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ORIGINAL ARTICLE

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P2X7 Receptor Inhibition Interrupts the Progression of Seizures in Immature Rats and Reduces Hippocampal Damage

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SUMMARY

Aims: Early-life seizures, particularly when prolonged, may be harmful to the brain. Current pharmacotherapy is often ineffective; therefore, novel neuro- and/or glio-transmitter systems should be explored for targeting. The P2X7 receptor is a cation-permeable channel with trophic and excitability effects on neurons and glia which is activated by high amounts of ATP that may be released in the setting of injury after severe seizures. Here, we tested the effects of A-438079, a potent and selective P2X7 receptor antagonist in a lesional model of early-life status epilepticus. **Methods:** Seizures were induced by intra-amygdala kainic acid in 10-day-old rat pups. Electrographic seizure severity, changes to P2X7 receptor expression, inflammatory responses and histological effects were evaluated. **Results:** Seizures induced by intra-amygdala kainic acid increased levels of P2X7 receptor protein and interleukin-1β and caused significant cell death within the ipsilateral hippocampus. A-438079 rapidly reached the brain following systemic injection in P10 rats. Intraperitoneal injection of A-438079 (5 and 15 mg/kg) 60 min after triggering seizures reduced seizure severity and neuronal death within the hippocampus. A-438079 had superior neuroprotective effects compared with an equally seizure-suppressive dose of phenobarbital (25 mg/kg). **Conclusions:** These results suggest P2X7 receptor antagonists may be suitable as frontline or adjunctive treatments of pediatric status epilepticus or other early-life seizures, particularly when associated with brain damage.

Introduction

Seizures during the first month of life (neonatal seizures) are more common than at any other time of life [1,2], while status epilepticus (SE) is the most common neurological emergency of childhood [1–3]. Although the immature brain is more susceptible to seizures, it is less vulnerable to cell death [4]. Nevertheless, the risk of neurological damage after SE in childhood [5] and increasing concerns that neonatal seizures are harmful [6,7] underscores the need for prompt and effective pharmacotherapy. Treatment for pediatric SE begins with benzodiazepines such as lorazepam, followed by phenobarbital or other anti-epileptic drugs [8]. Phenobarbital remains the frontline treatment for neonatal seizures [9,10]. Unfortunately, the efficacy of these treatments for seizures in the developing brain is often inadequate, which has been attributed to brain maturational aspects of the glutamate and γ-amino butyric acid (GABA) systems, chloride gradients, and loss of GABA receptor function [9,11].

If prolonged seizures are harmful to the immature brain and current therapies are ineffective, then alternate targets for achieving seizure suppression must be identified. An optimal approach might be to combine the necessary anti-excitatory effects with anti-inflammatory or neuroprotective properties. ATP is an important neuro- and glio-transmitter in the nervous system that mediates depolarizing responses via the P2X class of ligand-gated ion channels [12,13]. In addition to effects on neurotransmitter release [14–18], the P2X7 receptor (P2X7R) has important trophic actions on microglia [19] and astrocytes [20] or can promote glial cell death [21,22]. As a result, there has been significant interest in the role of the P2X7R in neurological disorders [23,24]. Recent work in adult rodent SE models has shown that drugs acting on the P2X7R can have potent anticonvulsant effects [25,26]. P2X7R inhibition reduced seizure severity, neuronal death, microglia activation and interleukin-1β (IL1β) levels when SE was triggered by intra-amygdala kainic acid (KA) in mice, a model associated with a focal hippocampal lesion [25,26]. In contrast, P2X7R signaling did not affect seizures after systemic KA or picrotoxin, which are nonlesional models [27]. These differences may be due to a high ATP requirement for the deleterious effects of P2X7R activation to manifest, and this may be

achieved only in the setting of neuronal activity sufficient to produce excitotoxic injury [24,28].

It is unknown whether P2X7R ligands have effects against seizures in the developing brain. To test this idea, we evaluated A-438079, a potent and selective brain permeable antagonist of the P2X7R [29] against SE induced by intra-amygdala microinjection of KA in postnatal day 10 (P10) rats [30], an age-appropriate model of a human full-term newborn to 1 year of age [4,31].

Materials and methods

Intra-amygdala KA-induced SE in P10 Rats

Animal procedures were performed as before [30] and were reviewed and approved by the RCSI Research Ethics Committee and conducted under license from the Department of Health and Children, Dublin, Ireland. Animals were maintained on a 12 h light:dark cycle with access to food and water provided *ad libitum*. Male or female Sprague–Dawley rat pups (P10; 17–23 g; Harlan, UK) were separated from the dam, anesthetized with isoflurane, and placed in a stereotaxic frame equipped with a neonatal rat adaptor. Body temperature was maintained at 37°C using a feedback-controlled heat blanket (Harvard Apparatus Ltd, Kent, UK). Following a midline scalp incision, three partial cranial holes were drilled on the skull surface for placement of Electroencephalogram (EEG) screw electrodes (Bilaney Consultants Ltd, Sevenoaks, UK; see Figure 1A). A complete craniectomy was drilled for the placement of a guide cannula for the intra-amygdala injection. Coordinates from bregma were AP: −1.4 mm and L: +2.9 mm. The guide cannula was lowered to rest on the brain surface and the entire assembly fixed with dental cement. Pups were then placed in an incubator at nest temperature (35 ± 1°C) to recover from surgery for 30 min prior to further experimentation.

For induction of status epilepticus and EEG recordings, pups were placed in a 40 cm × 20 cm × 20 cm open container. The cortical electrodes were connected by a 6-channel cable (Bilaney Consultants Ltd) to a Grass Comet lab-based EEG and EEG monitored using TWin EEG software (Natus Neurology Inc, Warwick, RI, USA). Baseline EEG traces were recorded for 10 min, and then, a 31-gauge internal cannula was inserted into the lumen of the guide cannula and lowered 3.5 mm below cortical surface to target the central and basolateral amygdala (Figure 1A). KA (2 μg in 0.2 μL of phosphate-buffered saline [PBS, pH 7.4]) was injected through the cannula using a 1-μL Hamilton syringe. EEG was recorded for 2 h after KA, and then, pups were disconnected and returned to the dam. Typical maternal separation time was 3 h per pup.

EEG data were exported to Labchart 7 (AD Instruments Ltd, Oxford, UK) for analysis. The effects of drug treatments on EEG were determined for the 60 min recording period after injection. Mean amplitude, total power and high-amplitude spikes were calculated. Each trace was also converted to its absolute value to calculate the mean amplitude. The total power was automatically measured by the software with the default frequency range of 0–500 Hz. The spike count represents the number of high-amplitude spikes >50 μV and was calculated with the spike histogram module.

Drug Treatments

Animals were randomized to treatment groups, drugs were delivered via an intraperitoneal injection in 0.3 mL volume, 1 h after KA injection. A-438079 (Tocris Bioscience, Bristol, UK) was dissolved in water and given at 0.5, 5, 15, and 50 mg/kg. Phenobarbital was used at 25 mg/kg and bumetanide (Sigma-Aldrich, Arklow, Ireland) at 0.15 and 1 mg/kg.

Histopathology and Immunohistochemistry

Rats were killed by barbiturate overdose and saline perfused to remove intravascular blood. Fluoro-jade B staining (FJB) was used to identify acutely degenerating neurons as described [25]. Briefly, fresh-frozen tissue sections at the level of the dorsal hippocampus were postfixed, dehydrated, immersed in 0.06% $KMnO_4$ solution followed by a rinse and then stained with 0.001% w/v FJB (Millipore Ireland B.V., Tullagreen, Ireland). Staining was examined using an epifluorescence microscope (Nikon 2000s), and positive cells within the hippocampus were counted in a blinded fashion under 20× magnification, as described [25]. Representative images were obtained using an Orca 285 camera and processed using Wasabi software (Hamamatsu Photonics Germany GmbH, Herrsching, Germany). Images were converted to grayscale and inverted such that degenerated neurons appeared dark on a light background.

Assessment of irreversible DNA fragmentation was performed using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique, according to manufacturer's instructions (Promega UK, Southampton, UK). Staining was examined and quantified as above.

For P2X7R immunohistochemistry, sections were permeabilized and blocked in goat serum followed by incubation with antibodies against P2X7R (1:200; Alomone Labs, Jerusalem, Israel), NeuN (1:500; Millipore Ireland B.V.), or synaptophysin (1:500; Sigma-Aldrich) overnight at 4°C. Sections were washed and incubated with goat polyclonal secondary antibodies coupled to AlexaFluor 488 or AlexaFluor 568 (BioSciences, Dun Laoghaire, Ireland). To confirm specificity, additional sections were incubated without the primary antibody. Nuclei were labeled by staining with 4′ 6-diamidino-2-phenylidole (DAPI; Vector Laboratories, Burlingame, CA, USA), and sections were examined under an epifluorescence microscope. A semiquantitative 4-point scoring system was used to assess neuronal survival in NeuN-stained tissue sections as follows: (0) no damage; (1) minor loss of NeuN staining in scattered neurons; (2) modest loss of NeuN staining in CA3; (3) loss of NeuN staining in entire CA3; (4) loss of NeuN staining throughout entire hippocampus including hilus and CA1 area.

Western Blotting and IL1β Analysis

Western blotting was performed as previously described [25]. Briefly, hippocampus was homogenized in lysis buffer containing a protease inhibitor cocktail and proteins separated using SDS-PAGE. Proteins were transferred to nitrocellulose membranes

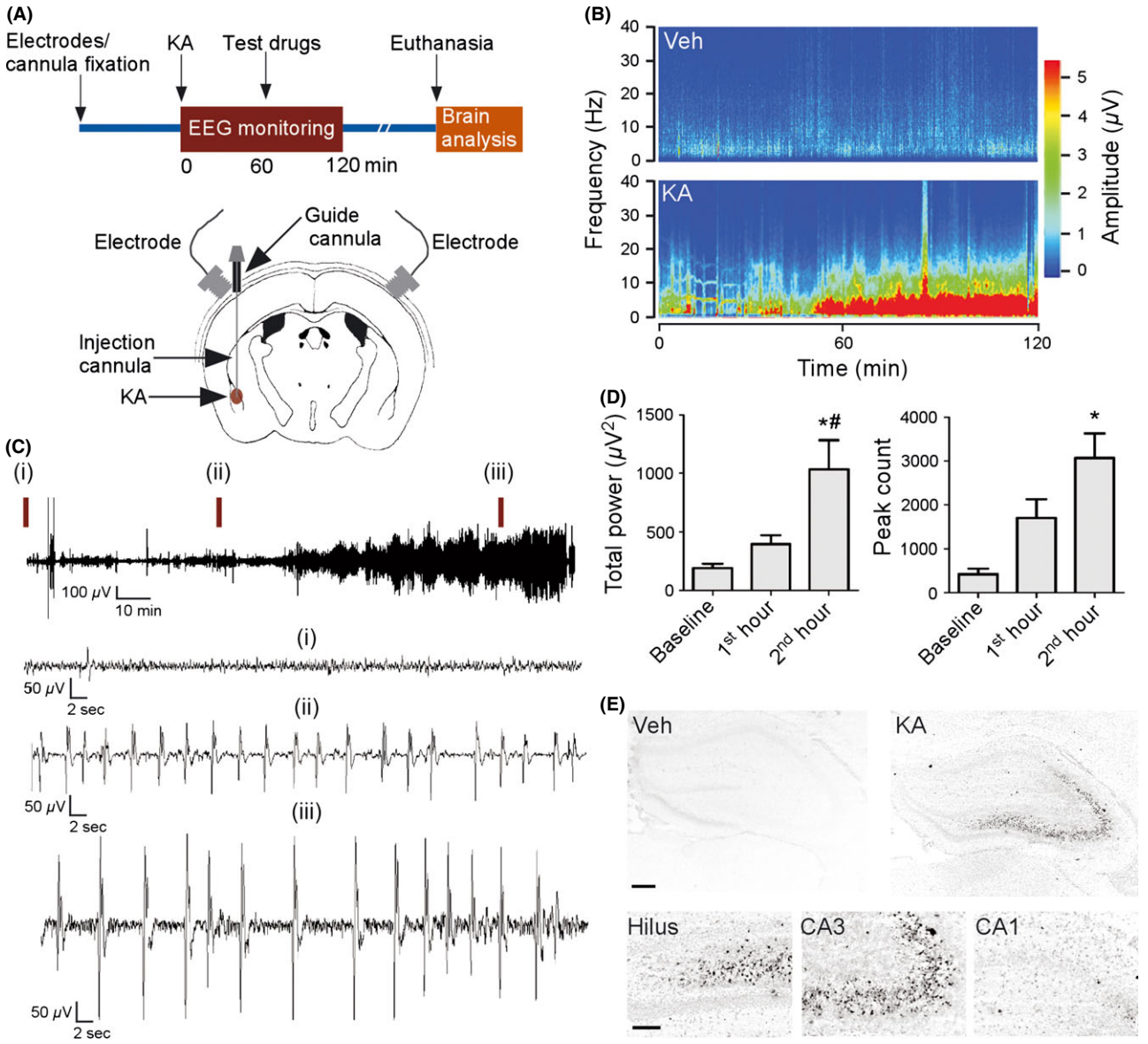

Figure 1 Status epilepticus and hippocampal damage following intra-amygdala kainic acid (KA) in P10 rat pups. (**A**) Schematic showing experimental paradigm. KA (or vehicle) is injected into the amygdala of freely moving pups followed by 2-h recordings. Test drugs were delivered after 60 min. Figure below depicts the stereotaxic level, surface-affixed recording electrodes and cannula placement for KA microinjection. (**B**) EEG heat map depicting typical frequency-amplitude data during status epilepticus (SE). Note onset of continuous seizures shortly before 60 min post-KA. (**C**) Representative EEG traces depicting electrographic seizure development following KA with increasing high-amplitude, high-frequency spikes. (**D**) Semiquantitative analysis of EEG total power and spikes in the model. Note temporal development of seizure severity in second hour compared to first. (**E**) Fluoro-jade B (FJB) staining of the ipsilateral hippocampus 72 h after SE. Damage extends from the hilus through the CA3 subfield as well as injury to the CA1-CA2 border. Scale bar in **E**, top; 250 μm, bottom; 60 μm. *$P < 0.05$ compared to baseline; #$P < 0.05$ compared to 1 h.

and incubated with primary antibodies against the rat form of the P2X7R (1:500; Alomone APR-004), IL1β (1:1000; Abcam AB9722, Cambridge, UK), or β-Actin (1:5000; Abcam AB8227) as a loading control. Proteins were visualized using a chemiluminescence kit, and bands captured using a Fujifilm LAS scanner. Semiquantitative analysis of band density was performed using Image J.

Measurement of Plasma and Brain Levels of A-438079

To confirm A-438079 reached the brain after systemic administration, P10 rat pups were injected with 5 mg/kg A-438079 and killed either 10 min, 30 min, or 2 h later (n = 4 per group). Blood samples were centrifuged at 1000 × g for 10 min to isolate the

plasma. Samples were analyzed using liquid chromatography–mass spectrometry (LC-MS/MS) by a service provider (Cyprotex, Macclesfield, UK). Briefly, protein was precipitated from 50 μL aliquots of the individual plasma or brain tissue homogenate, and A-438079 quantified by LC-MS/MS from a five-point standard curve.

Data Analysis

Data are presented as the mean ± standard error of the mean. Two-group comparisons were made using Student's *t* test and multigroup comparisons using analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Significance was accepted at $P < 0.05$.

Results

Intra-amygdala KA in P10 Rats Produces Prolonged Seizures and Damage to the Ipsilateral Hippocampus

Intra-amygdala microinjection of KA in P10 rat pups produced seizures resulting in damage to the ipsilateral hippocampus (Figure 1). Seizures typically began within a few minutes of KA injection, but EEG power remained only modestly elevated during the first 30–40 min before increasing and developing into continuous electrographic seizures from 45 to 50 min. Thereafter, seizures became continuous and severe (Figure 1B–D). Consistent with previous studies [30], the hippocampus from rat pups killed 72 h later displayed extensive FJB staining indicative of irreversible neuronal damage which was most prominent in the ipsilateral CA3 subfield but also incorporated the hilus, occasional dentate granule neurons, and CA1 pyramidal cells (Figure 1E).

Expression of P2X7R Following SE in P10 Rats

We next used Western blotting to detect protein for the receptor in hippocampal homogenates. The protein for the P2X7R was largely undetectable in the hippocampus at P1 and P5, whereas a band at the predicted molecular weight of ~75 kDa for the P2X7R was observed in P10 and P30 rats (Figure 2A).

Following SE in P10 rats, P2X7R protein levels increased rapidly and continued to increase at 24 and 72 h (Figure 2B). Confocal microscopy revealed increased P2X7R immunoreactivity within the hippocampus after SE (Figure 2C). This was prominent in the stratum lucidum of the CA3 subfield which corresponds to the mossy fiber terminals, a known site of P2X7R localization in the rat [14]. Supporting these observations, P2X7R staining was closely localized with immunoreactivity for the synaptic marker synaptophysin (Figure 2C). P2X7R activation is known to promote

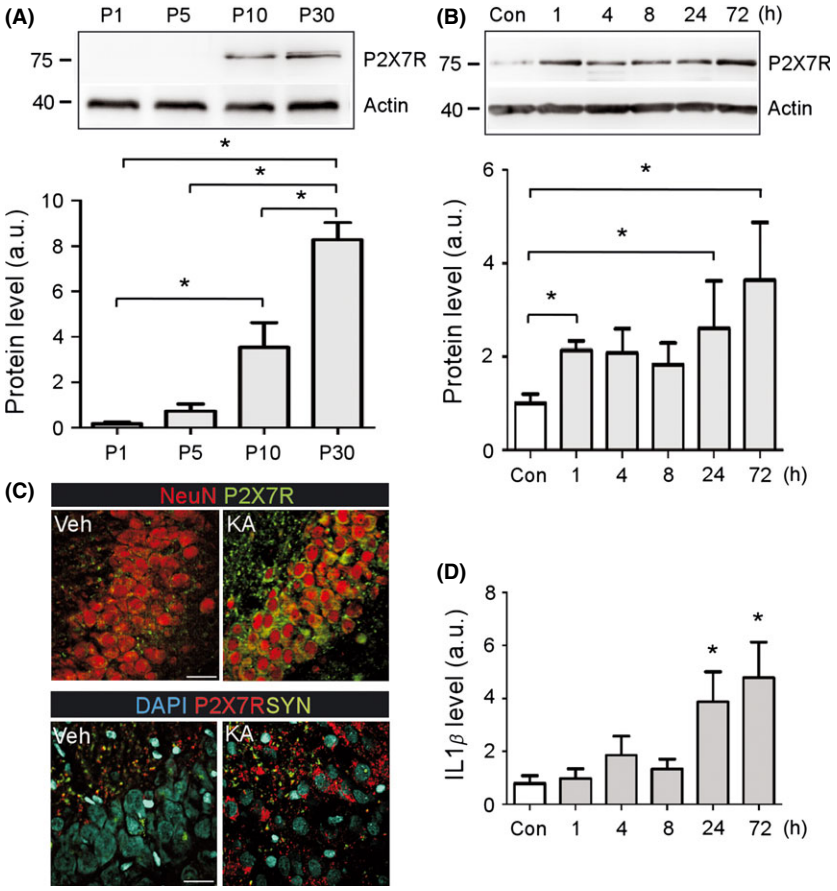

Figure 2 P2X7R protein levels following neonatal status epilepticus (SE). (**A**) Representative Western blot (n = 1 per lane) and graph (n = 4 per group) showing P2X7R protein levels in the rat hippocampus at postnatal day 1, 5, 10, and 30. (**B**) Representative Western blot (n = 1 per lane) and graph (n = 4 per group) showing P2X7R protein levels increase following neonatal SE. (**C**) Immunohistochemistry (4 h) showing (top) P2X7R (green) staining within the stratum lucidum (mossy fiber terminals) and around cell bodies of NeuN-positive cells and (bottom) P2X7R (red) with synaptophysin (syn, green). Scale bar, 50 μm. (**D**) Graph quantifying IL1β levels following neonatal SE (n = 5 per group). *$P < 0.05$ compared with control.

IL1β release [32], and we also detected an increase in IL1β protein levels after SE in the model (Figure 2D).

Dose-dependent Effects of A-438079 on SE in P10 Rats

To determine whether P2X7R antagonists had effects on SE, we injected rat pups 1 h after KA with A-438079 over the dose range 0.5–50 mg/kg and recorded effects using surface EEG over the next hour. At the lowest tested dose (0.5 mg/kg), A-438079 had no significant effect on seizure severity (Figure 3A,B). In contrast, seizure severity was reduced in rat pups given 5 or 15 mg/kg A-438079 (Figure 3A,B). At either dose, EEG amplitude, total power, and spike count were all reduced. At the highest tested dose (50 mg/kg), an effect on amplitude remained but spike count and overall EEG power were not significantly reduced compared to vehicle (Figure 3A,B).

A-438079 Reduces Seizure-induced Neuronal Death in P10 Rat Pups

We next examined neuronal damage in tissue sections obtained 72 h after SE. As expected, vehicle-treated SE pups displayed extensive FJB staining of damaged neurons within the ipsilateral hippocampus (Figure 4A,B). Seizure damage was significantly reduced in pups that received either 5 or 15 mg/kg A-438079 (Figure 4A,B). Damage in the 0.5 and 50 mg/kg groups was not different to vehicle-injected seizure pups (Figure 4B). Sections from pups given the lowest effective A-438079 dose (5 mg/kg) also showed reduced TUNEL staining, a marker of irreversible DNA fragmentation (Figure 4C), and a lower score for neuronal damage (Figure 4D). We also examined levels of IL1β in hippocampal lysates from rat pups 24 h after SE (Figure 4E). Rat pups subject to SE and treated with vehicle had significantly increased IL1β levels in the hippocampus ($P < 0.05$ compared with non-KA veh/A-438079 alone, n = 4). In contrast, IL1β levels in rats subject to SE and treated with A-438079 were not different to nonseizure controls (Figure 4E).

Plasma and Brain Levels of A-438079 Following Systemic Injection in P10 Rats

A-438079 has previously been reported to be available in the brain following systemic administration [29]. However, no study has established the brain levels of A-438079 after systemic injection in immature rats. We therefore injected groups of P10 rats with A-438079 (5 mg/kg, i.p.) and measured plasma and brain levels 10, 30, and 120 min later. Plasma levels of A-438079 reached ~2.3 μg/mL at the 10 min time point, declining rapidly thereafter (Figure 4F). Brain levels of A-438079 followed the same profile, peaking at the earliest tested time and declining rapidly thereafter (Figure 4G).

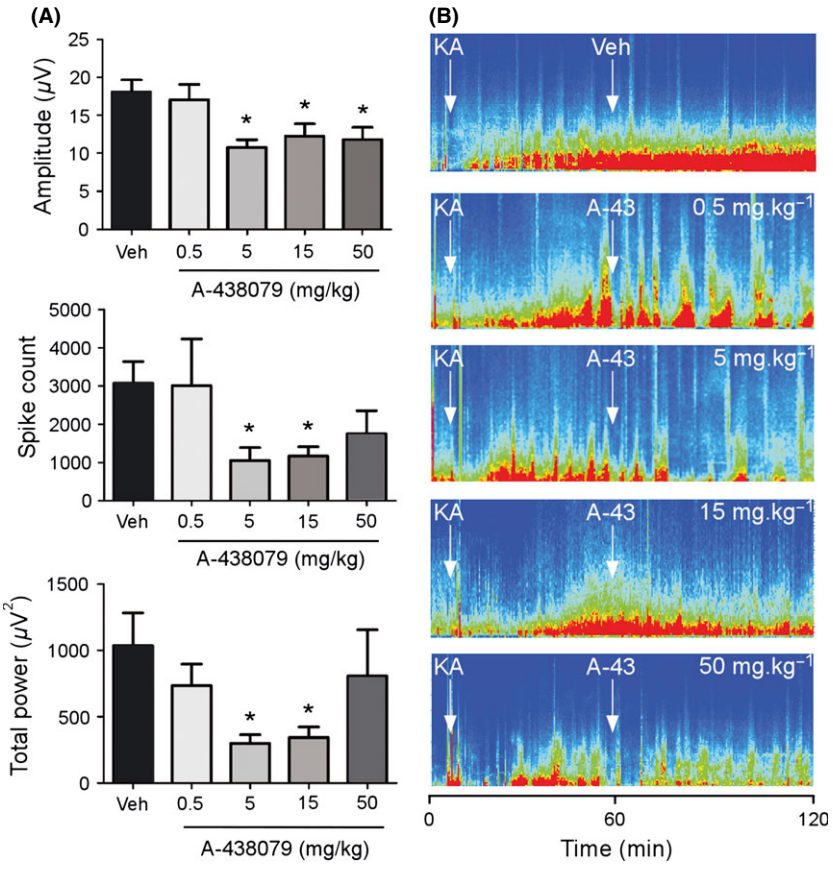

Figure 3 A-438079 suppresses neonatal status epilepticus (SE). (**A**) Graphs quantifying seizure parameters amplitude, spike count, and total power for rat pups injected 60 min after kainic acid (KA) with 0.5, 5, 15, and 50 mg/kg A-438079 compared with vehicle (Veh; n = 7–14 per group). *$P < 0.05$ compared to Veh. (**B**) Representative EEG heatmaps for each dose of A-438079 showing the seizure-suppressive effect mainly at 5 and 15 mg/kg (frequency and amplitude scale range same as for Figure 1B).

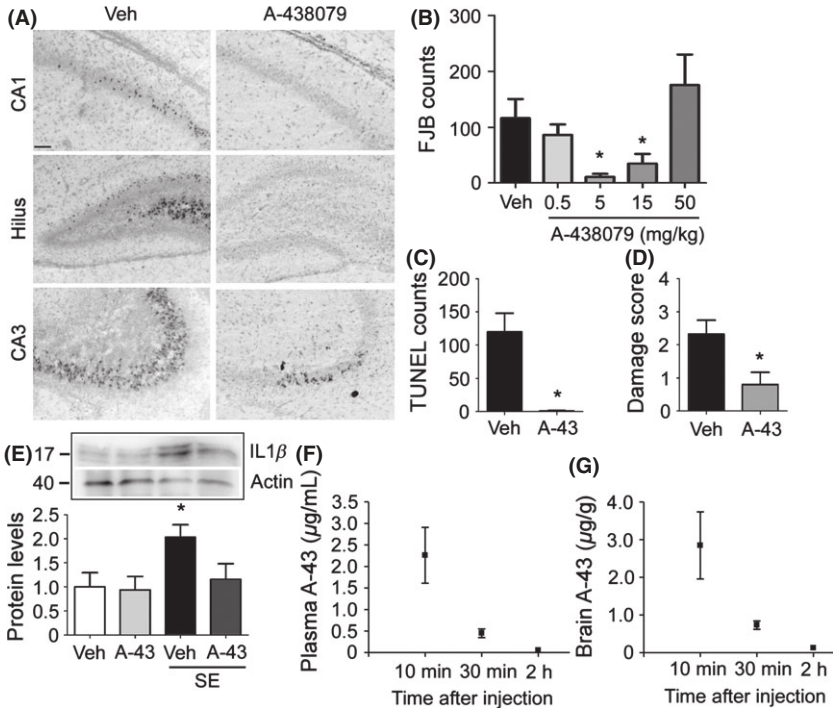

Figure 4 Effects of A-438079 on hippocampal injury after neonatal status epilepticus (SE). (**A**) Representative fluoro-jade B (FJB) staining at 72 h from vehicle-injected SE pups and animals that were given 5 mg/kg A-438079. Scale bar, 60 μm. (**B**) Graph showing FJB counts on sections from dorsal hippocampus showing protective effects of 5 and 15 mg/kg A-438079 (n = 5–9 per group). (**C, D**). Graphs showing that A-438079 (5 mg/kg) also reduced (**C**) TUNEL staining in the ipsilateral hippocampus and (**D**) improved neuronal survival compared to vehicle groups (n = 7–9 per group; (**E**) Representative Western blot (n = 1 per lane) showing induction of IL1β is reduced in rat pups treated with A-438079 (5 mg/kg). Data are representative of n = 4 independent experiments. (**F, G**) Plasma and brain levels of A-438079 measured after intraperitoneal injection of 5 mg/kg (n = 3–4 per lane). *$P < 0.05$ compared to vehicle.

Effects of Phenobarbital and Bumetanide on SE Induced by Intra-amygdala KA in P10 Rats

We next compared the effects of A-438079 to phenobarbital, the frontline treatment for neonatal seizures. As before, injection of A-438079 (5 mg/kg) 60 min after KA resulted in significantly reduced seizures in P10 rat pups (Figure 5A,B). Intraperitoneal injection of rat pups with 25 mg/kg phenobarbital, a dose within the range used in other studies of neonatal seizures [33,34], reduced seizure severity by over 50%, although the effect was not statistically superior to A-438079 (Figure 5A,B). No improvement in anticonvulsant effects was obtained with the combination of A-438079 plus phenobarbital (Figure 5A,B).

The NKCC1 (Na+, K+, 2Cl−) cotransporter is responsible for the high intracellular chloride concentration in the developing brain and has been implicated in excitatory effects of GABA [35]. Bumetanide is an inhibitor of the NKCC1 transporter that has been reported to inhibit seizures in nonlesional models of neonatal seizures in P10 rats [36] and is in clinical trials for neonatal seizures in the USA and Europe [37]. Accordingly, we investigated the effects of bumetanide in the intra-amygdala KA model. Intraperitoneal injection of P10 rat pups with either 0.15 or 1 mg/kg bumetanide had no significant effect on EEG amplitude, total power, or peak counts during seizures (Figure 5C and data not shown).

Analysis of FJB-stained tissue sections from the different groups of rats revealed that while hippocampal damage was reduced in rat pups injected with A-438079, seizure damage was not significantly reduced in the rats treated with either phenobarbital or bumetanide (Figure 5D,F). Staining sections for DNA fragmentation as a second marker of injury also revealed only A-438079 offered significant protection in the model (Figure 5E,F). Intraperitoneal injection of A-438079 alone had no effect on apoptosis in the brain at either 5 or 50 mg/kg (Figure 5E and data not shown).

Discussion

In the present study, we report potent anticonvulsant effects of a P2X7R antagonist in a model of early-life SE. We found A-438079 was centrally available after systemic injection and potently reduced seizures triggered by intra-amygdala KA in P10 rats. Although it was not more effective than phenobarbital at stopping seizures, A-438079 provided potent protection against hippocampal damage. These data suggest P2X7R antagonists may have potential applications for the treatment of severe early-life seizures.

Prolonged seizures in the developing brain are thought to be harmful, and current practice is to treat with anticonvulsants. Pharmacotherapy is often inadequate, however, with phenobarbital having limited efficacy against neonatal seizures and benzodiazepines often failing to curtail seizures during pediatric SE [8,10]. Thus, other neuromodulatory systems may represent suitable targets for the control of early-life seizures. Here, we investigated the P2X7R, an ATP-activated ion channel that gates depolarizing sodium and calcium entry into cells. Activation requires high amounts of ATP, and the main hypothesis pursued here was that if early-life seizures are sufficiently severe to produce hippocampal damage, the P2X7R would promote seizures and their pathophysiological consequences, including neuroinflammation. We found A-438079 at 5 and 15 mg/kg strongly reduced seizures triggered by intra-amygdala microinjection of KA in P10 rats. At 10 days old, rat pups are thought to represent the time from birth to 1 year in humans and are therefore suitable

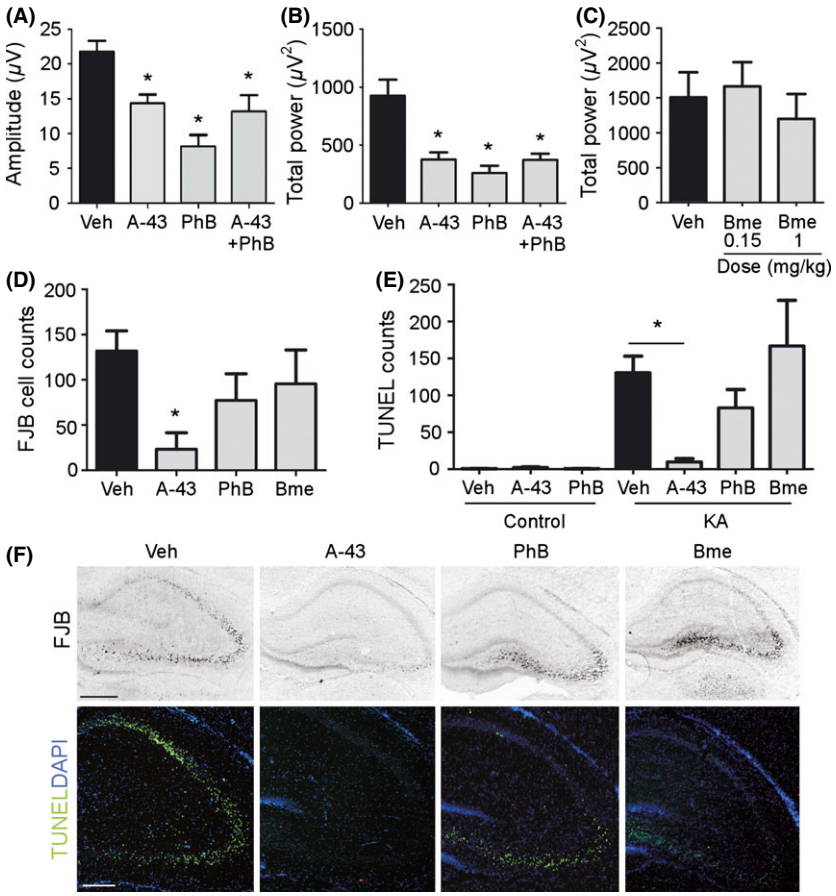

Figure 5 Comparison of A-438079 with phenobarbital and bumetanide. (**A, B**) Graphs showing EEG seizure parameters for rat pups during neonatal status epilepticus (SE) given either A-438079 (A-43, 5 mg/kg), phenobarbital (PhB, 25 mg/kg), or a combination of both (*$P < 0.05$ compared to vehicle; n = 5–6 per group). (**C**) Graph quantifying EEG data after neonatal SE in rat pups injected with bumetanide (Bme; n = 6–7 per group). (**D**) Fluoro-jade B (FJB) counts at the level of the dorsal hippocampus showing protective effects of A-438079, while damage persisted in rat pups given either phenobarbital or bumetanide (1 mg/kg) (n = 5–6 per group). (**E**) TUNEL counts at 72 h in rat pups given drug alone or 60 min after SE. Again, cell death is reduced in the A-438079 group but not following either phenobarbital or bumetanide (n = 6 per group). (**F**) Representative FJB-staining and TUNEL 72 h after SE in each group. Note the reduced damage in the A-438079-treated rat pups. Scale bar, top; 500 μm, bottom; 250 μm.

to model neonatal seizures and SE in the immature brain [4,31]. The seizures are sufficient to produce unilateral hippocampal sclerosis, and the model features a strong correlation between hippocampal damage and seizure severity [30]. In the design of our study, we allowed seizures to develop before delivering the drug, ensuring we evaluated in a realistic clinical scenario when seizures or damage may already have begun. To our knowledge, our study is the first to show an effect of a drug of this class on seizures in the developing brain, and the data extend reports of anticonvulsant effects of A-438079 in adult mice subject to SE triggered using the same focal-onset approach [25,26]. We also found that A-438079 was strongly neuroprotective, reducing hippocampal damage after SE in P10 rats by up to 80–90%. Thus, the present study provides preclinical data supporting the targeting of the ATP-gated P2X7R for the prevention of seizures and brain injury after prolonged early-life seizures.

Relatively little is known about the developmental maturation of ATP-gated signaling and the role of P2X7R in brain development. The absence of brain abnormalities in mice lacking P2X7R indicates the receptor is not required for normal brain development [17,25,27,38]. In the present study, we detected the P2X7R in hippocampal neurons and likely presynaptic terminals after seizures in P10 rat pups, supporting other data on the presence of the receptors in the rodent hippocampus [14,39,40].

Although we did not pursue the mechanism of seizure suppression by A-438079, it is likely to involve direct effects on neurons. The receptor is known to be located presynaptically in the hippocampus [14], and blockade of P2X7R may reduce neurotransmitter release [15–18]. P2X7R is expressed by microglia and activation results in transformation to an active state and release of IL1β [19,41,42], a potent convulsant in other early-life seizure models [43]. Data here support an effect of A-438079 inhibiting IL1β production, although we cannot exclude this being secondary to reduced seizures and tissue injury. The data fit with findings in adult models where IL1β induction and microglia activation was prevented by A-438079 [25]. By having dual effects, P2X7R antagonists may suppress both seizures and proinflammatory sequelae, which could be important in longer-term outcomes where inflammation has been implicated in epileptogenesis [44].

A common feature of the models where anticonvulsive effects are seen with P2X7R antagonists is the presence of early and significant seizure-induced lesions to the hippocampus [25,26]. This may release ATP or provoke glial and pro-inflammatory responses where the contribution of P2X7R activation is uniquely pathogenic [28]. Indeed, this may explain the lack of effects or even opposite response in nonlesional adult SE models [27]. The mechanism of the neuroprotection in the present study is likely to be mainly through seizure suppression, but P2X7R antagonists have been reported to have direct neuroprotective effects in nonseizure models [45–47].

In the present study, we also found phenobarbital had efficacy against SE in the intra-amygdala KA model, reducing seizure

severity by 60–70% although a complete dose-response analysis was not undertaken. This is quite similar to reported effects of phenobarbital in studies using systemic KA to induce SE in P10 [33,36] and older [48] rats. A surprising finding in the present study was the limited efficacy of phenobarbital against seizure-induced neuronal death in the model, despite the drug reducing seizure severity. The explanation for such uncoupling between seizures and damage with phenobarbital is uncertain. Phenobarbital may fail to prevent direct excitotoxicity in the model, or it is possible that cortical seizures were suppressed, while intrahippocampal seizures continued. Concern remains that high doses of anticonvulsants may promote neuronal apoptosis or suppress neurogenesis in the developing brain although consistent with other reports [34], we found phenobarbital was safe in P10 rats when given alone at 25 mg/kg. Notably, even at the highest dose tested (50 mg/kg), A-438079 alone did not promote cell death in P10 rats. Together, these findings suggest a continuing need to assess the safety of phenobarbital for the treatment of neonatal seizures and SE in the immature brain [49,50].

Bumetanide has been reported to inhibit neonatal seizures in P10 rats given systemic KA [36] as well as brief hypoxia-induced seizures, neither of which are lesional insults. Here, we did not observe seizure-suppressive effects of bumetanide in the P10 intra-amygdala KA model. A recent study also failed to detect an effect of bumetanide at 0.15 mg/kg against hypoxia-induced seizures when given alone [34]. This suggests bumetanide may not be effective against all types of seizures in the immature brain. Of course, we may have missed effects at other doses. The findings suggest the P10 rat intra-amygdala KA model may not capture effects of certain antiseizure compounds but nevertheless underscores the need for a range of models to be profiled [51]. Combining bumetanide with phenobarbital has been reported to produce synergistic effects against neonatal seizures [34], but we did not explore a combination of bumetanide with A-438079 in the present study.

Our study identified some potential limitations of A-438079. First, A-438079 passes into the brain following parenteral injection but is rapidly eliminated, possibly restricting the therapeutic window of this compound. This may explain why A-438079 did not completely suppress seizures at any dose. New P2X7R antagonists are in preclinical development with greater potency and extended CNS stability [52] which may provide superior seizure-suppressive effects and avoid some of the deleterious effects of directly targeting GABA and glutamate receptors [53]. However, translation of these results to clinical trials will likely require the drug to be an "add-on", for example to phenobarbital or where frontline treatment has failed. In our studies, we did not observe superior seizure suppression but addition of A-438079 may provide better neuroprotection than phenobarbital alone. We also observed a *U*-shaped dose response with A-438079, with the highest dose having weaker effects on seizures and the protective effects being lost. This suggests A-438079 may have off-target effects, an idea which could be pursued in future using P2X7R antagonists of a different chemical structure or with improved specificity. Third, a number of additional studies will be needed. This includes tests in another model(s), determining the role of IL1β reduction in the seizure or neuroprotective effects, and determining whether preventing SE by targeting P2X7R has effects on later-life cognitive impairments or epilepsy [30]. In summary, the present study identifies seizure-suppressive and neuroprotective effects of the P2X7R antagonist A-438079 against focal-onset SE in immature rats. These findings may help inform future preclinical test models and a novel focus for therapeutic approaches to prolonged early-life seizures.

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Conflict of Interest

The authors declare no conflict of interest.

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