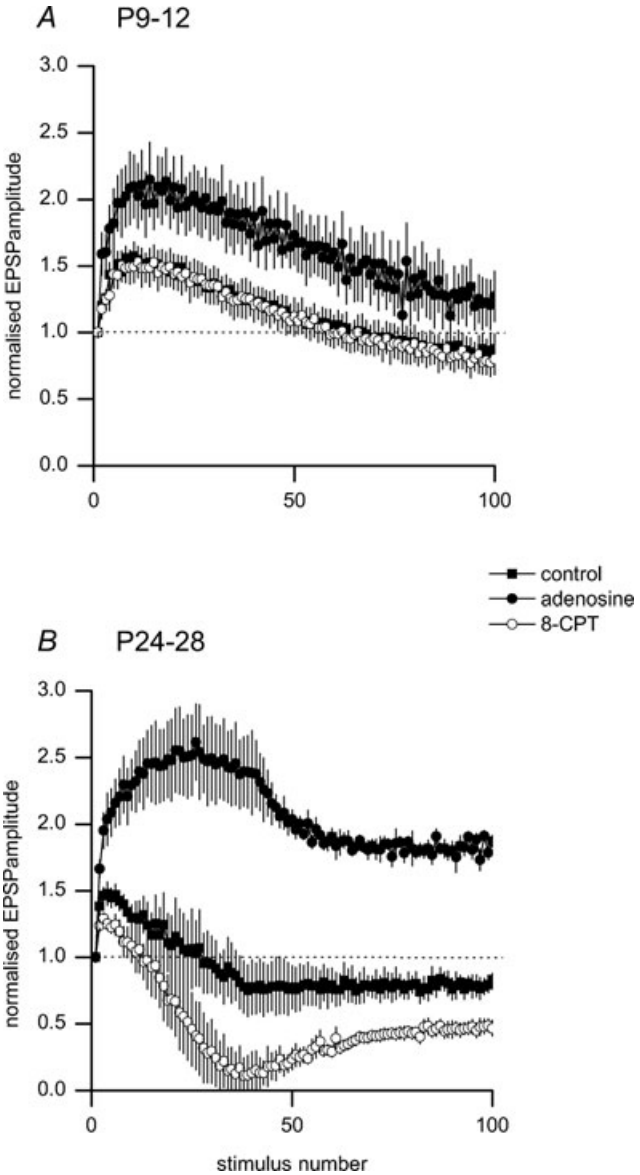

Figure 2. An A_1 receptor tone has marked effects on trains of activity
A, plot of normalised EPSP amplitude (relative to the first EPSP amplitude) against stimulus number in control, 100 μM adenosine and 2 μM 8-CPT for 6 parallel fibre–Purkinje cell synapses in slices from P9–12 rats. *B*, plot of normalised EPSP amplitude (relative to the first EPSP amplitude) against stimulus number in control, 100 μM adenosine and 2 μM 8-CPT for 5 parallel fibre–Purkinje cell synapses in slices from P24–28 rats. Stimulus frequency was 20 Hz.

concentrative transporters) followed by intracellular conversion to adenosine monophosphate by adenosine kinase. We have used pharmacological agents to reduce adenosine clearance and thus investigated the rate of basal adenosine production.

Adenosine metabolism. To investigate the role of metabolism, PF EPSPs recordings were made in the presence of EHNA. EHNA blocks the enzyme adenosine deaminase (Agarwal, 1982) and thus should produce extracellular adenosine accumulation and inhibition of PF EPSPs. Application of EHNA (20 μM) had little effect on PF EPSPS; the amplitude (1.2 ± 1.6% reduction, 5 slices, range 0 to 12%, Fig. 4*A*) and the paired pulse ratio (1.33 ± 0.3 *vs.* 1.34 ± 0.3) were not significantly changed.

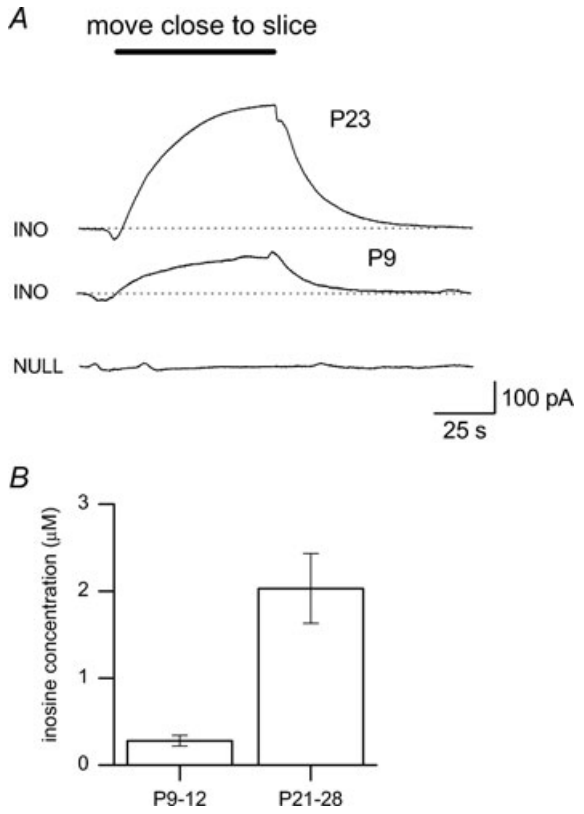

Figure 3. The concentration of extracellular adenosine metabolites is reduced in immature slices
A, example biosensor traces from experiments to measure the concentration of purines at the surface of slices from P23 and P9 rats (interleaved experiments). After the inosine (INO) biosensor was moved close to the slice surface there was a rapid increase in the baseline current of ~350 pA (P23) and ~100 pA (P9). Movement of the null sensor to the surface of the slice had no effect in P23 or P9 slices (P23 illustrated). *B*, plot of the mean concentration of inosine measured close to the slice surface (from 15 slices from P9–12 rats and 8 slices from P21–28 rats). The concentration of inosine was determined following calibration of the biosensor and assumes all the current arises from inosine.

Thus as suggested by the biosensor results, there appears to be little ongoing adenosine metabolism.

Equilibrative transporters. We have investigated the roles of the equilibrative transporters (ENT1 and ENT2) using the inhibiters NBTI and dipyridamole (Dunwiddie & Diao, 1994; Frenguelli *et al.* 2007). Application of 10 μM dipyridamole and 5 μM NBTI for prolonged periods (30 min) produced a mean reduction in EPSP amplitude of 16.9 ± 3.5% (31 slices). In 15 out of the 31 slices NBTI/dipyridamole had no effect on EPSP amplitude (Fig. 5*C*). In the remaining 16 slices, EPSP amplitude was

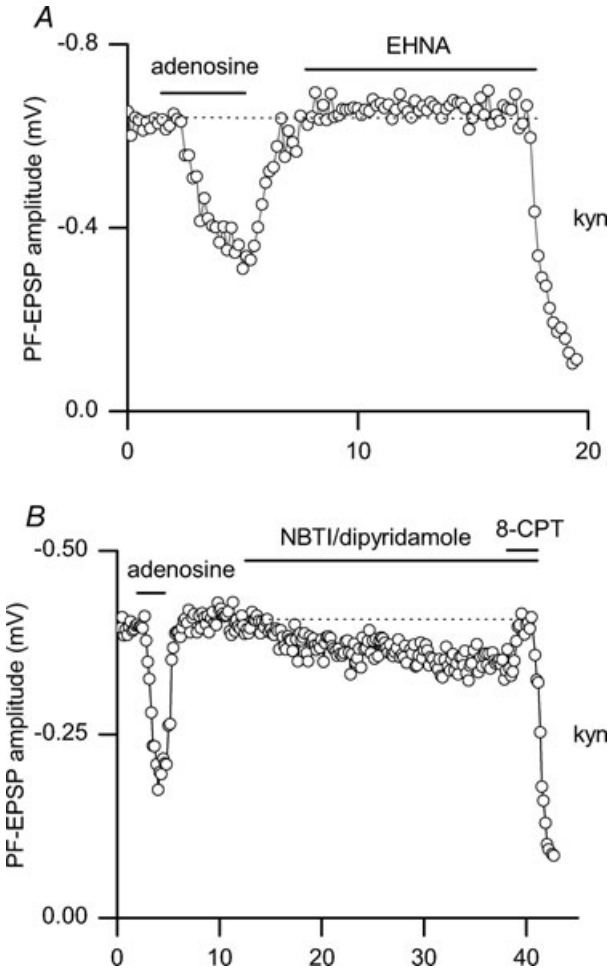

Figure 4. Inhibition of extracellular adenosine metabolism and equilibrative transport has little effect on synaptic transmission
A, although PF EPSPs were markedly reduced by adenosine (100 μM, ~50%), the adenosine deaminase inhibitor EHNA (20 μM) had no effect on PF EPSP amplitude. *B*, block of equilibrative transport with 5 μM NBTI and 10 μM dipyridamole caused a small reduction (~15%) in PF EPSP amplitude, which was reversed by blocking A_1 receptors with 8-CPT (1.5 μM). Addition of adenosine (100 μM) at the beginning of the experiment reduced EPSP amplitude by ~55%. The graphs in *A* and *B* plot the amplitude of individual PF EPSPs against time. PF EPSPs were blocked at the end of the experiment with 5 mM kynurenate (kyn).

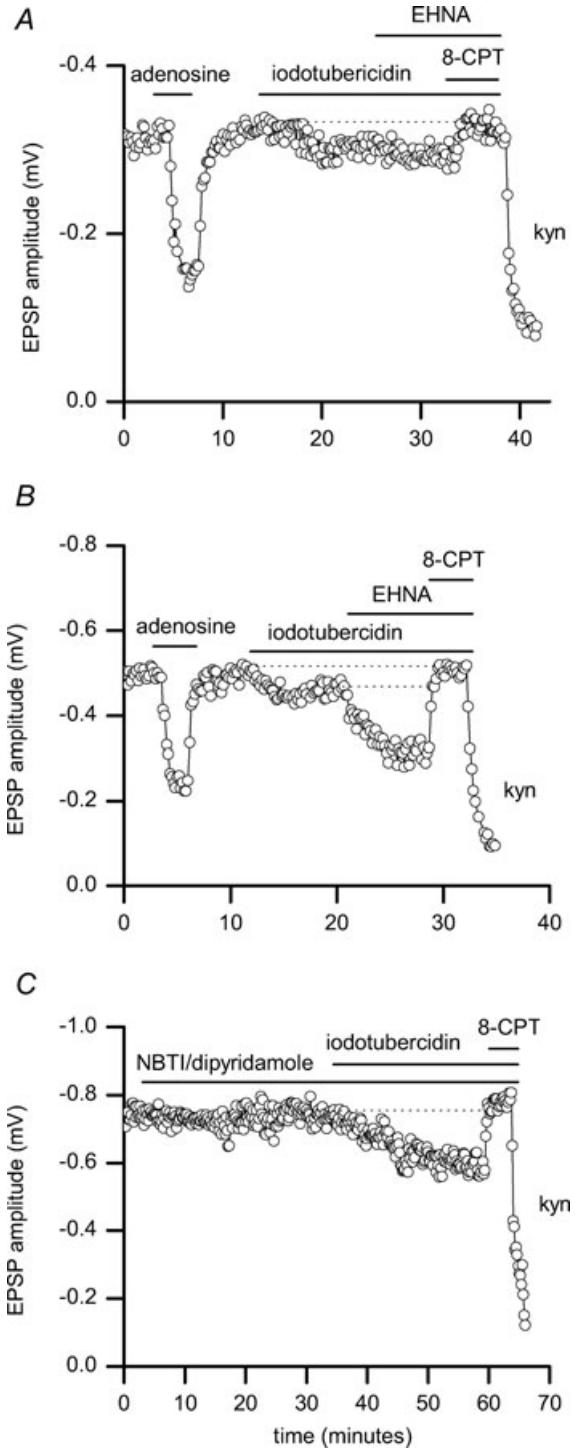

Figure 5. Inhibition of adenosine kinase has minor effects on synaptic transmission
A, the block of adenosine kinase with iodotubercidin (2 μM) produced a small reduction in PF EPSP amplitude (~20%). Block of adenosine deaminase (EHNA 20 μM) did not increase the block, which was reversed by blocking A_1 receptors with 8-CPT (1.5 μM). *B*, following addition of iodotubercidin (2 μM), block of extracellular adenosine metabolism (EHNA 20 μM) produced a marked increase in the inhibition of PF EPSP amplitude. The block was reversed by addition of 8-CPT (2 μM). *C*, pre-incubation with 5 μM NBTI and 10 μM dipyridamole did not occlude the effects of blocking adenosine kinase (2 μM iodotubercidin). The plots in *A*, *B* and *C* are of individual PF EPSPs against time with synaptic transmission blocked at the end of the experiment with kynurenate (kyn, 5 mM).

reduced by 32.7 ± 4% (Fig. 4*B*) with an increase in paired pulse ratio from 1.4 ± 0.05 to 1.6 ± 0.08. This inhibition was reversed by blocking A_1 receptors with 8-CPT (2 μM). There was no correlation between the reduction in EPSP amplitude produced by NBTI/dipyridamole and the presence or absence of an A_1 receptor tone. These results are similar to those observed at mature slices, where the actions of NBTI/dipyridamole were heterogeneous, although the mean inhibition was higher (~50%; Wall *et al.* 2007).

It is possible that any increase in extracellular adenosine concentration, following the block of equilibrative transport, is rapidly reduced by metabolism. Thus the adenosine deaminase inhibitor EHNA (20 μM) was applied in the presence of NBTI/dipyridamole (after 20–30 min pre-incubation). In six cells, pre-incubation with NBTI/dipyridamole had no effect on EPSP amplitude and nor did the addition of EHNA. In three cells, NBTI/dipyridamole decreased EPSP amplitude by 30 ± 0.5%, and addition of EHNA produced a further decrease in EPSP amplitude of 27 ± 3%. Thus some of the adenosine is metabolised by adenosine deaminase.

Adenosine kinase. Once transported into cells, adenosine is phosphorylated to form AMP by the enzyme adenosine kinase, thus maintaining low levels of intracellular adenosine and preventing efflux via equilibrative transporters (for review see Boison, 2006). To assess the importance of adenosine kinase activity, the adenosine kinase blocker iodotubercidin was applied. Iodotubercidin (2 μM) reduced PF EPSP amplitude (25 ± 3%) and increased the paired pulse ratio (1.3 ± 0.1 to 1.47 ± 0.1) in 14 out of 21 slices (Fig. 5*A*). The effects of iodotubercidin were completely reversed by blocking A_1 receptors with 8-CPT (1 μM). In the remaining seven slices, iodotubercidin had no effect on PF EPSP amplitude. The effects of iodotubercidin were significantly smaller than those observed in interleaved adult slices (68% inhibition, $n = 3$) and in published data (Wall *et al.* 2007).

To check if the elevated concentration of extracellular adenosine, produced by blocking adenosine kinase, is metabolised, we co-applied EHNA with iodotubercidin. The co-application of EHNA and iodotubercidin reduced PF EPSP amplitude by 50.7 ± 7.8% ($n = 6$), which was significantly ($P < 0.05$) higher than that observed with the block of adenosine kinase alone. This increased reduction in PF EPSP amplitude was reversed by addition

of 8-CPT (1 μM). To examine this in more detail we applied EHNA (20 μM), after first blocking adenosine kinase with iodotubercidin (2 μM). We found the effects of EHNA mixed, producing little or no effect in three slices (Fig. 5*A*) and producing a large block in another four slices (Fig. 5*B*).

We have tried to occlude the increase in adenosine tone produced by iodotubercidin by blocking equilibrative transport. Iodotubercidin would be expected to have no effect if adenosine translocation is blocked. In six slices, following application of NBTI/dipyridamole, block of adenosine kinase with iodotubercidin still reduced EPSP amplitude by $25.3 \pm 3.5\%$ (Fig. 5*C*). Thus, as observed with mature slices (Wall *et al.* 2007), NBTI/dipyridamole does not occlude the actions of iodotubercidin. In four slices, there was little or no adenosine transport as neither iodotubercidin nor NBTI/dipyridamole had any effect on synaptic transmission.

Adenosine release is delayed or reduced at immature parallel fibre–Purkinje cell synapses

Our results demonstrate that there is a low concentration of extracellular adenosine at parallel fibre–Purkinje cell synapses in slices from P9–12 rats. Since blocking clearance had little effect, there is little release of adenosine under basal conditions. To investigate the active release of adenosine we have used hypoxia and local electrical stimulation as release stimuli.

A 10 min period of hypoxia reversibly reduced PF EPSP amplitude, an effect which was reversed by blocking A_1 receptors with 8-CPT (1–2 μM). At steady state, PF EPSP amplitude was reduced by $64 \pm 7\%$ and the paired pulse ratio was increased from 1.25 ± 0.04 to 1.8 ± 0.1 ($n =$ 11 slices, Fig. 6*A*). The reduction in PF EPSP amplitude can be used to measure the speed of rise in extracellular adenosine concentration. The inhibition reached steady state after 5.4 ± 0.7 min (delay until start of depression 3.7 ± 0.6 min). The decrease in PF EPSP amplitude was fully reversed by reoxygenation after 5.3 ± 0.5 min ($n =$ 8 slices).

As a comparison, hypoxia (10 min) was also induced in P21–28 slices where it induced a marked depression in PF EPSP amplitude ($81.3 \pm 6.6\%$, $n = 5$ slices). However, following 10 min of hypoxia, the inhibition was often not completely reversed by blocking A_1 receptors with 8-CPT (complete reversal was only observed in 2 out of 5 slices). However, with shorter periods of hypoxia (5 min), reversal of block was observed in 6 out of 8 slices (mean inhibition $73 \pm 5.4\%$, $n = 6$ slices). The speed of depression was very rapid; the time to reach a steady state depression was 2.3 ± 0.3 min ($n = 8$ slices), which is double the rate of inhibition observed in young animals. The delay until the start of PF EPSP amplitude depression was 1.1 ± 0.4 min.

To investigate the kinetics of purine release in more detail we have used purine biosensors. ADO, INO and null sensors were placed on the surface of the molecular layer (sensors were bent to be parallel with the slice surface, see Wall & Dale 2007). A short period of hypoxia (10 min) produced a large current on ADO and INO sensors in both P9–12 and P21–28 rats (Fig. 6*C* and *D*). As no increase in baseline current was observed on null sensors, the current was produced by the release of purines (Fig. 6*C* inset). Since the currents on ADO and INO sensors were not different in rise or amplitude (after correction for calibration), the sensors detected adenosine metabolites (inosine and hypoxanthine). Assuming that all the metabolite is inosine, then the maximum concentration of inosine detected at the surface of the slice (following hypoxia) was 3.5 ± 0.4 μM for P9–12 slices ($n = 8$) and 5.5 ± 0.7 μM for P21–28 slices ($n = 8$). Although hypoxia appeared to release significantly ($P > 0.05$) more purines from older slices, this difference probably stems from the slower rate of purine release in P9–12 slices. In all P9–12 slices ($n = 8$), the sensor current had not reached steady state after 10 min of hypoxia and was still rising during reoxygenation (Fig. 6*C* and *D*). In contrast, the sensor current reached a plateau after 5.3 ± 0.5 min in P21–28 slices (Fig. 6*D*). The slow release in P9–12 slices resulted from a greater delay in the start of purine release combined with a much slower rise in purine concentration (experiments were interleaved so the bath inflow and outflow speed were exactly the same in mature and immature slices). The rising phase of the sensor current could be fitted with a single exponential to give a time constant of 9.1 ± 0.4 min for P9–12 slices and 1.5 ± 0.3 min for P21–28 slices. In some slices (both adult and young), following reoxygenation, the rate of purine release appeared enhanced before it began to fall. Such a phenomenon has been observed following ischemia in the hippocampus (termed post-hypoxic purine efflux, PPE, Frenguelli *et al.* 2003) and was more apparent if the biosensor was pushed into the slice ($n = 2$). After reoxygenation, the decay of the biosensor current could be fitted with a single exponential with a time constant of $\sim$15 min for both P9–12 and P21–28 slices.

In P21–28 rats, adenosine can be reliably released by trains of electrical stimulation in the molecular layer, a process which is both action potential and Ca^{2+} dependent (Fig. 6*E*; observed in 4 out of 4 slices in interleaved experiments, and see Wall & Dale (2007)). However it was not possible to observe such adenosine release (the response of the ADO sensor was the same as the null sensor) at immature synapses, even with prolonged high frequency stimulation (200 stimuli, 20–40 Hz, $n = 15$ slices, Fig. 6*E*). To check that it was possible to detect adenosine release in these slices, the stimulus strength was increased (above 25 V) to electroporate the cells under the stimulating electrode (Hamann & Attwell, 1996; Wall &

Dale, 2007). A large signal was then observed on the ADO sensor but not on the null sensor ($n = 3$, not illustrated).

Discussion

There is little or no tonic A_1 receptor activation at parallel fibre–Purkinje cell synapses in slices from P9–12 rats compared to synapses in slices from P21–28 rats (Wall *et al*. 2007). This low level of receptor activation does not result from either a lack of A_1 receptors or the presence of low affinity receptors. Thus during cerebellar development there is an increase in the level of tonic A_1 receptor activation with no obvious change in receptor properties. A similar developmental increase in A_1 receptor tone has been reported in the hippocampus (Psarropoulou *et al.* 1990) also with no change in receptor properties. In contrast, at the calyx of Held (in mice) there is a developmental reduction in both adenosine tone and receptor number (Kimura *et al.* 2003). Takahashi *et al.* (1995) have report a larger A_1 receptor activation in the cerebellum of postnatal day 10–15 rats (∼24%). However

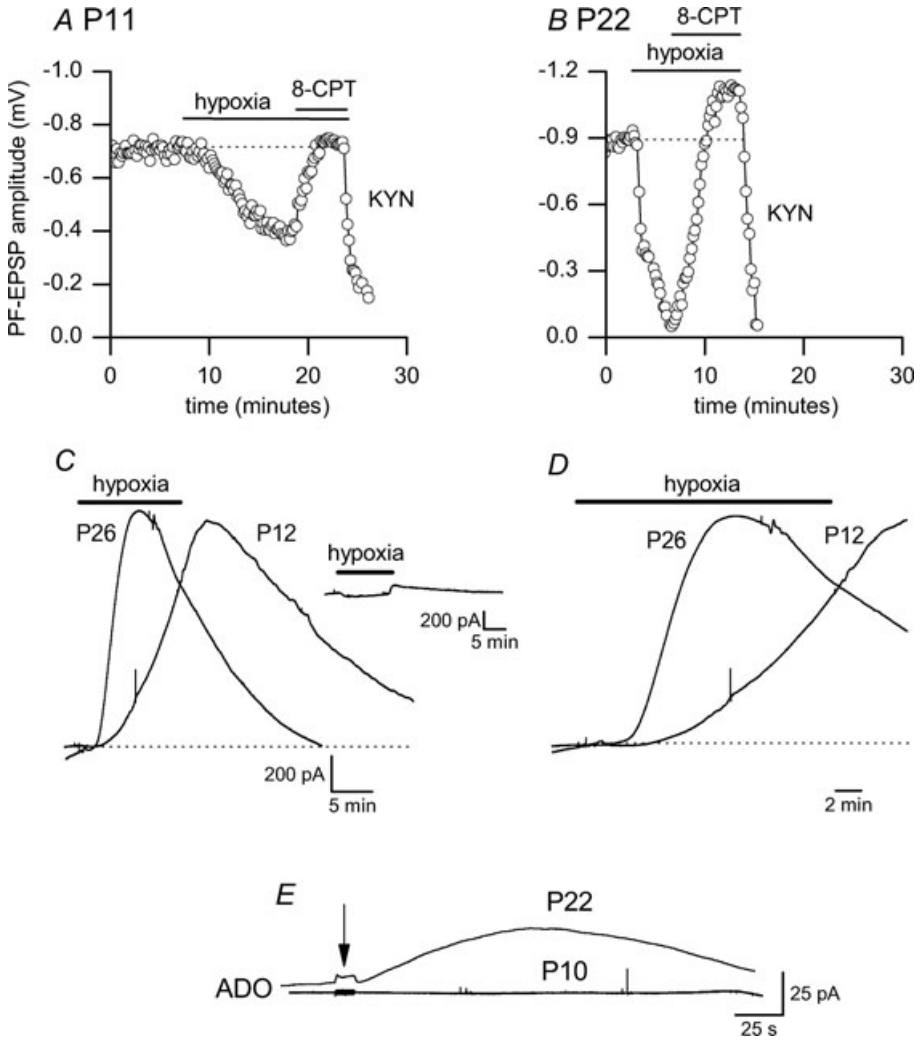

Figure 6. Hypoxia but not electrical stimulation releases adenosine in immature slices
A, the perfusion of a slice from a P11 rat with aCSF bubbled with N_2 (95%)–CO_2 (5%) for 10 min (hypoxia) caused a slow reduction in PF EPSP amplitude (∼45%), which was reversed by block of A_1 receptors with 8-CPT (2 μM). *B*, in an interleaved experiment (recording from a slice from a P22 rat), the application of aCSF bubbled with N_2–CO_2 caused a much more rapid and larger inhibition of PF EPSP amplitude, which again could be reversed with block of A_1 receptors (8-CPT, 2 μM). Note the clear presence of an A_1 receptor tone in the slice from the P22 rat (*B*). *C*, example traces from ADO biosensors placed on the surface of slices (molecular layer) from P12 and P26 rats (interleaved experiments). Perfusion with aCSF bubbled with N_2–CO_2 for 10 min caused an increase in extracellular purine concentration for both slices but the rise was much more rapid in the slice from the P26 rat. Inset, hypoxia causes a small drop in current on the null sensor. *D*, traces from *C* expanded to show the difference in rise-time of biosensor current. *E*, electrical stimulation (at arrow, 10 s, 20 Hz, 5 V) caused the release of purines in a slice from a P22 rat but no detectable release in a P10 slice.

these experiments were carried out at room temperature, where the clearance of adenosine may be reduced.

Clearly, the presence or absence of an A_1 receptor tone will have marked effects on synaptic transmission. Addition of exogenous adenosine enhanced facilitation at both immature and mature synapses during trains of stimuli. Removal of the A_1 receptor tone, at mature synapses resulted in almost complete synaptic depression. Developmental changes in other modulators (such as cannabinoids, Crepel, 2007) will also alter the properties of parallel fibre–Purkinje cell synaptic transmission during trains of activity.

Control of basal adenosine concentration at immature parallel fibre–Purkinje cell synapses

We have reduced the clearance of extracellular adenosine at immature parallel fibre–Purkinje cell synapses to investigate basal adenosine production. The small effect of blocking adenosine deaminase (with EHNA) suggests little ongoing extracellular metabolism. Similar small effects of EHNA have been observed in the hippocampus (Pak *et al.* 1994; Dunwiddie & Diao, 1994) and in mature cerebellar slices (Wall *et al.* 2007).

Adenosine kinase inhibition had minor effects on synaptic transmission (∼25% inhibition). Blockade of adenosine kinase causes an increase in the intracellular concentration of free adenosine and thus a resultant efflux of adenosine via equilibrative transporters (see Boison, 2006 for review). This is supported by the observation that blocking extracellular metabolism (with EHNA) can produce a reduction in PF EPSP amplitude following adenosine kinase inhibition. This also demonstrates that EHNA can reduce extracellular adenosine metabolism. The greater importance of adenosine kinase activity compared to extracellular metabolism has been observed in other brain regions (Pak *et al.* 1994; Lloyd & Fredholm, 1995) and in mature cerebellar slices (Wall *et al.* 2007).

The role of equilibrative transport was assessed by blocking ENT1 and ENT2. The effects on synaptic transmission were mixed with ∼50% of synapses inhibited (∼30%). Surprisingly, there was no correlation between the presence of an adenosine tone in control and the actions of NBTI/dipyridamole. As with mature slices, the application of NBTI/dipyridamole was unable to occlude the effects of adenosine kinase inhibition. Thus NBTI/dipyridamole was unable to block all adenosine transport. Such effects have also been observed at mature synapses (Wall *et al.* 2007) and may result from either expression of a transporter which is not blocked by NBTI/dipyridamole or high expression of ENT2, which is only weakly blocked in rat (Baldwin *et al.* 2004).

The blockade of adenosine breakdown, uptake and intracellular conversion to AMP all had relatively minor effects at immature synapses. The effects were also heterogeneous with synaptic transmission not effected at a large proportion of synapses. Thus the extracellular production of adenosine, under basal conditions, is low at immature synapses.

The active release of adenosine

We used hypoxia and electrical stimulation to actively release adenosine and investigate whether there are pools of releasable adenosine. Hypoxia produced robust adenosine release and marked PF EPSP inhibition at both immature and mature synapses. Biosensor recordings suggest that similar concentrations of adenosine are released but release is slower and delayed at immature synapses. At mature slices, adenosine release is very sensitive to hypoxia as the synaptic depression (and therefore adenosine release) starts almost as soon as the hypoxic aCSF reaches the bath. It seems unlikely that the increased extracellular concentration of adenosine present in mature synapses stems from the slices being continually hypoxic as the slices were perfused (from above and below) by highly oxygenated aCSF at a fast perfusion rate ($6\ \mathrm{ml\ min^{-1}}$) via gas tight tubing. Thus this mechanism of adenosine release appears to become sensitive to hypoxia during development.

It was not possible to measure adenosine release in response to electrical stimuli in P9–12 slices, which contrasts with the reliable release in slices from older rats (Wall & Dale, 2007). It is possible that this mechanism of adenosine release is not present at this developmental stage or perhaps the number of adenosine release sites is so small that the concentration of adenosine released is below the levels of detection (∼30 nM). As parallel fibre activity is required for adenosine release (Wall & Dale, 2007), the smaller number of parallel fibre synapses present in the immature molecular layer may produce undetectable adenosine release. However the contribution of action potential-dependent adenosine release to the basal extracellular adenosine tone is small at mature synapses, as TTX had only a very minor effect on the tone (Wall *et al.* 2007). Thus the lack of this form of adenosine release cannot explain the developmental difference in basal adenosine receptor activation.

In conclusion, the extracellular concentration of adenosine is low at immature parallel fibre–Purkinje cell synapses and interference with adenosine clearance suggests that little adenosine is being cycled between intra- and extracellular compartments under basal conditions. Reduced release in response to electrical stimulation and slowed release in response to hypoxia suggest that the mechanisms of adenosine release become more efficient with development. It is possible that changes in adenosine kinase expression could account for the change

in the amount of releasable adenosine (and thus lack of adenosine tone). Studer *et al.* (2006) showed that early in development, hippocampal adenosine kinase expression is mainly in neurones and shifts to glia during the second postnatal week. Adenosine kinase expression in neurones could account for the lack of intracellular adenosine stores and the reduced extracellular release. It would be interesting to use immunohistochemistry to confirm cerebellar adenosine kinase expression.

References

Altman J (1972). Postnatal development of the cerebellar cortex in the rat II. Phases in the maturation of Purkinje cells and of the molecular layer. *J Comp Neurol* **145**, 399–463.

Agarwal RP (1982). Inhibitors of adenosine deaminase. *Pharmacol Ther* **17**, 399–429.

Baldwin SA, Beal PR, Yao SYM, King AE, Cass CE & Young JD (2004). The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch* **447**,735–743.

Boison D (2006). Adenosine kinase, epilepsy and stroke: mechanisms and therapies. *Trends Pharmacol Sci* **27**, 652–658

Boison D (2007). Adenosine as a modulator of brain activity. *Drug News Perspect* **20**, 607–611

Clark BA & Barbour B (1997). Currents evoked in Bergmann glial cells by parallel fibre stimulation in rat cerebellar slices. *J Physiol* **502**, 335–350.

Craig CG & White TD (1993). N-methyl-D-aspartate and non-N-methyl-D-aspartate-evoked adenosine release from rat cortical slices: distinct purinergic sources and mechanisms of release. *J Neurochem* **60**, 1073–1080.

Crepel F (2007). Developmental changes in retrograde messengers involved in depolarization-induced suppression of excitation at parallel fibre-Purkinje cell synapses in rodents. *J Neurophysiol* **97**, 824–836.

Dale N (1998). Delayed production of adenosine underlies temporal modulation of swimming in frog embryo. *J Physiol* **511**, 265–272.

Descombes S, Avoli M & Psarropoulou C (1998). A comparison of the adenosine-mediated synaptic inhibition in the CA3 area of immature and adult rat hippocampus. *Brain Res Dev Brain Res* **110**, 51–59.

Dumas TC & Foster TC (1998). Late developmental changes in the ability of adenosine A1 receptors to regulate synaptic transmission in the hippocampus. *Brain Res Dev Brain Res* **105**, 137–9.

Dunwiddie TV & Diao L (1994). Extracellular adenosine concentration in hippocampal brain slices and the tonic inhibitory modulation of evoked excitatory responses. *J Pharmacol Exp Ther* **268**, 537–545.

Edwards, FA, Gibb AJ & Colquhoun D (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature* **359**, 144–147.

Frenguelli BG, Llaudet E & Dale N (2003). High-resolution real-time recording with microelectrode biosensors reveals novel aspects of adenosine release during hypoxia in rat hippocampal slices. *J Neurochem* **86**, 1506–15.

Frenguelli BG, Wigmore G, Llaudet E & Dale N (2007). Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *J Neurochem* **101**(5), 1400–1413.

Hamann M & Attwell D (1996). Non-synaptic release of ATP by electrical stimulation in slices of rat hippocampus, cerebellum and habenula. *Eur J Neurosci* **8**, 1500–1515.

Kimura M, Saitoh N & Takahashi T (2003). Adenosine A_1 receptor-mediated presynaptic inhibition at the calyx of Held of immature rats. *J Physiol* **553**, 415–426.

Kreitzer AC & Regehr WG (2000). Modulation of transmission during trains at a cerebellar synapse. *J Neurosci* **20**, 1348–1357.

Llaudet E, Botting N, Crayston J & Dale N (2003). A three enzyme microelectrode sensor for detecting purine release from central nervous system. *Biosens Bioelectron* **18**, 43–52.

Lloyd HG & Fredholm BB (1995). Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem Int* **26**, 387–395.

Noji T, Karaswa A & Kusaka H (2004). Adenosine uptake inhibitors. *Eur J Pharmacol* **495**, 1–16.

Pak MA, Haas HL, Decking UK & Schrader J (1994). Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. *Neuropharmacology* **33**, 1049–1053.

Pearson RA, Dale N, Llaudet E & Mobbs P (2005). ATP released via gap junction hemichannels from the pigment epithelium regulates neural retinal progenitor proliferation. *Neuron* **46**, 731–744.

Psarropoulou C, Kostopoulos G & Haas HL (1990). An electrophysiological study of the ontogenesis of adenosine receptors in the CA1 area of rat hippocampus. *Dev Brain Res* **55**, 147–150.

Studer FE, Fedele DE, Marowsky A, Schwerdel C, Wernli K, Vogt K, Fritschy JM & Boison D (2006). Shift of adenosine kinase expression from neurons to astrocytes during postnatal development suggests dual functionality of the enzyme. *Neuroscience* **142**, 125–137.

Sweeney MI (1996). Adenosine release and uptake in cerebellar granule neurons both occur via an equilibrative nucleoside carrier that is modulated by G proteins. *J Neurochem* **67**, 81–88.

Takahashi M, Kovalchuk Y & Attwell D (1995). Pre- and postsynaptic determinants of EPSC waveform at cerebellar fibre and parallel fibre to Purkinje cell synapses. *J Neurosci* **15**, 5693–5702.

Wall MJ & Dale N (2007). Auto-inhibition of parallel fibre-Purkinje cell synapses by activity dependent adenosine release. *J Physiol* **581**, 553–567.

Wall MJ, Atterbury A & Dale N (2007). Control of basal extracellular adenosine concentration in rat cerebellum. *J Physiol* **582**, 137–151.

Wall MJ & Dale N (2008). Activity-dependent release of adenosine: A critical re-evaluation of mechanism. *Curr Neuropharmacol* **6**, 329–337.

Weaver DR (1996). A1-adenosine receptor gene expression in fetal rat brain. *Brain Res Dev Brain Res* **94**, 205–223.

Wong AYC, Billups B, Johnston BJ, Evans RJ & Forsythe ID (2006). Endogenous activation of adenosine A1 receptors but not P2X receptors during high-frequency synaptic transmission at the calyx of Held. *J Neurophysiol* **95**, 3336–3342.

Yuan Y & Atchison WD (1999). Comparative effects of methylmercury on parallel-fibre and climbing fibre responses of rat cerebellar slices. *J Pharmacol Exp Ther* **288**, 1015–1025.

Author contributions

M.J.W. designed research. A.A. and M.J.W. performed research & analysed data. M.J.W. wrote paper.

Acknowledgements

We thank Wenjue Wu of Sarissa Biomedical Ltd for biosensor manufacture. We thank Professor Nicholas Dale for comments on early drafts of the manuscript. A.A. has a studentship funded by the BBSRC and Warwick Medical Research Fund.