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A New Model of Stroke and Ischemic Seizures in the Immature Mouse

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Ischemic brain injury from stroke is an important cause of disability in infants and children, but current experimental models for the disorder are complex. These preparations require occlusion of small intracerebral vessels or common carotid artery ligation combined with exposure to reduced levels of oxygen. Unilateral carotid artery ligation alone was sufficient to cause brain injury in more than 70% of 12-day-old CD1 mice. Using a blinded behavioral rating scale of seizure activity in mice, a direct, highly significant correlation between the severity of seizures over the 4-hour period after ligation and the severity of histologic brain injury 7 days later (Spearman's rho = 0.835, $P < 0.001$) was documented. This study presents the first model of stroke in immature mice produced by unilateral carotid artery ligation alone, and the first to demonstrate a clear correlation between acute ischemia-induced seizures and brain injury. This new model should be useful for examining the pathogenesis of stroke in the immature brain and the potential contribution of seizures to final outcome.

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Introduction

Stroke is an important cause of neurologic morbidity in infants and children, with an incidence of approximately 8 cases per 100,000 children per year [1]. Although the majority survive their stroke, approximately 75% of these children have sequelae including hemiparesis, epilepsy, learning disabilities, visual-field deficits, and mental retardation [2]. In pediatric patients, arterial stroke and cerebral sinovenous thrombosis present with seizures in 19-58% of the cases, depending on the study and patient group [2,3]. Children with ischemic middle cerebral artery stroke who present with seizures are at increased risk for poor neurologic and functional outcome [2]. One important category of pediatric stroke risk factors is structural abnormalities of the cerebral vasculature that result in abnormal cerebral blood flow. These vascular disorders, including cerebral hypoplasias, moyamoya disease, and others, frequently present with seizures and strokes [4,5]. Sturge-Weber syndrome is an example of a vascular malformation in which seizures may exacerbate brain injury resulting from impaired cerebral blood flow [6,7]. A simple animal model would be useful to study the pathogenesis of stroke in the immature brain and the potential role of seizures in the outcome.

Several animal models have been developed in rats and mice for the study of ischemic, hypoxic, and hypoxic-ischemic brain injury [8-13]. In immature rats, unilateral carotid ligation is not sufficient to produce brain injury and must be coupled with a period of hypoxia to produce brain injury [14]. Prior studies in immature mice have combined unilateral carotid ligation with a period of hypoxia to produce brain injury [15-18]. In mice, strain-related differences in the susceptibility to seizures or hypoxic-ischemic injury have been described [19-22]. To take advantage of these strain-related differences and the potential to use mice with genetic mutations in critical genes, we sought to develop a new model of brain ischemia and seizures in immature mice.

Materials and Methods

This protocol was approved by the Johns Hopkins University Animal Care and Use Committee. Unilateral carotid ligations were performed under isofluourane anesthesia in P12 CD1 mice (n = 28); littermates (n = 5) received sham surgeries. Mice were immediately placed in an incubator at 35°C. Seizure activity was scored by an observer blinded to

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ligation status, using a seizure rating scale for mice described by Morrison et al. [23]. Behavioral features characteristic of seizures were assessed continuously; every 5 minutes the animals were assigned a score for the highest level of seizure activity observed during that period, as follows: 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-minute interval scores were summed to produce a cumulative seizure score. This behavioral rating method was used to assess seizure activity because techniques for recording electroencephalogram in animals weighing less than 10 gm are unavailable. Litters were checked daily thereafter, but behavior was not formally assessed. Four animals (14%) lost weight and function and either died or were euthanized 3 to 5 days after surgery; all four of these mice had seizure scores >100.

One week later, mice were anesthetized with chloral hydrate and perfused with 4% formaldehyde. Gross brain injury was observed 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 [24]. Injury was scored from 0 to 4 for cortex (0: no injury; 1: 1-3 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); total score therefore ranged from 0 to 22. The average of the two investigators' scores was used for statistical analysis. Nonparametric regression was used to examine the relationship between seizure score rank and brain injury score rank.

Results

In the 28 ligated animals, seizure behavior was observed in 21 animals (75%). Typically, these resting pups would abruptly display intermittent rapid, tight circling interrupted by periods of normal ambulation. When this circling occurred repetitively, the seizure activity often progressed to repetitive forelimb clonus, repeated rearing and falling, and then to severe generalized tonic or tonic-clonic behavior. This activity is similar to seizure activity that we have observed in P12 CD1 mice given kainate injections. Seizure scores ranged from 0 to 116. Seizure behavior was first observed 30 to 120 minutes after the ligation, and most seizure behavior subsided by the end of the 4-hour observation period. No obvious seizure activity was observed at daily litter checks after the ligation.

Gross brain injury was evident in 71% of the ligated animals. A range of brain injury was observed that involved the cerebral cortex, striatum, hippocampus, and

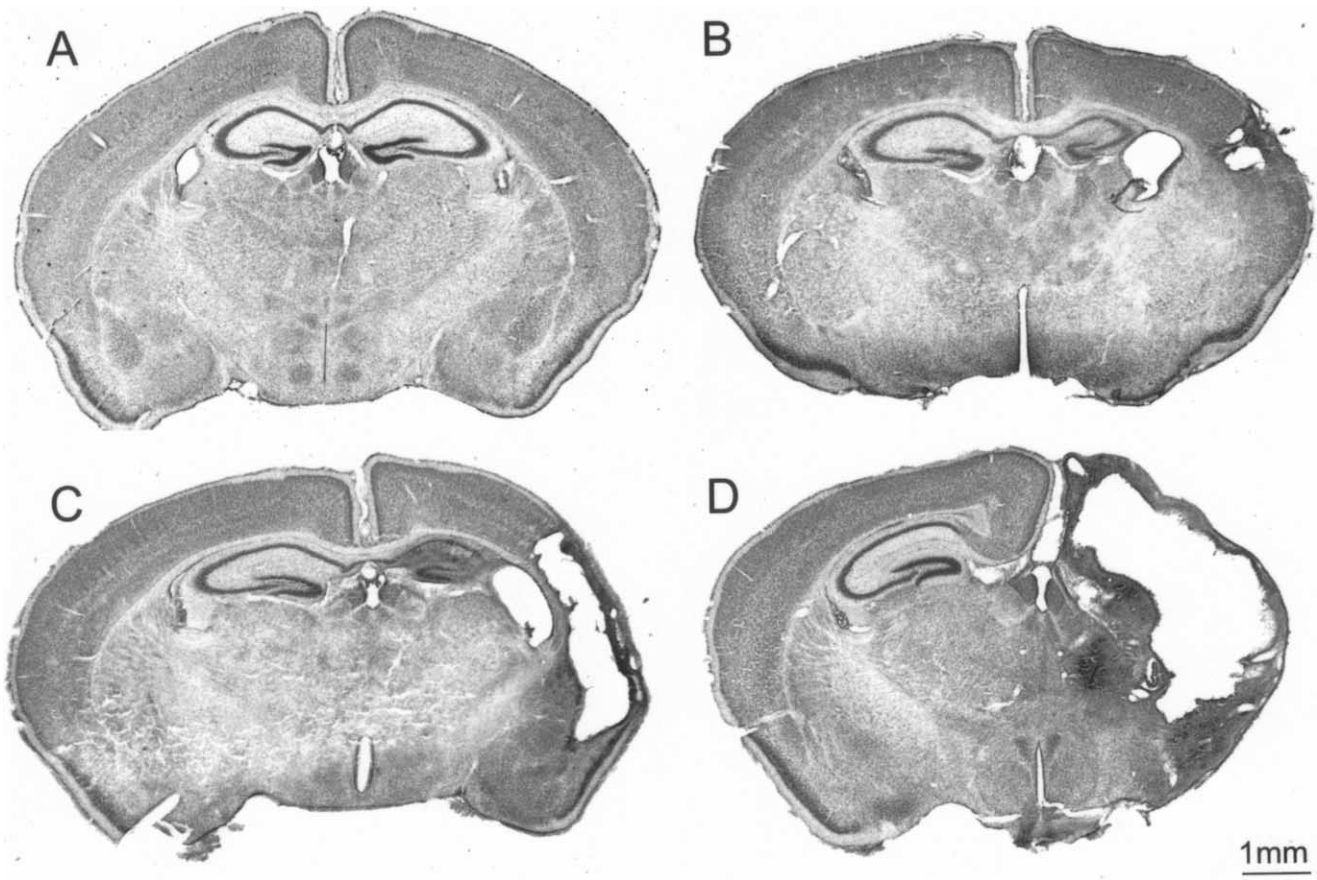

Figure 1. (A-D) CD1 mouse brain sections at the level of the hippocampus, stained with cresyl violet, that demonstrate the range of brain injury after carotid ligation. (A) This animal received a seizure score of 0 and a brain injury score of 0; no injury is observed. (B) This animal received a seizure score of 9 and a brain injury score of 11; a small confluent area of infarct in the cortex, moderate hippocampal atrophy, and mild thalamic atrophy are apparent in this section. (C) This animal received a seizure score of 18 and a brain injury score of 17.5; a larger confluent area of cortical infarct, hippocampal atrophy and necrosis, and atrophy of the striatum are apparent. (D) This animal received a seizure score of 66 and a brain injury score of 21; extensive necrotic infarct of the cortex, hippocampus, and striatum and atrophy and necrosis of the thalamus are observed. Note that brain injury scores are assigned based on review of multiple sections at different levels, not just the single level shown here.

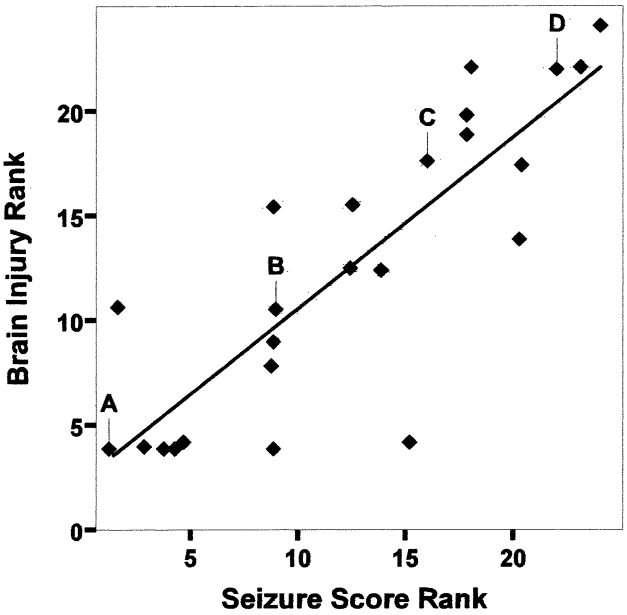

Figure 2. Scatterplot of brain injury rank vs seizure score rank. Data points labeled A-D correspond to animal brains depicted in Figure 1. Increased seizure activity correlated highly with increased brain injury (Spearman rank correlation = 0.835, $P < 0.001$*).*

thalamus (Fig 1A-D), 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 rating score (Spearman rank correlation = 0.835, $P < 0.001$). A scatterplot of brain injury rank vs seizure score rank is presented in Figure 2; increased seizure activity was associated with increased brain injury. This correlation was unchanged when the ligated animals were broken down by sex (Spearman rank correlation = 0.799, $P < 0.005$ females and 0.828, $P < 0.001$ males); there was no significant difference in brain injury or seizure score between males and females.

Cumulative seizure scores in control animals subjected to sham surgery ranged from 3 to 15. Sham animals received positive seizure scores primarily for scratching, with only one animal scored for circling behavior during one 5-minute period. None of these animals demonstrated the typical seizure progression that was frequently observed in the ligated group. Therefore, although some of the control animals were assigned low seizure scores, no convincing seizure behavior was observed in the control animals. There was no brain injury or mortality in the sham surgery group.

Discussion

These data demonstrate a fundamental difference in brain injury susceptibility between immature CD1 mice and immature rats, which require hypoxia superimposed on carotid ligation to cause ischemic injury. The reason for this difference in vulnerability is unclear. Unilateral carotid ligation alone in adult gerbils has been demonstrated to cause cerebral infarction in approximately 35% of the animals, and susceptibility to injury is related to individual variation in vascular anatomy [25]. Strain-related differences in seizure response or brain injury caused by hypoxia-ischemia suggest that genetic factors may play an important role in the vulnerability of immature CD1 mice to seizures and ischemic brain injury.

CD1 mice that received a unilateral carotid ligation on P12 frequently demonstrated seizure activity ranging from transient circling to prolonged tonic and tonic-clonic seizure activity. The cumulative seizure score was highly correlated with brain injury score. This immature CD1 mouse model can therefore be used to address the question of whether seizures exacerbate ischemic brain injury. The effect of seizures on ischemic/hypoxic-ischemic brain injury in rats has been examined with mixed results. Seizures have been observed after global hypoxia, global ischemia, or hypoxic/ischemic injury in adult and immature rats [26-28]. In adult rats, nonconvulsive seizures, periodic epileptiform discharges, and intermittent rhythmic delta activity have been reported after focal ischemia, but there was no significant correlation between seizure incidence or the occurrence of periodic epileptiform discharges and infarct volumes [29]. In immature animals, convulsive agents have been used in combination with cerebral hypoxia-ischemia. Status epilepticus induced by the γ-aminobutyric acid antagonist bicuculine after hypoxia-ischemia did not exacerbate brain damage in postnatal day 7 (P7) rats [30]. On the other hand, in P10 rats subjected to unilateral carotid ligation and brief hypoxia, seizures induced by kainate significantly exacerbated brain injury [31].

Clinical observations suggest that prolonged or frequent seizures in the setting of impaired blood flow may exacerbate the progression of ischemic brain injury in the developing brain. Release of glutamate and an increased metabolic demand resulting from prolonged seizure activity may further exacerbate brain injury in the setting of ischemia. If seizures in the developing brain exacerbate injury resulting from impaired brain perfusion, then aggressive treatment and prevention of seizures is warranted in at-risk children. Alternatively, if seizures are only a manifestation of the brain injury, then this would argue against aggressive seizure management and focus clinical attention on the management of impaired brain perfusion. Understanding the interaction between seizures and ischemia should provide new insights for the development of neuroprotective strategies for pediatric ischemic brain injury.

In summary, this study presents a new and practical mouse model of ischemia and seizures with important advantages over previous approaches. No hypoxia or drug is required in addition to the carotid ligation to produce either the seizures or the brain injury. Therefore this model more closely mimics the occurrence of ischemia, seizures, and brain injury in the pediatric patient. The severity of behavioral seizures is correlated with the severity of brain injury in this model, suggesting its potential usefulness for

better understanding the interactions between seizures and ischemia in producing injury in the developing brain.

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References

[1] **Giroud** M, Lemesle M, Gouyon JB, Nivelon JL, Milan C, Dumas R. Cerebrovascular disease in children under 16 years of age in the city of Dijon, France: A study of incidence and clinical features from 1985 to 1993. J Clin Epidemiol 1995;48:1343-8.

[2] **Delsing** BJ, Catsman-Berrevoets CE, Appel IM. Early prognostic indicators of outcome in ischemic childhood stroke. Pediatr Neurol 2001;24:283-9.

[3] **deVeber** G, Andrew M. Cerebral sinovenous thrombosis in children. N Engl J Med 2001;345:417-23.

[4] **Bojinova** V, Dimova P, Belopitova L. Clinical manifestations of cerebrovascular hypoplasias in childhood. J Child Neurol 2000;15:166-71.

[5] **Zafeiriou** DI, Ikeda H, Anastasiou A, et al. Familial moyamoya disease in a Greek family. Brain Dev 2003;25:288-90.

[6] **Coley** SC, Britton J, Clarke A. Status epilepticus and venous infarction in Sturge-Weber syndrome. Childs Nerv Syst 1998;14:693-6.

[7] **Aylett** SE, Neville BG, Cross JH, Boyd S, Chong WK, Kirkham FJ. Sturge-Weber syndrome: Cerebral haemodynamics during seizure activity. Dev Med Child Neurol 1999;41:480-5.

[8] **Hossmann** KA. Experimental models for the investigation of brain ischemia. Cardiovasc Res 1998;39:106-20.

[9] **Vannucci** RC, Connor JR, Mauger DT, et al. Rat model of perinatal hypoxic-ischemic brain damage. J Neurosci Res 1999;55:158-63.

[10] **Hoehn** M, Nicolay K, Franke C, van der Sanden B. Application of magnetic resonance to animal models of cerebral ischemia. J Magn Reson Imaging 2001;14:491-509.

[11] **Sarti** C, Pantoni L, Bartolini L, Inzitari D. Cognitive impairment and chronic cerebral hypoperfusion: What can be learned from experimental models. J Neurol Sci 2002;203-204:263-6.

[12] **Leker** RR, Constantini S. Experimental models in focal cerebral ischemia: Are we there yet? Acta Neurochir Suppl 2002;83:55-9.

[13] **Ginsberg** MD. Adventures in the pathophysiology of brain ischemia: Penumbra, gene expression, neuroprotection: The 2002 Thomas Willis Lecture. Stroke 2003;34:214-23.

[14] **Towfighi** J, Mauger D, Vannucci RC, Vannucci SJ. Influence of age on the cerebral lesions in an immature rat model of cerebral hypoxia-ischemia: A light microscopic study. Brain Res Dev Brain Res 1997;100:149-60.

[15] **Liu** XH, Kwon D, Schielke GP, Yang GY, Silverstein FS, Barks JD. Mice deficient in interleukin-1 converting enzyme are resistant to neonatal hypoxic-ischemic brain damage. J Cereb Blood Flow Metab 1999;19:1099-108.

[16] **Skoff** RP, Bessert DA, Barks JD, Song D, Cerghet M, Silverstein FS. Hypoxic-ischemic injury results in acute disruption of myelin gene expression and death of oligodendroglial precursors in neonatal mice. Int J Dev Neurosci 2001;19:197-208.

[17] **Gibson** ME, Han BH, Choi J, et al. BAX contributes to apoptotic-like death following neonatal hypoxia-ischemia: Evidence for distinct apoptosis pathways. Mol Med 2001;7:644-55.

[18] **Aden** U, Halldner L, Lagercrantz H, Dalmau I, Ledent C, Fredholm BB. Aggravated brain damage after hypoxic ischemia in immature adenosine A2A knockout mice. Stroke 2003;34:739-44.

[19] **Schauwecker** PE, Steward O. Genetic determinants of susceptibility to excitotoxic cell death: Implications for gene targeting approaches. Proc Natl Acad Sci USA 1997;94:4103-8.

[20] **Schauwecker** PE. Complications associated with genetic background effects in models of experimental epilepsy. Prog Brain Res 2002;135:139-48.

[21] **Sheldon** RA, Sedik C, Ferriero DM. Strain-related brain injury in neonatal mice subjected to hypoxia-ischemia. Brain Res 1998;810(1-2):114-22.

[22] **Mulle** C, Sailer A, Perez-Otano I, et al. Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice. Nature 1998;392(6676):601-5.

[23] **Morrison** RS, Wenzel HJ, Kinoshita Y, Robbins CA, Donehower LA, Schwartzkroin PA. Loss of the p53 tumor suppressor gene protects neurons from kainate-induced cell death. J Neurosci 1996;16:1337-45.

[24] **Matsushita** H, Johnston MV, Lange MS, Wilson MA. Protective effect of erythropoietin in neonatal hypoxic ischemia in mice. Neuroreport 2003;14:1757-61.

[25] **Donadio** MF, Kozlowski PB, Kaplan H, Wisniewski HM, Majkowski J. Brain vasculature and induced ischemia in seizure-prone and non-seizure-prone gerbils. Brain Res 1982;234:263-73.

[26] **Bentue-Ferrer** D, Bellissant E, Decombe R, Allain H. Temporal profile of aminergic neurotransmitter release in striatal dialysates in rats with post-ischemic seizures. Exp Brain Res 1994;97:437-43.

[27] **Krugers** HJ, Knollema S, Kemper RH, Ter Horst GJ, Korf J. Down-regulation of the hypothalamo-pituitary-adrenal axis reduces brain damage and number of seizures following hypoxia/ischaemia in rats. Brain Res 1995;690:41-7.

[28] **Holtzman** D, Togliatti A, Khait I, Jensen F. Creatine increases survival and suppresses seizures in the hypoxic immature rat. Pediatr Res 1998;44:410-4.

[29] **Hartings** JA, Williams AJ, Tortella FC. Occurrence of nonconvulsive seizures, periodic epileptiform discharges, and intermittent rhythmic delta activity in rat focal ischemia. Exp Neurol 2003;179:139-49.

[30] **Cataltepe** O, Vannucci RC, Heitjan DF, Towfighi J. Effect of status epilepticus on hypoxic-ischemic brain damage in the immature rat. Pediatr Res 1995;38:251-7.

[31] **Wirrell** EC, Armstrong EA, Osman LD, Yager JY. Prolonged seizures exacerbate perinatal hypoxic-ischemic brain damage. Pediatr Res 2001;50:445-54.