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## Adenosine is released during thalamic oscillations

## to provide negative feedback control

Mark J Wall<sup>1\*</sup>, Katie Puddefoot<sup>1</sup>, Wencheng Yin<sup>2</sup>, Chris Bingham<sup>2</sup>, Mohsen Seifi <sup>3</sup>, Jerome D Swinny <sup>4</sup> and Richard Teke Ngomba<sup>3\*</sup>

<sup>1</sup>School of Life Sciences, University of Warwick, Coventry, United Kingdom; <sup>2</sup>University of Lincoln, College of Science, <sup>3</sup>University of Lincoln, School of Pharmacy, United Kingdom Leicester School of Pharmacy, De Montfort University, Leicester, LE1 9BH, UK, and <sup>4</sup>School of Pharmacy & Biomedical Sciences, University of Portsmouth, St Michael's Building, White Swan Road, Portsmouth, PO12DT, UK

\*Address correspondence to:

\*Dr. Mark Wall: School of Life Sciences, University of Warwick, Gibbet Hill, Coventry' CV4 7AL, UK. Email: <u>Mark.wall@warwick.ac.uk</u>

\*Dr. Richard T Ngomba: University of Lincoln, School of Pharmacy, College of science, Joseph Banks Laboratories; LN6 7DL, UK e-mail: <u>richardngomba@hotmail.com</u> (<u>rngomba@lincoln.ac.uk</u>)

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 A<sub>1</sub> 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 nonepileptiform and epileptiform oscillations but can only be directly measured during epileptiform activity.

#### 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 A<sub>1</sub> 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,  $A_1$  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 A<sub>1</sub> receptors to feedback and reduce thalamic oscillatory activity.

## 1. Introduction

The thalamus is the principal portal for sensory information, and it is central to the corticothalamocortical (CTC) loop that generates rhythmic oscillations (Meeren et al., 2002, 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 (Blumenfield 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, 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 A<sub>1</sub> receptor being the most widely expressed in the brain (reviewed in Fredholm et al 2000). Activation of A<sub>1</sub> receptors inhibits synaptic transmission and hyperpolarises the membrane potential via the activation of K<sup>+</sup> 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 equilbrative 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  $A_1$  receptors with seizures inhibited by  $A_1$  receptor agonists and enhanced by  $A_1$  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 A<sub>1</sub> and A<sub>2</sub>a receptor antagonist) and selective adenosine receptor antagonists (A<sub>1</sub> and A<sub>2</sub>a) 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 A<sub>1</sub> receptor antagonists (Kovács et al 2017). The results from most of these experiments are difficult to interpret as the injected pharmacological agents will have <u>effects</u> 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 A<sub>1</sub> receptors in the rodent thalamus and examined how inhibiting A<sub>1</sub> 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-hour light-dark cycle with slices made 90 minutes after entering the light cycle. In

accordance with the U.K. Animals (Scientific Procedures) Act (1986), 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 Mg<sup>2+</sup>, low Ca<sup>2+</sup> aCSF, composed of (mM): 127 NaCl, 1.9 KCl, 8 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 D-glucose (pH 7.4 when bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, 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 hours in aCSF (1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>) 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 minutes, followed by 10 minutes 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 hours 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 antiadenosine 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-minute 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 hours at room temperature on a horizontal shaker. Secondary antibodies were all raised in donkey, and conjugated to either Alexa Fluor<sup>™</sup> 488 (Invitrogen, Eugene, OR, USA), indocarbocyanine (Cy<sup>™</sup>3) or DyLight<sup>™</sup> 549, or DyLight<sup>™</sup> 649 (Jackson Immunoresearch, West Grove, PA, USA), for 2 hours. Sections were then washed three times for 10 minute periods in TBS-Tx to remove unbound antibodies, mounted on glass microscope slides, air dried, and sealed with glass coverslips using Vectashield<sup>™</sup> antifade mounting medium (Vector Laboratories). The specificity of the A<sub>1</sub> 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 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 ~500  $\mu$ m and diameter of ~ 50  $\mu$ 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; Llaudet et al 2005): they show a linear response to increasing concentrations of analyte and have a fast response time (< 1 s Wall and Richardson, 2015). In each experiment, the biosensors were calibrated with analyte (10  $\mu$ M) 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  $\mu$ 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<sup>-1</sup> with aCSF at 32 ± 0.5°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 $\Omega$ ) 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  $\tau_{fast} = 3$  ms and  $\tau_{slow} = 10$  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 ~2-3 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).

#### 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  $\mu$ M or picrotoxin 25  $\mu$ M 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)-7*H*-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 ± SEM. For statistical analysis non-parametric tests were used (Mann Whitney).

## 3. Results

## 3.1 Adenosine A1 receptor distribution in the thalamus

Initially the distribution of adenosine A<sub>1</sub> receptor expression in the thalamus was examined before examining thalamic oscillations. A low power overview of A<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> receptor immunoreactive clusters were also colocalised with VGLUT1 immunopositive puncta (Fig. 1 C). There was no discernible association between the signal for adenosine A<sub>1</sub> receptors and VGLUT2 (SI Fig. 2). This data shows a concentrated expression of A<sub>1</sub> 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 et al 2018; Kyuyoung and Huguenard 2014; Sohal et al 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 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, Figure 2B), so they were

perfused from above and below; perfusion rates were high (5-6 ml<sup>-1</sup> 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 figure 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 recordings of oscillations and purine release. A single stimulus (1-4 V) delivered in the internal capsule (electrode positions displayed in Fig. 2A, B) under control conditions (aCSF with 1 mM Mg<sup>2+</sup> and 3 mM Ca<sup>2+</sup>) evoked activity in the thalamus (Fig. 2C, D). The amplitude of the activity was much smaller than reported from recordings using interface chambers presumably due to current loss through the solution had a similar pattern to what has previously been reported (Ulrich and Huguenard 1995).

#### 3.3 Activation of adenosine A1 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, Figure 3A arrow, 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  $\pm$  0.2, range 1 to 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<sup>2+</sup> 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 to 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  $\pm$  81.6 ms. Application of either adenosine (100  $\mu$ M, n = 5 slices) or the selective A<sub>1</sub> receptor agonist CPA (50 nM, n = 3 slices, not illustrated) reversibly inhibited the stimulated activity (Fig. 3A, 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 A1 receptor

antagonist 8CPT at 1-2  $\mu$ M (Fig. 3C). Although <u>the reported Ki for 8CPT for A<sub>1</sub> receptors is</u> <u>10.9 nM and is 1440 nM for A<sub>2</sub> receptors (Jacobson et al 1989), higher concentrations are</u> <u>routinely used in brain slices (for example see Qi et al 2017) compared to cell lines</u> <u>expressing recombinant receptors (Jacobson et al 1989).</u> Application of ATP (50-100  $\mu$ M) 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<sub>1</sub> receptor antagonist (2  $\mu$ M 8-CPT, Fig. 3D) together with the inhibitory effects of CPA show that the inhibition of oscillations was mainly via A<sub>1</sub> receptor activation.

Since oscillations were recorded in the VB thalamus, we investigated the effects of adenosine on the electrophysiological properties of VB thalamocortical neurons using current clamp recording. Application of adenosine (100  $\mu$ M, n = 6 slices) produced a hyperpolarisation of the membrane potential of TC neurons (Fig. 4A, membrane potential hyperpolarised from -65.7  $\pm$  2.7 mV to -71.4  $\pm$  2.2 mV, Fig. 4B) which was reversible upon wash (mean membrane potential on wash  $-65.6 \pm 2 \text{ mV}$ , 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  $\mu$ M) significantly increased the interval (from  $273 \pm 45$  ms to  $446 \pm 65$  ms, P = 0.001 Fig. 4E) between the bursts and also significantly reduced the number of spikes per burst (from  $4.8 \pm$ 0.5 to  $3.98 \pm 0.3$  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 A1 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  $\mu$ 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<sub>1</sub> 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<sub>2a</sub> 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<sub>2a</sub> 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<sub>1</sub> 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<sub>1</sub> 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 A1 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 and Dale 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  $\mu$ 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 A1 receptor activation modulates epileptiform oscillatory activity

We next investigated the role of adenosine A<sub>1</sub> receptors and adenosine release in epileptiform oscillations. Hypersynchronous epileptiform activity can be induced by reducing GABAergic inhibition coupled with a reduction in Mg<sup>2+</sup> concentration to enhance NMDA receptor activity (Ulrich and Huguenard 1995). Perfusion of slices with aCSF containing a reduced concentration of Mg<sup>2+</sup> together with a GABA<sub>A</sub> receptor antagonist induced significantly (p =1.77114E<sup>-06</sup>) more oscillatory bursts evoked by stimulation  $(11.7 \pm 1.44 \text{ vs} 2.23 \pm 0.2, n = 15)$ and n = 12) compared to control conditions and also significantly (P = 0.018328) shortened the latency to the first burst  $(438 \pm 16 \text{ vs} 621 \pm 81.6 \text{ ms}, \text{Fig. 7A})$ . 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  $\mu$ 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  $\mu$ M, n = 6) or CPA (50 nM, n = 3) either abolished or greatly reduced the number of bursts per stimulus (Fig. 7C, D) and also significantly reduced the frequency of spontaneous activity (for adenosine U = 36, Z = 2.8, P = 0.0021). Antagonism of A<sub>1</sub> receptors (with 2 µM CPT) significantly increased the number of bursts evoked per stimulus (Fig. 7E, F) without changing the latency to the first burst (Fig 7G). Application of 8CPT (2 µM) 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<sub>1</sub> receptors and dampen oscillations. However, recordings from TC neurons in aCSF containing low Mg<sup>2+</sup> and with a GABA<sub>A</sub> 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<sub>1</sub> receptor tone in the tissue under quiescent conditions.

#### 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, 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  $\mu$ M to 1.5  $\mu$ M with a mean of  $0.69 \pm 0.16 \mu M$  (*n* = 10). The biosensor currents had a slow rise-time (time to peak ~30-60s, mean  $42.3 \pm 5$  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<sub>1</sub> 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 hemi-channels) 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<sub>1</sub> 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 A1 receptors in thalamic oscillations

We have found that application of either adenosine or selective A<sub>1</sub> 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<sub>1</sub> 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. Wall et al 2022 final version

The effect of adenosine A<sub>1</sub> receptor agonists on the thalamic oscillations is consistent with the dense A<sub>1</sub> receptor expression in both the nRT and VB thalamus. The A<sub>1</sub> 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 (Fremeau 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 A1 receptor expression pattern.

Under control conditions, block of A1 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<sub>1</sub> 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<sub>1</sub> 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 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<sub>1</sub> receptors. Blocking this A<sub>1</sub> 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<sub>1</sub> 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 were 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, B) which maybe expected if more neurons were recruited.

#### Properties of purine release during epileptiform oscillations

Epileptiform oscillations were induced using aCSF that had 0.1 mM Mg<sup>2+</sup> and picrotoxin (25  $\mu$ M) to partially block GABA<sub>A</sub> 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  $\mu$ M adenosine, would be equivalent to ~ 0.1  $\mu$ M 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 and Dale 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 ~ 100  $\mu$ M from the purine release source, the rise time of the biosensor signal would be predicted to be  $\sim$ 20 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 detected currents associated with individual stimuli. If we extrapolate backwards, we can suggest that at the release sites the concentration of purines would be ~ 35  $\mu$ M. It is possible that the release sites are deep within the slice (slice is 400 µM 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> receptors with i.p. injection of specific agonists enhanced SWD frequency whereas A<sub>1</sub> receptor antagonists reduced SWD frequency (Germe et al 2014). There has been no study where adenosine or A<sub>1</sub> 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.

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## Legends

## Figure 1. Immunolocalization of $A_1$ 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  $A_1$  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 A<sub>1</sub> 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 A<sub>1</sub> 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 A<sub>1</sub> receptor (A1-R) immunopositive clusters colocalise with VGLUT1 immunopositive profiles (arrowheads) whilst another population colocalise with VGLUT1 signal (arrows).

(C1) In the VB adenosine A<sub>1</sub> 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 A<sub>1</sub> receptor immunopositive clusters colocalise with VGLUT1-immunoreactive puncta. (C4) is a magnified view of the boxed areas in (C1-3) indicating that adenosine A<sub>1</sub> 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

## 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 simulating 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 amplitude of the peak equivalent to the number of events within the oscillation.

## Fig. 3. Adenosine A<sub>1</sub> receptor activation dampens thalamic oscillations.

A, Example extracellular traces from a single slice in control, following application of adenosine (100  $\mu$ M) 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  $\mu$ M) was co-applied with the A<sub>1</sub>

receptor antagonist 8-CPT (1  $\mu$ M). D, Oscillations were blocked by ATP (50  $\mu$ M) an effect which was reversed by blocking A<sub>1</sub> receptors with 8 CPT (1  $\mu$ M).

#### Fig 4. Adenosine hyperpolarises TC neurons by activating adenosine A1 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. F, Graph plotting the probability distribution of burst intervals in control (sold 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. H, 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.

## Fig. 5. The number of oscillations is increased by the block of endogenous A<sub>1</sub> 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<sub>1</sub> receptor antagonist 8CPT (2  $\mu$ M). The antagonism of A<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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 = 1.55x10-4) G, Membrane potential traces in response to the injection of a naturalistic noisy current in control (top) and in 8 CPT (2 µM) 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  $\mu$ M) produced a current of 3.4 nA thus a limit of detection (for ~ 20 pA current) was ~ 60 nM.

#### Fig. 7. Epileptiform thalamic oscillations are modulated by A<sub>1</sub> receptors.

A, examples of successive traces of epileptiform thalamic oscillations (induced in 0.1 mM  $Mg^{2+}and 25 \mu M PTX$ ) with each stimulation producing multiple bursts of activity. Application of the AED (anti-epileptic drug) ethosuximide (500  $\mu$ M) reversibly abolished the oscillations. B, Spontaneous activity (there was no stimulation) recorded in 0.1 mM  $Mg^{2+}and 25 \mu M PTX$ . The activity was reversibly abolished by ethosuximide (500  $\mu$ M). C, examples of successive traces of epileptiform thalamic oscillations. Application of adenosine (100  $\mu$ M) reversibly abolished the oscillations 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  $\mu$ M), 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<sub>1</sub> 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<sub>1</sub> 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).

## Figure 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<sup>2+</sup>and 25  $\mu$ M 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  $\mu$ M 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<sub>1</sub> 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<sub>1</sub> receptors (8CPT) led to a current on the biosensor. The inset shows the spontaneous activity in control and in 8CPT.



Figure 1



Figure 2.



Figure 3.



Figure 4.



Figure 5.



Figure 6.



Figure 7.



Figure 8.



## SI Figure 1

Validation of the specificity of immunohistochemistry reactions.

(A) shows the respective immunohistochemistry patterns for parvalbumin and A1 receptor when both primary and secondary antibodies are used.

(B) shows the lack of any specific signal for the A1 receptor when the primary antibody is preadsorbed with the immunizing peptide prior to the immunohistochemical reaction.

(C) shows the lack of any specific signal for both parvalbumin and the A1 receptor when the primary antibody is omitted, thereby confirming the specificity of the secondary antibodies. Scale bars 50  $\mu$ m.



#### SI Figure 2

Lack of an association between immunohistochemistry for the A1 receptor and VGLUT2. (A) shows immunoreactivity for the A1 receptor (red) in the VB and its association with parvalbumin-immunopositive (PV) (blue) profiles.

(B) shows, in the same field of view, puncta immunopositive for VGLUT2 (green).

(C) shows, in the same field of view, the distribution of profiles immunopositive for the A1 receptor and VGLUT2, indicating the lack of any specific association.(D) is an overview of all panels.

Scale bars 2 µm.



## SI Figure 3

Effect of adenosine and A1 receptor antagonists on evoked oscillations in mice slices (A) Example extracellular traces from a single mouse slice in control, following application of adenosine (50  $\mu$ M) and in wash. Adenosine reduced the initial burst that immediately followed the stimulation and abolished the subsequent oscillatory activity (there was recovery with wash).

(B) Example extracellular traces from a single mouse slice in control, following application of adenosine (50  $\mu$ M) and in wash. Adenosine abolished oscillatory activity. Addition of 8CPT (A<sub>1</sub> receptor antagonist 1  $\mu$ M) led to increased oscillatory activity compared to control. Insets, show oscillations (in box) at an expanded time base.