Epilepsia, 53(Suppl. 1):109–115, 2012
doi: 10.1111/j.1528-1167.2012.03481.x

EPILEPSY-RELATED ABNORMALITIES/CHANGES IN BRAIN STRUCTURE

Synaptic connections of hilar basal dendrites of dentate granule cells in a neonatal hypoxia model of epilepsy

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Summary

Numerous animal models of epileptogenesis demonstrate neuroplastic changes in the hippocampus. These changes occur not only for the mature neurons and glia, but also for the newly generated granule cells in the dentate gyrus. One of these changes, the sprouting of mossy fiber axons, is derived predominantly from newborn granule cells in adult rats with pilocarpine-induced temporal lobe epilepsy. Newborn granule cells also mainly contribute to another neuroplastic change, hilar basal dendrites (HBDs), which are synaptically targeted by mossy fibers in the hilus. Both sprouted mossy fibers and HBDs contribute to recurrent excitatory circuitry that is hypothesized to be involved in increased seizure susceptibility and the development of spontaneous recurrent seizures (SRS) that occur following the initial pilocarpine-induced status epilepticus. Considering the putative role of these neuroplastic changes in epileptogenesis, a critical question is whether similar anatomic phenomena occur after epileptogenic insults to the immature brain, where the proportion of recently born granule cells is higher due to ongoing maturation. The current study aimed to determine if such neuroplastic changes could be observed in a standardized model of neonatal seizure-inducing hypoxia that results in development of SRS. We used immunoelectron microscopy for the immature neuronal marker doublecortin to label newborn neurons and their HBDs following neonatal hypoxia. Our goal was to determine whether synapses form on HBDs from neurons born after neonatal hypoxia. Our results show a robust synapse formation on HBDs from animals that experienced neonatal hypoxia, regardless of whether the animals experienced tonic–clonic seizures during the hypoxic event. In both cases, the axon terminals that synapse onto HBDs were identified as mossy fiber terminals, based on the appearance of dense core vesicles. No such synapses were observed on HBDs from newborn granule cells obtained from sham animals analyzed at the same time points. This aberrant circuit formation may provide an anatomic substrate for increased seizure susceptibility and the development of epilepsy.

KEY WORDS: **Immunoelectron microscopy, Doublecortin, Neurogenesis, Mossy fiber, Neuroplasticity.**

Hypoxia/ischemia is a major cause of neonatal encephalopathy and seizures, and can lead to epilepsy (Bergamasco et al., 1984; Volpe, 2000). Seizures are most prevalent in the neonatal period, with an incidence of 1–3.5/1,000 live births (Lanska et al., 1995; Saliba et al., 1999), and up to 60% of neonatal seizures are associated with a hypoxic event (Aicardi & Chevrie, 1970; Hauser et al., 1993; Gluckman et al., 2005; Shankaran et al., 2005). Approximately 25–30% of infants who experienced neonatal seizures will develop epilepsy (Holden et al., 1982; Bergamasco et al., 1984; Bernes & Kaplan, 1994). Therefore, clinical evidence indicates that seizure-inducing neonatal hypoxia can lead to epilepsy and other long-term neurologic impairment. Current therapies aim to treat the acute distress, as the cellular and molecular

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pathologic consequences for brain maturation and function are not yet adequately understood. In addition, neonatal seizures can be refractory to antiepileptic drugs (AEDs) and the neonatal brain responds variably to current AEDs, which are most commonly tested in adults. Clearly, the development of therapies and interventions to minimize the risk of long-term adverse sequelae of neonatal hypoxia-induced seizures will require a better understanding of the underlying pathophysiologic mechanisms.

In rats, a single episode of global hypoxia at postnatal day 10 (P10) induces spontaneous behavioral and electrographic seizures, and results in decreased seizure thresholds through adulthood (Silverstein & Jensen, 2007); spontaneous discharges begin 2 weeks after the initial hypoxic event and increase in frequency over time (Rakhade et al., 2011). This age in rat (P10) corresponds approximately to the time of birth in humans by various biochemical and anatomic parameters (Himwich, 1970; Romijn et al., 1991). Therefore, this model mimics the age specificity of the proepileptogenic effects of global hypoxia and the chronic increase in seizure susceptibility (or epilepsy risk) observed clinically.

Hippocampal slices from animals that experienced hypoxia at P10 exhibit various manifestations of hyperexcitability, including prolonged epileptiform afterdischarges in response to high frequency, high-intensity stimulation of Schaffer collateral axons (Sanchez et al., 2001) and ictal-like pattern of field bursting when exposed to Mg^{2+} free media (Jensen et al., 1998). Consequently, neonatal hypoxia-induced seizures result in apparent long-term changes in the function of hippocampal neurons and circuits that result in network hyperexcitability and promote seizure-like electrographic activity. Considering these findings, it is possible that neuroplastic changes in the hippocampus occur in response to neonatal hypoxia that may provide an anatomic substrate for increased hyperexcitability. However, few studies have examined this possibility in neonatal hypoxia, as gross examination suggested a lack of widespread neuronal damage (Jensen et al., 1991; Koh & Jensen, 2001; Sanchez et al., 2001; Rakhade et al., 2011), which may be a trigger for neuroplastic changes in epileptogenic models (Dudek et al., 2010).

The hippocampal dentate gyrus is a late developing structure, such that a majority of the granule cell neurons of the dentate gyrus are born and become synaptically integrated after birth (Altman & Das, 1965; Rakic & Nowakowski, 1981; Wenzel et al., 1981). The hippocampus is also highly susceptible to seizure-promoting insults; dentate gyrus reorganization via sprouting of mossy fibers, formation of hilar basal dendrites (HBDs), and aberrant synaptogenesis have long been postulated to facilitate hyperexcitability and/or generate spontaneous recurrent seizures (SRS) in adult chemoconvulsant models such as pilocarpine and kainic acid (Ribak et al., 2000; Scharfman et al., 2000; Austin & Buckmaster, 2004; Morgan & Soltesz, 2008; Thind et al., 2008; Toni et al., 2008). More recently, the formation of stable mossy fiber synapses onto sprouted HBDs from adult newborn granule cells has been suggested to play a role in the formation of epileptic recurrent excitatory circuitry (Buckmaster et al., 2002; Austin & Buckmaster, 2004; Shapiro et al., 2005; Shapiro & Ribak, 2006; Thind et al., 2008; Toni et al., 2008). Recent studies in the pilocarpine model demonstrated that periseizure born granule cells contribute the majority of mossy fiber and HBD sprouting, and are interconnected within a recurrent circuitry (Shapiro & Ribak, 2006; Shapiro et al., 2007; Danzer et al., 2010; Kron et al., 2010; McAuliffe et al., 2011; Murphy et al., 2011). Considering the high ratio of newborn to mature neurons in developing brains, it is possible that similar neuroplastic changes occur in newborn granule cells following neonatal hypoxia, another model that results in SRS (Rakhade et al., 2011). Therefore, the current study sought to determine whether neonatal hypoxia leads to aberrant synaptic connections on the HBDs of newborn granule cells.

Methods

Subjects

Rat pups were divided into three groups: (1) control (N = 6); (2) rats that experienced hypoxia but did not have tonic–clonic seizures during the hypoxia (hypoxia-no seizure; N = 7); and (3) rat pups that experienced hypoxia and had tonic–clonic seizures during the hypoxia (hypoxia-seizure; N = 7). Rats in the two hypoxia-treated groups were placed individually into a small cage within an airtight chamber. The inner cage rested on a heating pad calibrated to maintain rectal temperature at 33–34°C. The oxygen concentration in the airtight chamber was reduced to 5–7% for 15 min by pumping in 100% nitrogen gas. Animals were observed and videotaped during hypoxia treatment and the number of stereotyped behavioral seizures was counted. After 15 min, the chamber was reopened to room air, and pups were earmarked for identification by cutting out a small wedge of the ear with sharp scissors. Control rats were placed individually in the same type of airtight chamber and exposed continuously to room air for 15 min, and then earmarked for identification. Handling of the control group simulated every aspect of the hypoxia-treated animals except for the actual hypoxia exposure, and thus provides an important control for any stress effects from handling alone.

Seizure assessment

Seizure behaviors were assessed by counting myoclonic jerks and tonic–clonic convulsions. Any animal that had tonic–clonic convulsions during the 15 min of hypoxia were placed in the hypoxia-seizure group, whereas those that had no tonic–clonic convulsions were placed in the hypoxia-no seizure group.

Immunocytochemical analysis of doublecortin-labeled immature neurons

At 30 days after neonatal hypoxia (P40), rats were perfused as described previously (Foresti et al., 2009). Briefly, rats were anesthetized with Euthasol (Virbac Corporation, Ft. Worth, TX, U.S.A. and perfused through the heart with 0.1-M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1-M phosphate buffer. Brains were allowed to postfix in the skull for 48 h, and were removed and postfixed in 4% PFA at 4°C for an additional 48 h. Coronal, 50-μm–thick sections were cut using a vibratome and stored in 0.1-M PBS prior to immunohistochemical processing. Sections were incubated in 1.0% H_2O_2 for 1 h to block endogenous peroxidase activity. After rinsing in PBS, the sections were incubated free-floating in antibodies directed to doublecortin (DCX; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in 0.1-M phosphate buffer and 3% normal horse serum, for 24 h at 4°C. The sections were washed in PBS and incubated in anti-goat immunoglobulin G (IgG) (Vector Labs, Burlingame, CA, U.S.A.) for 1 h, rotating at room temperature. The sections were again washed and incubated for 1 h in the ABC kit (Vector Labs), rotating at room temperature. Sections were then rinsed and reacted in 3,3′-Diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, U.S.A.). The DAB reaction was halted by rinsing in PBS, three times, for 5 min each rinse. Hippocampi were then dissected out and prepared for immunoelectron microscopy processing, as described previously (Shapiro et al., 2005; Shapiro & Ribak, 2006). Briefly, the hippocampal sections of interest were osmicated for 1 h and then processed for embedding in plastic following dehydration through a series of ethanol and followed by propylene oxide. Sections containing the dentate gyrus and DCX-labeled granule cells with HBDs were sectioned with an ultramicrotome (Reichert Ultracut, Reichert, Inc., Depew, NY, U.S.A.). Ultrathin sections were cut from each block and collected on mesh grids to observe in the electron microscope. The mesh grids allow us to know the total area of sections examined for analysis. Before placing the grids in the microscope, the sections were stained with uranyl acetate and lead citrate. To ensure that the reviewer was blind to the condition of the animals, the grids were then coded and the code was not broken until the completion of all image analysis. After drying, the thin sections containing the cell body and dendrites of these granule cells were examined with a Philips (Eindhoven, The Netherlands) CM-10 transmission electron microscope. Analysis focused only on the region of sections at the hilus–granule cell layer border, and up to 50 μm into the hilus. Within this region, photographs of DCX-labeled HBDs were captured using a Gatan digital camera. We determined (1) the number of DCX-labeled basal dendrites per 1,000 μm^2 of tissue examined, (2) the number of synapses per DCX-labeled dendrite, and (3) the number of synapses per 1,000 μm^2 of DCX-labeled dendrites. The data were compared using analysis of variance (ANOVA) with post hoc paired comparisons.

Results

The electron dense immunoreaction product for DCX was located in dendrites and perikaryal cytoplasm of neurons located in the granule cell layer and the subgranular zone (Figs. 1–3). These elements are considered to be from newborn neurons as indicated by previous studies (Shapiro et al., 2005; Shapiro & Ribak, 2006), and the findings are similar to those published previously for 60-day-old rats (Shapiro & Ribak, 2006). The control tissue had no synapses on any DCX-labeled dendrites in the hilus. In contrast, both the hypoxia-no seizure and the hypoxia-seizure groups had synapses on DCX-labeled dendrites in the hilus (Figs. 2 and 3, respectively). It is noteworthy that in several cases from both hypoxia groups of animals, axon terminals with more than one active zone were observed on the DCX-labeled dendrites (Figs 2 and 3). Some of the axon terminals that formed synapses with DCX-labeled dendrites contained dense core vesicles. This finding suggested that these axon terminals arose from granule cells (Laatsch & Cowan, 1966; Chandy et al., 1995). These qualitative data were confirmed with the quantitative analysis below.

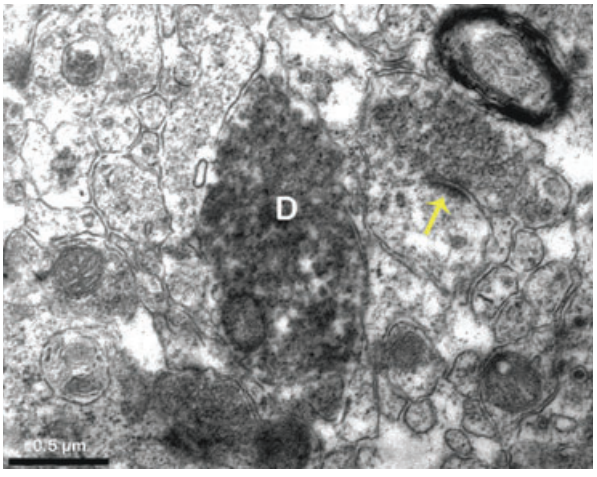

Figure 1.
Representative electron micrograph depicting DCX-labeled basal dendrites (D) in the hilus of a control rat. Note that no synapses were observed on any of the basal dendrites examined from the control animal. In addition, note the synapse (arrow) in the neuropil that is adjacent to the DCX-labeled dendrite (D) within this section. Scale bar = 0.5 μm.
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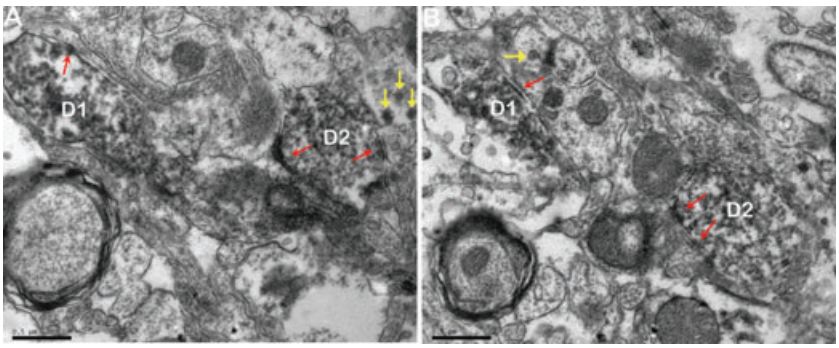

Figure 2.
Representative electron micrographs from a series of serial sections (separated by about four ultrathin sections) showing two DCX-labeled dendrites (D) in the hilus of a hypoxia-no seizure animal. In (**A**) one synapse (red arrow) is observed on the dendrite to the left of the image (D1) and two synapses (red arrows) are observed on the dendrite to the right of the image (D2). Note the dense core vesicles (yellow arrows) within axon terminals in figures (**A** and **B**) consistent with their identification as mossy fiber terminals. Note that in (**B**) the dense core vesicle is within the terminal that is synapsing (red arrow) onto the DCX-labeled dendrite to the left of the image (D1). In addition, note that in (**B**) the dendrite to the right of the image (D2) has two active zones (red arrows). Scale bar = 0.5 μm for both images.

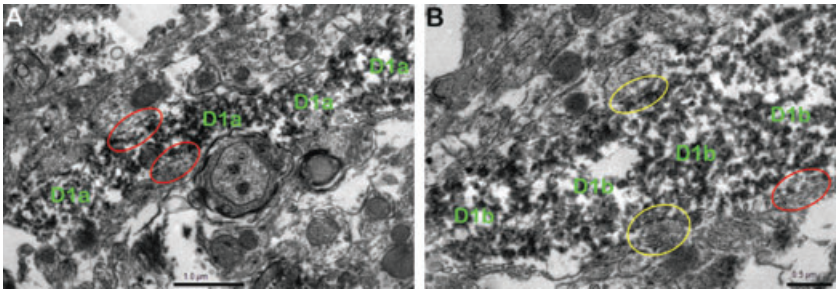

Figure 3.
Representative electron micrographs from a series of serial sections (separated by about three ultrathin sections) showing two adjacent portions of a long DCX-labeled dendrite (D1) in the hilus of a hypoxia-seizure animal. In (**A**) two different synaptic sites with single active zones (red circles) are observed on this thinner portion of the labeled dendrite (D1a). In (**B**) the thicker portion of this dendrite (D1b) has three different synaptic sites; one with a single active zone (red circle) and two with double active zones (yellow circles). Scale bars = 1.0 μm in (**A**) and 0.5 μm in (**B**).

Before counting the number of synapses found on DCX-labeled dendrites, we determined the frequency of these dendrites in the hilus in each of the three groups. The first step was to measure the mean area examined for each condition (Fig. 4). No significant differences were found in the area examined between any of the groups ($F_{2,17} = 0.352$, $p = 0.708$, NS). The second step in this analysis was to count the mean number of profiles of DCX-labeled dendrites per 1,000 μm^2 observed in each of the three groups (Fig. 4). The ANOVA revealed an overall significant difference for mean number of dendrites per 1,000 μm^2 ($F_{2,17} = 5.288$, $p < 0.02$). Post hoc paired comparisons using the Bonferroni test revealed that the mean number of HBDs per 1,000 μm^2 (Fig. 4) was significantly less for controls compared to the hypoxia-seizure group ($p < 0.05$). In addition, there was a trend toward fewer HBDs in controls compared to the hypoxia-no seizure group ($p = 0.064$, NS). No difference was observed in basal dendrites per 1,000 μm^2 between the two hypoxia groups (nonseizure and seizure, $p = 1.000$, NS). Because significantly fewer DCX-labeled basal dendrites were observed in the control group (despite an equal area of tissue examined), additional samples were obtained for analysis of the control group to ensure an equal chance of observing synapses on HBDs. Despite the addition of two to three basal dendrites per control animal, no synapses

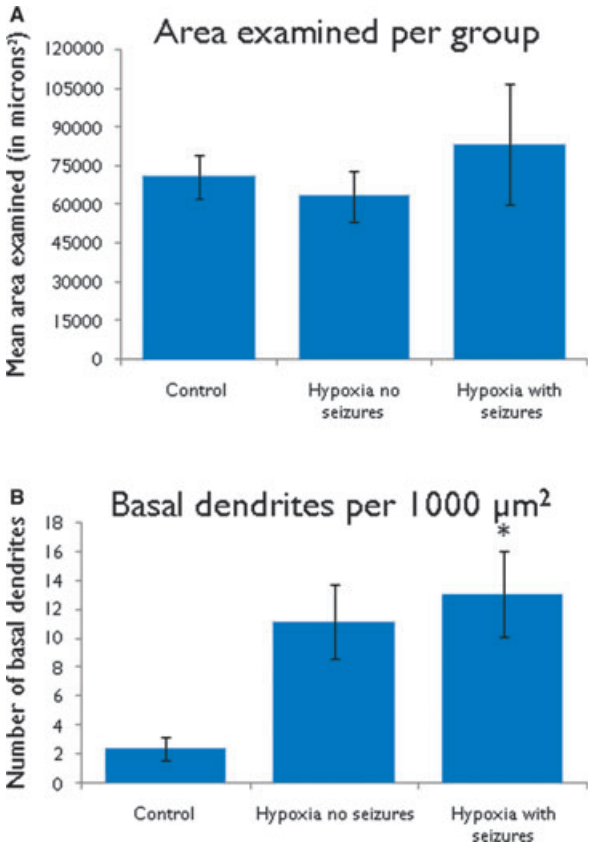

Figure 4.
Graph of means showing the mean area examined per group (**A**) and the mean number of basal dendrites observed per 1,000 μm^2 of tissue examined (**B**). No significant differences were observed between any of the groups for total area examined. Alternatively, significantly less basal dendrites were observed in the control group compared to the hypoxia-seizure group ($p < 0.05$) and a trend toward significance when comparing controls and hypoxia-no seizure group ($p = 0.064$). No significant differences were observed between the two hypoxia groups.
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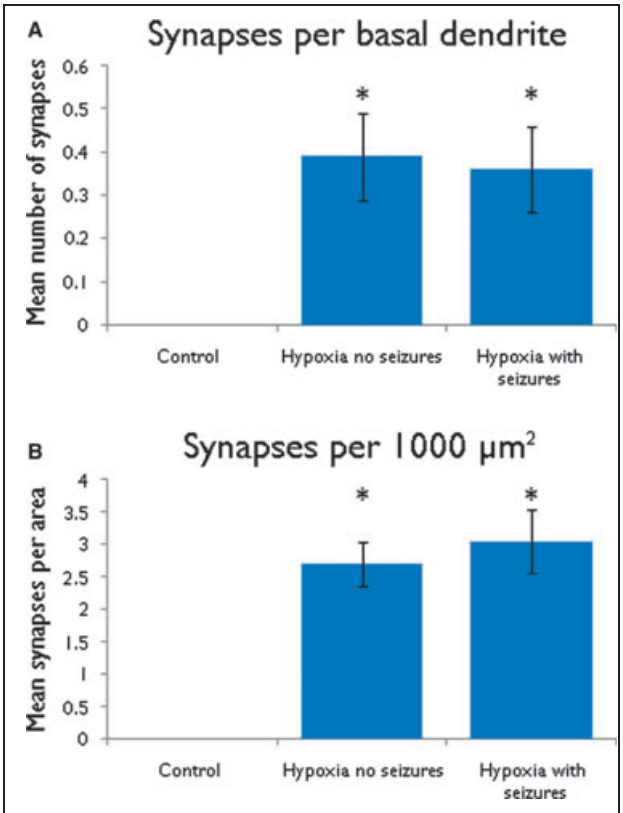

Figure 5.
Graph of means showing the mean number of synapses per basal dendrite (**A**) and per 1,000 μm^2 of tissue examined (**B**). For both sets of analysis, no synapses were observed on any of the DCX-labeled basal dendrites from the control animals and the results were significant compared to both, hypoxia-no seizure ($p < 0.026$ and 0.001, respectively) and hypoxia-seizure ($p < 0.038$ and 0.0001, respectively) groups. No significant differences were observed when comparing the two hypoxia groups.
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were observed on any of the DCX-labeled basal dendrites from the control group. These additional data were not included in the quantitative analysis, which was performed on the counts taken with the reviewer blind to the condition of the animal.

Significant differences were observed in comparing both synapses per DCX-labeled dendrite ($F_{2,17} = 5.454$, $p < 0.02$) and the synapses per 1,000 μm^2 ($F_{2,17} = 19.728$, $p < 0.0001$). Post hoc Bonferroni analysis revealed no differences between the two hypoxia groups (no seizure and seizure) for synapses per dendrite and synapses per 1,000 μm^2 ($p = 1.000$ for both). When comparing mean number of synapses per DCX-labeled dendrites to controls, controls had significantly fewer than the hypoxia-no seizure ($p < 0.026$) and hypoxia-seizure ($p < 0.038$) groups. Comparisons of synapses per 1,000 μm^2 showed that the control group had significantly fewer than both hypoxia groups ($p < 0.0001$ for both). These results are summarized in Fig. 5. Furthermore, synapses onto the DCX-labeled basal dendrites were observed in sections from most hypoxia animals (hypoxia-seizure group, six of seven animals, mean synapses per 1,000 $\mu m^2 = 2.69$; hypoxia-no seizure group, six of seven animals, mean synapses per 1,000 $\mu m^2 = 3.04$).

Discussion

Previous studies showed newborn neurons with DCX-labeled basal dendrites in young adult dentate gyrus

(Shapiro et al., 2005; Shapiro & Ribak, 2006). The current study demonstrated that DCX-labeled basal dendrites are a consistent feature of rodents at postnatal day 40 and that hypoxia enhances the appearance of DCX-labeled basal dendrites from newborn neurons. Moreover, the results show that significantly more DCX-labeled basal dendrites are observed in animals that experienced hypoxia with seizures, whereas only a trend toward a significant increase was observed for animals that experienced hypoxia with no seizures. Therefore, HBD sprouting is reported in at least three distinct models of SRS: chemoconvulsants such as pilocarpine and kainic acid (Spigelman et al., 1998; Ribak et al., 2000); p35 knockout mice (Wenzel et al., 2001; Patel et al., 2004); and neonatal hypoxia. It should be noted that the reports of SRS in the neonatal hypoxia model were made only for animals that experienced seizures during the hypoxic event (Rakhade et al., 2011). It remains to be seen if animals that experience hypoxia at P10 without seizures will also develop SRS.

A second major finding is that the hypoxic event was sufficient to promote synapse formation onto the DCX-labeled basal dendrites regardless of whether the animals had seizures. In contrast, no synapses were observed in the control group, consistent with previous findings (Shapiro & Ribak, 2006). We observed multiple axon terminals with >1 active zone on DCX-labeled basal dendrites from several of the animals from both hypoxia groups (Figs. 2 and 3); this finding is distinctly different from that of a previous study that examined DCX-labeled basal dendrites at 30 days following pilocarpine-induced status epilepticus in adult rats. In this latter study, few if any of the DCX-labeled basal dendrites were observed to have >1 active zone (Shapiro & Ribak, 2006). It is pertinent to note that another study, which examined synaptogenesis on basal dendrites of mature granule cells (that were not as immature as the DCX–labeled dendrites because spines were present) after pilocarpine-induced seizures, reported axon terminals with multiple active zones on the dendrites (Thind et al., 2008). Therefore, it is possible that the young age of the rats in the current study results in a more rapid sprouting/plastic response, especially considering the immaturity of the hippocampus at the time of hypoxia (P10). Of interest, both pilocarpine and hypoxia models result in the appearance of spontaneous seizures beginning at 2–3 weeks after the initial precipitating injury, with increasing frequency over time (Turski et al., 1984; Curia et al., 2008; Rakhade et al., 2011). Synapse formation onto basal dendrites is a common feature of both of these models of epileptogenesis and might be an anatomic substrate for recurrent excitation.

Previous studies showed dendritic and synaptic alterations of granule cells from hypoxic animals. A recent study indicated that hypoxia at P10 in mice enhances the dendritic complexity of newborn neurons born around the time of the insult (Pugh et al., 2011). Moreover, synaptic integration was enhanced in these mice but not in mice exposed to a chemical insult at the same age. It is notable that the enhanced dendritic structure in the newborn granule cells from the mice that experienced neonatal hypoxia was associated with an increase in the frequency of spontaneous γ-aminobutyric acid (GABA)–mediated synaptic currents (Pugh et al., 2011). A previous study also revealed alterations to hippocampal field recordings and enhanced epileptogenesis following neonatal hypoxia in rats (Sanchez et al., 2001). A study involving p35 knockout mice revealed numerous changes to dentate granule cells, including basal dendrites that contribute to an aberrant excitatory feedback circuit that may contribute to the SRS observed in these animals (Patel et al., 2004). Although synapses onto basal dendrites were not examined in these previous studies, the results of the current study, showing enhanced synaptogenesis onto HBDs of newborn granule cells, provides an additional type of dendritic alteration for granule cells following neonatal hypoxia. Future studies are needed to determine if the presence of synapses on such basal dendrites contributes to altered granule cell activity, network hyperexcitability, and epileptogenesis following neonatal hypoxia.

Acknowledgments

This work was supported by grant SDG2370076 from the American Heart Association to LAS. This material is the result of work supported with resources and the use of facilities at the Central Texas Veterans Health Care System, Temple, Texas. The authors would like to thank Kun Zhang and Amanda Ruch for their technical assistance. This article is dedicated to Dr. Jürgen Wenzel on the occasion of his retirement from a long and fulfilling career in studying the fine structure of the nervous system. One of the authors (CER) remembers meeting Jürgen at the University of California at Irvine in the early 1980s while he was on his sabbatical with Dr. Gary Lynch. A close friendship ensued as well as many discussions about the influence of seizures on hippocampal neuronal structure. All of the authors are grateful for the many contributions that Jürgen has made to the field of neuroanatomy.

Disclosure

The authors have no conflicts of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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