Epilepsia, 52(4):753–765, 2011
doi: 10.1111/j.1528-1167.2011.02992.x

FULL-LENGTH ORIGINAL RESEARCH

Development of later life spontaneous seizures in a rodent model of hypoxia-induced neonatal seizures

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Summary

Purpose: To study the development of epilepsy following hypoxia-induced neonatal seizures in Long-Evans rats and to establish the presence of spontaneous seizures in this model of early life seizures.

Methods: Long-Evans rat pups were subjected to hypoxia-induced neonatal seizures at postnatal day 10 (P10). Epidural cortical electroencephalography (EEG) and hippocampal depth electrodes were used to detect the presence of seizures in later adulthood (>P60). In addition, subdermal wire electrode recordings were used to monitor age at onset and progression of seizures in the juvenile period, at intervals between P10 and P60. Timm staining was performed to evaluate mossy fiber sprouting in the hippocampi of P100 adult rats that had experienced neonatal seizures.

Key Findings: In recordings made from adult rats (P60–180), the prevalence of epilepsy in cortical and hippocampal EEG recordings was 94.4% following early life hypoxic seizures. These spontaneous seizures were identified by characteristic spike and wave activity on EEG accompanied by behavioral arrest and facial automatisms (electroclinical seizures). Phenobarbital injection transiently abolished spontaneous seizures. EEG in the juvenile period (P10–60) showed that spontaneous seizures first occurred approximately 2 weeks after the initial episode of hypoxic seizures. Following this period, spontaneous seizure frequency and duration increased progressively with time. Furthermore, significantly increased sprouting of mossy fibers was observed in the CA3 pyramidal cell layer of the hippocampus in adult animals following hypoxia-induced neonatal seizures. Notably, Fluoro-Jade B staining confirmed that hypoxic seizures at P10 did not induce acute neuronal death.

Significance: The rodent model of hypoxia-induced neonatal seizures leads to the development of epilepsy in later life, accompanied by increased mossy fiber sprouting. In addition, this model appears to exhibit a seizure-free latent period, following which there is a progressive increase in the frequency of electroclinical seizures.

KEY WORDS: Neonatal seizures, Electroencephalography, Epilepsy, Infant, Animal model.

Seizures are a common neurologic disorder in the neonatal period, occurring in 1.8–5/1,000 live births in the United States and Canada (Hauser et al., 1993; Ronen et al., 2007). Hypoxic–ischemic encephalopathy (HIE) is the most common cause, and it occurs in approximately 1–2/1,000 live births, accounting for two thirds of cases of neonatal seizure (Tekgul et al., 2006; Ronen et al., 2007). Neonatal seizures can be clinically difficult to diagnose and may be exclusively electrographic (Mizrahi, 1987; Volpe, 2008). Furthermore, neonatal seizures are frequently refractory to currently available antiepileptic drugs (AEDs) (Sankar & Painter, 2005).

Accepted January 7, 2011; Early View publication March 2, 2011.

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HIE-associated neonatal seizures usually occur within the first 1–2 days of life and often remit after a few days (Volpe, 2008), but are associated with later life epilepsy, and neurologic and/or cognitive deficits (Tekgul et al., 2006; Ronen et al., 2007). Despite advances in neonatal care, recent prospective studies in North America have reported a high incidence of epilepsy (28% and 31%) as well as cognitive disabilities (30–43%) in infants who experienced neonatal seizures (Tekgul et al., 2006; Ronen et al., 2007).

We previously developed an experimental model of neonatal seizures that utilizes graded global hypoxia to induce seizures in postnatal day (P) 10–12 rat pups (Jensen et al., 1991, 1995). This enhanced susceptibility to seizures during the second postnatal week (P10–12) in Long-Evans rats coincides with a developmental stage of enhanced excitability and synaptic plasticity, and is analogous to the human neonatal period between 32 and 40 weeks of gestation (Talos et al., 2006; Rakhade & Jensen, 2009). We have

demonstrated that hypoxia-induced neonatal seizures involve the hippocampus and cortex, induce hippocampal hyperexcitability (Jensen et al., 1998; Rakhade et al., 2008), and produce long-term cognitive deficits (Jensen et al., 1992; Mikati et al., 2005). It is important to note that these acute and chronic effects following these early life seizures occur in the absence of cell death (Jensen et al., 1991; Koh & Jensen, 2001). Similar to humans, in the neonatal rodent, seizures can be resistant to conventional AEDs such as lorazepam and phenobarbital (Jensen et al., 1995; Dzhala et al., 2005, 2008). The refractoriness of these seizures, as well as their consequences, is likely to result from unique age-specific mechanisms (Silverstein & Jensen, 2007). For example, these early life seizures are associated with transient dysregulation of expression and function of the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate glutamate receptor (AMPAR) subunits (Sanchez et al., 2001, 2005a; Rakhade et al., 2008), and AMPAR antagonists have superior anticonvulsant efficacy in this model (Jensen et al., 1995; Koh et al., 2004).

It has been shown that hypoxia-induced early life seizures have long-term cognitive consequences (Jensen et al., 1998; Koh et al., 2004), but yet to be determined is whether this model generates spontaneous seizures in later life. Other models of infant and childhood epilepsy syndromes that exhibit the development of epilepsy include those induced by chemoconvulsants (Stafstrom et al., 1997; Smith et al., 1998; Holmes, 2005), hyperthermia (Dube et al., 2006), and hypoxia–ischemia (Kadam et al., 2010).

Despite an absence of neuronal death in this model of hypoxia-induced neonatal seizures, we showed increased susceptibility to seizures and seizure-induced neuronal injury in later life (Jensen et al., 1992; Jensen, 1999; Koh & Jensen, 2001). We hypothesized that hypoxia-induced neonatal seizures also promote the development of epilepsy in later life. In the present study, hypoxia-induced seizures initially result in a brief period (24–48 h) of continuing behavioral seizures. Histopathologic analysis of brain at 24 h, 48 h, 72 h, and 1 week following these early life seizures fails to show any significant increase in neuronal death. The acute seizures are followed by a 7–15 day latent period with subsequent development of increasingly frequent behavioral and electrographic seizures at juvenile and adult ages. In addition, these brief hypoxia-induced neonatal seizures also were associated with mossy fiber sprouting in stratum oriens of hippocampal area CA3. Taken together, these data suggest that neonatal seizures can result in development of long-term epilepsy and structural alterations in the hippocampal network.

Methods

Animals

Litters of male Long-Evans hooded rats (10 pups per litter; Charles River Laboratories, Wilmington, MA, U.S.A.) were housed in a facility with a 12/h light–dark cycle with unlimited food and water. P10 rat pups (18–22 g) were subjected to hypoxia and returned to their dams until P21, and at weaning placed in shared cages. All procedures were approved by and undertaken in accordance with the guidelines of the Animal Care and Use Committee at Children's Hospital (Boston, MA, U.S.A.) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Hypoxia-induced seizures

P10 rats were exposed to graded global hypoxia for 15 min in an airtight chamber, as described previously (Jensen et al., 1991). Briefly, oxygen concentration was maintained at 7% for 8 min, 5% for 6 min, and 4% for 1 min before termination of hypoxia. Litter mate controls were kept at room air. The entire rat litter was returned to their dams within 1 h after the experiment (additional information in Supporting Information).

Rat pups exposed to hypoxia were kept alive for up to 6 months, and video-electroencephalography (EEG) recordings were performed (n = 11/group) either during infancy and young adolescence or during adulthood (Fig. 1 and Supporting Information). Additional animals were subjected to hypoxia-induced seizures to assess cell death, and comparisons were made to littermate controls (n = 4/group).

Long-term video-EEG recordings with implanted cranial electrodes

Rat pups (n = 11) experiencing hypoxia-induced neonatal seizures survived into adulthood and were implanted with hippocampal and cortical electrodes to analyze seizure onset and progression and compared to littermate rat pups without hypoxia (n = 10).

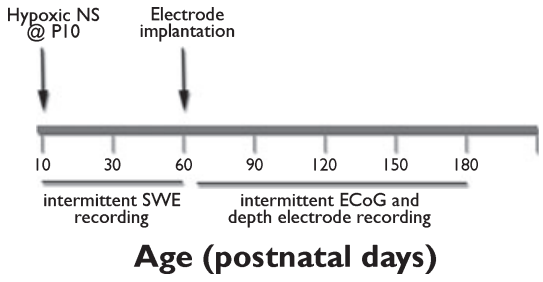

Figure 1.
Study design. Long-Evans rat pups were exposed to hypoxia-induced neonatal seizures at P10. Short-term video-EEG recordings were performed using subdermal wire electrodes during neonatal and adolescent period. Hippocampal depth electrodes and cortical electrodes were implanted at P55–60, and long-term sequential EEG recordings were obtained from P60 to 180.

At P55, rats were implanted unilaterally with cranial electrodes. Intracranial electrodes were implanted in hippocampus (AP −3.9, ML −3.0 from bregma, depth −2.3) as well as the cortical surface using epidural contact electrodes (Plastics One, Roanoke, VA, U.S.A.). Starting 1 week after electrode implantation and continuing up to P180 (6 months), video-EEG recordings were obtained at predetermined intervals for longitudinal studies (described in Supporting Information). A subgroup of adult rats was treated with phenobarbital (50 mg/kg, i.p.) to confirm the epileptic nature of the observed EEG abnormalities (n = 7). EEG power analysis was performed on 4 h epochs prior to and after injection of phenobarbital. EEG power was computed per 10 s interval by fast Fourier transform (FFT) of the EEG spectrum within 1–32 Hz.

Short video-EEG using subdermal wire electrodes

In the initial period of 7 weeks following P10 hypoxic seizures (P10–60), EEG recordings were acquired with Teflon-coated silver/silver chloride subdermal wire electrodes (SWEs) (Ives, 2005; Rotenberg et al., 2008). SWE implantation is minimally invasive and well tolerated by the young rat pups. Furthermore, SWEs could be removed following the EEG recordings so that pups could be returned to their dams, allowing multiple recording. Video-EEG recordings in pups were 2.5–3 h in duration and freely moving with a low torque commutator (Dragonfly Inc, Ridgeley, VA, U.S.A.) and connector assembly (John Ives, Manitock, ON, Canada, Supporting Information).

EEG studies were analyzed by two individuals (TH or PK) and all analyses were confirmed by study-blinded secondary review, including a board certified clinical neurophysiologist (SNR and AR). Seizures were defined by the appearance of sustained polyspike activity, significantly different than background rhythm, longer than 3 s, and associated with a behavioral correlate on video. Behavioral automatisms associated with abnormal EEG activity were used as benchmark for identifying electroclinical seizures. Average seizure frequency was calculated per hour of video-EEG recording, and seizure duration was measured as the time from first spike to last spike.

Timm staining

Timm staining was performed in brain tissue from P100 rats after hypoxia-induced neonatal seizures or age-matched littermate controls (Holmes et al., 1999; Huang et al., 1999). Slides from control and experimental animals were stained simultaneously and imaged with light intensity and filter settings maintained at a constant level (details described in Data S1).

Fluoro-Jade B immunostaining

Fluoro-Jade B immunostaining was performed in brain collected 24 h, 48 h, 72 h, and 1 week after hypoxic neonatal seizures in P10 rats to identify neuronal injury (Fluoro-Jade B, AB310; Millipore, Billerica, MA, U.S.A.) (Schmued et al., 2005; Meikle et al., 2007). Photomicrographs were obtained using a Nikon 80i microscope (Nikon Instruments Inc., Melville, NY, U.S.A.) and analyzed for Fluoro-Jade B staining in dying neuronal cells (details of staining procedures in Supporting Information).

Statistical analysis

Comparisons between the groups experiencing hypoxic seizures versus age-matched controls were performed using paired *t*-test and analysis of variance (ANOVA) with post hoc *t*-test for multiple group comparisons (SigmaPlot; Systat Inc., Chicago, IL, U.S.A.).

Results

Acute behavioral and EEG seizures during hypoxia

Consistent with prior results, exposure to graded global hypoxia for 15 min induced acute seizures in 93% of the rats (58 of 61 rat pups, more than five seizures during hypoxia). The semiology of these seizures consisted of myoclonic jerks of trunk and limbs, chewing, wet dog shakes, head bobbing, and tonic–clonic movements of head and limbs (Jensen et al., 1991; Koh et al., 2004). These behavioral changes were associated with electrographically recorded trains of polyspikes and sharp waves (Video S1 and Fig. S1). In a representative population of pups during hypoxia (n = 20), the average latency to the first behavioral seizure was 4.3 ± 0.7 min, and the average seizure number was 8.7 ± 1.2. The rat pups continued to exhibit short unprovoked behavioral seizures over 48 h following the initial hypoxic insult. Brain sections obtained 24 h, 48 h, 72 h, and 1 week after exposure to graded global hypoxia and coincident neonatal seizures did not show the presence of neuronal death and degeneration when stained with Fluoro-Jade B, a high affinity fluorescent marker for staining neurons undergoing degeneration (Fig. S2).

Depth electrode recordings from adult rats reveal spontaneous ictal activity following early life seizures

At 45 days following neonatal seizures, intracranial electrodes were successfully implanted in rats in right hippocampus and overlying cortex (n = 11) and in the same locations in normoxic littermate controls (n = 10). Video-EEG recordings were obtained from 8–16 h epochs on multiple days from P60 to P175. Seizures were defined as electrographic seizures recorded from the unilateral bipolar hippocampal electrodes and epidural cortical electrodes (Fig. 2), only when they consisted of spikes and sharp wave trains with amplitude two times greater than background and duration >3 s, and had an associated behavioral correlate, such as sudden behavioral arrest, staring episodes, head-jerking, and facial automatisms (Video S2). When epileptiform activity was observed in the hippocampal electrodes, there were accompanying widespread changes

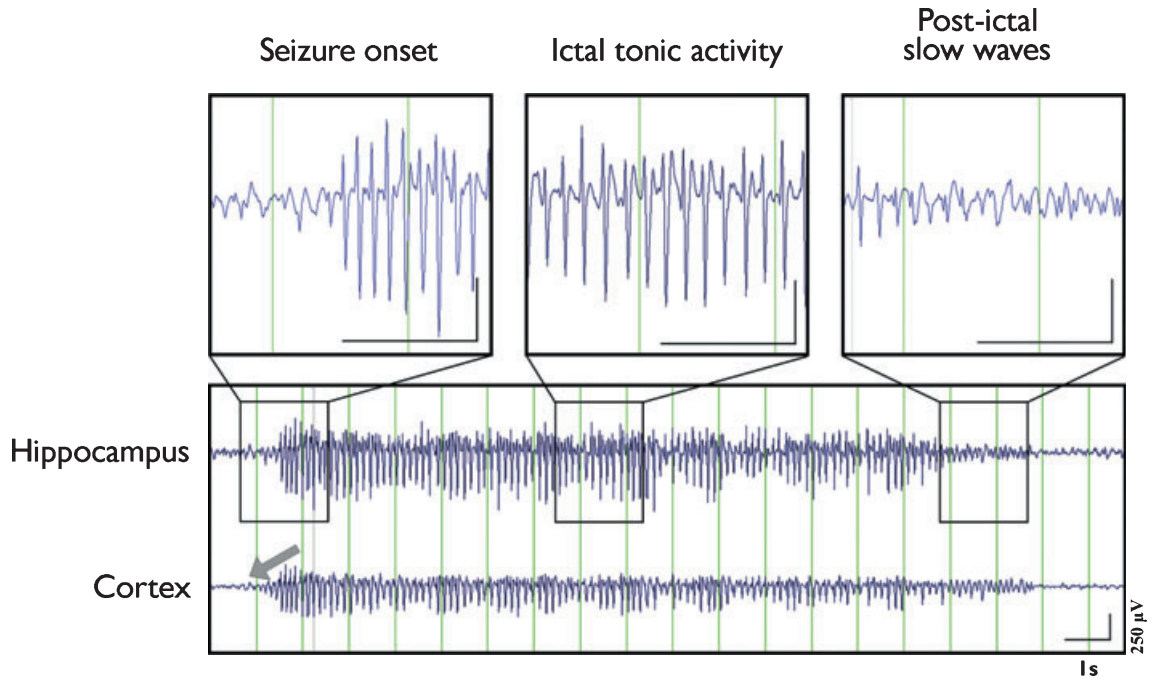

Figure 2.
Typical spontaneous electrographic seizure recorded from hippocampal electrodes in adult rats that have experienced hypoxic seizures during neonatal period. Seizures were defined as typical events that met electrographic and behavioral criteria. Hippocampal and cortical EEG recordings show baseline activity prior to onset of seizures. Arrow points to the onset of epileptiform discharges. These events were typically associated with behavioral arrest, and accompanied by staring, facial automatisms, and head jerking/pawing. Inset represents the EEG trace with an expanded time scale showing the seizure initiation with spikes, progression to high frequency, large amplitude spikes during tonic phase of seizure, and termination phase of seizure with smaller amplitude spike postictal slowing.

in the electrographic activity in the cortical leads as well (Fig. 2). The seizure epochs, shown in 10 s/page representation reveal the established patterns associated with epileptic seizures (Kadam et al., 2010), including incremental increase in frequency and amplitude of spike discharges, tonic spike wave discharges, and postictal slowing (Fig. 2). Following any one seizure, the EEG returned to baseline within seconds and coincided with resumption of baseline behavioral activity observed prior to the seizure.

Electroclinical seizures (behavioral automatisms with electrographic epileptiform activity) were observed in 11 rats experiencing early life seizures (one rat was excluded due to electrode-induced cerebral lesion–see Data S1). Spontaneous seizures were observed in the first recording performed after electrode implantation in nine rats following neonatal seizures at P10. Furthermore, at least one seizure occurred in 48 of 51 sessions of video-EEG recordings (94.4% of total) recorded for up to 8 h. In contrast, 12.5% of recordings from the normoxic controls showed abnormal activity on EEG. The presence of rare low frequency epileptiform discharges in control rats is consistent with previous reports in normal Long–Evans rats (Shaw, 2007).

With averaging of data recordings over 2–6 months of age, we noted that the average seizure frequency of 5.82 ± 1.05 seizures/h in posthypoxic neonatal seizure rats was significantly higher than the frequency of 0.10 ± 0.06 seizures/h observed in the normoxic controls ($n = 11$ hypoxic seizure rats; $n = 10$ controls, $p < 0.001$; Fig. 3A). Evaluation of the 1,215 seizures recorded from all animals showed that seizure duration ranged from 3–36.5 s, with a mean duration of 7.32 ± 0.12 s. The duration of 25.3% of recorded seizures was 3–5 s, whereas 58.2% of the recorded seizures were between 5 and 11 s, and all events were accompanied by previously described behavioral alterations. Only 4.9% of the seizures recorded across all age groups were longer than 15 s (Fig. 3B). Seizure durations in this range are similar to those in other rat models of acquired epilepsy (Cha et al., 2004; D'Ambrosio et al., 2009). Using graded thresholds for defining seizure duration, we observed a significant increase in the number of epileptic seizures observed after hypoxia-induced neonatal seizures compared to normoxic littermate controls for ranges of 3–10 s (Fig. S3).

Analysis of the progression of seizure frequency and duration in a subset of animals exposed to hypoxia-induced

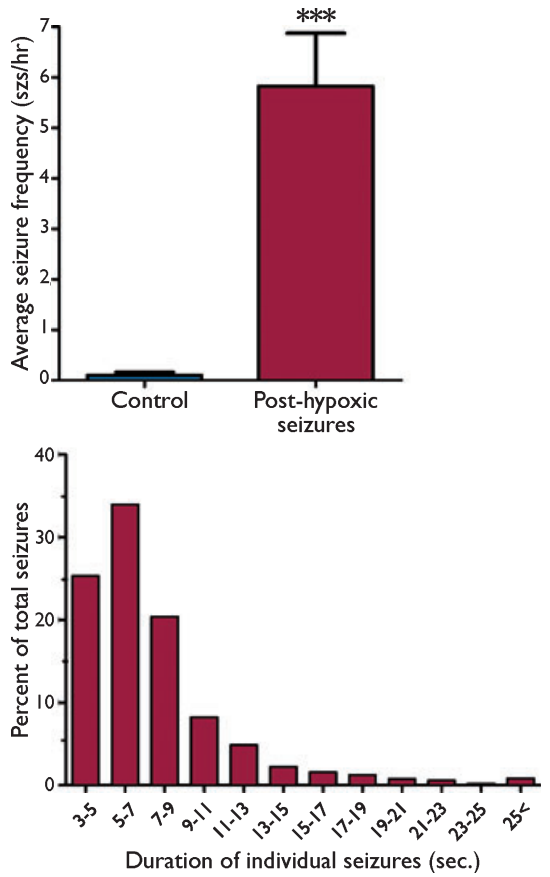

Figure 3.
Frequency and duration of seizure events in rats experiencing neonatal seizures and age-matched controls. (**A**) The frequency of seizures observed per hour of video-EEG recording analyzed from rats exposed to hypoxia-induced neonatal seizures at P10 and age-matched control rats is plotted. There is a significant increase in the number of seizure events observed in the rats exposed to neonatal seizures at P10, an average of 5.82 ± 1.0 seizures/h were recorded in rats exposed to neonatal seizures, compared with normoxic littermate controls (0.10 ± 0.06 seizures/h, n = 11 hypoxic seizure rats, n = 10 controls, $p < 0.001$). (**B**) Cumulative histogram of the duration of hippocampal EEG-recorded seizures in adult rats that had been subjected to graded global hypoxia at P10. Approximately 58.2% of all seizures recorded from the rats exposed to hypoxic neonatal seizures were in the 5–11 s duration time bin (n = 1215 seizures from 11 rats). The rats also exhibited some seizures with significantly longer durations, but the long epileptic seizures were infrequent.

neonatal seizures revealed that average seizure frequency increased by 234 ± 94% (n = 7, $p < 0.05$) between the first and last recorded video-EEG session per animal, whereas there was a trend but no significant increase in the seizure duration (Fig. 4). The increase in seizure frequency was observed consistently in a majority of animals that had experienced neonatal seizures (representative data, Fig. 4C). Similarly the average seizure duration in this experimental subject increased from 4.6 ± 0.9 to 12.9 ± 1.5 s (Fig. 4D).

Epileptiform EEG abnormalities in adult rats can be suppressed by phenobarbital

To validate the ictal nature of the events defined as seizures, we examined the effect of the anticonvulsant phenobarbital on the activity. Prior to injection of phenobarbital, spontaneous seizures and ictal EEG changes were correlated with increased EEG power in both the lower and higher frequency bins (Fig. 5A). A single phenobarbital dose (50 mg/kg, i.p.) decreased total power over 15–30 min sustained over >6 h, consistent with other models of ictal activity (Fig. 5B) (Dzhala et al., 2008; Raol et al., 2009). Spectral analysis demonstrated persistently increased power at higher frequencies during seizures (12–32 Hz, Fig. 5A,B). In contrast, EEG power plots from age-matched control rats prior to and following phenobarbital administration did not show increased EEG power in the high frequency bins (Fig. 5C,D). Compared to baseline seizure frequency, phenobarbital dramatically reduced seizure activity within the first hour to 14.0 ± 5.6% of baseline from the preceding 4 h, and to 2.4 ± 1.8% within 2 h in adults with previous neonatal seizures (Fig. 5E). FFT analysis revealed that the spectral power in the 7–20 Hz range was significantly greater during the seizure epochs relative to random EEG baseline epochs from the same individual (Fig. 5F). These results suggest that the abnormal, high frequency spikes in these rats are indeed epileptic seizures that can be controlled by anticonvulsant administration.

Neonatal seizures lead to increased mossy fiber sprouting in area CA3 of hippocampus in later life

Increased and aberrant mossy fiber sprouting is observed in cases of human temporal lobe epilepsy and many experimental models of epilepsy, and has been associated with epileptogenesis in animal models. The Timm staining in P100 rats after hypoxia-induced neonatal seizures revealed a significantly increased distribution of Timm granules in the CA3 region compared to controls (Fig. 6A,B), as well as significantly higher average Timm scores (2.71 ± 0.11) compared to littermate control scores (1.92 ± 0.14, n = 7,7, $p < 0.001$). Densitometric analysis of the relative intensities of Timm staining consistently showed that animals experiencing hypoxia-induced seizures had a significantly stronger staining intensity (228 ± 19.0%) compared to littermate controls (100 ± 10.8, n = 7, 7; $p < 0.001$) (Fig. 6D). This staining was most prominent in the septal regions of the hippocampus.

Evidence for a latent period following acute and subacute ictal activity induced by early life seizures

In order to examine the process of seizure development and progression, video-EEG monitoring was performed in

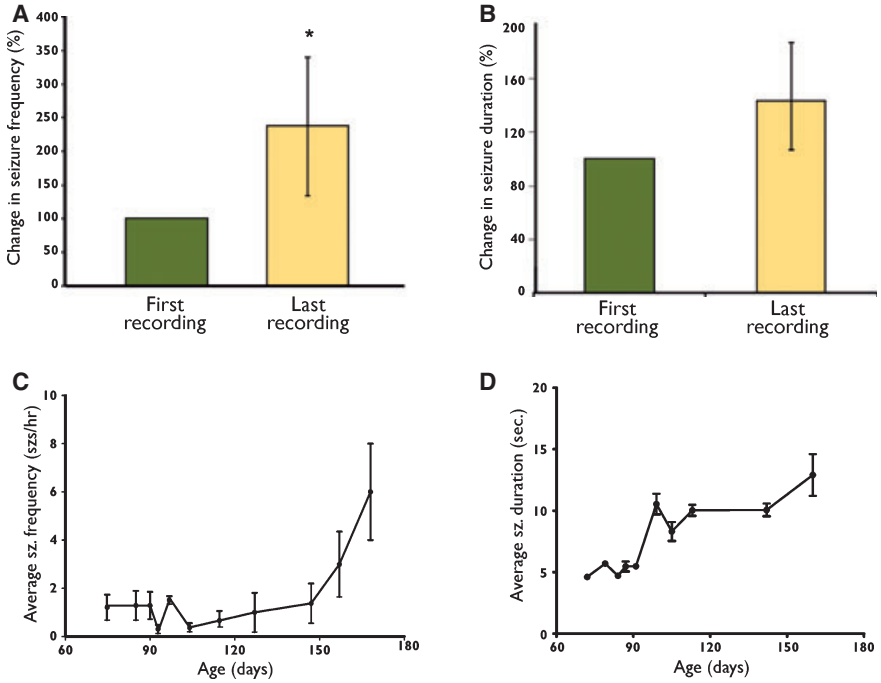

Figure 4.
Increase in severity of seizure frequency and duration with increasing age in Long-Evans rats exposed to neonatal seizures. Analysis of long-term EEG recording data from each individual rat that had been recorded sequentially for up to 6 months following initiation of hypoxic neonatal seizures, we observed a trend of increasing seizure frequency and duration with increasing age of the rats. The average frequency of seizures increased 234.2 ± 94% ($n = 7$, $p < 0.05$) in the last video-EEG recording compared to the first recording from the same individual subject (**A**); the seizure duration, however ,did not show a significant difference (**B**). Representative data from one subject shows that the average frequency of seizures increased from 1.8 ± 0.5 to 5.9 ± 1.2 seizures/h (**C**), the seizure duration in the subject increased from 4.6 ± 0.9 to 12.9 ± 1.5 s (**D**).

rat pups starting immediately after and extending up to 50 days from the hypoxic seizures. Five age windows were examined: the immediate 1–48 h post seizure (P10–12), the early juvenile period of 2–12 days (P13–22), mid-juvenile age (P23–31), late juvenile period (P32–37), and early adult (P38–56) (Fig. 7A).

In the immediate 48 h following hypoxia-induced seizures, 92% of the pups (10 of 11 pups) exhibited behavioral seizures accompanied by EEG changes, with an average duration of 5.4 ± 1.4 s (Fig. 7). The average frequency was 0.66 seizures/h, significantly less than during hypoxia (8.74 seizures/h, $n = 11$, $p < 0.05$). Following these subacute seizures, there was a relative cessation of spontaneous seizures in the early juvenile period at 2–12 days after hypoxic seizures, suggesting a latent period of epileptogenesis. During this time (P13–24), none of the video-EEG recordings from 11 rats revealed any abnormal behaviors. EEG recordings did reveal rare and short polyspike discharges lasting <1 s (data not shown), without behavioral correlate, and thus not defined as seizures.

Electroclinical seizures were observed again as early as P25 in 19% of the rats recorded from during the age-window from P25–31 (2 of 9 rats). The average seizure duration at this age was brief (3.2 ± 0.4 s), whereas no activity was seen in controls ($n = 5$, $p < 0.05$) (Table 1). The incidence of electroclinical seizures increased steadily up to 50% ($n = 6$, $p < 0.05$) by P32–37, and 55% by P37–55 ($n = 9$, $p < 0.05$). Behavioral correlates included cessation of activity and behavioral arrest, whisker movements, and head nodding/shaking in some cases. Seizure duration and frequency also increased at these time points; the average seizure duration and frequency at P32–37 was 5.2 ± 0.2 s and 2.26 ± 0.8 seizures/h, respectively, and at P38–55 was 6.42 ± 1.1 s and 2.8 ± 0.8 seizures/h, respectively (Fig. 7B,C). These data indicate that the incidence, frequency, and duration of seizures observed in individual animals after hypoxia-induced seizures increased with age following the hypoxic insult.

Discussion

The major focus of this study is the emergence of spontaneous seizure activity following early life hypoxia-induced seizures in the immature rat. We and others have reported acute seizures in this model (Rakhade & Jensen, 2009) and increased seizure susceptibility to ''second hit''

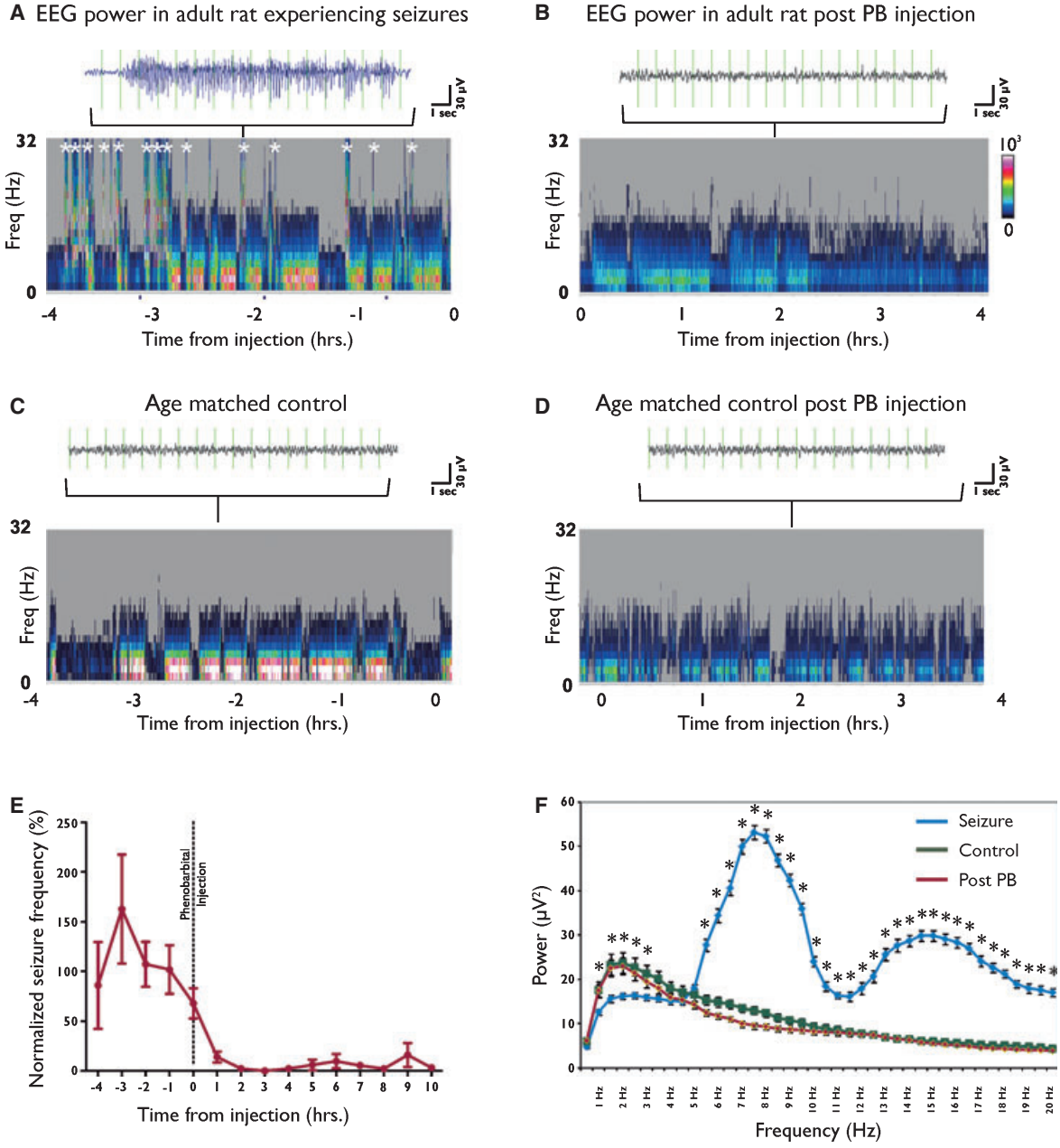

Figure 5.

Increase in EEG power parallels seizure occurrence and is reversed by phenobarbital. EEG data were analyzed for total power and power at various frequencies over an extended time period in a rat exposed to hypoxic seizures during the neonatal period. For each experimental condition the panel shows the power in 1 Hz frequency bins per 10 s interval, calculated by fast Fourier transform (FFT) (**A**) Seizure activity causes an increase in EEG power as evidenced by the intensity of the color and increase in high-frequency power (each segment marked with * corresponds to a similar increase in the power that is associated with the occurrence of an epileptiform event). (**B**) Administration of phenobarbital abolishes the occurrence of these epileptiform discharges. Power spectrograms from EEG recordings obtained from age-matched normoxic controls prior to administration of phenobarbital (**C**) and following phenobarbital administration (**D**) reveal the absence of these high intensity epileptiform events. (**E**) Representative data from seven different subjects that underwent these long-term recordings with phenobarbital injections administered intraperitoneally during the course of the recording. Normalized seizure frequency reduced to 14.0% of baseline seizure frequency within 1 h, and 2.4% of baseline seizure frequency within 2 h of phenobarbital administration. (**F**) FFT analysis revealed that the spectral power in the 7–20 Hz range was significantly greater during the seizure epochs relative to random EEG baseline epochs, and that the spectral power in EEG epochs following phenobarbital administration was attenuated to levels observed in baseline EEG epochs.

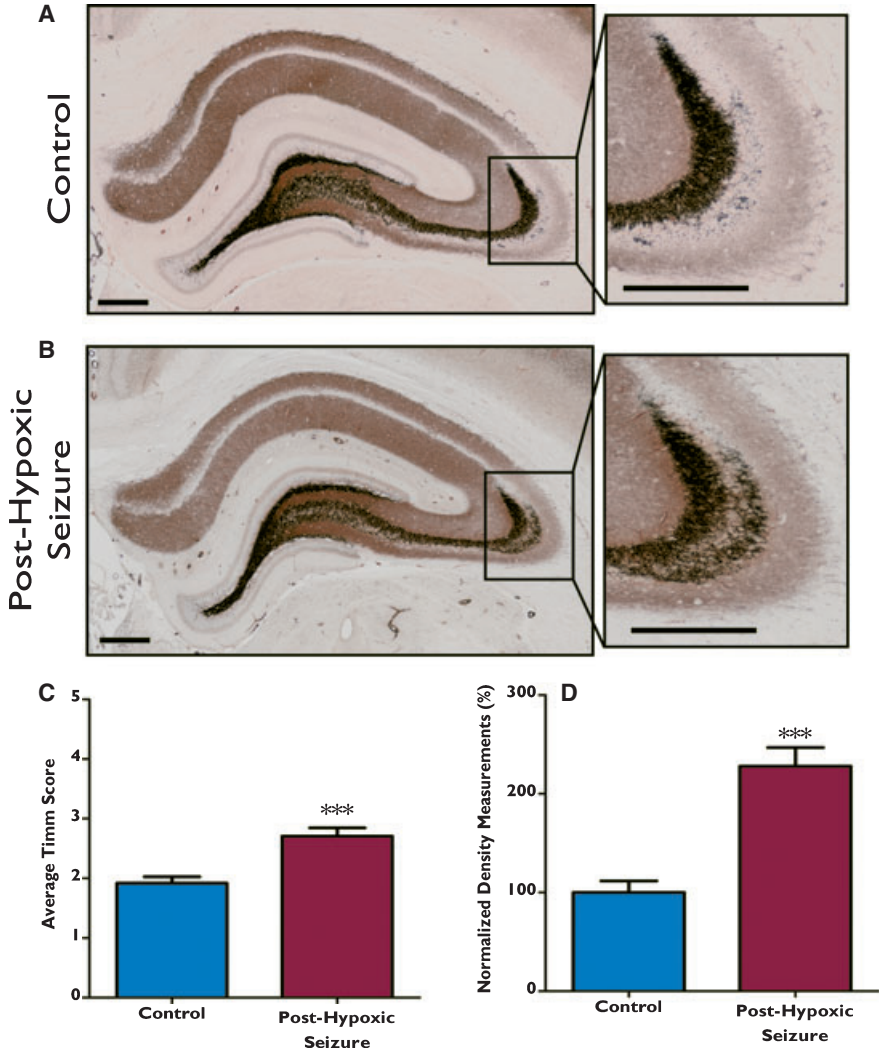

Figure 6.
Neonatal seizures led to increased mossy fiber sprouting from CA3 pyramidal cells. (**A**) Photomicrographs of Timm staining in hippocampi of adult rats that had experienced hypoxia-induced neonatal seizures at P10 show a marked increase in mossy fiber sprouting in the stratum pyramidale and stratum oriens layers near the hippocampal CA3 region, as compared to age-matched littermate controls (**B**). There is slight staining of pyramidal cells in the septal region of the hippocampus observed in control animals. Inset shows higher magnification views of the distribution of Timm granules in the stratum pyramidale and stratum oriens (scale bar = 500 μm). (**C**) Average Timm scores, calculated using a semiquantitative scoring scale described previously, from hippocampal sections from animals experiencing neonatal seizures were significantly higher (2.71 ± 0.11), as compared to scores in the littermate controls (1.92 ± 0.14, n = 7,7, $p < 0.001$). (**D**) Mean density measurements of Timm staining as measured in the stratum pyramidale-oriens, confirmed the increase in Timm granules, showing a 228 ± 19% increase as compared to littermate controls (100 ± 10.8, n = 7,7; $p < 0.001$).

seizures in later life (Koh et al., 2004). Enhanced susceptibility to provoked seizures demonstrates increased network excitability.

Herein we report for the first time that hypoxia-induced seizures during the neonatal period in rats led to recurrent, unprovoked, and spontaneous behavioral and electrographic (electroclinical) seizures in adulthood. In addition, this is the first report of a 2–3 week latent period in this model. Following this latent phase, there was a progressive intensification of seizure activity as evidenced by the increased seizure frequency and duration, as well as an increase in the cumulative number of individual rats exhibiting spontaneous epileptiform events. Furthermore, similar to other immature seizure models,

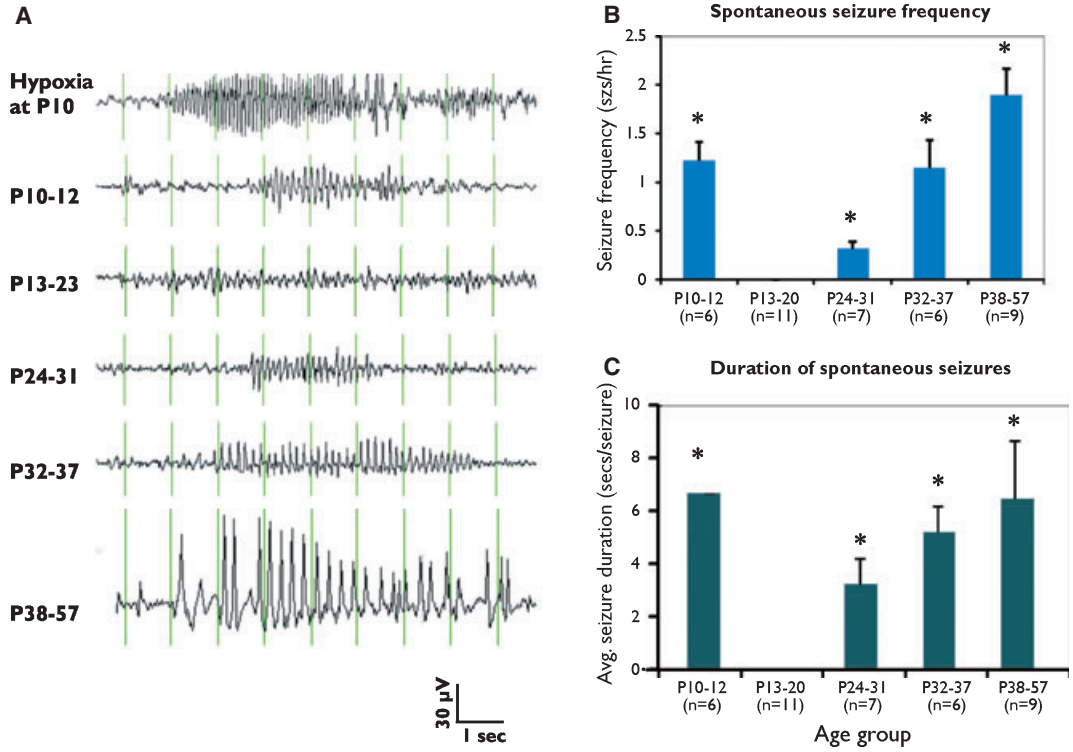

Figure 7.
EEG recordings with representations of ictal discharges (**A**) during hypoxia, (**B**) 0–2 days, (**C**) 3–10 days, (**D**) 14–21 days, (**E**) 22–27 days, and (**F**) 28–47 days following hypoxia. Abnormal activity was classified as seizures when activity was paroxysmal, rhythmic, increasing in amplitude, and >3 s in duration. Electrographic seizures associated with abnormal behavioral automatisms were observed within the first 48 h following hypoxia-induced seizures at P10. There was a decrease in the electrographic seizure activity between the ages of P13 and P20. The frequency of electrographic seizures associated with abnormal behavior increased again in the animals at P24–31 and continued to increase into adulthood.
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Table 1. Emergence of spontaneous seizures following hypoxia-induced neonatal seizures

Age window (postnatal days)	Percentage of animals with spontaneous seizures	Average number of seizures recorded/animal
P10–12 (n = 6)	92	4.2
P12–24 (n = 11)	0	0
P25–31 (n = 9)	21	8.3
P32–37 (n = 6)	50	9
P38–58 (n = 9)	50	11.2
P58–180 (n = 11)	100	22

EEG recordings were performed in rat pups exposed to neonatal seizures, and the percentage of pups exhibiting spontaneous recurrent seizures during specified time windows was calculated. The average number of seizures observed during the 3-h video-EEG recording epoch is tabulated.

we observed increased mossy fiber sprouting in hippocampal area CA3 in the adult brains after hypoxia-induced neonatal seizures at P10. Taken together, these data indicate that the hypoxia-induced early life seizures result in the development of epilepsy in rats and thus may represent a useful model for studying the epileptogenic process, as well as interventional strategies to prevent the long-term sequelae of this form of neonatal seizures.

Emergence of epilepsy following neonatal seizures in human infants

Hypoxic encephalopathy is one of the most common causes of neonatal seizures (Volpe, 2008) and can result in later life epilepsy and other neurodevelopmental sequelae (Berg & Shinnar, 1997; Ronen et al., 2007). The age of onset of seizures influences the long-term developmental challenges such as intellectual impairment (Hermann et al., 2002), learning disabilities (Soria et al., 2007), and medical refractoriness (Berg et al., 1996; Camfield & Camfield, 2002). Neonatal seizures can lead to development of both partial and generalized epilepsy in later life in 23–40% of cases (Ronen et al., 2007). Furthermore, children experiencing neonatal seizures have lower scores on measures of intellectual ability (Toet et al., 2005; Glass et al., 2009).

Development of an animal model of neonatal seizures with later life epilepsy

Herein we present a model suitable for the study of molecular mechanisms and therapeutic efficacy trials for both the previously reported neurobehavioral outcomes as well as the presently described epileptogenic effects. Depth electrode recordings of spontaneous recurrent seizures arising over 8 weeks and into adulthood demonstrated involvement of both hippocampal and cortical structures. The accompanying seizure semiology was primarily behavioral arrest with head jerking and wet dog shakes, rather than episodes of generalized tonic–clonic seizures or status epilepticus. Cortical ictal EEG patterns were present synchronously in both hemispheres, consistent with the fact that the original insult was global hypoxia. In addition, this model induced later life spontaneous electroclinical events predominantly 15 s or less in duration as opposed to recurrent episodes of prolonged status or generalized seizure activity. Other models of acquired epilepsy have similarly reported outcomes of either partial or brief seizures, including models involving prior traumatic brain injury (D'Ambrosio et al., 2009), hypoxia/ischemia, and stroke (Kadam et al., 2010), as well as febrile seizures (Dube et al., 2006). In particular, D'Ambrosio et al. have described the presence of short electroclinical epileptiform events with duration of 0.8–2 s being associated with behavioral arrest in both human subjects and rodents exposed to rostral parasagittal fluid percussion injury (D'Ambrosio et al., 2009). We showed that administration of phenobarbital temporarily abolished the seizure activity observed in the adult rats with previously exposure to hypoxia-induced seizures.

Our prior in vitro experiments performed in hippocampal slices during the subacute period have shown enhanced network excitability and changes in neurotransmitter function following these early life seizures (Sanchez et al., 2001, 2005a; Rakhade et al., 2008). Depth electrode recordings confirmed involvement of the hippocampus in initiating and/or maintaining epileptic seizures; the seizure semiology was similar to behavioral changes typically observed in seizures involving limbic areas (Ben Ari et al., 1981; D'Ambrosio et al., 2009).

Neonatal seizures lead to increased mossy fiber sprouting in hippocampus

Sprouting of axon terminals after prolonged or recurrent seizures has been described in a number of models including kainic acid (Tauck & Nadler, 1985; Cronin & Dudek, 1988), electrical stimulation (Sutula et al., 1988), and pentylenetetrazole kindling (Golarai et al., 1992). Mossy fiber sprouting has been observed in tissue from patients with temporal lobe epilepsy (Sutula et al., 1989; Babb, 1991). Indirect evidence from several studies suggest that these new neurites establish functional synaptic connections and they may contribute to the state of hyperexcitability that either provokes or facilitates abnormal discharges (Wuarin & Dudek, 1996). Alterations in the CA3 pyramidal region have been observed in other models of seizures in the immature brain, including seizures caused by PTZ kindling (Holmes et al., 1999), kainic acid (Huang et al., 1999), amygdala kindling (Represa & Ben-Ari, 1992), corticotropin releasing hormone (CRH)–induced status epilepticus (Ribak & Baram, 1996), and intrahippocampal injections of kainic acid at P7. This altered mossy fiber distribution may contribute to the enhanced synaptic activity seen at CA1:CA3 synapses following hypoxic neonatal seizures (Sanchez et al., 2005b; Rakhade et al., 2008) and may correlate with disturbance in cognitive function seen in later life (Crusio et al., 1987; Schwegler et al., 1988). The axons of the granule cells are elongating during the first 2 weeks of postnatal development (Amaral, 1979; Rahimi & Claiborne, 2007), and neonatal seizures during this window of development may alter the hippocampal circuitry that is being actively modulated.

Neonatal hypoxia is followed by a latent period of epileptogenesis and spontaneous seizures progressively increase with age

Most models of acquired epilepsy in adult brain suggest that epileptogenesis is progressive and that there is a ''latent period'' between the initial insult and the appearance of the epileptic seizures (Cherubini et al., 1983; Cronin & Dudek, 1988; Dudek et al., 2002; D'Ambrosio et al., 2004). Although an increasing number of studies in immature rats have shown that early life insults can lead to the development of epilepsy in later life (Holmes et al., 1998; Dube et al., 2006; Kadam et al., 2010), these studies have not focused on characterizing an intervening latent period. Our experimental model of hypoxia-induced seizures reveals a progression of epileptic activity following early life seizures, and we did not observe spontaneous electroclinical seizures between 2 and 15 days following the original hypoxia-induced seizures. Due to the age of the pups and their inability to be separated from their dam for >2–3 h, continuous EEG recordings were not possible, and hence we can only conclude that there was relatively less seizure activity in this period. The earliest electroclinical seizures, starting approximately around fourth postnatal week, were manifested by a behavioral arrest and showed that animals progressed to develop electroclinical events associated with repetitive motor activity/pawing and head-nodding/head shakes. The potential presence of the latent phase suggests that there may be a possibility of intervening during this phase to prevent the development of clinical epilepsy. Indeed, we have previously shown that the administration of specific anticonvulsant agents can attenuate the hippocampal hyperexcitability and induction of signaling cascades in this rodent model (Sanchez et al., 2005a; Rakhade et al., 2008).

Spontaneous recurrent seizures arise in the absence of neuronal death

Models of adult epileptogenesis, including those initiated by kainic acid– or pilocarpine-induced SE, result in acute neuronal death (Holmes, 2009). However, seizure-induced neuronal death is not a consistent feature in immature rodents. Previous studies performed in immature rats exposed to hyperthermia (Dube et al., 2006) and flurothyl-induced seizures (Schmid et al., 1999) have reported the development of spontaneous seizures despite the lack of cell death. In contrast, a recent study in an experimental model of perinatal stroke and hypoxic ischemic encephalopathy suggests that neuronal death (i.e., a clear infarct following neonatal stroke) is required for the development of epilepsy (Dudek et al., 2010; Kadam et al., 2010). We have previously shown that hypoxia-induced neonatal seizures do not result in neuronal death (Koh et al., 2004), although they evoke synaptic ''dysplasticity'' in the surviving neuronal network (Sanchez et al., 2005a; Rakhade et al., 2008). The second postnatal week is a critical period of development in the rat brain (Silverstein & Jensen, 2007). Insults such as pilocarpine injection (Liu et al., 1994), flurothyl inhalation (Sogawa et al., 2001), and hyperthermia (Dube et al., 2006) during this critical period can induce recurrent seizures in rats despite the lack of neuronal cell death. Furthermore, most of these models have shown considerable impairment in spatial learning as well as tests of social interaction when studied during young adolescence and adulthood (Baram et al., 2002; Karnam et al., 2009). The present data suggest that cellular death may not be an essential component for the development of cognitive, developmental, and epileptogenic effects of early life seizures. Another important observation is that the spontaneous seizures observed in later life in immature seizure models that lack cell death (Holmes et al., 1999; Dube et al., 2005) are generally of shorter duration and lower frequency than in those immature models with neuronal death (Kadam et al., 2010). It is possible that a relatively intact neuronal network may restrain excessive seizure activity, and may explain this difference. In addition, the absence of cell death allows study of the cellular and molecular alterations during a period of relative seizure silence (P12–24) without the confounds of cell death mechanisms.

Conclusions

The hypoxia-induced neonatal seizure model recapitulates a number of salient clinical features of hypoxic encephalopathy and neonatal seizures. In this model, seizures during the neonatal period appear to be sufficient to initiate epileptogenic processes and development of later life epilepsy. Furthermore, although there is a lack of cell death in this experimental model, there are structural alterations including increased mossy fiber sprouting in the hippocampal CA3 region. Our prior work has shown that the administration of AMPAR antagonists can attenuate the hippocampal hyperexcitability and induction of signaling cascades in this rodent model (Koh et al., 2004; Sanchez et al., 2005a; Rakhade et al., 2008). Given the present demonstration that this model results in spontaneous seizures in later life, future work can now address whether intervention following seizures can modify epileptogenesis.

Acknowledgments

The authors wish to thank Neil Marya, Andrew Murphy, and Victor Lan for help with animal handling. This work was supported by funding from National Institutes of Health NINDS NS 31718 (FEJ), Office of the Director, National Institutes of Health GMS DP1 OD003347 (FEJ), Parents Against Childhood Epilepsy (SNR and FEJ), and core support from the Mental Retardation Developmental Disorders Research Center Grant NIH NICHHD P30 HD18655.

Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Representative EEG recording from P10 rat pups before and during hypoxia obtained using subdermal wire electrodes.

Figure S2. Survey view of Fluoro-Jade B staining following hypoxia-induced neonatal seizures and following unilateral infarct after unilateral carotid artery ligation.

Figure S3. Calculation of seizure frequency in animals that have experienced neonatal seizures using graded duration criteria for definition of spontaneous seizures.

Table S1. Comparison of characteristics of normal cortical rhythms, interictal spikes, and spike wave seizures observed in adult Long-Evans rats.

Video S1. Representative video-EEG from P10 rat pup experiencing neonatal seizures on exposure to graded global hypoxia.

Video S2. Representative video-EEG from adult rat showing behavior associated with epileptic seizures—video-EEG recording from an adult Long-Evans rat that had experienced neonatal seizures at P10 shows the onset of spontaneous seizure activity.

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