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Impact of Transient Acute Hypoxia on the Developing Mouse EEG

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Abstract

Hypoxemic events are common in sick preterm and term infants and represent the most common cause of seizures in the newborn period. Neonatal seizures often lack clinical correlates and are only recognized by electroencephalogram (EEG). The mechanisms leading from a hypoxic/ischemic insult to acute seizures in neonates remain poorly understood. Further, the effects of hypoxia on EEG at various developmental stages have not been fully characterized in neonatal animals, in part due to technical challenges. We evaluated the impact of hypoxia on neonatal mouse EEG to define periods of increased susceptibility to seizures during postnatal development. Hippocampal and cortical electrodes were implanted stereotactically in C57BL/6 mice from postnatal age 3 (P3) to P15. Following recovery, EEG recording were obtained during baseline, acute hypoxia (4% FiO₂ for 4 min) and reoxygenation. In baseline recordings, maturation of EEG was characterized by the appearance of a more continuous background pattern that replaced alternating high and low amplitude activity. Clinical seizures during hypoxia were observed more frequently in younger animals (100% P3-4, 87.5% P5-6, 93% P7-8, 83% P9-10, 33% P11-12, 17% P15, $r^2=0.81$) and also occurred at higher FiO₂ in younger animals ($11.2\pm1.1\%$ P3-P6 vs. $8.9\pm0.8\%$ P7-12, $p<0.05$). Background attenuation followed the initial hypoxemic seizure; progressive return to baseline during reoxygenation was observed in survivors. Electrographic seizures without clinical manifestations were observed during reoxygenation, again more commonly in younger animals (83% P3-4, 86% P5-6, 75% P7-8, 71% P9-10, 20% P11-12, $r^2=0.82$). All P15 animals died with this duration and degree of hypoxia. Post-ictal abnormalities included burst attenuation and post-anoxic myoclonus and were more commonly seen in older animals. In summary, neonatal mice exposed to brief and severe hypoxia followed by rapid reoxygenation reliably develop seizures and the response to hypoxia varies with postnatal age and maturation.

Keywords

Neonatal; mouse; EEG; hypoxia; reoxygenation; seizures; development

INTRODUCTION

Seizures are more common in the neonatal period than any other time in life (Cowan, 2002) and both experimental (Cornejo et al., 2008; Lee et al., 2001; Kleen et al., 2011; Isaeva et al., 2009; Jensen et al., 1992; Holmes et al., 1998) and clinical evidence suggest that early life seizures negatively impact later neurodevelopment independently of the underlying pathology (Miller et al., 2002; Glass et al., 2009; McBride et al., 2000; van der Heide et al., 2012). In preterm infants, seizures during the first three days after birth are associated increased risk of adverse short-term and long-term neurodevelopmental outcomes (Vesoulis et al., 2013). In animal models, recurrent seizures during early postnatal development have been associated with learning deficits (Swann, 2005). While data in neonates suggest that the severity of the seizures impact these outcomes (Glass et al., 2009), the number of seizures needed to adversely impact outcomes is not known at this time. Interestingly, in P7 rats, even a single episode of early life seizures has been shown to impair hippocampus-dependent short-term memory (Cornejo et al., 2008).

Hypoxemic events are common in sick preterm and term infants and represent the most common cause of seizures in the newborn period (Tekgul et al., 2006; Garfinkle and Shevell, 2011; Yildiz et al., 2012). However, mechanisms leading from a hypoxic or hypoxic/ischemic insult to acute seizures in neonates remain poorly understood.

A critical problem encountered when investigating hypoxia-mediated brain injury and hypoxic seizures is the importance of the developmental stage in the response to a hypoxic insult. Immature brain characteristics including glutamate and GABA_A receptor ontogeny (Jensen, 2002; Insel et al., 1990; McDonald et al., 1990; Selip et al., 2012), nNOS distribution, and antioxidant capabilities (Ferriero, 2001; Jiang et al., 2004) contribute to the selective vulnerability of the developing brain to hypoxia.

Electroencephalography is a powerful physiological tool that is very sensitive to the maturational stage of the brain (Scraggs, 2012). However, there is limited information regarding EEG maturation during early postnatal development in the mouse. In addition, a comprehensive description of the effects of hypoxia on EEG characteristics through the early stages of postnatal development has not been performed. Previous studies have characterized acute hypoxic seizures in the early postnatal period in a rat model and have generated vitally important information regarding epileptogenesis in this age group (Rakhade and Jensen, 2009). Rat models have the advantage of larger brain mass facilitating stable EEG recordings. In addition, some aspects of the rat brain are closer to that of the human brain including a thicker cortex. However, one limitation of the rat model is that powerful techniques such as knock-outs or knock-ins are not easily applied, emphasizing the need to develop clinically relevant models in the mouse. A neonatal mouse model of hypoxia-induced seizure has the potential to facilitate the investigation of underlying mechanisms leading to hypoxia-induced seizures as well as the evaluation of novel therapies.

This study characterized the effects of hypoxia during postnatal development on mouse EEG with the goal to define the critical developmental window of susceptibility to hypoxia-

induced seizures. Understanding the effects of brain maturation on response to hypoxia is an important step in the investigation of the mechanisms involved in the development of acute seizures after exposure to hypoxia in the immature brain.

MATERIALS AND METHODS

Animals

All procedures were approved by the Animal Care and Use Committee of the University of Virginia and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines. Time-pregnant C57BL/6 mice were obtained at E16 from Charles River Laboratories (Wilmington, MA, USA) and housed with free access to food and water until pup delivery. Following their birth, pups were reared normally until day of surgery. Day of birth was considered as postnatal day 0.

Surgery and EEG monitoring procedure

Neonatal mice were anesthetized with isoflurane to effect via a specially adapted nose piece (5% for induction and 1.5-2% for maintenance) and surgery was performed in a clean, ventilated hood with sterile instruments and fields. Additional analgesia with subcutaneous bupivacaine was also used. The surgical procedure occurred at least 12h from experimental hypoxia and the duration of exposure to isoflurane was limited to 15-20 min. For EEG recording, monopolar insulated stainless steel wire electrodes (76 μ m bare diameter, 140 μ m coated, A-M Systems, Sequim, WA) were implanted in P3 to P15 mice using a modified stereotaxic frame to accommodate the small size of the animals: 2 in the dorsal hippocampus, 2 in the cortex and 1 ground. Coordinates from bregma for hippocampal electrodes in all age groups are shown in Table 1. Because of size, only 3 electrodes (2 hippocampal and ground) were implanted in the brain of P3-4 animals. Electrodes were secured in place and the skin was placed back over the implant after surgery completion using loctite 454 instant adhesive (Grainger, Lake Forest, Ill).

EEGs recorded from young animals are characterized by overall low amplitude signal and high amplitude movement artifact when compared to that of mature animals. To address this problem, pups were connected to a unity gain impedance matching head stage (TLC2274 Quad Low-Noise Rail-To-Rail Operational Amplifier, Texas Instruments, Dallas, TX) prior to amplification. The output signal was then amplified using a Grass amplifier (Model 12, Natus Neurology Incorporated – Grass Products, Warwick, RI), digitized and recorded for later review using a Stellate Harmonie system ((Natus Medical Incorporated, San Carlos, CA).

Animals were allowed to recover on a heating pad until the anesthetic wore off and behavior returned to baseline, after which they were placed back with their mother for a minimum of 12 hours. Animals were kept in a lit room for that duration as this was found to prevent the dam from damaging to the electrode assembly. Following the recovery period, mouse pups were exposed to hypoxia (described below). Following completion of the experiment, pups were euthanized for fixation of the brain in 4% paraformaldehyde. Brains were subsequently sectioned and mounted on slides to visually verify electrode placement.

Method for producing acute hypoxia

Mouse pups were placed in a heated custom-made Plexiglas chamber (Figure 1A) to allow for the uninterrupted visualization of the animal and facilitate the subsequent matching of behavior with fraction of inspired oxygen [FiO_2] and EEG. FiO_2 within the chamber was continuously monitored via an oxygen monitor (ProOX P110, Biospherix, Ltd, Lacona, NY). Hypoxia was produced by flushing the chamber with 60 liters/min of 100% N_2 and 0.415 liters/min for 100% O_2 . When the oxygen saturation in the chamber reached 12%, the N_2 flow was decreased to 10 liters/min while O_2 flow remained unchanged. Thereafter small manual adjustments were made as needed to maintain the FiO_2 at target level of 4% for the remainder of the 4 min period.

The optimal method for generating hypoxia-induced seizures in a neonatal mouse model was developed in the P7-8 mice as they most closely match the term human brain (Sheldon et al., 1998). Various duration and severity of hypoxia were tested in P7-P8 pups including: FiO_2 of 4% for 2, 3, 4 and 7 min as well as FiO_2 of 6% for 7, 10 and 15 min (5 animals per group). The experimental paradigm found to most reliably generate seizures was then applied to other age groups (P3-P15) to investigate the effects of postnatal development on response to hypoxia. Reoxygenation was achieved by opening and fanning of the chamber with room air and return to 21% was achieved in 100.7 ± 24.3 sec, FiO_2 reached 10% in 35.8 ± 4.8 sec. This paradigm was developed to study neonatal acute symptomatic seizures where seizures are typically observed in the first 12-24h after resuscitation once infants are fully reoxygenated (Gillam-Krakauer and Carter, 2012).

EEG review procedure and terminology

Video-EEGs were recorded for 20 min prior to experimental hypoxia (baseline), during hypoxia and for 20 min after reoxygenation onset as described above. In order to evaluate the effects of development on EEG maturation and response to hypoxia/reoxygenation, EEG data were grouped as follows: P3-4, P5-6, P7-8, P9-10, P11-P12 and P15.

All EEGs traces were reviewed concomitantly with video recordings to assess for movement artifacts. Artifacts and physiological noise were more evident in the younger age groups with improved noise-to-signal ratio beyond postnatal day 8. Of note, the review was not blinded to the animals' age groups as this could be approximated in the accompanying video. Independent reviews of all EEGs were initially performed by SZ, SK, and JK. A subsequent independent review was performed by HPG. JK and HPG have achieved board certification in clinical neurophysiology. We obtained a "very good" strength of agreement with an inter-rater agreement kappa of 0.942 (95% CI 0.829-1). Wherever disagreement occurred, EEGs were reviewed by all authors and consensus was obtained.

Spikes were defined as events with a rapid positive and/or negative components, mono-, bi- or multi-phasic and less than <70 msec in duration (White et al., 2010; Gastaut and Broughton, 1972). Sharp-waves were defined as pointed transient with a duration of 70 to 200 msec (Gastaut and Broughton, 1972; Levin and Luders, 2000; Selip et al., 2012; Levin and Luders, 2000; Gastaut and Broughton, 1972). Electrographic seizures were defined as the appearance of high frequency (>2Hz) rhythmic sharp wave discharges with amplitude at

least 3 times that of baseline and lasting greater than 10 sec with clear evolution (Mizrahi and Clancy, 2000; White et al., 2010; Kerjan et al., 2009). Clinical seizures were defined according to the Racine scale (Racine, 1972) by the presence of stereotyped behavior including mouth and facial movements, head nodding and forelimb clonus. During hypoxia, EEG recordings were evaluated also for background attenuation defined as the time at which background activity decreased to less than 50% from baseline. High amplitude activity was defined as EEG amplitude 50% greater than baseline.

EEG background evolution with postnatal development was assessed visually and power calculations were performed using a MatLab program provided by Dr J.G. Keating (Raol et al., 2009). Total power was determined by filtering the EEG using a 5Hz high-pass and a 60Hz low-pass filters. Data was binned in 2-sec intervals and power within a 4 min time window was calculated and graphically displayed using MatLab software. To quantify the effects of hypoxia and reoxygenation on EEG background activity, the mean and standard deviation of the EEG voltage trace was calculated from randomly selected EEG samples of 10 sec duration. EEG traces were selected from each section, including baseline, hypoxia and 3 time points during reoxygenation (just prior and just after the first reoxygenation seizure and 20 min post-seizure) after removing epochs containing movement artifacts. Results were normalized to baseline with each animal serving as their own control.

Statistics

Comparisons between the different age groups were performed using ANOVA with post-hoc t-test for multiple group comparisons. Linear regression analysis was used to test proportions. Data presented as mean $\pm$ SEM. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Baseline EEG characteristics in the developing neonatal mouse

Patterns of EEG activity recorded from hippocampal and cortical electrodes were similar and hippocampal patterns are discussed in detail below. Differences between the two recordings are highlighted when needed. Overall, the background EEG evolved from a discontinuous pattern (with periodic variation in amplitude) towards a continuous pattern with advancing postnatal age (Figure 2).

Early during development (P3-4 and P5-6), the EEG background was dominated by low amplitude mixed frequency activities lasting upwards of 20-40 sec (Figure 2A and 2B) with briefer periods of higher amplitude mixed frequency activity typically lasting from 2-4 sec in P3-4 animals (Figure 2Aa) and 6-8 sec in P5-6 animals (Figure 2Ba). Some longer episodes of high amplitude activity of up to 20 sec duration were occasionally observed. During these periods of prolonged higher amplitude activity, trains or bursts of spike wave activity were present. Frequent repetitive spike or spike-wave discharges were noted throughout the recording but were lower in amplitude and less frequent in P5-6 animals than in P3-4 animals (Figure 2Ab).

Longer periods of higher amplitude activity (lasting 2 to 18 sec) were observed in recordings from P7-8 animals (Figure 2Ca). The high amplitude activity had a sinusoidal pattern with gradual build-up and decline (Figure 2C). Spike wave discharges were observed during periods of higher amplitude and less frequently during periods of lower amplitude activity. A similar and synchronous pattern was observed in the cortical recording, however, the contrast between higher and lower amplitude activity was less apparent.

In P9-10 animals, the hippocampal background activity largely consisted of continuous moderate amplitude activity with minimal variability and some interspersed sharper activities (Figure 2D). Brief periods of lower amplitude activity lasting 1 to 2 seconds were observed (Figure 2Da).

In P11-12 animals (Figure 2E), the hippocampal background activity was continuous with moderate amplitude activities in a sinusoidal pattern present throughout the recording and with brief periods of lower amplitude activity interspersed with prolonged periods of higher amplitude activity. There were no spike discharges present during these recordings in either the hippocampal or the cortical recordings. There were some sharper appearing activities that did not stand out from the background.

The effects of postnatal development of total power of background EEG is shown in Figure 3. Consistent with the visual analysis discussed above, the power analysis demonstrates clear evolution to a more continuous pattern starting at P9-10 in the neonatal mouse.

Development of a model of hypoxia-induced seizures in the P7-8 mouse

Electrographic seizures, with no mortality, were reliably (100%) generated during the period of reoxygenation by exposing the P7-8 mouse to an FiO_2 of 4% for 3 and 4 min. Of note, shorter duration (2 min) of severe hypoxia (4% FiO_2) did not lead to seizures during the period of reoxygenation while longer duration (7 min) resulted in 100% mortality. Milder hypoxia (6% FiO_2 for 7, 10 and 15 min) did not reliably lead to seizures with seizures during the period of reoxygenation observed in only 20 to 40% of animals. Duration and latency of seizures for the combinations leading to seizures during the period of reoxygenation in more than 1 animal with no mortality are shown in figure 1B. These were similar except for 6% FiO_2 for 7 min where latency was significantly decreased. This optimized model (4% $\text{FiO}_2 \times 4$ min) was subsequently applied to the P3 through P15 mice to evaluate the effects of postnatal development on hypoxia-induced seizures.

A representative example of the effects of hypoxia and reoxygenation in a P7 mouse is shown in Figure 4. Following onset of hypoxia, a rapid decrease in chamber FiO_2 was noted (Figure 4A) and the chamber reached the target FiO_2 of 4% in 104 ± 25 sec. The effects of FiO_2 changes on EEG are shown in Figure 4B.

Effect of hypoxia in the developing neonatal mouse EEG

After onset of hypoxia, emergence of tachypnea was noted followed by acute change in behavior in all age groups. These behaviors included a period of active exploration of the environment and flight-or-fright-like response followed by rhythmic head and forelimb clonic jerks which suggested a seizure. Some animals, particularly P7 and older, were noted

to be standing against the wall of the small hypoxic chamber with subsequent fall on their backs (see supplemental material for examples of response to hypoxia in a P3 and a P7 mouse). This clinical seizure occurred at higher FiO_2 in younger animals (P3-6, FiO_2 $11.2 \pm 1.1\%$) compared to older animals (P7-12, FiO_2 $8.9 \pm 0.8\%$, $P < 0.001$, t-test). The presence of an associated electrographic seizure could not be conclusively ascertained in most animals due to the presence of movement artefacts and the lack of clear progression of the EEG spikes (Figure 5). The duration of hypoxic seizures was not statistically different among age groups (11 to 21 sec; Figure 7B and Table 3). Hypoxic seizures were observed more frequently in younger age groups: 100% of P3-4, 87.5% of P5-6, 93% of P7-8 (Figure 4B2), 83% of P9-10 compared to only 33% of P11-12 and 17% of P15 animals (Figure 7A, $r^2 = 0.81$). There was a single seizure observed during the 4 min hypoxic period in most animals. However, in the majority of animals additional motor behaviors suggestive of epileptic seizure were observed during the first half of hypoxia but were shorter than 10 sec and did not meet our definition criterion for an electrographic seizure (Figure 5).

Thereafter, behavioral arrest was noted for the remainder of the hypoxia period in all animals corresponding to significant background attenuation (Figures 4B3 and 5 and Table 3). There was no statistically significant difference in time to background attenuation from onset of hypoxia: 54.0 ± 2.7 sec (P3-4); 72.1 ± 13.2 sec (P5-6); 87.5 ± 8.0 sec (P7-8); 75.4 ± 4.8 sec (P9-10) and 77.0 ± 14.5 sec (P11-12; r^2 0.41). Background attenuation was interspersed by large amplitude spikes associated with whole body jerks decreasing in frequency with ongoing hypoxia. Of note all P15 animals died during exposure to hypoxia after becoming terminally apneic.

Effect of post-hypoxic reoxygenation on the developing mouse EEG

Electrographic seizures were observed on the EEG trace in P3-12 animals shortly after the onset of rapid reoxygenation (Table 2). The first seizure after onset of reoxygenation consisted of rhythmic spikes, preceded by low amplitude polyspike activity (Figure 6). There was no behavior accompaniment to the first EEG seizure except for some clonic jerks towards the end of the seizure in a minority of animals. Of note, animals had resumed regular breathing at the time of the first reoxygenation seizure but still exhibited behavioral arrest and were not tested for motor activity. Again seizures during reoxygenation were more common in younger animals: 83% of P3-4, 86% of P5-6, 75% of P7-8, 71% of P9-10 compared to only 20% of P11-15 (r^2 0.82, figure 7C). Most animals experienced only 1 seizure in the 20 min reoxygenation period except for P3-4 animals that experienced an average of 2.3 ± 1.9 seizures. These subsequent reoxygenation seizures were accompanied by clonus, whole body jerks and posturing. There was a trend towards increased seizure duration with advancing postnatal age as shown in figure 7D and table 2. Electrographic seizures were most often seen only in the hippocampal region with the observation of concomitant seizures in the cortical areas more common with advancing postnatal age. The seizure involved the cortex in addition to the hippocampus in 50% of P5-6, 56% P7-8, 80% of P9-10 and 100% of P11-12 animals. Cortical seizures when present were lower in amplitude and had shorter duration (Figure 8).

Post-ictal abnormalities included burst attenuation and post-anoxic myoclonus. A burst attenuation pattern was noted in the early reoxygenation phase in all mice P8 or younger and in 80% of P9-10 and 60% of P11-12 animals (Figure 9). Bursts were characterized by periods of higher amplitude activities consisting of spike waves discharges mixed with slow wave discharges. These bursts were not well organized and did not demonstrate evolving rhythmicity to qualify as seizures. These bursts of high amplitude activity were typically not accompanied by behavior changes. However, in the younger age groups (P3-8), some bursts of high amplitude activity were temporarily accompanied by clonus and posturing particularly in the late reoxygenation phase. The reoxygenation period was also characterized by frequent high amplitude sharp wave discharges some of which were accompanied by myoclonus jerks suggestive of post-anoxic myoclonus. These occurred in all age groups but were more frequent and pronounced in older animals.

Background baseline activity progressively returned to baseline by the end of the 20 min reoxygenation period in all age groups (Figure 10 and Table 3). However, none of the pups P3-P6 recovered their baseline behavior and they continued to demonstrate persistent decrease in activity at the end of the 20 min recording period. In older age groups, animal who survived acute hypoxia recovered their baseline behavior in 21%, 33% and 50% of P7-8, P9-10 and P11-12 animals respectively.

DISCUSSION

This study provides a first comprehensive description of the effects of postnatal development on EEG maturation in the mice. Similar to human EEG, early neonatal mice display an immature EEG pattern with prolonged periods of low amplitude activity and brief periods of high amplitude activity. With advancing development, the EEG background patterns become more continuous, starting at P9-10. Acute hypoxia exposure in this period results in both hypoxic and reoxygenation seizures with the period of highest susceptibility to hypoxic seizure occurring before postnatal day 10.

Neonates with acute symptomatic seizures are difficult to treat and in 40-50% of patients do not respond to mainstay therapies, including phenobarbital, phenytoin and benzodiazepines (Painter et al., 1999;Boylan et al., 2004;Boylan et al., 2002;Maytal et al., 1991). Equally concerning, these first line therapies may interfere with normal developmental pathways (Bittigau et al., 2003;Olney et al., 2004) and evidence point to detrimental long term neurodevelopmental outcomes from early life exposure to these drugs (Thorp et al., 1999;Sulzbacher et al., 1999;Sulzbacher et al., 1999;Kim et al., 2007;Maitre et al., 2013;Maitre et al., 2013). Newer therapies including levetiracetam and topiramate are increasingly used (Glass et al., 2012) but they are no class I randomized controlled trials documenting safety and efficacy in this population. Therefore, the development of safer and more effective therapies for neonatal seizures is the focus of intense research efforts (Sankar and Painter, 2005).

In order to achieve these goals, the development of relevant animal models is critical. In this study, we describe a neonatal mouse model critical for the study of acute symptomatic seizures in neonates. The C57Bl/6 mouse strain was chosen because of the large number of

genetically modified phenotype available using this background allowing for in depth investigation of the underlying mechanism leading to seizures after a hypoxic insult. In this model, exposure to severe and brief hypoxia results in profound background attenuation with slow return to baseline over 20 min, demonstrating the significant impact of even brief hypoxia exposure on EEG in neonatal animals. Further, this degree and duration of hypoxia reliably led to seizures in all age group both during hypoxia and during reoxygenation. Interestingly, seizures, both during hypoxia and reoxygenation, were more commonly observed in the younger age groups, emphasizing the critical nature of developmental stage when studying neonatal seizure pathophysiology. In addition to describing the effects of hypoxia on neonatal EEG, we also found that the EEG of neonatal mice demonstrates maturation of baseline over the first postnatal week, including the emergence of a more continuous EEG pattern.

Postnatal maturation of EEG is well described in human neonates including those born preterm (Vecchierini et al., 2007). In very preterm infants less than 28 weeks gestation, the EEG tracing is discontinuous with interburst intervals as long as 46 sec (Selton et al., 2000). With maturation of the infant, EEG background follows a predictable progression to a continuous pattern by 30-32 weeks gestation. Neonatal mice appear to have a similar predictable pattern of EEG maturation with progression from discontinuous pattern at P3-4 to continuous pattern emerging at P7-8. This emphasizes the importance of considering brain maturation in developing animal models. Wireless methods of EEG monitoring have been recently reported in neonatal rats (Zayachivsky et al., 2013). In this model, similar patterns of EEG maturation were described in P7 and older rats. These novel techniques offer the unprecedented advantage of continuous EEG monitoring in small animals but the implantable miniature telemetry system remains too large for neonatal mice at this time.

Investigation of EEG maturation and response to hypoxia has proved challenging due to the animal small size as well as low aggregate of neuronal activity rendering EEG recording particularly difficult to interpret because of high physiological noise. In order to address these issues, technological advancements were developed including customization of the stereotaxic frame and incorporation of a unity gain impedance matching head stage directly onto the electrode assembly. These advancements resulted in significant decrease in movement artifact and cable-related noise, allowing for the recording of high quality hippocampal and cortical EEG recordings. To our knowledge this is the 1st study describing the maturation of EEG background pattern and the effects of hypoxia in the immediate postnatal period in the mouse.

Although EEGs were performed at the same time of the day, one of the limitations of this study is that we did not use EMG monitoring to more formally document the sleep-wake cycle status of the animals during the recording of baseline EEG. Because eye-opening does not occur until postnatal day 11 or 12, our assessment of the sleep-wake status of the animals was limited to evaluation of behavior (presence of coordinated movements) and was therefore not optimal. We cannot definitely rule-out that some differences in the sleep-wake status of the animals may account for some of the differences seen in maturational patterns of background EEGs.

In neonates suffering from hypoxic-ischemic encephalopathy, clinical seizures are typically observed once the infant is fully oxygenated and may represent another limitation of this study. It should be emphasized that no data exists to prove or disprove the presence of electrographic only seizures during the hypoxic event or during the early reoxygenation phase. We observed seizures during the hypoxic exposure as well as early during reoxygenation phase making this model a suitable one to study acute seizure mechanisms. To strengthen the model and its applicability to human diseases, further studies are required including prolonged EEG monitoring of the pups as well as investigation of long term effects.

CONCLUSIONS

Neonatal mouse EEG matures over the first 2 postnatal weeks to reach a continuous background pattern by P10. Exposure to acute and brief hypoxia during this period of development leads to seizures during exposure to hypoxic as well as during early reoxygenation. These seizures are more commonly observed in animals P3-P10. Exposure to this degree and duration of hypoxia in older animals results in 100% mortality. Of note, early reoxygenation seizures are common and do not have clinical correlate in the neonatal period emphasizing the crucial role of continuous EEG monitoring when studying neonatal seizures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Model development

Panel A: Pictorial representation of the hypoxic chamber: 1= oximeter; 2 = custom Plexiglass chamber demonstrating a P7 C57BL/6 mouse with hippocampal and cortical electrodes implanted; 3 = head stage; 4 = heating pad. **Panel B:** Summary of seizure duration and latency in P7-8 mice under varied hypoxia conditions (n = 5 per condition). The optimal method for generating hypoxia-induced seizures was developed in the P7-8 mice. Of all the duration and depth of hypoxia tested (see table 3) FiO₂ of 4% (for 3 and 4 min) and 6% FiO₂ (for 7 min) were the only combinations reliably leading to seizures with no mortality. Exposure to FiO₂ of 4% for 3 and 4 min resulted in seizures in 100% of animals while exposure to FiO₂ of 6% for 7 min resulted in seizures in 40% of animals. The optimized paradigm (4% FiO₂ for 4 min) was subsequently applied to the P3 through P15 mice to evaluate the impact of brain maturation on EEG and EEG response to hypoxia as well as evaluate the effects of development on hypoxia-induced seizures. * P < 0.05 vs. 3 and 4 min (One-Way ANOVA).

Figure 2. Baseline characteristics of EEG in the developing mice

Representative 5 min EEG recordings are shown for P3-4 (**A**), P5-6 (**B**), P7-8 (**C**), P9-10 (**D**) and P11-12 (**E**) mice. Five second EEG segments (labeled a and b) are shown at a higher scale below the full length EEG. Note that the baseline EEG activity cycled between periods of higher amplitude (Aa, Ba, Ca) alternating with periods of lower amplitude (Ab, Bb, Cb) in P3-P8 age groups. Spike wave discharges can be seen in P3-6 animals and less frequently in P7-8 animals (see higher scale example in Ab). The high amplitude activity had a sinusoidal pattern with gradual build-up and decline in P5-10 animals (B, C, D). Periods of high amplitude activity lengthened with advancing postnatal age and a continuous background activity pattern was noted in P11-12 animals (E). Of note, motion artifacts were less prominent in older age groups. Hippocampal recordings shown.

Figure 3. Effect of postnatal development on background EEG power

Total power during postnatal development is shown demonstrating the appearance of a more continuous pattern starting at P9-10. 4 min background epochs devoid of artefacts were hand-selected selected for the power analysis. The data is filtered (5Hz high-pass, 60Hz low-pass) and binned in 2-sec intervals. Data graphically displayed using MatLab software.

Figure 4. Effects of hypoxia on EEG characteristics in the P7-8 mouse

Panel A: Chamber FiO₂ changes during hypoxia-reoxygenation in a representative P7 animal demonstrating that target FiO₂ is achieved rapidly. **Panel B:** Effects of FiO₂ changes on EEG in a representative P7 animal. **B1:** At baseline, the EEG background activity consists of high amplitude activity with a sinusoidal pattern interspersed with short periods of low amplitude activity. **B2:** At an average FiO₂ of $9.7 \pm 2.9\%$ (30.5 ± 10.2 sec from hypoxia onset), P7-8 animals exhibited a behavioral seizure consisting of myoclonic jerking of the head and limb. This behavior was accompanied by rhythmic spike discharges on EEG without clear progression lasting 19.0 ± 5.4 sec. **B3:** Subsequently, complete background suppression was observed within 88.8 ± 30.9 sec of hypoxia onset in P7-P8 pups. Note spiking during hypoxia. **B4:** Following reoxygenation, animals exhibited EEG only seizure with a latency of 115.7 ± 42.2 sec and duration of 24.1 ± 7.9 sec. **B5:** Background activity progressively returned to baseline over the 20 min recording of reoxygenation. Hippocampal recording shown.

Figure 5. Effects of postnatal age on EEG changes during hypoxia exposure

Mice were exposed to brief and severe hypoxia (4% FiO₂ for 4 min) in a custom-made Plexiglas chamber. As discussed above, following onset of hypoxia (arrow head), animals demonstrated acute changes in behavior coinciding with rhythmic spike discharges on EEG without clear progression (underlined sections). Additional events shorter than 10 sec can also be seen. Subsequently, attenuation of background EEG activity as well as loss of variability was observed in all age groups. Note large amplitude spikes decreasing in frequency with ongoing hypoxia. Hippocampal recordings shown.

Figure 6. Post-hypoxic seizure characteristics in the developing mouse

Panel A: Seizures during the reoxygenation phase were observed in 83% of P3-4, 86% of P5-6, 75% of P7-8, 71% of P9-10 and 20% of P11-12 animals (first reoxygenation seizure shown). These electrical seizures were typically not associated with clinical manifestations although in some animals rhythmic clonus was observed towards the end of the seizure. **Panel B:** Magnification of underlined EEG section in panel A.

Figure 7. Seizure characteristics during hypoxia and reoxygenation in the neonatal mouse
Hypoxic seizures were more common in younger age groups than older age groups (**Panel A**). However there was no significant difference in seizure duration by postnatal age (**Panel B**). Similarly, reoxygenation seizures were more common in younger versus older animals (**Panel C**). There was a trend towards longer reoxygenation seizures in older animals (Panel D) that reached statistical significance for group P3-4 vs. group P7-8 ($p < 0.001$, one-way ANOVA). Hippocampal recordings shown.

Figure 8. Generalization of hypoxic seizure in the neonatal mouse exposed to acute and severe hypoxia

33% of P5-6, 20% of P7-8, 25% of P9-10 and 100% of P11-12 animals had cortical seizures recorded along with hippocampal seizure in the immediate reoxygenation period. Examples of seizures recorded in both the hippocampal (**panel A**) and cortical (**panel B**) regions are shown.

Figure 9. Effects of reoxygenation on EEG characteristics in the developing mouse
Representative EEGs in P3 to P12 mice demonstrating EEG patterns in the early reoxygenation period. Burst attenuation and anoxic myoclonus was observed in all age groups but was most pronounced and prolonged in older animals.

Figure 10. Effect of hypoxia-reoxygenation on EEG background in the developing mouse
Background EEG activity was quantified by calculating the mean voltage and standard deviation of randomly selected 10 sec EEG traces from each experimental period (baseline; hypoxia, just prior to reoxygenation; and 3 time points during reoxygenation (just before and just after the first reoxygenation seizure and 20 min after onset of reoxygenation). All measurements were normalized to baseline with each animal serving as its own control. Data is expressed as % suppression from baseline.

Table 1

Bregma coordinates for hippocampal electrode placement in neonatal mice

Age group	Anterior-posterior	Media lateral	Depth from touch point
P3-4	−3.25	±1.75	−1.25
P5-6	−4.25	±2	−1.5
P7-8	−4.5	±2	−1.75
P9-10	−4.75	±2	−1.75
P11-12	−5	±2.5	−2
P15	−5.5	±2.75	−2.25

Table 2

Characteristics of hypoxic and post-hypoxic seizures in the developing mouse

Age groups	P3-4 (n=6)	P5-6 (n=6)	P7-8 (n=19)	P9-10 (n=6)	P11-12 (n=10)	P15 (n=6)
Mortality	0	0	0	0	50	100*
Hypoxic seizures (observed during the 4 min × 4% FiO ₂ period))						
%	100	87.5	95	83	33	17
FiO ₂ (%)	11.0 ± 1.7	12.5 ± 1.8	9.5 ± 0.8	7.8 ± 0.7	7.2 ± 1.4	8.1
Latency (s)	25.2 ± 1.9	30.3 ± 3.1	30.5 ± 2.8	37.2 ± 3.9	70.2 ± 20.4	22
Duration (s)	21.0 ± 2.3	18.0 ± 1.9	19.0 ± 1.5	12.8 ± 0.9	18.0 ± 4.2	11
Reoxygenation seizures (observed during the 20 min recording after onset of reoxygenation)						
%	83	86	75	71	20	N/A *
FiO ₂ (%)	14.5 ± 2.6	18.6 ± 0.4	16.9 ± 0.5	15.7 ± 1.8	18.6 ± 2.2	N/A
Latency (s)	76.8 ± 15.1	115.5 ± 16.5	110.5 ± 12.9	81.0 ± 3.6	149.3 ± 34.7	N/A
Duration (s)	25.4 ± 4.5	31.3 ± 4.9	29.0 ± 2.7	50.4 ± 8.4	44.0 ± 10.0	N/A

Hypoxia (4% FiO₂ × 4 min) was applied in all groups

* All P15 animals died during the 4 min exposure to hypoxia; Data presented as mean ± SEM

Table 3

Effect of hypoxia-reoxygenation on EEG background suppression in the developing mouse

Age groups	EEG background suppression (% from baseline)*			
	Hypoxia	Reoxygenation		
		Pre-seizure	Post-seizure	20 min
P3-4	45.8 ± 9.1	60.7 ± 12.8	86.1 ± 23.6	101.7 ± 28.7
P5-6	24.7 ± 9.1	37.5 ± 25.1	58.8 ± 16.0	99.9 ± 26.4
P7-8	23.0 ± 13.4	35.3 ± 24.7	69.9 ± 31.2	92.9 ± 26.0
P9-10	19.7 ± 13.3	27.3 ± 27.8	79.2 ± 22.4	92.9 ± 15.8
P11-12	14.7 ± 6.8	23.1 ± 1.9	89.7 ± 54.4	74.0 ± 0.5

* Mean and standard deviation of the EEG trace's voltage were calculated from 10 sec EEG samples taken at baseline, during hypoxic suppression, just prior and just after the first reoxygenation seizure and at 20 min of reoxygenation. Results were subsequently normalized to baseline with each animal serving as its own control.