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Strain Variability, Injury Distribution, and Seizure Onset in a Mouse Model of Stroke in the Immature Brain

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Ischemia · Mouse pup · Seizures · Strain · Brain injury

Abstract
Neonatal stroke is an important cause of neurologic morbidity and cerebral palsy. Recently, we have determined that in postnatal day 12 CD1 mice unilateral carotid ligation alone results in seizures and brain injury. We have shown that, in this model, seizure scores correlate with brain injury scores. We have applied this model to another strain of mice to assess strain-related differences in vulnerability to seizures and brain injury after unilateral carotid ligation. Under isoflurane anesthesia, unilateral right-sided carotid ligation was performed in postnatal day 12 C3HeB/FeJ mice followed by a 4-hour period of observation in a 35°C incubator. Seizure scores and brain jury scores were assigned and compared to scores in mice receiving sham surgery. Timing of seizure onset and regional distribution of brain injury were compared in the CD1 and C3HeB/FeJ mice. Unilateral carotid ligation in postnatal day 12 C3HeB/FeJ mice resulted in seizure behavior and brain injury in some animals, with similar time to seizure onset and regional injury distribution, but affected a significantly smaller percentage of C3HeB/FeJ pups than that observed in postnatal day 12 CD1 mice, indicating strain-related vulnerability in this model.

Introduction

Cerebral palsy has been broadly defined as 'a nonprogressive motor impairment syndrome caused by a problem in the developing brain' and affects at least 2 in 1,000 children in the United States [1]. About 33% of children with cerebral palsy have the hemiplegic form affecting the motor functions of one side of the body. In many of these children, hemiplegic cerebral palsy results from a stroke in the pre-, peri-, or postnatal period [2]. Neonatal stroke (defined as stroke in the first 30 days of life) occurs in approximately 1 per 4,000 term births [3]. Stroke in children (age >30 days) is also an important cause of neurologic morbidity with an estimated incidence of 8 per 100,000 children [4]. Although most survive their stroke, the majority of these children have sequelae including cerebral palsy. Stroke is one of the major causes of cerebral palsy.

Cerebral ischemia triggers the release of the excitatory neurotransmitter glutamate [5], and energy uncoupling produced by ischemia prevents the active removal of glutamate from the synaptic cleft [6]. Excess glutamate overstimulates postsynaptic receptors, thereby opening the associated ion channels. This allows sodium and calcium ions to enter the cell, while potassium flows outward [7]. Excessive calcium influx initiates a cascade of energy-consuming and ultimately lethal intracellular events. Cell death produced in this fashion is a primary mechanism

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of injury in the developing brain after ischemia and is referred to as excitotoxicity.

Various rodent and murine models have been developed for the study of ischemic, hypoxic, and hypoxic-ischemic brain injury [8–11]. In immature rats, unilateral carotid ligation alone does not produce brain injury and must be coupled with a period of hypoxia in order to produce brain injury [12]. Recent studies in postnatal day 7 (P7) rats have utilized either transient or permanent middle cerebral artery occlusion to study neonatal stroke. Prior studies in immature mice have combined unilateral carotid ligation with a period of hypoxia to produce hypoxic-ischemic brain injury [13–17]. Carotid ligation alone in adult gerbils is known to produce injury in about 35% of the animals [18].

Developmental mouse models of postnatal stroke are relevant for addressing the mechanisms of ischemic injury, neuroprotection, and regeneration. Mouse models are of special interest because in addition to their utility for pharmacologic studies, mice with selective gene deletions are available. Transgenic mice currently have a large role in stroke research. Expression of numerous genes has been shown to be induced by cerebral ischemia, including the heat shock protein hsp72 [19], antioxidants [20], JNK3 and the immediate early genes [21], and HIF-1α [22]. Transgenic mice are being used to investigate ischemia-induced responses in the brain, to clarify the mechanisms of neurodegeneration, and to identify targets for specific modulation of these cellular responses. However, several studies in various murine models of ischemia have emphasized the critical need to understand the relative susceptibilities of different mouse strains.

We have recently shown that P12 CD1 mice frequently exhibit seizure activity and brain injury after unilateral carotid ligation and that cumulative seizure score is highly correlated with brain injury score in this model [23]. No hypoxia was required to produce brain injury. Seizure behavior was observed in 75% of the animals after carotid ligation, with cumulative seizure scores that ranged from 0 to 116. Gross brain injury was noted in 71% of the ligated animals. Brain injury was seen in the cerebral cortex, striatum, hippocampus, and thalamus, and brain injury scores ranged from 0 to 22 out of a maximal score of 22. There was a positive correlation between seizure score and brain injury score (Spearman rank correlation = 0.835, $p < 0.001$), and this correlation was unchanged when the ligated animals were analyzed by sex.

The advantages of this unilateral carotid ligation mouse model of stroke in the developing brain include (1) the lack of requirement for hypoxia to produce injury; (2) the relative ease of carotid ligation compared to middle cerebral artery occlusion; (3) the occurrence of seizures, which is also a common phenomenon in pediatric stroke, and (4) the ability to utilize knockout mice to address questions of mechanism. One interesting feature of this model is that, in P12 CD1 mice, seizures in the first 4 h after injury correlate with brain injury 7 days later. We can therefore predict which animals will be significantly injured and this may be helpful in designing experiments to address mechanisms of injury and neuroprotection. We predicted that important strain-related vulnerabilities are likely present in this unilateral carotid ligation model of stroke in the developing brain. Here, we report the results of carotid ligations performed in P12 C3HeB/FeJ mice, compare the distribution of injury and time to onset of seizures in CD1 and C3HeB/FeJ mice, and discuss the implications of strain-related differences in mouse models of ischemia.

Experimental Procedures

This protocol was approved by the Johns Hopkins University Animal Care and Use Committee.

Seizure and Brain Injury Rating Studies

Unilateral carotid ligations were carried out as previously described [23] under isoflurane anesthesia in P12 C3HeB/FeJ mice (n = 22 from three litters of mice); littermates (n = 3, respectively) received sham surgeries. Mice were immediately placed in an incubator at 35°C. Seizure activity was scored by an observer blinded to ligation status, using a seizure rating scale for mice described by Morrison et al. [24]. Behavioral features characteristic of seizures were assessed continuously; every 5 min, the animals were assigned a score for the highest level of seizure activity observed during that period: 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-hour observation period, pups were returned to the cage with the dam, and the 5-min interval scores were summed to produce a cumulative seizure score.

One week later, mice were anesthetized with chloral hydrate and perfused with 4% formaldehyde. Gross brain injury was noted where present. Neuropathologic injury was examined in sections stained with cresyl violet. Two independent, blinded assessments of brain injury were made and the average of the two scores was assigned as the brain injury score, as previously described [25]. Injury was scored from 0 to 4 for the cortex (0 = no injury, 1 = 1–3 small groups of injured cells, 2 = 1 to several larger groups of injured cells, 3 = moderate confluent infarction, 4 = extensive confluent infarction) and 0–6 for the hippocampus, striatum, and thalamus (0–3 for no, mild, moderate, or extensive infarction and 0–3 for no, mild, moderate, or extensive atrophy); total score therefore ranged from 0 to 22. The average of the two investigators' scores was used for statistical analysis.

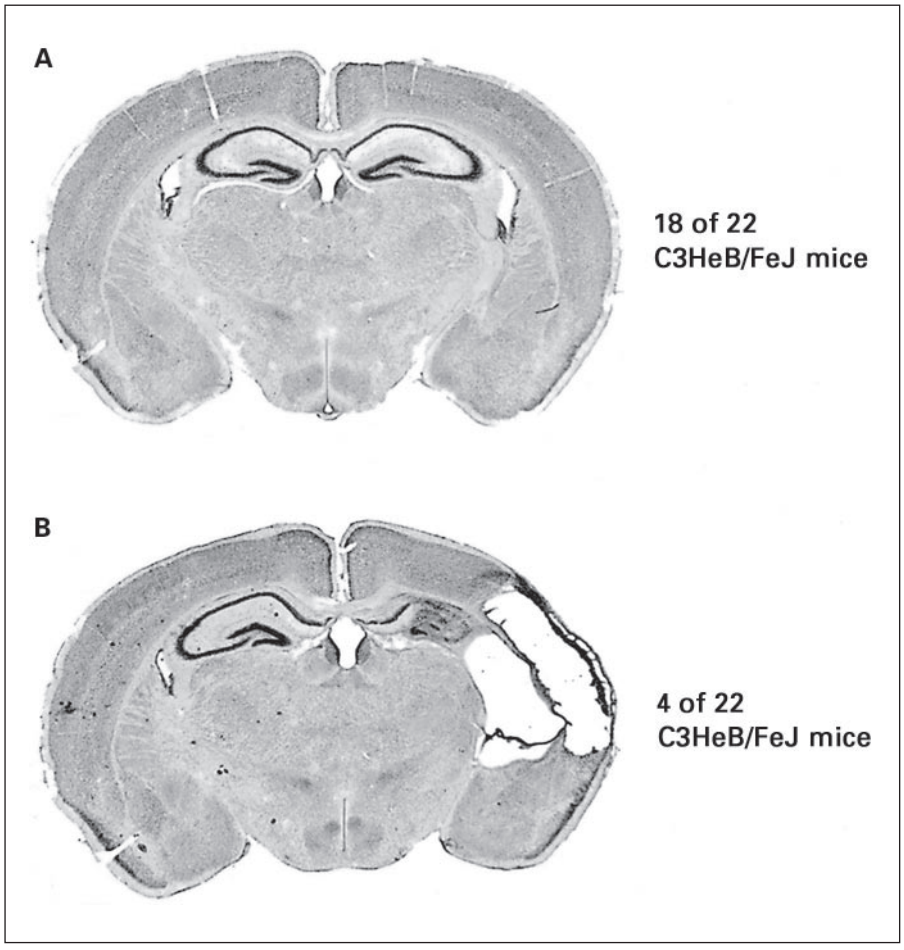

Fig. 1. Brain injury after carotid ligation in C3HeB/FeJ mice at P12. **A** Uninjured brain section at the level of the hippocampus. Eighty-two percent of the C3HeB/FeJ mice had no demonstrable injury at 7 days after ligation. **B** Brain section demonstrating brain injury in the cortex, hippocampus, and striatum typical of the 18% of P12 C3HeB/FeJ mice that were injured after unilateral carotid ligation.

Data Analysis

Nonparametric regression (Spearman rank correlation) was used to examine the relationship between seizure score rank and brain injury score rank. Animals that died prior to 7 days after ligation are reported in mortality figures, but were not included in any of the brain injury analyses. We compared data previously collected from P12 CD1 mice to those obtained in C3HeB/FeJ mice after right-sided carotid ligation. Mortality and the proportion of animals injured were compared using the Pearson χ^2. Brain injury score, seizure score, time to onset of seizures, and regional distribution (cortex, hippocampus, striatum, and thalamus) of brain injury were compared. Nonparametric statistical comparison (Kolmogorov-Smirnov Z test) was carried out using SPSS 12.0 for Windows (SPSS, Inc., Chicago, Ill., USA).

Results

In the P12 C3HeB/FeJ mice, gross brain injury was observed in 4 of 22 (18%) at 7 days (fig. 1, A = uninjured animal, B = brain injury in P12 C3HeB/FeJ mouse after unilateral carotid ligation). Brain injury scores ranged from 0 to 19.5 (table 1). One animal died 1 day after ligation. No injury was found in any of the C3HeB/FeJ sham-surgery animals. Comparing the overall median brain injury score and regional brain injury for the two strains, we found both to be significantly lower ($p < 0.01$, table 1) overall and in all four regions (cortex, hippocampus, striatum, and thalamus); this was due to the significantly decreased frequency of injury in the C3HeB/FeJ mice. However, in the small number of C3HeB/FeJ animals found to have brain injury, the regional distribution of brain injury was comparable to that observed previously in the CD1 mice.

The seizure score in the C3HeB/FeJ mice was found to correlate with the brain injury score (Spearman's rank correlation = 0.527, $p < 0.008$) although the correlation was weaker than that previously reported in CD1 mice (see Introduction). In the C3HeB/FeJ mice that demonstrated seizures, we observed a progression of seizure be-

Table 1. Comparison of strains

	C3HeB/FeJ mice (n = 22)	CD1 mice (n = 28)	Significance
Age at ligation	P12	P12	
Male, %	50	54	n.s.
Mortality, %	5	14	n.s.
Percent of animals that were injured	18	71	$p < 0.01$
Median brain injury score	0 (0–19.5)	13 (0–22)	$p < 0.001$
Median cortex injury score	0 (0–4)	3.5 (0–4)	$p < 0.01$
Median hippocampus injury score	0 (0–6)	5.0 (0–6)	$p < 0.01$
Median striatum injury score	0 (0–6)	2.5 (0–6)	$p < 0.01$
Median thalamus injury score	0 (0–3.5)	2.0 (0–6)	$p < 0.01$
Median seizure score	3.0 (0–36)	14.5 (0–116)	$p < 0.001$
Median time to seizure for animals with positive score, min	167.5 (30–235)	70 (10–230)	$p < 0.001$

CD1 data previously reported [23]. n.s. = Not significant. Figures in parentheses indicate ranges.

havior including intermittent, moderate intensity circling to the right, intense forceful circling to the right interspersed with periods of immobilization with head deviated to the right, rearing and falling, barrel rolling, tonic extension, and tonic-clonic movements, similar to the behavior we reported in CD1 mice. The animal that died and 2 of the 4 with injury had seizure scores greater than 10 (range 15–36). The remaining ligated animals had very low seizure scores (range 0–6) representing very brief circling or scratching. The median seizure score in the C3HeB/FeJ mice (table 1) was significantly lower than in the CD1 mice. The seizure scores in the three sham-surgery animals ranged from 0 to 3. For ligated animals with non-zero seizure scores, median time to onset of seizure behavior was longer in the C3HeB/FeJ (167 min, range 30–235) than in the CD1 mice (70 min, range 10–230, $p < 0.05$, table 1).

Discussion

The major finding in this study is that the vulnerability to ischemic brain injury, in the unilateral carotid ligation model, is significantly less in P12 C3HeB/FeJ mice than in P12 CD1 mice. Seizures were observed in a smaller percentage of C3HeB/FeJ mice, and the onset of seizures was later in this strain than in CD1 mice. The reason(s) for these differences in vulnerability remain(s) unclear. Different vascular anatomy may have a role; however, genetically mediated differences in the response to ischemia are also likely. The correlation between seizures and brain injury in this model is different from the kainate model of brain injury, in which the duration or severity of seizures does not correlate with subsequent cell death [26]. The regional distribution of injury in the P12 unilateral carotid ligation model was similar in both the C3HeB/FeJ and CD1 mouse strains. There also appears to be a threshold phenomenon in this model; animals from both strains demonstrated either very little to no injury or substantial, grossly apparent injury.

Strain differences have been reported in a variety of adult models of ischemia [27–31]. Sheldon et al. [32] reported on strain-related brain injury in neonatal mice subjected to hypoxia-ischemia by the Vannucci model, in which unilateral carotid ligation is combined with hypoxia of variable duration to produce brain injury. They found differential susceptibility to injury in P7 CD1, C57Bl/6, and 129Sv mice and outcrosses of these strains; CD1 mice had the highest median brain injury scores and the highest percentage of injured animals (88%) with 30 min of hypoxia. Only 28% of C57Bl/6 mice demonstrated injury with 30 min of hypoxia, but injury occurred more frequently with longer durations of hypoxia. The 129Sv mice had the highest mortality (46%) with 30 min of hypoxia; of the animals that survived, however, only 17% were injured [32]. In comparison to these findings in the mouse neonatal hypoxia-ischemia model, in the P12 CD1 mouse carotid ligation ischemia model, the CD1 mouse strain was also found to be highly susceptible to injury. The regional pattern of injury differs from that reported in the Vannucci model in mice, where it is reported that the hippocampus is most susceptible to in-

jury [32], whereas in P12 CD1 mice after carotid ligation both the cortex and hippocampus are highly susceptible to injury, and lesser injury was also seen in the striatum of all injured animals [23].

In some studies, strain-related differences in vulnerability to ischemic injury have been attributed to variations in cerebral vascular anatomy. Maeda et al. [28] reported anatomical differences in adult C57Bl/6 and 129Sv mice, specifically that C57Bl/6 mice have a larger vascular area perfused by the middle cerebral artery than the 129Sv mice. This finding may account for larger strokes in the C57Bl/6 mice after middle cerebral artery occlusion. High resolution MR angiography studies have revealed highly variable arterial structures in mice from different strains and within the same strain. Beckman [33] observed a unilateral anastomosis between the posterior cerebral and the superior cerebellar arteries on the right side of the circle of Willis in 4 of 6 C57Bl/6 mice, but no anastomosis in 4 of 6 CD1 mice. Barone et al. [34] observed differences in vascular anatomy in 3 strains of mice; the presence or absence of the posterior cerebral artery in these strains was related to the sensitivity to ischemia. These anatomical differences could account in part for the greater susceptibility of CD1 mice to focal ischemic injury, particularly when induced on the right side. These observations offer at least a partial explanation for the variability seen among strains.

On the other hand, Wellons et al. [31] found that although the C57Bl/6 mice had smaller posterior communicating arteries, they had significantly larger basilar arteries than Sv129 mice, perhaps accounting for similar regional cerebral blood flow they observed in these 2 strains. They suggest that the difference between these strains in sensitivity to injury after bilateral carotid occlusion is not attributable to either methodological factors or vascular anatomy. Similar findings in the permanent focal cerebral ischemia model in adult C57Bl/6J, Balb/C and 129X1/SvJ mice were reported by Majid et al. [30], who noted that the presence and patency of the posterior communicating arteries did not affect pre-ischemic or postischemic cerebral blood flow (measured by laser Doppler) or bear any relationship to ischemic injury.

Since vascular differences anatomy may not completely explain differences in vulnerability to ischemic insult, other genetically determined factors that vary in different mouse strains may contribute to the extent of injury after an ischemic insult. To date, the exact nature of these putative genetic differences has remained obscure. Several strain-specific differences have been found in physiologic processes relevant to ischemia. Mouse strain variation in maximal electroshock seizure threshold [35], electroconvulsive thresholds [36], endothelial dysfunction [37], blood pressure variability [37], and cerebral energy metabolism [38] have all been documented. Strain-specific differences have also been found in cellular maturational and molecular factors with the potential to contribute to ischemic brain injury, including microglial-macrophage synthesis of tumor necrosis factor after focal cerebral ischemia [39], antioxidant proteins [40], hippocampal expression of ionotropic glutamate receptors [41], and AMPA receptor regulation [42]. Some of these factors may contribute to strain-specific vulnerability to ischemic injury in mice, but their potential contributions have not yet been examined in this model.

When recognized in the neonatal period, stroke is frequently accompanied by seizures [3, 43]. In pediatric patients, arterial stroke and cerebral sinovenous thrombosis present with seizures in 19–58% of the cases, depending on the study and population [44, 45]. Children with ischemic middle cerebral artery stroke who present with seizures may be at increased risk for poor neurologic and functional outcome [45, 46].

Seizures induced by global hypoxia, global ischemia, or hypoxic-ischemic injury have been reported in adult and immature rats [47–49]. Studies examining interactions between seizures and hypoxic-ischemic brain injury in developing rats have produced conflicting results. Status epilepticus induced by the GABA antagonist bicuculline after hypoxia-ischemia did not exacerbate brain injury in P7 rats [50]. On the other hand, in P10 rats subjected to unilateral carotid ligation and brief hypoxia, seizures induced by kainate significantly worsened brain injury [51]. Furthermore, in P10–12 rats, hypoxia produces seizures, and those animals with seizures induced by hypoxia at P10 have increased seizure susceptibility in adulthood and hippocampal alterations [52, 53]. In the P12 CD1 and C3HeB/FeJ mice after carotid ligation, the seizure scores were found to correlate positively with the brain injury. If severe or prolonged seizures contribute to brain injury in the setting of ischemia, then genetic propensity for seizures could contribute to strain-related differences in vulnerability to brain injury in this stroke model. However, whether severe or prolonged seizures occurring in the setting of brain ischemia exacerbate or only reflect the injury is a controversial question.

The current studies are limited by the scoring system used to rate the brain injury. In future studies, it would be valuable to undertake quantitative morphometric studies of reductions in brain volume and counts of de-

generating cells labeled by TUNEL or activated caspase-3 immunohistochemistry in the mouse P12 carotid ligation ischemia model. Quantification of relative numbers of apoptotic cells in a rodent model of hypoxia-ischemia indicates that the cerebral cortex and basal ganglia contain high numbers of apoptotic cells for more than 7 days after hypoxia-ischemia [54]. Late cell death in the thalamus of the neonatal rat after hypoxia-ischemia is programmed cell death [55]. Activation of apoptosis-executing caspases in response to ischemia may be enhanced in the immature brain compared to the adult, in part as a result of the high level of developmentally regulated programmed cell death, which normally eliminates neurons that fail to make functional connections during this period [56].

During a 4-hour period of seizures away from the dam, it is possible that the P12 mice could become hypoglycemic, which might affect the degree of brain injury. This might be particularly true in animals with prolonged severe seizures. Future studies should measure serum glucose levels during the 4-hour observation period to determine whether and to what degree hypoglycemia is occurring. The method of seizure rating used in these studies, while it does provide a quantification of seizure severity, does not allow calculation of an animal's total seizure frequency or duration. Future studies could be designed to examine the question of status epilepticus more directly.

In conclusion, our studies in the immature mouse unilateral carotid ligation model confirm a strain-dependent susceptibility to ischemic brain injury, which is likely related to differences in vascular anatomy and/or genetic susceptibility. Much work, including cerebral blood flow studies, studies of other strains, and studies of age dependence of injury, remains to be done to characterize this new model of stroke in the developing brain. The model offers a promising new approach to stroke in the immature brain.

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