Neurobiology of Disease 116 (2018) 1–12

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Sleep dysfunction following neonatal ischemic seizures are differential by neonatal age of insult as determined by qEEG in a mouse model

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ARTICLE INFO

Keywords:
Hypoxic-ischemic encephalopathy
Ischemia
Neonatal seizures
Hyperactivity
Sleep structure
Delta power

ABSTRACT

Neonatal seizures associated with hypoxic-ischemic encephalopathy (HIE) pose a challenge in their acute clinical management and are often followed by long-term neurological consequences. We used a newly characterized CD-1 mouse model of neonatal ischemic seizures associated with age-dependent (P7 vs. P10) seizure severity and phenobarbital efficacy (i.e.; PB-resistant vs. PB-efficacious respectively) following unilateral carotid ligation. The long-term consequences following untreated neonatal seizures in P7 vs. P10 ligated pups were investigated using neurobehavioral testing, 24 h v- quantitative EEG -EMG (qEEG, qEMG), and western blot analyses in adult mice. Significant hyperactivity emerged in a small sub-set of mice in both age-groups associated with a failure to habituate during open-field (OF) testing. 24 h continuous qEEGs detected significantly altered sleep architecture due to long-wake cycles in both age-groups. Delta power (0.5–4 Hz) quantification during slow-wave-sleep (SWS) revealed significant SWS compensation in P10 ligates following periods of increased sleep pressure which the P7 ligate group failed to show. Theta/beta ratios deemed as negative correlation markers of attentional control were significantly higher only in the P10 ligates. These results indicate that neonatal age-dependent differences in the characteristics of ischemic neonatal seizures in CD-1 pups differentially modulate long-term outcomes, when evaluated with v-qEEG/EMG as adults.

1. Introduction

Neonatal seizures affect 1.5-3/1000 newborns and result in long-term neurological consequences (Volpe, 2001). Hypoxic-ischemic encephalopathy (HIE) is the predominant underlying etiology of neonatal seizures, with adverse long-term outcomes such as epilepsy, cerebral palsy, developmental delay, and intellectual disabilities (Pisani and Spagnoli, 2016;Tekgul et al., 2006;Vasudevan and Levene, 2013).

The roles of etiology and acute seizure severity on long-term outcomes of neonatal seizures have been debated clinically (Glass et al., 2011;Kwon et al., 2011). At 5 years of age, patients with mild HIE, graded by early EEG and clinical assessment, have significantly lower full-scale IQ, verbal IQ, and performance IQ than their peers (Murray et al., 2016). Neonates with a total electrographic seizure burden of > 40 min had a nine-fold increase in odds of neurological consequences (Kharoshankaya et al., 2016). More severe electrographic seizure burdens in HIE are significantly associated with abnormal outcomes. Few animal model studies have been done to determine the factors responsible for the long-term co-morbidities of neonatal seizures (Kang and Kadam, 2015). Even fewer use EEG to determine seizure burdens at both the acute and chronic stages.

Using electro-clinical, molecular, and behavioral assays in C57BL/6J P7 pups, (Rodriguez-Alvarez et al., 2015) showed increased long-term seizure susceptibility but minimal injury and no spatial memory deficits. Similarly, a model of perinatal hypoxia/ischemia in Sprague Dawley rat pups has shown chronically progressive epilepsy associated exclusively with injury (Kadam and Dudek, 2007). We have previously characterized a mouse model of neonatal ischemic seizures in CD-1 pups, reporting a significant age-dependent difference in acute seizure severity and phenobarbital refractoriness to the same ischemic insult (Kang et al., 2015). In contrast to chemoconvulsant models including kainic acid, this new model of neonatal ischemic seizures shows electrographic seizure burdens and brain injury profiles similar to those reported in HIE, highlighting clinical relevance (Kharoshankaya et al.,

Abbreviations: EEG, electroencephalogram; EMG, electromyogram; PB, phenobarbital

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https://doi.org/10.1016/j.nbd.2018.04.012
Received 16 January 2018; Received in revised form 10 April 2018; Accepted 18 April 2018
Available online 21 April 2018
0969-9961/

2016;Kang and Kadam, 2014). In the current study, the long-term comorbidities of hypoxic/ischemic insult were investigated using standard neurobehavioral tests and chronic 24 h v-qEEG/EMGs to investigate: 1) the long-term developmental consequences of untreated neonatal ischemic seizures at two distinct developmental ages (i.e.; P7 vs. P10); 2) the use of acute seizure parameters to predict acute and long-term outcomes; and 3) qEEG biomarkers identifying long-term neurophysiological outcomes by neonatal age of ischemic insult.

2. Materials and methods

2.1. Animals and acute surgical procedures

All animal care and procedures were carried out in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and the protocols used in this study were approved by the Committee on the Ethics of Animal Experiments of the Johns Hopkins University. All surgical procedures were performed under isoflurane anesthesia. All litters of CD-1 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA) with newly born litters of pups ($n = 10$; equal number for both sexes) arriving on postnatal day (PND) 3 day. Pups were allowed to acclimate in polycarbonate cages on a 12 h light-dark cycle. Food was provided ad libitum (see Table 1 for sample size and sex distribution and Fig. 1A for timelines).

2.1.1. Surgical procedure for ischemic insult and in-vivo synchronous video-EEG recording

Briefly, pups were anesthetized with 4% isoflurane for induction and maintained at 1.5% as previously described (Kang et al., 2015). Neonatal ischemia was induced by permanent double-ligation of the right common carotid artery. Sham-controls were treated identically except for the carotid ligation. Three sub-dermal EEG silver electrodes (1 recording, 1 reference, 1 ground) were implanted on the pups' skull overlying the parietal cortex using bregma as a reference. A third ground electrode was implanted over the rostrum. The electrodes (IVES EEG; Model # SWE-L25 – IVES EEG solutions, MA, USA) were fixed with minimal cyanoacrylate adhesive (KrazyGlue). Pups were allowed to recover from the anesthesia in a 36 °C isothermal chamber followed by continuous 3 h recordings of video-EEG. EEG and video data were acquired using Sirenia Acquisition software (Pinnacle Technology Inc.). Raw EEG traces with synchronous EMG and video were used to manually score behavioral state transitions within Sirenia (i.e.; sleep score module), blind to the treatment group identity. A seizure was defined as epileptiform discharges lasting ≥6 s. At the end of the recording session and following removal of the electrodes and application of topical anesthetic, the pups were returned to the dams. Pups were monitored daily for the next week to ensure post-surgery recovery.

2.1.2. Experimental design

Equal number of pups of both sexes from each litter ($n = 10$ each) at both P7 and P10 were randomly assigned to one of the two experimental groups: 1) sham: naïve; 2) ligation control. During the neonatal ligation, pups underwent permanent toe-clipping under anesthesia for all future identification for each litter. Experimenters used toe-clip identity only and remained blind to treatment category for both long-term behavioral studies and EEG recordings as adults. Experimenters scoring and analyzing qEEG were similarly blinded.

Table 1
Sample size by age and sex.

	Control (♂/♀)	Ligated (♂/♀)	Total (♂/♀)
P7	9 (4/5)	10 (5/5)	19 (9/10)
P10	10 (5/5)	10 (5/5)	20 (10/10)
Total	19 (9/10)	20 (10/10)	39 (19/20)

2.1.3. Animal housing

At PND 21–23, mice were weaned from the dam and housed 4–5 mice per cage, separated by sex. Housing was in polycarbonate cages on a 12 h light-dark cycle and food was provided ad libitum as before. After head mount implantation surgery at P120, animals were single-housed along with piece of bedding from its home cage and a water-gel pack to prevent dehydration during recovery. Mice were monitored daily for a week to ensure satisfactory post-surgery recovery.

2.2. Behavior tests

All procedures were conducted with minimal handling under strict supervision of the Behavior Core at the Brain Science Institute, Johns Hopkins University. Adult mice underwent testing at ages PND 80 ± 4 or 130 ± 4. Mice were habituated daily for 5 min prior to the initiation of behavior tests. Habituation period and the behavioral tests covered a period of two weeks, with at least 48 h breaks in between tests to minimize stress. All experimental groups went through the same tests, which were conducted during daylight hours (8 AM to 6 PM), in the same sequence: for P80 open-field, Y-maze, EPM, social interaction, and PPI; for P130, open-field, social interaction, and PPI. Each session was run in a sex-matched manner, and no cross-sex interaction or presence in same space occurred. Minimal standardization for the mouse cohorts other than age were employed (i.e.; body weight etc.) for the behavior tests conducted in this study, since heterogenization within experiments has been purported to improve reproducibility among experiments (Richter et al., 2010).

2.2.1. Open-field (OF) test

All testing procedures were carried out in a square PAS-Open field station (16"W × 16"D × 15H; San Diego Instruments, CA) equipped with photo-beam configuration. Data was acquired through PAS software based on the grid of invisible infrared light beams (16 × 16) set on the four sides of the walls of the station. Each station was air/humidity controlled and had ambient light. Mice were placed at the center of the station and remained in the enclosure for 25 min while the mice were allowed to explore the field freely. The station was cleaned with 70% ethanol after completion of each session. Total activity levels for the entire duration (25 min) and the 5 min habituation interval were quantitated. Each mouse underwent OF test session twice: once at PND 80 and again at PND 130.

2.2.1.1. Identification of hyperactive mice by OF. Identification of hyperactivity was based on beam breaks in OF test, and thus defined as the animals which met Rosner's Extreme Studentized Deviate test for multiple outliers (two-sided test, significance level set at 0.00001, maximum number of outliers allowed set at 3 out of total 39); increasing the maximum number of outliers allowed to 10 did not identify any other animals as an outlier. Further statistical justification was provided by Shapiro-Wilks test, a goodness-of-the-fit test allowing the comparison of sample distributions pre- and post-outlier removal. Inclusion of the three hyperactive animals resulted in a $p < 0.001$ by Shapiro-Wilks test, indicating the non-normal distribution of those 3 mice. In contrast, the same test justified the normal distribution (Shapiro-Wilks test, $p = 0.602$), after the exclusion of 3 hyperactive mice, and thus the identification of hyperactive animals as a cohort.

2.2.2. Y-maze

Y-maze testing for spatial memory consisted of two sessions. For the first session, mice were placed in a Y-shaped apparatus at one arm and were allowed to explore each arm. The sequence in which it navigated each arm was recorded, to examine if it preferred a new arm over the one it just exited from. Three days later, a different Y-shaped apparatus was used to conduct the second session. Mice were allowed to habituate