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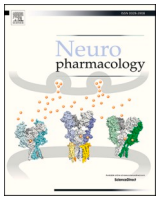

Antagonism of P2X7 receptors enhances lorazepam action in delaying seizure onset in an *in vitro* model of status epilepticus.

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ABSTRACT

Approximately 30% of patients with status epilepticus (SE) become refractory to two or more antiseizure medications (ASMs). There is thus a real need to identify novel targets against which to develop new ASMs for treating this clinical emergency. Among purinergic receptors, the ionotropic ATP–gated P2X7 receptor (P2X7R) has received attention as a potential ASM target. This study evaluated the effect of the selective P2X7R antagonist A740003 on acute seizures in the dentate gyrus (DG) of hippocampal brain slices, where P2X7Rs are highly expressed, with a view to establishing the potential of P2X7R antagonists as a therapy or adjunct with lorazepam (LZP) in refractory SE. Extracellular electrophysiological recordings were made from the DG of male mouse hippocampal slices. Spontaneous seizure-like events (SLEs) were induced by removing extracellular Mg^{2+} and sequentially adding the K^+ channel blocker 4-aminopyridine and the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline, during which the early and late application of A740003 and/or lorazepam was evaluated. Our study revealed that, in the absence of changes in mRNA for P2X7Rs or inflammatory markers, P2X7R antagonism did not reduce the frequency of SLEs. However, A740003 in conjunction with LZP delayed the onset of seizures. Furthermore, our results support the need for employing LZP before seizures become refractory during SE as delayed application of LZP increased seizure frequency. These studies reveal possible sites of intervention that could have a positive impact in patients with high risk of suffering SE.

1. Introduction

Thirty percent of the adult population with epilepsy experience poor seizure control with commercially-available antiseizure medications (ASMs) (Golyala and Kwan, 2017; Chen et al., 2018; Burman et al., 2022). There is therefore a need to find new molecular targets against which to develop novel ASMs. Possible targets may emerge from purinergic signalling, which is mediated by purine nucleotides and nucleosides such as ATP and adenosine, respectively. While adenosine is an important endogenous anticonvulsant, ATP is an extracellular-signalling molecule able to bidirectionally modulate seizure-activity by activating ionotropic P2X and some metabotropic P2Y receptors (Dale and Frenguelli, 2009; Dossi et al., 2018; Beamer et al., 2021; Engel et al., 2021). In particular, among all P2 receptors, a potential therapeutic role of the P2X7 receptor (P2X7R) in refractory epilepsy has been proposed (Song et al., 2019; Engel et al., 2021; Beamer et al., 2022; Guerra Leal et al., 2022).

P2X7Rs are expressed in the hippocampus with the highest expression localized in the dentate gyrus (DG) compared with other hippocampal subregions such as CA3 or CA1 (Kaczmarek-Hajek et al., 2018; Ramirez-Fernandez et al., 2020). Functional P2X7Rs in the DG have previously been reported especially in the neural progenitor cells (NPCs) localised in the subgranular zone of the DG (Messemer et al., 2013) and in astrocytes of the DG (Leeson et al., 2018; Khan et al., 2019). In the DG, P2X7Rs have several physiological roles ranging from the Ca^{2+}-dependent regulation of proliferation during neurogenesis, to the initiation of cell death upon excessive activation (Leeson et al., 2019).

In this latter regard, increased expression of hippocampal P2X7R levels have been described in the DG after status epilepticus (SE), a clinical emergency characterised by seizures lasting longer than 5 min, in different wild-type and P2X7R mutant mouse models: (1) an *in vivo* model of SE induced by intra-amygdala kainic acid injection (Engel et al., 2012; Jimenez-Pacheco et al., 2016; Kaczmarek-Hajek et al., 2018; Morgan et al., 2020; Ramirez-Fernandez et al., 2020; Beamer et al., 2022), (2) isolated microglia from an *in vivo* mouse model of intra-peritoneal kainic acid injection (Rappold et al., 2006) and (3) *in vivo* rat and mouse models of SE induced by intra-peritoneal or

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intra-cerebroventricular pilocarpine injection (Dona et al., 2009; Rozmer et al., 2017).

Given this evidence for increased P2X7R expression in response to seizure activity, several studies have proposed that a combination of a P2X7R antagonist (such as A438079, BBG, AFC-5128, JNJ-47965567 and tanshinone IIA sulfonate) with other ASMs (for instance, lorazepam, ceftriaxone or carbamazepine) could be used as a possible adjunct therapy to prevent or delay seizure onset (Engel et al., 2012; Soni et al., 2015; Fischer et al., 2016). Nevertheless, contradictory results have been published when using P2X7 as a possible adjunct ASM after SE or during seizure activity (see Supplementary Table 1).

To simplify the question as to P2X7R involvement in seizure activity, and to test their targeting as adjuncts in SE, we examined the role of P2X7Rs in the absence or presence of a commonly-used ASM for SE, lorazepam, specifically in the DG during seizure activity and after pharmacologically-induced SE using an *in vitro* model in acutely-prepared rodent brain slices (Lopatář et al., 2011; Hall and Frenguelli, 2018). We found that, while there was no obvious role for P2X7Rs in acute seizure activity, or changes in mRNA for P2X7Rs or inflammatory markers, co-application of a P2X7R antagonist with lorazepam delayed entry into a SE-like state. These observations suggest that antagonists of P2X7Rs may be useful adjuncts to therapies for status epilepticus.

2. Material and methods

2.1. Drugs and chemicals

8-cyclopentyltheophylline, (8-CPT; C102), 4-aminopyridine (4-AP; 275875) and lorazepam (LZP, 846-49-1), $CaCl_2$ and $MgCl_2$ were purchased from Sigma Aldrich. N-[1-[[(Cyanoamino)(5-quinolinylamino) methylene]amino]-2,2-dimethylpropyl]-3,4-dimethoxybenzeneacetamide (A740003; 3701) was purchased from Tocris Biosciences. All salts for the artificial cerebrospinal fluid (aCSF), NaCl, KCl, NaH_2PO_4, $NaHCO_3$, $MgSO_4$, and D-glucose, were purchased from Fisher Scientific.

2.2. Hippocampal slice preparation

Male mice of 6–13 weeks old were killed by an overdose of isoflurane and then decapitated after cervical dislocation in accordance with Schedule 1 of the UK Government Animals (Scientific procedures) Act. 1986 and with local Ethical Review procedures, and with approval from the University of Warwick Animal Welfare and Ethical Review Board (AWERB), approval number G13-19. The brain was quickly removed after decapitation and placed into ice-cold physiological saline containing (mM): NaCl (130), KCl (2.7), $CaCl_2$ (0.5), $MgCl_2$ (3.5), NaH_2PO_4 (1), $NaHCO_3$ (18), glucose (15), and pH of 7.4 gassed with 95% O_2/5% CO_2. Parasagittal hippocampal brain slices (400 μm) were cut on a vibratome (vibrating microtome 7000smz-2, Campden Instruments Ltd.) at 4 °C. Then, brain slices were placed in aCSF containing (mM): NaCl (130), KCl (2.7), $CaCl_2$ (2), $MgCl_2$ (1), NaH_2PO_4 (1), $NaHCO_3$ (18), glucose (15), pH of 7.4 gassed with 95% O_2/5% CO_2 and kept at 34 °C upon a mesh support in an incubation chamber (250 ml) for 1–1.5 h prior to recording. (Hall and Frenguelli, 2018; Privitera et al., 2020).

2.3. Electrophysiological recordings and drug application

Post recovery, individual slices were placed on a submerged mesh in a recording chamber, secured with a platinum harp with nylon threads and were perfused with oxygenated aCSF at a rate of ~6.0 ml/min and maintained at 32 ± 0.5 °C. The mesh ensured adequate perfusion of the slice, allowing aCSF to flow below and above the slice. Slices underwent a period of electrical stimulation of the perforant path in the molecular layer in the DG prior to experiments to ensure viability of the slice. A concentric bipolar electrode (30205 FHC, stainless steel outer pole diameter 200 μm, platinum-iridium inner pole diameter 25 μm, length 75 mm, tip rounded) was placed in the entorhinal cortex. Extracellular field excitatory postsynaptic potentials (fEPSP) were evoked using square-wave pulses (0.1 ms in duration) at 10 s intervals. A glass microelectrode filled with aCSF, as described previously (Frenguelli et al., 2007), was used to record neuronal activity (both evoked and spontaneous) and was placed in the molecular layer of the DG. Since P2X7Rs are blocked by cations, we use low cationic solutions (0 mM Mg^{2+}, 0.5 mM Ca^{2+}) to enhance the activation of P2X7Rs. Stimulus parameters and acquisition of fEPSPs were under the control of WinLTP software (WinLTP; Anderson et al., 2007). Once tissue viability was established, electrical stimulation was switched off to allow spontaneous seizures to be observed. Spontaneous activity was captured and analysed using Spike2 software (Spike2 Software CED, Cambridge, UK). Sample rate for the electrophysiology data was 10,000 Hz. A 15–20 min baseline was recorded prior to the bath-application of 0 mM Mg^{2+} aCSF. Three seizure-inducing solutions were used to induced spontaneous seizure-like activity each of which were bath-perfused following this protocol: (1) 30 min in 0 mM Mg^{2+} aCSF, followed by (2) 20 min in 4-AP (50 μM) + 0 mM Mg^{2+} aCSF and (3) 60 min in 8-CPT (1 μM) + 4-AP (50 μM) + 0 mM Mg^{2+} aCSF, the latter of which induced SE-like activity. All pharmacological agents were applied at the desired concentration via the corresponding seizure-inducing solution. Recordings lasted between 1 h 30 min - 2 h 30 min h per slice.

2.4. RNA extraction and real time quantitative PCR

For the assessment of potential changes in mRNA expression of P2X7Rs and inflammatory markers, brain slices (400 μm) were incubated in a chamber with 0 mM Mg^{2+} where 4-AP (50 μM) and 8-CPT (1 μM) were successively added. Control slices were kept in regular aCSF. Both chambers were maintained at 33 °C in a water bath and slices were continuously oxygenated with 95% O_2/5% CO_2. At specific times (15 min, 30 min, 1 h and 2 h after the addition of 8-CPT) slices were removed and the Invitrogen™ TRIzol™ protocol was followed to isolate high quality total RNA from hippocampal slices. For extra RNA purification, RNeasy® Lipid Tissue Mini Kit (Cat # 74804, Qiagen, Valencia, CA, USA) was used following the recommended protocol. Pure mRNA was then re-dissolved in RNase-free water. Quantification of mRNA was measured by Nanodrop (Thermo Fisher Scientific™). Final mRNA samples were then treated with DNase I (RNase-free, New England BioLabs Inc.) to avoid genomic DNA contamination and cDNA was synthesised using LunaScript™ RT SuperMix Kit (New England BioLabs Inc.). Finally, qPCR was performed on cDNA using SYBR™ Green PCR Master Mix (Applied Biosystems™) according to the manufacturer's instructions using Mx3005P qPCR System (Agilent Technologies). Samples were run using the following programme: (1) Pre-incubation (95 °C, 15 min), (2) Amplification, 50 cycles (95 °C, 15 s, followed by 55 °C, 20 s, and 72 °C, 40 s), (3) Melting Curve (95 °C, 10 s, followed by 65 °C, 15 s, and 95 °C, 30 s). Each sample was processed in triplicates. Primer sequences were as follows: β-actin (*ACTB)*: forward, 5′-gggtgtgatggtgggaatgg-3'; reverse 5′-ggacttgggattccggttgg-3'; P2X7R (*P2rx7)*: forward, 5′-ctggcaggtgtgttccata-3'; reverse 5′-gtgctctttgtagaacggtt-3' (Jimenez-Pacheco et al., 2016); IL-1β (*Il1b)*: forward, 5′-tgaagttgacggaccccaaa-3'; reverse 5′-taacaccagcacctcttcga-3'; Iba1 (*Aif1)*: forward, 5′-tggaggggatcaacaagcaa-3'; reverse 5′-accccaagtttctccagcat-3' (Jimenez-Mateos et al., 2015). Primer specificity was confirmed using Primer-BLAST (National Center for Biotechnology Information, NCBI). Relative quantification of mRNA transcript levels were assessed by normalising to β-actin using the standard $2^{-\Delta\Delta CT}$ method (Rao et al., 2013). Further normalisation of the data was done by dividing the average of the controls per sample (from each day) to calculate the fold change.

2.5. Signal acquisition, processing, and analysis

For the quantification of drug effects on epileptiform activity, two

parameters were determined: spontaneous seizure-like event (SLE) frequency (in Hz) and amplitude (in mV). This analysis was performed using the available tools of the Spike2 software.

For statistical analysis, paired t-tests were used to compare control (defined as seizure activity recorded 10 min before drug application) with A740003 or LZP in the same slice. Otherwise, two sample t-tests were used to compare between different slices, in the presence of A740004, LZP or A740003 + LZP, with the control slices. Statistical significance is given as *$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$. One way ANOVA was used for multiple comparisons followed by Holm-Bonferroni correction.

Graphs were drawn and statistical analyses were performed in OriginPro 2019 software. In boxplot graphs (box = mean ± SEM and whiskers = ± SD), individual symbols represent individual slices. Bar graphs represents mean ± SEM and the control condition is represented by 100%, dotted black line. For the electrophysiological measurements, "n" values refer to the number of slices per experimental condition and "N" numbers refer to the number of mice.

3. Results

3.1. Pharmacologically-induced seizure-like events (SLEs) in hippocampal slices

The protocol to induce seizure activity is shown in Fig. 1A: 30 min in 0 mM Mg^{2+} medium followed by 20 min in 4-AP (50 μM) + 0 mM Mg^{2+} medium and 60 min in 8-CPT (1 μM) + 4-AP (50 μM) + 0 mM Mg^{2+} medium. To simplify the nomenclature, we refer to "4-AP medium" and "8-CPT medium" the seizure-inducing solutions composed of 4-AP + 0 mM Mg^{2+} and by 8-CPT + 4-AP + 0 mM Mg^{2+}, respectively. The type of spontaneous seizures induced varied between bursting activity (periods of electrophysiological activity with intervening long periods of quiescence), inter-ictal activity (periodic ictal spiking activity interrupted by periods of electrical quiescence) and SE-like activity (prolonged intense spiking that lacked periods of quiescence).

Removal of extracellular Mg^{2+} for at least 10 min resulted in seizure-like events (SLEs) in the DG, with bursting activity recorded in 74% of the slices analysed (n_{slices} = 23). Subsequent application of the K^+ channel blocker 4-AP 30 min after the perfusion of nominally Mg^{2+}-free aCSF caused a profound exacerbation of pharmacologically-induced spontaneous SLEs with SLEs being more intense (frequency 1.94 ± 0.70 Hz) compared with SLE frequency in 0 mM Mg^{2+} medium (1.05 ± 0.39 Hz, Supplementary Table 2). Inter-ictal activity was predominantly seen in 4-AP medium (39% of 23 slices analysed). The most robust seizure-inducing solution was 8-CPT medium where 82% of the total slices analysed (n_{slices} = 23) showed a highly reproducible SE-like activity where the average of the spontaneous SLEs frequency was higher than in 0 mM Mg^{2+} and 4-AP medium (3.76 ± 1.04 Hz, Supplementary Table 2).

3.2. Pro-seizure effect of late application of lorazepam after in vitro pharmacological SE

To establish whether our SE-like seizure activity resembled SE activity in humans, in which resistance to benzodiazepines emerges over time (Burman et al., 2019), we tested the effects of late application of lorazepam, 60 min after pharmacologically-induced SE, with a concentration range (1–30 μM) commonly used by others (Shen et al., 2002, 2005; Sanna et al., 2005; Wang et al., 2021; Crocetti et al., 2022). We compared seizure activity 10 min before drug application with that in the 10 min after drug application in the same slice (Fig. 1A, panels a1 and a2, respectively). To ensure that over this period seizure activity in control slices was stable and suitable for assessing any drug-induced changes, we compared these 10 min epochs in the absence of any drugs for the frequency and amplitude of the SLEs. This analysis showed that over this time window seizure activity was stable (Fig. 1B and C).

Having established the stability of the SE-like activity, we applied the $GABA_A$ receptor positive allosteric modulator lorazepam (LZP) 60 min after pharmacologically-induced SE (by 8-CPT medium) to test its effects during SE-like activity. In contrast to the expected attenuation of seizure activity by lorazepam, we found that the number of spontaneous SLEs increased in an LZP concentration-dependent manner (LZP: 1, 10 30 μM) compared with the control condition (10 min before LZP application) (Fig. 2A, B, C, respectively). Thus, while there was no change in SLE

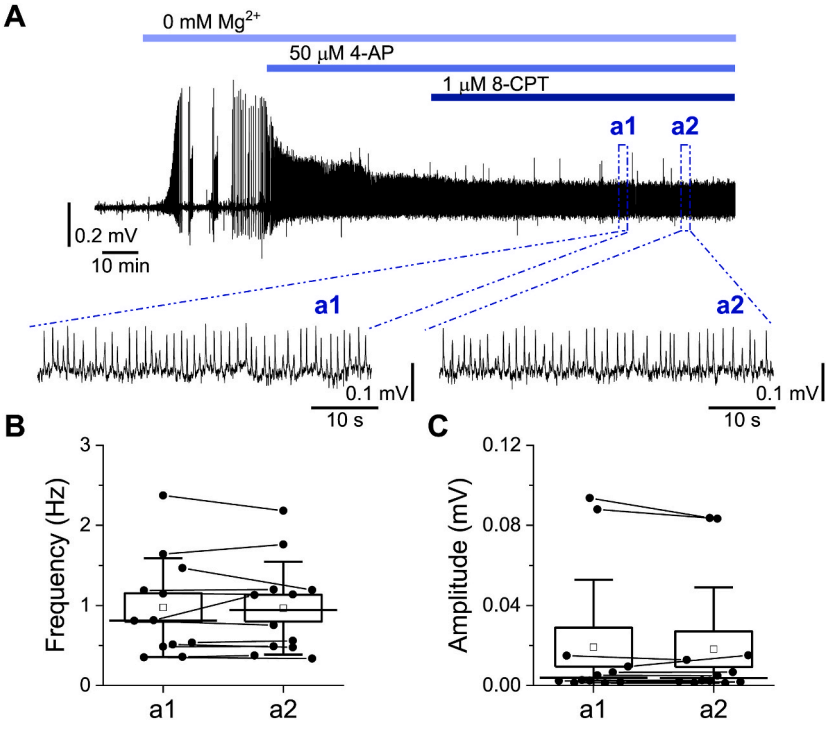

Fig. 1. Representative seizure profile and stability of late seizure activity.
(A) Protocol to induce spontaneous seizure–like activity in the dentate gyrus: 30 min of 0 mM Mg^{2+} aCSF, followed by 4-AP (50 μM) application. After 20 min of 4-AP, 8-CPT (1 μM) was added to the medium, which elicited status epilepticus–like activity. (B) Frequency and (C) amplitude of the spontaneous seizure–like events during the two 10 min comparison time periods (10 min before any drug application, panel a1) and after any drug application time (60 min after SE–like activity was induced, panel a2). (B) and (C) show that the time gap between both periods did not affect the seizure activity. Boxplots (box = mean ± SEM and whiskers = ± SD), mean is shown as the central square and median as a line. Paired sample *t*-test was used to compare a2 with internal control (a1) from the same slice. Neither frequency nor amplitude differed over this time period. n_{slices} = 12, N_{mice} = 12. Statistical analysis is shown in Supplementary Table 3.

amplitude (Fig. 3A and B), lorazepam provoked an increase in the frequency of SLEs (Fig. 3C, mean ± SEM; control: 0.42 ± 0.06 Hz; LZP: 1 μM 0.46 ± 0.07 Hz; 10 μM 0.70 ± 0.18 Hz and 30 μM 0.58 ± 0.14 Hz). The change of frequency was significantly different in two of the three LZP concentration groups compared with the control group (Fig. 3D). Overall, LZP application 60 min after SE-like activity was initiated increased the frequency of the seizure activity by 16% (1 μM LZP), 38% (10 μM LZP) and 75% (30 μM LZP) compared to the control group (represented by 100%). We conclude that our *in vitro* model of SE–like activity, like prolonged SE in humans, develops resistance to the inhibitory effects of lorazepam.

3.3. Early application of lorazepam delayed the onset of the seizures and reduced acute seizure activity

Since late application (60 min after *in vitro* SE) of LZP resulted in resistance to the inhibitory effects of LZP, and indeed in an enhancement of the SE-like activity, we tested whether early application of LZP resulted in the anticipated attenuation of seizure activity. To do this, we added LZP (1, 10 or 30 μM) to all seizure-inducing solutions, except those solutions used for control slices (Fig. 4).

Our data showed that the early application of LZP resulted in a concentration-dependent delay in the onset of the seizures, defined as the time when the first SLEs appeared, compared with the control group (Fig. 5A). This delay achieved statistical significance for 30 μM LZP (Fig. 5A; seizure onset delay, mean ± SEM, 663 ± 103 s in control group vs. 1374 ± 228 s in 30 μM LZP group). Expressed as a percentage of the time to the onset of SLEs in control slices, LZP delayed the onset of seizure activity in a concentration-dependent manner by 58%, 93% and 155% compared with the control (100%) versus LZP at 1, 10, 30 μM, respectively, and was statistically significant at 30 μM LZP (Fig. 5B). Furthermore, in 4-AP medium, the frequency of the seizure activity (Fig. 5C) was reduced by 42% and 43% when 10 μM and 30 μM LZP was bath-applied, respectively (Fig. 5D), with no effect on SLE amplitude (Fig. 5E). The same LZP concentrations in 8-CPT medium provoked only a minor change in the frequency of SLEs (Fig. 5F and G), with no effect on SLE amplitude (Fig. 5H).

3.4. No obvious contribution of P2X7Rs to seizure activity in the mouse dentate gyrus

Given our *in vitro* observations and published clinical findings regarding resistance to the inhibitory effects of benzodiazepines after prolonged seizure activity, we explored a possible alternative strategy for seizure suppression after protracted seizures: the P2X7 receptor (P2X7R). In order to activate P2X7Rs, high concentrations of extracellular ATP (>100 μM) or long exposure to ATP is required (Linden et al., 2019). Such situations may arise when synaptic transmission is enhanced, for example during epileptic seizures. Moreover, since endogenous protein expression of P2X7Rs is higher in the DG than in other hippocampal subregions (Kaczmarek-Hajek et al., 2018; Ramirez-Fernandez et al., 2020) and overexpression of hippocampal P2X7Rs has been reported after *in vivo* SE induced by intra-amygdala kainic acid injection (Engel et al., 2012; Jimenez-Pacheco et al., 2016), the potential involvement of P2X7Rs in long-duration seizure activity is plausible. We thus wanted to establish whether P2X7Rs might be at least partially responsible for the seizure-like activity in the DG after pharmacologically-induced SE *in vitro*.

Using the protocol described previously (Fig. 1A) the selective competitive P2X7 antagonist A740003 (Honore et al., 2006; Donnelly-Roberts et al., 2009) was added to the 8-CPT medium at a concentration (10 μM) previously used by others to selectively antagonise P2X7R-mediated responses in a wide variety of experimental settings ((Deeb et al., 2012; Schulz et al., 2012; Facci et al., 2014; Facci et al., 2018; Zhang et al., 2023); Fig. 6A). However, under these conditions (Fig. 6A), A740003 did not change either the frequency (Fig. 6B) or the amplitude (Fig. 6C) of the SLEs induced by 8-CPT medium compared with the internal slice control (SLEs interval analysed 10 min before drug application, Fig. 6A, panel a1 vs panel a2). These observations are consistent with the lack of an appreciable P2XR component in seizure activity in the rat CA1 region (Lopatář et al., 2011).

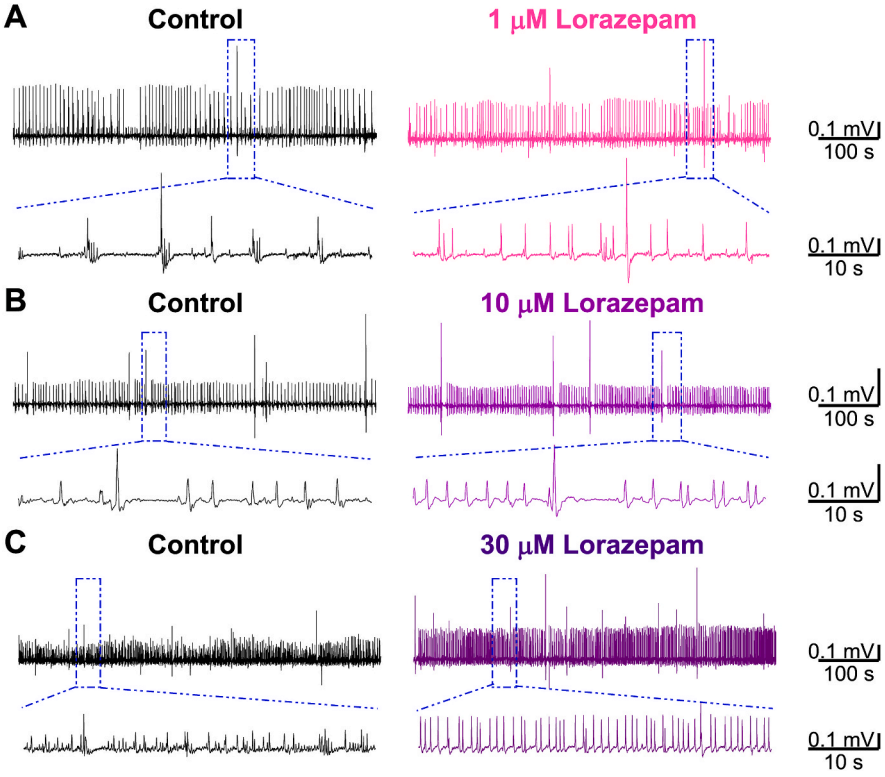

Fig. 2. Late application of lorazepam enhances seizure-like events in the dentate gyrus 60 min after pharmacological SE was induced.
Mouse hippocampal slices (400 μm) were incubated with 0 mM Mg^{2+}. 4-AP (50 μM) and 8-CPT (1 μM) were added consecutively after 30 min and 20 min of incubation, respectively. Control periods per slice were selected 10 min before lorazepam (LZP) was bath-perfused. (A–C) Concentration–response experiments for increasing concentrations of LZP (1, 10 and 30 μM). LZP was added 60 min after SE was induced by 8-CPT medium. Blue boxes mark the source of the expanded traces. Note the increase in the frequency of the SLE with increasing concentrations of LZP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

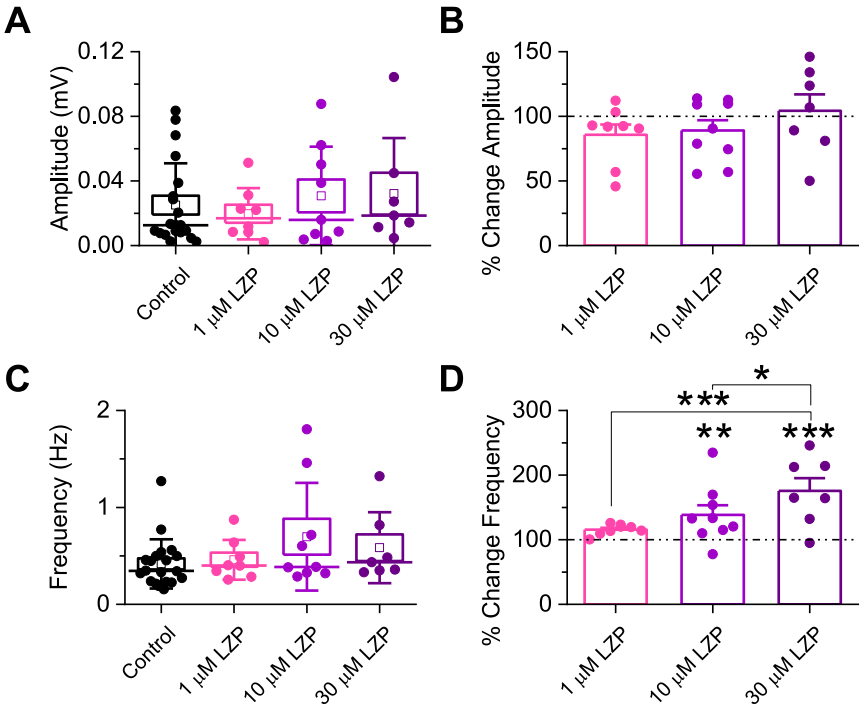

Fig. 3. Seizure activity was enhanced by application of lorazepam 60 min after SE–like activity was evoked in the dentate gyrus.
(A–D) Graphs showing: (A) amplitude in mV, (B) change in the amplitude (%), (C) frequency (in Hz) and (D) change in the frequency (%). For boxplots (box = mean ± SEM and whiskers = ± SD), mean is shown as the central open square and the median as a horizontal line. Bar graphs represents mean ± SEM and the control condition (100%) is shown as a broken line. For control, 10 min of seizure activity before LZP application per slice was analysed. While LZP increased the frequency of the SLEs, the amplitude of the SLEs remained unchanged. One way ANOVA with Holm-Bonferroni correction test was used for the statistical analysis. Statistical significance when *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and $p < \alpha$. Individual symbols represent brain slices ($n_{Control} = 20$; $n_{1\ \mu M\ LZP} = 8$; $n_{10\ \mu M\ LZP} = 9$; $n_{30\ \mu M\ LZP} = 7$; $N_{mice} = 15$). Average frequency and amplitude values are shown in Supplementary Table 2 and statistical analysis is shown in Supplementary Table 3.

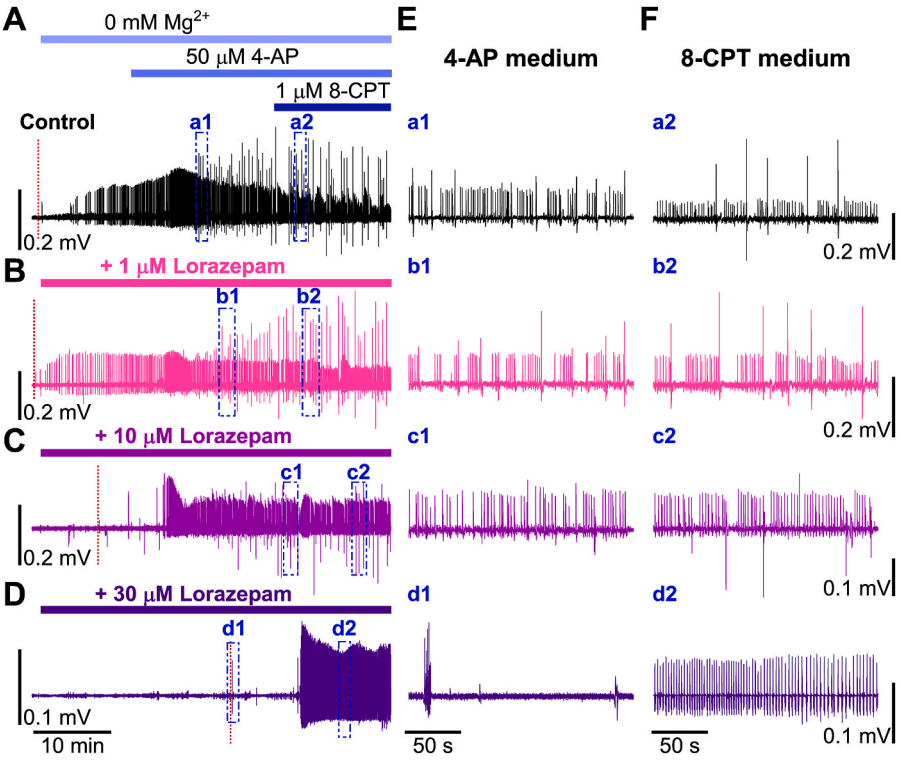

Fig. 4. Early application of lorazepam delays seizure onset in a concentration-dependent manner.
(A–D) Representative traces of hippocampal brain slices (400 μm) in the absence (A, control) and in the presence of different concentrations of LZP: (B) 1 μM; (C) 10 μM; (D) 30 μM. Spontaneous seizure-like activity was induced by 50 μM 4-AP/1 μM 8-CPT/0 mM Mg^{2+}. The broken vertical red line marks the first seizure-like event, and the blue boxes mark the source of the expanded traces showed in (E, F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. The selective P2X7 antagonist A740003 in combination with lorazepam delayed the onset of the seizures in an in vitro model of SE resistant to the inhibitory effects of lorazepam

Our data demonstrate that our *in vitro* model of prolonged seizure activity becomes resistant to the inhibitory effects of LZP an hour after SE onset (Fig. 2) compared to early application which delays seizure onset (Fig. 4). We therefore investigated whether the selective P2X7 antagonist A740003 (10 μM) applied early (i.e., before spontaneous SLEs were pharmacologically elicited) could reduce seizure–like activity in the DG (Fig. 7A and B). Furthermore, a possible therapy targeting P2X7Rs as a novel adjunct treatment with LZP for clinical LZP–resistant SE, combining A740003 and LZP was also tested (Fig. 7C).

A740003 (10 μM) alone did not affect seizure onset compared to

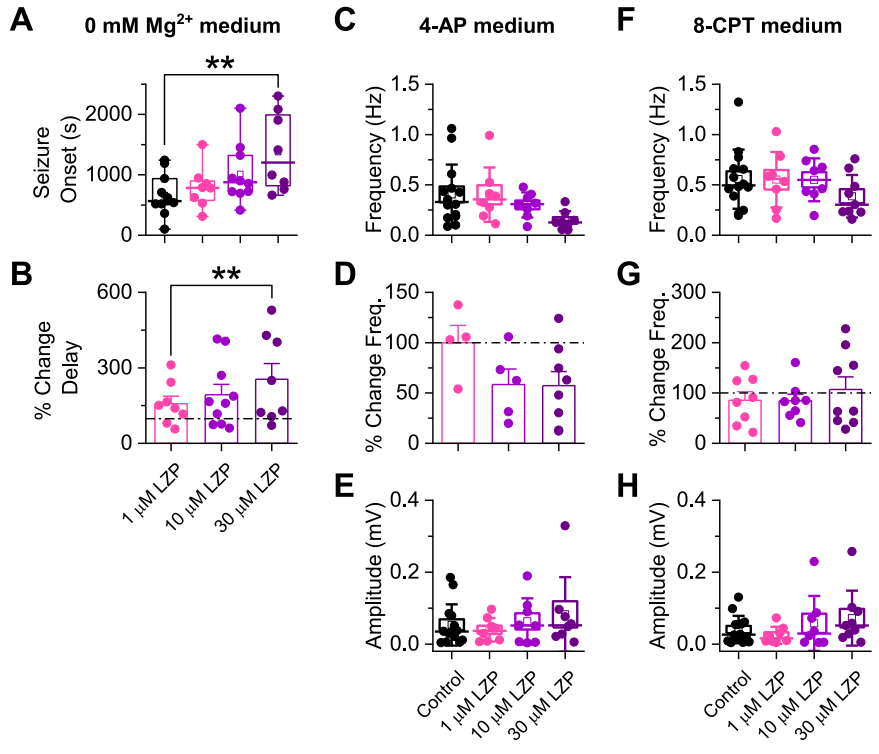

Fig. 5. Early application of lorazepam delays the onset of the seizures in the DG, but not seizure frequency or seizure event amplitude.
(A, B) Delay of seizure onset in seconds and as a percentage of the control onset delay (control is represented by 100%, broken black line). Time zero was considered when Mg^{2+} was removed from the medium. The frequency (in Hz) and amplitude (in mV) of the SLEs were analysed when seizure-like activity was induced by: (C, D) 50 μM 4-AP/0 mM Mg^{2+} and (E, F) 1 μM 8-CPT/50 μM 4-AP/0 mM Mg^{2+}. For boxplots (box = mean ± SEM and whiskers = ± SD), mean is shown as the central open square and median as a horizontal line. Bar graph represents mean ± SEM. LZP 30 μM significantly delayed the onset of the seizures. One way ANOVA with Holm-Bonferroni correction test was used for statistical analysis Individual symbols represent slices ($n_{Control} = 14$; $n_{1\ \mu M\ LZP} = 8$; $n_{10\ \mu M\ LZP} = 8$; $n_{30\ \mu M\ LZP} = 9$, $N_{mice} = 14$). Statistical significance when: *$p < 0.05$, **$p < 0.01$ and when $p < \alpha$. Average frequency and amplitude values are shown in Supplementary Table 2 and statistical analysis is shown in Supplementary Table 3.

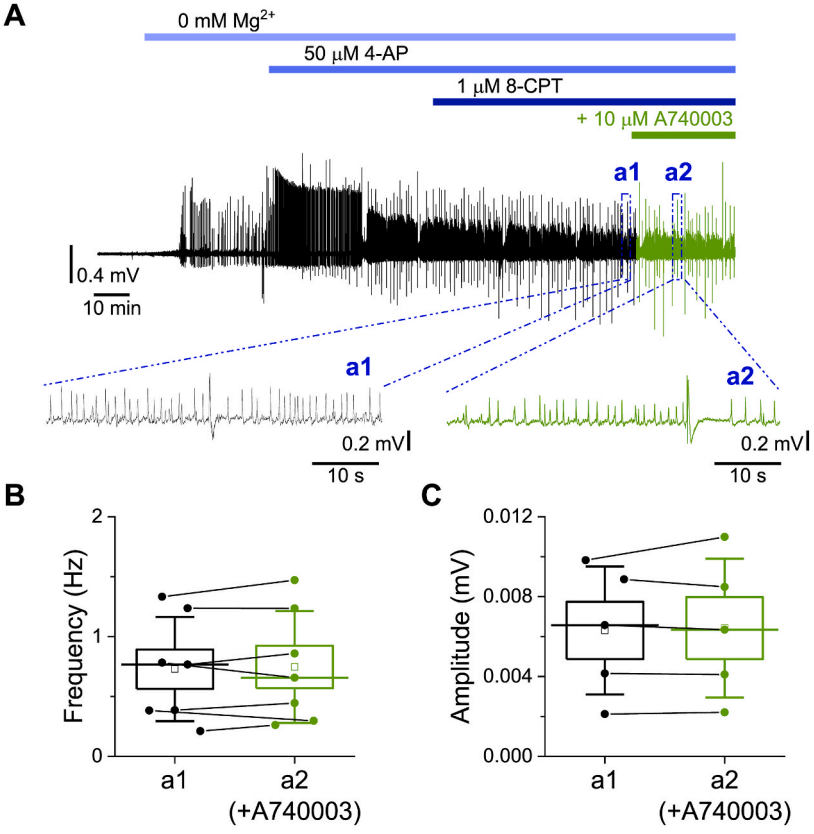

Fig. 6. No obvious contribution of P2X7Rs to seizure activity in the mouse dentate gyrus.
(A) A combination of 0 mM Mg^{2+} ±50 μM 4-AP ± 1 μM 8-CPT induced different patterns of seizure activity in the DG (horizontal blue lines indicate the protocol duration). (B) The selective P2X7R antagonist A740003 (10 μM) was added 1 h after SE-like activity was induced (green). The blue boxes mark the source of the expanded traces below (a1 and a2), before and after A740003 application. (B, C) Graphs showing frequency (Hz) and amplitude (mV) of the spontaneous seizures (box as mean ± SEM, whiskers, ±SD, the open square indicates the mean, and the line indicates the median). Neither frequency nor amplitude were significantly different. $n_{Time\ Control} = 12$, $n_{A740003} = 7$, $N_{total\ mice} = 19$. Average frequency and amplitude values are shown in Supplementary Table 2 and statistical analysis is shown in Supplementary Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

parallel control slices (805.0 ± 93.7 s vs 875.2 ± 160.8 s; on average, 107.4% of control; Fig. 7B, D, E). However, the combination of A740003 (10 μM) with LZP (10 μM), which did not result in a significant increase in the time to seizure onset (Fig. 5A and B), significantly delayed the appearance of the first SLE compared to parallel control slices (605.7 ± 91.0 s vs 1094.7 ± 163.5 s; on average, 204.5% of control; Fig. 7C, D, E).

Even though no effect of A740003 was seen on seizure frequency

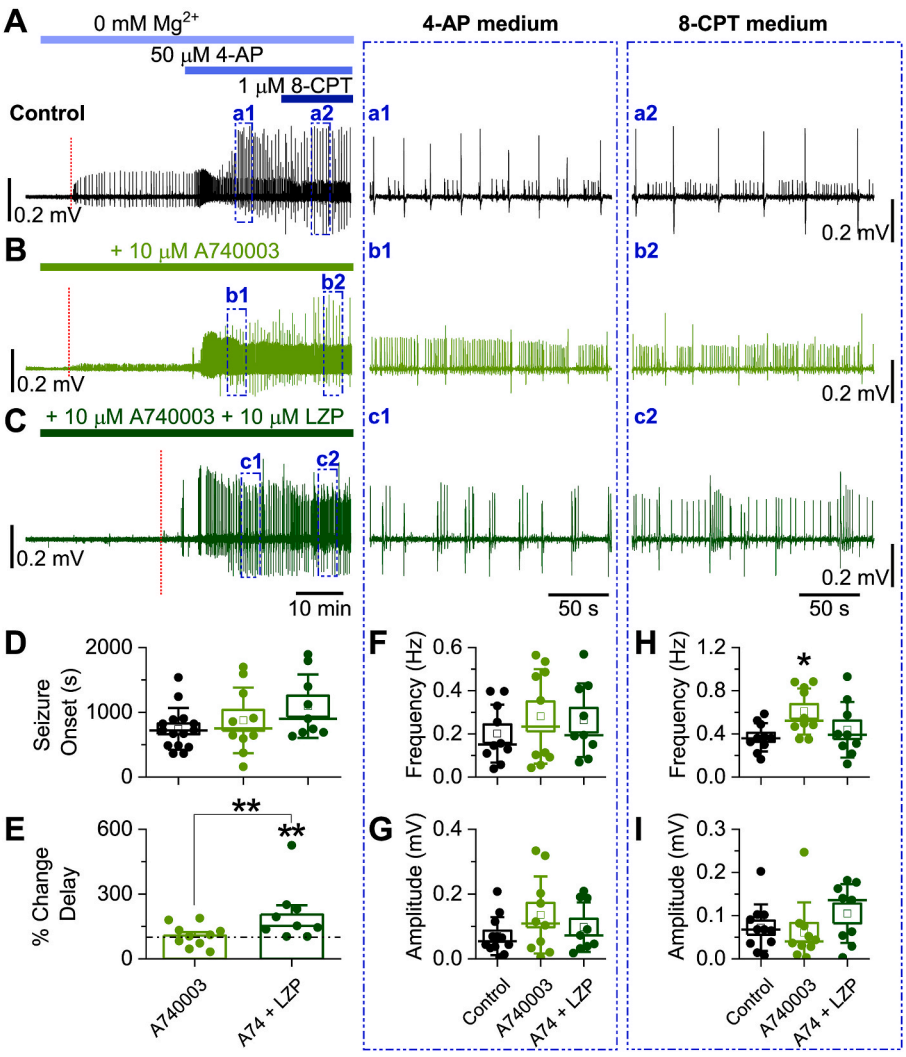

Fig. 7. A combination of A740003 and lorazepam delayed seizure onset in the DG during SE.
(A–C) Representative traces from the molecular layer of the DG when seizure-like activity was induced with 0 mM Mg^{2+} ±50 μM 4-AP ± 1 μM 8-CPT (horizontal blue line indicates the duration of the different protocols) in hippocampal brain slices (400 μm). (A) Control, (B) when the slices were incubated with 10 μM A740003 for the entire duration of the experiment (light green traces) or (C) combined with 10 μM lorazepam (LZP). The broken vertical red line indicates the first SLEs recorded, and the blue boxes mark the source of the expanded traces on the right. (D, E) Graphs showing the delay of the onset of the SLEs in seconds (D) or the percentage of the delay compared with control slices (control is represented by 100%, horizontal broken dotted black line) (E). (F, G) Frequency (in Hz) and amplitude (in mV) of the SLEs when seizure-like activity was induced by: (F, G) 50 μM 4-AP/0 mM Mg^{2+} and (H, I) 1 μM 8-CPT/50 μM 4-AP/0 mM Mg^{2+}. For boxplots (box = mean ± SEM and whiskers = ± SD), mean is shown as the central open square and median as a line. Bar graph represents mean ± SEM. One way ANOVA with Holm-Bonferroni correction test was used as statistical test Statistical significance when: *p < 0.05, **p < 0.01 and when p < α. Individual symbols represent slices ($n_{Control} = 10$; $n_{A740003} = 10$; $n_{A74+LZP} = 9$; $N_{mice} = 10$). Average frequency and amplitude values are shown in Supplementary Table 2 and statistical analysis is shown in Supplementary Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 7F) or amplitude (Fig. 7G) when seizure–like activity was induced by 4-AP medium, unexpectedly, A740003 significantly increased the seizure frequency during SE-like activity induced by 8-CPT (Fig. 7H, from 0.37 ± 0.04 Hz in control group to 0.61 ± 0.07 Hz in the A74 group), without affecting amplitude (Fig. 7I). This is in contrast to the late application of A740003 (10 μM), which had no effect on seizure parameters (Fig. 6). Finally, co-application of LZP with A740003 had no effect on seizure parameters in 4-AP medium (Fig. 7 F, G), but prevented the increase in seizure frequency cause by A740003 in 8-CPT medium (Fig. 7H) with no effect on the amplitude of SLEs (Fig. 7I).

3.6. No evidence for changes in P2X7R expression or inflammatory markers in pharmacological SE

While there were effects of P2X7R antagonism on seizure activity (delaying seizure onset when combined with LZP (Fig. 7E); increasing SE seizure frequency after prolonged application (Fig. 7H)), they were not as pronounced as would have been expected given previous reports of strong anti-seizure activity in a variety of *in vivo* experimental models of epilepsy (Supplementary Table 1). Of the many differences between the *in vitro* and *in vivo* settings, one difference may be changes in the expression of P2X7Rs, or the development of an inflammatory response after the *in vivo* induction of seizures that could have a bearing on the efficacy of P2X7R antagonists.

Accordingly, we used qPCR to determine hippocampal levels of mRNA for *P2rx7*, the microglial marker *Aif1*, which encodes Iba1, and *Il1b* which codes for the pro-inflammatory cytokine IL-1β, at various time points after the induction of seizure activity in the *in vitro* hippocampal slice. Between 15 min and 2 h after the start of the seizure protocol depicted in Supplementary Fig. 1A, there were no obvious changes in the expression of mRNA for *P2rx7*, *Aif1*, or *Il1b* (Supplementary Figs. 1B, C, D, respectively). Thus, over the timescale of these *in vitro* studies, prominent changes in the expression of P2X7Rs and inflammatory markers are unlikely to occur, which may contribute to the subtle effects of P2X7R antagonism in this model.

4. Discussion

Increased P2X7R protein level was observed in post-mortem neocortical brain tissue from patients with temporal lobe epilepsy (Jimenez-Pacheco et al., 2013; Barros-Barbosa et al., 2016; Song et al., 2019). Given this, and evidence from *in vivo* models of epilepsy, there is considerable interest in the potential benefits of P2X7R antagonists as a new class of ASM to treat refractory SE (Engel et al., 2021; Beamer et al., 2022; Guerra Leal et al., 2022). In an attempt to recapitulate the reported effects of P2X7Rs in *in vivo* epilepsy models, we made electrophysiological recordings from the DG of mouse hippocampal slices, an area where the endogenous expression of P2X7Rs is higher than in other hippocampal sub-regions (Dona et al., 2009; Engel et al., 2012; Kaczmarek-Hajek et al., 2018; Ramirez-Fernandez et al., 2020). Although we observed insensitivity to the inhibitory effects of benzodiazepines (lorazepam; LZP) in our *in vitro* model of status epilepticus, our data suggests

that the sole use of a P2X7R antagonist would play only a minor or negligible role in seizure-like activity in the DG during LZP inhibition-resistant SE, but may, in combination with LZP delay entry into ASM-resistant SE.

4.1. Bidirectional effect of LZP on seizure-like activity in the DG depends on LZP application time

In the present study seizure-like activity was pharmacologically induced in hippocampal slices by removing Mg^{2+} from the extracellular medium and adding the K^+ channel blocker 4-AP to the aCSF, two well-known protocols to induce epileptiform activity (Avoli et al., 1988; Dreier and Heinemann, 1991; Gloveli et al., 1995; Pacico and Mingorance-Le Meur, 2014). The former increases neuronal excitability by alleviating the Mg^{2+} block of the NMDA receptor ion channel, while the latter, by slowing action potential repolarisation, increased the frequency of the seizure-like events and resulted in a mixed seizure pattern consisting of a combination of bursting and continuous spike activity. To mimic a clinically-relevant *in vitro* model of SE, where adenosinergic inhibitory mechanisms may break down (Young and Dragunow, 1994), the adenosine A_1 receptor antagonist 8-CPT was added to the Mg^{2+}-free plus 4-AP medium. As previously demonstrated by our and other groups (Avsar and Empson, 2004; Etherington and Frenguelli, 2004; Hall and Frenguelli, 2018), preventing the anti-seizure effect of adenosine via adenosine A_1 receptors caused robust and highly reproducible continuous spiking activity across all brain slices, and is reminiscent of SE-like activity, which is confirmed by the resistance to LZP inhibition that develops within an hour of SE-like activity.

Benzodiazepines, such as LZP, are the first-line treatment for SE, commonly defined as seizures lasting longer than 5 min. Under these conditions, LZP is usually administered as a 4 mg intravenous bolus, with a second 4 mg bolus delivered after 5 min if the seizures have not stopped (Crawshaw and Cock, 2020). This dose equates to peak plasma levels of LZP of 100 ng/ml (Dundee et al., 1978) in adults (~300 nM). Much higher plasma LZP values have been reported in neonates (in excess of 700 ng/ml; ~2 μM) (McDermott et al., 1992). Given the prolonged half-life of LZP (~15 h) (Greenblatt et al., 1979b), plasma LZP values double with repeated dosing (Greenblatt et al., 1979a). Of this intravenous LZP, approximately 17% is detected in cerebrospinal fluid (Ochs et al., 1980).

Approximately 30% of patients with SE are resistant to benzodiazepines (BZPs) (Kwan and Brodie, 2000; Burman et al., 2022). BZPs are positive modulators of chloride (Cl^-) permeable $GABA_A$ receptors ($GABA_A$Rs), which are frontline targets for epilepsy and SE. Clinical data demonstrates that the selection of the appropriate time window for BZP administration is crucial to improve seizure control and patient outcome: while an earlier administration can prevent seizure enhancement, a late administration promotes an increase in BZP–resistance in the patient (Gainza-Lein et al., 2018; Gainza-Lein et al., 2019; Trau et al., 2020). *In vitro* studies in the hippocampal CA1 region have also confirmed the clinical data: while early application of diazepam (3 μM) had anti-seizure effects and prevented SLE generation, application of diazepam 60 min after the induction of SE-like activity caused an increase in seizure activity (Burman et al., 2019). Focusing on the DG, with its high expression of P2X7Rs, we observed a concentration-dependent delay to the onset of the seizures provoked solely by the omission of Mg^{2+} from the extracellular medium. In contrast, and in keeping with the observations of Burman et al. (2019), a late application of LZP (60 min after SE-like activity was pharmacologically-induced) enhanced epileptiform activity. These observations suggest the *in vitro* development of resistance to LZP's inhibitory actions, and indeed the development of a $GABA_A$R-dependent pro-seizure state in the DG over a period of 60 min.

Two hypotheses for LZP's lack of inhibitory effect and pro-convulsive actions when used in late SE have been proposed: i) $GABA_A$Rs are rapidly internalized during SE and thus BZP such as LZP are less effective at inhibiting seizure activity (Goodkin et al., 2005, 2008; Naylor et al., 2005; Deeb et al., 2012), and ii) the intense neuronal activity during SE causes Cl^- influx into neurons via $GABA_A$Rs, which, together with the reduced expression or activity of the K^+/Cl^- co-transporter 2 (KCC2) results in elevated intraneuronal Cl^- concentration. This causes a shift in the reversal potential for $GABA_A$R-mediated synaptic responses such that $GABA_A$Rs switch from hyperpolarising and inhibitory to becoming depolarising and excitatory, which has been observed after SE in CA1 pyramidal neurons, DG granule cells and DG fast-spiking basket cells (Pathak et al., 2007; Yu et al., 2013; Burman et al., 2019). Thus, $GABA_A$R internalization and reduced Cl^- extrusion capability may drive BZP resistance and indeed excitation. Accordingly, clinical data has shown that earlier administration of BZPs are effective in preventing seizures, potentially due to being delivered prior to changes in $GABA_A$R trafficking (Naylor, 2023). Moreover, the predicted exacerbation of clinical seizure activity by late application of BZPs (Glykys and Staley, 2015; Burman et al., 2019, 2022) has a precedent in the seizures provoked by LZP and midazolam in neonates, where GABA may be depolarising (Ng et al., 2002). Thus, *in vitro* models of SE that can observe both resistance to the inhibitory effects of benzodiazepines, and benzodiazepine-induced exacerbation of seizure activity, may be especially useful in the development of new treatments for SE and its ASM-refractory variants.

4.2. Lack of involvement of P2X7Rs to seizure activity in the dentate gyrus

P2X7Rs are activated when a high concentration of extracellular ATP (>100 μM) is reached. Such situations may be possible when synaptic transmission is enhanced as occurs during epileptic seizures, or when the extracellular space shrinks due to cell swelling. The present study demonstrated that early application of the selective P2X7R antagonist A740003 did not modify the onset of the seizures, nor the seizure activity induced by 4-AP (50 μM)/Mg^{2+}-free aCSF in the DG in acute brain slices. Similarly, the late application of A740003 (60 min after induced SE-like activity) did not reduce seizure activity in the DG. Instead, and paradoxically, P2X7R antagonism may have enhanced seizure activity when slices were in their equivalent of SE.

This data corroborates similar results where other groups showed little or no contribution of P2X7Rs to seizure activity in brain slices treated with several P2X7 antagonists such as A740003, A438079, BBG (Lopatář et al., 2011, 2015; Klaft et al., 2012; Abiega et al., 2016; Nieoczym et al., 2017). The lack of effect of P2X7 antagonist on seizures not only has been seen in *in vitro* models but also in *in vivo* models. For instance, a lack of anti-convulsive effects of A-438079, JNJ-47965567, AFC-5128, BBG, PPADS have been detected in different models of seizures, SE and absence epilepsy (Avignone et al., 2008; Fischer et al., 2016; Doğan et al., 2020).

Critically, there are conflicting results between different models (Supplementary Table 1). For example, an anti-seizure effect of P2X7R antagonists (such as A740003, A438079, BBG or PPADS) has been described in different models of SE induced by intra-amygdala KA injection or by coriaria lactone (Engel et al., 2012; Jimenez-Pacheco et al., 2013; Huang et al., 2017; Alves et al., 2022).

Why there are such disparities regarding the role of P2X7Rs in epilepsy is unclear, but there are a number of potential explanations. For example, different animal models (even different animal species) and drug administration methods can increase the variability in the results between laboratories (Loscher, 2017). For instance, comparing two chemiconvulsant-induced SE models, P2X7 knockout mice displayed both reduced seizure severity after intra-amygdala KA-induced SE (Engel et al., 2012), and increased seizure activity in an intra-peritoneal pilocarpine model of SE (Kim and Kang, 2011).

Such discrepancies may arise through differences in neuroinflammation provoked by the epilepsy model. Indeed, the severity of seizures and their sensitivity to P2X7R modulation may be influenced by

the neuroinflammatory status of the tissue, which could explain why the effects of P2X7R manipulation on seizures vary depending on the specific model used (Supplementary Table 1). Potentially, the effects of P2X7R antagonists or genetic modifications may only be manifested when an inflammatory response is activated in the tissue. In line with this, the administration of the pro-inflammatory agent lipopolysaccharide (LPS) prior to treatment in a mouse model of SE induced by intra-amygdala injection of kainic acid, not only led to an increase in P2X7R expression in the brain but also reduced the mice's responsiveness to anticonvulsants (Beamer et al., 2022). The lack of anti-seizure action of A740003 in the present study may reflect an absence of such a neuroinflammatory response in acute brain slices. This lack of an inflammatory response is evidenced by no changes in the expression of *Aif1*, which codes for microglial marker Iba1, and *Il1b* which codes for the pro-inflammatory cytokine IL-1β (Supplementary Fig. 1).

Alternatively, since P2X7Rs are activated by high concentrations of ATP, the question remains whether sufficient ATP is released into the extracellular space of the DG in this chemical model of seizures. While one would expect high concentrations to be achieved in the narrow, and potentially shrinking, confines of the synaptic cleft during intense seizure activity, the rapid conversion by ecto-nucleotidases from ATP to its metabolites (e.g., ADP, AMP, adenosine) may preclude an appreciable activation of the P2X7, especially given its low affinity for ATP compared to other members of the P2X family (EC_{50} of 300–400 μM).

While an antiseizure activity of the P2X7R antagonist was not observed, surprisingly however, the early application of A740003 enhanced the frequency of the SLEs in the DG during SE-like activity. The same paradoxical pro-seizure effect of A740003 was previously observed by Lopatář et al. where other P2X7-selective antagonists such as BBG and A438079 induced a small increase in seizure duration in the CA1 region from acutely-prepared rat hippocampal slices (Lopatář et al., 2011, 2015). Remarkably, this phenomenon has also been shown *in vivo* where an increase in seizure activity was observed when mice were administered P2X7R antagonists (A438079, A740003 and OxATP) in a pilocarpine model of seizures (Kim and Kang, 2011). Similarly, other authors have shown that AZ10606120 (a negative allosteric modulator of P2X7Rs) and BBG (a competitive P2X7R antagonist) increased the number of seizures and their severity in the DG after pilocarpine-induced SE (Rozmer et al., 2017). Why blocking P2X7Rs might have a pro-seizure effect in the DG is not clear. According to Rozmer and colleagues, (Rozmer et al., 2017), P2X7R could have a protective role in the neuronal precursor cells (NPCs) in the DG after SE by preventing NPCs migration and avoiding the creation of aberrant neuronal circuits that could impede entry into chronic epilepsy. Alternatively, given the P2X7R's dual role in regulating glutamate and GABA release in the DG via P2X7Rs expressed on astrocytes (Leeson et al., 2018; Khan et al., 2019), it is possible that disruption of this release by a P2X7R antagonist could tip the balance of their actions towards excitation. Whether these factors might play a role in acute experiments *in vitro* remains to be seen.

4.3. P2X7R antagonism as a possible adjunct therapy with LZP to delay seizure onset

While P2X7R antagonism had no inhibitory effect on its own on seizure activity, and if anything a slight excitatory influence when applied throughout the experiment, it has been suggested that P2X7R antagonists (such as A438079, BBG, AFC-5128 or JNJ-47965567) could be used as an adjunct therapy with other ASMs such as LZP, ceftriaxone or carbamazepine (Engel et al., 2012; Soni et al., 2015; Fischer et al., 2016). We tested this in our model of seizure-like activity in the dentate gyrus. Our data showed that a combination of LZP (10 μM), which had no effect on its own, together with A740003 (10 μM) increased the latency to the first spontaneous seizure in the DG. In addition, the inclusion of LZP together with the P2X7R antagonist prevented the apparent increase in seizure frequency observed in the presence of the antagonist alone. Thus, it is possible that the combination of LZP before the switch to GABAergic excitation, and early application of a P2X7R antagonist, potentially before any receptor desensitization has occurred, may be effective in staving off entry into a pro-epileptic state after conditions such as acute brain injury. This suggestion is not completely implausible given the realisation that, new treatments for post-traumatic epilepsy and SE are unlikely to be applied as monotherapies and are most likely used as adjunctive therapy in combination with ASMs already in clinical use. Therefore, P2X7R antagonists in combination with ASMs could be an alternative for seizure control during pharmacoresistant epilepsy and to prevent the onset of future seizures in patients with high risk of suffering SE.

5. Conclusions

Our model of *in vitro* status epilepticus (SE) shared properties with that observed *in vivo*, namely intense and prolonged seizure activity, and, moreover, entry into a state characterised by resistance to the inhibitory effects of a commonly used ASM for SE: lorazepam. In addition, lorazepam intensified SE-like activity. While these are observations are of interest, patch-clamp studies of individual DG cells are required to establish whether this intensification arises through the appearance of depolarising GABAergic excitation as a consequence of altered Cl^- gradients across the neuronal membrane. Nonetheless, the present model is relevant to the study of mechanisms of drug refractoriness in SE, and indeed the adverse neurological events associated with benzodiazepines that have been seen in neonates. In contrast to reports of the involvement of P2X7Rs in seizure activity, P2X7R antagonism alone did not delay the onset of seizure activity, reduce seizure activity, or prevent entry into SE-like state, and indeed may have provoked seizure activity in SE. These largely negative observations may be due to the absence of any change in mRNAs coding for the P2X7R and inflammatory markers. However, the early application of the P2X7R antagonist, in combination with a previously ineffective concentration of lorazepam, delayed the onset of seizure activity. While at present the mechanistic basis for these observations are unclear, our findings may be of relevance to attempts to develop novel ASMs and prophylactic strategies for patients with a high risk of suffering from SE, for example, after traumatic brain injury.

CRediT authorship contribution statement

Monica Garcia-Durillo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Bruno G. Frenguelli:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest: The Corresponding Author, Bruno G. Frenguelli, is the Editor-in-Chief of Neuropharmacology

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved experimental animals has been conducted

with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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