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Effects of P2X7 receptor antagonists on hypoxia-induced neonatal seizures in mice

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ABSTRACT

Neonatal seizures are a common consequence of hypoxic/ischemic encephalopathy (HIE). Phenobarbital remains the frontline treatment for neonatal seizures but is often ineffective. The P2X7 receptor (P2X7R) is a cell surface-expressed ionotropic receptor activated by high amounts of ATP which may be released during seizures or as a consequence of tissue injury. Here, we explored the role of the P2X7R in a mouse model of neonatal seizures induced by hypoxia. Exposure of postnatal day 7 (P7) mouse pups to global hypoxia (5% O_2 for 15 min) produced electrographically-defined seizures with behavioural correlates that persisted after restitution of normoxia. Expression of the P2X7R showed age-dependent increases in the hippocampus and neocortex of developing mice and was present in human neonatal brain. P2X7R transcript and protein levels were increased 24 h after neonatal hypoxia-induced seizures in mouse pups. EEG recordings in pups determined that injection of the P2X7R antagonist A-438079 (25 mg/kg^{-1}, intraperitoneal) reduced electrographic seizure number, EEG power and spiking during hypoxia. A-438079 did not reduce post-hypoxia seizures. Caspase-1 processing and molecular markers of inflammation and microglia were reduced in A438079-treated mice. Electrographic seizure-suppressive effects were also observed with a second P2X7R antagonist, JNJ-47965567, in the same model. The present study shows hypoxia-induced seizures alter expression of purinergic and neuroinflammatory signalling components and suggest potential applications but also limitations of the P2X7R as a target for the treatment of HIE and other causes of neonatal seizures.

1. Introduction

Seizures are a common and serious co-morbidity in neonates suffering from conditions such as hypoxic-ischemic encephalopathy (HIE), stroke and meningitis (Vasudevan and Levene, 2013). The incidence of neonatal seizures is 1–5/1000 in term new-borns and 10–130/1000 in preterm new-borns (Silverstein and Jensen, 2007; Vasudevan and Levene, 2013). Both animal studies and clinical findings suggest neonatal seizures impose a severe metabolic burden and/or directly result in neuronal injury (Dzhala et al., 2000; Glass et al., 2009; Hall et al., 1998; Jensen, 2009; Yager et al., 2002). As a result, routine clinical practice is to treat neonatal seizures. Phenobarbital, a γ-amino butyric acid receptor A ($GABA_A$-R) agonist is the most common frontline drug for neonatal seizures but is often ineffective (Chapman et al., 2012; Low et al., 2016; Painter et al., 1999). There are also concerns that altering GABAergic function during this critical developmental period has harmful effects on brain development (Bittigau et al., 2002; Ikonomidou and Turski, 2010). In more recent years, bumetanide, an antagonist of the sodium-potassium-chloride cotransporter 1 (NKCC1) emerged as a leading candidate for neonatal seizure therapy (Ben-Ari, 2002; Dzhala et al., 2005). However, one multicentre clinical trial was recently stopped due to concerns with efficacy and ototoxicity (Pressler et al., 2015). Among non-

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pharmacologic treatments, brain cooling is beneficial for protection against death and disability in HIE but efficacy against neonatal seizures remains unproven (Azzopardi et al., 2015; Low et al., 2012; Rossetti, 2011). Consequently, there is an urgent need to identify novel drug targets for the treatment of neonatal seizures (Donovan et al., 2016).

P2X receptors are ligand-gated cation-nonselective ionotropic receptors that orchestrate responses to extracellular adenosine triphosphate (ATP). The source of ATP release for P2X receptors is thought to be a combination of activity-dependent release as a neuro-/glio-transmitter and via damaged cells, for example after hypoxia/ischemia or prolonged seizures (Burnstock, 2008; Eltzschig et al., 2013). Of the seven P2X receptors, there has been most interest in targeting the P2X7R for seizure control (Engel et al., 2016). The P2X7R is sensitive only to high amounts of ATP (high μM, low mM range) and is therefore activated under mainly pathological conditions (Sperlagh and Illes, 2014). P2X7R stimulation is a primary mechanism for microglial activation and inflammasome-mediated caspase-1-dependent interleukin-1β (IL-1β) release. This pathway is activated in the brains of neonates who experienced HIE-induced neonatal seizures (Schiering et al., 2014). The P2X7R may also function pre-synaptically to regulate neurotransmitter release (Sperlagh and Illes, 2014). Recent studies show that pharmacologic and genetic targeting of the P2X7R suppresses chemoconvulsant-induced status epilepticus in select rodent models (Engel et al., 2012; Fischer et al., 2016; Jimenez-Pacheco et al., 2013) and reduces spontaneous seizures in epileptic mice (Jimenez-Pacheco et al., 2016). Notably, the potent and specific P2X7R antagonist A-438079 reduced seizures and hippocampal damage triggered by intra-amygdala kainic acid in postnatal day 10 (P10) rats, a model of early-life status epilepticus (Mesuret et al., 2014).

It is unknown whether P2X7R antagonists affect neonatal seizures induced by aetiologically-relevant stimuli such as low oxygen. We recently developed an age-appropriate, non-invasive model of hypoxia-induced neonatal seizures in P7 mice (Rodriguez-Alvarez et al., 2015). The model features electro-clinical seizures during and after brief global hypoxia and sub-therapeutic responses to phenobarbital. Here, we investigated P2X receptor expression in experimental and human samples from neonates with HIE and epilepsy and pharmacologic targeting of the P2X7R in this model.

2. Materials and methods

2.1. Mouse model of neonatal hypoxia-induced seizures

Animal experiments were performed in accordance with the guidelines of the European Communities Council Directives (86/609/EU and 2010/63/EU) and were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC #765) under license from the Department of Health, Dublin, Ireland (B100/4524) and Health Products Regulatory Authority (AE19127/P013/I036). Litters of male and female C57Bl/6 J mice (Harlan) were kept with their dams and maintained in a barrier-controlled facility on a 12 h light:dark cycle with unlimited access to food and water. Procedures for inducing hypoxia in mouse pups were undertaken as previously described (Jensen, 1995; Rodriguez-Alvarez et al., 2015). Under normothermic conditions (33 ± 2 °C/80% humidity), pups were placed in an air-tight chamber for 15 min and exposed to a premixed gas containing 5% O_2/95% N_2 to produce global hypoxia. Non-hypoxic control pups underwent the same procedure but were exposed to 21% O_2 for an equivalent time. All animals were observed and videotaped during and for up to 60 min after hypoxia and behavioural seizures were scored using a modified Morrison scale for scoring hypoxia/ischemia-induced seizures in neonatal mice (Comi et al., 2004; Morrison et al., 1996): Score 0, normal behaviour; 1a, immobility; 1b, immobility and myoclonic jerks; 2, rigid posture; 3, circling, repetitive swimming and pedalling movements, head bobbing and tail extension; 4, spasms, forelimb clonic–tonic seizures, loss of posture with hyperventilation; 5, repeated stage 4.

For EEG recordings, mouse pups were separated from the dam and placed in a stereotaxic frame equipped with a neonatal rodent adaptor under anaesthesia (isoflurane/oxygen mixture; 5% for induction, 3% for maintenance). Body temperature was maintained using a feedback controlled heat blanket (Harvard Apparatus Ltd. U.K.). Following a midline scalp incision, three partial craniectomies were performed and skull-mounted electrodes (stainless steel screws soldered to a Teflon-insulated stainless steel wire; E/363/20, Bilaney Consultants Ltd. UK) were affixed to the skull surface, with one overlying each temporal area and one as a reference above the cerebellum. The electrode assembly was fixed using dental cement. EEG was recorded using a Grass Comet XL digital EEG-amplifier and digitalized with TWin EEG software using Notch filter (70 Hz) (Natus/Grass Technologies Ltd. Warwick, RI, USA). Pups were returned to an incubator at nest temperature (33 ± 2 °C) to recover from surgery. After 1 h of recovery, hypoxia-induced seizures were induced as above. Pups subject to acute EEG procedures were euthanized on completion of recordings. For analysis of EEG data, files were exported to LabChart Pro software (V7, ADInstruments Ltd.). Seizure-like activity was defined as bilateral electrographic seizures comprising the appearance of several polyspike discharges, spiking with abrupt alteration relative to baseline rhythm, of high frequency (>5 Hz baseline), high amplitude (>2 times baseline) and lasting >5 s with clear evolution (Mizrahi and Clancy, 2000; White et al., 2010). Electrographic data was binned into 30 s or 5 min intervals (epochs) prior to analysis. The number, time to onset (relative to hypoxia) and duration of seizures (measured as the time from first spike to last spike) were calculated per hypoxic and post-hypoxic periods of video-EEG recordings. EEG total power and high-amplitude spikes were also analysed in both periods. The total power (range 0–50 Hz) was automatically calculated by LabChart software and normalized to the baseline (pre-hypoxia induction) for each animal. To compute the contribution of the EEG total power into the different frequency bands, total power was fractionated into five frequency bands, as follows: 0–3.99 Hz (δ), 4–7.99 Hz (θ), 8–12.99 Hz (α), 13–29.99 Hz (β) and 30–80 Hz (γ). Spikes were counted automatically as events in the EEG recordings with rapid positive and/or negative components (mono-, bi- or multi-phasic) lasting less than 200 msec (2.5 S. D). Spike event count is related to the frequency of the discharge and represents the number of high-amplitude spikes >50 μV. These spikes occurred during and between seizures (ictal and interictal periods). Power spectral density heat maps were generated with the LabChart program (module: Spectrum view) filtering the EEG in the frequency domain from 0 Hz to 40 Hz and in the amplitude domain from 0 to 5 μV. EEG heat maps were set to fast Fourier transform (FFT) size:1K; Data window: rainbow spectrum: Window overlap: 50%). Lastly, artifacts caused by incorrect electrode placement or animal movement were identified as noise and excluded from the analysis manually.

2.2. Drug treatments

Pups from the same litter were randomly assigned to vehicle or drug. All drugs were freshly prepared immediately before use and delivered via an intraperitoneal injection in 0.1 mL volume. A subgroup of P7 mice were equipped for EEG recordings as above and then, 5 min before hypoxia, pups received an intraperitoneal