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Anticonvulsant effect of flupirtine in an animal model of neonatal hypoxic-ischemic encephalopathy

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ABSTRACT

Research studies suggest that neonatal seizures, which are most commonly associated with hypoxic-ischemic injury, may contribute to brain injury and adverse neurologic outcome. Unfortunately, neonatal seizures are often resistant to treatment with current anticonvulsants. In the present study, we evaluated the efficacy of flupirtine, administered at clinically relevant time-points, for the treatment of neonatal seizures in an animal model of hypoxic-ischemic injury that closely replicates features of the human syndrome. We also compared the efficacy of flupirtine to that of phenobarbital, the current first-line drug for neonatal seizures. Flupirtine is a KCNQ potassium channel opener. KCNQ channels play an important role in controlling brain excitability during early development. In this study, hypoxic-ischemic injury was induced in neonatal rats, and synchronized video-EEG records were acquired at various time-points during the experiment to identify seizures. The results revealed that flupirtine, administered either 5 min after the first electroclinical seizure, or following completion of 2 h of hypoxia, i.e., during the immediate reperfusion period, reduced the number of rats with electroclinical seizures, and also the frequency and total duration of electroclinical seizures. Further, daily dosing of flupirtine decreased the seizure burden over 3 days following HI-induction, and modified the natural evolution of acute seizures. Moreover, compared to a therapeutic dose of phenobarbital, which was modestly effective against electroclinical seizures, flupirtine showed greater efficacy. Our results indicate that flupirtine is an extremely effective treatment for neonatal seizures in rats and provide evidence for a trial of this medication in newborn humans.

1. Introduction

The probability of occurrence of seizures during the neonatal period is higher than any other age group (Hauser et al., 1993). The incidence of seizures in newborns is estimated at 1.8–3.5 per 1000 live births (Lanska et al., 1995; Saliba et al., 1999). Approximately 60% of neonatal seizures are associated with a hypoxic-ischemic event (Ronen et al., 2007; Tekgul et al., 2006). Survivors of neonatal hypoxic-ischemic encephalopathy (HIE) often develop brain injury and neurologic disabilities (e.g. cognitive deficits and epilepsy) in later life (Bergamasco et al., 1984; Robertson and Finer, 1988). Studies in humans and animal models suggest that seizures contribute to brain damage and adverse neurologic outcome (Dzhala et al., 2000; Glass et al., 2009; McBride et al., 2000; Miller et al., 2002; Wirrell et al., 2001). In clinics across the world, phenobarbital is the drug of choice for the treatment of neonatal seizures (Blume et al., 2009; Carmo and Barr, 2005). However, phenobarbital, an agonist of γ-aminobutyric acid (GABA), is not completely effective in controlling seizures; it does not stop seizures in 50% of patients. Moreover, it has only a limited efficacy in treating electrographic seizures (Foster and Lewis, 2007; Painter et al., 1999; Sankar and Painter, 2005). Frequently, clinical seizures will respond to phenobarbital but then electrical seizures will persist. In neonates, electrographic seizure activity has been shown to correlate with poor neurodevelopmental outcome (McBride et al., 2000), and treatment of electrographic seizures in addition to clinical seizures has been demonstrated to significantly reduce the severity of brain injury (van Rooij et al., 2010). Further, in developing rats and children, phenobarbital treatment has been linked to neuronal and white matter apoptosis, chronic changes in gene expression, and synaptic and cognitive impairment (Bittigau

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Abbreviations

GABA	γ-aminobutyric acid
h	hour
HI	hypoxia-ischemia
HIE	hypoxic-ischemic encephalopathy
min	minutes
P7	postnatal day 7
sec	second

et al., 2002; Farwell et al., 1990; Forcelli et al., 2012; Kaushal et al., 2016; Raol et al., 2005). The GABAergic inhibitory system of the immature brain is underdeveloped as compared to that of the adult brain (Ben-Ari, 2002; Brooks-Kayal and Pritchett, 1993; Gibbs et al., 1996; Kapur and Macdonald, 1999), which may partly explain the suboptimal efficacy of phenobarbital in the treatment of neonatal seizures. Thus, development of a safe treatment that is effective against both clinical and purely electrographic seizures is critical.

In the kainic acid and flurothyl models of neonatal seizures, flupirtine is very effective and more efficacious than phenobarbital and diazepam in stopping seizures (Raol et al., 2009). Flupirtine (ethyl-*N*-[2-amino-6-(4-fluorophenylmethyl-amino)pyridin-3-yl] carbamate) has been used as an analgesic in Europe for the last 30 years. Flupirtine shifts the voltage required to open KCNQ type of potassium channels to a more negative potential, resulting in an increased threshold for generating a neuronal action potential (Klinger et al., 2012; Martire et al., 2004; Wladyka and Kunze, 2006). KCNQ channels are voltage gated, depolarization activated potassium channels whose expression in the brain begins before birth (Brown and Passmore, 2009; Devaux et al., 2004; Okada et al., 2003). These channels play a very important role in controlling over excitation during early life when the GABA-mediated inhibition is weak (Pena and Alavez-Perez, 2006; Peters et al., 2005). Mutations in genes encoding KCNQ2/3 channels result in benign familial neonatal epilepsy (BFNE), a relatively mild condition in which seizures resolve spontaneously within a few weeks after the onset and majority of patients have normal outcome, and KCNQ2 encephalopathy, in which seizures are usually pharmacoresistant and patients have an epileptic encephalopathy with severe to moderate intellectual disability (Kato et al., 2013; Saitsu et al., 2012; Singh et al., 2003; Weckhuysen et al., 2012). Flupirtine has been shown also to activate G-protein-regulated inwardly rectifying K^+ channels (GIRK) (Jakob and Krieglstein, 1997; Kornhuber et al., 1999; Montandon et al., 2016; Sattler et al., 2008) (but see (Klinger et al., 2012)), which has been suggested to indirectly inhibit NMDA receptor activity (Klinger et al., 2012; Kornhuber et al., 1999) (but see (Jakob and Krieglstein, 1997)). Recent studies suggest that flupirtine may also shift the gating of $GABA_A$ receptors to lower GABA concentration, an action that is more pronounced in dorsal horn neurons than in hippocampal neurons (Klinger et al., 2012). In our recent study, we showed that flupirtine given to 10-day-old rats before exposure to global hypoxia prevented development of electroclinical seizures (behavioral seizures with an EEG correlate) during a hypoxic episode (Sampath et al., 2015). In humans, however, the treatment is usually started after a seizure is observed. In neonates with HIE, the median age for the detection of purely electrographic seizures has been reported as 13.1 h (Lynch et al., 2015), but clinical seizures have been observed as early as 4 h of age in babies in whom the injury occurred before labor (Filan et al., 2005). These seizures often continue to occur for 48 h or more (Ahn et al., 1998; Filan et al., 2005).

In the current study, we examined the efficacy of flupirtine to stop seizures when given at clinically relevant time points. The rat model of HIE that we used replicates the etiology and characteristics of seizures in human newborns (Sampath et al., 2014). We also compared the efficacy of flupirtine with phenobarbital, the first-line choice for the treatment of neonatal seizures. Our result suggests that flupirtine is highly efficacious against HI-induced neonatal seizures, and is effective even when given following 2 h of hypoxia and multiple seizures. In our study, phenobarbital at a clinically therapeutic dose was only modestly effective in reducing and preventing seizures. This is similar to the situation in human neonates, supporting the use of our experiment as a model for the human case.

2. Methods

2.1. Animals

All procedures involving animals were performed in accordance with the NIH guidelines for the care and use of laboratory animals, and according to the protocol approved by the Institutional Animal Care and use Committee (IACUC) of the University of Colorado Anschutz Medical Campus (UC-AMC). In addition, all efforts were made to reduce animal suffering and the number of animals used. Timed pregnant Sprague-Dawley rats were obtained from Charles River laboratories (Wilmington, MA). The pregnant rats were at the 14^{th} day of gestation (E14) on arrival at the vivarium and delivered the pups at E22 or E23. Only male pups were used for the study.

2.2. Hypoxia-ischemia protocol

HI was induced in postnatal day 7 (P7) rats by Rice-Vannucci method (Rice et al., 1981; Sampath et al., 2014). Under isoflurane anesthesia (2–4% for induction and 1–1.5% maintenance), the right common carotid artery was identified and double ligated with a 4–0 polyglycolic acid suture. The neck incision was closed with 4–0 nylon Dermalon sutures. The entire surgical procedure lasted for 10–12 min. Following the ligation, the pups were housed with the dam in a warm cage for 1.5–2.25 h before they were exposed to hypoxia. The pups were exposed to hypoxic environment (8–8.3% oxygen) for 2 h. The oxygen content of the chamber was monitored using an oxygen sensor (Dräger Pac 7000, Pittsburg, PA). During the hypoxia, the temperature was maintained at 36.5° Celsius and humidity between 60 and 70%. Following HI-induction, the pups were treated with analgesic (0.1 mg/kg buprenorphine hydrochloride) once every 12 h for 48 h.

2.3. Electrode implantation

To record EEG, the electrode implantation was performed according to our published protocol (Sampath et al., 2014). Briefly, P6 rats were implanted bilaterally in the parietal cortex with a silver electrode (A-M Systems, Carlsborg, WA). The active electrode in each hemisphere was referenced to a separate silver electrode positioned near lambda in the same hemisphere. The implantation procedure was performed under isoflurane anesthesia (2–4% for induction and 1–1.5% for maintenance). After the surgery, the rats were returned to the dam and treated with analgesic (0.1 mg/kg buprenorphine hydrochloride) once every 12 h for 48 h.

2.4. Video-EEG monitoring

Synchronized, time locked video and EEG signals were recorded using the Stellate Harmonie system (Natus Medical, San Carlos, CA, U.S.A.). The EEG signal was digitized at 1000 Hz and stored on a