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A CHRONIC HISTOPATHOLOGICAL AND ELECTROPHYSIOLOGICAL ANALYSIS OF A RODENT HYPOXIC–ISCHEMIC BRAIN INJURY MODEL AND ITS USE AS A MODEL OF EPILEPSY

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Abstract—Ischemic brain injury is one of the leading causes of epilepsy in the elderly, and there are currently no adult rodent models of global ischemia, unilateral hemispheric ischemia, or focal ischemia that report the occurrence of spontaneous motor seizures following ischemic brain injury. The rodent hypoxic–ischemic (H–I) model of brain injury in adult rats is a model of unilateral hemispheric ischemic injury. Recent studies have shown that an H–I injury in perinatal rats causes hippocampal mossy fiber sprouting and epilepsy. These experiments aimed to test the hypothesis that a unilateral H–I injury leading to severe neuronal loss in young-adult rats also causes mossy fiber sprouting and spontaneous motor seizures many months after the injury, and that the mossy fiber sprouting induced by the H–I injury forms new functional recurrent excitatory synapses. The right common carotid artery of 30-day old rats was permanently ligated, and the rats were placed into a chamber with 8% oxygen for 30 min. A quantitative stereologic analysis revealed that the ipsilateral hippocampus had significant hilar and CA1 pyramidal neuronal loss compared with the contralateral and sham-control hippocampi. The septal region from the ipsilateral and contralateral hippocampus had small but significantly increased amounts of Timm staining in the inner molecular layer compared with the sham-control hippocampi. Three of 20 lesioned animals (15%) were observed to have at least one spontaneous motor seizure 6–12 months after treatment. Approximately 50% of the ipsilateral and contralateral hippocampal slices displayed abnormal electrophysiological responses in the dentate gyrus, manifest as all-or-none bursts to hilar stimulation. This study suggests that H–I injury is associated with synaptic reorganization in the lesioned region of the hippocampus, and that new recurrent excitatory circuits can predispose the hippocampus to abnormal electrophysiological activity and spontaneous motor seizures.

Key words: epilepsy, sprouting, recurrent excitation, population spikes, epileptiform bursts.

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Abbreviations: ANOVA, analysis of variance; AP-5, DL-2-amino-5-phosphonovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3,(1H,4H)-dione; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; H–I, hypoxia-ischemia; IML, inner molecular layer; P, postnatal day; SNK, Student-Newman-Keuls.

One common chronic complication following ischemic brain injury is the later development of chronic spontaneous recurrent seizures (Pohlmann-Eden et al., 1996; Olsen, 2001). Factors associated with ischemic brain injury and epilepsy are poorly defined, but it appears that large cortical lesions have a strong association with the later appearance of seizures (Berges et al., 2000; Lamy et al., 2003). Currently, the occurrence of chronic spontaneous recurrent motor seizures has been observed in only one rodent model of focal ischemia (cortical photothrombosis, Karhunen et al., 2007), but not in focal ischemia induced by middle cerebral artery occlusion (Karhunen et al., 2003, 2006) nor in global ischemia (Epsztein et al., 2006).

Global forebrain ischemia following cardiopulmonary arrest in humans has been shown to cause delayed hippocampal neuronal loss (Petito et al., 1987). The rodent model commonly used to analyze this type of brain injury is the four-vessel occlusion model (Pulsinelli and Brierley, 1979; Pulsinelli et al., 1982). An earlier alternate to this model was implemented by Levine (1960), and modified by Rice et al. (1981); it used a combination of hypoxia and ischemia to cause brain injury (relative oligemia rather than a true ischemia; see Ginsberg and Busto, 1989 for review). This hypoxia-ischemia (H–I) model is commonly used in postnatal day (P) 7 rats to induce a brain injury similar to neonatal H–I in humans. Unilateral H–I lesions in P7 rats have recently been shown to cause mossy fiber sprouting in the inner molecular layer (IML) of the dentate gyrus in both the lesioned and unlesioned hippocampi (Williams et al., 2004). Chronic spontaneous motor seizures were observed months after the H–I lesion in approximately 40% of the injured rats (Williams et al., 2004). Towfighi et al. (1997) reported that neuronal susceptibility to an H–I injury shifts as a function of age. The most susceptible hippocampal neurons in H–I-injured P7 rats are thought to be the CA3 pyramidal neurons, followed by the CA1 pyramidal neurons, and then the dentate gyrus and the hilus (Towfighi and Mauger, 1998). In contrast, 30-day old rats subjected to an H–I insult showed that CA1 and the hilus appeared to be the more susceptible neuronal populations, with relative sparing of CA3 and the dentate gyrus. The pattern of neuronal loss in 30-day H–I rats is similar to what is seen in adult rats that undergo global forebrain ischemia by the four-vessel occlusion method and in humans after global forebrain ischemia following cardiopulmonary arrest (Pulsinelli and Brierley, 1979; Pulsinelli et al., 1982; Petito et al., 1987). The pattern of injury in the hippocampus after a hypoxic and/or ischemic insult has many general similarities to the histopathological abnormalities associated

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with temporal lobe epilepsy; one of the central features of human temporal lobe epilepsy is Ammon's horn sclerosis, where neurons in the hilus and in the CA3 and/or CA1 areas are lost (Babb and Brown, 1987; Franck and Roberts, 1990).

In human temporal lobe epilepsy (Houser et al., 1990), and in both the kainate and pilocarpine models of status epilepticus with subsequent epileptogenesis in rats (Nadler et al., 1980; Mello et al., 1993), Timm staining and other anatomical techniques have shown that the mossy fibers of the dentate granule cells innervate the IML of the dentate gyrus, a condition that is not usually present in normal animals or humans (Buckmaster and Dudek, 1997a, b,1999). Onodera and coworkers (1990) showed that the four-vessel occlusion model of global forebrain ischemia is associated with abnormal Timm staining in the IML, although the amount of Timm staining in the IML was substantially less than in the kainate- or pilocarpine-models of temporal lobe epilepsy. The presence of mossy fiber sprouting in the hippocampus may act as a marker of the formation of new recurrent synapses in other areas of the brain and of the potential for the development of epilepsy (Gorter et al., 2001).

The hypothesis that Timm stain in the IML and associated mossy fiber sprouting leads to enhanced recurrent excitation, and could contribute to epileptogenesis in the dentate gyrus, was advanced by the experiments of Tauck and Nadler (1985). They showed that mossy fiber sprouting in kainate-treated rats was associated with paired-pulse potentiation and multiple population spikes to hilar stimulation, which would be expected to activate primarily the mossy fiber axons of the dentate granule cells. Subsequent experiments revealed that in most preparations from kainate-treated rats, the responses to hilar and perforant path stimulation were relatively normal or only slightly abnormal in standard media (Cronin et al., 1992; Patrylo and Dudek, 1998); however, when $GABA_A$-mediated inhibition was blocked with bicuculline, some preparations with mossy fiber sprouting showed bursts to hilar stimulation, while those without sprouting did not show bursts. Some of these bursts had a long and variable latency when evoked at low stimulus intensities, a characteristic expected of circuits with recurrent excitation (Traub and Wong, 1982; Miles and Wong, 1986, 1987; Christian and Dudek, 1988a,b). If synaptic reorganization occurs after H–I and is similar to the kainate model, then local inhibitory circuits may mask recurrent excitation, and one method used to reveal these new excitatory synapses is to block or depress inhibition and/or raise the concentration of extracellular potassium (Traub and Wong, 1982; Miles and Wong, 1986, 1987; Christian and Dudek, 1988a,b; Cronin et al., 1992; Wuarin and Dudek, 1996, 2001; Patrylo and Dudek, 1998; Hardison et al., 2000; Lynch and Sutula, 2000). Another method used to demonstrate the formation of new recurrent excitatory synapses is focal flash-photolysis of caged-glutamate, which has been used to map neuronal circuitry and the formation of these new circuits (Callaway and Katz, 1993; Katz and Dalva, 1994; Dalva and Katz, 1994; Wuarin and Dudek, 1996, 2001; Molnar and Nadler, 1999).

Studies that have examined neuronal and network excitability after global forebrain ischemia have had varied results. Mody and coworkers (1995) reported that inhibition was intact in granule cells 3 months after 15 min of global ischemia, and that hyperexcitability was not present. Furthermore, a decrease in the excitability of CA1/CA2 pyramidal neurons was observed in rats 10–12 months after global ischemia (Arabadzisz et al., 2002).Hyperexcitability, a reduced threshold for burst generation, and interictal epileptiform discharges have been reported in the CA3 region chronically after global ischemia (Congar et al., 2000; Wu et al., 2005; Epsztein et al., 2006). However, chronic spontaneous seizures after global forebrain ischemia have not been reported in the literature.

The goal of this study was to use an H–I model in 30-day-old rats to create a large unilateral lesion in the cortex and hippocampus. This would in turn allow an evaluation and comparison of the potential for mossy fiber sprouting in the IML and chronic spontaneous motor seizures in this model (i.e. 30-day H–I) with the perinatal model of H–I and with the four-vessel occlusion model of global forebrain ischemia. This study tested the hypothesis that the neuronal loss caused by an H–I injury at 30 days of age would be associated with synaptic reorganization in the hippocampus, and that these new circuits would lead to abnormal electrophysiological responses in the dentate gyrus. We performed a septo-temporal analysis of Timm staining in the IML and a quantitative stereologic assessment of the hippocampus to address the first part of this hypothesis. The second part of the hypothesis was examined with *in vitro* extracellular and whole-cell patch-clamp recordings with focal flash photolysis of caged glutamate in hippocampal slices from H–I injured rats and age-matched controls many months after the H–I lesion. We further hypothesized that these rats following an H–I injury at 30 days of age would develop chronic spontaneous motor seizures, and that this model of unilateral hemispheric brain injury may have use as a model of epilepsy.

EXPERIMENTAL PROCEDURES

Surgical preparation

A modified version of the Levine preparation, as described by Rice and coworkers (1981), was used to create an H–I injury in the right hemisphere. Sprague–Dawley rats (both male and female, 30 days of age, $n=29$ H–I-treated animals and 13 sham-surgical controls) were anesthetized using a 2% isoflurane/oxygen mixture. The ventral midline of the neck was surgically prepared and infused with bupivacaine (0.5%, 0.5 ml). A 1-cm ventral-midline incision was made, and the right common carotid artery exposed and permanently double ligated with 4–0 Dexon (U.S. Surgical, North Haven, CT, USA). The skin was closed with 4–0 Dermalon (U.S. Surgical), and the rats were allowed to recover in a heated cage. For the sham controls, the carotid artery was exposed but not ligated. After 2 h of recovery, the H–I treated rats were placed in an airtight chamber where the temperature and humidity were maintained at 37 °C and 90%, respectively. The chamber was then filled with an 8% oxygen and 92% nitrogen mixture. The oxygen content of the chamber was monitored with an oxygen-