Combined electrophysiological and biosensor approaches to study purinergic regulation of epileptiform activity in cortical tissue.

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Abstract:

Background: Cortical brain slices offer a readily accessible experimental model of a region of the brain commonly affected by epilepsy. The diversity of recording techniques, seizure-promoting protocols and mutant mouse models provides a rich diversity of avenues of investigation, which is facilitated by the regular arrangement of distinct neuronal populations and afferent fibre pathways, particularly in the hippocampus.

New method & Results: We have been interested in the regulation of seizure activity in hippocampal and neocortical slices by the purines, adenosine and ATP. Via the use of microelectrode biosensors we have been able to measure the release of these important neuroactive compounds simultaneously with on-going epileptiform activity, even of brief durations. In addition, detailed numerical analysis and computational modelling has produced new insights into the kinetics and spatial distribution of elevations in purine concentration that occur during seizure activity.

Comparison and Conclusions: Such an approach allows the spatio-temporal characteristics of neurotransmitter/neuromodulator release to be directly correlated with electrophysiological measures of synaptic and seizure activity, and can provide greater insight into the role of purines in epilepsy.

Keywords: epilepsy; seizure; epileptiform activity; biosensor; adenosine; ATP

Highlights:

- The purines adenosine and ATP are important endogenous neuroactive compounds
- They are released during seizures where they influence neuronal activity
- Enzyme-based biosensors can be used to detect purines in real-time during seizures
- Sensors can be used with electro/optical techniques to correlate release and activity
- Together with modelling, new insights in purine release and action can be determined

1. Introduction:

Brain slices have a long and venerable history in experimental neuroscience. Their use was promoted in large part by the late, great Henry McIlwain who, from the 1950s onwards, produced an impressive series of papers on this versatile preparation. These studies initially used biochemical techniques to investigate brain metabolism, but later adopted electrophysiological approaches with which to study functional aspects of brain physiology *in vitro* (McIlwain, 1984). Of relevance to the present review are his contributions to the effects of convulsant drugs on brain energy metabolism and the release of the purines such as adenosine and ATP, some of which we have discussed in a previous review (Dale and Frenguelli, 2009).

The purines have, to some extent, been overlooked by sections of the epilepsy community in favour of the more readily accessible and experimentally-amenable fast synaptic transmission mediated by glutamate and GABA receptors. This is not surprising: since epilepsy results from an imbalance between excitation and inhibition, then where better to look than at the major players of excitation and inhibition? This approach has certainly been successful in generating antiepileptic drugs that target GABA receptors in particular. However, there is another naturally occurring inhibitory substance in the brain - adenosine – the release of which has been measured via microdialysis from the hippocampi of humans during seizures (During and Spencer, 1992). From these studies the time-course of ictal adenosine release suggested to the authors that adenosine may be responsible for both seizure arrest and post-ictal refractoriness to subsequent seizures. Studies using microelectrode biosensors for adenosine have borne out these prescient predictions and confirm the value of direct measurement of substances of interest.

Adenosine is a metabolite of ATP, the primary cellular energy source and a neurotransmitter in its own right (Burnstock, 2006). Accordingly, adenosine can arise in the extracellular space via two distinct mechanisms: following the intracellular breakdown of ATP and equilibrative transport of adenosine across the plasma membrane into the extracellular space, or via the release of ATP, potentially via vesicular or channel-mediated mechanisms, and subsequent extracellular metabolism to adenosine via a wide array of ecto-nucleotidases (Dale and Frenguelli, 2009; Zimmermann et al., 2012; Wall and Dale, 2013). A third, somewhat provocative, suggestion is that adenosine is released as a neurotransmitter in its own right (Klyuch et al., 2012b). Here we run into the first problem – the ubiquitous nature of ATP and adenosine and the many cellular sources and release conduits for adenosine *per se* or a precursor, including cAMP (Figure 1). The second issue is that, until recently, there were no means by which to measure rapidly and directly the release of adenosine. Valuable inferences as to the presence and effects of adenosine in brain tissue have been obtained through the use of receptor antagonists, of which there are good examples for each of the four known adenosine receptors (A_1, A_{2A}, A_{2B}, A_3), and from which we know that the A_1 receptor is anticonvulsant. However the limited temporal and spatial resolution of microdialysis techniques, which require an additional and time-consuming HPLC identification step, precluded the ready detection of adenosine release during brief events such as seizures.

2. The development of microelectrode biosensors

In the late 1990s Nicholas Dale at the University of St Andrews developed a means to measure directly adenosine release from the *Xenopus* spinal cord during fictive locomotion (Dale, 1998). The technique involved a solution of adenosine metabolising enzymes contained within microdialysis tubing, which was placed against the isolated *Xenopus* spinal cord. Adenosine produced by the spinal cord during fictive swimming diffused into the microdialysis tubing where it

was sequentially metabolised to uric acid and hydrogen peroxide, the latter of which was oxidised on polarised platinum wires in the tubing to give rise to an oxidation current linearly related to the initial adenosine concentration (Dale, 1998). This experimental demonstration allowed him to confirm that extracellular adenosine levels rose gradually during locomotor activity and terminated the ATP P2Y1 receptor-driven neuronal activity that initiates swimming. Subsequent studies in hippocampal slices revealed for the first time the direct release of adenosine during hypoxia and allowed an estimation of the IC_{50} in the low micromolar range for the inhibitory effect of adenosine against excitatory synaptic transmission (Dale et al., 2000).

These initial studies using the first generation microdialysis tubing-based biosensors indicated the value of being able to measure directly and in real time the release of neuroactive compounds simultaneously with on-going electrophysiological activity. Subsequent developments in biosensor technology saw the enzymes immobilised in a polymer layer on the surface of 25 – 50 µm platinum/iridium (Pt/Ir) wires (Figure 2) (Llaudet et al., 2003; Llaudet et al., 2005), allowing for the insertion of biosensors into discrete brain regions thereby improving spatial resolution and allowing the mapping of differential release from closely spaced anatomical regions. Moreover, the miniature nature of the sensors facilitates simultaneous electrophysiological (both extracellular and whole-cell patch clamp; see below) as well as optical measurements to be made (Sims and Dale, 2014). This latter imaging study allowed the conclusion to be drawn that the consumption of ATP by the Na^+/K^+ ATPase at synapses generated localised adenosine release – a plausible mechanism by which the release of adenosine is directly coupled to synaptic activity (Sims and Dale, 2014). These developments and some of their applications by us and others are described in a recent review (Dale and Frenguelli, 2012).

3. Practicalities of biosensor use

Biosensors are an invaluable tool for directly measuring localised changes in the extracellular concentration of analytes, in real time, during brain activity. However the data produced must be treated with caution and appropriate controls must be used. The major issues concerning the use of biosensors and the interpretation of the resultant signals can be summarised under the following headings:

3.1 Practical Considerations:

For *in vitro* (and *in vivo*) studies, sensors are held by micromanipulators and positioned into the region of interest under optical magnification. The exposed sensor tip (typically 500 µm in length; Figure 2) should be fully embedded into the tissue to avoid a sensor/air interface and to maximise the contact area with the tissue. Typically we use brain slices that are submerged in aCSF, though a fluid meniscus around the sensor in an interface chamber should suffice. *In vivo* a fluid pool could be created around the sensor using dental wax and an appropriate medium (Dale et al., 2002), whilst in cell culture the incubation medium serves this purpose (Rubini et al., 2009; Wall et al., 2010; Huckstepp et al., 2010). Prior to first use of the sensors they should be hydrated in the recording medium by placing them in the recording chamber. They should be kept hydrated for the remainder of their lifetime.

Insertion of sensors into tissue will cause damage. This is minimised by the narrow diameter of the sensors (typically 25 – 50 µm for Pt/Ir sensors, but as thin as 7 µm for carbon fibre sensors). Time should be left, usually 30 mins, for any insertion-associated signals to dissipate. Time should also be allowed for the Faradic current associated with the sensor to fade. This is an exponential

process and time should be left until the sensor current is seen to be stable and upon which any biological signals can be clearly observed and measured.

To improve sensor stability careful control over recording chamber fluid level and temperature is required. We achieve this by using peristaltic pumps to control inflow and suction from the chamber, and a feedback-based heating system for the perfusion medium, respectively. Pre-warming the perfusion medium in a water bath can help avoid a large temperature differential across the heating elements and can contribute to a more stable bath temperature, as well as reducing out-gassing from the perfusion medium. It is good practise to check for stability by placing sensors into the recording chamber (either away from or in the absence of tissue). The sensor signal should not fluctuate if: the fluid level and bath temperature are stable; all connections are sound and dry, and appropriate earthing of the set-up is achieved. This is also the configuration in which the sensors are calibrated with known concentrations of exogenous analytes, for example 10 µM for adenosine and ATP.

Under such stable conditions, we routinely make sensor recordings from individual slices for several hours with no ill-effect to basal neuronal properties as far as we can discern. Whilst the sensors are polarized to +500 to 600 mV with respect to the Ag/AgCl reference electrode, this potential dissipates over a short distance (of nm) and indeed it is possible to record field excitatory postsynaptic potentials (Frenguelli et al., 2003) and electrographic seizure activity (Etherington et al., 2009) on sensors suggesting that the neuropil around the sensors has not been inactivated. The ability to make such recordings directly from the sensors also indicates that any damage caused by sensor insertion is not extensive and does not preclude the detection of neuronal

activity in the close vicinity of the sensors. Additional conventional stimulating and recording electrodes can be inserted in the vicinity of the sensors (Figure 2). However, it should be appreciated that afferent fibres may have been damaged by the sensors and their insertion likely provokes the release of substances that might interfere with synaptic transmission (K^+, adenosine, glutamate). Thus, attempts to record synaptic activity should be delayed until the bulk of the insertion signal has dissipated. We have noticed that electrically-evoked seizures are shorter in duration when sensors are embedded into the slice and this may reflect the severing of afferent fibers and the reduction of both neuronal connectivity and the propagation of seizure activity across the slice (Etherington et al., 2009).

3.2 Specificity:

3.2.1 The range of detectable analytes

The sensors rely on an enzymatic cascade that results in the generation of hydrogen peroxide (H_2O_2) which is oxidised on a polarised (+500-600 mV) Pt/Ir microelectrode to generate an oxidation current that is linearly related to the concentration of the analyte of interest, typically over the low-mid nM to high µM range. The reliance upon an oxidase in principle means that a biosensor could be made for any analyte, which at some stage in their metabolism, requires an oxidase. Thus, sensors currently exist for adenosine, inosine, hypoxathine/xanthine, ATP, glutamate, D-serine, acetylcholine/choline, glucose and lactate. Whilst a GABA sensor would no doubt be of value to the epilepsy field, there is no direct means to oxidise GABA to H_2O_2 without generating glutamate and hence an oxidation current via the necessary use of glutamate oxidase. Whilst such a need may be reduced by the ability to use evoked, spontaneous or miniature excitatory or inhibitory postsynaptic currents to detect the release of glutamate and GABA,

respectively, recordings of synaptic currents can have limitations in terms of: the limited duration of patch-clamp recordings; the infrequency of miniature events; potential non-linearity or desensitization/deactivation of postsynaptic receptors, and the restriction of measuring synaptic, vs extrasynaptic or nonsynaptic release, although it is appreciated that holding current measurements, coupled with $GABA_A$ receptor antagonists, can be used as an index of the extracellular GABA concentration.

3.2.2 Identifying signals and avoiding artefacts

In principle any cellular reaction capable of generating H_2O_2 or the release of any electro-active substance oxidised at +500 mV, for example 5-HT, can give rise to a current on the sensor. Such extraneous signals could erroneously be construed as deriving from the release of the analyte of interest. Thus, it is important to be certain that what is measured by the biosensor reflects the extracellular presence of the analyte of interest i.e. that an ATP biosensor is detecting ATP. Fortunately there are a number of ways in which confidence can be instilled as to what is actually being detected by the biosensor.

3.2.2.1 A selectivity layer excludes electroactive substances

The first of these specificity measures involves a selectivity layer coated onto the surface of the Pt/Ir microelectrode which excludes electroactive substances such as 5-HT and dopamine (Figure 2). The patency of this layer can easily be tested via the perfusion of 5-HT (at 10 µM) onto the sensor whilst it is placed in the recording chamber. The 5-HT should give no, or a very small current (<150 pA) on the sensor. We routinely perform this procedure at the end of every experiment after removal of the sensors from the tissue.

3.2.2.2 The importance of the null sensor and the value of differential recordings

The second means by which the specificity of the signal can be determined is via the use of a null sensor. The null sensor is exactly the same as the biosensor but lacks enzymes and thus cannot detect the analyte of interest. This controls for non-specific electroactive interferents and for changes in pH, ionic environment, perfusion flow, temperature etc. which would produce practically identical signals on both the biosensor and the null sensor. The signal from the null sensor can be digitally subtracted from the biosensor thus taking into account any extraneous currents not related to the analyte of interest. It is good practice to include a null sensor in recordings from tissue (Figure 2) to avoid doubt as to the source of the signal on the enzyme-containing sensor.

Interestingly, a recent innovation has seen the development of a null sensor for the null sensor! The fact that the null can detect electoactive interferents such as reactive oxygen species (ROS) has led to the creation of a sensor in which the embedding of superoxide dismutase and catalyse eliminate ROS prior to their detection on the surface sensor (Ficker et al., 2014). This then allows subtraction of the ROS sensor signal from the null sensor giving rise to a signal attributable to the presence of extracellular ROS, in this case evoked in spinal cord by the activation of P2X7 receptors (Ficker et al., 2014).

An extension of this dual sensor approach is to perform differential recordings between two sensors that differ in one enzyme in the cascade. This is only applicable where biosensors utilise a multi enzyme cascade such as the sensors for adenosine and inosine. Under these circumstances

the contribution of extracellular inosine, as detected on the inosine biosensor which lacks adenosine deaminase, is subtracted from the signal generated by the adenosine biosensor (Dale et al., 2000; Pearson et al., 2001; Frenguelli et al., 2003; Pearson and Frenguelli, 2004; Dulla et al., 2005; Pearson et al., 2006). Thus a net adenosine signal is produced and a "true" adenosine concentration can be given. However, we have found that this approach is likely most amenable to global challenges such as hypoxia or oxygen/glucose deprivation and not electrically-evoked release (Wall and Dale, 2007) or seizure activity (Etherington et al., 2009) where neuronal activity may be highly localised and thus the two sensors may report varying amounts of purine release rendering differential recordings meaningless (see below). In these cases we report the signal generated as that with the null subtracted and ascribe the concentration as μM' (μM prime) to indicate that the signal reflects a composite of adenosine and its metabolites.

3.2.2.3 Inactivating sensors to confirm the analyte

A further control for the specificity of the signal detected on the sensor is to either poison the biosensor so it cannot detect the analyte, or remove a co-factor necessary for the generation of H_2O_2: both these manipulations should abolish the signal if it is produced by the analyte. For example, the adenosine deaminase inhibitors EHNA (see below) or coformycin prevents the adenosine biosensor detecting adenosine by blocking the first enzyme in the cascade (Dale, 1998; Dale et al., 2000; Frenguelli et al., 2003; Wall and Dale, 2007; Wall and Richardson, 2015).

Since the ATP biosensor requires glycerol as a cofactor to detect ATP, the absence of glycerol should cause a loss of the signals if they are produced by ATP. In reality, it is easier to start experiments without glycerol and then wash glycerol in and see the appearance of the signals, as

glycerol is very difficult to wash out from the sensor. If the correct analyte is being detected, then it should be possible to modulate the signals in a predictable manner. For example, signals on an ATP biosensor, if produced by ATP, would be predicted to be enhanced if the extracellular breakdown of ATP was inhibited, since more ATP will reach the biosensor. This was the case in the hippocampus where the ecto-ATPase inhibitor, ARL67156 enhanced the ATP signals measured in response to DHPG application (see below and (Lopatar et al., 2015). Conversely ATP signals should be reduced or abolished if the ecto-nucleotidase apyrase is applied.

3.2.2.4 Independent tests as a reality check for sensor signals

If there is evidence for changes in the concentration of an analyte from biosensor measurements, it is good practise to confirm this with independent evidence. For example, a train of impulses in both the cerebellum and hippocampus causes adenosine release (as detected by a biosensor). This was confirmed by measuring fEPSPs and showing that following a train of stimuli or theta burst stimulation they are depressed, an effect which is prevented by blocking adenosine A_1 receptors with an antagonist (Wall and Dale, 2007; Wall and Dale, 2013; Diogenes et al., 2014). A similar result has been produced with adenosine release produced by hypoxia and oxygen/glucose deprivation (Dale et al., 2000; Pearson et al., 2006) and following seizures (Etherington et al., 2009; Wall and Richardson, 2015), both of which inhibit excitatory synaptic transmission in a manner sensitive to adenosine A_1 receptor antagonists.

3.3 Sensitivity

3.3.1 Calibration of signals

As indicated previously the biosensors effectively measure over a wide physiological range, typically tens of nM to tens of µM, although for some the range is greatly extended (eg lactate to 800 µM and glucose to 40 mM). Once the identity of the analyte is confirmed the concentration of analyte measured during an experiment needs to be quantified. This is achieved by comparing the amplitude of the measured signal on the biosensor during an experiment, to the biosensor calibration which, for an ATP biosensor, is usually achieved by bath applying 10 µM exogenous ATP. Thus, if a 100 pA signal is measured on an ATP biosensor during an experiment and the calibration for 10 µM ATP gives 1000 pA, then the signal represents the measurement of 1 µM ATP. The calibration is often carried out before the biosensors are inserted into the tissue, and after the experiment when they have been removed from the tissue, so that any loss in biosensor sensitivity can be measured. The second calibration is routinely used to calculate the amount of analyte detected during experiments, whereas the first can inform whether the sensor requires cycling to improve sensitivity (see below).

It is important that the calibration is conducted in the same solution as that in which the experiment was performed. This way the sensor can be tested in a solution with identical composition, pH and temperature so that the enzymes will behave similarly in the two situations. We calibrate with sensors in the recording chamber through which experimental solution continues to be perfused. For experiments in which appreciable tissue pH changes are anticipated, a calibration curve (to, for example, 10 µM analyte) at a range of known pHs should be conducted to take into account any influence of pH on the enzymes. Similarly, to avoid the possibility of experimental drugs influencing the sensitivity of sensors, it is worthwhile calibrating in the presence and absence of the drug, especially if high concentrations of drugs are used. This can

indicate whether the drug is directly oxidised on the polarised Pt/Ir wire (which should give a signal on the null) or whether the drug inhibits any of the enzymes in the sensor.

The sensitivity of the biosensor can change during an experiment (see below), especially if prolonged, and thus it is not possible to accurately calculate the absolute concentration of analyte but instead an approximate figure is obtained. Note also the caveats above with respect to adenosine measurements when no differential (adenosine-inosine) measurements are being performed.

3.3.2 Measurements of basal concentrations or the background tone

Measuring an increase in the concentration of an analyte in response to activity (ie above basal) is relatively straight forward but biosensors can also, with caution and in selected circumstances, be used to infer the basal extracellular concentration (or tone) of an analyte in tissue. This can be achieved in two ways. Firstly when biosensors are removed from tissue they will no longer measure the concentration of an analyte within the tissue and thus the baseline current will fall. If the signal from the null sensor (when it is also removed from the tissue) is subtracted from the biosensor signal to control for changes in the ionic environment, then this fall in current can be used as an index of the tone of analyte in the tissue. This method has been used to show changes in extracellular adenosine tone in the hippocampus when animals are sleep deprived (Schmitt et al., 2012; Sims et al., 2013) and in the influence of genetic manipulations of adenosine kinase on extracellular adenosine (Diogenes et al., 2014).

In contrast, the increase in current that is measured when a biosensor is inserted into tissue cannot be used to measure the extracellular tone of an analyte, as clearly cells will be damaged during insertion leading to the release of ATP, adenosine, glutamate etc. Thus, it is important to wait for at least 20-30 minutes after biosensor insertion before starting experiments, so that any released substances are washed away. A tone can also be measured by moving a biosensor from a distal location to a point close to the slice surface (Wall et al., 2007). This approach does not damage the tissue but relies on the analyte diffusing out of the tissue. Another approach is to leave the biosensor within the tissue and inhibit the biosensor so it cannot detect the analyte and then measure the resultant fall in baseline current. This can be achieved by applying EHNA for example, to block the adenosine biosensor. Also if the tone of adenosine is produced by extracellular ATP metabolism, then blocking ATP metabolism with agents such as ARL67156 and POM-1 should reduce the baseline current (Dulla et al., 2005; Frenguelli et al., 2007; Wall et al., 2007; Wall et al., 2008). If the extracellular tone is activity-dependent then blocking activity (with tetrodotoxin, glutamate receptor antagonists etc.) should also produce a fall in baseline current.

3.4 Stability

The biosensors can lose some sensitivity during experiments, due to the actions of proteases in the tissue, which can damage the enzyme layer, and also through fouling of the surface of the Pt/Ir wire hampering access of H_2O_2. This latter component is offset by the presence of an outer anti-fouling layer (Figure 2) and fouling itself can often be reversed by a process of “cycling” where a positive going (to +500 mV) and then a negative going (to -500 mV) voltage ramp is applied to the biosensor over a few seconds for 10 – 12 cycles. In our experience we find that most sensors can be used several times if treated carefully, cycled upon each use and stored in PBS to prevent them from drying out.

3.5 Speed:

In our experience sensors typically respond within a few seconds of the start of physiological or pathological activity. This delay is not due to inefficiencies in enzymic conversion or diffusional barriers within the sensor to the analyte or to H_2O_2. In a recent publication (Wall and Richardson, 2015) we showed that the response time of multi-enzyme adenosine biosensors to brief (200 ms) puffer application of adenosine was in the order of 1 s, but that detectable adenosine release in response to seizure activity occurred ~30 s after seizure onset. This delay may therefore represent the reaching of a threshold of activity sufficient to provoke the release of a compound such as adenosine or ATP, and/or may reflect delays associated with the compound overcoming local uptake and metabolism and the spread to the sensors. In this regard it is unlikely that sensors detect exclusively, or even at all, the synaptic release of compounds but instead potentially spillover of the analyte beyond local buffering and may indeed reflect that signal associated with volume transmission of neurotransmitters/modulators.

Such delays do not detract from the ability to correlate, in our case, purine release with ongoing synaptic or physiological activity. For example the *in vivo* ATP release from sensory afferents was shown to be phase locked to respiratory rhythm (cycle period of about 1s) (Gourine et al., 2008). In addition, the differences in the kinetics of release on the initiation and termination of a stimulus clearly reveal that distinct processes are occurring during the release and removal phases of the signal. In this regard microelectrode biosensors allowed a surge of adenosine release to be observed upon reoxygenation after hypoxia or oxygen/glucose deprivation, and permitted

hysteresis in the relationship between adenosine release and the depression and recovery of synaptic transmission to be detected (Frenguelli et al., 2003).

3.6 Size:

Typically we use sensors made with Pt/Ir wire of 50 µm in diameter and 500 µm in length. This allows penetration of the entire thickness of brain slices at an acute angle to ensure that most of the sensing surface is in contact with the tissue. These sensors have a sensitivity of several nA/10 µM of analyte providing a good trade-off between sensor diameter (and damage to the tissue) with sensitivity. Thinner Pt/Ir sensors are available, most commonly 25 µm, but the thinnest sensors currently available are 7 µm diameter carbon fibres (Yang et al., 2012). These carbon fibre sensors retain good sensitivity (1 nA/10 µM analyte) likely by virtue of the patterned surface of the carbon fibre providing greater surface area for enzyme deposition. These carbon fibre sensors are very flexible and may not be suitable for tissue penetration unless a guide cannula is used, but instead could be placed on the surface of tissue. Whilst the nominal diameter of the most frequently-used sensors are the values given above, deposition of the various enzyme and selectivity layers does add to the diameter of the sensors, but not to the extent that it hampers penetration of tissue.

3.7 Future perspectives:

The currently-available microelectrode biosensors are a considerable advance on the first generation sensors based around enzymes in solution within barrels formed by microdialysis tubing (Dale, 1998; Dale et al., 2000). These sensors were not suited to tissue penetration and so this need to do so was met by immobilising enzymes in a gel layer on the surface of thin Pt/Ir wire.

The subsequent appreciation that harsh experimental conditions (eg in response to the anoxic depolarization during prolonged oxygen glucose deprivation) provoked the release of electroactive interferents led to the incorporation of the selectivity layers on the sensors (Frenguelli et al., 2007). Thus, developments are led in part by the need to improve the technical and experimental capabilities of the sensors, and by demands for additional sensing capacity.

We foresee future developments to revolve around further miniaturisation of the sensors, for example to the level of carbon nanotubes, that might allow single-, and indeed within-cell sensing. Additionally the need for chronic (hours, days, weeks) sensing in freely-moving animals will drive improvements in enzyme stability and anti-fouling of the sensors, whilst the desire to simultaneously sample multiple analytes may see silicon or ceramic microelectrode array sensors, which may allow independent sampling through various cortical layers. The analyte range, stability, sensitivity or specificity of sensors may be enhanced by advances in protein engineering or synthetic biology through the modification or creation of enzymes capable of being incorporated into gel layers and still retaining optimal activity. From a clinical perspective, the value of purines as potential biomarkers of disease in complex fluids such as whole blood has led to the development of mediated biosensors where the Pt/Ir electrode is held at a much lower potential (0 to -50 mV) where there are fewer substrates for amperometric reduction in this case. Trials are currently under way to test their value in a number of life-threatening conditions.

Readers interested in current and future developments in microelectrode biosensors are referred to an excellent recent textbook on this topic edited by Stéphane Marinesco and Nicholas Dale (Marinesco and Dale, 2013).

4. The application of biosensors to study seizures

Utilising the methods and approaches described in the previous section, we have used microelectrode biosensors, in conjunction with electrophysiological and pharmacological tools, to investigate the release and effects of endogenous adenosine and ATP during seizure activity in a number of contexts. This work has been conducted in wild-type and genetically-modified rodent parasagittal hippocampal and neocortical slices. A thickness of 600 μm is required for hippocampal slices, as opposed to the standard 300 – 400 μm, as we find thinner rat slices do not support electrically-evoked seizure activity (Etherington and Frenguelli, 2004) or that induced by the GI mGluR agonist DHPG (Lopatar et al., 2015). We avoid hypoxia in the core of the slice by suspending the slice upon a mesh in the recording chamber and perfusing the submerged tissue on both sides, using gas-impermeable tubing and perfusing aCSF at ~6 ml/min (Etherington and Frenguelli, 2004; Wall and Richardson, 2015). However, 450 μm thick mouse slices can support seizure-like events provided they are cut midway between the sagittal and horizontal orientation, potentially via better preservation of the entorhinal cortex and trisynaptic pathway within the plane of the slice (unpublished observations). For necortical slices, the greater recurrent network connectivity means that standard 300-400 μm thick slices can support spontaneous seizures (Wall and Richardson, 2015).

Promotion of seizure activity in hippocampal slices can involve brief high-frequency stimulation (2s, 60 Hz) of the Schaffer collateral-commissural pathway in area CA1 in nominally Mg^{2+}-free aCSF (Figure 3) (Pearson et al., 2003; Etherington and Frenguelli, 2004; Etherington et al., 2009; Lopatar et al., 2011), perfusion of slices with nominally Mg^{2+}-free and high K^+ aCSF (0 mM Mg^{2+}/6 mM K^+), (Lopatar et al., 2011) or low Mg^{2+} (0.1 mM) and the K^+ channel blocker 4-AP (10 – 50 μM) (Avoli et al., 2002). For neocortical slices, we have found that perfusion with nominally Mg^{2+}-free aCSF

reliably induces spontaneous seizures (Wall and Richardson, 2015). It is however important to slowly reduce the concentration of magnesium in the aCSF to avoid the production of spreading depression or mixed states (combination of spreading depression and seizure activity). The advantage of evoking seizures by electrical stimulation, rather than acquiring spontaneous events, is that electrical stimulation permits accurate timing of highly reproducible seizure events (Figure 3), typically of 20 s in duration. This permits within-slice comparisons of the effects of various drugs (Etherington and Frenguelli, 2004; Etherington et al., 2009; Lopatar et al., 2011). This protocol also results in a transient (4 – 6 min) depression of electrically-evoked field excitatory postsynaptic potentials (fEPSPs), which is greatly attenuated by A_1 receptor antagonists (Figure 4). This is despite the fact that electrically-evoked seizure duration is at least doubled and seizure intensity, in terms of spike frequency, is greatly increased. These observations, and similar observations made by others (Dragunow, 1986; Dunwiddie, 1999; Boison, 2012), suggest that seizures cause the release of endogenous adenosine which acts, via A_1 receptors, to both reduce the intensity of seizures and to bring them to an abrupt halt (Etherington and Frenguelli, 2004; Etherington et al., 2009; Lopatar et al., 2011; Wall and Richardson, 2015). Indeed, antagonism of adenosine A_1 receptors during conditions of heightened excitability (eg low extracellular Mg^{2+}) can *per se* provoke intense and prolonged electrographic seizure activity *in vitro* (Figure 4D) whilst *in vivo* can convert self-terminating seizure activity in into *status epilepticus* (Young and Dragunow, 1994).

5. Insight into the regulation of basal and seizure-induced purine release.

The observations above imply that as well as being released by seizure activity and providing a potent negative feedback mechanism for controlling seizure intensity and duration, there is also a basal extracellular tone of adenosine in the tissue that raises the threshold for ictogenesis. This

adenosine tone likely results, at least in part, from localised low-level network activity. This is supported by data from paired patch clamp recording from layer 5 pyramidal cells which show considerable heterogeneity in the basal extracellular adenosine concentration present at synapses within the same slice and even between reciprocal connections (Kerr et al., 2013).

Interestingly, this basal adenosine tone seems to be at least partly dependent on the presence and metabolism of extracellular ATP: studies using ecto-nucleotidase inhibitors such as ARL 67156 (Dulla et al., 2005; Frenguelli et al., 2007) or POM1 (Wall et al., 2008) reveal a decrease of the adenosine sensor signal of ~100 nM, which may indicate an astrocytic origin for the adenosine tone (Pascual et al., 2005). Furthermore, this adenosine tone can also be reduced by hypocapnia, which promotes seizure activity in hippocampal slices (Dulla et al., 2005). The fact that hyperventilation-induced hypocapnia is used to provoke seizures as a diagnostic test (Mendez and Brenner, 2006) and enhance electroconvulsive seizures, as are A_1 receptor antagonists such as caffeine, theophylline and aminophylline (Loo et al., 2010), strongly suggests an important tonic inhibitory role for endogenous adenosine in the regulation of human seizures. Moreover, successful attempts to terminate human epileptic seizures, even after their initiation, have been made using 5 % CO_2 inhalation (Tolner et al., 2011). Indeed, we have shown in hippocampal slices that hypercapnia is a powerful stimulus for adenosine release and adenosine A_1 receptor-dependent suppression of seizure activity (Dulla et al., 2005).

In addition to pH-dependent regulation of extracellular adenosine (Dulla et al., 2005; Dulla et al., 2009) an additional critical regulator is adenosine kinase (ADK), an enzyme that converts adenosine to AMP thereby maintaining an inward adenosine gradient across the plasma

membrane (Boison, 2008). That this enzyme plays a crucial role in determining extracellular levels of adenosine is evidenced by the fact that mutant mice expressing elevated or reduced levels of forebrain ADK show differential release of adenosine and adenosine-dependent transient inhibition of the fEPSP in response to theta-burst stimulation (Figure 5) (Diogenes et al., 2014). Furthermore, inhibition of ADK with iodotubercidin results in accumulation of extracellular adenosine, inhibition of synaptic transmission and the abolition of seizure activity in hippocampal slices (Etherington et al., 2009). This involvement of ADK in regulating extracellular adenosine and the threshold for ictogenesis is particularly pertinent in the context of chronic epilepsy in which astrogliosis is a prominent feature since ADK is predominately located in astrocytes (Studer et al., 2006; Etherington et al., 2009). Thus, the astrogliosis precipitated by brain injury begets a vicious cycle in which ADK expression is elevated, extracellular adenosine is lowered, the threshold for seizures is reduced, chronic seizures cause more brain damage and so on, and has led to the ADK hypothesis of epileptogenesis (Boison, 2008). Indeed, ADK over-expressing mice exhibited spontaneous seizures and fatal kainic acid-induced status epilepticus (KASE), whereas ADK-deficient mice showed reduced KASE-induced seizures and brain damage and were protected against subsequent KASE-induced epileptogenesis (Li et al., 2008). Accordingly, ADK and the adenosine-dependent changes that contribute to astrogliosis are promising targets in both injury-induced epileptogenesis and indeed established but refractory epilepsy (Aronica et al., 2013).

Given the profound influence of adenosine acting via adenosine A_1 receptors in the suppression of seizures, an obvious question is to what extent does this result from the prior release of ATP? Indeed, given the wide array of excitatory and inhibitory receptors for ATP (Burnstock et al., 2011), and the potential for their involvement in epilepsy (Kumaria et al., 2008), we recently investigated the possible release and role of ATP receptors hippocampal slices in different models (Lopatar et

al., 2011; Lopatar et al., 2015). In the first model, using brief high-frequency stimulation of the Schaffer pathway in nominally Mg^{2+}-free aCSF, we observed that broad spectrum antagonism of ATP P2X (ionotropic) and P2Y (GPCR) receptors had only a minor inhibitory effect on the duration and intensity of electrically-evoked seizures. This minor role was echoed by ATP biosensor measurements, which showed little appreciable release of ATP during either evoked seizure activity or spontaneous seizures caused by the elevation of extracellular K^+ to 6 mM, both of which were capable of provoking considerable adenosine release (Figure 6). Thus, under these conditions there seems to be both little ATP release and little P2 receptor involvement in modulating epileptiform activity, observations confirmed by others (Klaft et al., 2012). However, these findings do not preclude the potential importance of ATP release and P2 receptors in other seizure models or conditions (Burnstock et al., 2011).

Indeed, in a more recent study, we have observed that the application of the GI mGluR agonist DHPG elicits burst firing in area CA1, which is associated with adenosine release from areas CA1 and CA3, but ATP release from only area CA3 (Figure 7) (Lopatar et al., 2015). Importantly, ATP release, but not the release of adenosine, was greatly suppressed by either probenecid or a low concentration of carbenoxolone, suggesting both a spatial and mechanistic dissociation between ATP and adenosine release, and the release of ATP via pannexin-1 hemichannels. We went on to show that ATP drove burst firing via the activation of ATP P2Y1 receptors, whereas the timing and intensity of burst firing was regulated by adenosine A_1 receptors. These observations suggest bidirectional actions of the purines reminiscent of the observations made in *Xenopus* spinal cord.

Attempts to define the cellular source and molecular conduit for adenosine and ATP release under physiological and pathological conditions have been fraught with difficulty due to the large variety of potential cellular and molecular sources (Figure 1). Accordingly, there is evidence (reviewed in (Dale and Frenguelli, 2009)) to include both neurones (Lovatt et al., 2012; Wall and Dale, 2013) and astrocytes (Pascual et al., 2005; Wall and Dale, 2013) as sources of purines, and equilibrative adenosine transporters, vesicular release, connexin and pannexin gap junction hemichannels and maxi anion channels as effective conduits that can be recruited, potentially in isolation or in combination, depending upon the prevailing stimuli. With judicious use of the more selective pharmacological tools, molecular reagents to knock down protein expression or activity, appropriate mutant mice, and direct measurement of the kinetics of purine release, it may be possible to tease apart potentially overlapping sources. This has been attempted recently in the context of K^+ depolarisation of hippocampal slices and the release of adenosine, ATP and glutamate (Heinrich et al., 2012), and in stimulated adenosine release in the cerebellum and hippocampus (Klyuch et al., 2012a; Wall and Dale, 2013).

6. Analysis of biosensor signals and modelling of adenosine release kinetics

To better address the kinetics of purine release during seizure activity we have recently applied computational methods that allow pertinent release parameters to be extracted from the biosensor signals observed during spontaneous seizures. It is desirable to accurately measure the amplitude of the biosensor current produced by each seizure and to thus estimate the increase in extracellular purine concentration. However, this is often difficult as spontaneous seizures can occur so frequently that the biosensor currents overlap and thus each increase in biosensor current sits on the decay of the previous biosensor current (Figure 8A). This problem can be overcome using the method of Richardson and Silberberg (Richardson and Silberberg, 2008).

Signals are deconvolved by removing the long decay component, which reflects the slow loss of purines from the slice surface (Klyuch et al., 2011; Wall and Richardson, 2015). The sharper biosensor signals can then be cropped out and reconvolved to yield isolated waveforms from which the amplitude can be accurately measured (Figure 8B) and from which the concentration of purine calculated. From such measurements it is clear that amount of purine released by each seizure progressively reduces, likely reflecting the depletion of intracellular purine stores (Pearson et al., 2001; Pearson et al., 2003; Klyuch et al., 2011).

Depletion of adenosine release has been observed *in vivo* and so is not merely an artefact of the slice preparation (Pearson et al., 2003). We have considered the implications of this depletion, which likely revolves around the diminution of the ATP pool during metabolic stress or seizure activity, and have recently demonstrated that the provision of ribose (1 mM) and adenine (50 µM) in the slice incubation medium for ~3 hours restores tissue ATP levels to those found *in vivo* (zur Nedden et al., 2011). This results in greater adenosine release in response to physiological (zur Nedden et al., 2011) and pathological stimuli (zur Nedden et al., 2014) with consequent raising of the threshold for the induction of long-term potentiation and the acceleration of the depression of glutamatergic transmission by oxygen/glucose deprivation and a slowing of the recovery, respectively.

Since adenosine biosensors detect both adenosine and its metabolites (inosine and hypoxanthine; Figure 2) the biosensor currents produced during seizures could be due to direct detection of adenosine, detection of metabolites or a combination of both. It may not be important to determine the precise composition of the purines detected as long as it can be shown that any

metabolites detected arise from the extracellular metabolism of adenosine and are not directly released themselves (for example see (Wall et al., 2010) where inosine is directly released). To estimate the component of the biosensor signal directly attributable to adenosine, we have used the adenosine deaminase inhibitor EHNA. This blocks the biosensor's ability to detect adenosine and also prevents the endogenous metabolism of adenosine to inosine within the slice. Since this reduced biosensor signals by ~70-80 % it confirmed that the majority of the purines detected during seizures arise from extracellular adenosine rather than direct release of inosine or hypoxanthine (Figure 8C, (Wall and Richardson, 2015)).

Biosensors have additionally allowed us to test whether there is a homogeneous increase in extracellular purine concentration across the tissue during seizures or whether instead release is patchy and depends on local network activity. This was made possible by the biosensor's ability to also act like an extracellular electrode and detect local network activity. Measurements with multiple biosensors, particularly at the onset of seizure activity, show considerable heterogeneity in the concentration of purine detected (Wall and Richardson, 2015). In some examples (Figure 9A, B) purines released by activity around one biosensor could not be detected by another biosensor ~0.5 mm away, where the network was inactive. This highly localised seizure-induced purine release precludes the use of differential measurements to separate an increase in the concentration of adenosine from an increase in adenosine metabolite concentration. As discussed previously. Since the amount of purine released will vary across the slice, subtraction of the signal on an inosine biosensor from the adenosine biosensor signal, to reveal the adenosine concentration, will be invalid unless the network activity around each biosensor is the same. In the example shown in Figure 9, if biosensor A was an inosine biosensor and B an adenosine biosensor, it would be incorrectly assumed that all of the initial signal on biosensor B is due to adenosine as

no inosine is detected. However such differential measurements are possible when the challenge to the slice is uniform, for example during oxygen/glucose deprivation, and a net adenosine signal can be determined (Frenguelli et al., 2003). This highly localised signal does allow us to begin to make estimates of the diffusion of purines during seizures and the sphere of influence over which adenosine exerts its actions ((Wall and Richardson, 2015); Figure 9C). Using a simple mathematical model, we could estimate an upper bound on the distance that purines travels in the extracellular space. This model suggests that the concentration of adenosine (and its metabolites) decreases rapidly with distance, with, for example, only 2 % remaining at a distance of 100 μm from the release site (Figure 9C). This, together with the experimental evidence in Figure 9B, suggests that the extracellular adenosine concentration becomes uncorrelated with global seizure activity over very short distances in tissue, which are certainly less than 500 μm (Wall and Richardson, 2015). Thus adenosine, acting via A_1 receptors, provides a negative feedback mechanism which can be fine-tuned depending on the local network activity.

7. Summary

Neocortical and hippocampal brain slices offer the means to gain valuable insight into the underlying neurobiology of seizure activity in clinically-relevant brain areas. The many and varied approaches that can be deployed to probe the circuitry and neurochemistry of epileptiform activity have allowed the generation of testable hypotheses and identification of tractable drug targets. Given the highly dynamic, transient and often erratic nature of seizures there remains a need for the monitoring of such activity in real time. Biosensors, together with modelling, optical imaging and electrophysiology, offer a means by which to monitor on-going seizure activity and to complement existing techniques with knowledge of the release of neuroactive compounds.

8. Acknowledgments:

The support of Epilepsy Research UK is gratefully acknowledged. We thank Professor Nicholas Dale for helpful comments on the manuscript.

9. Conflict of interest

BGF is a Non-Executive Director of Sarissa Biomedical Ltd, a company set up by Prof Dale to commercialise enzyme-based biosensors.

Figure Legends:

Figure 1. Generic scheme for the range of sources of extracellular adenosine and ATP. 5'N, cytosolic 5'-nucleotidase; AC, adenylate cyclase; AD adenosine deaminase; AdK, adenylate kinase; ADK, adenosine kinase; AL, adenylosuccinate lyase; AS, adenylosuccinate synthase; e5'N, ecto 5'-nucleotidase; eAP, ectoalkaline phosphatase; ePD, ecto-phosphodiesterase; HGPRT, hypoxanthine phospho-ribosyl-transferase; NP, nucleoside phosphorylase; NPP, nucleotide pyrophosphatase/phosphodiesterase; NTPD, nucleoside triphosphate diphosphohydrolase; SAHase, S-adenosyl-Lhomocysteine hydrolase; XO, xanthine oxidase. In red is the primary route of adenosine metabolism and the enzymic cascade utilised by the adenosine biosensor. Circles on the plasma membrane reflect transporters; the invagination represents vesicular release whilst membrane channels provide an additional release conduit for purines. Modified from (Dale and Frenguelli, 2009).

Figure 2. Microelectrode biosensor design (top) and placement in the hippocampal slice (below) with (right) enzyme cascades allowing detection of adenosine (upper right panel) and ATP (lower right panel). The enzymes utilised preclude cross-reactivity of adenosine and ATP between the sensors. Ado, adenosine biosensor; stim, twisted bipolar stimulating electrode within a plastic sleeve (~100 µm diameter); rec, glass recording microelectrode. Sensors in the slice measure 50 µm in diameter and the exposed sensing element is 500 µm in length. The null sensor lacks enzymes and is used to detect any non-specific electroactive interferents, the contribution of which can be subtracted from the signal detected on the purine sensors. Modified from (Dale and Frenguelli, 2009). Microelectrode design figure courtesy of Sarissa Biomedical.

Figure 3. Model of repetitive and reproducible electrically-evoked seizures in 600 µm rat hippocampal slices bathed in nominally Mg^{2+}-free aCSF. A) Continuous electrophysiological recording showing evoked fEPSPs (periodic downward deflections) and time of high frequency stimulation (HFS; arrow; 2s 60 Hz). Note transient depression of fEPSP after HFS. B) Expanded seizures taken at times indicated showing consistency of electrographic activity over time. Short black bars above the traces indicate the 2 s period of HFS. C) Time course of fEPSP slope for experiment depicted in A. Inset fEPSPs (a-f) taken at times indicated. From (Etherington and Frenguelli, 2004).

Figure 4. Adenosine A_1 receptors mediate profound anticonvulsant actions. A) Electrically-evoked seizures as per Figure 3 in the absence (left) and presence (right) of the A_1 receptor antagonist 8-cyclopentyltheophylline (8-CPT; 1 µM). Seizures are longer and more intense in the presence of the A_1 antagonist. Short black bars above the traces indicate the 2 s period of HFS. Diamond on the extreme left of the trace indicates the fEPSP immediately preceding HFS. B) Mean time-course of seizure-induced fEPSP depression in the absence (filled square) and presence of 8-CPT (grey circles). Light grey bar represents mean duration of seizures under control conditions and dark grey bar the additional seizure duration in 8-CPT. Note the seizures are twice as long in 8-CPT, but the depression of the fEPSP is greatly attenuated. Inset fEPSPs taken (a) before, (b) at peak and (c) after seizure-induced synaptic depression in the absence (left) and presence (right) of 8-CPT. C) Release of adenosine, as measured via an adenosine (Ado) sensor, in response to a single electrically-evoked seizure (open triangle) and the consequent inhibition of the simultaneously-recorded fEPSP. D) Application of 8-CPT (arrow) results in spontaneous seizure activity and a large rise in extracellular adenosine, which is sustained by continued interictal activity (filled triangles). Note the preservation of the fEPSP despite the high levels of adenosine. Electrographic seizure

activity can be observed on the adenosine sensor. All experiments conducted in nominally Mg^{2+}-free aCSF. Taken from (Etherington and Frenguelli, 2004; Etherington et al., 2009).

Figure 5. Adenosine kinase (ADK) regulates activity-dependent adenosine release. Top panel: Adenosine (Ado) sensor signals in response to theta-burst stimulation (TBS; arrowhead) in slices from wild-type (WT), ADK deficient (Fb-Adk-def) and ADK overexpressing (Adk-tg) mice. Lower panel: Time course of TBS-induced synaptic depression and potentiation. Note that the depression and recovery of transmission was longer in slices from mice deficient for ADK (filled circles), which released more adenosine (upper panel), whereas the depression and recovery was quicker in slices from mice overexpressing ADK (filled triangles), which released less adenosine (upper panel). WT mice (filled squares) showed an intermediate phenotype. Inset are fEPSPs for each of the genotypes taken at the times indicated. Scale bars for fEPSPs measure 0.5 mV and 5 ms. Adenosine sensor traces are displayed as mean ± SEM. From (Diogenes et al., 2014).

Figure 6. No obvious ATP release following electrically-evoked or high K^+-induced seizure activity in hippocampal area CA1. High-frequency stimulation (2 s, 60 Hz; delivered at the arrow on the lower fEPSP trace) was delivered to a slice in nominally Mg^{2+}-free aCSF. Whilst this elicited a robust signal on the adenosine (ADO) sensor (upper trace) no signal was detected on the ATP sensor. Provoking a spontaneous seizure through the elevation of extracellular K^+ from 3 mM to 6 mM (prior to which periodic stimulation of the Schaffer pathway was halted) resulted in a prominent signal on the adenosine sensor, but no signal on the ATP sensor. Taken from (Lopatar et al., 2011).

Figure 7. The GI mGluR agonist S-DHPG elicited bursting activity and the region-specific release of ATP in hippocampal slices. A) DHPG induced ATP release in area CA3 (black trace) but not in the CA1 region (grey trace). B) The ecto-ATPase inhibitor ARL 67156 caused a further increase in ATP in area CA3 (upper trace labelled "ATP") above that induced by DHPG alone (the pre-DHPG sensor baseline is depicted by the lower broken line). ARL 67156 also elicited a change in bursting pattern. An example is shown in the middle trace with two DHPG-induced bursts before (left) and after (right) ARL 67156 application. This change is quantified in C) with a graph showing an increase in the duration of DHPG-induced bursts after ARL 67156 administration in six individual slices (filled circles) and the mean ± SEM for the data (open diamond; *$p < 0.02$; paired t-test). ATP traces in A and B are displayed as mean ± SEM. Figure taken from (Lopatar et al., 2015).

Figure 8. Analysis of biosensor signals during seizure activity A) ADO biosensor and extracellular (ext) recording during seizure activity in a neocortical slice. The trace from the null sensor (not shown) has been subtracted from the ADO biosensor trace (ADO-null). Following each burst of activity a large current is produced on the ADO biosensor showing an increase in extracellular purine concentration. The downward deflections on the biosensor are local field potentials as the biosensor also acts as an extracellular electrode. B) The solid line shows the model to fit the data in (A). The dotted line shows each separated purine waveform, with the baseline removed, allowing accurate measurement of biosensor current amplitude. Inset log plot of the ADO biosensor waveform in response to the first burst of activity. The decay is well described by a single exponential. C) ADO biosensor and extracellular (ext) recording during seizure activity. Application of the adenosine deaminase inhibitor EHNA (20 μM), which reduces the ability of the sensors to detect adenosine, reduced the amplitude of purine waveforms by ~75 % confirming

that the purines detected arise from either adenosine or adenosine metabolites. Modified from (Wall and Richardson, 2015).

Figure 9. Increases in extracellular purine concentration during seizure activity are highly localised. A) positioning of two biosensors (labelled A and B) 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 below in (B). Network activity was induced by nominally Mg^{2+}-free aCSF. Initially the network was only active around biosensor B (fast deflections, arrows) and not around biosensor A. The resultant increases in extracellular purine concentration, measured by biosensor B, were not detected by biosensor A. 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. C) Data from a mathematical model showing that purine concentration decreases rapidly with distance because of diffusive dilution. At the release site the concentration (*C*) is 100 % (*C* = 100 %) and the rise time of the biosensor signal is 2.5 s (t = 2.5s). With increasing distance from the release sites the amount detected decreases rapidly and the rise of the biosensor signal becomes slower. At 100 µm from the release sites only 2 % is detected and the rise of the biosensor signal is 22 s. Modified from (Wall and Richardson, 2015).

Figure 1

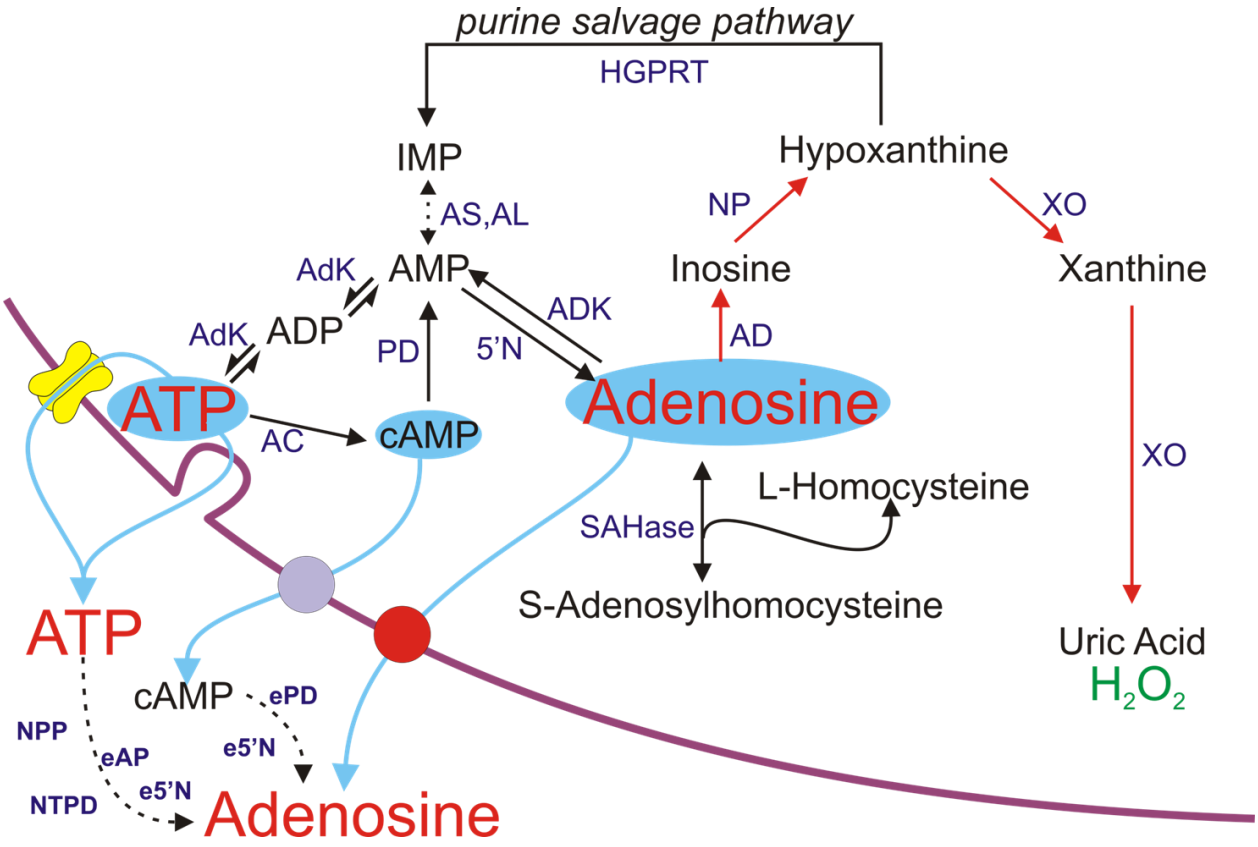
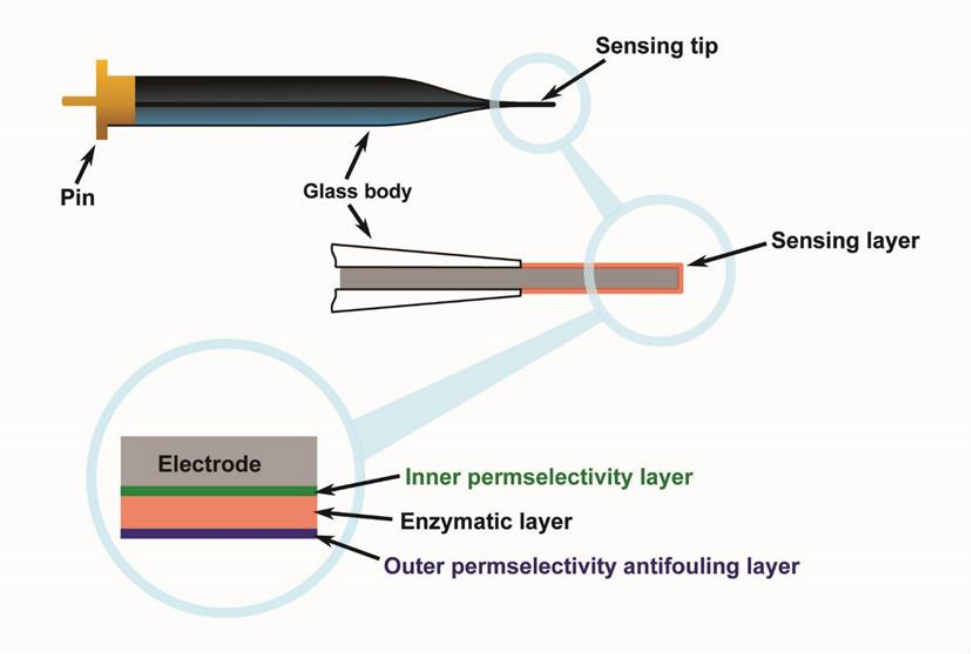
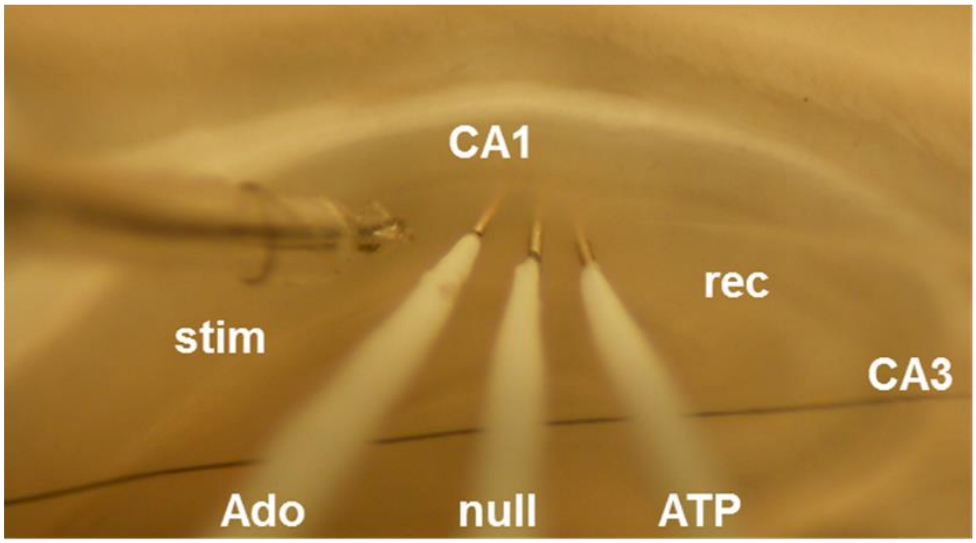
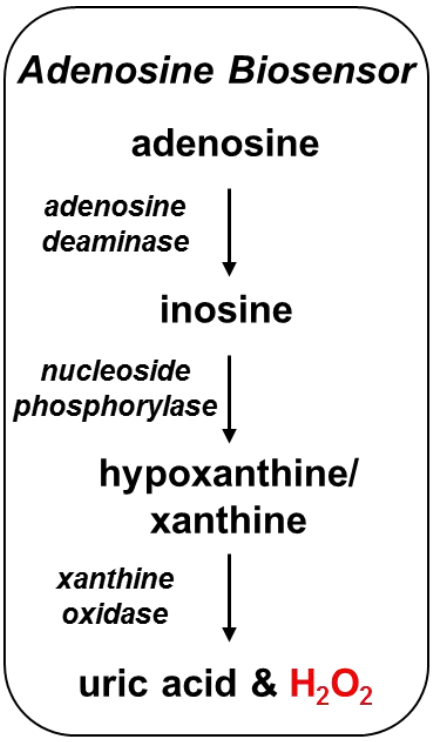

purine salvage pathway
HGPRT
IMP
Hypoxanthine
AS,AL
NP
XO
AdK
AMP
Inosine
Xanthine
AdK
ADP
PD
5'N
ADK
AD
ATP
AC
cAMP
Adenosine
XO
L-Homocysteine
SAHase
S-Adenosylhomocysteine
ATP
Uric Acid
H_2O_2
cAMP
ePD
NPP
eAP
e5'N
e5'N
NTPD
Adenosine

Figure 2


Sensing tip
Pin
Glass body
Sensing layer
Electrode
Inner permselectivity layer
Enzymatic layer
Outer permselectivity antifouling layer


CA1
rec
stim
CA3
Ado
null
ATP


Adenosine Biosensor
adenosine
adenosine deaminase
inosine
nucleoside phosphorylase
hypoxanthine/ xanthine
xanthine oxidase
uric acid & H2O2

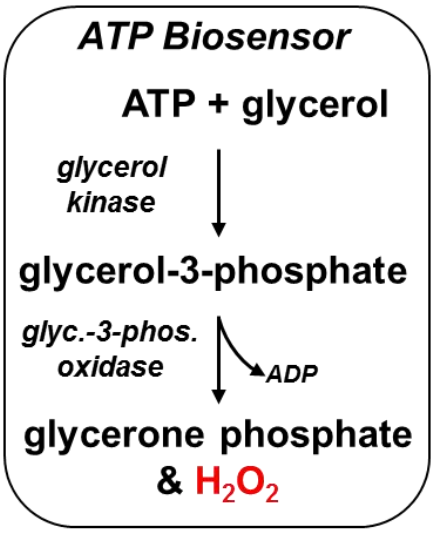

ATP Biosensor
ATP + glycerol
glycerol kinase
glycerol-3-phosphate
glyc.-3-phos. oxidase
ADP
glycerone phosphate & H2O2

Figure 3

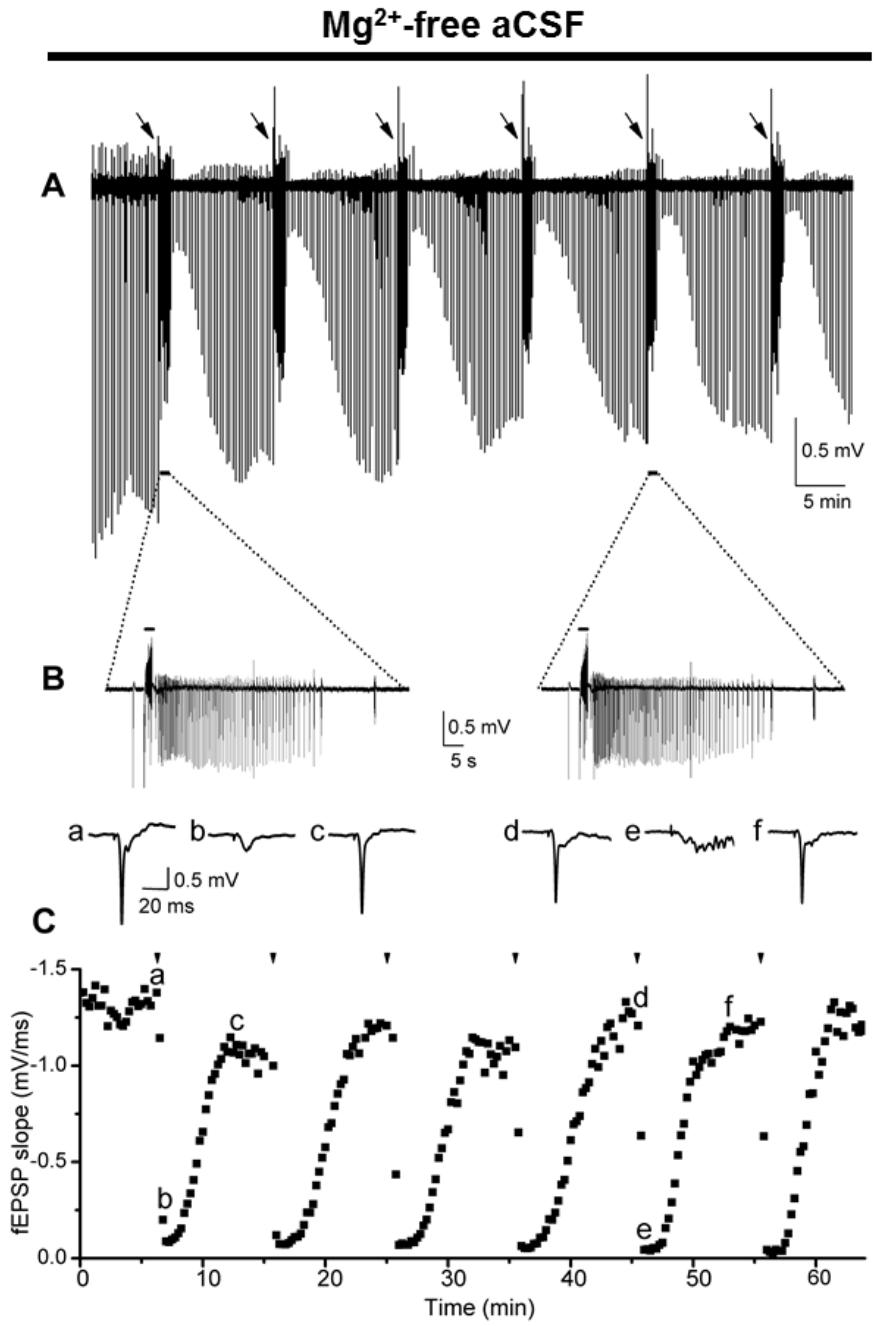

Mg2+-free aCSF
A
0.5 mV
5 min
B
0.5 mV
5 s
a
b
c
d
e
f
0.5 mV
20 ms
C
fEPSP slope (mV/ms)
-1.5
-1.0
-0.5
0.0
0
10
20
30
40
50
60
Time (min)

Figure 4

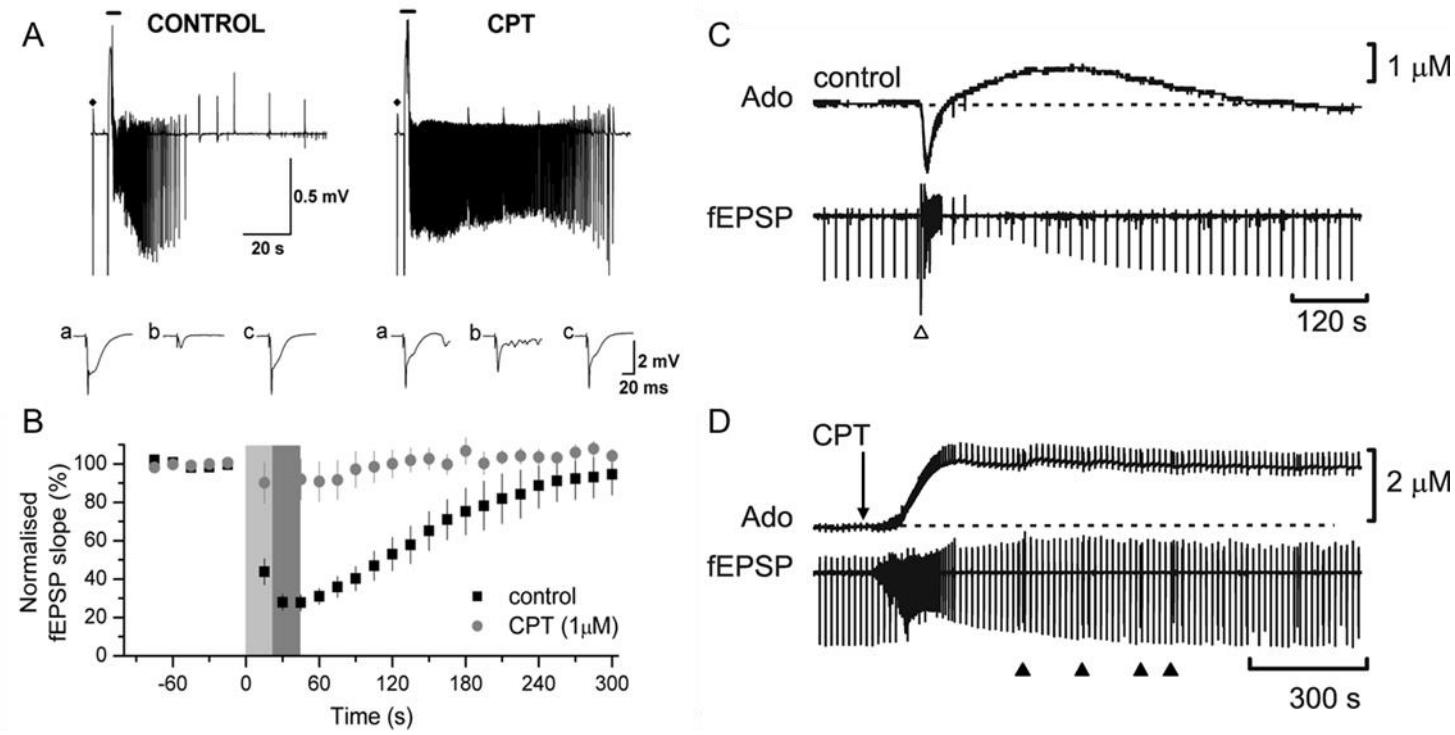

A
CONTROL
CPT
0.5 mV
20 s
a
b
c
2 mV
20 ms
B
Normalised fEPSP slope (%)
Time (s)
control
CPT (1μM)
C
control
Ado
fEPSP
1 μM
120 s
D
CPT
Ado
fEPSP
2 μM
300 s

Figure 5

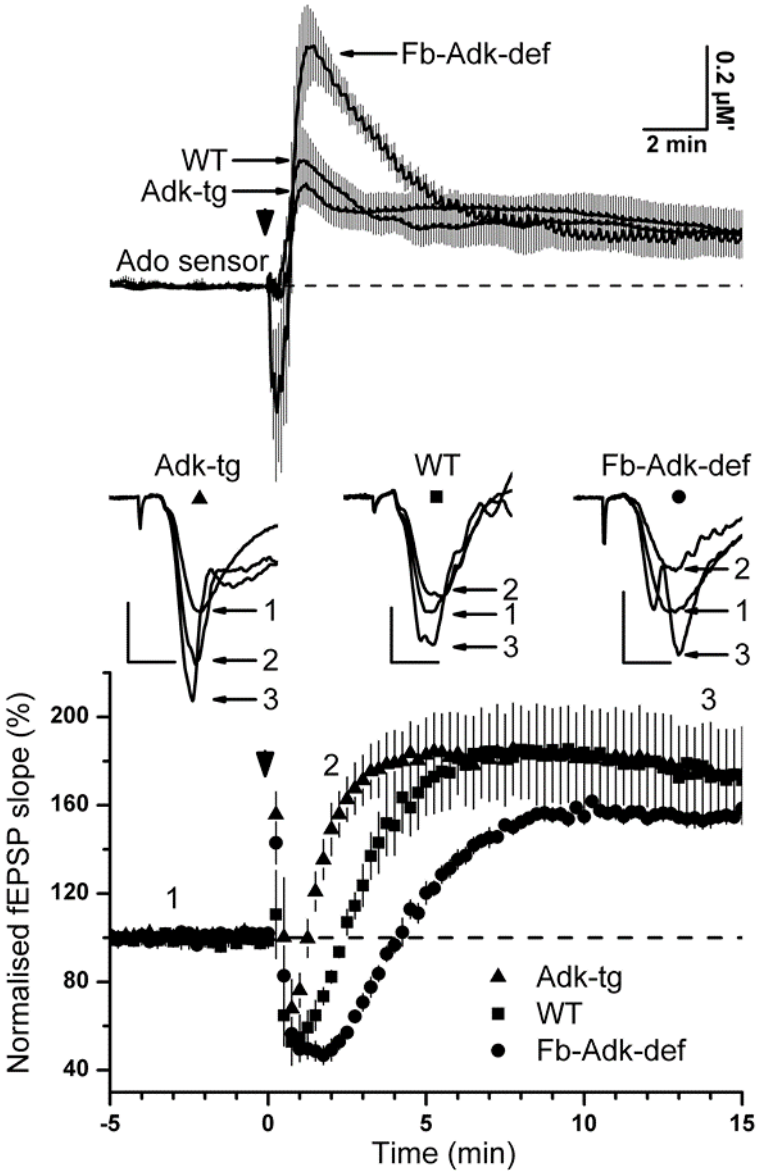

Fb-Adk-def
0.2 µM
2 min
WT
Adk-tg
Ado sensor
Adk-tg
WT
Fb-Adk-def
1
2
3
Normalised fEPSP slope (%)
200
160
120
80
40
-5
0
5
10
15
Time (min)
Adk-tg
WT
Fb-Adk-def

Figure 6

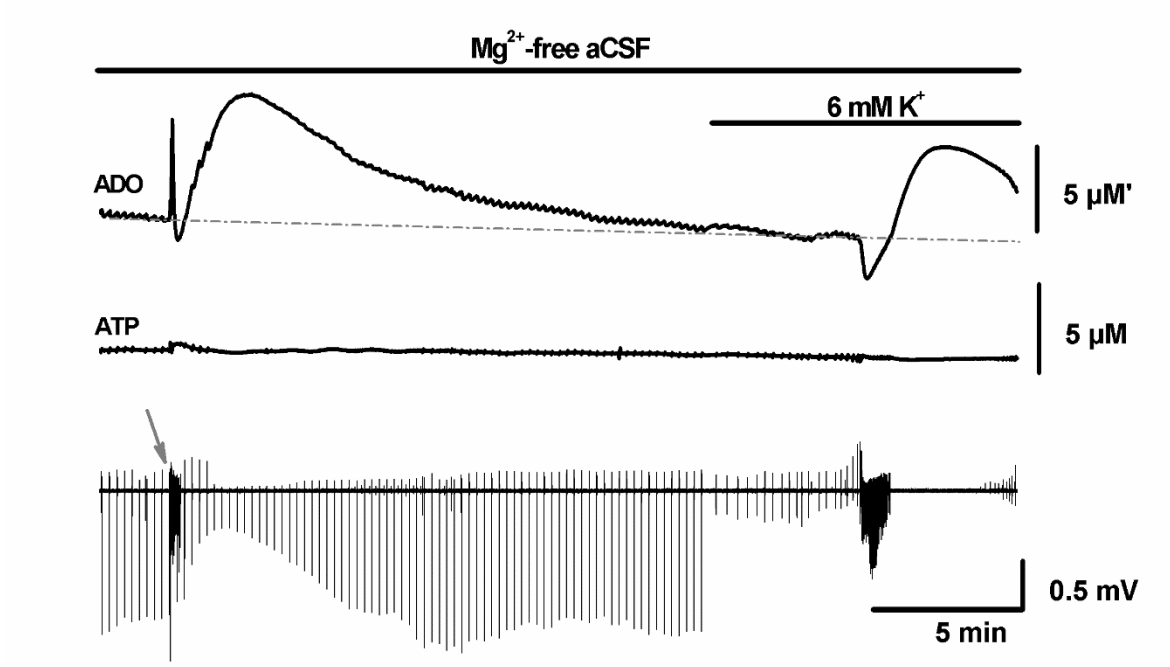


Figure 7

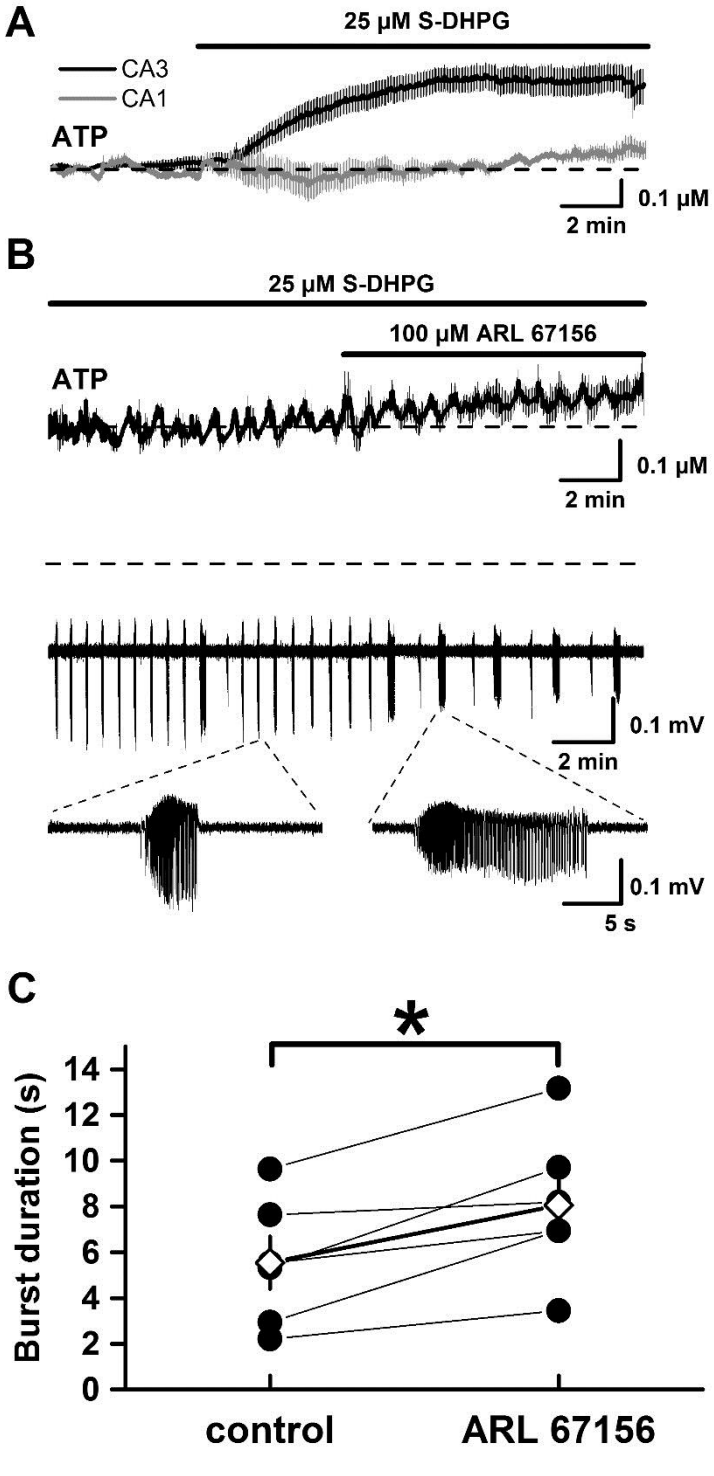

A
25 µM S-DHPG
CA3
CA1
ATP
0.1 µM
2 min
B
25 µM S-DHPG
100 µM ARL 67156
ATP
0.1 µM
2 min
0.1 mV
2 min
0.1 mV
5 s
C
*
Burst duration (s)
0
2
4
6
8
10
12
14
control
ARL 67156

Figure 8

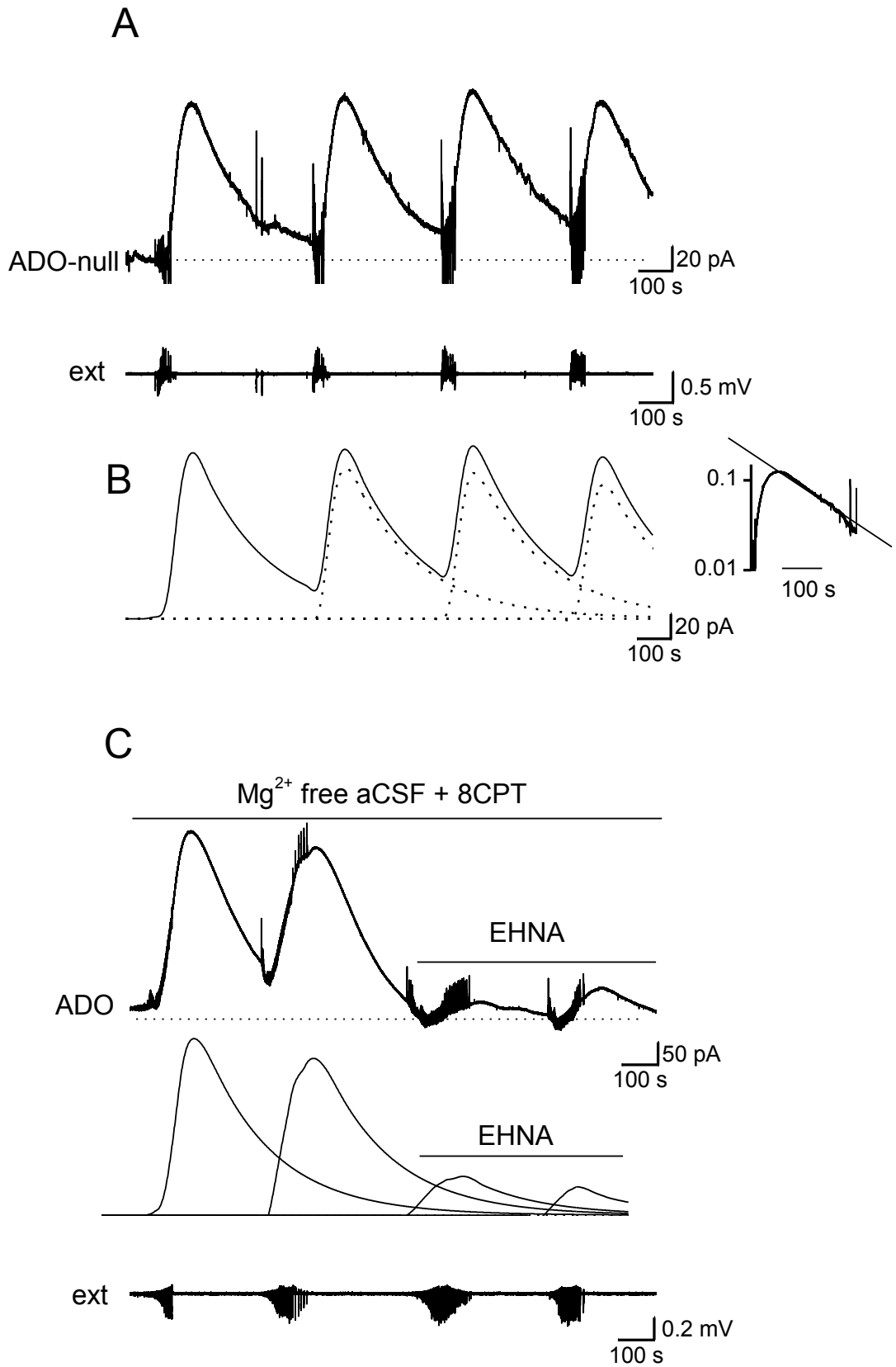
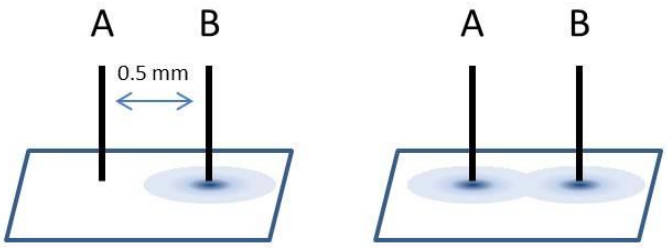
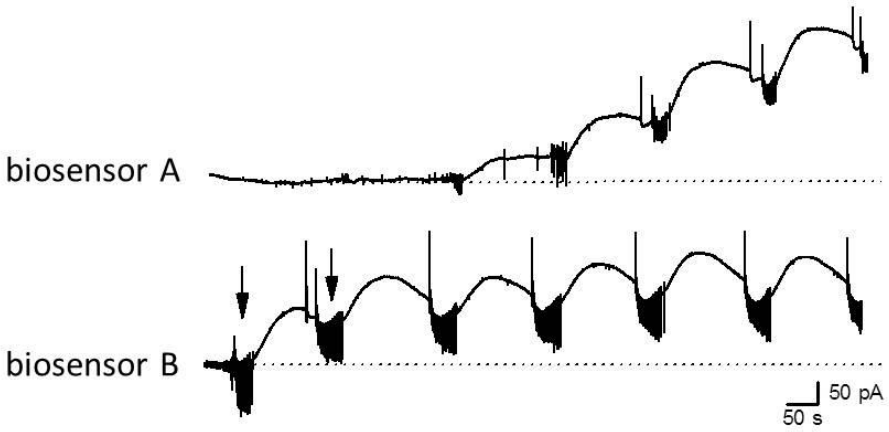

A
ADO-null
20 pA
100 s
ext
0.5 mV
100 s
B
0.1
0.01
100 s
20 pA
100 s
C
Mg2+ free aCSF + 8CPT
EHNA
ADO
50 pA
100 s
EHNA
ext
0.2 mV
100 s

Figure 9

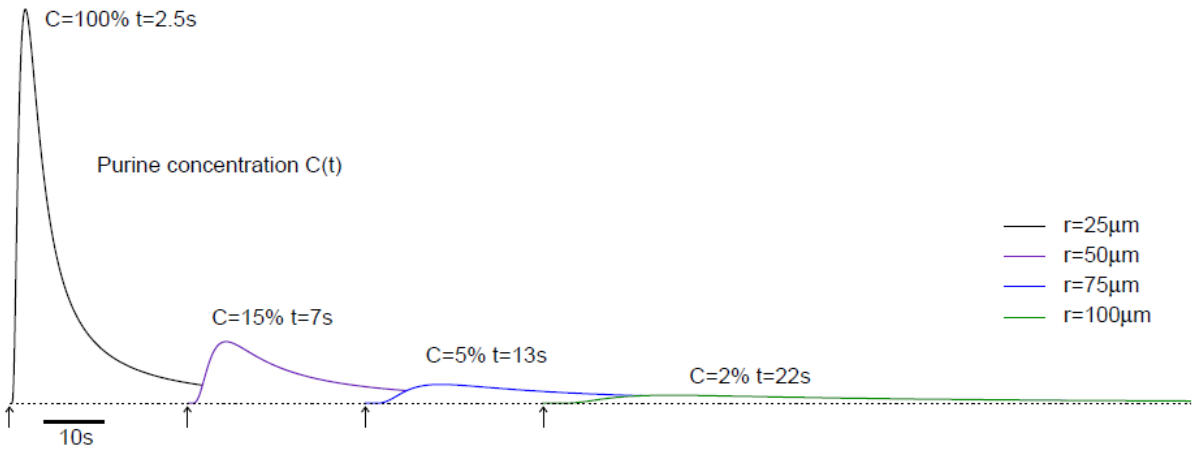
A


A
B
0.5 mm
A
B

B


biosensor A
biosensor B
50 pA
50 s

C


C=100% t=2.5s
Purine concentration C(t)
C=15% t=7s
C=5% t=13s
C=2% t=22s
r=25µm
r=50µm
r=75µm
r=100µm
10s

References

Aronica, E., Sandau, U.S., Iyer, A. & Boison, D. (2013) Glial adenosine kinase - A neuropathological marker of the epileptic brain. *Neurochem.Int.*.

Avoli, M., D'Antuono, M., Louvel, J., Kohling, R., Biagini, G., Pumain, R., D'Arcangelo, G. & Tancredi, V. (2002) Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro. *Prog.Neurobiol.*, **68**, 167-207.

Boison, D. (2008) The adenosine kinase hypothesis of epileptogenesis. *Prog.Neurobiol.*, **84**, 249-262.

Boison, D. (2012) Adenosine dysfunction in epilepsy. *Glia*, **60**, 1234-1243.

Burnstock, G. (2006) Historical review: ATP as a neurotransmitter. *Trends Pharmacol.Sci.*, **27**, 166-176.

Burnstock, G., Krugel, U., Abbracchio, M.P. & Illes, P. (2011) Purinergic signalling: from normal behaviour to pathological brain function. *Prog.Neurobiol.*, **95**, 229-274.

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

Dale, N. & Frenguelli, B.G. (2009) Release of adenosine and ATP during ischemia and epilepsy. *Current Neuropharmacology*, 160-179.

Dale, N. & Frenguelli, B.G. (2012) Measurement of purine release with microelectrode biosensors. *Purinergic.Signal.*, **8**, 27-40.

Dale, N., Gourine, A.V., Llaudet, E., Bulmer, D., Thomas, T. & Spyer, K.M. (2002) Rapid adenosine release in the nucleus tractus solitarii during defence response in rats: real-time measurement in vivo. *J.Physiol.*, **544**, 149-160.

Dale, N., Pearson, T. & Frenguelli, B.G. (2000) Direct measurement of adenosine release during hypoxia in the CA1 region of the rat hippocampal slice. *J.Physiol.*, **526**, 143-155.

Diogenes, M.J., Neves-Tome, R., Fucile, S., Martinello, K., Scianni, M., Theofilas, P., Lopatar, J., Ribeiro, J.A., Maggi, L., Frenguelli, B.G., Limatola, C., Boison, D. & Sebastiao, A.M. (2014) Homeostatic Control of Synaptic Activity by Endogenous Adenosine is Mediated by Adenosine Kinase. *Cereb.Cortex*, **24**, 67-80.

Dragunow, M. (1986) Endogenous anticonvulsant substances. *Neurosci.Biobehav.Rev.*, **10**, 229-244.

Dulla, C., Dobelis, P., Pearson, T., Frenguelli, B.G., Staley, K.J. & Masino, S.A. (2005) Adenosine and ATP link PCO2 to cortical excitability via pH. *Neuron*, **48**, 1011-1023.

Dulla, C.G., Frenguelli, B.G., Staley, K.J. & Masino, S.A. (2009) Intracellular acidification causes adenosine release during States of hyperexcitability in the hippocampus. *J.Neurophysiol.*, **102**, 1984-1993.

Dunwiddie, T.V. (1999) Adenosine and suppression of seizures. *Adv.Neurol.*, **79**, 1001-1010.

During, M.J. & Spencer, D.D. (1992) Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Ann.Neurol.*, **32**, 618-624.

Etherington, L.A. & Frenguelli, B.G. (2004) Endogenous adenosine modulates epileptiform activity in rat hippocampus in a receptor subtype-dependent manner. *Eur.J.Neurosci.*, **19**, 2539-2550.

Etherington, L.A., Patterson, G.E., Meechan, L., Boison, D., Irving, A.J., Dale, N. & Frenguelli, B.G. (2009) Astrocytic adenosine kinase regulates basal synaptic adenosine levels and seizure activity but not activity-dependent adenosine release in the hippocampus. *Neuropharmacol.*, **56**, 429-437.

Ficker, C., Rozmer, K., Kato, E., Ando, R.D., Schumann, L., Krugel, U., Franke, H., Sperlagh, B., Riedel, T. & Illes, P. (2014) Astrocyte-neuron interaction in the substantia gelatinosa of the spinal cord dorsal horn via P2X7 receptor-mediated release of glutamate and reactive oxygen species. *Glia*, **62**, 1671-1686.

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

Gourine, A.V., Dale, N., Korsak, A., Llaudet, E., Tian, F., Huckstepp, R. & Spyer, K.M. (2008) Release of ATP and glutamate in the nucleus tractus solitarii mediate pulmonary stretch receptor (Breuer-Hering) reflex pathway. *J.Physiol*, **586**, 3963-3978.

Heinrich, A., Ando, R.D., Turi, G., Rozsa, B. & Sperlagh, B. (2012) K+ depolarization evokes ATP, adenosine and glutamate release from glia in rat hippocampus: a microelectrode biosensor study. *Br.J.Pharmacol.*, **167**, 1003-1020.

Huckstepp, R.T., Eason, R., Sachdev, A. & Dale, N. (2010) CO2-dependent opening of connexin 26 and related beta connexins. *J.Physiol*, **588**, 3921-3931.

Kerr, M.I., Wall, M.J. & Richardson, M.J. (2013) Adenosine A1 receptor activation mediates the developmental shift at layer 5 pyramidal cell synapses and is a determinant of mature synaptic strength. *J.Physiol*, **591**, 3371-3380.

Klaft, Z.J., Schulz, S.B., Maslarova, A., Gabriel, S., Heinemann, U. & Gerevich, Z. (2012) Extracellular ATP differentially affects epileptiform activity via purinergic P2X7 and adenosine A1 receptors in naive and chronic epileptic rats. *Epilepsia*, **53**, 1978-1986.

Klyuch, B.P., Dale, N. & Wall, M.J. (2012a) Receptor-mediated modulation of activity-dependent adenosine release in rat cerebellum. *Neuropharmacol.*, **62**, 815-824.

Klyuch, B.P., Dale, N. & Wall, M.J. (2012b) Deletion of ecto-5'-nucleotidase (CD73) reveals direct action potential-dependent adenosine release. *J.Neurosci.*, **32**, 3842-3847.

Klyuch, B.P., Richardson, M.J., Dale, N. & Wall, M.J. (2011) The dynamics of single spike-evoked adenosine release in the cerebellum. *J.Physiol*, **589**, 283-295.

Kumaria, A., Tolias, C.M. & Burnstock, G. (2008) ATP signalling in epilepsy. *Purinergic.Signal.*, **4**, 339-346.

Li, T., Ren, G., Lusardi, T., Wilz, A., Lan, J.Q., Iwasato, T., Itohara, S., Simon, R.P. & Boison, D. (2008) Adenosine kinase is a target for the prediction and prevention of epileptogenesis in mice. *J.Clin.Invest*, **118**, 571-582.

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

Llaudet, E., Hatz, S., Droniou, M. & Dale, N. (2005) Microelectrode biosensor for real-time measurement of ATP in biological tissue. *Anal.Chem.*, **77**, 3267-3273.

Loo, C., Simpson, B. & MacPherson, R. (2010) Augmentation strategies in electroconvulsive therapy. *J.ECT.*, **26**, 202-207.

Lopatar, J., Dale, N. & Frenguelli, B.G. (2015) Pannexin-1-mediated ATP release from area CA3 drives mGlu5-dependent neuronal oscillations. *Neuropharmacol.*, **93**, 219-228.

Lopatar, J., Dale, N. & Frenguelli, B.G. (2011) Minor contribution of ATP P2 receptors to electrically-evoked seizure activity in hippocampal slices: evidence from purine biosensors and P2 receptor agonists and antagonists. *Neuropharmacol.*.

Lovatt, D., Xu, Q., Liu, W., Takano, T., Smith, N.A., Schnermann, J., Tieu, K. & Nedergaard, M. (2012) Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. *Proc.Natl.Acad.Sci.U.S.A*.

Marinesco, S. & Dale, N. (2013) *Microelectrode Biosensors* Neuromethods, Vol 80, Springer.

McIlwain, H. (1984) Cerebral Subsystems as Biological Entities. In Dingledine, R. (ed), *Brain Slices*. Plenum, New York, pp. 1-5.

Mendez, O.E. & Brenner, R.P. (2006) Increasing the yield of EEG. *J.Clin.Neurophysiol.*, **23**, 282-293.

Pascual, O., Casper, K.B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J.Y., Takano, H., Moss, S.J., McCarthy, K. & Haydon, P.G. (2005) Astrocytic purinergic signaling coordinates synaptic networks. *Science*, **310**, 113-116.

Pearson, T., Currie, A.J., Etherington, L.A., Gadalla, A.E., Damian, K., Llaudet, E., Dale, N. & Frenguelli, B.G. (2003) Plasticity of purine release during cerebral ischemia: clinical implications? *J.Cell Mol.Med.*, **7**, 362-375.

Pearson, T., Damian, K., Lynas, R. & Frenguelli, B.G. (2006) Sustained elevation of extracellular adenosine and activation of A1 receptors underlie the post-ischaemic inhibition of neuronal function in rat hippocampus *in vitro*. *J.Neurochem.*, **97**, 1357-1368.

Pearson, T. & Frenguelli, B.G. (2004) Adrenoceptor subtype-specific acceleration of the hypoxic depression of excitatory synaptic transmission in area CA1 of rat hippocampus. *Eur.J.Neurosci.*, **20**, 1555-1565.

Pearson, T., Nuritova, F., Caldwell, D., Dale, N. & Frenguelli, B.G. (2001) A depletable pool of adenosine in area CA1 of the rat hippocampus. *J.Neurosci.*, **21**, 2298-2307.

Richardson, M.J. & Silberberg, G. (2008) Measurement and analysis of postsynaptic potentials using a novel voltage-deconvolution method. *J.Neurophysiol.*, **99**, 1020-1031.

Rubini, P., Milosevic, J., Engelhardt, J., Al-Khrasani, M., Franke, H., Heinrich, A., Sperlagh, B., Schwarz, S.C., Schwarz, J., Norenberg, W. & Illes, P. (2009) Increase of intracellular Ca2+ by adenine and uracil nucleotides in human midbrain-derived neuronal progenitor cells. *Cell Calcium*, **45**, 485-498.

Schmitt, L.I., Sims, R.E., Dale, N. & Haydon, P.G. (2012) Wakefulness affects synaptic and network activity by increasing extracellular astrocyte-derived adenosine. *J.Neurosci.*, **32**, 4417-4425.

Sims, R.E. & Dale, N. (2014) Activity-dependent adenosine release may be linked to activation of Na(+)-K(+) ATPase: an in vitro rat study. *PLoS.One.*, **9**, e87481.

Sims, R.E., Wu, H.H. & Dale, N. (2013) Sleep-wake sensitive mechanisms of adenosine release in the basal forebrain of rodents: an in vitro study. *PLoS.One.*, **8**, e53814.

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

Tolner, E.A., Hochman, D.W., Hassinen, P., Otahal, J., Gaily, E., Haglund, M.M., Kubova, H., Schuchmann, S., Vanhatalo, S. & Kaila, K. (2011) Five percent CO(2) is a potent, fast-acting inhalation anticonvulsant. *Epilepsia*, **52**, 104-114.

Wall, M., Eason, R. & Dale, N. (2010) Biosensor measurement of purine release from cerebellar cultures and slices. *Purinergic.Signal.*, **6**, 339-348.

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

Wall, M.J. & Dale, N. (2013) Neuronal transporter and astrocytic ATP exocytosis underlie activity-dependent adenosine release in the hippocampus. *J.Physiol*, **591**, 3853-3871.

Wall, M.J. & Dale, N. (2007) Auto-inhibition of rat parallel fibre-Purkinje cell synapses by activity-dependent adenosine release. *J.Physiol*, **581**, 553-565.

Wall, M.J. & Richardson, M.J. (2015) Localized adenosine signaling provides fine-tuned negative feedback over a wide dynamic range of neocortical network activities. *J.Neurophysiol.*, **113**, 871-882.

Wall, M.J., Wigmore, G., Lopatar, 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. *Neuropharmacol.*, **55**, 1251-1258.

Yang, G.K., Squires, P.E., Tian, F., Kieffer, T.J., Kwok, Y.N. & Dale, N. (2012) Glucose decreases extracellular adenosine levels in isolated mouse and rat pancreatic islets. *Islets.*, **4**, 64-70.

Young, D. & Dragunow, M. (1994) Status epilepticus may be caused by loss of adenosine anticonvulsant mechanisms. *Neuroscience*, **58**, 245-261.

Zimmermann, H., Zebisch, M. & Strater, N. (2012) Cellular function and molecular structure of ecto-nucleotidases. *Purinergic.Signal.*, **8**, 437-502.

zur Nedden, S., Doney, A.S. & Frenguelli, B.G. (2014) Modulation of intracellular ATP determines adenosine release and functional outcome in response to metabolic stress in rat hippocampal slices and cerebellar granule cells. *J.Neurochem.*, **128**, 111-124.

zur Nedden, S., Hawley, S.A., Pentland, N., Hardie, D.G., Doney, A.S. & Frenguelli, B.G. (2011) Intracellular ATP influences synaptic plasticity in area CA1 of rat hippocampus via metabolism to adenosine and activity-dependent activation of adenosine A1 receptors. *Journal of Neuroscience*, **31**, 6221-6234.