

Adenosine is released during thalamic oscillations to provide negative feedback control

Mark J. Wall^{a,*}, Katie Puddefoot^a, Wencheng Yin^b, Chris Bingham^b, Mohsen Seifi^c, Jerome D. Swinny^d, Richard Teke Ngomba^{e,**}

^a School of Life Sciences, University of Warwick, Coventry, CV4 7AL, UK

^b University of Lincoln, College of Science, School of Engineering LN6 7TS, UK

^c Leicester School of Pharmacy, De Montfort University, Leicester, LE1 9BH, UK

^d School of Pharmacy & Biomedical Sciences, University of Portsmouth, St Michael's Building, White Swan Road, Portsmouth, PO12DT, UK

^e University of Lincoln, School of Pharmacy, LN6 7DL, UK

ABSTRACT

Physiological oscillations in the cortico-thalamo-cortical loop occur during processes such as sleep, but these can become dysfunctional in pathological conditions such as absence epilepsy. The purine neuromodulator adenosine can act as an endogenous anticonvulsant: it is released into the extracellular space during convulsive seizures to activate A₁ receptors suppressing on-going activity and delaying the occurrence of the next seizure. However, the role of adenosine in thalamic physiological and epileptiform oscillations is less clear. Here we have combined immunohistochemistry, electrophysiology, and fixed potential amperometry (FPA) biosensor measurements to characterise the release and actions of adenosine in thalamic oscillations measured in rodent slices. In the thalamus, A₁ receptors are highly expressed particularly in the ventral basal (VB) thalamus and reticular thalamic nucleus (nRT) supporting a role for adenosine signalling in controlling oscillations. In agreement with previous studies, both adenosine and adenosine A₁ receptor agonists inhibited thalamic oscillations in control (spindle-like) and in epileptic conditions. Here we have shown for the first time that both control and epileptiform oscillations are enhanced (i.e., increased number of oscillatory cycles) by blocking A₁ receptors consistent with adenosine release occurring during oscillations. Although increases in extracellular adenosine could not be directly detected during control oscillations, clear increases in adenosine concentration could be detected with a biosensor during epileptiform oscillation activity. Thus, adenosine is released during thalamic oscillations and acts via A₁ receptors to feedback and reduce thalamic oscillatory activity.

Key point summary

- Oscillations in the thalamus occur during sleep and light anaesthesia and are enhanced in pathological conditions such as absence epilepsy.
- Adenosine is a well characterised neuromodulator that provides negative feedback control of neural circuits via activation of A₁ receptors. The role of adenosine in convulsive seizures is well established but its role in non-epileptiform and epileptiform thalamic oscillations is less clear.
- Here we have used electrophysiology combined with microelectrode biosensor recording and immunohistochemistry to investigate adenosine signalling in the thalamus and its role in controlling thalamic oscillations in vitro.
- We have found that endogenous adenosine plays a role in controlling both non-epileptiform and epileptiform oscillations but can only be directly measured during epileptiform activity.

1. Introduction

The thalamus is the principal portal for sensory information, and it is central to the cortico-thalamocortical (CTC) loop that generates rhythmic oscillations (Meeren et al., 2005; Buzsáki, 2006; Polack et al., 2009). In normal physiology, this loop sustains oscillations as observed during sleep states but in pathological conditions, such as absence epilepsy, these rhythms appear to become dysfunctional leading to non-convulsive seizures. These seizures are characterised by spike-and-wave discharges (SWDs) observed in rodent models and in human patients (Blumenfeld, 2005; Crunelli and Leresche, 2002; Huguenard and McCormick, 2007). The anatomical structure which controls these oscillations is comprised of thalamic relay neurons in both the first-order and higher-order nuclei which send excitatory glutamatergic projections to the neocortex. Neurons in both thalamocortical and layer VI corticothalamic pathways also send excitatory collaterals onto neurons in the reticular thalamic nucleus (nRT), which, in turn,

* Corresponding author. School of Life Sciences, University of Warwick, Gibbet Hill, Coventry, CV4 7AL, UK.

** Corresponding author. University of Lincoln, School of Pharmacy, College of science, Joseph Banks Laboratories, LN6 7DL, UK.

E-mail addresses: mark.wall@warwick.ac.uk (M.J. Wall), richardngomba@hotmail.com, mgomba@lincoln.ac.uk (R.T. Ngomba).

provide a GABAergic input to all ventral basal thalamic neurons (Zhang and Jones, 2004; Blumenfeld, 2005). Despite knowledge of the circuit structure and cellular physiological basis for the oscillations, the mechanisms that control these oscillations and prevent them from becoming pathological have not yet been fully defined.

The purine neuromodulator adenosine acts via multiple subtypes of cell surface G-protein coupled receptors to modulate neural activity, with the high affinity inhibitory A₁ receptor being the most widely expressed in the brain (reviewed in Fredholm et al., 2000). Activation of A₁ receptors inhibits synaptic transmission and hyperpolarises the membrane potential via the activation of K⁺ channels (reviewed in Dunwiddie and Masino, 2001; Borea et al., 2018). Adenosine is released into the extracellular space to activate its receptors by multiple mechanisms which include: direct release via equilibrative nucleoside transporters (ENTs, Lovatt et al., 2012; Wall and Dale, 2013); release in the form of ATP from neurons (by exocytosis Pankratov et al., 2007) and from glial cells (by exocytosis and via hemi channels Newman, 2004; Pascual et al., 2005; Wall and Dale, 2013) to be metabolised in the extracellular space to from adenosine (Masino et al., 2002). There is also evidence that adenosine may be released directly as a neurotransmitter in the cerebellum (Klyuch et al., 2012).

Adenosine is released during convulsive epileptic events both to provide negative feedback to terminate bursts of activity and delay the next event (During and Spencer, 1992; Boison, 2008). These effects are mainly via the activation of A₁ receptors with seizures inhibited by A₁ receptor agonists and enhanced by A₁ receptor antagonists (reviewed in Weltha et al., 2019). Not much is known about the release of adenosine in the thalamus during activity but there is evidence that activation of adenosine receptors can modulate non-convulsive (absence) seizures. For example, injection of adenosine (i.p) increases the frequency of spike and wave discharges (SWDs) recorded in WAG/Rij rats, a model of absence epilepsy (Ilbay et al., 2001). In the GAERs model of absence epilepsy, caffeine (a mixed A₁ and A_{2a} receptor antagonist) and selective adenosine receptor antagonists (A₁ and A_{2a}) reduce the frequency of SWDs (Germé et al., 2015). Similar effects have also been reported for theophylline (a mixed adenosine receptor antagonist) on SWDs in WAG/Rij rats (Ates et al., 2004). However, it has also been reported that the suppressing effects of ketones on SWDs in WAG/Rij rats is attenuated by A₁ receptor antagonists (Kovács et al., 2017). The results from most of these experiments are difficult to interpret as the injected pharmacological agents will have effects not only in the thalamus but also in other brain structures, which may modify the frequency of SWDs.

The *in vitro* thalamic preparation is a powerful assay that allows for the probing of neurotransmitter and neuromodulator effects on the key synapses in the thalamic circuitry, including glutamatergic input from the cortex or from the thalamus to the nRT. In rodents (rats and mice) it has until recently been proposed that GABAergic inhibition in the thalamus originates entirely from the nRT (Diamond et al., 2008; Jones, 2009) but see (Simko and Markram, 2021). In isolated slices, Ulrich and Huguenard (1995) showed that glutamatergic and GABAergic synaptic transmission are inhibited by adenosine in both the nRT and VB thalamus and that adenosine inhibits thalamic oscillations. However, they did not investigate whether adenosine is released during oscillations to feedback and control activity.

We have investigated the distribution of A₁ receptors in the rodent thalamus and examined how inhibiting A₁ receptors effects isolated thalamic oscillations in control (spindles) and in epileptiform conditions. We have combined electrophysiology with biosensor recordings to directly measure adenosine release in the thalamus. We have found that adenosine can be released during thalamic oscillations and acts to feedback and suppress oscillatory activity.

2. Methods

2.1. Preparation of thalamocortical slices

All animal care and experimental procedures were reviewed and approved by the institutional animal welfare and ethical review body (AWERB) at the University of Warwick.

Coronal brain slices (400 µm) were initially prepared from male C57 BL/6J mice (3–4 weeks) and then from Sprague Dawley rats, at postnatal days 18–21. Rats and mice were kept on a 12-h light-dark cycle with slices made 90 min after entering the light cycle. In accordance with the U.K. Animals (Scientific Procedures) Act (1986), rats and mice were culled by cervical dislocation and then decapitated. The brain was rapidly removed, the rostral and caudal regions of the brain were trimmed and then brain was stuck down so the rostral end of the brain faced the blade. Slices were cut with a Microm HM 650V microslicer in cold (2–4 °C) high Mg²⁺, low Ca²⁺ aCSF, composed of (mM): 127 NaCl, 1.9 KCl, 8 MgCl₂, 0.5 CaCl₂, 1.2 KH₂PO₄, 26 NaHCO₃, 10 D-glucose (pH 7.4 when bubbled with 95% O₂ and 5% CO₂, 300 mOSM. Slices were kept for recording when the connections between the striatum and thalamus appeared intact (2–3 slices per animal). These slices were cut in half, trimmed and then stored at 34 °C for 1–6 h in aCSF (1 mM MgCl₂, 2 mM CaCl₂) before recording.

2.2. Extracellular recording of thalamic oscillations from coronal brain slices

A slice 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 (Tygon) was gas tight (to prevent loss of oxygen). For extracellular recording, an aCSF filled microelectrode was placed on the surface of the ventrobasal thalamus. To evoke thalamic oscillations, a bipolar stimulating electrode was placed on the surface of the internal capsule with oscillations evoked with a 200 µs duration stimulus of 1–4 V amplitude delivered every 10–20s (initially at 10 s to find a connection and then at 20 s during the experiments). Extracellular recordings were made using a differential model 3000 amplifier (AM systems, WA USA) with oscillations evoked with an isolated pulse stimulator model 2100 (AM Systems, WA). 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), (see also Kyuyoung and Huguenard, 2014).

2.3. Analysis of thalamic oscillations

Data and statistical analyses were performed using bespoke MATLAB R2009b scripts. A threshold was set at a value above the variance of the background noise and the number of bursts, spikes per burst and latencies were measured. The maximum period for a spike was defined as 200 ms from stimulation and the minimum number of spikes that was classified as burst was defined as 3 spikes (see Kleiman-Weiner et al., 2009; Kyuyoung and Huguenard, 2014).

2.4. Immunohistochemistry methods

Immunohistochemical analyses were replicated in five male C57 BL/6J mice, aged 3 months, using methodology according to (Jackson et al., 2021). Animal tissue was fixed by inducing anaesthesia with isoflurane and maintained with pentobarbitone (1.25 mg/kg of bodyweight, IP) and transcardially perfused with 0.9% saline solution for 2 min, followed by 10 min of a fixative consisting of 1% formaldehyde and 15% v/v saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4. After the perfusion, the brains were dissected and post-fixed over night at room temperature in the same fixative solution. The following day, the tissue was rinsed in 0.1 M PB, after which 60 µm coronal sections were

prepared using a VT 1000 vibrating microtome (Leica, Wetzlar, Germany). The sections were thoroughly washed in 0.1 M PB to remove any residual fixative and then stored in a solution containing 0.1 M PB and 0.05% w/v sodium azide until further processing. To minimise the non-specific binding of secondary antibodies, tissue sections were pre-incubated in a TBS-Tx solution containing 20% normal serum from the species that the secondary antibodies were raised in (Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature on a horizontal shaker.

Tissue sections were incubated with multiple primary antibodies diluted in TBS-Tx at 4 °C overnight on a horizontal shaker. The primary antibodies used were: rabbit anti-adenosine A₁ receptor (Alomone, AAR-006), goat and parvalbumin (Swant, PVG-213), guinea pig anti vesicular glutamate transporter 1 (Frontier Institute, VGLUT1-GP-Af1650) and 1, mouse anti vesicular glutamate transporter 2 (NeuroMab, 75-067). On the following day, tissue sections were washed in TBS-Tx three times for 10-min periods to remove unbound antibodies. Tissue sections were then incubated in a cocktail containing appropriate secondary

antibodies targeted at the Fc region of primary antibodies, for 2 h at room temperature on a horizontal shaker. Secondary antibodies were all raised in donkey, and conjugated to either Alexa Fluor™ 488 (Invitrogen, Eugene, OR, USA), indocarbocyanine (Cy™3) or DyLight™ 549, or DyLight™ 649 (Jackson ImmunoResearch, West Grove, PA, USA), for 2 h. Sections were then washed three times for 10 min periods in TBS-Tx to remove unbound antibodies, mounted on glass microscope slides, air dried, and sealed with glass coverslips using Vectashield™ antifade mounting medium (Vector Laboratories). The specificity of the A₁ receptor antibody was assessed by preadsorbing it with the immunizing peptide prior to incubating the tissue sections. The specificity of the secondary antibodies was assessed by omitting the primary antibodies (SI Fig. 1).

2.5. Microscopy

Tissue samples were examined with a confocal laser-scanning microscope (LSM 880 with AiryScan; Zeiss, Oberkochen, Germany) using

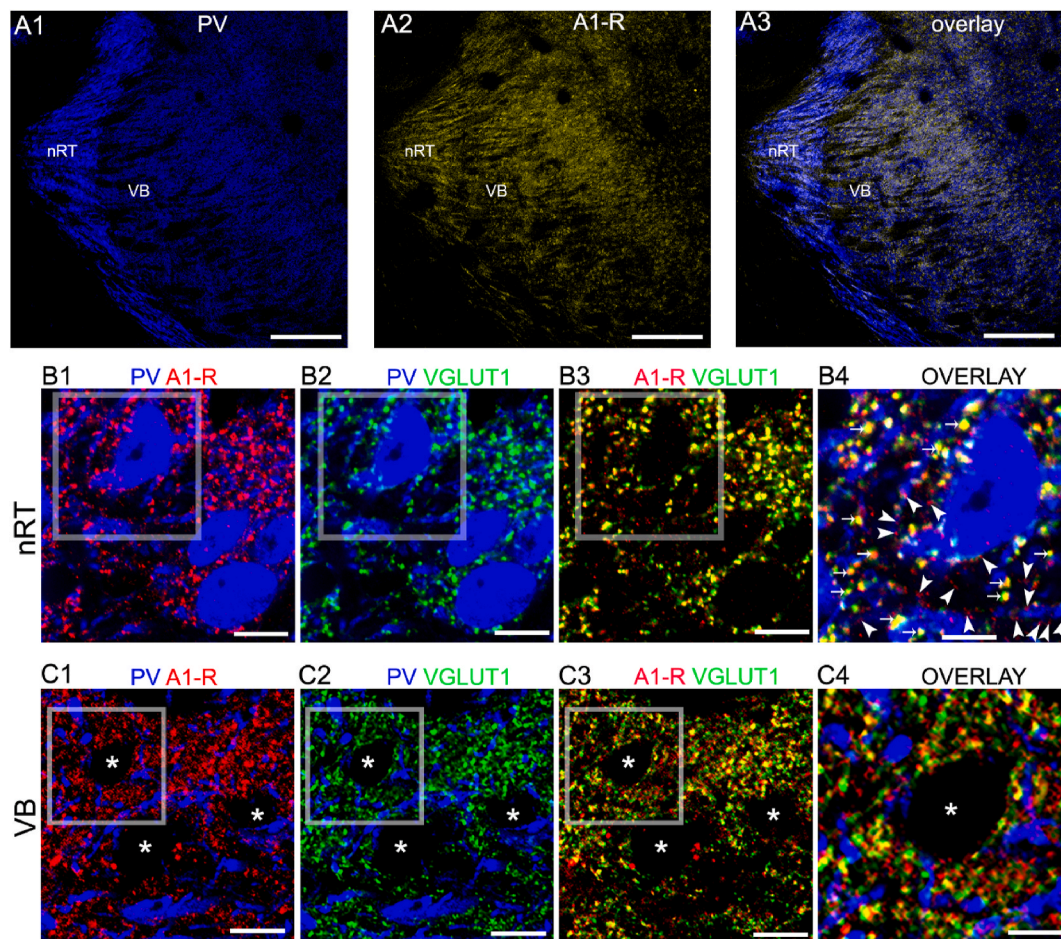


Fig. 1. Immunolocalization of A₁ receptors in the thalamus (A1) Immunoreactivity for the calcium binding protein parvalbumin (PV) which identifies the principal neurons of the thalamic reticular nucleus (nRT) and their projections to the ventrobasal complex (VB). (A2) in the same field of view (FOV), immunoreactivity for the adenosine A₁ receptor (A1-R) is distributed throughout the nRT and VB. (A3) is an overlay of (A1-2).

(B1) High resolution view of the nRT showing that adenosine A₁ receptor immunoreactivity (A1-R) in the nRT presents as individual clusters closely associated with PV-immunopositive profiles. (B2) in the same FOV, putative cortico-thalamic inputs, identified by immunoreactivity for the vesicular glutamate transporter 1 (VGLUT1) decorate PV-immunopositive profiles. (B3) shows that the large majority of adenosine A₁ receptor (A1-R) immunopositive clusters colocalise with VGLUT1-immunoreactive puncta. (B4) is a magnified view of the boxed areas in (B1-3) indicating that a proportion with adenosine A₁ receptor (A1-R) immunoreactive clusters contact PV-immunopositive profiles (arrowheads) whilst another population colocalise with VGLUT1 signal (arrows).

(C1) In the VB adenosine A₁ receptor (A1-R) immunoreactivity presents as individual clusters partly associated with PV-immunopositive profiles and surrounding putative VB neurons (asterisks). (C2) shows the distribution of VGLUT1-immunopositive clusters and PV-immunopositive profiles in the same FOV. (C3) shows that the large majority of adenosine A₁ receptor immunopositive clusters colocalise with VGLUT1-immunoreactive puncta. (C4) is a magnified view of the boxed areas in (C1-3) indicating that adenosine A₁ receptor immunoreactive clusters colocalise with VGLUT1 puncta contacting putative VB neurons.

Scale bars: (A) 300 µm; (B, C1-3) 10 µm; (B, C4) 5 µm.

either a Plan Apochromat 20x (NA 0.8; pixel size 0.42 μm) objective, Plan Apochromat 40x DIC oil objective (NA 1.3; pixel size 0.29 μm), Plan Apochromat 63x DIC oil objective (NA 1.4; pixel size 0.13 μm) objective or a Plan Apochromat 100x DIC oil objective (NA 1.46; pixel size 0.08 μm). Detection was with PMT detectors. All images presented represent a single optical section. Images were acquired using sequential acquisition of the different channels to avoid cross-talk between fluorophores. Pinholes were adjusted to 1.0 Airy unit. In all cases where multiple images were captured from the same immunohistochemical reaction, laser power, pinhole, and exposure settings were captured once on tissue from a representative control section and maintained throughout imaging. Images were processed with Zen software (Zeiss) and exported into bitmap images for processing in ImageJ and Adobe Photoshop (Adobe Systems, San Jose, Ca, USA). Only brightness and contrast were adjusted for the whole frame, and no part of any frame was enhanced or modified in any way.

2.6. Fixed potential amperometry (FPA) microelectrode biosensor characteristics

Microelectrode biosensors (Sarissa Biomedical Ltd, Coventry UK) consist of enzymes trapped within a matrix around a Pt or Pt/Ir (90/10) wire (Llaudet et al., 2003). Biosensors were cylindrical with an exposed length of $\sim 500 \mu\text{m}$ and diameter of $\sim 50 \mu\text{m}$. Three 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 and baseline drift. Secondly, biosensors containing adenosine deaminase, nucleoside phosphorylase and xanthine oxidase (responsive to adenosine, inosine and hypoxanthine: ADO biosensors); Thirdly, biosensors for ATP which consisted of the entrapped enzymes glycerol kinase and glycerol-3-phosphate oxidase (Llaudet et al., 2005). For the experiments in which ATP biosensors were used, glycerol (2 mM) was included in the aCSF as it is a co-substrate for ATP detection. This concentration of glycerol has been used in many previous experiments and has no significant effect on the recorded activity (see Frenguelli and Wall, 2016 for review).

A full description of biosensor properties has previously been published (Llaudet et al. 2003, 2005): they show a linear response to increasing concentrations of analyte and have a fast response time ($<1 \text{ s}$ Wall and Richardson, 2015). In each experiment, the biosensors were calibrated with analyte (10 μM) before measurements were made, to check for loss of sensitivity. In the experiments, the composition of purines detected by ADO biosensors was not fully defined. Since ADO biosensors have an approximately 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 $\mu\text{M}'$ or nM' of purines (as outlined in Pearson et al., 2001; Klyuch et al., 2012). Biosensor signals were acquired at 1 kHz with a Micro CED (Mark 2) interface using Spike (Vs 6.1) software.

2.7. Whole cell patch clamp recording

A slice was transferred to the recording chamber and perfused at 3 ml min^{-1} with aCSF at $32 \pm 0.5^\circ\text{C}$. Slices were visualized using IR-DIC optics with an Olympus BX151W microscope (Scientifica) and a CCD camera (Hitachi). Whole-cell current clamp recordings were made from thalamocortical neurons in ventrobasal thalamus using patch pipettes (5–10 M Ω) 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 (290 mOSM, pH 7.2). Current clamp 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 10 (Molecular Devices, USA). To extract the electrophysiological properties of recorded thalamocortical neurons, both step (50 pA steps from $-200/150$ to $50/100$) and more naturalistic, fluctuating currents were injected

in control and after the application of either adenosine or 8CPT and in wash. To measure the firing rate, a current waveform, designed to provoke naturalistic fluctuating voltages, was constructed using the summed numerical output of two Ornstein–Uhlenbeck processes (Uhlenbeck and Ornstein, 1930) with time constants $\tau_{\text{fast}} = 3 \text{ ms}$ and $\tau_{\text{slow}} = 10 \text{ ms}$. This current waveform, which mimics the stochastic actions of AMPA and GABA-receptor channel activation, is injected into neurons and the resulting voltage recorded (a fluctuating, naturalistic trace of 30s duration). The firing rate was measured from voltage traces evoked by injecting a current waveform of the same gain for all recordings (firing rate $\sim 2\text{--}3 \text{ Hz}$). Action potentials were detected by a manually set threshold and the interval between action potentials and bursts were measured (Badel et al., 2008; Harrison et al., 2015; Hill et al., 2019; Kaufmann et al 2016).

2.8. Epileptiform activity

Epileptiform thalamic oscillations were induced with aCSF which contained a reduced concentration of Mg^{2+} (0.1 mM) and with partial inhibition of GABA $_A$ receptors (either bicuculline 5 μM or picrotoxin 25 μM based on Ulrich and Huguenard, 1995).

2.9. Drugs

Drugs were made up as stock solutions (1–10 mM) in distilled water and then diluted in aCSF. Adenosine, 8-cyclopentyltheophylline (8CPT), N6-Cyclopentyladenosine (CPA), (–)-bicuculline methiodide and picrotoxin were obtained from Sigma-Aldrich (Dorset, UK). Ethosuximide (Sigma Aldrich) was dissolved in ethanol. The final concentration of ethanol in the aCSF was less than 0.01%. 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261) was obtained from Tocris (Bristol, UK).

2.10. Statistics

All quoted data is mean $\pm$ SEM. For statistical analysis non-parametric tests were used (Mann Whitney).

3. Results

3.1. Adenosine A $_1$ receptor distribution in the thalamus

Initially the distribution of adenosine A $_1$ receptor expression in the thalamus was examined before examining thalamic oscillations. A low power overview of A $_1$ receptor immunoreactivity in the thalamus revealed signal distributed throughout the reticular nucleus (nRT) and ventrobasal (VB) complex (Fig. 1A). High resolution inspection of the nRT revealed A $_1$ receptor immunoreactive puncta, which, for the large majority, colocalised with clusters immunopositive for the vesicular glutamate transporter 1 (VGLUT1) and forming appositions to PV-positive somata and dendrites (Fig. 1 B). Likewise, within the VB complex, A $_1$ receptor immunoreactive clusters were also colocalised with VGLUT1 immunopositive puncta (Fig. 1 C). There was no discernible association between the signal for adenosine A $_1$ receptors and VGLUT2 (SI Fig. 2). This data shows a concentrated expression of A $_1$ receptors in the VB and nRT which would be expected to influence thalamic oscillations.

3.2. Measuring thalamic oscillations in a submerged chamber

In previous studies, thalamic oscillations have been recorded in vitro from corticothalamic slices in an interface chamber (for example see Ulrich and Huguenard, 1995; Ulrich and Huguenard, 1995; Kyuyoung and Huguenard, 2014; Sohal, 2003 etc). However, to make biosensor measurements, the brain slices must be fully submerged (Llaudet et al., 2003). We took a number of approaches to achieve reliable recordings of

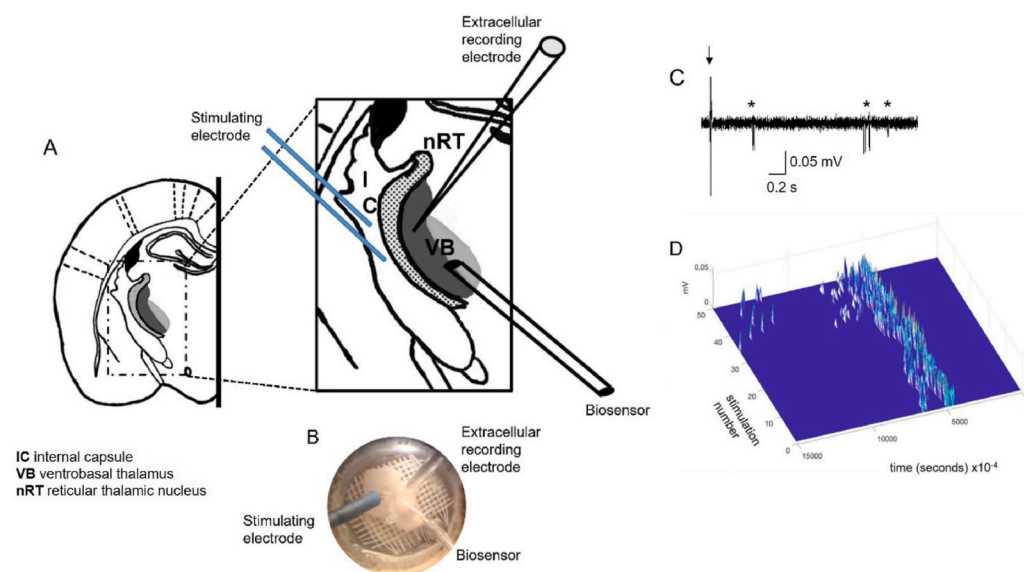


Fig. 2. The submerged thalamocortical slice preparation and measurement of thalamic oscillations. A, Diagram of thalamo-cortical slice illustrating electrode placements with thalamus recording area shown magnified. Bipolar stimulating electrode(s) were placed on the surface of the internal capsule and the extracellular recording electrode was placed in line with the stimulating electrode in the VB region of the thalamus. Biosensors were laid on the surface of the VB near the recording electrode. B, Photograph of the slice placed on a grid, so it is perfused with aCSF from above and below, showing positions of recording and stimulating electrodes and biosensor. C, Example extracellular trace showing stimulation (arrow) followed by oscillations (*). D, 3D projection of evoked oscillations from a single experiment (~50 stimulations) produced from a bespoke MATLAB script. Stimulation occurs at time zero (on the right-hand side). The first oscillation occurs at around 0.5 s, as denoted by a peak, with the ampli-

tude of the peak equivalent to the number of events within the oscillation.

thalamic oscillations from slices in a submerged chamber: slices were placed on a raised grid (Etherington and Frenguelli, 2004; Wall and Richardson, 2015; reviewed in Frenguelli and Wall, 2016, Fig. 2B), so they were perfused from above and below; perfusion rates were high (5–6 ml⁻¹ for example see Oren et al., 2006; Hájos and Mody, 2009 on how this improves the health of submerged slices) and the tubing was gas-tight (Frenguelli and Wall, 2016). In initial experiments, recordings were made from slices from mice. However, it was found to be

technically difficult to reliably evoke oscillations and record purine release with biosensors simultaneously. In many recordings the biosensor had to be repositioned parallel to the axis of the nRT (see Fig. 2) in order to detect purine release. This was very difficult in the small slices from mice without losing recordings of the oscillations, as the biosensor was not pushed into the tissue but instead was bent so the sensing region was parallel to the slice surface. Thus, we moved to making recordings from larger rat slices which allowed more reliable

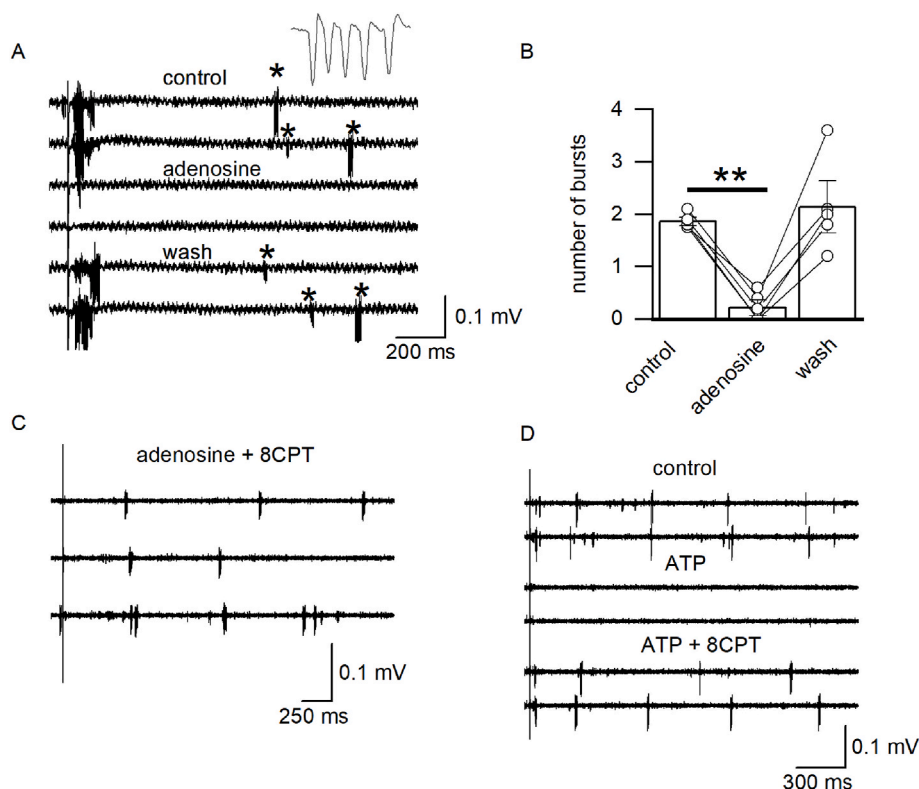


Fig. 3. Adenosine A₁ receptor activation dampens thalamic oscillations. A, Example extracellular traces from a single slice in control, following application of adenosine (100 μM) and in wash. Adenosine abolished the initial burst that immediately followed the stimulation and also abolished the subsequent oscillatory activity (there was recovery with wash). Inset, oscillation shown at an expanded time base to illustrate the characteristic waveform B, Summary of data from 5 slices, with each data point the mean number of events recorded in each slice. Adenosine significantly (Mann Whitney, U = 25, Z = 2.52, P = 0.01167) reduced the number of bursts (from 1.86 ± 0.08 to 0.22 ± 0.15 bursts) evoked following stimulation, an effect that was reversible upon wash (2.1 ± 0.5 bursts). C, The evoked oscillations persisted when adenosine (100 μM) was co-applied with the A₁ receptor antagonist 8-CPT (1 μM). D, Oscillations were blocked by ATP (50 μM) an effect which was reversed by blocking A₁ receptors with 8 CPT (1 μM).

recordings of oscillations and purine release. A single stimulus (1–4 V) delivered in the internal capsule (electrode positions displayed in Fig. 2A and B) under control conditions (aCSF with 1 mM Mg^{2+} and 3 mM Ca^{2+}) evoked activity in the thalamus (Fig. 2C and D). The amplitude of the activity was much smaller than reported from recordings using interface chambers, presumably due to current loss through the solution, but had a similar pattern to what has previously been reported (Ulrich and Huguenard, 1995).

3.3. Activation of adenosine A_1 receptors inhibits thalamic oscillations

The activity evoked by stimulation in the internal capsule typically consisted of a burst of activity immediately following the stimulus (which represents the activation of direct glutamatergic connections onto VB thalamic neurons, Fig. 3A, Huguenard and Prince, 1994) followed by 1–4 bursts of activity at a longer latency (Fig. 3A asterisks, mean number of bursts evoked 2.2 ± 0.2 , range 1–4 bursts, $n = 12$ slices). These oscillations are similar to the activity observed during sleep spindles and result from an interplay of RTN GABAergic connections to the VB removing T-type Ca^{2+} current inactivation from TC neurons, resulting in rebound TC neuron firing that reactivates the RTN GABAergic connections onto the VB (Huguenard and Prince, 1994).

Each burst consisted of a number of spike-like events (3–7 spikes per burst at a frequency of ~ 0.3 – 0.5 kHz, Fig. 3A inset) with the mean latency to the first burst of 621 ± 81.6 ms. Application of either adenosine (100 μM , $n = 5$ slices) or the selective A_1 receptor agonist CPA (50 nM, $n = 3$ slices, not illustrated) reversibly inhibited the stimulated activity (Fig. 3A and B): abolishing the activity immediately following the stimulus and also the later bursts of activity. This is consistent with previous data that showed that adenosine inhibits the glutamatergic transmission to both VB and NRT thalamic neurons and blocks epileptiform thalamic activity (Ulrich and Huguenard, 1995). This effect of adenosine could be prevented by pre-application of the adenosine A_1 receptor antagonist 8CPT at 1–2 μM (Fig. 3C). Although the reported K_i for 8CPT for A_1 receptors is 10.9 nM and is 1440 nM for A_2 receptors (Jacobson et al., 1989), higher concentrations are routinely used in brain slices (for example see Qi et al., 2017) compared to cell lines expressing recombinant receptors (Jacobson et al., 1989). Application of ATP (50–100 μM) also abolished activity consistent with its extracellular breakdown to adenosine ($n = 5$ slices, Fig. 3D). The effects of adenosine and ATP were reversed by application of an A_1 receptor antagonist (2 μM 8-CPT, Fig. 3D) together with the inhibitory effects of CPA show that the inhibition of oscillations was mainly via A_1 receptor activation.

Since oscillations were recorded in the VB thalamus, we investigated

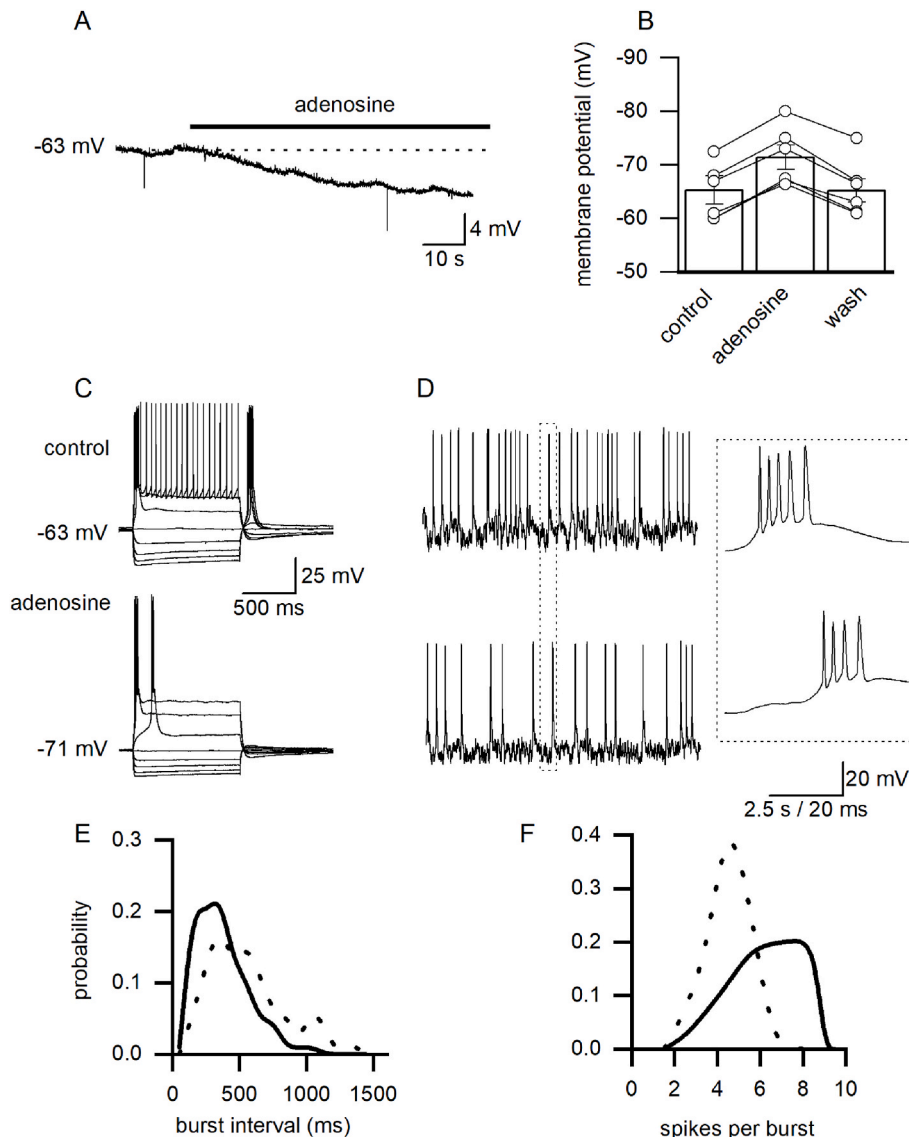


Fig. 4. Adenosine hyperpolarises TC neurons by activating adenosine A_1 receptors. A, Recording of the membrane potential from a TC neuron in the VB thalamus. The membrane potential was hyperpolarised by 100 μM adenosine from -63 to -74 mV. B, Graph summarising data from 6 slices. The membrane potential was significantly (Mann Whitney U = 34.5, Z = 2.57 P = 0.00649) hyperpolarised by adenosine (from -65.3 ± 2.7 mV to -71.4 ± 2.2 mV) which recovered upon wash (-65.2 ± 2.1 mV). C, Voltage traces in VB TC neuron in response to 50 pA current steps (from -200 pA to 150 pA). Adenosine reduced the spiking at positive steps and abolished rebound spiking following the hyperpolarising steps. D, Membrane potential traces in response to the injection of a naturalistic noisy current in control (top) and in 100 μM adenosine. The insets show the same burst in control and in the presence of adenosine illustrating the delay in firing and the reduction in the number of action potentials in the burst. E, Graph plotting the probability distribution of burst intervals in control (solid line) and in adenosine (dotted line) measured during naturalistic noisy current injection. The burst interval data is pooled from 6 neurons, is binned into 100 ms bins and the number within each bin divided by the total number of bursts to give the probability. The curve is shifted to the right in adenosine denoting an increase in burst interval. F, Graph plotting the probability of different numbers of spikes per burst. The spike number per burst is pooled from 6 neurons, is binned into 1 spike bins and the number within each bin divided by the total number of bursts to give the probability. The curve is shifted to the left in adenosine denoting a decrease in the number of spikes in each burst.

the effects of adenosine on the electrophysiological properties of VB thalamocortical neurons using current clamp recording. Application of adenosine (100 μ M, $n = 6$ slices) produced a hyperpolarisation of the membrane potential of TC neurons (Fig. 4A, membrane potential hyperpolarised from -65.7 ± 2.7 mV to -71.4 ± 2.2 mV, Fig. 4B) which was reversible upon wash (mean membrane potential on wash -65.6 ± 2 mV, Fig. 4B). The hyperpolarisation of the membrane potential lead to a fall in the rate of firing and a loss of rebound firing following the termination of hyperpolarising steps (Fig. 4C). To investigate the effects of adenosine on TC neuron firing under more realistic conditions, we injected a naturalistic noisy current into TC neurons and measured the firing rate (see methods). The firing induced by the fluctuating current consisted of discrete bursts (Fig. 4D, inset). Application of adenosine (100 μ M) significantly increased the interval (from 273 ± 45 ms to 446 ± 65 ms, $P = 0.001$ Fig. 4E) between the bursts and also significantly reduced the number of spikes per burst (from 4.8 ± 0.5 to 3.98 ± 0.3 spikes, $P = 0.0023$, Fig. 4F). Together with the inhibitory effects on synaptic transmission to nRT and VB neurons (Ulrich and Huguenard, 1995) this hyperpolarisation will contribute to the loss of thalamic oscillations.

3.4. Activation of A_1 receptors by endogenous adenosine dampens thalamic oscillations

To investigate whether the concentration of endogenous adenosine present in the slices was sufficient, to activate A_1 receptors and dampen oscillations, we applied the selective A_1 receptor antagonist 8CPT (2 μ M). Application of 8CPT significantly increased the number of bursts following the stimulation (Fig. 5A–C) without changing the properties of the bursts that were present before its application (Fig. 5A, D, E). The additional oscillations induced by blocking A_1 receptors occurred after the existing bursts; there was no significant change in the latency of the bursts which were present before 8CPT application (Fig. 5A, D). This suggests that removing the A_1 receptor tone allows the circuit to continue oscillating for an increased number of cycles. Application of 8CPT also increased the amplitude of the burst of activity that immediately followed the stimulus, suggesting a direct effect on the synapses onto VB thalamus neurons (Fig. 5F). In contrast, application of the A_{2a} receptor antagonist SCH 58261 (50 nM) had no significant effect on the oscillations ($n = 4$, not illustrated, $P = 0.568$) suggesting little role for A_{2a} receptors. We next investigated whether 8CPT changed the firing pattern of VB TC cells under quiescent conditions, to determine if the basal level of adenosine in the slice was sufficient to activate A_1 receptors and hyperpolarise the membrane potential. There was no significant change in either the membrane potential (-67.9 ± 2.3 vs -67.8 ± 2.2 mV) or the firing rate of TC neurons (mean burst interval in naturalistic current injection 255 ± 58 vs 245 ± 54 ms) following the application of 8 CPT ($n = 6$, Fig. 5G, $P = 0.67$ and $P = 0.187$ Mann Whitney) suggesting that the concentration of adenosine in the tissue is low when there is no activity. This is the first study to show that an adenosine A_1 receptor tone is present in the in vitro thalamus and is sufficient to modulate the number of oscillations evoked by each stimulus.

3.5. Adenosine cannot be detected by biosensors during thalamic oscillations under physiological conditions

The effects of the A_1 receptor antagonist 8CPT on thalamic oscillations and the lack of effect of 8CPT on TC neurons in quiescent conditions suggests that adenosine release could occur during thalamic activity. To directly detect this adenosine release, we used adenosine microelectrode biosensors (Llaudet et al., 2003). In initial studies we inserted the biosensor and the null sensor into the thalamus (as in Wall and Dale, 2013 and see Frenguelli and Wall, 2016). However, we found that this often led to a loss of oscillations (recorded on an extracellular electrode) and thus instead we bent the sensors so that the sensing area

of the sensors (null and biosensor) was parallel to the slice surface. The sensors were then positioned close to the slice surface and therefore there was no damage to the tissue. This recording configuration has previously been used to measure activity dependent adenosine release in cerebellar slices (for example see Wall et al., 2007; Klyuch et al., 2012). Oscillations were first recorded via an extracellular electrode placed in the VB thalamus, to determine where in the slice the neural circuit was complete. The biosensor and null sensor were then placed as close to the extracellular electrode as possible (~ 100 μ m, always slightly closer to the stimulating electrode). This ensured that network activity was occurring in the area close to the position of the biosensor and the null sensor. This is important as the concentration of adenosine measured from a release site falls markedly with distance due to dilution (Wall and Richardson, 2015). In some experiments the stimulation artefacts could be observed on the biosensor current trace, showing that the biosensor was positioned very close to the slice surface (Fig. 6A). In 8 slices (from 5 rats) there was no significant difference between the signal on the adenosine biosensor and on the null sensor (Fig. 6A, subtraction of the null signal from the biosensor signal did not reveal an increase in baseline current). This does not necessarily mean that no adenosine is released during activity, but it could be that the concentration is below the level of detection by the biosensor. A similar inability to directly measure adenosine release with biosensors has been reported in the neocortex with an excitant solution, although 8CPT significantly enhanced activity suggesting adenosine release is occurring (Wall and Richardson, 2015).

3.6. Endogenous A_1 receptor activation modulates epileptiform oscillatory activity

We next investigated the role of adenosine A_1 receptors and adenosine release in epileptiform oscillations. Hypersynchronous epileptiform activity can be induced by reducing GABAergic inhibition coupled with a reduction in Mg^{2+} concentration to enhance NMDA receptor activity (Ulrich and Huguenard, 1995). Perfusion of slices with aCSF containing a reduced concentration of Mg^{2+} together with a GABA_A receptor antagonist induced significantly ($p = 1.77114 \times 10^{-6}$) more oscillatory bursts evoked by stimulation (11.7 ± 1.44 vs 2.23 ± 0.2 , $n = 15$ and $n = 12$) compared to control conditions and also significantly ($P = 0.018328$) shortened the latency to the first burst (438 ± 16 vs 621 ± 81.6 ms, Fig. 7A). Under epileptiform conditions there were often spontaneous events, which occurred without the requirement for a stimulus (Fig. 7B). Both the evoked (Fig. 7A) and spontaneous oscillations (Fig. 7B) could either be abolished or greatly reduced in frequency and amplitude by ethosuximide (500 μ M, $n = 4$), a drug used to treat absence epilepsy (Brown et al., 1975; Malafosse et al., 1994; McNamara, 1995). Application of either adenosine (100 μ M, $n = 6$) or CPA (50 nM, $n = 3$) either abolished or greatly reduced the number of bursts per stimulus (Fig. 7C and D) and also significantly reduced the frequency of spontaneous activity (for adenosine $U = 36$, $Z = 2.8$, $P = 0.0021$). Antagonism of A_1 receptors (with 2 μ M CPT) significantly increased the number of bursts evoked per stimulus (Fig. 7E and F) without changing the latency to the first burst (Fig. 7G). Application of 8CPT (2 μ M) also significantly reduced the interval between spontaneous bursts of activity (Fig. 7H, $U = 16$, $Z = 2.2$, $P = 0.025$). These effects of 8CPT are consistent with the presence of sufficient concentrations of endogenous adenosine to activate A_1 receptors and dampen oscillations. However, recordings from TC neurons in aCSF containing low Mg^{2+} and with a GABA_A receptor antagonist showed no consistent effect of 8CPT on either membrane potential (-67.8 ± 3 mV vs -65.6 ± 5 mV $n = 6$) or the firing rate (Fig. 7I) suggesting that there is little adenosine A_1 receptor tone in the tissue under quiescent conditions.

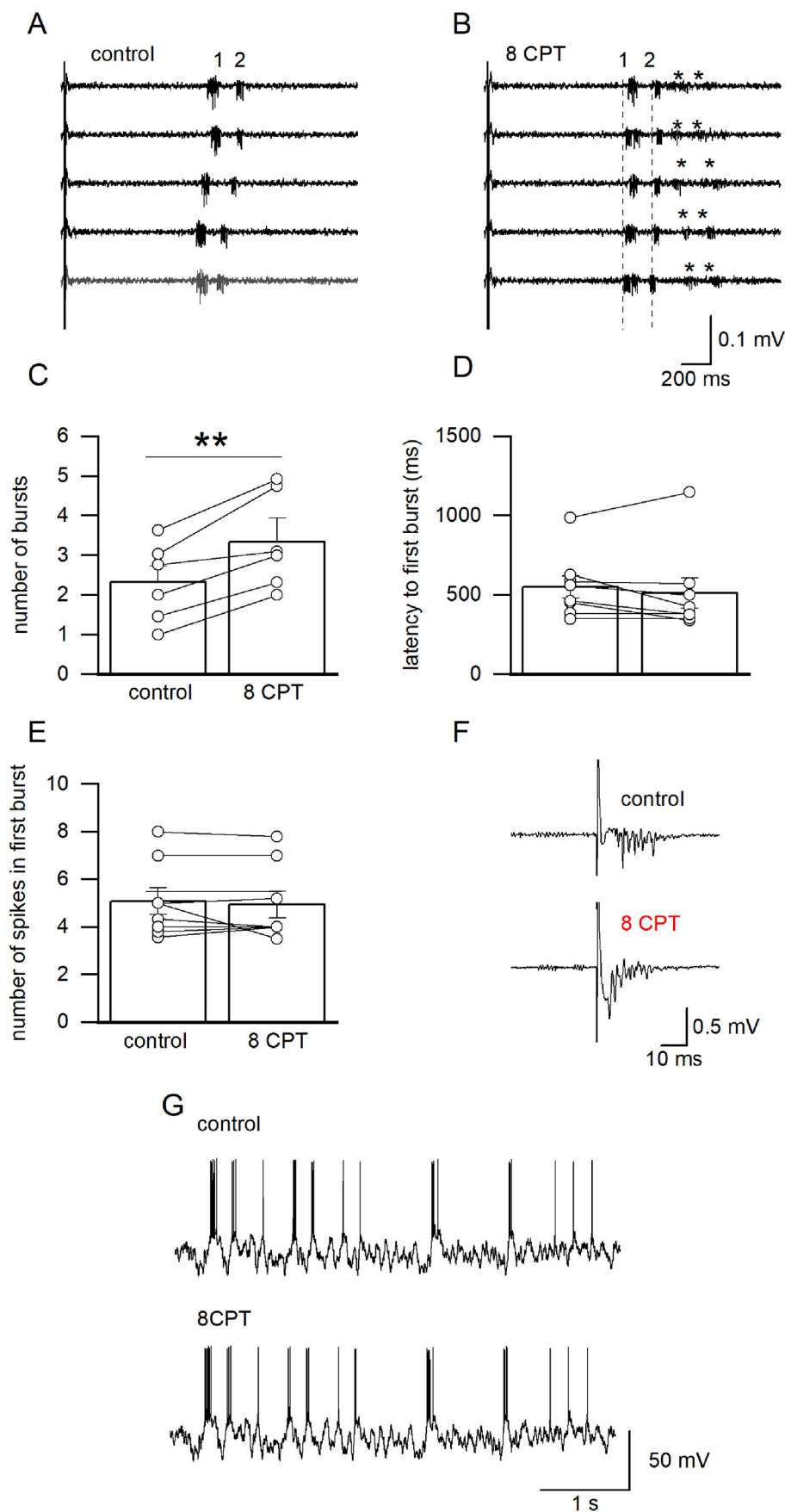


Fig. 5. The number of oscillations is increased by the block of endogenous A_1 receptor activation. A, Examples of successive traces of thalamic oscillations, with each stimulation producing 2 bursts (labelled 1 and 2). B, Successive traces from the same recording as (A) in the presence of the A_1 receptor antagonist 8CPT (2 μ M). The antagonism of A_1 receptors increased the number of bursts (from 2 to 4, shown by *) but had no effect on the latency to the initial bursts (dotted lines are the mean latency of the first and second burst in control) compared to control. C, Graph summarising data from 8 slices with the mean number of bursts per stimulation significantly (Mann Whitney U = 11, Z = -2.56 P = 0.0075) increased by application of 8CPT (from 2.33 ± 0.4 to 3.34 ± 0.6). D, Graph summarising data on the latency to first oscillatory burst in control and in 8CPT. Block of A_1 receptors had no significant effect (Mann Whitney U = 42, Z = 0.9977, P = 0.328) on the latency to the first burst (551 ± 70 ms to 512 ± 94 ms). E, Graph summarising data on the number of spikes in the first oscillatory burst in control and in 8CPT. Block of A_1 receptors had no significant effect (Mann Whitney U = 34.5, Z = 0.213, P = 0.816) on the spike number in the first burst (5.1 ± 0.56 vs 4.94 ± 0.56). F, Examples of the first burst of activity immediately following the stimulus in control and in 8CPT from the same slice. There was a significant increase in the area of this initial burst in 8 CPT (Mann Whitney U = 0, Z = -3.3, P = 1.55×10^{-4}). G, Membrane potential traces in response to the injection of a naturalistic noisy current in control (top) and in 8 CPT (2 μ M) showing very similar sub threshold and firing activity.

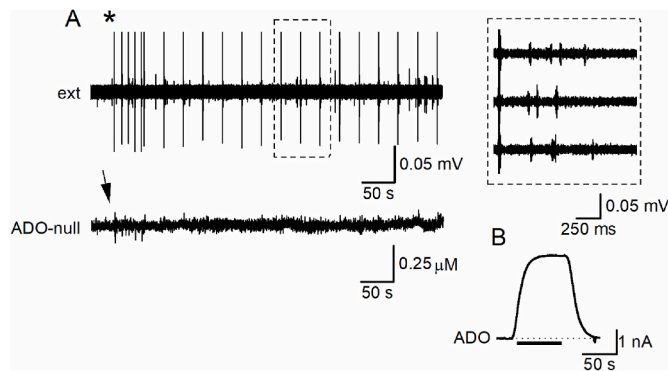


Fig. 6. Adenosine release is not directly detected during non-epileptiform oscillations. A, Trace from extracellular electrode placed in VB thalamus (ext) and from an adenosine biosensor (Ado) trace with the trace from the null sensor subtracted (ADO-null). The large upward and downward deflections on the ext trace are the stimulations. Inset, the region denoted by the box at a higher gain and with an expanded time base to illustrate that each stimulation produces 3–4 bursts of oscillatory activity. The biosensor trace shows no change in the baseline current during stimulation, indicating no detection of purine release. Stimulation of oscillations starts at the (*) initially at an interval of 10 s and are picked up by the biosensor (arrow) and then are continued at an interval of 20 s. B, The calibration trace for the biosensor. Adenosine (10 μ M) produced a current of 3.4 nA thus a limit of detection (for ~ 20 pA current) was ~ 60 nM.

3.7. Adenosine can be detected with biosensors during epileptiform activity

Adenosine biosensors placed in close apposition to the surface of the VB thalamus could detect purine release during epileptiform thalamic activity in 10 out of 15 slices (in 5 there was no difference between null and biosensor). There was a positive shift in the adenosine biosensor baseline with no equivalent shift in the baseline of the null sensor (Fig. 8A and B). The biosensor waveform was not smooth but often consisted of a number of peaks (arrows on Fig. 8A) suggesting the biosensor is detecting multiple release events possibly from different sources. The concentration of purines detected ranged from 0.2 μ M to 1.5 μ M with a mean of 0.69 ± 0.16 μ M ($n = 10$). The biosensor currents had a slow rise-time (time to peak ~ 30 –60 s, mean 42.3 ± 5 s) and appeared to result from the slow accumulation of extracellular purines, as there was no clear association between individual stimulations and the biosensor current (Fig. 8A). However, when stimulation was stopped the biosensor current returned to baseline (Fig. 8B, D). The biosensor current was abolished if slices were incubated in the A_1 receptor agonist CPA (50 nM, Fig. 8C, $n = 3$). CPA is not broken down to adenosine and thus is not detected by the biosensor. This demonstrates that the increase in extracellular purine concentration is not just a consequence of the electrical stimulus but requires neural activity. The concentration of adenosine in the extracellular space can be increased by a number of mechanisms that include the release of ATP (via exocytosis or hemichannels) which is subsequently metabolised to adenosine. To investigate whether this was the case for the release during thalamic oscillations, we used ATP biosensors to detect ATP release. In 6 slices, although there was a clear signal on the adenosine biosensor, no current was measured on the ATP biosensor (Fig. 8D). The adenosine biosensor current increased during stimulation and returned to baseline when the stimulation was stopped (Fig. 8B, D). This was the case even when spontaneous activity continued after the stimulation had stopped. To investigate whether adenosine could be detected in response to the oscillations without electrical stimulation, we recorded without stimulation. There was no clear biosensor current unless 8CPT was applied to block A_1 receptors. This markedly enhanced spontaneous activity (as noted above) and also induced a current on the adenosine biosensor ($n = 3$, Fig. 8E). This suggests that oscillations alone, as long as they occur

at a sufficiently high enough frequency, can induce detectable purine release.

In conclusion

Our data is consistent with adenosine release during oscillations that feeds back to suppress oscillations via the activation of A_1 receptors.

4. Discussion

As far as we are aware, this is the first measurement of thalamic oscillations using a submerged chamber and the first direct measurement of adenosine release in the thalamus during oscillations. A submerged chamber was required for these experiments to carry out the biosensor measurements, since the biosensor sensing area has to be fully submerged in order to function (Llaudet et al., 2003). We have taken several approaches to ensure the health of the slices with fast perfusion rates, gas tight tubing and placing the slice on a grid, so the slice was perfused from above and below. The lack of adenosine A_1 receptor tone in quiescent conditions verifies that the slices are not hypoxic. One of the difficulties encountered was the small amplitude of the oscillatory activity (often less than 0.1 mV compared to much larger events recorded in interface chambers) because of the current loss in the submerged chamber. However, by minimising electrical noise, the oscillations could be reliably detected.

Role of adenosine A_1 receptors in thalamic oscillations

We have found that application of either adenosine or selective A_1 receptor agonists abolished both spindle and epileptiform oscillations (as previously reported by Ulrich and Huguenard, 1995 for epileptiform oscillations). Previous studies have shown that adenosine A_1 receptors inhibit GABAergic and glutamatergic synaptic transmission on to both nRT and VB thalamocortical neurons (Ulrich and Huguenard, 1995). We have also shown that adenosine directly hyperpolarises VB TC neurons and reduces their firing rate. In response to a naturalistic current injection, adenosine increases the interval between bursts of action potentials and also decreases the number of action potentials fired in each burst.

The effect of adenosine A_1 receptor agonists on the thalamic oscillations is consistent with the dense A_1 receptor expression in both the nRT and VB thalamus. The A_1 receptors appear co-localised with the glutamate transporter VGLUT-1 but not with VGLUT-2. In the thalamus VGLUT1 appears to be mainly expressed at synapses derived from cortical afferents (Freneau et al., 2001). One possible caveat to interpreting this data is the immunohistochemistry was carried out using slices from mice and the physiology was carried out in slices from both rats and mice. We used rat slices as they allowed greater success in simultaneously recording purine release with a biosensor and recording oscillations. The small size of mouse slices made it technically difficult to position and reposition the biosensor and maintain oscillation recordings. In slices from mice we observed similar effects of adenosine and adenosine receptor antagonists to that observed in rat slices (SFig. 3) consistent with a similar A_1 receptor expression pattern.

Under control conditions, block of A_1 receptors increased the number of oscillatory bursts evoked by each stimulus, without affecting the properties of the existing bursts before antagonist application. Blocking of A_1 receptors also enhanced the initial activity immediately following the stimulus, consistent with the removal of A_1 receptor inhibition of synaptic transmission on to VB neurons (Ulrich and Huguenard, 1995). How does the removal of A_1 receptor activation increase the number of oscillations following stimulation? There are at least 3 possible mechanisms that may either occur independently or together. Firstly, the marked increase in the initial excitation onto VB neurons (see Fig. 3F) could in itself lead to a greater number of oscillations. This mechanism alone seems unlikely to account for the observations, as the number of

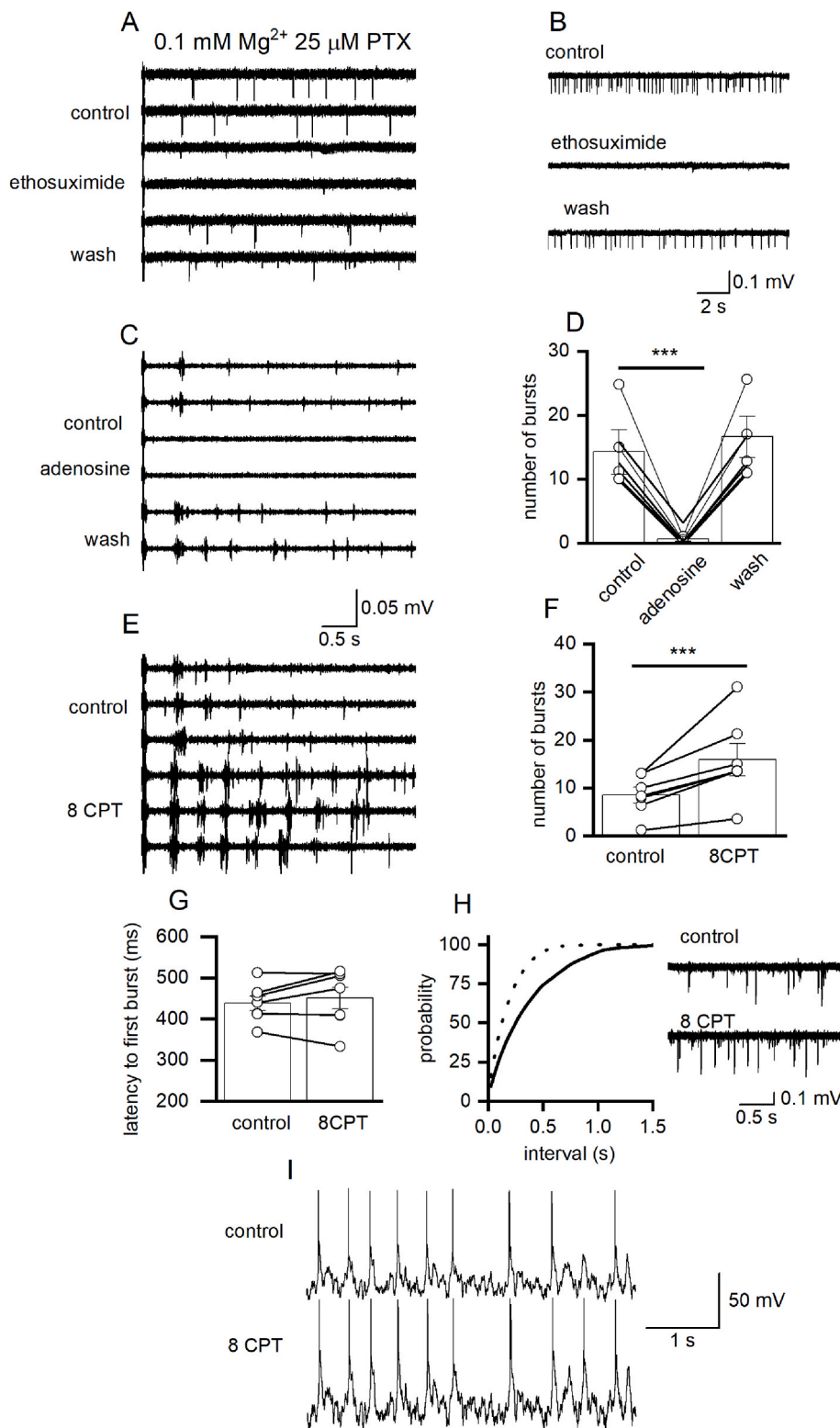


Fig. 7. Epileptiform thalamic oscillations are modulated by A_1 receptors. **A**, examples of successive traces of epileptiform thalamic oscillations (induced in 0.1 mM Mg^{2+} and 25 μM PTX) with each stimulation producing multiple bursts of activity. Application of the AED (anti-epileptic drug) ethosuximide (500 μM) reversibly abolished the oscillations. **B**, Spontaneous activity (there was no stimulation) recorded in 0.1 mM Mg^{2+} and 25 μM PTX. The activity was reversibly abolished by ethosuximide (500 μM). **C**, examples of successive traces of epileptiform thalamic oscillations. Application of adenosine (100 μM) reversibly abolished the oscillations. **D**, Graph summarising data on the number of bursts following each stimulation in control, adenosine and wash for 6 slices. Adenosine significantly (Mann Whitney $U = 36$, $Z = 2.82$, $P = 0.00477$) reduced the number of bursts evoked by each stimulus (from 14.25 ± 3.5 to 0.62 ± 0.38) an effect which recovered in wash (16.6 ± 3.3). The data points are the mean number of bursts evoked in each slice. **E**, Examples of successive traces of epileptiform thalamic oscillations in control and after application of 8CPT (2 μM), which markedly increased the number of bursts per stimulation. **F**, Graph summarising data on the number of bursts following each stimulation in control and after A_1 receptors were blocked with 8CPT in 8 slices. The number of bursts was significantly ($p = 0.001408$) increased (from 8.5 ± 1.7 to 15.9 ± 3.4). **G**, Graph summarising data on the latency to the first burst in control and in 8CPT. Block of A_1 receptors had no significant effect (Mann Whitney $U = 28$, $Z = -0.36$, $P = 0.713$) on the latency to the first burst in epileptiform activity (438 ± 17.4 ms to 451 ± 26.1 ms). **H**, Cumulative probability plot on interval between spontaneous bursts in control (solid line) and in 8CPT (dotted line).

oscillations did not scale with stimulus strength, appearing to be all or none. Secondly, adenosine released by the activity induced by each stimulation activates A_1 receptors. Blocking this A_1 receptor activation enhances synaptic transmission and also moves the neuronal membrane potential of thalamic neurons closer to threshold, thus allowing the oscillations to persist longer. Finally, removal of A_1 receptor activation could induce oscillations in neurons that were either below threshold or did not receive enough synaptic excitation/inhibition and thus were

quiescent before receptor antagonism. This is almost certainly happening, as we found that in some slices the extracellular electrode could be moved to areas of the VB where there was previously no activity, but it was present after 8CPT application. However, there was no clear increase in the amplitude of the recorded oscillations (for example see Fig. 3A and B) which maybe expected if more neurons were recruited.

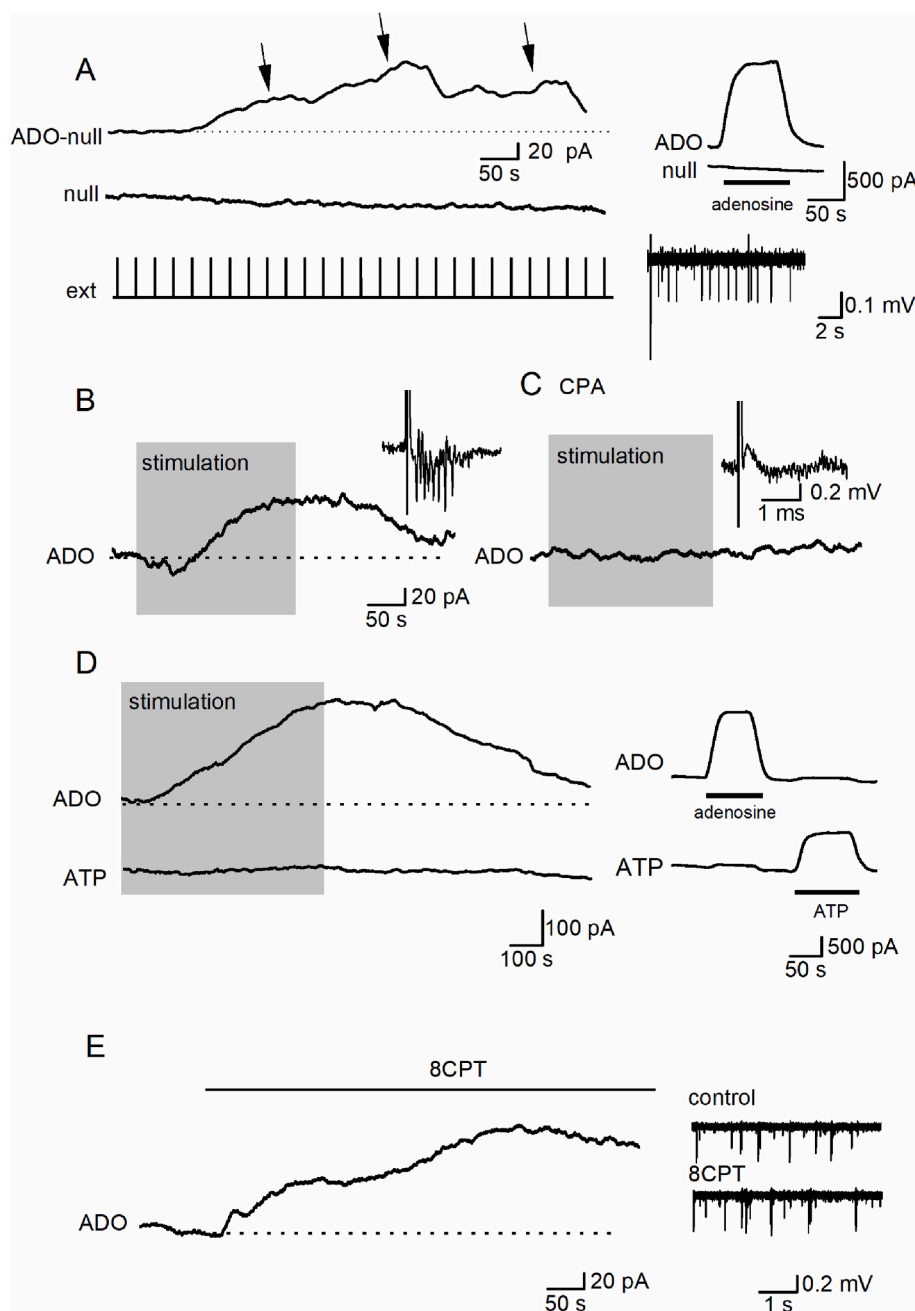


Fig. 8. Direct detection of adenosine release during epileptiform thalamic oscillation activity A, Example biosensor traces (ADO-null and null) during thalamic oscillations (0.1 mM Mg^{2+} and 25 μM PTX), stimulation was started at the beginning of the trace (stimulation every 10 s, marked on ext trace). There is a clear increase in current amplitude measured by the ADO biosensor with no shift in the baseline current on the null sensor, showing the detection of purines. The inset shows the calibration (10 μM adenosine) for the ADO biosensor and the null sensor and an example of oscillations following an individual stimulation. B, ADO biosensor trace with stimulation (every 10s) of thalamic oscillations occurring within the period denoted by the grey box. The current on the ADO biosensor rises during stimulation and then falls back to baseline when the stimulation is stopped. The inset shows the initial burst produced by the stimulation (*). C, ADO biosensor trace (from same slice as B) with stimulation of thalamic oscillations occurring within the period denoted by the grey box in the presence of the A_1 receptor agonist CPA (50 nM). There is no increase in current on the ADO biosensor. The inset shows that the initial burst of activity is blocked. D, ADO biosensor trace from an experiment in which there is spontaneous thalamic oscillations. Block of A_1 receptors (8CPT) led to a current on the biosensor. The inset shows the spontaneous activity in control and in 8CPT.

Properties of purine release during epileptiform oscillations

Epileptiform oscillations were induced using aCSF that had 0.1 mM Mg^{2+} and picrotoxin (25 μM) to partially block GABA_A receptors. Under these conditions, there were many more oscillations induced by each stimulus and they could occur spontaneously independent of stimulation. These oscillations could be inhibited by the anti-seizure drug ethosuximide which acts by inhibiting the T-type calcium channel, required for thalamic neuron rebound firing following GABAergic hyperpolarisation (Coulter et al., 1989). Purine release (adenosine and/or metabolites) could only be detected during epileptiform activity and not during the thalamic oscillations recorded under physiological conditions. This does not mean that no adenosine (or other purines) are released during physiological oscillations but instead the concentration of adenosine (and its metabolites) in the area around the biosensor maybe below the limit of detection. It is clear that A_1 receptors are

activated, since blocking A_1 receptors with an antagonist enhances oscillations. Adenosine A_1 receptors have a very high affinity for adenosine (Dunwiddie and Diao, 1994) and thus low concentrations of adenosine would be sufficient to activate the receptors. We can estimate a limit of biosensor detection: a minimal measurable biosensor current is ~ 20 pA, which with a biosensor sensitivity of 2500 pA for 10 μM adenosine, would be equivalent to ~ 0.1 μM purines. The concentration of purines measured during epileptiform oscillations was around 0.7 μM (equivalent to a biosensor current of ~ 100 pA). The rise-time of the biosensor currents was very slow (30–50 s to reach a peak) and individual oscillations did not induce detectable rises in biosensor current. The slow rise is not a consequence of the biosensor response time as we have previously shown that fast application of analytes can induce sub-second biosensor response times (Wall and Richardson, 2015). It is not a consequence of the biosensor being placed on the surface of the slice per se, since biosensor currents recorded in the cerebellum had a rise time in

the range of 10–15 s (Wall et al., 2007). One possibility is that the slow rise is a consequence of the release process itself which may have slow kinetics alternatively the slow rise time may be a consequence of the diffusion distance between the release sites and the biosensor. Using a diffusion simulation for purines, Wall and Richardson (2015) showed that if the biosensor was $\sim 100\ \mu\text{M}$ from the purine release source, the rise time of the biosensor signal would be predicted to be $\sim 20\ \text{s}$ with only $\sim 2\%$ of the purine released being detected by the biosensor. This filtering of the biosensor waveform would lead to the inability to detect currents associated with individual stimuli. If we extrapolate backwards, we can suggest that at the release sites the concentration of purines would be $\sim 35\ \mu\text{M}$. It is possible that the release sites are deep within the slice (slice is $400\ \mu\text{M}$ thick) or the release does not occur in VB. In some experiments we moved the biosensor into the nRT but did not detect any increases in purine release. In our experiments we have not used differential measurements (with ADO and INO biosensors) and thus the composition of the purines detected is not known. Since block of A_1 receptors enhances oscillations, it is clear that adenosine is released. However, it may be adenosine metabolites (inosine and hypoxanthine) that are actually detected by the biosensor as the adenosine is rapidly metabolised.

Role of adenosine in thalamic oscillations and spike wave discharges

Our data shows that thalamocortical circuitry can be modulated by local release of adenosine. Although we could not directly detect ATP release, there is strong evidence that astrocytes can release ATP, which is then degraded to adenosine to act on neural adenosine A_1 receptors to regulate activity (reviewed in Halassa, 2011). Genetic inhibition of astrocytic ATP release revealed that astrocytes modulate sleep rhythmogenesis, sleep behavior, and sleep-related cognitive functioning.

In pathological states, both our data and the data from Ulrich and Huguenard (1995) suggest that activation of A_1 receptors in the thalamus would be expected to suppress absence seizures, if they arise from thalamocortical oscillations. However, data from previous studies using rodent models of epilepsy have shown that activation of A_1 receptors can enhance the frequency of spike and wave discharges (SWDs). For example, Ilbay et al. (2001) have shown that i.p. injection of adenosine enhances the frequency of SWDs in WAG/Rij rats. However, the changes in SWD frequency were associated with large changes in blood pressure and body temperature and occurred with large concentrations of adenosine (30–120 mg/kg of adenosine). In the GAERS model, activating A_1 receptors with i.p. injection of specific agonists enhanced SWD frequency whereas A_1 receptor antagonists reduced SWD frequency (Germé et al., 2015). There has been no study where adenosine or A_1 receptor agonists have been directly infused into the thalamus and thus the contrast in effects may result from peripheral effects or effects in brain regions other than the thalamus.

CRedit authorship contribution statement

Mark J. Wall: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing – original draft, Writing – review & editing, Funding acquisition, Investigation. **Katie Puddefoot:** Investigation. **Wencheng Yin:** Conceptualization, Formal analysis. **Chris Bingham:** Conceptualization, Formal analysis. **Mohsen Seifi:** Investigation. **Jerome D. Swinny:** Data curation, Methodology, Writing – original draft, Writing – review & editing, Investigation. **Richard Teke Ngomba:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing – original draft, Writing – review & editing, Funding acquisition, Investigation.

Acknowledgements

Work was perceived by MJW and RTN. Physiology experiments were performed by MJW, RTN and KP, with immunohistochemistry

experiments carried out by JDS and MS. Physiology data was analysed by RTN, MJW, WY and CB. Paper was written by MJW and RTN. KP was an MIBTP funded PhD student. **The work was funded by an ERUK pilot grant (PGE 1720) to MJW and RTN.** The authors would like to thank Prof. Tom E. Salt (emeritus University College London) for reading an earlier version of the manuscript and for expert scientific comments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuropharm.2022.109172>.

References

- Ates, N., Sahin, D., Ilbay, G., 2004 Oct. Theophylline, a methylxanthine derivative, suppresses absence epileptic seizures in WAG/Rij rats. *Epilepsy Behav.* 5 (5), 645–648. <https://doi.org/10.1016/j.yebeh.2004.06.001>. PMID: 15380114.
- Badel, L., Lefort, S., Brette, Petersen, C.C.H., Gerstner, W., Richardson, M.J.E., 2008. Dynamic I-V curves are reliable predictors of naturalistic pyramidal-neuron voltage traces. *J. Neurophysiol.* 99, 656–666.
- Boison, D., 2008 Feb. Adenosine as a neuromodulator in neurological diseases. *Curr. Opin. Pharmacol.* 8 (1), 2–7. <https://doi.org/10.1016/j.coph.2007.09.002>. Epub 2007 Oct 17. PMID: 17942368; PMCID: PMC2950121.
- Blumenfeld, H., 2005. Cellular and network mechanisms of spike-wave seizures. *Epilepsia* 46 (Suppl. 412 9), 21–33. <https://doi.org/10.1111/j.1528-1167.2005.00311.x>. PMID: 16302873.
- Brown, T.R., Dreifuss, F.E., Dyken, P.R., Goode, D.J., Penry, J.R., Porter, R.J., White, B.G., White, P.T., 1975. Ethosuximide in the treatment of absence (petit mal) seizures. *Neurology* 25, 515–524.
- Borea, P.A., Gessi, S., Merighi, S., Vincenzi, F., Varani, K., 2018 Jul 1. Pharmacology of adenosine receptors: the state of the art. *Physiol. Rev.* 98 (3), 1591–1625. <https://doi.org/10.1152/physrev.00049.2017>. PMID: 29848236.
- Buzsáki, G., 2006. *Rhythms of the Brain*. Oxford UP, Oxford; New York.
- Coulter, D.A., Huguenard, J.R., Prince, D.A., 1989 Jun. Characterization of ethosuximide reduction of low-threshold calcium current in thalamic neurons. *Ann. Neurol.* 25 (6), 582–593. <https://doi.org/10.1002/ana.410250610>. PMID: 2545161.
- Crunelli, V., Leresche, N., 2002 May. Childhood absence epilepsy: genes, channels, neurons and networks. *Nat. Rev. Neurosci.* 3 (5), 371–382. <https://doi.org/10.1038/nrn811>. PMID: 11988776.
- Diamond, M.E., von Heimendahl, M., Knutsen, P.M., Kleinfeld, D., Ahissar, E., 2008 Aug. 'Where' and 'what' in the whisker sensorimotor system. *Nat. Rev. Neurosci.* 9 (8), 601–612. <https://doi.org/10.1038/nrn2411>. Erratum in: *Nat. Rev. Neurosci.* 2008 Sep;9(9):709. PMID: 18641667.
- Dunwiddie, T.V., Diao, L., 1994 Feb. Extracellular adenosine concentrations in hippocampal brain slices and the tonic inhibitory modulation of evoked excitatory responses. *J. Pharmacol. Exp. Therapeut.* 268 (2), 537–545. PMID: 8113965.
- Dunwiddie, T.V., Masino, S.A., 2001. The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* 24, 31–55. <https://doi.org/10.1146/annurev.neuro.24.1.31>. PMID: 11283304.
- During, M.J., Spencer, D.D., 1992 Nov. Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Ann. Neurol.* 32 (5), 618–624. <https://doi.org/10.1002/ana.410320504>. PMID: 1449242.
- Etherington, L.A., Frenguelli, B.G., 2004 May. Endogenous adenosine modulates epileptiform activity in rat hippocampus in a receptor subtype-dependent manner. *Eur. J. Neurosci.* 19 (9), 2539–2550. <https://doi.org/10.1111/j.0953-816X.2004.03355.x>. PMID: 15128407.
- Fredholm, B.B., Arslan, G., Halldner, L., Kull, B., Schulte, G., Wasserman, W., 2000 Nov. Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 362 (4–5), 364–374. <https://doi.org/10.1007/s002100000313>. PMID: 11111830.
- Freneau Jr., R.T., Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Bellocchio, E.E., Fortin, D., Storm-Mathisen, J., Edwards, R.H., 2001 Aug 2. The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron* 31 (2), 247–260. [https://doi.org/10.1016/s0896-6273\(01\)00344-0](https://doi.org/10.1016/s0896-6273(01)00344-0). PMID: 11502256.
- Frenguelli, B.G., Wall, M.J., 2016 Feb 15. Combined electrophysiological and biosensor approaches to study purinergic regulation of epileptiform activity in cortical tissue. *J. Neurosci. Methods* 260, 202–214. <https://doi.org/10.1016/j.jneumeth.2015.09.011>. Epub 2015 Sep 14. PMID: 26381061.
- Germé, K., Faure, J.B., Koning, E., Nehlig, A., 2015 Feb. Effect of caffeine and adenosine receptor ligands on the expression of spike-and-wave discharges in Genetic Absence Epilepsy Rats from Strasbourg (GAERS). *Epilepsy Res.* 110, 105–114. <https://doi.org/10.1016/j.eplepsyres.2014.11.022>. Epub 2014 Dec 9. PMID: 25616462.
- Halassa, M.M., 2011 Apr. Thalamocortical dynamics of sleep: roles of purinergic neuromodulation. *Semin. Cell Dev. Biol.* 22 (2), 245–251. <https://doi.org/10.1016/j.semcdb.2011.02.008>. Epub 2011 Feb 15. PMID: 21329763; PMCID: PMC3070804.
- Hajos, N., Mody, I., 2009 Oct 15. Establishing a physiological environment for visualized in vitro brain slice recordings by increasing oxygen supply and modifying aCSF content. *J. Neurosci. Methods* 183 (2), 107–113. <https://doi.org/10.1016/j.jneumeth.2009.06.005>. Epub 2009 Jun 12. PMID: 19524611; PMCID: PMC2753642.

- Harrison, P.M., Badel, L., Wall, M.J., Richardson, M.J.E., 2015. Experimentally verified parameter sets for modelling heterogeneous neocortical pyramidal cell populations. *PLoS Comput. Biol.* 11, e1004165.
- Hill, E., Karikari, T.K., Moffat, K.G., Richardson, M.J.E., Wall, M.J., 2019 Oct 15. Introduction of Tau oligomers into cortical neurons alters action potential dynamics and disrupts synaptic transmission and plasticity. *ENEURO*.0166-19.2019 eNeuro 6 (5). <https://doi.org/10.1523/ENEURO.0166-19.2019>. PMID: 31554666; PMCID: PMC6794083.
- Huguenard, J.R., McCormick, D.A., 2007 Jul. Thalamic synchrony and dynamic regulation of global forebrain oscillations. *Trends Neurosci.* 30 (7), 350–356. <https://doi.org/10.1016/j.tins.2007.05.007>. Epub 2007 Jun 4. PMID: 17544519.
- Huguenard, J.R., Prince, D.A., 1994 Sep. Intrathalamic rhythmicity studied in vitro: nominal T-current modulation causes robust antioscillatory effects. *J. Neurosci.* 14 (9), 5485–5502. <https://doi.org/10.1523/JNEUROSCI.14-09-05485.1994>. PMID: 8083749; PMCID: PMC6577071.
- Ilbay, G., Sahin, D., Karson, A., Ates, N., 2001 Aug. Effects of adenosine administration on spike-wave discharge frequency in genetically epileptic rats. *Clin. Exp. Pharmacol. Physiol.* 28 (8), 643–646. <https://doi.org/10.1046/j.1440-1681.2001.03499.x>. PMID: 11473530.
- Jackson, T., Seiff, M., Górecki, D.C., Swinny, J.D., 2021 Jun. Specific dystrophins selectively associate with inhibitory and excitatory synapses of the mouse cerebellum and their loss alters expression of P2X7 purinoceptors and pro-inflammatory mediators. *Cell. Mol. Neurobiol.* 8 <https://doi.org/10.1007/s10571-021-01110-6>. Epub ahead of print. PMID: 34101068.
- Jacobson, K.A., Kiriasis, L., Barone, S., Bradbury, B.J., Kammula, U., Campagne, J.M., Secunda, S., Daly, J.W., Neumeyer, J.L., Pfeleiderer, W., 1989 Aug. Sulfur-containing 1,3-dialkylxanthine derivatives as selective antagonists at A1-adenosine receptors. *J. Med. Chem.* 32 (8), 1873–1879. <https://doi.org/10.1021/jm00128a031>. PMID: 2754711; PMCID: PMC3479653.
- Jones, E.G., 2009 Mar. Synchrony in the interconnected circuitry of the thalamus and cerebral cortex. *Ann. N. Y. Acad. Sci.* 1157, 10–23. <https://doi.org/10.1111/j.1749-6632.2009.04534.x>. PMID: 19351352.
- Kaufmann, T., Harrison, P., Richardson, M., Pinheiro, T., Wall, M., 2016. Intracellular soluble α -synuclein oligomers reduce pyramidal cell excitability. *J. Physiol.* 594 (10), 2751–2772.
- Kleiman-Weiner, M., Beenhakker, M.P., Segal, W.A., Huguenard, J.R., 2009 Jul. Synergistic roles of GABA_A receptors and SK channels in regulating thalamocortical oscillations. *J. Neurophysiol.* 102 (1), 203–213. <https://doi.org/10.1152/jn.91158.2008>. Epub 2009 Apr 22. PMID: 19386752; PMCID: PMC2712277.
- Kovács, Z., D'Agostino, D.P., Dobolyi, A., Ari, C., 2017 Jul 25. Adenosine A1 receptor antagonism abolished the anti-seizure effects of exogenous ketone supplementation in Wistar Albino Glaxo Rijswijk rats. *Front. Mol. Neurosci.* 10, 235. <https://doi.org/10.3389/fnmol.2017.00235>. PMID: 28790891; PMCID: PMC5524776.
- Klyuch, B.P., Dale, N., Wall, M.J., 2012a. Receptor-mediated modulation of activity-dependent adenosine release in rat cerebellum. *Neuropharmacology* 62 (2), 815–824. <https://doi.org/10.1016/j.neuropharm.2011.09.007>, 2012 Feb.
- Klyuch, B.P., Dale, N., Wall, M.J., 2012 Mar 14. Deletion of ecto-5'-nucleotidase (CD73) reveals direct action potential-dependent adenosine release. *J. Neurosci.* 32 (11), 3842–3847. <https://doi.org/10.1523/JNEUROSCI.6052-11.2012>. PMID: 22423104; PMCID: PMC6703466.
- Kyuyoung, C.L., Huguenard, J.R., 2014 Jan 8. Modulation of short-term plasticity in the corticothalamic circuit by group III metabotropic glutamate receptors. *J. Neurosci.* 34 (2), 675–687. <https://doi.org/10.1523/JNEUROSCI.1477-13.2014>. PMID: 24403165; PMCID: PMC3870944.
- Llaudet, E., Botting, N.P., Crayston, J.A., et al., 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 May 15. Microelectrode biosensor for real-time measurement of ATP in biological tissue. *Anal. Chem.* 77 (10), 3267–3273. <https://doi.org/10.1021/ac048106q>. PMID: 15889918.
- Lovatt, D., Xu, Q., Liu, W., Takano, T., Smith, N.A., Schnerrmann, J., Tieu, K., Nedergaard, M., 2012 Apr 17. Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. *Proc. Natl. Acad. Sci. U. S. A.* 109 (16), 6265–6270. <https://doi.org/10.1073/pnas.1120997109>. Epub 2012 Mar 15. PMID: 22421436; PMCID: PMC3341061.
- Malafosse, A., Genton, P., Hirsch, E., Marescaux, C., Broglia, D., Bernasconi, R., 1994. Idiopathic Generalized Epilepsies. Libbey, London).
- Masino, S.A., Diao, L., Illes, P., Zahniser, N.R., Larson, G.A., Johansson, B., Fredholm, B.B., Dunwiddie, T.V., 2002 Oct. Modulation of hippocampal glutamatergic transmission by ATP is dependent on adenosine a(1) receptors. *J. Pharmacol. Exp. Therapeut.* 303 (1), 356–363. <https://doi.org/10.1124/jpet.102.036731>. PMID: 12235271.
- McNamara, J.O., 1995. Drugs effective in the therapy of the epilepsies. In: Hardman, J. G., Goodman Gilman, A., Limbird, L.E. (Eds.), *The Pharmacological Basis of Therapeutics*. McGraw-Hill, New York, pp. 461–486.
- Meeren, H., van Luijckelaar, G., Lopes da Silva, F., Coenen, A., 2005 Mar. Evolving concepts on the pathophysiology of absence seizures: the cortical focus theory. *Arch. Neurol.* 62 (3), 371–376. <https://doi.org/10.1001/archneur.62.3.371>. PMID: 15767501.
- Newman, E.A., 2004 Aug 15. Glial modulation of synaptic transmission in the retina. *Glia* 47 (3), 268–274. <https://doi.org/10.1002/glia.20030>. PMID: 15252816; PMCID: PMC2322937.
- Oren, I., Mann, E.O., Paulsen, O., Hájos, N., 2006. Synaptic currents in anatomically identified CA3 neurons during hippocampal gamma oscillations in vitro. *J. Neurosci.* 26, 9923–9934.
- Pankratov, Y., Lalo, U., Verkhratsky, A., North, R.A., 2007 Mar. Quantal release of ATP in mouse cortex. *J. Gen. Physiol.* 129 (3), 257–265. <https://doi.org/10.1085/jgp.200609693>. PMID: 17325196; PMCID: PMC2151610.
- 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 Oct 7. Astrocytic purinergic signaling coordinates synaptic networks. *Science* 310 (5745), 113–116. <https://doi.org/10.1126/science.1116916>. PMID: 16210541.
- Pearson, T., Nuritova, F., Caldwell, D., Dale, N., Frenguelli, B.G., 2001 Apr 1. A depletable pool of adenosine in area CA1 of the rat hippocampus. *J. Neurosci.* 21 (7), 2298–2307. <https://doi.org/10.1523/JNEUROSCI.21-07-02298.2001>. PMID: 11264305; PMCID: PMC6762415.
- Polack, P.O., Mahon, S., Chavez, M., Charpier, S., 2009 Sep. Inactivation of the somatosensory cortex prevents paroxysmal oscillations in cortical and related thalamic neurons in a genetic model of absence epilepsy. *Cerebr. Cortex* 19 (9), 2078–2091. <https://doi.org/10.1093/cercor/bhn237>. Epub 2009 Mar 10. PMID: 19276326.
- Qi, G., van Aerde, K., Abel, T., Feldmeyer, D., 2017 Sep 1. Adenosine differentially modulates synaptic transmission of excitatory and inhibitory microcircuits in layer 4 of rat barrel cortex. *Cerebr. Cortex* 27 (9), 4411–4422. <https://doi.org/10.1093/cercor/bhw243>. PMID: 27522071; PMCID: PMC6433180.
- Simko, J., Markram, H., 2021 Nov. Morphology, physiology and synaptic connectivity of local interneurons in the mouse somatosensory thalamus. *J. Physiol.* 599 (22), 5085–5101. <https://doi.org/10.1113/JP281711>. Epub 2021 Oct 23. PMID: 34591324.
- Sohal, Huguenard, 2003. Inhibitory Interconnections Control Burst Pattern and Emergent Network Synchrony in Reticular Thalamus. *J. Neurosci.* 23, 8978.
- Uhlenbeck, G.E., Ornstein, L.S., 1930. On the theory of the Brownian motion. *Phys. Rev.* 36, 823–841.
- Ulrich, D., Huguenard, J.R., 1995 Oct. Purinergic inhibition of GABA and glutamate release in the thalamus: implications for thalamic network activity. *Neuron* 15 (4), 909–918. [https://doi.org/10.1016/0896-6273\(95\)90181-7](https://doi.org/10.1016/0896-6273(95)90181-7). PMID: 7576639.
- 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., Atterbury, A., Dale, N., 2007. Control of basal extracellular adenosine concentration in rat cerebellum. *J. Physiol.* 582, 137–153.
- Wall, M.J., Richardson, M.J., 2015. Localized adenosine signaling provides fine-tuned negative feedback over a wide dynamic range of neocortical network activities. *Epub* 2014 Nov 12 *J. Neurophysiol.* 113 (3), 871–882. <https://doi.org/10.1152/jn.00620.2014>, 2015 Feb 1.
- Weltha, L., Reemmer, J., Boison, D., 2019 Sep. The role of adenosine in epilepsy. *Brain Res. Bull.* 151, 46–54. <https://doi.org/10.1016/j.brainresbull.2018.11.008>. Epub 2018 Nov 20. PMID: 30468847; PMCID: PMC6527499.
- Zhang, L., Jones, E.G., 2004 Feb. Corticothalamic inhibition in the thalamic reticular nucleus. *J. Neurophysiol.* 91 (2), 759–766. <https://doi.org/10.1152/jn.00624.2003>. Epub 2003 Oct 29. PMID: 14586030.