Localised adenosine signalling provides fine-tuned negative feedback over a wide dynamic range of neocortical network activities

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Abstract

Although the patterns of activity produced by neocortical networks are now better understood, how these states are activated, sustained and terminated still remains unclear. Negative feedback by the endogenous neuromodulator adenosine may potentially play an important role, as it can be released by activity and there is dense A₁ receptor expression in the neocortex. Using electrophysiology, biosensors and modelling, we have investigated the properties of adenosine signalling during physiological and pathological network activity in rat neocortical slices. Both low and high-rate network activities were reduced by A₁ receptor activation and enhanced by block of A₁ receptors, consistent with activity-dependent adenosine release. Since the A₁ receptors were neither saturated nor completely unoccupied during either low or high-rate activity, adenosine signalling provides a negative feedback mechanism with a wide dynamic range. Modelling and biosensor experiments show that during high-rate activity increases in extracellular adenosine concentration are highly localised and are uncorrelated over short distances that are certainly less than 500 μm. Modelling also predicts that the slow time course of the purine waveform cannot be from diffusion from distal release sites but more likely results from uptake and metabolism. The inability to directly measure adenosine release during low-rate activity, although it is present, is probably a consequence of small localised increases in adenosine concentration which are rapidly diminished by diffusion and active removal mechanisms. Saturation of such removal mechanisms when higher concentrations of adenosine are released, results in the accumulation of inosine, explaining the strong purine signal during high-rate activity.
The neocortex supports a range of different network activities which underlie its importance in cognitive function. These range from slow wave oscillations (Steriade et al. 1993) to sustained bursts of high frequency activity that occur in pathological states such as epilepsy (Grenier et al. 2003). Acute in-vitro slice preparations have been developed which, through manipulation of artificial cerebrospinal fluid (aCSF) composition, provide powerful experimental tools for analysing the roles synaptic and cellular components play in supporting these states (for example see Sanchez-Vives and McCormick 2000; Silberberg et al 2004). However, the precise nature of these states is still unclear (Parga and Abbot 2007; Frohlich and McCormick 2010) and in particular there remains intense debate as to the generative mechanism of their dynamics. One candidate mechanism is negative feedback provided by the activity-dependent release of an endogenous neuromodulator such as adenosine.

The purine adenosine is a potent neuromodulator, central to many CNS processes, including sleep, memory formation and locomotion, and can be neuroprotective (depending on brain region and stage of development) during epilepsy, hypoxia and ischemia (reviewed in Fredholm 1996; Boison 2009; Dale and Frenguelli 2009). Adenosine activates cell surface G-protein coupled receptors, with the widely distributed high affinity A1 receptor inhibiting transmitter release and hyperpolarising the membrane potential (reviewed in Fredholm et al. 2000). Once released, adenosine is rapidly removed by uptake and is subsequently converted either to AMP by adenosine kinase (primarily in glial cells) or to inosine and hypoxanthine by adenosine deaminase and purine nucleoside phosphorylase (reviewed in Dunwiddie and Masino 2001). Trains of action potentials increase the concentration of extracellular adenosine (activity-dependent adenosine release) in many brain regions including the hippocampus (Mitchell et al. 1993; Lovatt et al 2012; Wall and Dale 2013), Calyx of Held.
(Kimura et al. 2003; Wong et al. 2006), supraoptic nucleus (Oliet and Poulain 1999), striatum (Pajski and Venton 2010) and cerebellum (Wall and Dale 2007). It has been known for many years that adenosine release occurs during high-rate pathological activity, such as epileptic seizures, where it acts to reduce the frequency and duration of subsequent seizures (During and Spencer 1992; Boison 2008; Dale and Frenguelli 2009). Recent studies have shown that only a small number of action potentials at physiological frequencies are required to increase the extracellular adenosine concentration sufficiently to inhibit synaptic transmission (Lovatt et al. 2013; Wall and Dale 2013). Thus, during low-rate activity states, the release of adenosine and activation of $A_1$ receptors could control the degree of network activation, terminate events and prevent physiological activity becoming pathological.

We hypothesize that adenosine plays an important role in controlling neocortical network activity because $A_1$ adenosine receptors are strongly expressed by thick tufted layer-V pyramidal cells (Rivkees et al. 1995; Ochiishi et al. 1999), where they potently inhibit synaptic transmission (Kerr et al. 2013) and hyperpolarise the membrane potential (van Aerde et al. 2013). In this study we have investigated the role of adenosine in physiological and pathological neocortical network activity induced by either excitant solution (as a model of the up-down states observed in-vivo) or Mg$^{2+}$-free aCSF (as a model of higher rate pathological, epileptic-like activity). Our results demonstrate that adenosine provides highly localized negative-feedback control that is effective over a wide range of activity intensities.
Materials and Methods

Preparation of neocortical slices

Sagittal slices of hind-limb somatosensory neocortex (350-400 µm) were prepared from male Wistar rats, at postnatal days 18-25 (Kerr et al. 2013). Rats were kept on a 12 hour light-dark cycle with slices made 90 minutes after entering the light cycle. In accordance with the U.K. Animals (Scientific Procedures) Act (1986), male rats were killed by cervical dislocation and decapitated. The brain was removed, cut down the mid line and the two sides of the brain stuck down. Slices were cut around the midline with a Microm HM 650V microslicer in cold (2-4°C) high Mg\(^2+\), low Ca\(^2+\) aCSF, composed of (mM): 127 NaCl, 1.9 KCl, 8 MgCl\(_2\), 0.5 CaCl\(_2\), 1.2 KH\(_2\)PO\(_4\), 26 NaHCO\(_3\), 10 D-glucose (pH 7.4 when bubbled with 95% O\(_2\) and 5% CO\(_2\), 300 mOSM). Slices were trimmed so that only the neocortex was present, stored at 34°C for 30 minutes in aCSF (1 mM MgCl\(_2\), 2 mM CaCl\(_2\)) and then at room temperature for 1-6 hours.

Patch-clamp recording from layer-V neurones

A slice (350 µm) was transferred to the recording chamber and perfused at 3 ml min\(^{-1}\) with aCSF at 32 ± 0.5°C. Slices were visualized using IR-DIC optics with an Olympus BX51W1 microscope and Hitachi CCD camera (Scientifica, Bedford UK). Whole-cell current clamp recordings were made from layer-V pyramidal cells using patch pipettes (5–10 MΩ) manufactured from thick walled glass (Harvard Apparatus, Edenbridge UK) and containing (mM): potassium gluconate 135, NaCl 7, HEPES 10, EGTA 0.5, phosphocreatine 10, MgATP 2, NaGTP 0.3 and biocytin 1 mg ml\(^{-1}\) (290 mOSM, pH 7.2). Layer-V pyramidal cells were identified by their position in the slice, current-voltage
relationship and morphology. Voltage recordings were obtained using an Axon Multiclamp 700B amplifier (Molecular Devices, USA) and digitised at 20 KHz. Data acquisition and analysis was performed using Pclamp 9 (Molecular Devices). In some experiments whole-cell patch clamp recording was combined either with extracellular recording or biosensor measurements (see below).

**Extracellular and biosensor recording from neocortical slices**

A slice (400 µm) was transferred to the recording chamber, submerged in aCSF and perfused at 6 ml/min (32°C). The slice was placed on a grid allowing perfusion above and below the tissue and all tubing was gas tight (to prevent hypoxia). For extracellular recording, two aCSF filled microelectrodes were placed on the surface of layer-V (1-3 mm apart). Extracellular recordings were made using ISO-DAM amplifiers (WPI, Stevenage UK). Signals were filtered at 3 kHz and digitised on line (10 kHz) with a Micro CED (Mark 2) interface controlled by Spike software (Vs 6.1, Cambridge Electronic Design, Cambridge UK). Standard cylindrical microelectrode biosensors were inserted into the slice, so that biosensors went through the slice and disk biosensors were gently pushed into the slice surface.

**Biosensor characteristics**

Biosensors (Sarissa Biomedical Ltd, Coventry UK) consisted of enzymes trapped within a matrix around a Pt or Pt/Ir (90/10) wire (Llaudet et al. 2003). Biosensors were of two types: cylinders which had an exposed length of ~500 µm and diameter of ~ 50 µm and disk sensors which had a diameter of 125 µM. Four types of sensor were used in this study:
Firstly, null sensors, possessing the matrix but no enzymes, to control for non-specific electro-active interferents;

Secondly, biosensors containing adenosine deaminase, nucleoside phosphorylase and xanthine oxidase (responsive to adenosine, inosine and hypoxanthine: ADO biosensors);

Thirdly, biosensors containing nucleoside phosphorylase and xanthine oxidase (responsive to inosine and hypoxanthine: INO biosensors);

Finally, biosensors containing xanthine oxidase (responsive to hypoxanthine: HYP biosensors).

A full description of biosensor properties has previously been published (Llaudet et al. 2003). After each experiment, the biosensors were calibrated with analyte (10 μM). In some experiments, the composition of purines detected by ADO biosensors was not fully defined. Since ADO biosensors have an equal sensitivity to adenosine, inosine and hypoxanthine (Llaudet et al. 2003; Wall et al. 2007), the total concentration of purines detected was related to the calibration to adenosine to give μM’ or nM’ of purines (as outlined in Pearson et al 2001; Klyuch et al. 2011). Biosensor signals were acquired at 1 kHz with a Micro CED (Mark 2) interface using Spike (Vs 6.1) software.

**Purine diffusion model**

We construct a basic mathematical model that can be used to provide an upper bound on the distance that adenosine travels once released into the extracellular space. To this end, we neglect removal by cellular uptake and combine together the adenosine, inosine and hypoxanthine concentrations into a single purine concentration \( C(t) \), which obeys diffusive dynamics
\[ \frac{\partial C}{\partial t} = D \nabla^2 C \]  

**Equation 1**

where \( D \) is the diffusion constant. Two scenarios are considered: (i) the *in-vitro* slice preparation, of thickness \( h \), with a zero-concentration condition at the top and bottom slice surfaces and (ii) free diffusion, which is more similar to the *in-vivo* case. For the first case we consider that the variable \( z \) measures the distance normal to the surfaces of the slice at \( z = h/2 \) and \(-h/2\), and \( r \) measures a distance parallel to the slice surface. The Fourier-series solution for an initial sheet-like concentration, uniform in \( x \) and \( y \) at the centre of the slice (Klyuch et al. 2011) can be adapted to the case of an initial point-like source at position \( x=y=z=0 \), by simply multiplying that result with a 2D Gaussian that is a function of the variable \( r^2 = x^2 + y^2 \). The resulting space- and time-dependent concentration can be written as

\[ C = \exp\left(-\frac{r^2}{4Dt}\right) \frac{2}{h} \sum_{m=0}^{\infty} \exp\left(-t/\tau_m\right) \cos\left(\frac{(2m+1)\pi z}{h}\right) \]  

**Equation 2**

where the time constants \( \tau_m \) are defined as

\[ \tau_m = \frac{h^2}{(2m+1)^2 \pi^2 D}. \]  

**Equation 3**

Equation 2 gives the concentration for an initial short release event of unit amplitude at time \( t=0 \) within the slice at the origin. Setting \( z=0 \) in the above equation gives the concentration at a distance \( r \) from the release site in the centre of the slice. The time constant, \( \tau_0 = h^2/\pi^2 D \), of the longest decay can be used to estimate the effective diffusion
constant in tissue. The solution given by Equation 2 can also be written in terms of Gaussians using the method of images. This is a method used in the physics of charged particles that makes use of the linearity of the problem. It accounts for the zero-concentration boundaries through the addition to the point source at \( x=y=z=0 \) of fictitious sources at distances \( z=h, 2h, 3h, \) etc from the centre. These have freely diffusing concentrations (3D-Gaussian solution) that have alternating signs. However, the first term of such a series solution is simply identical to the diffusion in the \textit{in-vivo} geometry (with no slice surfaces) where here \( r^2=x^2+y^2+z^2 \)

\[
C = \frac{\exp\left(-r^2/4Dt\right)}{(4\pi Dt)^{3/2}} \quad \text{Equation 4}
\]

This equation behaves closely to that for the slice geometry (Eq 2) at times \( < h^2/4D \), which are before any purine molecules are likely to have reached the slice surface. In plotting the purine concentrations \( C(t) \), the waveforms were filtered with an additional exponential filter of time constant 1 second to mimic the rise time constant on the purine biosensors.

**Deconvolution and reconvolution of purine waveforms**

The amplitude of closely spaced purine waveforms can be difficult to measure as subsequent pulses sit on the decay of proceeding ones. Following Richardson and Silberberg (2008) closely spaced purine pulses were deconvolved as in Klyuch et al (2011) by removing the long decay \( \tau_0 \) component. The resulting sharper, well-spaced pulses could then be cropped and reconvolved to yield isolated purine waveforms from which the amplitude could be directly measured.
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, inosine, hypoxanthine and 8-cyclopentyltheophylline (8CPT) were purchased from Sigma (Poole, Uk). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was purchased from Tocris-Cookson (Bristol UK).

Data analysis

The data is presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student’s t-test. The significance level was set at p<0.05.

Induction of cortical network activity

In control conditions, neocortical slices are essentially quiescent with spontaneous network activity extremely infrequent. Thus, in common with previous studies, network activity was induced by altering the ionic composition of the extracellular solution (aCSF). Two different extracellular solutions were used to excite network activity: excitant solution to induce up-down state like activity (aCSF with 0.5 mM Mg$^{2+}$, K+ 6.5 mM, 1.5 mM Ca$^{2+}$; Sanchez-Vives and McCormick, 2000; Silberberg et al. 2004) and zero Mg$^{2+}$ aCSF (0 mM Mg$^{2+}$, 3.5 K+, 2 mM Ca$^{2+}$) to induce strong epileptiform-like activity (Mody et al. 1987; Traub et al. 1994; Gulyás-Kovács et al. 2002). It took 10 to 15 minutes for network activity to commence and there was a progression in the level of excitation until steady state conditions occurred, reflecting the slow time to alter ionic concentrations deep within the slices. Network activity was defined as co-ordinated activity on two electrodes, which includes extracellular electrodes, biosensors (as they
also act as extracellular electrodes, Dale et al. 2000) and intracellular recordings from pyramidal cells.

Results

Adenosine $A_1$ receptors modulate low-rate neocortical network activity

Perfusion of slices with excitant solution induced co-ordinated network activity, defined as correlated activity on both extracellular electrodes (Figure 1A). In 16 out of 18 slices (88%) activity consisted of single and small groups (2-5) of population spikes (Figure 1A), with a population spike defined as an isolated change in potential (Figure 1A, inset). In 2 slices, longer bursts of activity were also observed. The frequency of activity was $0.1 \pm 0.02$ Hz ($n=18$) which is ~6 events per minute. Whole-cell patch clamp recordings from layer-V pyramidal cells revealed that a single extracellular population spike was equivalent to a short lived membrane depolarisation (~200-300 ms) with 1-5 superimposed action potentials (frequency 10-25 Hz, Figure 1B, $n=5$). Groups of these depolarised states could occur together, on occasion, with a high frequency component, similar to that induced by zero Mg$^{2+}$aCSF (see later).

To investigate whether the network activity induced by excitant solution can be modulated by adenosine receptor activation, adenosine (100 $\mu$M) was applied. Adenosine either abolished or greatly reduced the frequency of co-ordinated activity (activity abolished in 6 slices, frequency reduced from 0.1 to 0.001 Hz in 2 slices). Infrequent activity could sometimes be observed on individual electrodes but this was no longer co-ordinated across the network (Figure 1A). The effects of adenosine could be reversed in wash or by the application of the $A_1$ receptor antagonist 8 CPT (2 $\mu$M, $n=5$).
To investigate whether A<sub>1</sub> receptors are activated and functional during network activity, the A<sub>1</sub> receptor antagonist 8CPT was applied. Blocking A<sub>1</sub> receptors significantly (P < 0.01) increased the frequency of network events (from 0.084 ± 0.03 to 0.145 ± 0.05 Hz; a reduction in inter-event interval from ~12 to 7 s, n = 9 slices, Figure 1C). Examination of inter-event intervals revealed an increase in the probability of an event occurring within the first few seconds of the previous event, suggesting that the activation of A<sub>1</sub> receptors plays a role in terminating and subsequently suppressing activity (Figure 1C, inset). In some slices (n = 4) blockade of A<sub>1</sub> receptors with 8CPT also induced bursts of sustained activity, which were similar to that observed in zero Mg<sup>2+</sup> aCSF (Figure 1D, mean duration 88 ± 33 s, see later) suggesting that activation of A<sub>1</sub> receptors can prevent transition from physiological to pathological states. The increase in network excitability following block of A<sub>1</sub> receptors is consistent with adenosine being released into the extracellular space by activity.

**Actions of adenosine during high-rate pathological network activation**

To induce high-rate network activity, slices were perfused with aCSF which did not contain magnesium (zero Mg<sup>2+</sup> aCSF). This solution reliably induced spontaneous activity (13 out of 13 slices) with activity patterns that ranged from frequent population spikes to sustained bursts of activity (mean burst length 32 ± 3 s, burst frequency 0.02 ± 0.01 Hz, ~ 1 burst per minute, Figure 2A). Whole-cell patch clamp recordings from layer-V pyramidal cells revealed that each burst consisted of an initial period of continuous action potential firing (10-100 Hz) followed by individual bursts made up of 1-5 action potentials (fired at 10-25 Hz) superimposed on short depolarisations (Figure 2B, n = 5).
To confirm that neocortical network activity induced by zero Mg\(^{2+}\) aCSF could be modulated by adenosine receptor activation (see O'Shaughnessy et al. 1988), adenosine (100 μM) was applied. In 7 out of 7 slices, application of adenosine greatly reduced network activity (Figure 2A). The frequency of bursts was reduced from 0.017 ± 0.01 Hz to 0.005 ± 0.0023 Hz, less than 1 burst every 3 minutes. Burst duration was decreased from 30.5 ± 4.44 to 8.2 ± 5 s, with in some slices, bursts converted to isolated population spikes. The effects of adenosine could be reversed in wash or by application of the A\(_1\) receptor antagonist 8CPT (1-2 μM, Figure 2A, n = 4).

To investigate whether there is activation of A\(_1\) receptors during the network activity induced by zero Mg\(^{2+}\) aCSF, the A\(_1\) receptor antagonist 8CPT was applied. In 8 out of 8 slices, 8CPT (1-2 μM) caused an increase in network excitation, a fall in the time interval between bursts and an increase in the frequency of events within a burst (with events considered separate if they occurred more than 0.5 s apart, Figure 2C). The interval between bursts was reduced from 16.5 ± 0.8 s to 4.6 ± 1.7 s, with the frequency of events within a burst increasing from 0.34 ± 0.2 Hz to 0.83 ± 0.3 Hz). In some slices, discrete bursts of activity became almost continuous with no obvious quiescent periods between bursts. This suggests that endogenous activation of A\(_1\) receptors plays a role in the termination of activity preventing continual network activation. Thus, as with many former studies (particularly in the hippocampus) pathological network activity is enhanced by blocking A\(_1\) receptors and the quiet periods between bursts are at least in part caused by the actions of adenosine.

Measuring adenosine release directly with biosensors during low-rate activity
Low-rate network activity induced by excitant solution was enhanced by blocking $A_1$ receptors, which is consistent with an increase in the concentration of extracellular adenosine during activity. To investigate this, ADO biosensors and null sensors were placed within layer V, to directly measure changes in extracellular adenosine concentration during activity. Although excitant solution always induced activity, in the majority of slices (11 out of 14) this did not increase the ADO biosensor current compared to the null sensor (Figure 3A, B). There was also no clear increase in ADO biosensor current immediately following a single population spike or a group of population spikes (Figure 3A, B). The fast deflections present on the ADO biosensor occur at the same time as the extracellular electrode (and null sensor) and have the same duration, and thus are produced by electrical activity (action potentials and EPSPs) rather than purine detection. Although the detection of electrical events by the biosensor shows that the network is active around the biosensor, there was no detectable increase in the concentration of extracellular adenosine (or metabolites) during activity. Thus if adenosine is being released the concentration may be lower than the limits of detection ($\sim 25$ nM, see later) or there may be local release and the adenosine is rapidly removed from the extracellular space.

To confirm the validity of our results we carried out a number of controls. Firstly, to confirm that the damage produced by biosensor insertion did not prevent adenosine detection, biosensors were placed parallel to the slice surface (Klyuch et al. 2011; Wall and Dale 2013). This did not reveal a current on the ADO biosensor during excitant-induced activity ($n = 5$, data not shown). To confirm that an increase in extracellular adenosine concentration (or metabolite concentration, as the biosensor also detects adenosine metabolites, see later) could be detected either the $\text{Mg}^{2+}$ concentration of excitant solution was lowered (to between 0.3 and 0 mM) or $A_1$ receptors were blocked.
with 8CPT. This reliably produced large biosensor currents (Figure 3C) but these were associated with bursts of activity rather than single or groups of population spikes. To check whether the extracellular concentration of adenosine (or its metabolites), is increased in other cortical layers, ADO biosensors were placed into layer 2/3. This did not reveal biosensor currents during excitant-induced activity, although zero Mg\textsuperscript{2+} aCSF induced large ADO biosensor currents (5 out of 5 slices) similarly to what was seen in layer 5 (data not shown).

Adenosine release can be directly measured during high-rate activity
In most slices, the activity induced by excitant solution (single or small groups of population spikes) did not lead to a directly detectable increase in extracellular adenosine (or metabolite) concentration. However, in a small minority of slices (3 out of 14) small but unambiguous increases in ADO biosensor current were observed, which were absent on the null sensor (Figure 4A). When examined carefully, it was found that the activity consisted of population spikes followed by high frequency activity (Figure 4A, inset). For further investigation, we combined whole-cell patch clamp recordings low-rate excitant solution, with the Mg\textsuperscript{2+} varied from 0.5 to 0.3 to induce different strengths of activity. When pyramidal-cell activity was equivalent to single population spikes (0.5 mM Mg\textsuperscript{2+}) there was no detectable increase in extracellular purine concentration (\(n = 7\), Figure 4B). However, when Mg\textsuperscript{2+} was lowered (to 0.3 mM) clear biosensor signals could be observed (\(n = 4\), Figure 4C, D). In most of the pyramidal cell recordings activity appeared prolonged and the depolarisation larger than with 0.5 mM Mg\textsuperscript{2+} indicative of greater network activity (Figure 4C, D). Thus although the form of activity required to produce detectable concentrations of adenosine is not radically different to when adenosine cannot be measured, greater network activity is required.
In figure 4C although a large biosensor current was produced by the initial activity (arrows) subsequent activity was not detected on the biosensor and there was no change in the gradient of the biosensor current. This suggests adenosine (or metabolites) may only be detected locally, where the network is active.

Properties of adenosine release induced by zero Mg$^{2+}$ aCSF

We used the data from pathological high-rate network activity combined with simulations, to analyse the properties of adenosine release, diffusion and metabolism. In zero Mg$^{2+}$ aCSF, each burst of neural activity induced a current on the ADO biosensor with no effect on the null sensor baseline current (Figure 5A, $n = 6$). Deconvolution-reconvolution analysis (see methods) of the overlapping events on the ADO biosensor allowed for separation into component waveforms, facilitating measurement of amplitude and kinetics (Figure 5B). The mean amplitude of the current produced by the first burst of neural activity was $422 \pm 200$ pA which is equivalent to $3.4 \pm 1.1 \mu$M of purines (range 0.1 to 13 $\mu$M, $n = 14$). The large variability in the amount of purines detected presumably stems from differences in the amount of neural activity (number of cells activated and the duration of the activity) and distance from release sites.

Since ADO biosensors respond not only to adenosine but also to inosine and hypoxanthine (adenosine metabolites, Llaudet et al. 2003) with approximately equal sensitivity, the ADO biosensor signal could be produced by adenosine alone, adenosine metabolites or a combination of both. Thus the adenosine deaminase inhibitor EHNA (Agarwal et al. 1977) was used to determine the proportion of the purines detected that arise from the extracellular metabolism of adenosine (Wall and Dale 2007; Klyuch et al. 2011; Wall and Dale 2013). EHNA prevents the conversion of adenosine to inosine (by adenosine deaminase, ADA) in the slice and also prevents ADO biosensors detecting
adenosine, with no effect on the detection of inosine or hypoxanthine (Wall and Dale 2007). If adenosine is directly detected or any inosine (or hypoxanthine) detected arises from the metabolism of adenosine by ADA, then EHNA will reduce the amplitude of the biosensor signal (Wall and Dale 2007; Klyuch et al. 2011; Wall and Dale 2013). Conversely, if the biosensor signals arise from the direct release of inosine or hypoxanthine into the extracellular space, then EHNA will have no effect on the signal amplitude (Wall et al. 2010). Following the induction of network activity (with zero Mg\(^{2+}\) aCSF) and resultant purine release, EHNA (20 \(\mu\)M) was applied (2 \(\mu\)M of the A\(_1\) receptor antagonist 8CPT was also present to prevent accumulation of adenosine reducing neural activity). EHNA reduced the ADO biosensor signal by 74 ± 5 % (Figure 5C, \(n = 5\)) and thus either adenosine is directly detected or the biosensor signal arises from the metabolism of adenosine by ADA in the slice.

The ADO biosensor waveform had a slow time to rise of 108 ± 10 s (\(n = 10\), Figure 5D). This slow rise was not determined by the response time of the biosensor, as it is much slower than biosensor calibration traces (rise typically ~10-15 s, Figure 5D), which are limited by the rate of bath exchange rather than speed of biosensor response. For fast applications of adenosine the biosensor response time is of the order of 1 s (Figure 5D, bottom panel). We also observed that the signal on the ADO biosensor is delayed and did not start to rise until around the middle of bursts of activity (Figure 5E, also see Figure 4C). The delay and slow rise suggest that either adenosine or its metabolites have to diffuse to the biosensor from distant release sites or sufficient adenosine has to be released to saturate efficient removal mechanisms and reach the biosensor, directly or in the form of metabolites. The decay of the ADO biosensor waveform was also slow (decay time constant of ~ 200 s, \(n = 5\) see figures 5A, B and
compare with 5D bottom panel) which is consistent with the slow loss of purines from
the slice surface into the bath medium (Klyuch et al. 2011).

The slow purine waveform is not the result of diffusion

We used mathematical models and simulations to investigate whether it is feasible for
the slow (100 s) rise of the ADO biosensor signal to result from the diffusion of
adenosine or its metabolites from distal release sites. The role of this model is not to
capture adenosine transport in detail, but to provide an upper bound on the distance
adenosine will travel by ignoring removal mechanisms. We examined two models: a
model that accounts for the in-vitro configuration (Eq 2) of the slice (with top and
bottom surfaces open to the bathing medium) and a model that has free diffusion (Eq 4)
and is therefore similar to the in-vivo geometry. Following Klyuch et al (2011) we used
the 200 s decay time constant of the waveform to provide a model-dependent estimate
of the effective diffusion constant of adenosine in the neocortex as 80 \( \mu \text{m}^2/\text{s} \), which is
lower than that estimated for the cerebellum (~250 \( \mu \text{m}^2/\text{s} \)) and also than that expected
on the grounds of tortuous diffusion in the extracellular space, potentially reflecting the
action of distinct transport mechanisms in the neocortex. Model plots (Fig 6A) of the
purine-concentration waveform at four distances from a site of brief release show a
rapid decrease in amplitude with distance and increase in the rise time. For a slice
thickness of 400 \( \mu \text{m} \) there was little difference between the model that included the
effects of the slice surfaces (Eq 2) or simply free diffusion (Eq 4) up to times of the order
of 100s. The Gaussian distribution for free diffusion (Eq 4) can therefore be used to
estimate the time to peak amplitude, and gives \( t=r^2/6D \). From this result it is
straightforward to show that the peak amplitude decreases rapidly with distance from source in proportion to $1/r^3$. These observations are shown in Fig 6B-D where it is seen that to observe a rise time of the order of 100s would require the source to be 220$\mu$m distant (Fig 6B,C), but at that distance the amplitude is 500 times less than that at 25$\mu$m (Fig 6D). Applying this to our experimental data suggests that, although on average 3.4 $\mu$M of adenosine (or metabolites) were measured for each burst of activity, in order to have such a slow rise time the concentration at 25 $\mu$m from the site of release would be $\sim 1.7$ mM. Such a high concentration is extremely unlikely, and was never measured during our experimentation (highest concentration measured was 13 $\mu$M). The conclusion therefore is that the slow rise on the biosensor is not due to diffusion from a distal source, but rather to other transport or delayed release mechanisms.

We additionally tested the model prediction that adenosine (and metabolites) will only be detected close to release sites by using multiple biosensors. We applied Mg$^{2+}$ free aCSF and looked for differences in responses between 2 biosensors (placed $\sim 0.5$ mm apart in layer V, Figure 7A). In most experiments, purine currents and electrical events (fast deflections) were measured on both biosensors. However, there was considerable variation in the concentration of purine detected by each biosensor consistent with a heterogeneous distribution of purine concentrations across the slice. In the example illustrated in figure 7B, as network activity begins, there are initially no purine currents or fast deflections, due to electrical activity present on biosensor (1) but clear purine currents are present on biosensor (2) along with deflections (arrows) due to network activity. Thus the network is inactive around biosensor (1) and the increase in purine concentration that occurs around biosensor (2) is not detected by biosensor (1). Since the biosensors are approximately 500 $\mu$m apart, this agrees with the model predictions that virtually all of the purine released will be diluted below the level of
detection once they diffuse ~300 μm. However, once network activity becomes co-
ordinated across the slice and is detected on both biosensors, then purines are
measured on both biosensors (Fig 7B). However there is no clear correlation between
the amplitude of the purine waveforms present on both biosensors. These results show
that adenosine and its metabolites have concentrations that are a function of the local
activity and vary on a scale that is certainly less than 500 μm.

Adenosine is metabolised before biosensor detection

Both experimental data and modelling suggest that the slow biosensor response time is
a consequence of the release process rather than a long diffusion distance. It is possible
that adenosine is metabolised before biosensor detection, since the ADO biosensor will
detect adenosine and its metabolites, and this could contribute to the slow response
time. To investigate this, differential measurements were made using ADO, INO and HYP
biosensors (for example see Wall et al. 2007). If adenosine was directly detected, and
there was no metabolism in the tissue, there would only be a signal on the ADO
biosensor. If adenosine was metabolised to inosine this would produce currents on ADO
and INO biosensors and if adenosine was metabolised through to hypoxanthine this
would produce currents on all 3 biosensors. Subtracting the calibrated biosensor
signals allows for an estimation of extracellular adenosine, inosine and hypoxanthine
concentrations during activity (Wall and Dale 2007).

The three biosensors were inserted into layer V of the neocortex and network
activity was induced by zero Mg²⁺ aCSF. During network activity, currents were
measured on all 3 biosensors in 8 out of 10 slices (Figure 8A, in 2 slices there was no
signal on the HYP biosensor). Addition of EHNA (20 μM), to block adenosine deaminase,
reduced the signal on all 3 biosensors (81 ± 5 % n = 3, Figure 8B) demonstrating that
the signals either arises directly from adenosine or following adenosine metabolism. The signal on the HYP biosensor directly demonstrates an increase in the extracellular concentration of hypoxanthine during activity. Thus a proportion of adenosine is metabolised to hypoxanthine by adenosine deaminase and purine nucleoside phosphorylase. To determine whether adenosine was directly detected, currents on ADO and INO biosensors were compared. It would be predicted that the rise of the signal on the ADO biosensor would be faster than the INO biosensor signal, as there is a metabolism step before inosine is detected. However there was no significant difference in the rise time of the currents (Figure 8C) on ADO and INO biosensors. Examination of the signals on ADO and INO biosensors within recordings from individual slices revealed no clear correlation. Sometimes large amplitude signals occurred on the ADO biosensor with little on the INO biosensor and vice versa (for example the INO biosensor currents are larger than ADO biosensor currents in Figure 8 A). This is consistent with each biosensor only detecting the purines released by local network activity, with little or no overlap on what is measured by each sensor. Thus in most slices, little adenosine is directly detected and instead most of the adenosine is metabolised before biosensor detection.

**Metabolism of exogenous adenosine**

To investigate adenosine metabolism in more detail, adenosine was applied to neocortical slices and the purines produced were measured using ADO, INO and HYP biosensors inserted into layer V. Biosensor signals, scaled by calibration, were subtracted to estimate the concentrations of adenosine, inosine and hypoxanthine in the tissue (as in Wall et al. 2007; Frenguelli et al. 2007). Constant duration adenosine applications (50 μM, 5 minutes) produced, 3 ± 0.2 μM adenosine in the tissue (n = 5),
which represents ~ 6 % of the applied adenosine (Figure 9A). The rise-time (~300-400 s) of the ADO biosensor current was significantly slower than the rise of the calibration trace (~ 10-15 s). Thus the ADO biosensor is only detecting the adenosine that has diffused into the slice, rather than measuring adenosine in the bath. A similar slow rise-time of adenosine current was also observed with disk sensors, which have a sensing area only at the end of the biosensor which is pushed into the slice surface ($n = 3$).

During diffusion through the slice, the adenosine produced $0.7 \pm 0.2 \mu M$ inosine and $0.4 \pm 0.1 \mu M$ hypoxanthine ($n = 5$ slices). In some slices (3 out of 5) there was a clear delay (75 ± 5 s) between the initial detection of adenosine (ADO biosensor) and the detection of inosine (Figure 9B). In all slices, the currents measured with INO biosensors were markedly slower to rise than currents measured on ADO biosensors (Figure 9B, inset). The slow rise on the INO sensor was not a consequence of slower inosine diffusion in the tissue (compared to adenosine) as the rise-time for bath application of inosine (50 $\mu M$) was the same for applied adenosine (see figure 9A inset). When inosine was applied, a similar amount of inosine was detected in the tissue ($3.0 \pm 0.8 \mu M$) compared to adenosine application, although more hypoxanthine was detected (2.0 ± 0.1 $\mu M$).

Interestingly there was no clear delay between the currents on the INO and HYP biosensors (Figure 9C). This is consistent with most of the adenosine being removed before it reaches the biosensor and the slow production of metabolites.

Discussion

We have investigated the properties of adenosine signalling in the neocortex using in-vitro models of low-rate activity (excitant solution) and high-rate pathological activity (zero Mg$^{2+}$ aCSF). Both of these patterns of network activity were strongly attenuated by activation of adenosine A$_1$ receptors and were enhanced by blocking A$_1$ receptors.
with an antagonist. Although this has been previously reported for high-rate activity (O'Shaughnessy et al. 1988) this is the first report for a role for adenosine in controlling low-rate activity and shows that neocortical A₁ receptors were neither fully occupied (saturated) or unoccupied during either low or high-rate activity. As the extracellular concentration of adenosine sits towards the middle of the concentration-response for A₁ receptors, small changes in adenosine concentration will have large effects on network activity. Thus neocortical adenosinergic signalling is a negative-feedback mechanism with a wide dynamic range and is able to control network activities that vary from low frequency single events to prolonged high frequency bursts.

**Localised increases in extracellular adenosine concentration**

During pathological, high-rate activity sufficient adenosine was released into the extracellular space to be detected with a biosensor, as also observed for hippocampal seizures (Dale and Frenguelli 2009; Etherington et al. 2009). Experimental data from multiple biosensors supported by computational modelling, show that the increase in extracellular adenosine and metabolite concentration was not homogeneous across the neocortex but was instead highly localised. Adenosine did not flood as a wave across tissue, but instead the concentration of adenosine was determined by local network activity, with increases in extracellular adenosine concentration becoming uncorrelated over very short distances, which are certainly less than 500 µm.

During low-rate activity (single and groups of population spikes), adenosine (and metabolites) could not be directly measured with a biosensor. However, when A₁ receptors were blocked, there was an increase in the probability of a network event occurring within a short interval after the previous event, which is consistent with the
pulsatile release of adenosine. Since the Kd for A<sub>1</sub> receptors is low (between 70-300 nM; Dunwiddie and Masino 2001; Fredholm et al. 2001) and the concentration of adenosine falls very rapidly during diffusion, if adenosine release is very proximal to the receptors, it is feasible for the localised release of adenosine to modulate activity but not be detected by a biosensor. The minimal detectable increase in the extracellular concentration of adenosine (or metabolite) is ~25 nM (assuming a sensitivity of the ADO biosensor of at least ~2000 pA for 10 μM adenosine, which would give currents in the order of ~2-5 pA). In the hippocampus, an increase in extracellular adenosine (or metabolite) concentration could be detected with a biosensor after a single population spike (Wall and Dale 2013). However, the currents measured were small (~ 2 pA) and only observed in a minority of slices. Similar release may occur in the neocortex, but if the number of activated fibres/cells is smaller than in the hippocampus and there is a longer diffusion distance between the release sites and the biosensor, then the concentration of adenosine released will fall below the limits of biosensor detection.

Instead of pulsatile adenosine release, there could be a basal homogeneous extracellular tone of adenosine, which acts to dampen down network excitability. Because modelling and experimental data suggest that adenosine is rapidly diluted during diffusion, a homogeneous global extracellular tone would require the coordinated release of adenosine from multiple release sites across the tissue. It is much more likely that the extracellular concentration of adenosine will vary depending on local network activity. This is in keeping with previous experimental evidence for such local variation seen by the effects of blocking A<sub>1</sub> receptors on synaptic transmission between pairs of layer-V pyramidal cells: There was marked variation in the degree of A<sub>1</sub> receptor activation between synapses in slices from the same animal and even between reciprocal synapses within the same slice (Kerr et al. 2013). Thus adenosine
signalling provides a targeted negative feedback mechanism for the fine, local control of network activity.

With pathological high-rate activity, inosine not adenosine is detected by biosensors

Evidence from differential biosensor measurements suggest that the major purines detected during high-rate pathological activity were the adenosine metabolites inosine and hypoxanthine rather than adenosine itself. Blocking adenosine deaminase (ADA) markedly reduced the amplitude of currents on all biosensors and thus the inosine and hypoxanthine arise from adenosine metabolism (Wall and Dale 2007; Klyuch et al. 2011). There was no clear difference in the rise of currents measured on ADO and INO biosensors. If adenosine was directly detected, then the signal on the INO biosensor would be expected to be slower or delayed compared to the signal on the ADO biosensor, as metabolism of adenosine would have to occur before inosine detection. This was confirmed in experiments where adenosine was exogenously applied to slices and the signal on the INO biosensor was much slower than the ADO biosensor signal. A similar delay in inosine production has also been observed in the cerebellum and in the hippocampus (Wall et al. 2007; Frenguelli et al. 2007). Once produced, inosine is metabolised to hypoxanthine by purine nucleoside phosphorylase (PNP) that, like ADA, is mainly an intracellular enzyme (Bzowska et al. 2000). Therefore adenosine is transported into cells, metabolised and then effluxed back into the extracellular space before detection. The slow rise of the currents on all biosensors (in the order of 100 s) is consistent with these intervening metabolic and uptake/efflux steps.
Modes of adenosine removal

Our data is consistent with a small amount of adenosine being released during low-rate network activity, which is rapidly diluted and actively removed before biosensor detection. Although the model included no active removal processes, just diffusion, it showed that the concentration of adenosine rapidly falls due to diffusion, providing an upper range on the distance of adenosine transport. Addition of active removal mechanisms would produce an even more rapid fall in concentration. Previous studies suggest that the major method of active adenosine removal, under basal conditions, is uptake into cells, by equilibrative nucleoside transporters (ENTs), and subsequent conversion to AMP by adenosine kinase (ADK, Dunwiddie and Masino 2001). Thus blocking either ADK or ENTs increases the extracellular concentration of adenosine and inhibits synaptic transmission via A₁ receptor activation (Wall et al 2007; Atterbury and Wall 2009; Etherington et al. 2009). In contrast, metabolism to inosine by adenosine deaminase (ADA) plays only a minor role in adenosine removal when there is little network activity. Thus, blocking ADA activity has little effect on the extracellular concentration of adenosine and synaptic transmission (Pak et al. 1994; Zhu and Krnjevic 1994; Wall et al 2007; Atterbury and Wall 2009). If however ADK is blocked, then inhibition of ADA has a much larger effect on adenosine removal (for example see Atterbury and Wall 2009). Thus it is predicted that with small amounts of adenosine released, the fall in concentration is dominated by diffusion with subsequent conversion of adenosine to AMP by ADK.

Our data from pathological high-rate network activity is consistent with a more significant role for ADA in adenosine removal (Lloyd and Fredholm 1995; Latini and Pedata 2001). With high-rate activity, the large amount of adenosine released saturates ADK ($K_{m} \sim 2\text{um}$, Phillips and Newsholme 1979) and some adenosine escapes...
conversion to AMP and is instead deaminated by ADA ($K_m \sim 47$ um, Geiger and Nagy 1986) to produce inosine. Since the concentration of extracellular adenosine rapidly falls, the high concentration of inosine inside the cell leads to efflux, down the concentration gradient, and detection by the biosensor. This is consistent with previous studies in the hippocampus where, for example blocking ADK increased the basal concentrations of adenosine but had little effect on the purine efflux produced by seizures since it is saturated (Etherington et al. 2009). Thus even during high-rate neocortical activity adenosine is not directly measured. The extracellular concentration falls rapidly due to diffusion and uptake and only an indirect measurement of adenosine is possible when one of the removal mechanisms becomes saturated. The efficiency of adenosine removal/inactivation ensures the localisation of signalling and thus allows fine control over small regions of the network.
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Legends

Figure 1. Low-rate neocortical network activity is modulated by adenosine. A, co-ordinated network activity measured on two extracellular electrodes. Adenosine (100 μM) abolished network activity which recovered in wash. Inset, a single extracellular population spike (*), at an expanded time base. B, Whole-cell patch clamp recording from a layer V pyramidal cell (intra) with simultaneous extracellular recording of a population spike (ext). C, Co-ordinated network activity on two extracellular electrodes. The A₁ receptor antagonist 8CPT (1 μM) increased burst duration and frequency. Inset, plot of cumulative probability for event interval from data in (C). D, Co-ordinated network activity on two extracellular electrodes. The A₁ receptor antagonist 8CPT (1 μM) induced a prolonged burst of activity. Network activity was induced by incubation in excitant solution.

Figure 2. Adenosine modulates pathological high-rate network activity. A, Co-ordinated activity on two extracellular electrodes. Adenosine (100 μM) reduced the frequency of burst occurrence, burst duration and spike amplitude. Addition of the A₁ receptor antagonist 8CPT (1 μM) reversed the effects of adenosine. B, Whole-cell patch clamp recording from a layer-V pyramidal cell (intra) with simultaneous extracellular recording (ext) during a burst of activity. C, Co-ordinated activity on two extracellular electrodes. Application of 8 CPT (1 μM) greatly increased activity with no pauses between bursts. Network activity was induced by incubation in zero Mg²⁺ aCSF. The marked differences in activity illustrated in (A) and (C) reflects variations across slices.
Figure 3. Biosensors do not reliably detect an increase in extracellular purine concentration with activity induced by excitant solution. A, Co-ordinated activity on an adenosine biosensor (ADO) and an extracellular electrode (ext) induced by excitant solution. There was no change in the amplitude of the biosensor baseline current during activity (same as null trace which is not illustrated). The fast deflections on the ADO biosensor trace are due to detection of electrical activity not changes in purine concentration. B, Co-ordinated activity, consisting of groups of population spikes, recorded on an adenosine biosensor (ADO) and an extracellular electrode (ext). There was no increase in biosensor current following each group of population spikes. C, When activity was induced with excitant solution there was no detectable increase in extracellular purine concentration measured by the adenosine biosensor (ADO, top panel). However when Mg\textsuperscript{2+} free aCSF was applied to the same slice, a robust current was measured on the ADO biosensor with no increase in current on the null sensor (bottom panel).

Figure 4. Network activity required for the biosensor detection of adenosine or metabolites. A, Co-ordinated activity (induced by excitant solution) recorded on an adenosine biosensor, null sensor and an extracellular electrode (ext). There was an increase in ADO biosensor current following each group of population spikes. Inset, a single event from (A, ext) consists of a population spike followed by a train of high frequency activity. B, Co-ordinated activity (induced by excitant solution) recorded on an adenosine biosensor (ADO) and an intracellular recording from layer-V pyramidal cell (intra). Short lived depolarised states (equivalent to population spikes) did not produce an increase in ADO biosensor current. Inset, the period (*) at an expanded
time-base to illustrate a single burst of intracellular activity. C, Co-ordinated activity recorded on an adenosine biosensor (ADO) and an intracellular recording from layer-V pyramidal cell (intra). In this recording there was a clear increase in the ADO biosensor current following initial network activity (arrows). Inset, the period (*) at an expanded time-base to illustrate the form of intracellular activity. Although more action potentials are fired in (B), activity in (C) is prolonged with greater depolarisation and more EPSPs and thus represents greater network activation. D, Co-ordinated activity recorded on an adenosine biosensor (ADO) and an intracellular recording from layer-V pyramidal cell (intra). In this recording there was a clear increase in the ADO biosensor current following activity, which consists of a number of EPSPs indicating network activation. Inset, the period (*) at an expanded time-base to illustrate the form of intracellular activity (bottom panel). The spikes in the inset panels have been truncated.

**Figure 5. Purine release during pathological network activation**

A, Adenosine biosensor (ADO) and null sensor during network activity. Although there are large currents following each burst on the ADO biosensor there are no currents on the null sensor. B, adenosine biosensor (ADO) and extracellular recording (ext) during network activity. With model (black line) and data (gray line). The dotted line shows each separated purine waveform, with the baseline removed, allowing accurate measurement of biosensor current amplitude. Inset, log plot of the adenosine biosensor waveform in response to the first burst of activity. The decay is well described by a single exponential confirming validity of deconvolution procedure. C, Separated ADO waveforms and extracellular recording (ext) during network activity. Application of the adenosine deaminase inhibitor EHNA (20 μM) reduced the amplitude of ADO biosensor waveforms by ~75 % confirming that the purines detected either arise from adenosine
or adenosine metabolites. D, Top panel: expanded portion of (A), illustrating the slow rise of the ADO biosensor waveform in comparison to the calibration trace (bath application of 10 μM adenosine). Bottom panel: response of an ADO biosensor to the rapid application of adenosine (200 ms puff). E, Expanded portion from (B) illustrating how the ADO biosensor waveform does not start to rise until about half way through a ~30 second burst of network activity.

**Figure 6. Purine concentration decreases rapidly with distance due to diffusive dilution.** (A) Purine waveforms (solution to Eq 2) at four distances (25, 50, 75, 100 μm) from a site of brief adenosine release. The waveform amplitude at 100 μm is 2% that at 25 μm, and has a time-to-peak of 22 s. (B) The peak amplitude drops rapidly with distance with little visible difference between the free diffusion case (Eq 4) and that for neocortical slices (Eq 2) for the range shown. (C) The time-to-peak amplitude grows with the square of distance: a 100s time-to-peak occurs at a distance of 220 μm. Again, little difference can be seen between the results for free diffusion (Eq 4) and the slice geometry (Eq 2). (D) At a distance where the time to peak is 100 s the relative concentration is two-thousandths that at 25μm suggesting that the slow time to peak is unlikely to be due to diffusion from a distant source.

**Figure 7. Increases in purine concentration are localised.** A, positioning of 2 biosensors (labelled 1 and 2) in layer V of the neocortex, with diagrams illustrating the localisation of increases in extracellular purine concentration during network activity as interpreted from the biosensor traces in (B). Network activity was induced by zero Mg\(^{2+}\) aCSF. Initially the network (fast deflections are electrical activity) was only active around biosensor 1 and not around biosensor 2. The resultant increases in extracellular
purine concentration, measured by biosensor 1, were not detected by biosensor 2 (Aa).

When the network was active around both biosensors then an increase in purine concentration was detected on both biosensors, although there was no relationship between the concentrations of purines measured (Ab).

**Figure 8. The major purines detected are adenosine metabolites.** A, separated waveforms from ADO, INO and HYP biosensors with trace from extracellular recording electrode (ext) during pathological network activity, induced by zero Mg$^{2+}$ aCSF. The traces have been corrected for biosensor sensitivity (measured from calibration). B, graph plotting the concentration of hypoxanthine against burst number. EHNA blocked hypoxanthine detection, thus the hypoxanthine arises from adenosine metabolism. C, Superimposed traces from ADO and INO biosensors following a single burst of activity. After normalisation, the waveforms had virtually identical kinetics. This strongly suggests that adenosine metabolites are detected rather than adenosine itself.

**Figure 9. Neocortical adenosine metabolism.** A, Traces from ADO, INO and HYP biosensors following bath application of 50 µM adenosine and then 50 µM inosine. The currents have been corrected for biosensor sensitivity (from the calibration). The grey line is the calibration trace for the ADO biosensor (10 µM adenosine, no tissue present) illustrating the slow rise and decay of the signals measured in tissue. Inset, responses from the ADO biosensor to application of adenosine and to inosine normalised and superimposed to show a similar speed of response. B, The traces from (A) have been corrected for biosensor sensitivity and then subtracted to give the adenosine, inosine and hypoxanthine concentration profiles following bath application of adenosine. Inset, the traces from (B) normalised, to illustrate differences in the time-course of adenosine,
inosine and hypoxanthine waveforms. C, The traces from (A) have been corrected for biosensor sensitivity and then subtracted to give the inosine and hypoxanthine concentration profiles following bath application of inosine. Inset, the traces from (B) normalised and aligned to illustrate the time-courses of inosine and hypoxanthine detection. The black line is the inosine response from the ADO biosensor illustrating that the ADO biosensor has a comparable response time to the INO and HYP biosensor.
Figure 1, 1 column
Figure 2, 1 column
Figure 3, 1 column
Figure 4, 1 column

A

excitant solution

ADO

null

d

ext

B

excitant solution

ADO

intra

C

excitant solution (0.3 mM Mg$^{2+}$)

ADO

intra

D

excitant solution (0.3 mM Mg$^{2+}$)

ADO

intra
Figure 5, 1 column
Figure 7, 2 columns
Figure 8, 1 column
figure 9, 2 columns