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A population of immature cerebellar parallel fibre synapses are insensitive to adenosine but are inhibited by hypoxia.

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Abstract

The purine adenosine plays an important role in a number of physiological and pathological processes and is neuroprotective during hypoxia and ischemia. The major effect of adenosine is to suppress network activity via the activation of A_1 receptors. Here we report that in immature cerebellar slices, the activation of A_1 receptors has variable effects on parallel fibre synaptic transmission, ranging from zero depression to an almost complete abolition of transmission. Concentration-response curves suggest that the heterogeneity of inhibition stems from differences in A_1 receptor properties which could include coupling to downstream effectors. There is less variation in the effects of adenosine at parallel fibre synapses in slices from older rats and thus adenosine signalling appears developmentally regulated.

In the cerebellum, hypoxia increases the concentration of extracellular adenosine leading to the activation of A_1 receptors (at adenosine-sensitive parallel fibre synapses) and the suppression of glutamate release. It would be predicted that the synapses that were insensitive to adenosine would be less depressed by hypoxia and thus maintain function during metabolic stress. However those synapses which were insensitive to adenosine were rapidly inhibited by hypoxia via a mechanism which was not reversed by blocking A_1 receptors. Thus another mechanism must be responsible for the hypoxia-mediated depression at these synapses. These different mechanisms of depression maybe important for cell survival and for maintenance of cerebellar function following oxygen starvation.

1. Introduction

Adenosine plays important roles in a number of physiological and pathological processes in the brain (for review see Dunwiddie and Masino, 2001; Dale and Frenguelli, 2009). Adenosine accumulates during hypoxia and ischemia and has a neuroprotective role (Goldberg et al. 1988; Von Lubitz et al 1988; Cunha 2005) reducing the brains energy requirements during periods of metabolic stress. The actions of adenosine are well characterised, it acts via multiple cell surface G-protein coupled receptors: A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al. 2000). The major effect of adenosine is to dampen down network excitation via A_1 receptor activation. Adenosine A_1 receptors inhibit Ca^{2+} influx via voltage gated Ca^{2+} channels leading to reduced transmitter release (Gundlfinger et al. 2007) and A_1 receptors activate inwardly rectifying K^+ channels (GIRKs) leading to the hyperpolarisation of the membrane potential (Lüscher et al. 1997).

In many brain regions A_1 receptors are present on the terminals of glutamatergic neurones where they reduce glutamate release. In the cerebellum, A_1 receptors are present on both parallel fibre (Kocsis et al. 1984; Takahashi et al. 1995; Dittman and Regher, 1996; Wall et al. 2007) and climbing fibre terminals (Takahashi et al. 1995) where they reduce the release of glutamate. In a previous study, we discovered that a proportion of parallel fibre synapses in immature cerebellar slices were either weakly modulated by adenosine or not affected at all (Atterbury and Wall, 2009). Here we have investigated these synapses in more detail and found that other than the difference in modulation by adenosine, these synapses appear functionally identical to synapses which are strongly modulated by adenosine. We suggest that the difference in modulation stems from variation in adenosine receptor properties or

coupling to effectors rather than differences in adenosine metabolism or uptake.

These synapses are still depressed during hypoxia although the mechanism does not appear to involve the activation of A_1 receptors by the increased extracellular adenosine concentration.

2. Methods

2.1 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 U.K. Animals (Scientific Procedures) Act (1986), male rats were killed by cervical dislocation and then decapitated. The cerebellum was rapidly removed and slices were cut on a Microm HM 650V microslicer in cold (2-4ºC) high Mg^{2+}, low Ca^{2+} 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 and 5% CO_2, 300 mOSM). Slices were stored in normal aCSF (1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$) at room temperature for 1-6 hours before recording.

2.2 Extracellular recording

An individual slice was transferred to a recording chamber, submerged in normal aCSF and perfused at 6 ml/min (30-32°C). The slice was placed upon a suspended grid to allow perfusion of the slice from above and below and thus 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, Fisher). For the stimulation

of parallel fibres, square voltage pulses (2- 5V, 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, 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 and Atchison, 1999). A typical extracellular field potential consisted of an initial component which persisted in 5 mM kynurenate but was blocked by 1 μM TTX (parallel fibre volley) followed by a component which could be blocked by 1 μM TTX and greatly reduced by 5 mM kynurenate. This component (fEPSP) probably consists of parallel fibre-mediated glutamatergic excitatory synaptic potentials which maybe combined with action potentials in Purkinje cells and interneurones (Clark and Barbour, 1997). Parallel fibre fEPSP amplitude was estimated from the kynurenate-sensitive potential, which was measured by subtracting the potential that remained in kynurenate from control potentials. Confirmation of PF fEPSP identity was achieved by evoking pairs of fEPSPs (interval 50 ms) and observing facilitation (20-30%) and by examining the pharmacological profile (inhibition by mGlu4 receptor agonists and by glutamate receptor antagonists). The firing rate of Purkinje cells was measured using an aCSF filled patch pipette placed in the Purkinje cell layer. Recordings that were obviously from multiple units (different spike amplitudes) were discarded. Extracellular recordings were made using an ISO-DAM extracellular amplifier (WPI, Stevenage UK), filtered at 1 kHz and digitised on line (10 kHz) with a Micro CED (Mark 2, Cambridge UK) interface controlled by Spike (Vs 6.1).

2.3 Drugs

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, baclofen, N-cyclopentyladenosine (CPA), tetrodotoxin (TTX) and 8-cyclopentyltheophylline (8CPT) were purchased from Sigma (Poole, UK). Kynurenic acid, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and L-AP4 (L-(+)-2-Amino-4-phosphonobutyric acid) were purchased from Ascent Scientific (Bristol, UK).

2.4 *Statistics*

All quoted data is mean ± SEM. For statistical analysis, paired and unpaired student's *t* tests were performed. Data was described with either sigmoid curves or linear fits using Origin 8.5 (Silverdale Scientific Ltd, Stoke Mandeville UK)

3. Results

3.1 Adenosine has heterogeneous effects on synaptic transmission at immature parallel fibre synapses.

In a previous study (Wall and Atterbury, 2009) we reported that at a proportion of parallel fibre synapses in cerebellar slices from immature rats (postnatal days 9-12), adenosine had little effect on synaptic transmission. In that study such synapses were not analysed further. Here we investigate the modulation of parallel fibre synapses by adenosine in more detail. In 167 slices, the mean reduction in PF-fEPSP (parallel fibre field excitatory postsynaptic potential) amplitude produced by the application of

100 μM adenosine was 48 ± 2 %, which was accompanied by a significant ($P<0.01$) increase in paired pulse facilitation (interval 50 ms) from 1.38 ± 0.02 to 1.67 ± 0.07. There was considerable variation (Fig. 1B) in the reduction of fEPSP amplitude produced by adenosine (co-efficient of variance 0.68) ranging from no effect in 21 slices (Fig. 1A, B), to an almost complete abolition of transmission in 3 slices (> 90 % reduction in fEPSP amplitude). The reduction in fEPSP amplitude strongly correlated with the increase in paired pulse ratio ($r = 0.7$, Fig. 1C) suggesting that the inhibition was presynaptic (Dunwiddie and Haas, 1985).

We investigated whether the degree of adenosine-mediated inhibition correlated with any synaptic properties. In all slices, synaptic transmission showed paired pulse facilitation before adenosine application (50 ms interval, paired pulse ratio 1.38 ± 0.02) and fEPSP amplitude could be greatly reduced (> 80 % reduction) following block of glutamate receptors with either 5 mM kynurenic acid or 10 μM CNQX. There was no correlation: between the reduction in fEPSP amplitude (100 μM adenosine) and the initial (before adenosine application) fEPSP amplitude ($r = 0.03$, not illustrated), between the initial amount of paired pulse facilitation (before adenosine application) and reduction in fEPSP amplitude ($r = 0.04$, Fig. 1D) and between the magnitude of adenosine inhibition and the age of rat from which the cerebellar slices were made (P8-14, $r = 0.04$, not illustrated). Dunwiddie (1985) showed that the amount of inhibition produced by adenosine (on hippocampal fEPSPs) was reduced with larger stimulation voltages, although the effects of adenosine on paired pulse facilitation were independent of stimulation strength (Dunwiddie and Haas, 1985). In our experiments, the smallest stimulation voltage was used in order to produce a measurable fEPSP and thus the stimulation voltage was relatively constant across experiments. Also the effects of adenosine on fEPSP amplitude correlates with

the effects on the paired pulse ratio (Fig. 1C). Metabotropic glutamate receptors of the mGlu4 subtype are only expressed by parallel fibres in the cerebellum (Mateos et al. 1998) and thus inhibition of fEPSPs by their activation with the agonist L-AP4 confirms that parallel fibre synapses are activated. In all slices tested, L-AP4 (50 μM) significantly reduced fEPSP amplitude (mean inhibition 58 ± 2, *n* = 40 slices), whether or not adenosine had any effect on fEPSP amplitude. There was no correlation between the reduction in fEPSP amplitude produced by L-AP4 and the inhibition produced by 100 μM adenosine (Fig. 1A, E). Parallel fibres also express $GABA_B$ receptors, which inhibit transmitter release (Batchelor and Garthwaite, 1992; Wall and Dale, 2007). In 9 slices, where adenosine (100 μM) produced little or no inhibition (< 20 % reduction in fEPSP amplitude), activation of $GABA_B$ receptors with 10-25 μM baclofen produced an almost complete abolition of synaptic transmission (mean reduction in amplitude 97 ± 3 %, Fig. 1A). Thus synapses which were either weakly modulated or unaffected by adenosine were still strongly inhibited by either $GABA_B$ receptor or mGlu4 receptor activation.

3.2 There is less variation in adenosine inhibition in slices from older rats

To investigate if the adenosine-insensitivity of parallel fibre synapses persists during cerebellar development, recordings were made from parallel fibre synapses in slices from older rats (average age 23 ± 0.2 postnatal days). The average inhibition produced by 100 μM adenosine was 59 ± 2 %, with the paired pulse ratio increased from 1.5 ± 0.02 to 2 ± 0.08 (*n* =59 slices and see Wall et al. 2007). Although the degree of inhibition was variable, ranging from 27 to 100 % (CV 0.29), in a sample of 59 slices no synapses were completely insensitive to

adenosine (Fig. 2A). As with immature synapses there was no relationship between the degree of inhibition with fEPSP amplitude (r = 0.002) or initial paired pulse ratio (r = 0.04). In 14 slices, the inhibition produced by 100 μM adenosine was compared to that produced by 50 μM L-AP4 (mean inhibition 62 ± 4 %) and there was only a weak relationship (r = 0.14, Fig. 2B). Thus although there is still variation in the effects of adenosine across synapses in older animals, it appears less than at immature synapses.

3.3 Neither adenosine nor L-AP4 modulate Purkinje cell firing rate

It has been suggested that the field response evoked by parallel fibre stimulation consists of a combination of EPSPs and action potentials in Purkinje cells, interneurones and Bergmann glia (Clark and Barbour, 1997). If the field response is contaminated with population spikes this could explain the variation in effects between baclofen, L-AP4 and adenosine. If baclofen and L-AP4 have some postsynaptic effect (moving the cell away from threshold) resulting in a loss of population spikes this could explain their greater efficacy and consistent depressant action on synaptic transmission. This seems unlikely as mGlu4 receptors only appear to be expressed on parallel fibres (and not on postsynaptic cells, Mateos et al. 1998) and the stimulation strengths are low and are unlikely to activate enough parallel fibres to fire a Purkinje cell. However to exclude this possibility, the firing rate of spontaneously active Purkinje cells was measured in control and following the application of adenosine (100 μM), L-AP4 (50 μM) and baclofen (25 μM). Neither adenosine nor L-AP4 had any effect on the firing rate of Purkinje cells (control

instantaneous firing rate, 13.0 ± 1.6 Hz, adenosine 13.1 ± 1.6, L-AP4 13.5 ± 1.6) although baclofen (7.6 ± 1.6 Hz) significantly ($P<0.01$) reduced the firing rate (Fig. 3). Thus the more consistent effect of L-AP4 on synaptic transmission compared to adenosine does not appear to stem from a postsynaptic action.

3.4 The effects of adenosine are blocked by the A_1 receptor antagonist 8CPT

We have investigated whether the effects of adenosine are only through the activation of A_1 receptors. Recent evidence suggests that A_{2A} receptors (Brooks et al. 2008) may be expressed in the cerebellum and as they can facilitate synaptic transmission (Quarta et al. 2004) the variation in synaptic inhibition block could stem from the combined activation of inhibitory A_1 receptors and facilitatory A_{2A} receptors. However in 20 slices, we found that applying adenosine in the presence of the selective A_1 receptor antagonist 8CPT (1-2 μM) had no effect (data not shown), even if adenosine had little effect on fEPSP amplitude in control. Thus the lack of effect on fEPSP amplitude cannot be explained by activation of multiple receptor subtypes with opposing effects.

3.5 Concentration responses at synapses sensitive and insensitive to adenosine inhibition

Usually 100 μM adenosine would activate the majority of synaptic A_1 receptors and thus be close to the top of the concentration-response curve (Takahashi et al. 1995;

Kimura et al. 2003). However, at synapses were 100 μM adenosine had little effect on fEPSP amplitude, the concentration-response curve may be shifted to the right. Thus the effects of applying higher concentrations (300 μM) of adenosine were investigated. Slices could be divided into 2 groups: in the first group 300 μM adenosine produced little or no extra inhibition compared to 100 μM adenosine (n = 8, 54 ± 8 % Vs 50 ± 11 % inhibition, Fig. 4A) with effects comparable to L-AP4 (50 μM). In the second group, 300 μM adenosine had a much larger effect on fEPSP amplitude than 100 μM adenosine (12 ± 3.1 Vs 36 ± 9 %, n = 8, Fig. 4B). This large increase in inhibition produced by 300 μM adenosine (~ 3 times that produced by 100 μM) suggests that at these synapses 100 μM adenosine is not at the top of the concentration-response curve. This could be the result of rapid extracellular adenosine breakdown so that the concentration of adenosine reaching the receptors is low or perhaps at some synapses there is expression of low potency receptors. To test for changes in receptor properties, concentration-response curves were constructed for the non-hydrolysable A_1 receptor agonist CPA. Adenosine (100 μM) was first applied to identify synapses where there was either little or no inhibition (n = 6, mean reduction in fEPSP amplitude 15.8 ± 5 %) or synapses where adenosine produced at least a 50 % reduction in fEPSP amplitude (mean inhibition 68 ± 5 %, n = 7). The identity of all synapses was confirmed by applying L-AP4 (50 μM) which markedly reduced fEPSP amplitude (< 50 % inhibition). Concentration response curves were then constructed by sequentially adding increasing concentrations of CPA (0.001 μM to 100 μM). At synapses were adenosine produced a marked reduction in fEPSP amplitude, CPA almost completely inhibited synaptic transmission (90 ± 4.4 % block, Fig. 4C, E, F) with an IC_{50} of 50 nM (in agreement with previous studies, Wall and Atterbury, 2009). The effects of CPA could only be reversed with large concentrations of 8CPT (10-20

μM). At synapses where adenosine had little or no effect on fEPSP amplitude, the concentration response curve was shifted to the right (IC_{50} of 250 nM, Fig 3D, E) with a maximal inhibition (% inhibition of fEPSP amplitude with 100 μM CPA, 53 ± 0.6 %, Fig. 4F). The effects of CPA were readily reversed by 8CPT (2 μM) suggesting that the affinity of the receptor for CPA was reduced. Thus synapses show variation in the potency of CPA suggesting that the variation stems from receptor properties (and possibly downstream signalling) rather than differences in adenosine uptake and metabolism.

3.6 Changes in the presynaptic volley can account for adenosine inhibition at insensitive synapses

Adenosine has a small effect on the presynaptic volley, reducing it by ~ 10 % (Dunwiddie and Miller, 1993; Dittman and Regher, 1996; Wall et al. 2008). We have investigated whether this reduction in volley amplitude is sufficient to account for the reduction in fEPSP amplitude at synapses sensitive and relatively insensitive to adenosine inhibition. For each slice we varied the stimulus strength and then constructed a plot of volley amplitude against fEPSP amplitude. We then applied adenosine (100 μM) and L-AP4 (50 μM) to inhibit transmission. If the reduction in volley amplitude (produced by adenosine and L-AP4) can account for the reduction in fEPSP amplitude then, when plotted, the point will fall upon the volley Vs fEPSP amplitude curve. If the reduction in volley amplitude is too small to account for the reduction in fEPSP amplitude, then the point will fall below the line. We confirmed the identity of the volley: it persists following application of the glutamate receptor antagonist kynurenate (5 mM) and is blocked by TTX (0.5 μM, n = 5). In the

majority of slices (9 out of 12), the reduction in volley amplitude (produced by either adenosine or L-AP4) was much too small to account for the reduction in fEPSP amplitude and the points were well below the line (Fig. 5A, B, C also reported in hippocampus Dunwiddie, 1984). However, in 3 out of 12 slices where the effect of adenosine on fEPSP amplitude was small (~10-15 %) the reduction in fEPSP amplitude could be accounted for by the change in volley amplitude (with the point falling on the curve, Fig 5C, D, E). This suggests that the mechanisms of synaptic inhibition may differ between sensitive and insensitive synapses.

3.7 Effects of hypoxia on synapses sensitive and insensitive to adenosine

Starvation of O_2 (hypoxia) produces a marked increase in extracellular adenosine concentration in brain tissue, activating presynaptic A_1 receptors leading to the inhibition of transmitter release (Fowler, 1990; Winn et al. 1981; Katchman and Hershkowitz, 1993; Frenguelli et al. 2003). It has been reported that hypoxia-mediated adenosine release in the cerebellum depresses parallel fibre synaptic transmission, an effect reversed by blocking A_1 receptors (Atterbury and Wall, 2009). However these experiments were performed in slices where adenosine (100 μM) produced a large inhibition of transmission (< 50 % reduction in PF fEPSP amplitude). We have investigated if parallel fibre synapses, where adenosine has little effect on synaptic transmission, are either resistant or show a smaller response to hypoxia.

In confirmation of previous data (Atterbury and Wall, 2009), at synapses where adenosine (100 μM) had a large effect on fEPSP amplitude (mean inhibition 68 ± 4 %, n = 6 slices) hypoxia (aCSF bubbled with N_2/CO_2) induced a slow depression

of transmission (steady state reduction in fEPSP amplitude 43 ± 3 %, delay to onset of inhibition 161 ± 37 s, time from start of depression to maximum inhibition 327 ± 34 s, Fig. 6A). This inhibition was accompanied by a significant ($P<0.01$) increase in paired pulse ratio (from 1.29 ± 0.05 to 1.7 ± 0.07, Fig. 6B) but there was no significant change in the amplitude of the presynaptic volley (0.47 ± 0.07 Vs 0.47 ± 0.06 mV). Thus there is a reduction in transmitter release with no change in the number of active parallel fibres. The effects of hypoxia were almost completely reversed (85 ± 3 %) by blocking A_1 receptors with 8-CPT (2 μM, Fig. 6A). Thus in these slices, the major short-term effect of hypoxia is to activate presynaptic A_1 receptors leading to reduced parallel fibre glutamate release.

In slices where 100 μM adenosine had a significantly ($P<0.001$) smaller effect on fEPSP amplitude (mean inhibition 19 ± 3 % Vs ~68 %, $n = 5$), hypoxia still induced depression (mean inhibition of fEPSP amplitude 62 ± 5 %, Fig. 6C). This inhibition was significantly ($P<0.01$) larger than observed with synapses that were sensitive to adenosine and it was quicker to occur (delay 70.6 ± 6.2 vs 161 ± 37 s and time from start of depression to maximum inhibition 75 ± 44 Vs 327 ± 34 s, Fig. 6C). In 3 out of 5 slices the inhibition was accompanied by a large increase in the paired pulse ratio (Fig. 5D), in the other slices there was little change in the ratio (overall change in paired pulse ratio from 1.34 ± 0.06 to 1.78 ± 0.17). There was no significant ($P = 0.4$) change in the amplitude of the presynaptic volley during hypoxia (0.37 ± 0.1 Vs 0.44 ± 0.13 mV) and hence no change in the number of active parallel fibres. The inhibition produced by hypoxia was not reversed by 8 CPT (transient increase in fEPSP amplitude 21 ± 6 %, Fig. 6C) and thus most of the inhibition was not produced by extracellular adenosine acting via A_1 receptors. **As activation of either mGlu4 or $GABA_B$ receptors will inhibit parallel fibre transmitter release, mGlu4 and**

$GABA_B$ receptor antagonists were applied (in the presence of 8CPT) to investigate whether they could reverse the effects of hypoxia. Neither MSOP (100 μM, mGlu4R antagonist, *n* = 3) or CGP5248 (20 μM, $GABA_B$ receptor antagonist, *n* = 4) had any effect on fEPSP amplitude. The reduction in fEPSP amplitude was almost completely removed by washing back in oxygenated aCSF.

4. Discussion

We have found considerable variation in the reduction of fEPSP amplitude produced by the application of adenosine to parallel fibre synapses in slices from immature rats (confirming previous results Atterbury and Wall, 2009). Differences in adenosine-sensitivity were observed in slices from the same rat and in recordings from different molecular layers in the same slice. **Future experiments will investigate whether these insensitive and sensitive synapses occur in specific cerebellar lobes.** The observation that activation of other presynaptic receptors present on parallel fibre terminals (mGluR4 and $GABA_B$) produces consistent amounts of inhibition shows that the variation is specific to adenosine signalling. Furthermore, the consistent inhibitory effects of L-AP4 confirm that all the fEPSPs recorded result from parallel fibre stimulation (Mateos et al. 1998).

Since recordings of parallel fibre fEPSPs were made using an extracellular electrode, the precise composition of the population of postsynaptic cells recorded from is not known. The major postsynaptic cell type in the molecular layer is the Purkinje cell (1000s of parallel fibres make synapses onto Purkinje cell dendrites) but field recordings will also potentially include EPSPs from molecular layer

interneurones (Liu and Cull-Candy, 2000) and from Bergmann glia (Clark and Barbour, 1997; Bellamy and Ogden, 2005; Bellamy, 2007). One hypothesis for the variation in adenosine inhibition is that parallel fibres synapses onto different postsynaptic cell types have different sensitivities to adenosine and that the variation in fEPSP inhibition stems from differences in the proportion of sensitive and insensitive synapses recorded from. The evidence for such a hypothesis is relatively weak. Parallel fibre synapses onto Purkinje cells are modulated by adenosine (IC_{50} 1.1 μM, maximum block ~80 %, 100 μM adenosine close to the top of the concentration response curve, Takahashi et al. 1995). There is currently no data for the effects of CPA (non-hydrolysable and non-transported A_1 receptor agonist) on parallel fibre to Purkinje cell synapses using whole cell patch clamp recording. Synaptic transmission between parallel fibres and Bergmann glia is also modulated by the activation of A_1 receptors (CPA has an EC_{50} of 490 nM and produces a maximal inhibition of ~71 %, Bellamy, 2007). The sensitivity of parallel fibre-molecular layer interneuron synapses to adenosine (and CPA) inhibition is currently not known. We found that synapses that were sensitive to adenosine had an EC_{50} for CPA of ~ 50 nM and a maximal inhibition of 100%. It is possible to hypothesise that fEPSPs recorded from an almost pure population of parallel fibre-Purkinje cell synapses are sensitive to adenosine (most synapses) whereas fEPSPs arising from either mixtures of postsynaptic cell types or from relatively pure populations of Bergmann glia/ interneurones are less sensitive to adenosine. Conversely, it is also possible that different parallel fibre synapses onto Purkinje cells have different sensitivities to adenosine.

4.1 What determines the degree of inhibition produced by adenosine?

Application of 100 μM adenosine produced different amounts of inhibition at different parallel fibre synapses. This variation could be the result of different amounts of adenosine reaching the synaptic receptors due to differences in adenosine metabolism and uptake. The reported K_d value for adenosine at A_1 receptors is ~600-700 nM (hippocampus, Dunwiddie and Diao, 1994) and thus 100 μM adenosine should saturate the receptors and produce a maximal effect. However adenosine is rapidly removed from the extracellular space (by uptake and by metabolism) and thus only a small fraction of exogenous adenosine actually reaches the receptors. Although differences in the metabolism and uptake of adenosine would explain the variation this is not supported by the experiments using the non-hydrolysable adenosine analogue CPA (which is also not transported) which suggest that the differences in sensitivity lies at the level of the A_1 receptor. The rightward shift in the concentration-response curve and reduction in maximal inhibition shows that the potency of CPA at the receptor is reduced. This could reflect differences in both receptor affinity and efficacy. Changes in the number of receptors expressed at the synapse could explain the drop in maximal response but would not explain the rightward shift in concentration-response curve. Adenosine A_1 receptors show very slow desensitisation and internalisation (reviewed by Palmer and Stiles, 1997) and this coupled with the low adenosine tone measured in slices (Atterbury and Wall, 2009) suggests that that receptor desensitisation/internalisation is unlikely to explain the difference between synapses. It is possible that the efficacy of downstream signalling from the receptor to the Ca^{2+} channels/release machinery or K^+ channels is reduced. The A_1 receptor subtype couples to both G_i- and G_o-proteins in different

systems, enabling a number of transduction mechanisms to inhibit synaptic transmission. **Differences in the complement of presynaptic voltage-gated Ca^{2+} channels involved in transmitter release between individual parallel fibres could underlie the variation in inhibition, if A_1 receptors only inhibit certain Ca^{2+} channel subtypes. It has been reported that there are multiple subtypes of Ca^{2+} channel involved in parallel fibre transmitter release (N, P and Q Doroshenko et al 1997).** At most synapses the actions of adenosine cannot be accounted for by an action on the presynaptic volley (also reported by Dundwiddie 1984). However in synapses where the effects of adenosine on fEPSP amplitude were small, the inhibition could be explained by changes in volley amplitude. The volley is a compound action potential which scales with stimulus strength. Thus at these synapses the reduction in transmitter release may simply stem from a reduction in the number of active fibres. Adenosine can activate GIRK K^+ channels which could cause action potential failures (by hyperpolarisation) or could reduce Ca^{2+} influx via voltage gated Ca^{2+} channels which may otherwise contribute to depolarisation.

4.2 Hypoxia

Hypoxia produces a rapid accumulation of extracellular adenosine which activates presynaptic A_1 receptors leading to membrane potential hyperpolarisation and the inhibition of excitatory synapses (reviewed in Krnjević 2008; Frenguelli and Dale, 2009). This helps to reduce the brains energy requirements enabling it to maintain the minimal metabolism required for survival and is thus neuroprotective in adult brain (Krnjević 2008; Cunha 2005). In the cerebellum, parallel fibre synapses (that were sensitive to adenosine) were

markedly depressed by hypoxia, as a result of the increased concentration of extracellular adenosine activating A_1 receptors and reducing glutamate release (also see Atterbury and Wall, 2009). It might be expected that synapses that were not sensitive to inhibition by adenosine would be much more resistant to hypoxia, equivalent to hypoxia induced in the presence of an A_1 receptor antagonist where there is only slow and partial depression (for example see Gribkoff et al 1990). Surprisingly, these synapses were not resistant to hypoxia but instead appeared more sensitive (a quicker onset of depression and a larger steady state reduction in fEPSP amplitude). This inhibition was not produced by the activation of A_1 receptors, as it was not reversed by A_1 receptor antagonists (which only had a small transient action). However the reduction in EPSP amplitude could be reversed following reoxygenation.

What is the mechanism of hypoxia-mediated depression at these synapses? The mean increase in paired-pulse ratio suggests that some of the effects are presynaptic (reduction in glutamate release), although the effects of hypoxia on paired-pulse ratio were not consistent, with no effect at some synapses. There was no change in the presynaptic volley and thus the number of active parallel fibres was not reduced. Receptors such as mGluR4 and $GABA_B$ are present on parallel fibre terminals and could reduce the release of glutamate if activated (Mateos et al. 1998; Batchelor and Garthwaite, 1992). In the hippocampus, following block of A_1 receptors, part of the remaining hypoxia-induced depression can be relieved with a group III mGluR antagonist (MPPG, de Mendonça and Ribeiro 1997b). There is also evidence that activation of mGluR (Groups I and III) can attenuate the action of adenosine via A1 receptors (de Mendonça and Ribeiro 1997a). However, hypoxia-induced depression was not

reduced by either mGlu4 or $GABA_B$ receptor antagonists and thus they do not appear to be involved. There are a number of ion channels that can be acutely modulated by hypoxia including Ca^{2+} channels and K^+ channels which could underlie the inhibition (see Krnjević 2008 for review).

Why are there multiple mechanisms of hypoxia-induced depression at immature parallel fibre synapses? It has been suggested that, unlike adult brain, the activation of A_1 receptors maybe ineffective or even detrimental to neuronal survival following hypoxia in the immature brain (reviewed in Cunha 2005). Thus the neurones less-sensitive to adenosine may be preferentially protected following a hypoxic insult and thus may play an important role in maintaining cerebellar function. Future work is required to determine the precise anatomical location of these neurones in the cerebellar vermis providing a clue to their function.

4.3 Conclusions

There is variation in the inhibition of synaptic transmission at immature parallel fibre synapses produced by A_1 receptor activation. Some synapses are potently inhibited while others appear resistant. This variation could stem from differences in receptor expression, affinity and efficacy and maybe developmentally regulated as less variation is apparent in slices from older rats. Hypoxia depress all synapses whether sensitive or insensitive to adenosine but by different mechanisms and this maybe important for maintaining cerebellar function during metabolic stress.

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References

Atterbury, A., Wall, M.J., 2009. Adenosine signalling at immature parallel fibre-Purkinje cell synapses in rat cerebellum. J. Physiol. 587, 4497-508.

Batchelor, A.M., Garthwaite, J., 1992. GABAB Receptors in the Parallel Fibre Pathway of Rat Cerebellum. Eur. J. Neurosci. 4, 1059-1064.

Bellamy, T.C., 2007. Presynaptic modulation of parallel fibre signalling to Bergmann glia. Neuropharm. 52, 368-75.

Bellamy, T.C., Ogden, D., 2005. Short-term plasticity of Bergmann glial cell extrasynaptic currents during parallel fiber stimulation in rat cerebellum. Glia. 52, 325-35.

Brooks, D.J., Doder, M., Osman, S., Luthra, S.K., Hirani, E., Hume, S., Kase, H., Kilborn, J., Martindill, S., Mori, A., 2008. Positron emission tomography analysis of [11C]KW-6002 binding to human and rat adenosine A_{2A} receptors in the brain. Synapse 62, 671-81.

Clark, B.A., Barbour, B., 1997. Currents evoked in Bergmann glial cells by parallel fibre stimulation in rat cerebellar slices. J. Physiol. 502, 335-50.

Cunha, R.A., 2005. Neuroprotection by adenosine in the brain: From A(1) receptor activation to A (2A) receptor blockade. Purinergic Signal. 2005 1:111-34.

Dale, N., Frenguelli, B.G., 2009. Release of Adenosine and ATP during ischemia and epilepsy. Curr. Neuropharmacol. 7:160-179.

de Mendonça, A., Ribeiro, J.A., 1997a. Influence of metabotropic glutamate receptor agonists on the inhibitory effects of adenosine A1 receptor activation in the rat hippocampus. Br. J. Pharmacol. 121, 1541-8.

de Mendonca, A., Riberio, J.A., 1997b. Contribution of metabotropic glutamate receptors to the depression of excitatory postsynaptic potentials during hypoxia. Neuroreport 8, 3667-3671.

Dittman, J.S., Regehr, W.G., 1996. Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. J. Neurosci. 16, 1623-1633.

Doroshenko, P.A., Woppmann, A., Miljanich, G., Augustine, G.J. 1997 Pharmacologically distinct presynaptic calcium channels in cerebellar excitatory and inhibitory synapses. Neuropharm 36, 865-872.

Dunwiddie, T.V., 1984. Interactions between the effects of adenosine and calcium on synaptic responses in rat hippocampus in vitro. J. Physiol. 350:545-59.

Dunwiddie, T.V., Haas, H.L., 1985. Adenosine increases synaptic facilitation in the in vitro rat hippocampus: evidence for a presynaptic site of action. J. Physiol. 369:365-77.

Dunwiddie, T.V., Diao, L., 1994. Extracellular adenosine concentrations in hippocampal brain slices and the tonic inhibitory modulation of evoked excitatory responses. J. Pharmacol. Exp. Ther. 268, 537-45.

Dunwiddie, T.V., Masino, S,A., 2001. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci. 24:31-55.

Dunwiddie, T.V., Miller, K.K., 1993. Effects of adenosine and cadmium on presynaptic fiber spikes in the CA1 region of rat hippocampus in vitro. Neuropharm 32:1061-1068.

Fowler, J.C., 1990. Adenosine antagonists alter the synaptic response to in vitro ischemia in the rat hippocampus. Brain. Res. 509, 331 334.

Fredholm, B.B., Arslan, G., Halldner, L., Kull, B., Schulte, G., Wasserman, W., 2000. Structure and function of adenosine receptors and their genes. Naunyn Schmiedebergs Arch. Pharmacol. 362, 364-74.

Frenguelli, B.G., 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, 1506–1515.

Frenguelli, B., Dale, N., 2009. Release of adenosine and ATP during ischemia and epilepsy. Curr. Neuropharmacol. 7, 160-179.

Goldberg, M., Monyer, H., Weiss J., Choi, D., 1988. Adenosine reduces cortical neuronal injury induced by oxygen or glucose deprivation in vitro. Neurosci. Lett. 89, 323–327.

Gribkoff, V.K., Bauman, L.A., VanderMaelen, C.P., 1990. The adenosine antagonist 8-cyclopentyltheophylline reduces the depression of hippocampal neuronal responses during hypoxia. Brain Research 512, 353-357.

Gundlfinger, A., Bischofberger, J., Johenning, F.W., Torvinen, M., Schmitz, D., Breustedt, J., 2007. Adenosine modulates transmission at the hippocampal mossy fibre synapse via direct inhibition of presynaptic calcium channels. J. Physiol. 582, 263-77.

Katchman, A.N., Hershkowitz, N., 1993. Adenosine antagonists prevent hypoxia-induced depression of excitatory but not inhibitory synaptic currents. Neurosci. Lett. 159, 123-126.

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.

Kocsis, J.D., Eng, D.L., Bhisitkul, R.B., 1984. Adenosine selectively blocks parallel-fiber-mediated synaptic potentials in rat cerebellar cortex. Proc. Natl. Acad. Sci. USA.81:6531-4.

Krnjević K., 2008. Electrophysiology of cerebral ischemia. Neuropharmacology 55, 319-333.

Liu, S.J., Cull-Candy, S.G., 2000. Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. Nature 405, 454-458.

Lüscher, C., Jan, L.Y., Stoffel, M., Malenka, R.C., Nicoll, R,A., 1997. G protein-coupled inwardly rectifying K^+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron. 19, 687-9.

Mateos, J.M., Azkue, J., Sarría, R., Kuhn, R., Grandes, P., Knöpfel, T., 1998. Localization of the mGlu4a metabotropic glutamate receptor in rat cerebellar cortex. Histochem. Cell. Biol. 109, 135-9.

Palmer, T.M., Stiles, G.L., 1997. Structure-function analysis of inhibitory adenosine receptor regulation. Neuropharm. 36, 1141-1147.

Quarta, D., Borycz, J., Solinas, M., Patkar, K., Hockemeyer, J., Ciruela, F., Lluis, C., Franco, R., Woods, A.S., Goldberg, S.R., Ferré, S., 2004. Adenosine receptor-mediated modulation of dopamine release in the nucleus accumbens depends on glutamate neurotransmission and N-methyl-D-aspartate receptor stimulation. J. Neurochem. 91, 873-80.

Takahashi, M., Kovalchuk, Y., Attwell, D., 1995. Pre- and postsynaptic determinants of EPSC waveform at cerebellar climbing fiber and parallel fiber to Purkinje cell synapses. J. Neurosci. 15, 5693-702.

Von Lubitz, D., Dambrosia, J., Kempski, O., Redmond, D., 1988. Cyclohexyladenosine protects against neuronal death following ischemia in the CA1 region of gerbil hippocampus, Stroke, 19, 1133-1139.

Wall, M.J., Atterbury, A., Dale, N., 2007. Control of basal extracellular adenosine concentration in rat cerebellum. J. Physiol. 582, 137-51.

Wall, M.J., Dale, N., 2007. Auto-inhibition of rat parallel fibre-Purkinje cell synapses by activity-dependent adenosine release. J. Physiol. 581, 553-65.

Wall, M.J., Wigmore, G., Lopatár, J., Frenguelli, B.G., Dale, N., 2008. The novel NTPDase inhibitor sodium polyoxotungstate (POM-1) inhibits ATP breakdown but also blocks central synaptic transmission, an action independent of NTPDase inhibition. Neuropharm. 2008 55:1251-8.

Winn, H.R., Rubio, R., Berne, R.M., 1981. Brain adenosine concentration during hypoxia in rats, Am. J. Physiol. 241, 235-242.

Yuan, Y., Atchison, W.D., 1999. Comparative effects of methylmercury on parallel-fiber and climbing-fiber responses of rat cerebellar slices. J. Pharmacol. Exp. Ther. 288, 1015-25.

Legends

Fig. 1. Heterogeneous actions of adenosine at parallel fibre synapses in cerebellar slices from P9-12 rats. A: Graph plotting the amplitude of individual parallel fibre fEPSPs against time. Application of 100 μM adenosine had no effect on fEPSP amplitude but baclofen (25 μM, $GABA_B$ receptor agonist) and L-AP4 (50 μM, mGluR4 agonist) greatly reduced fEPSP amplitude. At the end of the experiment, fEPSPs were blocked with 5 mM kynurenate. B: Graph illustrating the distribution of adenosine inhibition (% reduction in fEPSP amplitude) in 167 slices (dotted line is the mean inhibition, 47.7 %). Inset, superimposed average fEPSPs (15 fEPSPs) in control and in 100 μM adenosine recorded from 2 slices illustrating the range of inhibition observed. C: Graph plotting the percentage increase in paired pulse ratio (produced by 100 μM adenosine) against percentage reduction in fEPSP amplitude produced by 100 μM adenosine. The data can be described by a linear fit (slope 0.4, R = 0.7). D: Graph plotting initial fEPSP paired-pulse ratio against percentage reduction in fEPSP amplitude produce by adenosine (100 μM, the linear fit has an r value of 0.03). E: Graph plotting the percentage reduction in fEPSP amplitude produced by 50 μM L-AP4 against the percentage reduction in fEPSP amplitude produced by adenosine (100 μM). The linear fit has a slope of 0.35.

Fig. 2. Actions of adenosine at parallel fibre synapse in cerebellar slices from older rats (P21-28). A: Graph illustrating the distribution of adenosine inhibition (% reduction in fEPSP amplitude) in 59 slices (dotted line is the mean inhibition, 59 %) from P21-28 rats. B: Graph plotting the percentage reduction in fEPSP amplitude

produced by 50 μM L-AP4 against the percentage reduction in fEPSP amplitude produced by adenosine (100 μM). The linear fit has a slope of -0.03.

Fig. 3. Adenosine and L-AP4 do not change the firing rate of Purkinje cells recorded in slices from P9-12 rats. A: Graph plotting the instantaneous firing rate (Hz) of a Purkinje cell against time. Application of adenosine (100 μM) or L-AP4 (50 μM) had no effect on the firing rate although addition of baclofen (25 μM) significantly reduced the firing rate. The dotted line is the mean firing rate in control (16. 4 Hz). B: Graph summarising Purkinje cell firing rate data from 5 slices.

Fig. 4. Variation in adenosine receptors properties in slices from P9-12 rats. A: Graph plotting the normalised amplitude of individual fEPSPs against time (fEPSPs were normalised by dividing by mean fEPSP amplitude in control). Application of 100 followed by 300 μM adenosine produced a similar percentage (~55 %) reduction of fEPSP amplitude. Application of L-AP4 (50 μM) also reduced fEPSP amplitude confirming that EPSPs were from parallel fibre synapses. B: Graph plotting the normalised amplitude of individual PF fEPSPs against time (fEPSPs were normalised by dividing by mean fEPSP amplitude in control). In this slice, 300 μM adenosine produced a much larger reduction in fEPSP amplitude (~35 %) than 100 μM adenosine (15 %). Application of L-AP4 (50 μM) also reduced fEPSP amplitude confirming that fEPSPs were from parallel fibre synapses. C: Graph plotting the amplitude of individual PF EPSPs against time for synapses sensitive to adenosine (100 μM adenosine abolished EPSPs). The amplitude of fEPSPs has been normalised to the average fEPSP amplitude in control. Increasing concentrations of the A_1 receptor agonist CPA (from 0.01 to 1 μM) produced greater reduction in EPSP

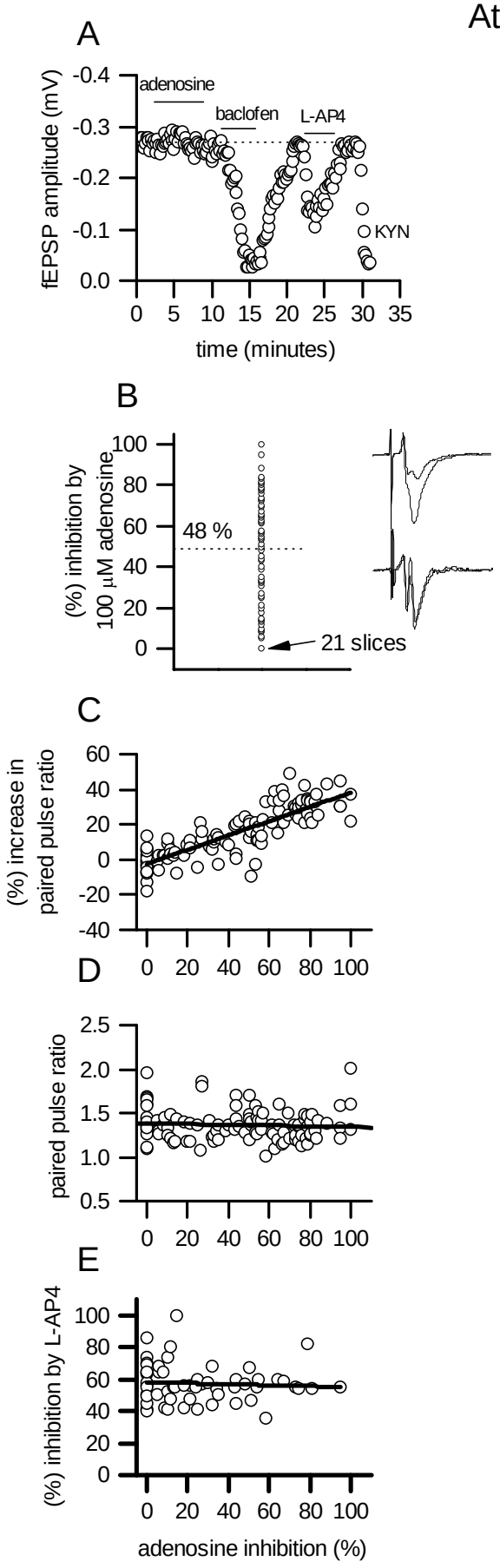
amplitude. Application of 10 μM 8 CPT (in the presence of 1 μM CPA) reversed the inhibition. Inset, superimposed average fEPSPs in control (20 fEPSPs) and in the presence of 100 μM adenosine for same slice as (C). D: Graph plotting the amplitude of individual PF fEPSPs against time for synapses relatively insensitive to adenosine (100 μM adenosine reduced EPSP amplitude by ~30 %). The amplitude of fEPSPs has been normalised to the average fEPSP amplitude in control. Increasing concentrations of the A_1 receptor agonist CPA (from 0.01 to 10 μM) produced greater reductions in fEPSP amplitude. Application of 2 μM 8 CPT (in the presence of 10 μM CPA) reversed the inhibition Inset, superimposed average fEPSPs in control (15 fEPSPs) and in the presence of 100 μM adenosine. E: Log concentration-response curves plotting percentage inhibition (relative to maxium inhibition) against log concentration of CPA (μM). The open circles were from synapses where application of adenosine (100 μM) produced at least 50 % inhibition (n = 7). The points are fitted with a sigmoid curve which gives an EC_{50} of 50 nM. The filled circles were from slices (n = 5) where the inhibition from 100 μM adenosine was < 50 %. The points are fitted with a sigmoid curve which gives an EC_{50} of 250 nM. F: Bar chart plotting inhibition produced by supra-maximal concentrations of CPA (up to 100 μM) for synapses which are sensitive and insensitive to adenosine inhibition.

Fig. 5. A reduction in volley amplitude can only account for the reduction in fEPSP amplitude at a small minority of synapses where adenosine has little effect. A: Graph plotting the amplitude of individual PF EPSPs against time. Both adenosine (100 μM) and L-AP4 (50 μM) produced a marked reduction in EPSP amplitude. B: Graph plotting the amplitude of the presynaptic volley against time for the same slice as (A).

Both adenosine (100 μM) and L-AP4 (50 μM) produced a small reversible reduction in volley amplitude. C: Graph plotting mean EPSP amplitude against mean presynaptic volley amplitude for the same slice in (A and B). To determine the relationship between presynaptic volley and EPSP amplitude, the stimulus strength was increased (from minimal stimulation strength) and the mean volley amplitude was plotted against mean EPSP amplitude for each stimulus. If the effects of adenosine and L-AP4 on fEPSP amplitude can be accounted for by the reduction in volley amplitude then fEPSP amplitude plotted against volley amplitude in adenosine and L-AP4 should fall on the line. The points fall well below the line (filled symbols) and thus the reduction in fEPSP amplitude cannot be accounted for by a fall in volley amplitude and by implication cannot be account for by a reduction in active parallel fibres. D: Graph plotting the amplitude of individual PF fEPSPs against time. Adenosine (100 μM) had little effect but L-AP4 (50 μM) produced a marked reduction in fEPSP amplitude. E: Graph plotting the amplitude of the presynaptic volley against time for the same slice as (D). Both adenosine (100 μM) and L-AP4 (50 μM) produced a small reversible reduction in volley amplitude. F: Graph plotting mean fEPSP amplitude against mean presynaptic volley amplitude for the same slice in (D and E). The relationship between volley and EPSP amplitude was constructed by changing the stimulus strength (as above). The amplitude of fEPSPs plotted against volley amplitude in adenosine (100 μM, filled symbol) falls upon the line and thus the reduction in fEPSP amplitude produced by adenosine can be accounted for by a fall in volley amplitude and by implication a reduction in active parallel fibres. In contrast fEPSP amplitude plotted against volley amplitude in L-AP4 (filled symbol) falls far below the line. Recordings were made in slices from P9-12 rats.

Fig. 6. Hypoxia has variable effects on parallel fibre synaptic transmission in slices from P9-12 rats. A: Graph plotting the normalised amplitude of individual parallel fibre fEPSPs against time (fEPSPs were normalised by dividing by the mean control amplitude). Hypoxia (N_2/CO_2) induced a reduction in fEPSP amplitude (~ 47%) which is almost completely reversed by blocking A_1 receptors with 8 CPT (2 μM). Inset, superimposed averages of 15 fEPSPs in control and in 100 μM adenosine from same experiment as (A, adenosine sensitivity measured before hypoxia was induced). Adenosine reduced fEPSP amplitude by ~ 80 %. B: Graph plotting the paired pulse ratio against time for fEPSPs in (A, paired pulse interval 50 ms). Hypoxia markedly increased the paired pulse ratio which was reversed by 8 CPT. C: Graph plotting the normalised amplitude of individual parallel fibre fEPSPs against time (fEPSPs were normalised by dividing by the mean control amplitude). Hypoxia induced a rapid reduction in EPSP amplitude (71 %) which was only partially and transiently reversed by blocking A_1 receptors with 8 CPT (2 μM) but was fully reversed by washing in oxygenated aCSF. Inset, superimposed averages of 15 fEPSPs in control and in adenosine (100 μM) from same experiment as (C, adenosine sensitivity measured before hypoxia was induced). Adenosine reduced fEPSP amplitude by ~ 20 %. D: Graph plotting the paired pulse ratio against time for fEPSPs in (C, paired pulse ratio 50 ms). Hypoxia markedly increased the paired pulse ratio which was only partially and transiently reduced by blocking A_1 receptors with 8 CPT (2 μM) but was fully reversed by reoxygenation.

Figure 1
Atterbury and Wall



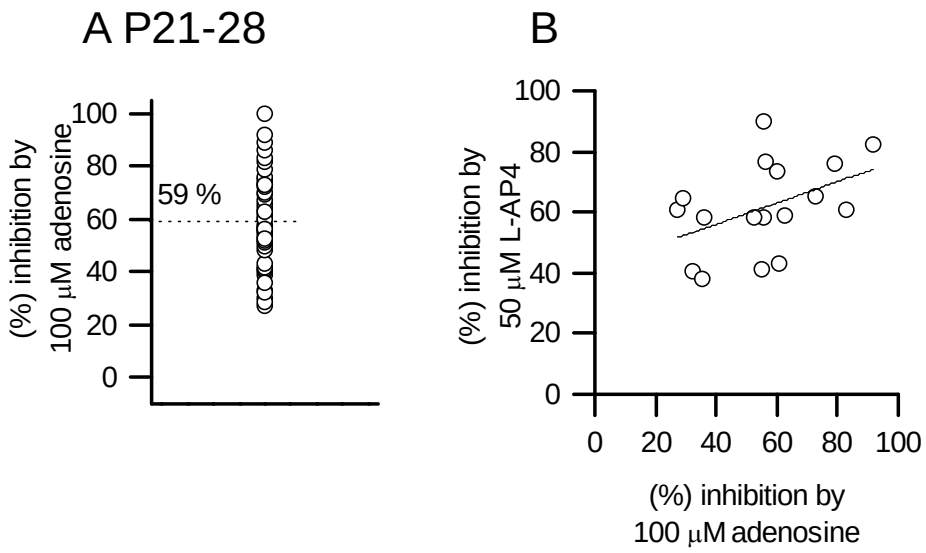


Figure 2 Atterbury and Wall

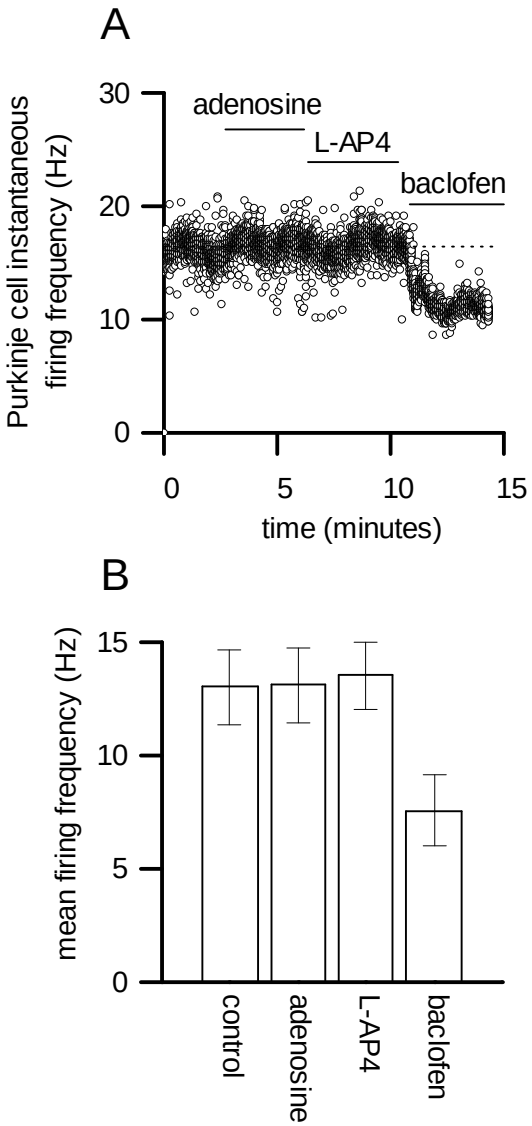


Figure 3 Atterbury and Wall

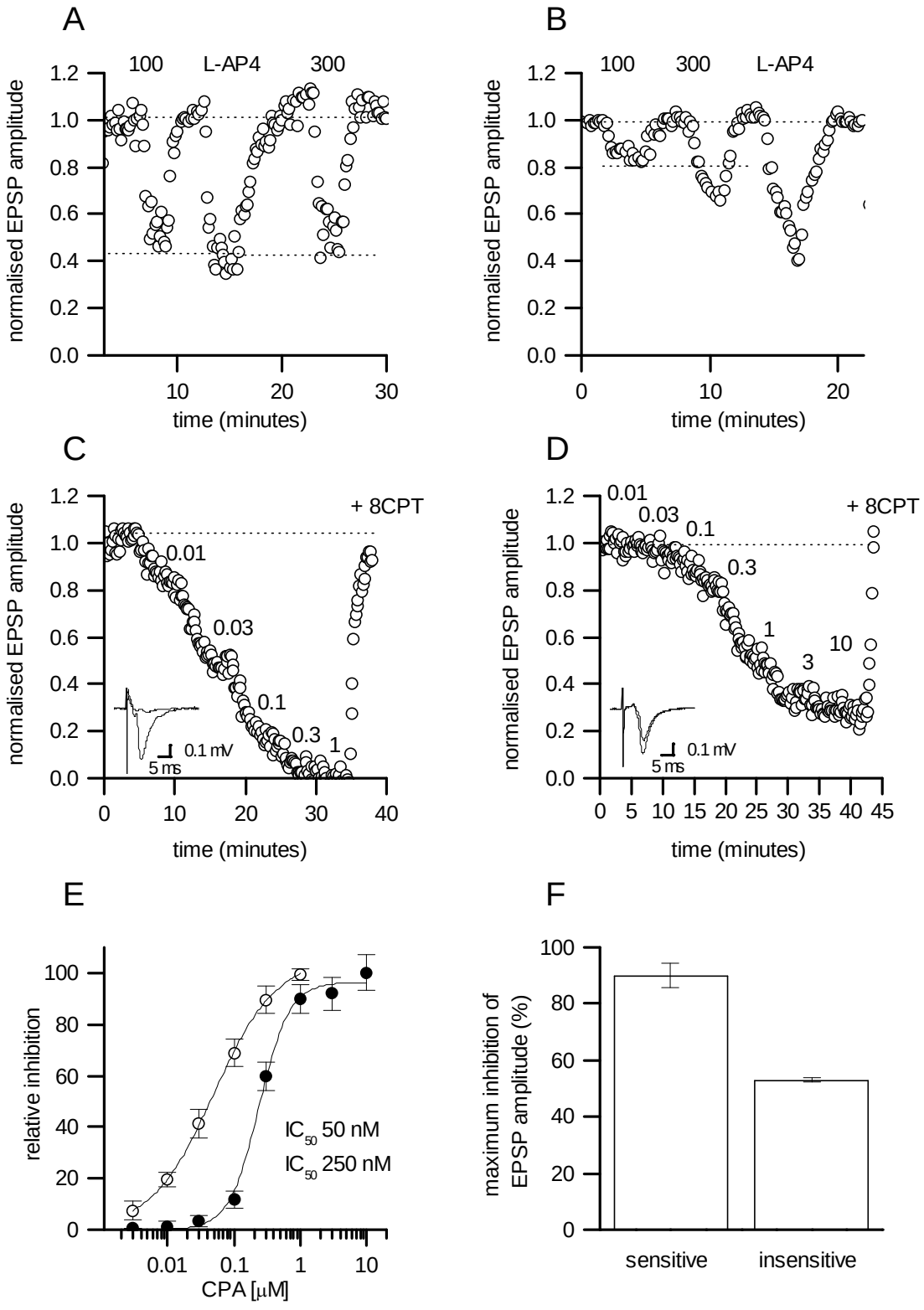


Figure 4 Atterbury and Wall

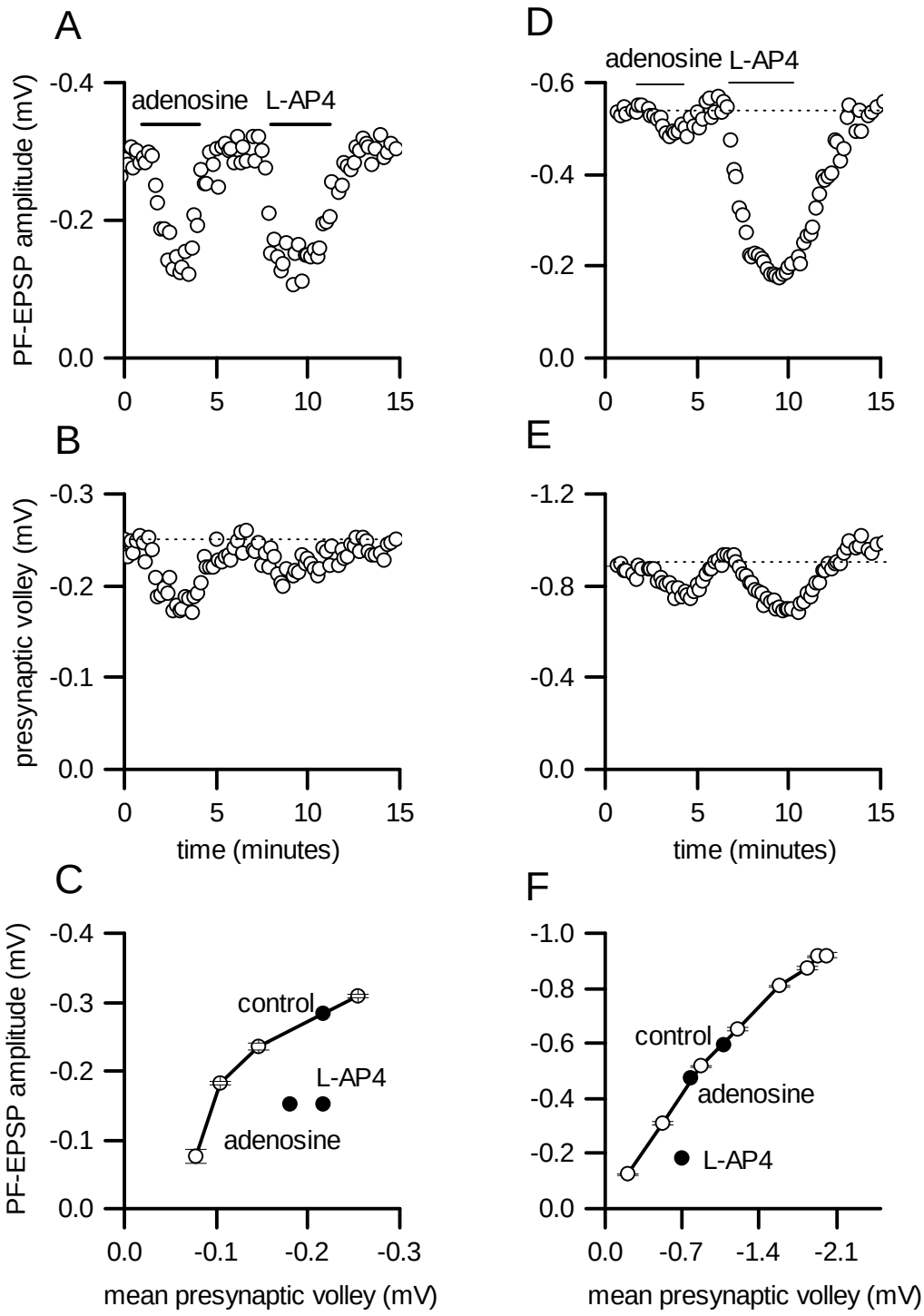


Figure 5 Atterbury and Wall

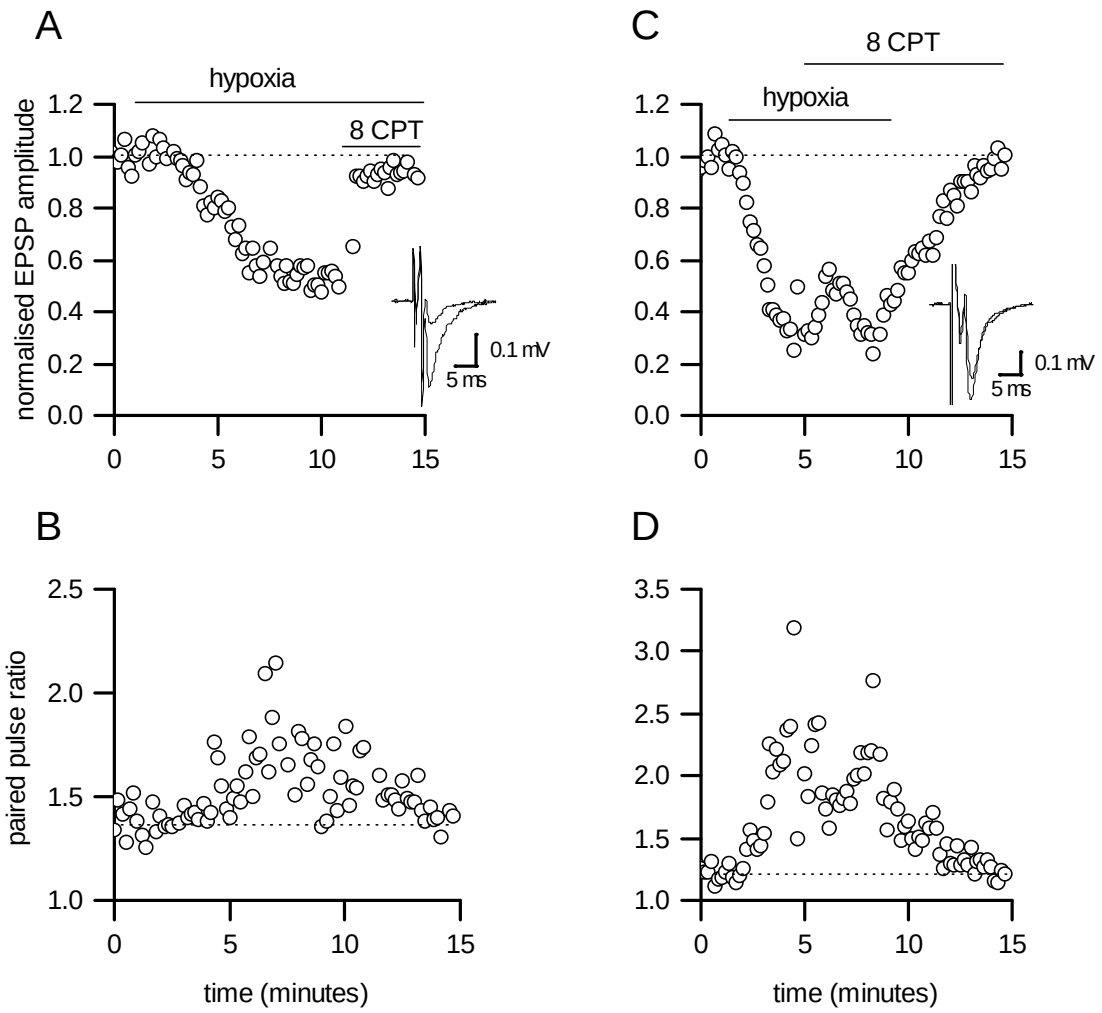


Figure 6 Atterbury and Wall