Ischemic injury suppresses hypoxia-induced electrographic seizures and the background EEG in a rat model of perinatal hypoxic-ischemic encephalopathy

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Abstract

The relationship between neonatal seizures, abnormalities of the electroencephalogram (EEG), brain injury, and long-term neurological outcome (e.g., epilepsy) remains controversial. The effects of hypoxia alone (Ha) and hypoxia-ischemia (HI) were studied in neonatal rats at postnatal day 7; both models generate EEG seizures *during* the 2-h hypoxia treatment, but only HI causes an infarct with severe neuronal degeneration. Single-channel, differential recordings of acute EEG seizures and background suppression were recorded with a novel miniature telemetry device during the hypoxia treatment and analyzed quantitatively. The waveforms of electrographic seizures (and their behavioral correlates) appeared virtually identical in both models, and were identified as discrete events with high power in the traditional delta (0.1-4 Hz) and/or alpha (8-12 Hz) bands. Although the EEG patterns during seizures were similar in Ha- and HI-treated animals at the beginning of the hypoxic insult, Ha caused a more severe electrographic seizure profile than HI near the end. Analyses of power spectral density and seizure frequency profiles indicated that the electrographic seizures progressively increased during the 2-h Ha treatment, while HI led to a progressive decrease in the seizures with significant suppression of the EEG background. These data show that: (1) the hypoxia component of these two models drives the seizures; (2) the seizures during Ha are substantially more robust than those during HI, possibly because ongoing neuronal damage blunts the electrographic activity, and (3) a progressive decrease in background EEG, rather than the presence of electrographic seizures, indicates neuronal degeneration during perinatal HI.
Seizures are relatively common in neonates, and are a potential harbinger of intractable epilepsy, cerebral palsy, cognitive deficits and other negative neurological outcomes. Prolonged periods of hypoxia alone (Ha; e.g., birth asphyxia or apnea) or hypoxia-ischemia (HI; e.g., perinatal stroke) are thought to be a major cause of neonatal seizures, but metabolic disturbances, encephalitis, and genetic abnormalities can also lead to seizures in neonates (Sarnat and Sarnat, 1976; Finer et al., 1981; Watanabe et al., 1982; Corey et al., 1988; Mercuri et al., 1999; Marin-Padilla, 2000; Nelson and Lynch, 2004; Malm, 2009; Sorge and Sorge, 2010). Although considerable EEG data are available on neonatal seizures, their predictive value is poor because outcomes range from benign to devastating. A definitive causal link between the characteristics of the neonatal EEG and the subsequent neurological outcome is difficult to establish in humans. Thus, it is unclear whether acute neonatal seizures are causes of - or merely symptoms of - other processes that lead to a negative outcome, making it difficult to gauge the necessity and efficacy of early clinical interventions to suppress seizures during the critical “sub-acute” period after neonatal insults (Monod et al., 1972; Tharp et al., 1981; Scher and Beggarly, 1989; Williams et al., 1992; Holmes and Lombroso, 1993; Murray et al., 2009; Korotchikova et al., 2011; Walsh et al., 2011). Furthermore, other events besides seizures, such as background abnormalities in the EEG, have been proposed to be better predictors of outcome (Monod et al., 1972; Tharp et al., 1981; Watanabe et al., 1982; Holmes and Lombroso 1993), but well-designed prospective studies on the neonatal EEG are limited. The EEG has generally not been classified quantitatively in rodent models of Ha and/or HI. Although it is unclear which features of the EEG are associated with overt brain damage and subsequent poor outcome, prolonged repetitive seizures and/or background abnormalities are the two main hypothetical indicators of a negative outcome.
In the present study, Ha and HI were used as two complimentary animal models of neonatal seizures in postnatal day 7 (P7) rat pups. These models are thought to have similar behavioral seizures, but little is known about the quantitative electrographic properties of neonatal seizures and how they are altered during the acute treatment period. Previous observations indicate that Ha causes little or no obvious neuronal death (Rice, et al, 1981; Jensen et al., 1991), while HI leads to an overt hemispheric lesion (Rice et al., 1981). In the present study, we hypothesized that both animal models (Ha and HI) would show robust electrographic seizures during treatment, but the two groups would have different EEG features. One hypothesis is that because brain injury can cause severe electrographic seizures, the occurrence of ongoing brain injury during HI would exacerbate the seizure activity. Another hypothesis is that injury-induced changes in cortical neurons (e.g., depolarization inactivation) progressively suppress the seizures as the injury evolves, at least at the site of the insult. Previous studies with long-term, continuous, radio-telemetric EEG recordings - using a variation of the HI model employed here - showed that the presence of a macroscopic infarct is necessary for development of epilepsy in Sprague-Dawley rats; however, rats exposed to HI treatment at P7 with acute behavioral seizures but no infarct consistently did not manifest the spontaneous recurrent seizures that define acquired epilepsy (Kadam et al., 2010). Therefore, electrographic recordings in these two models (i.e., Ha with seizures but no infarct vs HI with seizures plus a large infarct) during the actual treatments could hypothetically identify the electrographic features that could serve as biomarkers that predict subsequent epileptogenesis.

**Methods**

**Miniature telemetry**

Recent reports have described EEG activity with tethered recordings from immature rat pups using HI or Ha models (Sampath et al., 2014; Zanelli et al., 2014). With tethered systems,
however, the recording leads can alter the animal’s posture and restrict movement. Deformation of the soft skull by torque from the cables probably augments movement artefact and can injure the brain. In the present study, single-channel wireless technology was used for the recordings, which facilitated continuous quantitative EEG analysis of electrographic seizures and the background activity in the EEG signal (Zayachkivsky et al., 2013).

Implantation of telemetry unit

Pregnant Sprague-Dawley rat dams were obtained from Charles-River (Wilmington, MA). Rat pups were born in the University of Utah animal facility. Pups were reared with the dam and implanted at 6-7 days of age (postnatal day 6-7; P6-7) with the miniature wireless telemetry system (Zayachkivsky et al., 2012). During this procedure, animals were anesthetized with 4% isoflurane and a maintenance dose of 2% (MWI Veterinary Supply, Meridian, ID). The stereotaxic unit was sprayed with 70% alcohol, and surgical tools were sterilized by autoclaving and then maintained in 70% ethanol. The rat pup was placed in the stereotaxic unit using small-animal ear bars (David Kopf Instruments). An incision was made on the top of the head with a scalpel, and the skin was clamped with hemostats. The periosteum was then removed from the skull, and surface bleeding was cauterized. Two holes for electrodes were made using a surgical drill with a 0.7 mm burr (Fine Science Tools), 2 mm lateral from midline of the skull, 2 mm apart on the right side. The electrode wires of the transmitter system were trimmed to appropriate length and fed through the craniotomies with a target depth at the level of the dura. The transmitter was attached to the skull using a cyanoacrylate gel compound (Loctite 454) with accelerator (Loctite 7452). Additional cyanoacrylate was applied around the unit and the exposed areas of the skull to stabilize the implant. The skin was then sutured around the implant with Vicryl 4-0 coated polyglactin 910 suture (Ethicon). Animals were injected with 0.5 ml of lactated ringers (sub-cutaneous), treated with local anesthetic (Marcaine) and allowed to recover for 24 h with the dam.
Neonatal hypoxia (Ha) and hypoxia-ischemia (HI) treatments

The Ha and HI procedures included both male and female rat pups at P7-8 previously implanted with the telemetry units at P6 (see above). Animals were anesthetized with 2% isoflurane. The ventral midline of the neck was locally anesthetized with marcaine (0.5%, 0.2 mL). A 2-cm incision was made in the animal’s neck and the right common carotid artery was exposed by blunt dissection. In animals in the HI group (n=12), the carotid artery was occluded with micro-aneurism clamps, and cut by cauterization. Clamps were removed after cauterization of the artery. In animals in the Ha group (n=9), the carotid was not cut. In the control group (n=6), sham surgery was conducted, but the animals were not exposed to hypoxia or ischemia. The skin was then sutured and the rat pups were allowed to recover in a cage with the dam for 2 h. After a 2-h recovery, the pups were placed in the hypoxia chamber with feedback-controlled temperature control (37°C) and recording chambers (designed and built in-house). Each recording chamber was designed to have an independent gas input and output and a separate antenna for recording. The chambers were then filled with 8% oxygen and 92% nitrogen gas mixture at positive pressure using a pressure-control manifold. Temperature was verified independently from feedback control using a Vernier Instruments thermocouple. The rats in both groups were exposed to this mixture for 2 h, and were then allowed to recover with the dam and littermates. During the treatment, EEG was continuously recorded (Fig. 1). After treatment, the pups were given 0.5 ml lactated ringers. Animals were returned to the dam and euthanized 72 h after Ha or HI to verify the presence or absence of the lesion.

Data acquisition and recording

The wireless device amplified differential signals from two integrated wire electrodes with a bandwidth of 0.1 – 120 Hz and transmitted the EEG signal to the receiver bases through
capacitive coupling. The signals from multiple receivers (one per animal) were then digitized by a Biopac MP150 (Goleta, CA) analog-to-digital converter, sampled at 500 Hz, and stored on a PC computer using Acknowledge 4.1.1 (Biopac) software.

Signal analysis

EEG recordings from rat pups during treatment were analyzed in both the temporal and frequency domains. First, events were manually separated and classified into categories based on high power in different EEG-frequency bands using Acknowledge 4.1.1 software (Biopac, Goleta, CA). Based on the difference between experimental and control groups, three categories were established – abnormal delta, abnormal alpha and background (defined as the interictal period). The root mean square (RMS, square root of the arithmetic mean of the squares of the values) power was calculated for each event in these categories and was plotted as a function of time (Matlab, Mathworks, Natick, MA).

Signal power was normalized to the background power recorded in the control animals. The data were then separated into 10-min bins for seizures and 5-min bins for background, and the means of each bin were plotted over time. Statistical differences were determined between HI and Ha events over the full 2-h treatment and during each hour of the treatment. In cases where multiple comparisons were conducted, the Levene test was used to determine whether variances were uniform within groups. If the variance was uniform within groups, ANOVA was then performed to determine the differences in mean power (P>0.05 to reject) with a Games-Howell post-hoc test. If the variance was not uniform between groups, Welch ANOVA was performed (P>0.05 to reject) with a Games-Howell post-hoc test. Fast Fourier Transforms (FFTs) were performed to analyze EEG data in the frequency domain from 0 to 20 Hz. Power spectral densities (PSDs) were estimated from the FFT using 256 Hann-window segments based on the Welch method (NeuroExplorer, Littleton, MA) and normalized by PSD=10*log_{10}
(FFT). Power levels at all frequencies in 0.1 to 20 Hz were plotted with 95% confidence intervals.

Histology and Flouro-Jade B staining

The brains were sectioned into 40-µm sections, mounted on gelatin-subbed slides and stained with Flouro-Jade B, a marker of neuronal degeneration. Slides were treated with 100% ethanol for 3 min, followed by 1 min in 70% ethanol and 1 min in distilled water. They were then treated in 0.06% potassium permanganate for 10 min, washed with water three times and incubated in 0.001% Flouro-Jade B for 30 min. Sections were rinsed with distilled water and dried. On the following day, the slides were cover-slipped with DPX mounting medium (Sigma) and imaged on a Zeiss upright microscope.

Results

Power in the delta and alpha bands characterized electrographic seizures, which were qualitatively similar during Ha and HI.

To determine whether Ha and HI induce specific electrographic abnormalities, the EEG was first qualitatively examined during the two different treatment protocols: Ha and HI. During Ha and HI treatment, the EEG patterns during seizures appeared similar but dramatically different from the activity observed in untreated control animals (Fig. 2). During both Ha and HI, two distinct types of seizure-like activity and two different seizure patterns were apparent in the EEG (Fig. 3 and 4). Seizure-like activity was present both as discrete events (Fig. 3A) at a single frequency band (i.e., either alpha or delta) or as a combination of the two frequency bands with seizure discharges occurring in an alternating pattern of alpha and delta bands (Fig.
One seizure type showed high-amplitude discharges in the delta band (0.1 – 4 Hz); these electrical events occurred during classic convulsive-like behavior (Fig. 3B and 4). The discharges were 30 sec to several minutes in duration. High power in the alpha band (8-13 Hz, Fig. 3 and 4) characterized another type of seizure-like discharge, which also could be observed during either Ha or HI treatment and as either single- or multi-component events. Behaviors that appeared similar to tonic seizures accompanied the alpha-band type of electrical event. Therefore, the seizure-like electrical activity, which each had a discrete onset and termination, could be defined and quantified according to their respective frequency bands (i.e., alpha and delta); furthermore, the types of electrical activity were quite similar in both Ha or HI.

Specific behaviors accompanied the distinct forms of electrographic seizure-like activity.

Animals that were treated with Ha (n=9) or HI (n=12) presented with similar behaviors during treatment, and these behaviors corresponded closely to the two forms of electrical activity (i.e., alpha and delta frequency bands). Ha- and HI-induced abnormal behaviors began within minutes of administration of the hypoxic gas mixture. Both treatment groups (i.e., Ha and HI) exhibited abnormal behaviors that could be classified into three categories: (1) convulsions (2) “tonic-like” convulsions, and (3) complete behavioral arrest. The seizure behaviors appeared different than those defined by the standard Racine scale (Racine, 1972), presumably because of the immature state of the motor system of the pups. Untreated, EEG-implanted animals (i.e., controls) did not exhibit any of the above-described behaviors. The behaviors in untreated control animals included body flexion, extension and regular myoclonic jerks, which were identical in both EEG-implanted and non-implanted, untreated animals. No convulsive activity was detected in animals that were not exposed to hypoxia. After the administration of Ha or HI, the animals recovered within a few hours and behaved normally. Three animals died during administration of HI; nonetheless, their seizure activity was analyzed. Because the behavioral
profile of each experimental-treatment group was largely similar, the present study focused on
the EEG-recorded electrical activity from the two treatment groups.

**Overall seizure power progressively increased during Ha but slowly decreased during HI.**

The main *qualitative* difference between the EEG signals from the Ha and HI groups
appeared to be the *amount of electrical activity* during the second hour (Fig. 2); that is, all of the
seizures were *similar in waveform*, but those during HI treatment appeared to be of *lower
amplitude* during the second hour. Therefore, the raw electrographic data were analyzed in their
entirety (i.e., putative seizures, in addition to background activity) in the frequency domain.

Because the first and second hours of electrical activity appeared to be qualitatively different
during either treatment, the 2-h period of recording was arbitrarily divided in half to compare the
EEG features in the frequency domain. The power spectral density (PSD) was then estimated,
averaged across animals, and plotted with 95% confidence intervals. During the first hour (Fig.
4A), the frequency profiles during Ha and HI treatments were similar. The EEG had more power
throughout the delta band, and substantially more power in the alpha band compared to control
(Fig. 4A), which presumably represents the occurrence of the two types of seizure activity.

Power in both the delta (p=0.054 1st hour; p<0.001 2nd hour) and alpha bands (p=0.009 1st hour;
p=0.017 2nd hour) was greater in the Ha-treated animals than in the control animals. Similarly,
the power was also greater in the delta (p=0.054 1st hour; p<0.001 2nd hour) and alpha bands
(p=0.049 1st hour; p=0.002 2nd hour) in the HI-treated animals compared to controls. However, a
significant decrease in power was observed in both the alpha (p=0.998 1st hour; p<0.001 2nd
hour) and delta bands (p=0.889 1st hour; p=0.014 2nd hour) during the second hour of HI,
compared to Ha (Fig. 4B). As expected, in control animals, power in the alpha (p=0.98) and
delta bands (p=0.29) was not significantly different during the first and second hours (not
shown). Therefore, when analyzed in the frequency domain, overall power across the frequency
bands was higher in the Ha and HI animals than control animals, which corresponded to the
seizure-like activity (described above) in the EEG. Power was similar in the Ha and HI animals during the first hour, but greater in the Ha group compared to the HI animals during the second hour. These data from frequency-domain analyses, therefore, suggested that seizure activity increased during the second hour of the 2-h treatment period in the Ha animals, but decreased during the second hour of hypoxia in the HI animals.

During the second hour of hypoxia, seizure frequency increased in Ha but decreased in HI rat pups.

EEG traces were visually inspected, and discrete abnormal events were separated from segments of background activity between these events (Fig. 5). The abnormal discharges were then separated into events with dominant power in the delta (0.1-4 Hz) and alpha (8-13 Hz) frequency bands. The average number of events across animals in the Ha group was 28.9±5.2 (mean ± standard deviation) during the first hour and 35.6±13.8 during the second hour; the difference was not significant (p=0.12). In the HI-treated group, however, the number of events during the first hour (24±9.6) was significantly greater (p=0.02) than during the second hour (15±4.9). Across groups, the number of seizure-like events during the first hour of Ha and the first hour of HI was not significantly different (p=0.16); however, the second hour of HI had significantly fewer seizures than the second hour of Ha (p=0.004) (Fig. 3). Therefore, the number of seizures appeared to be similar for the Ha and HI groups at the beginning of the exposure to the hypoxic gas mixture, but then seizure frequency increased over the 2-h period in the Ha animals while it decreased in the HI animals.

To investigate the differences in electrographic profiles of Ha- and HI-treated animals, the RMS power properties of each abnormal event were examined as a function of time during treatment. RMS data from all animals were binned in 10-min intervals for alpha and delta bands and 5-min bins for background EEG over the entire 2-h period of treatment. RMS power was dominated by high-power delta events in both groups (Fig. 6 A-D). RMS power of these events
was not significantly different between groups during the first hour of treatment (p=0.899); however, during the second hour, differences became apparent (p= 0.014). During the second hour, the RMS power of the delta events in HI animals dropped below that of the Ha-treated rats, while in Ha animals the power profile steadily increased over time and was significantly greater than during the first hour (p<0.001). The pattern of the temporal distribution of alpha events was different from that of the delta events. The differences between Ha and HI treatment became apparent 20-30 min after the onset of the treatment period (Fig. 6E, F); however, mean RMS power was significantly different between the two treatment groups only during the second hour (p<0.001). In Ha animals, the RMS power of alpha events remained relatively stable over time, exhibiting a slight increase by the second hour.

The mean RMS power profile of background EEG in Ha-treated animals increased slightly through the first hour of treatment, but decreased back to the initial power level by the end of the second hour (Fig. 7). The mean RMS power of HI-treated animals was similar to the pattern of alpha events, with differences in RMS power between groups beginning at 20-30 min after the start of treatment and steadily decreasing over time. The quantitative decrease in RMS power during this time was consistent with qualitative observation of amplitude suppression in the EEG (Fig. 2). These data suggest that the time-dependent decrease in RMS power in background EEG at 30 min after the beginning of the period of hypoxic gas exposure in the HI group reflects impending and/or ongoing neuronal injury (see below).
degeneration present in all of the above brain structures. Therefore, Flouro-Jade B staining confirmed that Ha was associated with no detectable neuronal death, while HI led to dramatic brain lesions.

Discussion

The key results were that (1) acute electrographic seizures during both Ha or HI at P7 involved prolonged periods of increased EEG amplitude in two distinct, classically defined frequency bands associated with specific, abnormal behaviors; (2) because the EEG seizures appeared identical at the onset of Ha and HI, the 8%-oxygen hypoxic gas treatment appeared to generate the acute seizure activity during both Ha and HI; (3) electrographic seizures continuously increased during Ha (i.e., hypoxia without ischemia or injury), but decreased during HI (i.e., hypoxia with ischemia); and, (4) suppressed EEG background occurred during HI-induced brain damage (even during repetitive electrographic seizures), but not during Ha-induced seizures.

Unique features of acute seizure activity in Ha and HI

The EEG events that were defined as seizures were not present in control animals, had large amplitudes and prolonged durations (10’s of seconds), and were consistently associated with abnormal behaviors: convulsive-like behaviors (delta discharge) and tonic shivering (alpha discharge). Other researchers have described acute Ha- and HI-induced seizures (Jensen et al., 1991; Cuaycong et al., 2011; Zanelli et al., 2014; Sampath et al., 2014); however, in these previous studies, the seizure events were primarily analyzed in qualitative terms during either relatively brief exposures (i.e., minutes) to hypoxia (Jensen et al., 1991; Zanelli et al., 2014) or without a direct comparison between Ha and HI (Sampath et al., 2014). The use of tethered recordings and the atypical waveforms of the events during the seizures raised concerns about movement artifacts contaminating EEG signals (Zanelli et al., 2014), and reduced the ability to analyze the entire EEG signal. Instead, components of the signal were selected for analysis.
Miniature telemetry allowed quantitative analyses of the entire raw EEG, so all of the electrographic seizures that occurred during Ha and HI were incorporated into the analysis. Although the Ha- and HI-induced electrographic seizures lasted 10’s of seconds, they otherwise differed substantially from chemoconvulsant-induced electrographic seizures during status epilepticus (e.g., Dzhala et al., 2005; Lehmkuhle et al., 2009; Raol et al., 2009; Zayachkovsky et al., 2013). The Ha/HI-induced seizures, unlike status epilepticus, were not self-sustaining and terminated immediately after re-introduction of normal air into the treatment chamber. The Ha/HI-induced seizures also clearly differed from the spontaneous recurrent seizures characteristic of acquired epilepsy (Williams et al., 2009; Kadam et al., 2010); they lacked a progressive increase in amplitude at seizure onset, traditional tonic and clonic components, an evolution in the waveforms from onset to termination, and post-ictal depression.

The differential frequency components of the neonatal seizure discharges recorded during Ha/HI with miniature telemetry, such as rhythmic alpha and delta, have not been described previously in rodent models of neonatal seizures (e.g., Sampath et al., 2014; Zanelli et al., 2014), but were quite similar to those previously described in human neonates by Watanabe and colleagues (1980, 1982, 1999).

**Hypoxia drives acute electrographic neonatal seizures in both Ha and HI models:**

**Implications for brain damage and acquired epileptogenesis**

The properties of the electrographic seizures observed during Ha and HI treatments were virtually identical. The treatment protocol for HI was exactly the same as for Ha, except that the right carotid artery was ligated in the HI group, which consistently led to an HI-induced encephalopathy with a frank infarct ipsilateral to the carotid ligation. The increased resistance to oxygen deprivation in immature animals can explain the lack of overt brain damage after Ha treatment in immature animals (Luhmann et al., 1993, 1997); however, in the HI animals, addition of the ischemia from unilateral ligation of the carotid artery causes severe brain injury. The robust electrographic seizures in the Ha-treated rats strongly suggest that the 2-hr 8%-
oxygen, hypoxic-gas treatment drives the acute seizures in both the Ha and HI models. The HI model used here was similar (although not identical) to the one used by Kadam and collaborators (2010), where all of the HI-treated rats had similar acute behavioral seizures during the HI-treatment protocol; however, only about 50% of the HI-treated rats developed epilepsy, and all of the rats with epilepsy were later shown to have had a clearly defined, macroscopic infarct. The other half of the animals did not develop epilepsy (documented by prolonged, continuous video-EEG), and they had no macroscopic lesions. Although these data suggested that neuronal death associated with the infarct was required for the development of epilepsy, another possibility was that the animals with infarcts also had more frequent and/or intense acute electrographic seizures during HI treatment than those without the infarct.

Because of modifications of the HI protocol in the present study, 91% (11/12) HI animals had a confirmed infarct and 0% of Ha (0/9) animals had an infarct. Therefore, the observation here that the acute electrographic seizures became less intense and frequent during the 2-hr hypoxia protocol in the HI group provides further evidence that neuronal damage - not the severity of the acute neonatal seizures - was the cause of the subsequent epilepsy. These data are consistent with clinical observations of Watanabe and coworkers (1980, 1982, 1999), who reported that rhythmic, high-amplitude delta and alpha events (i.e., seizures) did not predict clinical outcome in human neonates. A report by Glass and collaborators (2011) also supports this concept.

**Neonatal seizures and the development of brain damage**

The electrographic seizures progressively increased from the first to the second hour during Ha; however, although the electrographic seizures in HI were similar to Ha during the first hour, the seizures decreased in frequency and power during the second hour. These data suggest that the progressive and ongoing neuronal damage during HI slowly suppresses seizures over time, which may be due to waves of neuronal depolarization and/or necrotic loss of neurons. The question of whether ongoing neuronal damage during HI blunts the electrographic seizures is a separate issue from the question of whether the acute
electrographic seizures augment neuronal death. Previous work that compared HI-mediated
brain damage with versus without kainate-induced seizures reported that seizure activity
exacerbates neuronal damage (Wirrell et al., 2001). Thus, although neonatal seizures alone do
not cause overt neuronal death, seizures may worsen the neuronal damage associated with a
prior or ongoing brain insult.

**EEG background activity as a prognostic indicator for determination of outcome**

Because of technical difficulties recording from immature rodents, most studies on
neonatal seizures have used behavioral measures and older animals (Jensen et al., 1991; Koh
and Jensen, 2001; Koh et al., 2004; Raol et al., 2009; Aujla et al., 2009), and most
electrophysiological studies on epileptiform activity from neonatal animals have been conducted
in vitro with brain slices (Dzhala et al., 2005). Therefore, electrographic analyses of HI
encephalopathy derive primarily from the human clinical literature and from large-animal
models. Human clinical data strongly suggest that EEG background abnormalities are better
predictors of outcome than neonatal seizures (Monod et al., 1972; Tharp et al., 1981; Watanabe
et al., 1982; Pezzani et al., 1986; Takeuchi and Watanabe, 1989; Tharp et al, 1989; Shinnar et
al., 1990; Clancy and Legido, 1991; Legido et al., 1991; Holmes and Lombroso, 1993; Volpe,
2001, Korotchikova et al., 2011). Seizures that occur over suppressed EEG background tend to
be associated with negative outcomes that include epilepsy, cerebral palsy and intellectual
disabilities (Connell et al., 1989); when superimposed over normal background, seizure activity
has been associated with a positive outcome (Rowe et al., 1985; Scher et al., 1989, Volpe,
2001). Markedly depressed background EEG has been strongly associated with development of
West syndrome and subsequent epilepsy (Watanabe et al., 1980, 1982; Takeuchi and
Watanabe, 1987; Kato et al., 2010), consistent with the hypothesis that neonatal insults
associated with frank brain damage are more likely to lead to epilepsy.

**Localization of electrographic abnormalities**
One of the limitations of this study is the use of a single-channel EEG. Although more electrodes are always better than fewer electrodes, a single-channel recording from two appropriately placed electrodes can readily address the issues we raised in this manuscript. The single-channel approach does not provide information relevant to the spatial localization of seizure activity. Here, however, the spatial location of the injury was known and is similar between animals; thus, the single-channel approach was adequate to study changes in the electrical activity during HI-induced brain injury at the site with the HI-induced injury. The use of differential recording significantly reduced noise and signal artifacts, but also made interpretation difficult in relation to which area(s) generated the seizures and the depressed background. The one-channel approach described here has similarities to amplitude-integrated EEG, except that the analyses used here are more extensive and quantitative. Future research with miniature telemetry (Zayachkivsky et al., 2013) may facilitate studies on neonatal seizures versus suppression of EEG background in animal models.

**Relationships between brain damage and EEG**

These data point to the potential importance of EEG background suppression as a biomarker for neonatal brain injury, compared to seizures, which are often followed by normal neurological outcomes. The beginning of EEG background suppression and the initial decrease in alpha power may reflect reduction of phosphocreatine in the affected brain area (Vannucci, 1990), a possible biochemical marker for metabolic changes preceding neuronal degeneration, followed by a decrease in power of delta-band events. The onset of background suppression may potentially serve as a clinical intervention point that may be temporally superior to imaging. Collection and analysis of imaging data under clinical conditions requires more time than electrographic recordings of background suppression, which can be obtained and detected in minutes. Further work with more recording sites and better control of lesion size should shed light on the relationship between seizure activity, background suppression, and brain damage. The closest human clinical equivalent to pure hypoxia-induced events in rats would most likely
be hypocalcemia-induced seizures (Watanabe et al., 1982), where seizures did not lead to
negative outcomes when they occurred with normal background EEG.
References


Figure Legends

**Figure 1.** Hypoxia and hypoxia-ischemia (Ha/HI) treatment and recording protocol. *A,* Before the administration of Ha/HI, the rat pups were implanted with a miniature telemetry transmitter at P6 and allowed to recover overnight. On the next day, the right common carotid artery was cauterized (HI) or a sham surgery was performed (Ha and control animals). The hypoxia-gas mixture was administered to the Ha and HI groups (not the control group) after the rat pups were allowed 2 h for recovery. *B,* The EEG baseline was monitored for 30 min before administration of the 8%-oxygen hypoxic-gas mixture, and for 2 h during the Ha/HI treatment with the 8%-oxygen gas.

**Figure 2.** EEG activity during the treatment protocol for the hypoxia alone (Ha), hypoxia-ischemia (HI) and untreated-control groups. Prior to onset of administration of the hypoxic-gas mixture (red arrows), the Ha (*A*) and HI (*B*) animals had normal EEG patterns (Trace #1 in *A* and *B*). During the 2-h administration of the 8%-oxygen, hypoxic-gas mixture, Ha- and HI-treated animals had distinct and consistent patterns of EEG activity (see *A* and *B*, respectively). High-voltage, abnormal seizure-like activity with a normal background characterized the EEG of the Ha group (*A*), while the EEG in the HI-treated animals (*B*) contained high-voltage discharges *with suppressed background* (compare Traces #2 and #3 in *A* and *B*). *C,* Untreated controls had normal EEG background with some typical oscillatory discharges (see Traces #1 to #3). Therefore, unlike the control animals, both Ha- and HI-treated animals exhibited robust seizure activity; however, only the HI-treated animals showed *background suppression.*

Histological studies showed that only the HI animals had macroscopic lesions (see below).
Figure 3. High-amplitude EEG seizure-like discharges, recorded during either Ha or HI treatment, occurred as single-component or multi-component events. Two dominant types of seizure-like discharges, which could be detected on the basis of power in two specific frequency bands (alpha [8-13 Hz] and delta [0.1 to 2 Hz]), occurred as single or isolated events (A) and or as events with two or more distinct components (B). The seizure-like discharges (1) with power in the alpha band were associated with tonic-like seizures (i.e., shivering) (A, 2) and (2) with delta-band power correlated with traditional convulsive seizures (B, 2). Band-pass filtering of the EEG discharges separated the two seizure types (B). When the signal was filtered using a Blackman band-pass filter in the frequency ranges of 0.1-4 Hz and 8-12 Hz, the two seizure types could be separated. Power in the two different bands was computed and plotted in 3-sec bins. Total number of abnormal events during the first and second hours of administration of Ha and HI (C). Bar graph shows the average number of abnormal events in delta and alpha frequency bands during the first and second hours of treatment.

Figure 4. Comparison of the frequency-domain profile of Ha, HI and untreated controls during the first and second hours of treatment. Power spectral density (PSD) profiles of the raw EEG power for the three treatment groups were averaged and plotted with 95% confidence intervals (actual data in A1 and B1; normalized data in A2 and B2). During the first hour of treatment (A), both Ha and HI had the greatest increase in power in the alpha frequency band (8-13 Hz). During the second hour (B), overall power was still substantially increased above controls (n=6) in both groups, but the increase was less for the HI (n=12) group than the Ha (n=9) group. Thus, the power in the two seizure-related bands, particularly in the alpha band, was substantially lower for HI than for the Ha group during the second hour of hypoxic-gas (i.e., 8% oxygen).
Figure 5. Separation and analysis of the EEG events. A, With visual analysis of the EEG traces, the seizure-like events could be readily classified and separated from background activity. The seizures were categorized based on their frequency band (delta, 0.1-4 Hz; alpha, 8-12 Hz). The time-of-onset of each seizure or inter-seizure interval (i.e., the background EEG) was used as a basis for analysis in the temporal domain. Temporal expansions of the EEG records are shown in panels B.

Figure 6. Temporal distribution of RMS power of the seizure events during administration of Ha and HI. Each seizure event was plotted based on the time of the start of each event and its measured intensity as RMS power (closed circles). Time was then divided into 10-min bins and the means of each bin were connected to form the line on each graph. A, When all of the seizure events were grouped together, the temporal profiles for Ha and HI were similar until about 90 min after the start of the treatment protocol. B, For clarity, the group data have been re-plotted to show mean values and standard error of the mean (see also D and F). C, D, Events in the delta frequency band appeared to dominate this profile for “all seizures” (shown in A). E,F, Alpha-event power showed a significant decrease in HI animals compared to Ha animals (p=0.002), as early as 20 min after the start of the treatment protocol. A single alpha event occurred during the last two binning periods in the HI group (E), thus the plotted mean is absent from the graph in last two bins (F).

Figure 7. Temporal distribution of the background RMS power during administration of Ha and HI. RMS power of background EEG (i.e., between seizures = interictal periods) was plotted based on the start time of each event and the corresponding RMS power. A, Time was then divided into 5-min bins, and the mean values of each bin were plotted as a line on the graph. The power of the EEG background in Ha-treated animals was significantly higher than in HI animals during the first (p<0.001) and second (p<0.001) hours. B, As in the previous figure, the
mean values and the standard errors of the mean of the RMS power for each 5-min bin were plotted as a function of time, which more clearly illustrates the difference between Ha- and HI-treated animals. The decrease of background power occurred as early as 20-30 min after the onset of the treatment.

Figure 8. Typical lesion in the HI-treated animal compared to an animal treated with Ha. No lesion was apparent after Ha treatment (A,B), while HI-treated animals had a macroscopically identifiable lesion 96 h after the insult (C,D). In addition to the lack of any apparent lesions, the Ha-treated animals had no Flouro-Jade B positive cells (B). Flouro-Jade B stained degenerating neurons were clearly apparent inside the lesion of HI-treated animals (D).
A. Ha/HI administration

- Telemetry implantation
- Recovery 20-24 h
- Carotid cauterized
- Recovery 2 h
- 8% oxygen for 2 h

P6  P7

Age

B. Recording

- 30 min Baseline
- 120 min Ha/HI administration
A  Hypoxia (Ha)

B  Hypoxia-ischemia (HI)

C  Untreated
A. ALL Seizures

B. ALL Seizures

C. Delta Events

D. Delta Events

E. Alpha Events

F. Alpha Events

Hypoxia (Ha)

Hypoxia-ischemia (HI)
A  |  Background

RMS Power  vs  Time (h)

Hypoxia (Ha)
Hypoxia-ischemia (HI)

B  |  Background

RMS Power  vs  Time (h)