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Impact of age and strain on ischemic brain injury and seizures after carotid ligation in immature mice

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ABSTRACT

Stroke is an important cause of neurologic injury in the neonatal period and frequently results in lifelong neurologic impairments. We reported previously that unilateral carotid ligation on postnatal day (P)12 in CD1 mice causes acute behavioral seizures and unilateral brain injury and provides a model for neonatal stroke in human infants. In the present study we confirmed that behavioral seizures observed after ligation on P12 in the CD1 strain are associated with rhythmic ictal discharges that show temporal progression on electrocorticograms. We also examined the effects of carotid ligation performed at different ages in CD1 mice or performed in the C57Bl/6 strain. The right common carotid was ligated at P7, P10, P12 or P21 in CD1 mice or at P12 in C57Bl/6 mice. Littermate controls received sham surgery. Seizures were rated for 4 h after surgery; brain injury was scored one week later. In a separate group of P12 CD1 mice, electrocorticographic activity was recorded continuously for 4 h after carotid ligation or sham surgery. Brain injury and cumulative seizure score varied significantly with age ($p < 0.001$) and strain ($p < 0.001$). In CD1 mice, injury was greatest after ligation on P10 to P12 and seizure score was maximal at P12. Seizure scores were significantly correlated with injury after ligation on P10 or P12. C57Bl/6 mice, like C3Heb/FeJ mice examined previously, were much less vulnerable to seizures and injury than CD1 mice after ligation on P12. This study demonstrates that carotid ligation in the CD1 mouse on P12 causes acute electrographic rhythmic discharges that correlate with behavioral seizures. We also found that the age at which ligation is performed and genetic strain have a strong influence on the severity of injury.

1. Introduction

Stroke is a significant cause of neurologic morbidity in neonates (approximately 1 in 4000 term births) (Lynch et al., 2002; Lynch and Nelson, 2001), infants, and children (approximately 8 cases per 100,000 children per year) (deVeber, 2002). Neonatal stroke, pediatric arterial stroke and pediatric cerebral sinovenous thrombosis often present with acute seizures (Lynch and Nelson, 2001; deVeber et al., 2000; Azam, 1998). Approximately 75% of pediatric stroke survivors have permanent neurologic deficits including hemiparesis, epilepsy, learning disabilities, visual-field deficits, and mental retardation (Koelfen et al., 1995), and those that present with acute seizures are at increased risk for poor functional outcome (Delsing et al., 2001).

Mouse models of postnatal stroke are of particular interest for investigating mechanisms of ischemic injury, neuroprotection or neuroregeneration, and for evaluating genetic or cell-based interventions. Stroke injuries are dynamic pathophysiological processes, in which the initial ischemic insult can trigger glutamate release and a cascade of biochemical reactions that lead to seizures and energy failure. Membrane depolarization, overactivation of NMDA receptors, and stimulation of voltage-sensitive ion channels lead to excessive intracellular calcium accumulation and eventually cell death (Johnston et al., 2001).

We previously observed that after unilateral carotid artery ligation under normoxic conditions, postnatal day (P)12 CD1 mice exhibit seizure-related behavior and brain injury (Comi et al., 2004). Brain injury was moderate to severe and involved the cerebral

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Abbreviations: P, postnatal day; ECoG, electrocorticogram; NMDA, *N*-methyl-D-aspartate.

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cortex, hippocampus, striatum, and thalamus. Seizure severity was strongly correlated with the extent of brain injury. Furthermore, we have shown that vulnerability to ischemic seizures and injury is less in P12 C3HeB/FeJ mice than in P12 CD1 mice (Comi et al., 2005).

Based on prior reports of age and strain-related differences in vulnerability to hypoxic-ischemic insult in rats and mice (Sheldon et al., 1998; Towfighi et al., 1997), we expected similar differences in vulnerability in this unilateral carotid ligation model of stroke in the developing brain. We report here the impact of unilateral right common carotid ligation in P7, P10 and P21 CD1 mice as well as in P12 C57Bl/6 mice and compare these findings to our previous observations in P12 CD1 and C3HeB/FeJ mice. We also determined the electrophysiological correlates of acute seizure behavior in this model by examining electrocorticographic (ECoG) activity after unilateral carotid ligation on P12.

2. Experimental procedures

2.1. Carotid ligation

Unilateral right common carotid ligation or sham surgery (comparable anesthesia time with skin incision but no artery dissection or ligation) was carried out under isofluorane anesthesia (4% induction, 1.2% maintenance) in P7 CD1 (n = 25 ligated and 5 shams from 3 litters), P10 CD1 (n = 23 ligated and 7 shams from 3 litters), and P21 CD1 mice (n = 25 ligated and 5 shams from 3 litters), and in P12 C57Bl/6 mice (n = 20 ligated and 2 shams from 3 litters; day of birth = P1). In each litter, 1 or 2 animals were selected randomly at the time of surgery as shams. A 2% solution of lidocaine was applied to the surgical site at the time of suturing. Mice were immediately placed in an incubator at 35 °C. Previously reported data for P12 CD1 mice (n = 28 ligated and 5 shams from 3 litters) and for P12 C3HeB/FeJ mice (n = 22 ligated and 3 shams from 3 litters) (Comi et al., 2004; Comi et al., 2005) are included here for direct comparison to the new data.

2.2. Seizure rating

Observers unaware of ligation status scored seizure activity using a seizure rating scale for mice described previously (Comi et al., 2004). The observers assessed the behavioral features characteristic of seizures continuously for 4 h after surgery. Every 5 min, each animal was assigned a score for the highest level of seizure activity observed during that interval (0 = normal behavior, 1 = immobility, 2 = rigid posture, 3 = repetitive scratching, circling or head bobbing, 4 = forelimb clonus, rearing and falling, 5 = mice that exhibited level four behavior repeatedly, and 6 = severe tonic-clonic behavior). At the end of the 4 h observation period, pups were returned to the cage with their respective dam and the 5-min interval scores were summed to obtain a cumulative seizure score. One week later, mice were anesthetized with chloral hydrate and perfused with 4% formaldehyde.

This protocol was approved by the Johns Hopkins University Animal Care and Use Committee, in compliance with local, national and international standards on animal welfare.

2.3. Histopathologic evaluation of brain injury

Neuropathologic injury was examined in coronal brain sections stained with cresyl violet. Two independent assessments of brain injury were made, and the average of the two scores was assigned as the brain injury score, as previously described (Nakajima et al., 2000), with minor modifications (Hagberg et al., 2004). Injury was scored from 0 to 4 for cortex (0: no injury, 1: one to three small groups of injured cells, 2: one to several larger groups of injured cells, 3: moderate confluent infarction, 4: extensive confluent infarction) and 0 to 6 for hippocampus, striatum, and thalamus (0–3 for no, mild, moderate or extensive infarction and 0–3 for no, mild, moderate or extensive atrophy). Regional scores were summed to obtain a total brain injury score for each subject, which ranged from 0 to 22.

Kruskal–Wallace analysis with Dunn's multiple comparison test was used to determine the effect of age or strain upon brain injury or seizure score. Data from prior studies of P12 CD1 and C3Heb/FeJ mice (Comi et al., 2004; Comi et al., 2005) were included in the analyses for comparison with the mice in the present study. Non-parametric regression was used to examine the relationship between seizure score and brain injury score. Chi-square or Fisher's exact test was used to compare the percent injured in each group. p values less than 0.05 were considered significant.

2.4. Electrocorticographic recording

Unilateral common carotid artery ligation (n = 8) or sham surgery (n = 2) was performed under isoflurane anesthesia in CD1 mice at P12. Immediately after ligation, stainless steel electrodes (Plastics One, Inc., Roanoke, VA) were placed in parietal cortex 2 mm lateral to the midline on each side, secured by cyanoacrylate adhesive. A third electrode was placed in the cerebellum to serve as ground. The animals recovered from anesthesia in an incubator at 35 °C for approximately 20 min. ECoG signal was then recorded continuously for 4 h, using Grass-Telefactor data acquisition software (PolyView v2.5, Astro-Med, West Warwick, RI), with analog signal conditioning (500-fold amplification and band-pass filtering at 0.3–70 Hz) prior to digital conversion and storage for offline analysis. Behavior was continuously observed and video recorded, noting the time and score for any seizure behavior as described above.

Analysis of ECoG data was performed using Reviewer, a data review module of the PolyView software. The ECoG signal was low-pass filtered using a second-order Butterworth filter with a 30 Hz cutoff frequency to eliminate environmental artifact and 60 Hz noise. The ECoG recordings were visually inspected, and episodes of rhythmic discharges were counted and analyzed to determine discharge rate. We also examined the temporal correlation between behavioral seizure scores and electrocorticographic episodes of rhythmic discharges.

3. Results

3.1. Electrocorticographic activity after unilateral carotid ligation

Electrocorticographic recordings performed in eight P12 CD1 mice after unilateral carotid ligation revealed rhythmic discharges in 7 of these animals. Six out of 8 ligated mice (No. 1, 2, 4, 5, 6 and 7) displayed paroxysmal rhythmic discharges during the first hour of ECoG recording (which began approximately 20 min after surgery), mice 1 and 6 displayed such activity during the second hour, mice 2, 5 and 6 during the third hour, and mice 6 and 8 showed similar patterns during the fourth hour. The number of recorded episodes of electrographic ictal activity per animal ranged from 3 to 12, and the episode duration ranged from 10 to 400 s. The ictal discharge rate ranged from 1 to 4 per second. No such activity was observed in the 2 non-ligated control mice. One of the ligated mice was withdrawn at the end of the second hour of recording due to dislodged electrodes; another died during the second hour of recording.

ECoG activity was recorded for 4 h in the remaining 6 ligated mice and 2 controls. During some portions of the recordings, when repetitive circling or tonic-clonic seizure behavior occurred, motion artifact obscured ECoG data. Immediately before and after such behaviors, however, the mice were immobile, often with the forelegs extended and the head turned to the right. Paroxysmal rhythmic electrophysiological discharges were observed during these periods of relative inactivity. In Fig. 1, a typical 20 s ECoG recording in a sham P12 mouse (a) is compared with the temporal evolution of rhythmic discharges before and during an epoch of behavioral seizure activity in a ligated mouse (b). This animal had 7 behavioral seizure episodes within the 4 h recording period; seizure scores during these episodes ranged from 3 to 6. Most lasted from 4 to 9 min, but the final episode was prolonged, lasting 35 min. The behavioral seizure episodes were separated by quiet intervals lasting 30 to 60 min. One such episode began 3 h 19 min after the beginning of the recording, during normal quiet behavior; during this episode the recording shown in Fig. 1b was obtained. The behavioral seizure episode began with twitches every 3 to 4 s for 33 s, followed by a 29 s period of circling behavior and then a generalized tonic clonic episode that lasted nearly a minute. ECoG activity was then suppressed for 12 s; the record in Fig. 1b begins at that point. During a period of immobile behavior we observed evolution of the ECoG rhythmic discharge activity for more than a minute before the animal entered another tonic clonic episode that lasted 50 s (beginning at the asterisk at 3:22:26). At the end of this tonic clonic episode (3:23:15), ECoG activity was again suppressed.

3.2. Effect of age and strain on neuropathologic score

Overall, 132 of 143 ligated animals survived to perfusion for analysis of injury, and the mortality rate was not significantly different among the age and strain groups (Table 1). All sham