Pannexin1-mediated ATP release from area CA3 drives mGlu5-dependent neuronal oscillations

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Abstract

The activation of Group I metabotropic glutamate receptors (GI mGluRs) in the hippocampus results in the appearance of persistent bursts of synchronised neuronal activity. Such activity is known to cause the release of the purines ATP and its neuroactive metabolite, adenosine. We have investigated the role of the purines in GI mGluR-induced oscillations in hippocampal areas CA3 and CA1 using pharmacological techniques and microelectrode biosensors for ATP and adenosine. The GI mGluR agonist DHPG induced both persistent oscillations in neuronal activity and the release of adenosine in areas CA1 and CA3. In contrast, the DHPG-induced release of ATP was only observed in area CA3. Whilst adenosine acting at adenosine A_1 receptors suppressed DHPG-induced burst activity, the activation of mGlu5 and $P2Y_1$ ATP receptors were necessary for the induction of DHPG-induced oscillations. Selective inhibition of pannexin-1 hemichannels with a low concentration of carbenoxolone (10 μM) or probenecid (1 mM) did not affect adenosine release in area CA3, but prevented both ATP release in area CA3 and DHPG-induced bursting. These data reveal key aspects of GI mGluR-dependent neuronal activity that are subject to bidirectional regulation by ATP and adenosine in the initiation and pacing of burst firing, respectively, and which have implications for the role of GI mGluRs in seizure activity and neurodevelopmental disorders.

Keywords: DHPG, Group I mGluR, ATP, adenosine, hemichannels, pannexin-1, connexins, seizures, epileptiform activity

Highlights:

- GI mGluR activation induces rhythmic bursting and the release of adenosine and ATP
- Adenosine regulates the timing and intensity of bursting via A_1 receptors
- ATP $P2Y_1$ receptors are required for the initiation of bursting
- Panx1 blockers prevent bursting and ATP, but not adenosine, release in area CA3
- Purines regulate mGluR-dependent bursting in a bidirectional manner

1 Introduction

Group I metabotropic glutamate receptor (GI mGluR) activation is implicated in various synchronised neuronal processes, ranging from perception-pertinent 40 Hz oscillations (Whittington et al., 1995) to hypersynchronous neuronal activity as seen in seizures (Moldrich et al., 2003). Indeed, activation of GI mGluRs has been shown to produce epileptiform activity in hippocampal slices (Sayin and Rutecki, 2003; Taylor et al., 1995), seizures *in vivo* (Sacaan and Schoepp, 1992) and can lead to protein synthesis-dependent long-term modification of network excitability and the subsequent transition from inter-ictal activity to seizure-like events (Wong et al., 2004). Furthermore, enhanced GI mGluR activity, in particular of mGlu5, is responsible for increased cortical excitability and burst frequency in mouse models of Fragile X syndrome (Hays et al., 2011), a developmental disorder characterised by intellectual disability, autism spectrum disorder and epilepsy (Kidd et al., 2014).

Epileptic seizures in humans (During and Spencer, 1992) and epileptiform activity *in vivo* (Schrader et al., 1980) and *in vitro* (Etherington et al., 2009) are accompanied by the release of the neuromodulator adenosine which, via the inhibitory actions of adenosine A_1 receptors, attenuates the intensity and duration of seizures (Dale and Frenguelli, 2009). Adenosine can be released *per se* or as a product of extracellular ATP metabolism (Zimmermann et al., 2012). ATP and other modulators can be released as a consequence of Ca^{2+} oscillations which can be triggered by GI mGluR activation. These Ca^{2+} oscillations have been observed both in neurones (Jaffe and Brown, 1994) and astrocytes (Pasti et al., 1997).

We have recently assessed the role of ATP and its P2 receptors in the Mg^{2+}-free model of electrically-induced epileptiform activity and observed only a minor excitatory role of P2 receptors in this phenomenon. This conclusion was supported by the failure to observe ATP release during epileptiform activity using microelectrode biosensors (Lopatar et al., 2011). Given the ability for GI mGluR activation to provoke seizure activity and trigger Ca^{2+} oscillations, which in turn can cause ATP release, we investigated the potential for purinergic regulation of GI mGluR-induced bursting in acute hippocampal slices using pharmacological approaches and real-time detection of purine release using microelectrode biosensors.

We show that the activation of GI mGluRs, and in particular mGlu5, by DHPG induced burst firing in area CA1. DHPG also caused a large increase in extracellular adenosine which is, however, independent of neuronal activity triggered by DHPG, but which exerts a powerful inhibitory influence on DHPG-induced bursting via adenosine A_1 receptors. This adenosine is released primarily as adenosine, although sub-micromolar CA3 region-specific levels of ATP likely triggers bursting via the activation of $P2Y_1$, but not $P2X_7$ receptors. ATP release was sensitive to probenecid and a low concentration of carbenoxolone indicative of ATP release via the pannexin-1 hemichannel. Our data extend our understanding of the consequence for neuronal function of GI mGluR activation by incorporating key purine steps in this process.

2 Materials and Methods

2.1 Drugs and chemicals

(S)-3,5-Dihydroxyphenylglycine (DHPG) was obtained from Tocris or Ascent Scientific. 2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS 2179), 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), tetrodotoxin (TTX), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine (A438079) and 6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156) were from Tocris. 8-Cyclopentyltheophylline (8-CPT), probenecid and carbenoxolone were purchased from Sigma Aldrich. Salts and glucose for the aCSF were purchased from Fisher Scientific. DHPG, MRS2179, TTX, MPEP, A438079, ARL 67156, carbenoxolone were all dissolved in distilled H_2O at stock concentrations at least 200 times the final concentration. Probenecid was dissolved in DMSO to give a final concentration of 0.2 % DMSO and 8-CPT was dissolved in 0.1 M NaOH at a 10 mM stock concentration.

2.2 Preparation of hippocampal slices

17-23 day old male Sprague-Dawley rats were used and were killed by cervical dislocation and then decapitated in accordance with Schedule 1 of the UK Government Animals Act (Scientific procedures) 1986 and with Local Ethical Review procedures. The brain was quickly removed and put into ice-cold aCSF containing in mM: 124 NaCl, 26 $NaHCO_3$, 10 D-glucose, 1.3 NaH_2PO_4, 2 $CaCl_2$, 2 KCl and 1 $MgSO_4$ with additional 10 mM $MgCl_2$. 600 or 400 µm parasagittal hippocampal slices were cut on a vibratome (Microm, HM 650 V) and kept at 34

°C in standard aCSF (containing 1 mM Mg^{2+} solution; pH 7.4) gassed with 95% O_2/5% CO_2 for at least 1 hour before use.

2.3 Electrophysiological recordings and drug application

After the recovery period, slices were submerged in a recording chamber, secured with a platinum harp with nylon threads, and were perfused with oxygenated aCSF at a flow rate of 6-7 mL/min and maintained throughout experiments at 30 °C. Extracellular field excitatory postsynaptic potentials (fEPSPs) and epileptiform activity were recorded (10 kHz sampling; 1 Hz to 3 kHz filtering) from stratum radiatum using an aCSF-filled glass microelectrode of ~1 MΩ resistance. To elicit fEPSPs as a measure of slice viability and recording stability, the Schaffer collateral-commissural pathway was stimulated at 0.067 Hz. Electrical stimulation of the Schaffer pathway at the level of stratum radiatum was provided via a bipolar stimulating electrode made from twisted Teflon-coated tungsten wire (50 µm in diameter). Stimulus parameters and acquisition and analysis of fEPSPs were under the control of LTP software (Anderson and Collingridge, 2001, 2007). Epileptiform activity was recorded and analysed using Spike 2 software. All pharmacological agents were bath-applied. Hippocampal slices were pre-incubated with drugs of interest for 15 minutes after which time DHPG was applied.

2.4 Electrophysiological data analyses

The fEPSP was used as an index of slice viability and recording stability and involved the measurement of the negative-going slope of the fEPSP typically over a 1 ms time range.

Periodic stimulation of the Schaffer pathway was stopped prior to the induction of epileptiform activity by DHPG. The analysis of epileptiform activity was as described previously (Lopatar et al., 2011): extracellular recordings were rectified, in that negative-going activity was multiplied by -1 to give positive-going signals. For the most part epileptiform activity was quantified in terms of burst frequency (i.e. the number of bursts per minute) as bursting behaviour was a common feature of the activity induced by DHPG and readily quantifiable across slices and treatments. Bursts were defined as periods of sustained repetitive activity of at least twice the amplitude of the background noise and separated by periods of quiescence. In the case of experiments involving the adenosine A_1R antagonist 8-CPT, which provoked increased epileptiform activity, the intensity of activity was additionally measured in terms of the number of spikes in a given period and the inter-burst interval. To avoid double counting of rectified spikes a threshold inter-spike interval of 50 ms was applied. When ARL 67156 was used, the duration of bursting was measured.

2.5 Adenosine and ATP biosensor measurements:

Adenosine, ATP and null biosensors (Pt/Ir wire of 50 µm in diameter and 500 µm in length) were purchased from Sarissa Biomedical Ltd (Coventry, UK) and were inserted through the thickness of the slice in either area CA3 or area CA1 (Figure 1A). The ATP biosensor requires glycerol (2 mM) to be present in the extracellular perfusion medium, which had no discernable effect on the fEPSP (data not shown). The biosensors contain specific enzymatic cascades able to break down ATP (Llaudet et al., 2005) or adenosine (Llaudet et al., 2003) to produce hydrogen peroxide (Figure 1B). The hydrogen peroxide is oxidised on the surface of the polarised Pt/Ir wire to give rise to a current linearly-related to the concentration of the

measured analyte. Null sensors contain no enzymes and measure only non-specific electroactive signals. Pairs of sensors (ATP and null; adenosine and null) were inserted into the stratum radiatum of the hippocampal CA1 or CA3 regions (Figure 1A). At least one hour was given for the signal associated with sensor insertion to dissipate before experiments started. After each experiment, sensors were withdrawn from slices and calibrated with 10 µM adenosine or 10 µM ATP. The values from adenosine biosensors are given as µM´ (µM prime) to reflect that the adenosine signal is a composite signal of adenosine and its metabolites (Frenguelli et al., 2007). A 10 µM serotonin solution was also used to assess the patency of the electro-active interferent screening layer of the sensors. Biosensor measurements were only accepted and further processed if serotonin response did not exceed 150 pA. The current response of the simultaneously-recorded null sensors was subtracted from ATP and adenosine signal to reveal net purine signal. Biosensor reading was taken once the release had stabilized which was usually 10 – 15 minutes after DHPG or other drug application. A one-minute rolling average of biosensor measurements around the 15^{th} minute after drug application was taken for quantification across slices and treatments. Sensor recordings were made using Spike 2 software at a sampling rate of 1 kHz.

2.6 Malachite green phosphate assay

Slices were placed for 15 minutes in 500 ml phosphate-free aCSF to remove the majority of inorganic phosphate from the slices. Afterwards, slices were transferred onto a mesh support in small beakers containing 33 ml of phosphate-free aCSF where they were pre-treated with carbenoxolone and with DHPG. Ten minutes after DHPG application, slices were transferred into a 24-well plate and 0.5 ml of the appropriate solution was added. 95 % O_2/5

% CO_2 was blown across the surface of the wells to ensure constant oxygen supply. In order to maintain the slice viability, we restricted our incubations in the wells to 15 minutes after which time the solution was removed and was further used to assess inorganic phosphate using malachite green assay as described previously (Carter and Karl, 1982; Frenguelli et al., 2007; Wall et al., 2008). Probenecid, but not carbenoxolone, skewed the calibration curve and thus probenecid was not used in this assay.

2.7 Statistical analyses

Biosensor traces were smoothed (rolling average, 1s) before pooling together. All values are expressed as mean ± S.E.M. N values represent number of slices per condition. No more than two slices were used from the same animal per condition. A one way ANOVA was used when more than two groups were to be compared. Graphs were drawn and statistical analyses were performed in OriginPro 8.5 software. Statistical significance was taken as $p < 0.05$.

3 Results

3.1 Activation of group I metabotropic glutamate receptors induces epileptiform bursting and the release of adenosine from hippocampal areas CA1 and CA3

Treatment of 600 µm hippocampal slices with the group I metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine (S-DHPG, 25-50 µM) induced repetitive discharges at a frequency of 3.7 ± 0.6 bursts/minute (bpm; n = 14, Figure 1C) and a burst duration ranging from ~ 0.5 - 5 s, which remained constant during DHPG application (1.75 ± 0.15 s for first 5 mins in DHPG vs 1.88 ± 0.22 s for the last 5 mins in DHPG, n = 14; paired t-test, p = 0.33). This bursting pattern was not different from the pattern obtained using 50 – 100 µM (R,S)-3,5-DHPG (4.2 ± 0.8 bpm, p = 0.4, unpaired t-test, n = 11, data not shown) which confirmed the specificity of the S enantiomer as shown before (Baker et al., 1995), and allowed us to use the more specific S enantiomer at the lower concentration.

Slice thickness was an important factor influencing the frequency of bursting. While in 600 µm slices the DHPG-induced activity occurred in every slice, in 400 µm slices the oscillations were not as pronounced or were not observed (average burst frequency 1.3 ± 0.6 bpm; $p < 0.05$; n = 6; data not shown), suggesting a need for a critical volume of network connectivity for DHPG-induced epileptiform activity, as we have previously observed for electrically-evoked epileptiform activity (Etherington and Frenguelli, 2004). Accordingly, subsequent experiments utilised 600 µm slices. The DHPG-induced activity persisted even when the agonist was washed out (Figure 1D) thus confirming its long-lasting effect on neuronal excitability (Merlin and Wong, 1997).

Since epileptiform activity is followed by an increase in extracellular levels of adenosine (Dale and Frenguelli, 2009), we tested whether DHPG-induced bursting was associated with adenosine release by utilising enzyme-based microelectrode adenosine biosensors (Frenguelli et al., 2003; Llaudet et al., 2003). Adenosine release was detected from both areas CA1 and CA3 of hippocampal slices following challenge with DHPG: 0.74 ± 0.22 µM' (n = 6) for area CA1 (data not shown) and 2.2 ± 0.8 µM' (n = 6) for area CA3 (Figure 1C). Similar to the effect of DHPG on bursting, the elevation of extracellular adenosine was long-lasting (Figure 1D): ten min after washout of DHPG adenosine levels in area CA3 were still at approximately 50 % of their peak value (1.18 ± 0.43 µM'; n = 3). In the two washout experiments performed in area CA1 adenosine levels ten min after washout of DHPG remained at ~20 – 60 % of their peak values (from 1.81 to 1.46 µM' and from 0.78 to 0.33 µM', respectively). The persistence of extracellular adenosine may reflect release associated with the continued epileptiform activity.

3.2 DHPG-induced bursting in area CA1 is activity-dependent and paced by A_1 receptors

In order to establish whether the release of adenosine induced by DHPG had physiological consequences, we applied the adenosine A_1 receptor antagonist 8-CPT (1 µM) once the bursting and extracellular adenosine levels had stabilized. Antagonism of inhibitory A_1 receptors (Figure 2A) caused a six-fold increase in the frequency of epileptiform activity from 3.1 ± 1.0 bpm in standard conditions to 18.2 ± 4.3 bpm in the presence of 8-CPT ($p < 0.05$, paired t-test, n = 4) suggesting an important role for adenosine A_1 receptors in limiting seizure activity. As additional indices of epileptiform activity we measured the intensity of

activity in terms of the number of spikes, and the interval between bursts in the 3 mins before and after application of 8-CPT. Antagonism of A_1 receptors provoked a large increase in the number of spikes over this period (192 ± 25 spikes vs 466 ± 59 spikes, respectively; $p < 0.01$, paired t-test, n = 4), and, in the majority of slices (3/4), dramatically reduced the interval between epileptiform events from 28.6 ± 9.0 s to 3.1 ± 1.2 s (n = 3). Thus, antagonism of adenosine A_1 receptors disrupts the timing and intensity of DHPG-induced bursting and provokes a state of near continuous epileptiform activity which has been described by others as the in vitro equivalent of status epilepticus (Avsar and Empson, 2004).

This increased epileptiform activity resulted in a further rise in extracellular adenosine of 1.3 ± 0.4 μM' in the three experiments in which adenosine sensors were used. The combination of DHPG and 8-CPT evoked overall release of 3.2 ± 0.8 μM' in area CA3 (n = 3). These observations suggest that, as per other seizure models, adenosine A_1 receptors provide a powerful inhibitory influence on epileptiform activity induced by GI mGluR activation and moreover, impose a strict timing pattern on neuronal bursting activity.

A_{2A} receptors are present in the hippocampus and respond to astrocytic mGlu5 activation by increasing synaptic strength (Panatier et al., 2011). To establish whether the activation of excitatory A_{2A} receptors contributed to DHPG-induced bursting we applied ZM 241385 (0.1 μM), a potent A_{2A} receptor antagonist, and compared DHPG-induced bursting in the presence and absence of ZM 241385 (Figure 2B). An excitatory contribution of A_{2A} receptors to DHPG-induced bursting would be inferred by a reduction in burst frequency in the presence of ZM 241385. However, no significant difference was detected between burst

frequency in the absence (11.1 ± 4.4 bpm) and presence (11.8 ± 3.9 bpm) of ZM 241385 (p = 0.61, paired t-test, n = 3; data not shown). These data exclude A_{2A} receptors as modulators of DHPG-induced synchronous activity.

DHPG caused an increase in extracellular adenosine even when bursting did not occur (in 400 µm slices; n = 2; 2.3 and 0.24 µM'; data not shown), and was observed even before bursting occurred in 3/6 slices (e.g. Figure 1C, D). This suggested a direct action of GI mGluR activation on adenosine release independent of synaptic activity. To test this, slices were pre-incubated with the voltage-gated sodium channel blocker tetrodotoxin (TTX; 0.75 – 1 µM) for 10 minutes prior to application of DHPG. Figure 2C demonstrates that slices were indeed capable of releasing adenosine directly upon GI mGluR activation in the absence of neuronal activity. After the DHPG-induced adenosine release stabilised, there was no difference (p = 0.48, unpaired t-test) in peak adenosine levels in the CA3 region between TTX-treated slices (1.3 ± 0.4 µM'; n = 3) and control slices (2.2 ± 0.8 µM'; n = 6; Figure 2D) although there were apparent differences in the rate of adenosine release with TTX-treated slices releasing adenosine more rapidly than control slices. This latter observation suggests some activity-dependent inhibitory influence on adenosine release.

As DHPG is an agonist at both mGlu1 and mGlu5 receptors, we further tested which of the two receptors was responsible for the DHPG-induced recurrent neuronal activity and adenosine release. Slices were pre-incubated with the mGlu5 receptor antagonist MPEP (Gasparini et al., 1999) at 5 µM (Jung et al., 2005). Slices were exposed to MPEP for 10 minutes after which DHPG was applied (Figure 2E). Although MPEP reduced, but did not

significantly affect the peak of adenosine release (1.65 ± 0.3 µM'; n = 5; Figure 2F), it significantly decreased the frequency of bursting (from 3.4 ± 0.5 bpm in control conditions, n = 14, to 0.6 ± 0.3 bpm in the presence of MPEP, n = 6, $p < 0.05$, unpaired t-test), indicative of an important role of mGlu5 receptor in provoking sustained DHPG-induced bursting (Merlin, 2002).

3.3 DHPG-induced bursting activity provokes ATP release in area CA3, but not area CA1

GI mGluR activation causes intracellular Ca^{2+} elevations in astrocytes and neurones. Astrocytic Ca^{2+} waves can be spread via ATP as an extracellular mediator (Leybaert and Sanderson, 2012). Accordingly, we wanted to test if the oscillations elicited by GI mGluR activation in the present study involved ATP release. ATP biosensors were inserted into area CA1 or CA3 and the oscillations were induced by bath-application of S-DHPG (25 – 50 µM). DHPG-induced ATP release was only observed in the CA3 region of the hippocampus (0.36 ± 0.05 µM, n = 13), while no appreciable ATP was detected in the CA1 region (Figure 3A). In contrast to the prolonged presence of extracellular adenosine after removal of DHPG (Figure 1D), ATP levels declined rapidly on washout of DHPG, falling by ~95 % from peak values by 10 min (0.0267 ± 0.008 µM; n = 3; data not shown). Given that ATP release could only be observed in area CA3, only area CA3 was examined in subsequent experiments. This inter-regional difference in adenosine and ATP release could be explained by ATP being important for the pace-making activity residing in the CA3 pyramidal neurones (Wong and Prince, 1981).

We confirmed the nature of the released substance to be ATP two-fold: (i) by application of the ecto-ATPase inhibitor ARL 67156 (100 μM), which enhances the spread of intercellular Ca^{2+} waves (Leybaert and Sanderson, 2012), and by employing the malachite green phosphate assay which can serve as an indirect measure of the presence of ATP via the metabolism of ATP to yield the inorganic phosphate detected by the malachite green assay (Carter and Karl, 1982; Frenguelli et al., 2007; Wall et al., 2008). When the ecto-ATPase inhibitor ARL 67156 was used, a small but consistent increase in extracellular ATP upon ARL 67156 application was seen in area CA3 (Figure 3B, n = 5). This ARL 67156-induced augmentation of extracellular ATP level was associated with an increase in burst duration (from 5.5 ± 1.1 s to 8.1 ± 1.3 s, $p < 0.02$, paired t-test, n = 6, Figure 3C), which contrasted with the stability of burst duration under control conditions over this time period. These data suggest a permissive role of ATP in DHPG-induced bursting.

Under basal conditions the malachite green phosphate assay revealed the accumulation over 15 minutes of ~7-8 μM of inorganic phosphate (Pi) in slices incubated in 0.5 mL phosphate-free aCSF. This basal Pi production was more than doubled in the presence of 25 μM S-DHPG (from 7.4 ± 0.4 μM in Pi-free aCSF (n = 7) to 17.9 ± 2.7 μM in the presence of DHPG, (n = 12), $p < 0.01$, one-way ANOVA; data not shown). Since the most likely source of this phosphate is via the metabolism of extracellular ATP these results support the observation of DHPG-induced ATP release as measured with biosensors.

3.4 Hemichannel blockers prevent ATP, but not adenosine release in area CA3 and DHPG-induced bursting in area CA1

Given the widespread distribution of connexin and pannexin-1 (Panx1) hemichannels on neurones and astrocytes (Cisneros-Mejorado et al., 2014; De Bock et al., 2014) and the strong evidence for a contribution of connexin and pannexin-1 hemichannel-mediated ATP release during neuronal and astrocytic activity (Garre et al., 2010; Santiago et al., 2011; Stout et al., 2002; Suadicani et al., 2012), we decided to test the hypothesis that GI mGluR-evoked ATP release occurs via hemichannels.

Slices were first pre-incubated in the known hemichannel blocker, carbenoxolone, which blocks gap junction hemichannels in a concentration-dependent manner. At higher concentrations (100 μM) it is considered a general gap junction blocker while at lower concentrations (10 μM) it is a relatively selective Panx1 blocker (Bruzzone et al., 2005; Silverman et al., 2008). Pre-incubation of hippocampal slices with carbenoxolone at a Panx1-selective concentration (10 μM) almost completely blocked DHPG-induced bursting (Figures 4A, F; n = 9). In order to confirm our carbenoxolone data, we used probenecid (1 mM), which, although it has actions at the renal organic anion transporter, is widely used as a Panx1 blocker (Silverman et al., 2008). DHPG application in the presence of probenecid did not evoke synchronised bursting activity in the majority of experiments (6/7; Figures 4B, F). Furthermore, little ATP was detected in area CA3 using biosensors in the presence of either carbenoxolone (n = 5) or probenecid (n = 5; Figures 4A, B, C).

Using the malachite green phosphate assay, pre-incubation of slices with the hemichannel blocker carbenoxolone (10 μM) for 15 minutes prior to the DHPG application resulted in a significant decrease in DHPG-induced phosphate production from 17.9 ± 2.7 μM to 11.7 ±

0.9 µM, n = 12, $p < 0.01$, one-way ANOVA; data not shown), consistent with the ATP biosensor data. Taking into account basal phosphate levels (7.4 ± 0.4 µM; n = 7) the reduction by carbenoxolone represents a 60 % decrease in tissue phosphate production. These data suggest an involvement of Panx1 hemichannels in both ATP release and initiation of DHPG-induced bursting. In contrast, adenosine release from area CA3 was unaffected by either carbenoxolone (n = 4) or probenecid (n = 3) compared to controls (n = 6; Figure 4D) and suggests a further mechanistic dissociation between ATP and adenosine release and the direct and parallel release of adenosine *per se* following activation of GI mGluRs.

3.5 Activation of $P2Y_1$ receptors by ATP is required for triggering DHPG-induced oscillations

We investigated the functional consequences of DHPG-induced ATP release via the use of P2 receptor antagonists. Since previous studies have suggested a relationship between $P2X_7$ receptors and Panx1 channels in mediating ATP release and intercellular calcium signalling (De Bock et al., 2014), we tested the $P2X_7$ receptor antagonist A438079 against DHPG-induced bursting. A438079 (1 µM) had no effect on DHPG-induced bursting (5.2 ± 0.8 bpm; n = 3; Figure 4F) compared to control slices (3.7 ± 0.6 bpm; n = 14).

In contrast, $P2Y_1$ receptor activation, which plays an important role in intercellular calcium waves amongst neurones and astrocytes (Leybaert and Sanderson, 2012), was necessary for DHPG-induced bursting. Hippocampal slices pre-incubated with the selective $P2Y_1$ receptor antagonist MRS 2179 (10 µM) showed almost no bursting in response to DHPG (Figures 4E, F, n = 7). In 5/7 experiments, DHPG triggered only a single burst shortly after DHPG

application, but no further repetitive bursts. This behaviour indicates that $P2Y_1$ receptor activation is a necessary step in maintaining the bursting activity triggered by GI mGluR activation, and suggests that potentially astrocytic $P2Y_1$ receptors play a key role in synchronising neuronal activity (Figure 5).

4 Discussion

In this study we have shown that the activation of GI mGluRs, and in particular mGlu5, in hippocampal slices leads to bursting triggered by the release of ATP via Panx1 channels in area CA3 and the subsequent activation of $P2Y_1$ receptors. In contrast, the GI mGluR-induced release of adenosine in area CA3 was independent of prior ATP release and regulated GI mGluR-evoked burst firing via adenosine A_1 receptors. These observations reveal a profound and novel purinergic bidirectional modulation of GI mGluR-mediated neuronal activity that has implications for neurological, psychiatric and neurodevelopmental conditions in which GI mGluRs are implicated (D'Antoni et al., 2014).

4.1 Purine release during mGluR-dependent oscillations

Repetitive oscillations of the neuronal network, which were sustained for tens of minutes, were produced by the group I mGluR agonist DHPG and were present even when the agonist was washed out. This has been reported by others (Merlin and Wong, 1997), and was proposed to be the GI mGluR basis for epileptogenesis (Bianchi et al., 2009). When adenosine biosensors were used, adenosine release could be observed (measuring ~2 µM in CA3) in response to DHPG-induced activity. Adenosine release in response to GI mGluR activation has previously been observed *in vivo* (Bennett et al., 2000) and *in vitro* (Sims et al., 2013), and is in line with the wide body of evidence that epileptic seizures or *in vitro* epileptiform activity is accompanied by a release of adenosine that limits seizure activity (Dale and Frenguelli, 2009).

Adenosine could be released prior to DHPG-induced bursting, in 400 µm slices not supporting bursting activity, or even when neuronal activity was completely prevented by tetrodotoxin. This assumes a more direct link between GI mGluR activation and the release of adenosine. Such appreciable and parallel release of an inhibitory modulator might be viewed as a homeostatic mechanism to limit the hyperexcitability of the network (Diogenes et al., 2014), or to impose a rhythm upon burst firing. However, whilst the mGlu5-selective antagonist MPEP prevented DHPG-induced oscillations, it was without effect on adenosine release suggesting a dissociation in the actions of the GI mGluRs, which may explain the release of adenosine in the absence of, or prior to, bursting.

Since astrocytes do not require neuronal activity for Ca^{2+} oscillations to occur, as has been shown in astrocytic cultures (Bowser and Khakh, 2007) or in slices (Aguado et al., 2002), one might speculate that astrocytic Ca^{2+} waves, which are known to provoke ATP release, could potentially contribute to the adenosine release detected on sensors. Indeed, we could detect the release of ATP in response to DHPG, which was corroborated by an increase in phosphate production as measured by the malachite green phosphate assay, and an increase in the ATP sensor signal in response to the ecto-ATPase inhibitor ARL 67156. This manipulation resulted in an increase in the duration of bursts, which may have arisen through either increased activation of excitatory P2 receptors (Lopatar et al., 2011), or decreased production of the inhibitory neuromodulator adenosine (Dale and Frenguelli, 2009).

The biosensor data showed that the ATP release is region-specific: while no appreciable ATP could be detected in the CA1 region, the sensors detected ATP in the CA3 region, although in quantities (< 400 nM) that cannot explain the amount of adenosine produced in the same region (~ 5 times that of ATP). Rather, it is likely that the release of adenosine and ATP are two separate processes. This conclusion is supported by the differential effects of the hemichannel blockers probenecid and carbenoxolone on ATP and adenosine release, and reminiscent of previous findings regarding temporal and mechanistic differences in ATP and adenosine release during oxygen/glucose deprivation in hippocampal slices (Frenguelli et al., 2007).

4.2 Source of purine release during mGluR-dependent oscillations

A variety of conduits have been described as mediating the release of ATP during neuronal and astrocytic activity. These include $P2X_7$ receptors and hemichannels (De Bock et al., 2014; Leybaert and Sanderson, 2012; Penuela et al., 2013). Whereas the $P2X_7$ receptor antagonist A438079 had no effect on DHPG-induced bursting, the fact that probenecid, as well as a low concentration of carbenoxolone, strongly suppressed both DHPG-induced neuronal activity and the release of ATP is suggestive of the requirement of Pannexin-1 hemichannels for the induction of this activity. Pannexin-1 hemichannels, unlike connexins, which form junctions between two adjacent cells, preferentially form a channel with the extracellular environment permeable to ATP (Iglesias et al., 2009; Leybaert and Sanderson, 2012; Locovei et al., 2006; Suadicani et al., 2012). Furthermore, Panx1 is activated by elevated extracellular K^+ (Scemes and Spray, 2012; Silverman et al., 2009) which would be expected during the synchronised neuronal bursting induced by DHPG. These data suggest that DHPG-evoked

release of ATP originates via Panx1 channels to either cause direct excitatory actions on neuronal activity or to provoke release of glutamate (Figure 5).

4.3 Purinergic regulation of mGluR-dependent oscillations

Given the evidence for DHPG-induced ATP release in area CA3, we asked as to what physiological role this ATP may have. Previous work has shown that $P2Y_1$ receptor activation results in the initiation of Ca^{2+} waves amongst astrocytes (Leybaert and Sanderson, 2012). Accordingly, we pretreated slices with the selective $P2Y_1$ receptor antagonist MRS 2179 prior to application of DHPG. In the presence of MRS 2179, a single burst was observed upon DHPG application, but repetitive burst firing was prevented. This suggests that ATP, via $P2Y_1$ receptor activation, triggers glutamate release to maintain recurrent synchronous activity following GI mGluR activation. $P2Y_1$ receptor-dependent glutamate release has been observed previously (Domercq et al., 2006; Jourdain et al., 2007) and our observations indicate that this release may be necessary to sustain GI mGluR-dependent oscillations. To our knowledge this is the first indication of a role of $P2Y_1$ receptors in GI mGluR-dependent neuronal activity.

In contrast, the appearance of adenosine in the extracellular space in both areas CA1 and CA3, which may have arisen directly as adenosine or following extracellular metabolism of ATP, had a pronounced inhibitory effect, via adenosine A_1 receptors, on burst firing evoked by GI mGluR activation. Furthermore, antagonism of A_1 receptors converted the regular DHPG-induced burst firing into an altogether more intense and more frequent burst pattern.

Thus, adenosine A_1 receptors may be important in setting the timing of GI mGluR-dependent oscillations. A modulatory role for adenosine A_1 receptors in cortical oscillations has been described previously (Pietersen et al., 2009), including for oscillations dependent upon release of adenosine from astrocytes (Fellin et al., 2009).

Previous studies in our lab have demonstrated the competing actions of ATP at $P2Y_1$ receptors (Brown and Dale, 2002) and adenosine at A_1 receptors (Brown and Dale, 2000) in the initiation and termination of motor pattern episodes in the frog embryo spinal cord (Dale, 1998, 2002; Dale and Gilday, 1996). Taken together, these observations suggest that the purines exert highly orchestrated actions in an evolutionary-conserved manner across diverse receptor groups in a wide variety of cellular and systems-levels processes.

4.4 Conclusions

In summary, with a combination of pharmacological agents and biosensors to detect ATP and adenosine we have shown that ATP, released specifically in the hippocampal CA3 region via Panx1 hemichannels, may be the agent triggering GI mGluR-dependent burst firing. We further conclude that adenosine is released independently of neuronal activity and that the released adenosine activates A_1 receptors via which it sets the pace for neuronal activity. This previously unappreciated bidirectional regulation of GI mGluR-dependent neuronal activity by the purines adenosine and ATP has implications for the range of physiological and pathological conditions in which GI mGluRs are involved and provides further support for the

contention that receptors for the purines adenosine and ATP may be targets for anti-epileptic drugs (Boison et al., 2013; Franke and Illes, 2014; Henshall et al., 2013).

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Conflict of Interest: ND and BGF are Director and Founder, and Non-Executive Director, respectively, of Sarissa Biomedical Ltd, the company which makes and supplies the adenosine and ATP biosensors purchased for use in this study. JL has been employed on an ad hoc basis by Sarissa Biomedical Ltd to manufacture biosensors.

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Figure legends

Figure 1: Group I mGluR activation triggers long-lasting bursts of neuronal activity and the release of adenosine in hippocampal slices. A) Arrangement of recording (Rec) and stimulating (Stim) electrodes and biosensors for the detection of ATP or adenosine (Ado); pairs of biosensors (ATP/Ado and null; 50 µm in diameter and 500 µm in length) were inserted into the hippocampal CA3 or CA1 region of hippocampal slices. B) Enzymatic cascades involved in signal detection in ATP (Llaudet et al., 2005) and Ado biosensors (Llaudet et al., 2003). Null sensors lack any enzymes. Glycerol (2 mM) is required by the ATP sensor and was present in all experiments C) Bath-application of 25 µM (S)-DHPG induced repetitive bursts (middle trace, with two events expanded below). Adenosine release was observed from area CA3 using the biosensors (top trace). D) A 5 minute application of DHPG resulted in a long-lasting elevation of extracellular adenosine (Ado) levels in area CA3 (top trace) as well as sustained bursting (middle trace and with two events expanded below).

Figure 2: Pharmacological characterization of DHPG-induced bursting in area CA1 and adenosine release from area CA3.

A) Antagonism of adenosine A_1 receptors using 8-CPT (1 µM) greatly increased DHPG-induced burst frequency and intensity in area CA1 (middle trace, with examples of burst activity expanded below) and resulted in additional extracellular adenosine release in area CA3 (Ado; top trace). Real time gap in trace reflects experimental artefact. B) No effect of adenosine A_{2A} receptor antagonist ZM241385 (100 nM) on burst frequency (CA1; middle trace and burst examples below) or released adenosine (CA3; Ado; top trace). C) TTX completely prevented DHPG-induced bursting (CA1; middle trace) but not adenosine release

(CA3; Ado; top trace). D) Pooled data of CA3 adenosine release upon DHPG application in the presence (n = 3) or absence (n = 6) of TTX. E) Pre-incubation of slices with MPEP, an antagonist of mGlu5, diminished CA1 bursting while F) leaving adenosine levels in area CA3 unchanged compared to controls (n = 5 for MPEP; n = 6 for controls).

Figure 3: GI mGluR activation elicits region-specific release of ATP

A) DHPG induced ATP release in area CA3 (black trace; mean ± SEM; n = 13), but not in the CA1 region (gray trace; mean ± SEM; n = 7). B) Preventing extracellular breakdown of ATP by ARL 67156 resulted in a further increase in ATP in area CA3 (top trace, mean ± SEM; n = 5) above that induced by DHPG (pre–DHPG sensor baseline depicted by lower broken line) and a change in bursting pattern (example shown in middle trace with two DHPG-induced bursts before, left, and after, right, ARL 67156 application). C) Graph showing an increase in the duration of DHPG-induced bursts after ARL 67156 administration in six individual slices (filled circles) and the mean ± SEM for the data (open diamond; * $p < 0.02$; paired t-test; n = 6).

Figure 4: ATP released via Pannexin-1 hemichannels induces DHPG-induced bursts via $P2Y_1$ receptors

Pre-incubation of slices with two hemichannel blockers, carbenoxolone (A, 10 µM) or probenecid (B, 1 mM) resulted in decreased ATP production in area CA3 (top traces) and almost a complete abolition of bursting (middle and lower traces). C) Pooled traces of CA3-specific DHPG-evoked ATP release under control conditions (black trace; n = 13), and with pre-treatment with carbenoxolone (dark grey trace; n = 5) and probenecid (gray trace; n = 5). D) No appreciable change in adenosine levels in area CA3 were observed upon DHPG application when slices were pre-treated with the hemichannel blockers carbenoxolone

(dark grey trace; n = 4) or probenecid (gray trace; n = 3) compared to control (black trace; n = 6), Pre-incubation of slices with the $P2Y_1$ receptor antagonist MRS 2179 (10 μM) completely abolished DHPG-induced repetitive bursting (n = 7). F) Graph summarizing the effects of the hemichannel blockers carbenoxolone (carbenox; n = 9), probenecid (proben; n = 7), the $P2Y_1R$ antagonist MRS 2179 (n = 7), and the $P2X_7R$ antagonist A438079 (n = 3) on the frequency of DHPG-induced bursting compared to control slices (n = 14). Probenecid did not prevent bursting in 1 out of 7 experiments. (** $p < 0.006$; *** $p < 0.0001$; One-way ANOVA)

Figure 5: Putative scheme for bidirectional purinergic regulation of DHPG-induced bursting. Activation of GI mGluRs results in the release of ATP via Pannexin-1 (Panx1) hemichannels and direct release of glutamate and adenosine, potentially from astrocytes. This ATP promotes further glutamate release to excite surrounding neurones via ionotropic glutamate receptors (iGluR) giving rise to TTX-sensitive synaptic activity, upon which an adenosine A_1 receptor-dependent oscillatory rhythm is imposed. This working model does not exclude additional excitatory actions of ATP at postsynaptic P2 receptors, the involvement of neuronal Panx1 hemichannels, or a role for GI mGluRs on pre- and postsynaptic neurones.

Figure 1

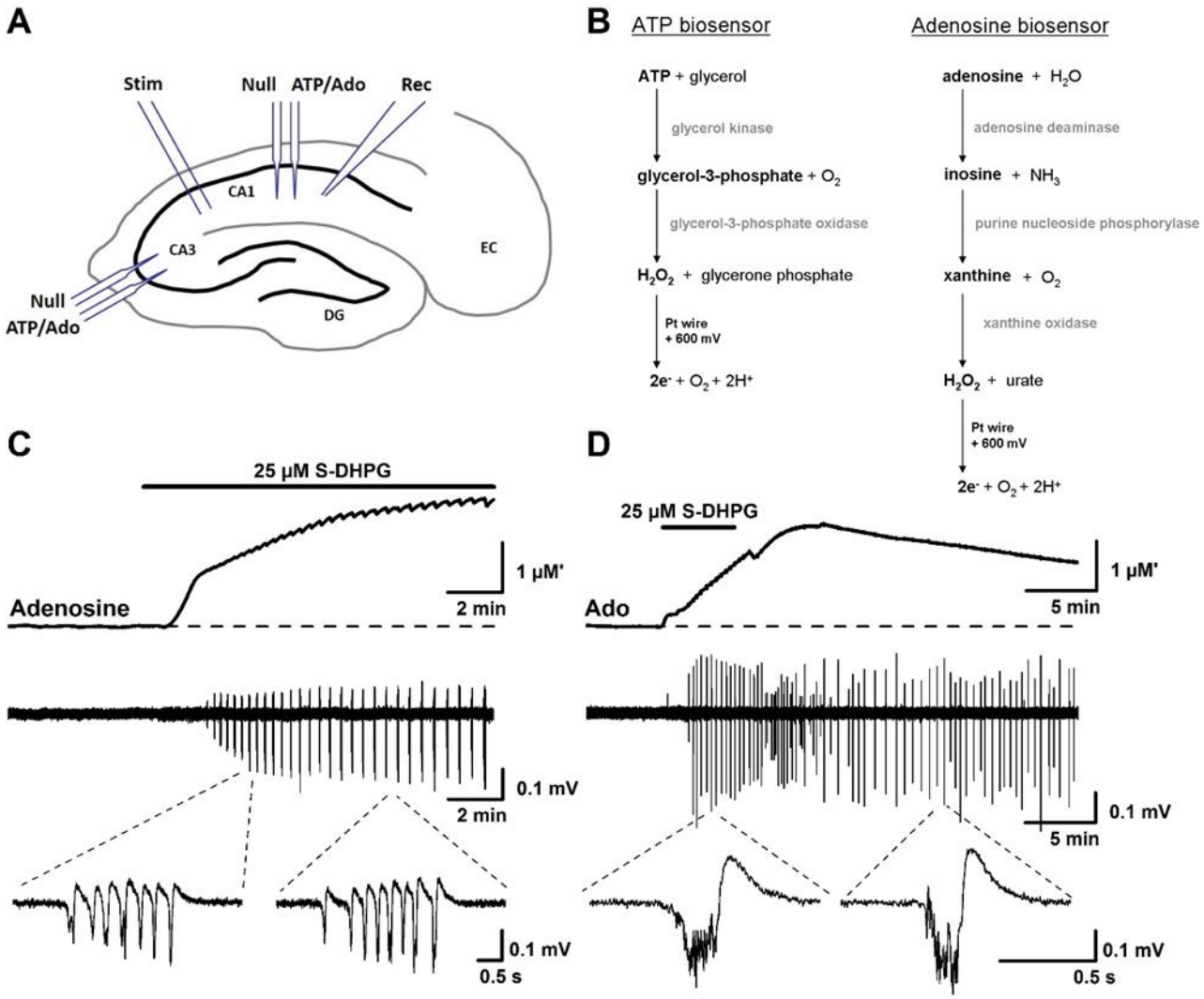
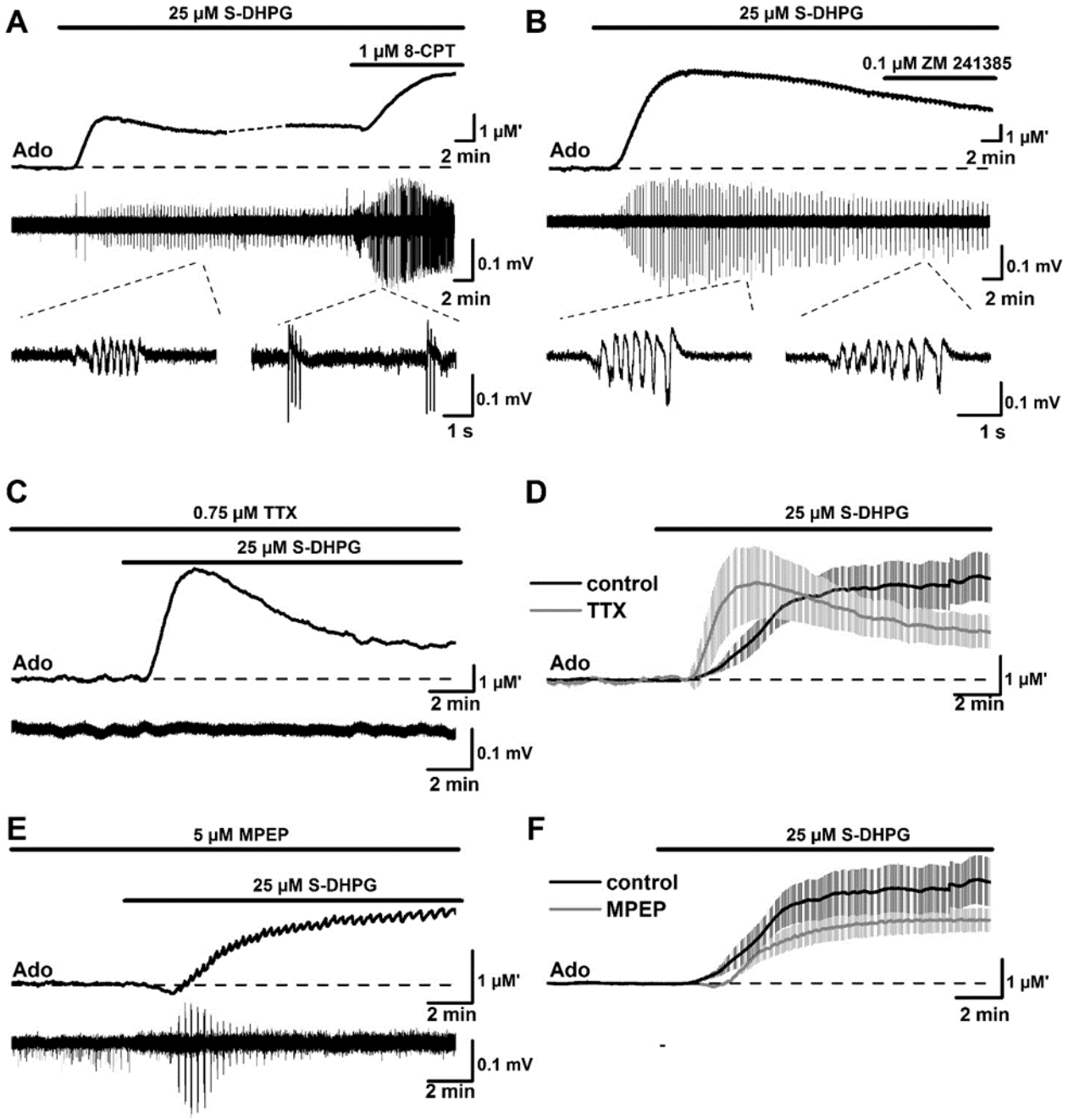

A
Stim
Null
ATP/Ado
Rec
CA1
CA3
EC
DG
Null
ATP/Ado
B
ATP biosensor
ATP + glycerol
glycerol kinase
glycerol-3-phosphate + O2
glycerol-3-phosphate oxidase
H2O2 + glycerone phosphate
Pt wire
+ 600 mV
2e- + O2 + 2H+
Adenosine biosensor
adenosine + H2O
adenosine deaminase
inosine + NH3
purine nucleoside phosphorylase
xanthine + O2
xanthine oxidase
H2O2 + urate
Pt wire
+ 600 mV
2e- + O2 + 2H+
C
25 µM S-DHPG
Adenosine
1 µM'
2 min
0.1 mV
2 min
0.1 mV
0.5 s
D
25 µM S-DHPG
Ado
1 µM'
5 min
0.1 mV
5 min
0.1 mV
0.5 s

Figure 2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25 µM S-DHPG
1 µM 8-CPT
Ado
1 µM'
2 min
0.1 mV
2 min
0.1 mV
1 s
B
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0.1 µM ZM 241385
Ado
1 µM'
2 min
0.1 mV
2 min
0.1 mV
1 s
C
0.75 µM TTX
25 µM S-DHPG
Ado
1 µM'
2 min
0.1 mV
2 min
D
25 µM S-DHPG
control
TTX
Ado
1 µM'
2 min
E
5 µM MPEP
25 µM S-DHPG
Ado
1 µM'
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2 min
F
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control
MPEP
Ado
1 µM'
2 min

Figure 3

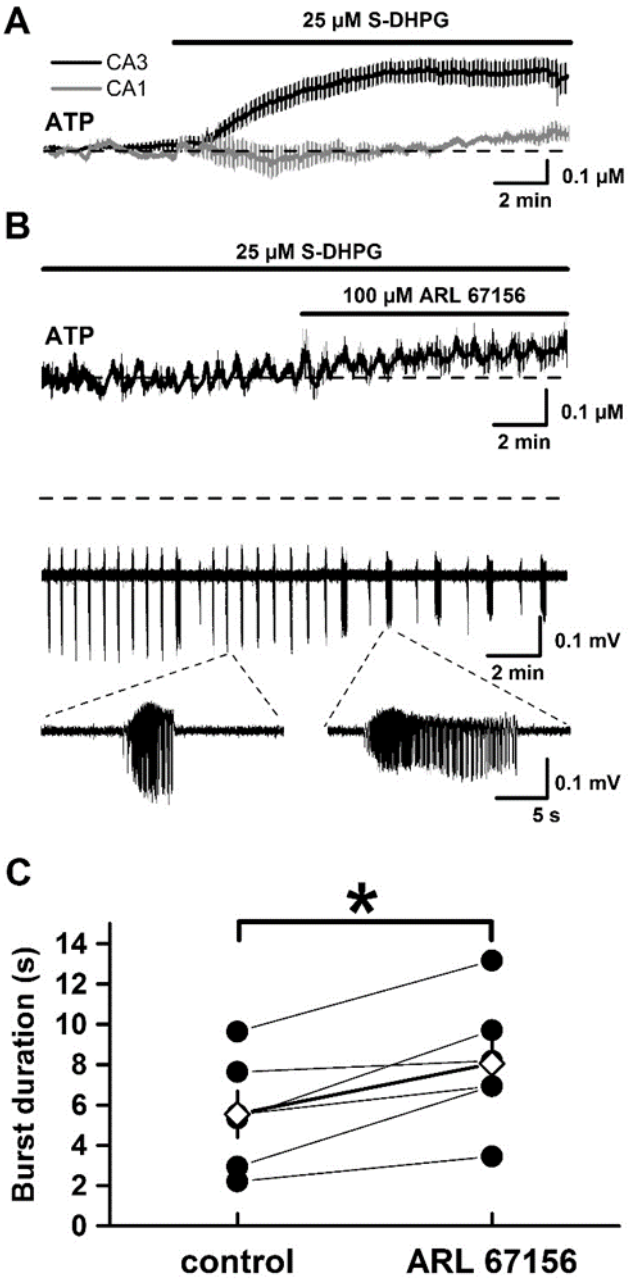
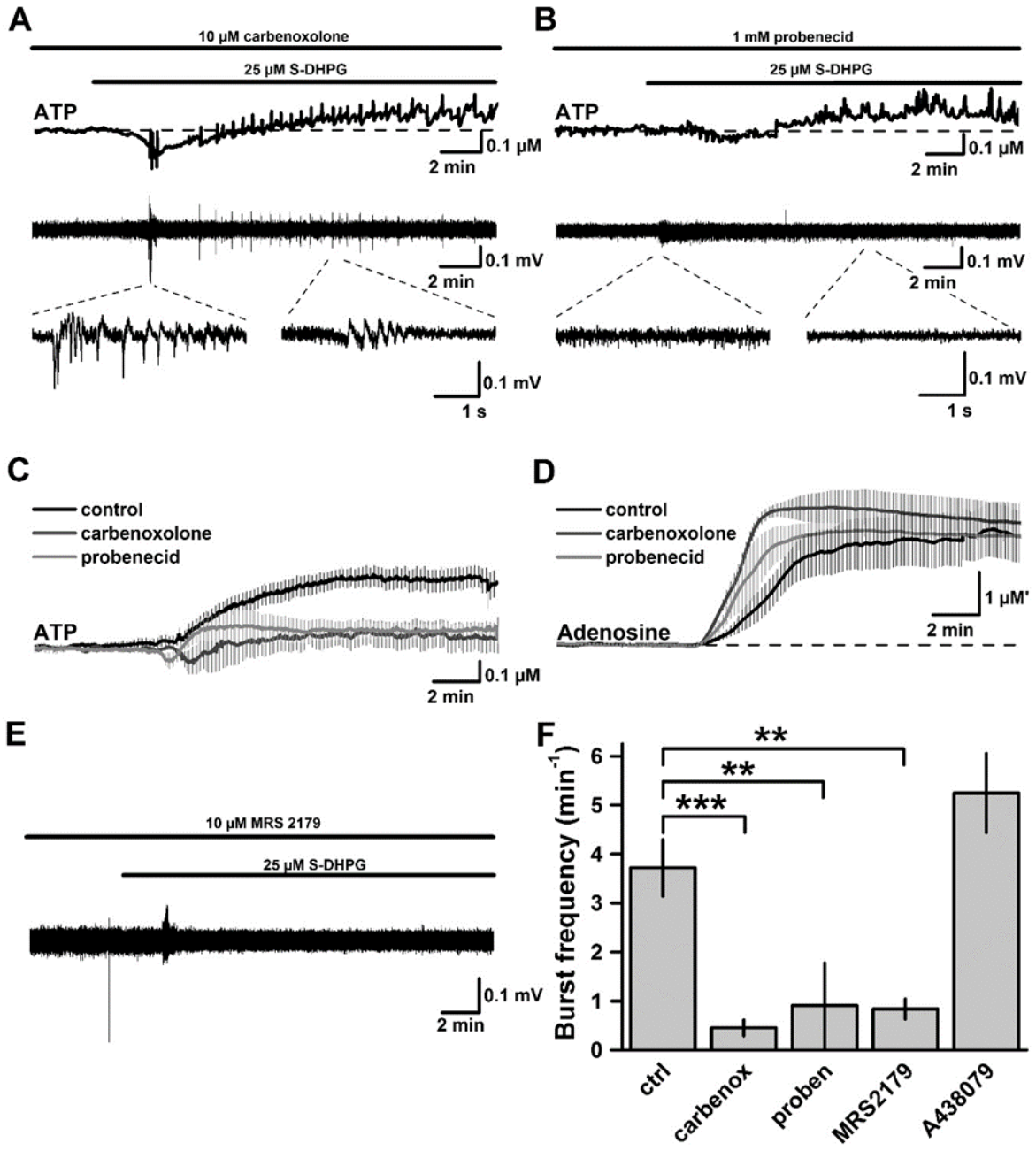

A
25 μM S-DHPG
CA3
CA1
ATP
0.1 μM
2 min
B
25 μM S-DHPG
100 μM ARL 67156
ATP
0.1 μM
2 min
0.1 mV
2 min
0.1 mV
5 s
C
*
Burst duration (s)
14
12
10
8
6
4
2
0
control
ARL 67156

Figure 4


A
10 μM carbenoxolone
25 μM S-DHPG
ATP
0.1 μM
2 min
0.1 mV
2 min
0.1 mV
1 s
B
1 mM probenecid
25 μM S-DHPG
ATP
0.1 μM
2 min
0.1 mV
2 min
0.1 mV
1 s
C
control
carbenoxolone
probenecid
ATP
0.1 μM
2 min
D
control
carbenoxolone
probenecid
1 μM'
Adenosine
2 min
E
10 μM MRS 2179
25 μM S-DHPG
0.1 mV
2 min
F
Burst frequency (min-1)
0
1
2
3
4
5
6
**
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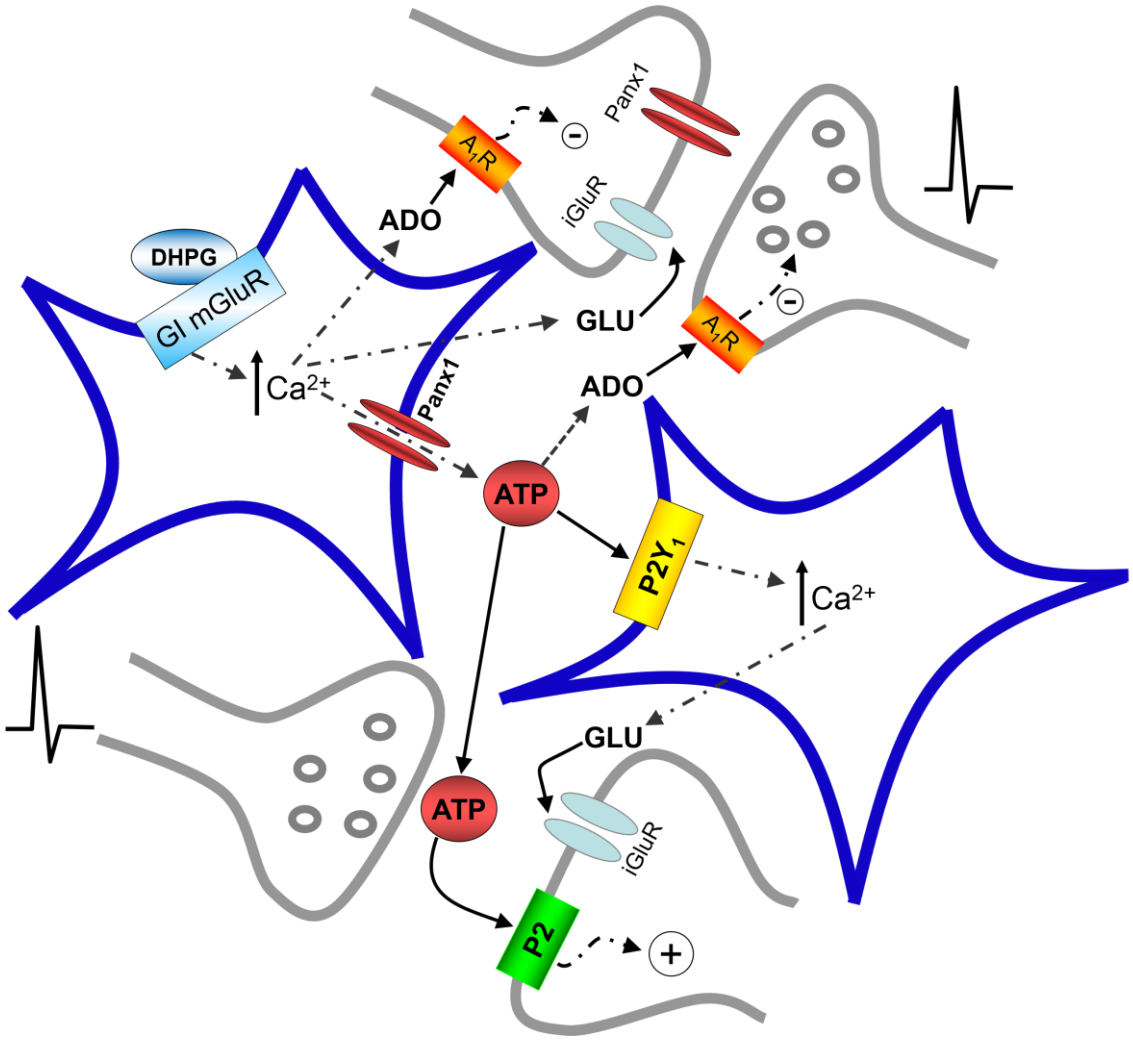
ctrl
carbenox
proben
MRS2179
A438079

Figure 5


Panx1
A1R
ADO
iGluR
DHPG
Gl mGluR
GLU
Ca2+
Panx1
A1R
ADO
ATP
P2Y1
Ca2+
GLU
ATP
iGluR
P2