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Flupirtine effectively prevents development of acute neonatal seizures in an animal model of global hypoxia

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Abstract

Current first-line drugs for the treatment of neonatal seizures have limited efficacy and are associated with side effects. Uncontrolled seizures may exacerbate brain injury and contribute to later-life neurological disability. Therefore, it is critical to develop a treatment for neonatal seizures that is effective and safe. In early-life, when the γ -aminobutyric acid (GABA) inhibitory system is not fully developed, potassium channels play an important role in controlling excitability. An earlier study demonstrated that flupirtine, a KCNQ potassium channel opener, is more efficacious than diazepam and phenobarbital for the treatment of chemoconvulsant-induced neonatal seizures. In newborns, seizures are most commonly associated with hypoxicischemic encephalopathy (HIE). Thus, in the present study, we examined the efficacy of flupirtine to treat neonatal seizures in an animal model of global hypoxia. Our results showed that flupirtine dose dependently blocks the occurrence of behavioral seizures in pups during hypoxia. Additionally, flupirtine inhibits the development of hypoxia-induced clinical seizures and associated epileptiform discharges, as well as purely electrographic (subclinical) seizures. These results suggest that flupirtine is an effective anti-seizure drug, and that further studies should be conducted to determine the time window within which its administration can effectively treat neonatal seizures.

Keywords

Neonatal Seizures; Video- EEG monitoring; hypoxia; flupirtine; Potassium channel opener

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1. Introduction

Seizures are common in human neonates and are most frequently associated with hypoxia-ischemia [38]. Survivors of neonatal hypoxic-ischemic encephalopathy (HIE) often experience neurodevelopmental disabilities and seizures in later life [4, 11, 26]. Studies in both human neonates and animal models suggest that seizures themselves may independently contribute to brain injury and poor neurological outcome [5, 18, 29, 31] (but also see [25, 41]). Unfortunately, neonatal seizures are often resistant to treatment with approved antiepileptic drugs [15]. Throughout the world phenobarbital, an agonist of γ -aminobutyric acid (GABA), the major inhibitory neurotransmitter in the adult brain, is the most commonly used drug for treating neonatal seizures [6, 10]. The GABAergic system of the immature brain is underdeveloped compared to that of the mature brain, making it a sub-optimal target for the treatment of neonatal seizures [3, 7, 17, 21]. Evidence from clinical and basic science research studies suggests that KCNQ potassium channels play a very important role in controlling excitation in early-life [33, 35, 36, 39]. Flupirtine, a KCNQ channel opener [12, 23, 28, 42], has been used clinically as an analgesic in Europe for over two decades with a good safety record. Our earlier study demonstrated that flupirtine is more efficacious than diazepam and phenobarbital for the treatment of chemoconvulsant-induced neonatal seizures [37]. In the current study, we evaluated the efficacy of flupirtine in the treatment of hypoxia-induced neonatal seizures in an animal model. The results suggest that flupirtine is effective in preventing development of both electroclinical and purely electrographic seizures during hypoxia.

2. Materials and methods

All animal procedures 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). 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 14th day of gestation (E14) on arrival at the vivarium and delivered the pups at E22 or E23. The pups from both sexes were used for the study.

2.1. Hypoxia protocol

Ten day old (P10) rat pups were exposed to global hypoxia according to a published protocol [24]. The oxygen concentration of the chamber was maintained at 7% for 8 minutes, 6% for 4 minutes, 5% for 2 minutes and 4% for 1 minute. The oxygen was balanced with nitrogen and the concentration of oxygen in the chamber was monitored using an oxygen sensor (Drager Pac 7000, Pittsburg, PA). The total hypoxia time varied slightly between the experiments, instead of exactly 15 minutes, as it took some time to equilibrate the chamber with the desired concentration of oxygen. During hypoxia, the temperature of the chamber was maintained at 36 - 37 °C, and the humidity at ~80%.

2.2. Flupirtine treatment protocol

In order to minimize the number of rats with electrodes for electroencephalography (EEG), as surgery for electrode implantation can cause short-term pain and distress to pups, a two-step approach was taken to examine the efficacy of flupirtine on behavioral and electrographic seizures. First, we identified a smallest drug dose that was most effective in preventing development of behavioral seizures during hypoxia (rats were not implanted to record EEG). For this, the rat pups were treated by intraperitoneal (i.p.) injection with either 25, 35, 45, and 50 mg/kg body weight flupirtine, or the vehicle (dimethyl sulfoxide and saline; 3:7 vol/vol) 15 to 30 minutes before exposure to hypoxic environment, and monitored for signs of behavioral seizures during hypoxia. The flupirtine doses were selected based on our previous study where we observed that the 50 mg/kg flupirtine was effective in preventing chemoconvulsant seizures [37]. The smallest flupirtine dose that was most efficacious in preventing behavioral seizure was then chosen (35 mg/kg, i.p.) to determine if it also treats EEG correlate of behavioral (clinical) seizure, and purely electrographic (subclinical) seizures.

2.3. Electrode implantation for the Video-EEG recording

At P9, the rat pups were implanted with the electrodes to record the electrical activity of the brain. One silver wire electrode (0.01" outer diameter; AM systems, Carlsborg, WA) was placed in each hemisphere of the brain over the parietal cortex. A silver electrode placed over the right and left side of the brain behind the lambdoid suture served as reference and ground electrodes respectively. The electrode assembly was held in place on the rat skull with tissue adhesive (3M Vetbond, St Paul MN) and dental acrylic cement. The entire 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 hours for 48 hours.

2.4. Seizure monitoring and analysis

Behavioral seizures—The P10 rats were continuously monitored during hypoxia by an investigator (Yogendra Raol) and the occurrences of behavioral seizures were noted manually. The behavioral seizures (the rats were not implanted with electrodes to record EEG) consisted of head shakes, and clonic and tonic limb movements.

Electroclinical seizures—At P10, the rats were connected to an EEG monitoring unit (Stellate Harmonie system, Natus Medical, San Carlos, CA) to record EEG signals time-locked with digital video. The EEG signal was digitized at 1000 Hz and stored on a hard disk for offline analysis. Following a 20 to 30 minutes of baseline recording, the pups were given either vehicle or 35 mg/kg flupirtine by i.p. injection. After 15 minutes of video-EEG recording following the treatment, the pups were exposed to graded hypoxia. The pups were continuously monitored by video-EEG during the hypoxia. The video-EEG records were reviewed by a board certified clinical epileptologist (Andrew White) who was blinded to the treatment paradigm. Electroclinical seizures were defined by an EEG pattern that differed from background in either amplitude, frequency, or both, evolved over time, and contained spikes or sharp waves lasting for 10 s or more and were associated with a change in the rat's

behavior. Electrographic seizures were defined as seizures observed in the EEG record that were not associated with a behavioral correlate on video.

2.5. Statistical analysis

GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. Fisher's exact test and the Mann Whitney test were used to determine the statistical significance of effects of flupirtine treatment on the frequency of rats developing seizures, and the amount of time spent in electroclinical seizures, respectively during hypoxia. P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Flupirtine blocks development of behavioral seizures during hypoxia

To study the effect of flupirtine on hypoxia-induced seizures, the P10 rats were given various doses of the drug or vehicle 15-30 minutes before exposure to hypoxia. All of the pups that were treated with vehicle ($n = 12$; males (M) = 7, females (F) = 5) developed behavioral seizures during hypoxia (Figure 1). The behavioral seizures consisted of head shakes, and clonic and tonic limb movements. Five out of eight pups ($n = 8$; 3M, 5F) treated with 25 mg/kg flupirtine did not exhibit any type of behavioral seizure; the remaining three rats developed head shakes during hypoxia (Figure 1). None of the rats treated with 35 ($n = 5$; 3M, 2F), 45 ($n = 3$; 2M, 1F) or 50 ($n = 5$; 3M, 2F) mg/kg flupirtine developed behavioral seizures during exposure to the graded hypoxia (Figure 1).

3.2. Flupirtine prevents development of electroclinical seizures during hypoxia

In neonates there is often dissociation between clinical (behavioral) seizures and EEG phenomena, known as electroclinical uncoupling [19]. Because of this phenomenon, some drugs effectively treat the behavioral component of the neonatal seizure without stopping the corresponding electrographic seizure. To determine the effects of flupirtine on electroclinical seizures (behavioral seizures with an EEG correlate), the P10 rats were treated with either 35 mg/kg flupirtine, the lowest dose that prevented behavioral seizures in the maximum number of rats in the current study (Figure 1), or vehicle 15 minutes before hypoxia. All vehicle treated rats experienced electroclinical seizures (Figure 2B) at varying frequency ($n = 6$; 4F, 2M; range = 1 – 10; mean $\pm$ SD = 0.33 ± 0.18 seizures/minute of hypoxia) during hypoxia that lasted on average for 19 minutes. Purely electrographic seizures (EEG seizures without a behavioral correlate; Figure 2C) were observed in three out of six vehicle treated rats during hypoxia (range = 0 – 6; mean $\pm$ SD = 0.076 ± 0.13 EEG only seizures/minute of hypoxia). The mean total duration of these electrographic seizures was 56.83 seconds ($\pm$ SD = 128 seconds). None of the 35 mg/kg flupirtine treated rats ($n = 6$; 3F, 3M) developed electroclinical seizures during hypoxia (Figure 3A, the average hypoxia duration was 18.2 minutes). In contrast, vehicle treated rats were seizing on an average for 14.61% time (calculated as (time in seizure $\times$ 100) $\div$ total duration of hypoxia) they were in hypoxic environment (Figure 3B). Further, none of the flupirtine treated rats developed purely electrographic seizures during hypoxia. A Behavioral seizure, as defined in the Materials and Methods Section, without an electrographic correlate was not observed in any rats.

Flupirtine treatment in rats (given before hypoxia) resulted in visually obvious suppression in EEG activities that was interspersed with burst-like activity (Figure 4B). These burst suppression-like patterns appeared within seconds of flupirtine injection. The burst-like activity lasted on an average for 5 seconds, occurred approximately every 15 seconds, and continued to appear even during hypoxia. The burst suppression-like pattern appeared similar to what has been observed with the use of anesthetics such as isoflurane [1, 14], and high doses of anticonvulsants [27].

4. Discussion

The current study demonstrates that flupirtine, a potassium channel opener, effectively blocks hypoxia-induced neonatal seizures. In neonates it is often observed that some drugs effectively treat the behavioral (clinical) component of the seizure without stopping the electrographic discharge [19]. Using time-locked video-EEG recording technique we show that flupirtine not only prevents the occurrence of behavioral seizures during hypoxia, it also treats the electrographic (subclinical) component. Further, unlike 50% of vehicle treated rats that developed purely electrographic seizures (at very low frequency), none of the flupirtine treated rats experienced such seizures. These observations are in agreement with the results of our previous study, wherein flupirtine effectively blocked chemoconvulsant-induced neonatal seizures [37]. Another study has shown that pre-administration of flupirtine increases the latency to first seizure, and reduces the duration and severity of febrile seizures [43].

Flupirtine shifts the voltage dependent activation of KCNQ channels towards hyperpolarized potentials [28, 42] resulting in an increased threshold for generating neuronal action potentials. Recent studies suggest that flupirtine also shifts the gating of GABA_A receptors to lower GABA concentrations [22, 23]. KCNQ channels (KCNQ1-5) are voltage-gated, depolarization activated potassium channels and are expressed in the nervous system [8, 20]. Mutations in KCNQ2/3 channels have been associated with benign familial neonatal seizures, a syndrome in which seizures resolve spontaneously within a few weeks after the onset, and KCNQ2 encephalopathy, a type of epilepsy in which seizures are usually pharmacoresistant and in which patients have an epileptic encephalopathy with moderate to severe intellectual disability. Certain mutations observed in patients with KCNQ2 encephalopathy result in an increase in threshold potential for the channel opening, slowing of the activation kinetics, and reduction in current amplitude [34]. Retigabine, an analogue of flupirtine, shifted the voltage activation of mutated KCNQ2 channels to more hyperpolarized potentials, and also increased the current amplitude [34]. Retigabine has been approved by the United States Food and Drug Administration (FDA) for the treatment of partial epilepsies in adult patients. However, retigabine has been associated with side effects such as blue discoloration in the skin and retinal abnormalities. Since flupirtine has a similar mechanism of action on KCNQ channels as retigabine, we believe that flupirtine will also effectively treat seizures observed in infants with KCNQ2 encephalopathy.

Flupirtine was first approved for use as a non-opioid analgesic in Europe in the 1980's. In the United States, flupirtine has not been approved for any indication by the FDA. Flupirtine is usually associated with mild side-effects, such as dizziness, dry mouth, nausea, pruritis,

fatigue, and heartburn. In recent years, however, concerns have been raised regarding association of long-term use of flupirtine and hepatotoxicity [13, 30]. In the present study, we observed that 35 mg/kg of flupirtine induced a burst suppression-like EEG pattern in the normal rats. Burst suppression is an EEG pattern in which high voltage activity alternates with the periods of severely attenuated EEG, and is often observed with deep levels of anesthesia or high doses of anticonvulsants such as phenobarbital [1, 14, 27]. In fact, induction of the burst suppression pattern by intravenous volatile anesthetics has been used in clinics to terminate refractory status epilepticus. Sedatives and anesthetics that severely reduce neuronal activity have been shown to cause apoptosis and behavioral impairments in later life in normal animals [40], however, in animal models of neonatal HIE, anesthetics such as isoflurane reduce brain injury and improve neurological outcome [9].

One of the limitations of the current study is the use of a protocol in which the animal is given the drug prior to the insult. In the case of a hypoxic-ischemic incident in a neonate, it is likely that the drug would have to be administered subsequent to the insult. There are other instances, such as during infant cardiac surgery, where prophylactic drugs could be given to reduce occurrence of seizures and brain injury. EEG seizures have been observed in 14 to 20% neonates following cardiac surgery with cardiopulmonary bypass [16, 32], and the use of benzodiazepines during the surgery may reduce seizure occurrence [2]. Further, this study provides a strong proof that flupirtine effectively treats hypoxia-induced neonatal seizures, and hence further studies should be carried out to determine its efficacy when given after the injury or insult has occurred, for example, during the reperfusion period following HI.

5. Conclusion

Our data clearly suggest that flupirtine is effective in treating hypoxia-induced neonatal seizures. Studies to determine the therapeutic window within which flupirtine can stop hypoxiaischemia induced neonatal seizures and the comparison of its efficacy with that of phenobarbital are currently underway in our laboratory. Further, it will be both interesting and important to study the effects of treatment of neonatal seizures on hypoxia-ischemia induced brain injury and adverse neurodevelopmental outcome.

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HIGHLIGHTS

- We examined efficacy of flupirtine to treat hypoxia-induced neonatal seizures.
- Flupirtine blocks development of behavioral seizures during hypoxia.
- Flupirtine treats clinical seizures and associated epileptiform activity.

Figure 1.

Flupirtine pretreatment effectively blocks hypoxia-induced behavioral seizures. All the P10 rat pups pretreated with 50 (5/5), 45 (3/3) or 35 (5/5) mg/kg flupirtine that were exposed to graded global hypoxia remained seizure free, i.e., none of them developed behavioral seizures during hypoxia. None of the rats pretreated with vehicle (12/12) and 60% rats pretreated with 25mg/kg flupirtine (5/8) remained seizure free. Statistical comparisons were made individually between the vehicle treated group and each of the flupirtine treated groups. * $p < 0.005$, Fisher's exact test. Hy = Hypoxia, Veh = Vehicle, Flup = Flupirtine.

Figure 2.

Representative EEG tracings from P10 rats during exposure to graded hypoxia. (A) The tracing shows an example of inter-ictal EEG during hypoxia. A magnified excerpt of a part of the EEG, marked by a bar above the tracing (1), is provided under the compressed EEG tracing. (B) Electroclinical seizure during the hypoxia. The EEG ictal activity was associated with a brief clonus and tonic extension of hindlimbs. The enlarged excerpt of a part of the EEG during tonic component of the behavioral seizure activity (2) is provided under the compressed EEG tracing. (C) The representative EEG from a different rat shows

purely electrographic seizure (no behavioral component associated) during the hypoxia. The magnified part of the electrographic seizure (3) shows synchronized spike activity during the seizure. (D) The representative tracing shows EEG during hypoxia from a rat treated with 35 mg/kg flupirtine. The magnified excerpt (4) shows very low voltage background activity. S = second, V = volts.

Figure 3.

Flupirtine pretreatment effectively blocks hypoxia-induced electroclinical seizures. (A) All the P10 rat pups treated with vehicle 15 minutes before exposure to hypoxic environment developed electroclinical seizures during hypoxia. In contrast none of the rats treated with 35 mg/kg of flupirtine exhibited electroclinical seizures. (B) The box and whisker plot shows the percent of time spent by the rats treated with vehicle or 35 mg/kg of flupirtine in electroclinical seizures during hypoxia. $n = 6$ for both vehicle and flupirtine treatment groups. *Statistically different from vehicle treated rats exposed to hypoxia. $*P < 0.005$, Fisher's exact test for the data presented in A, and Mann Whitney test for the data presented in B was used to determine the statistical significance. Hy = Hypoxia, Veh = Vehicle, Flup = Flupirtine.

Figure 4.

Representative EEG tracings from a P10 rat injected with flupirtine. (A) The tracing shows baseline EEG obtained before the rat was injected with a drug and exposed to hypoxia. A magnified trace of EEG (1) is provided under the compact EEG tracing for a better clarity. (B) The EEG shows a burst suppression-like pattern following flupirtine (35 mg/kg) treatment that appeared before the rat was exposed to hypoxia. The EEG trace at the slower time scale (2) provides a magnified view of the burst suppression-like pattern. S = second, V = volts.