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Increased Excitability and Excitatory Synaptic Transmission During *in Vitro* Ischemia in the Neonatal Mouse Hippocampus

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Abstract

Objective—The present study tested the hypothesis that exposure to *in vitro* hypoxia-ischemia alters membrane properties and excitability as well as excitatory synaptic transmission of CA1 pyramidal neurons in the neonatal mouse.

Methods—Experiments were conducted in hippocampal slices in P7-P9 C57Bl/6 mice using whole-cell patch clamp in current- and voltage-clamp mode. Passive (membrane potential V_m , input resistance (R_{in}) and active (action potential (AP) threshold and amplitude) membrane properties of CA1 pyramidal neurons were assessed at baseline, during 10 min *in vitro* ischemia (oxygen-glucose deprivation (OGD)) and during reoxygenation. Spontaneous and miniature excitatory post-synaptic currents (s and mEPSCs) were studied under similar conditions.

Results—OGD caused significant depolarization of CA1 pyramidal neurons as well as decrease in AP threshold and increase in AP amplitude. These changes were blocked by the application of tetrodotoxin, indicating Na^+ channels involvement. Following 10 min of reoxygenation, significant membrane hyperpolarization was noted and it was associated with a decrease in R_{in} . AP threshold and amplitude returned to baseline during that stage. sEPSC and mEPSC frequency increased during both OGD and reoxygenation but their amplitude remained unchanged. Additionally, we found that OGD decreases I_h (hyperpolarization activated depolarizing current) in CA1 neurons from neonatal mice and this effect persists during reoxygenation.

Significance—These results indicate that *in vitro* ischemia leads to changes in membrane excitability mediated by sodium and potassium channels. Further, it results in enhanced neurotransmitter release from presynaptic terminals. These changes are likely to represent one of the mechanisms of hypoxia/ischemia-mediated seizures in the neonatal period.

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Keywords

electrophysiology; excitability; excitatory postsynaptic current; seizure; neonatal brain

Introduction

Neonatal hypoxic-ischemic brain injury is the most common cause of seizures in the newborn period (Tekgul et al., 2006; Vasudevan and Levene, 2013). Early life seizures are an important marker of subsequent neurodevelopmental impairments, including development of post-neonatal epilepsy and cerebral palsy (Brunquell et al., 2002; Legido et al., 1991; Nelson and Ellenberg, 1979; Sheth, 1999; Tekgul et al., 2006; Vasudevan and Levene, 2013). Indeed mounting experimental evidence suggests that neonatal seizures are not only a marker of severe underlying disorder but also can contribute to brain injury (Holmes, 2005; Miller et al., 2002; Scher, 2003). While we understand a lot about the underlying developmental characteristics favoring excitability in the neonatal brain, little is known of the precise mechanisms leading from a hypoxic insult to seizures in the immature brain.

A large amount of data supports the presence of an underlying higher excitability in the immature brain due to the asynchronous developmental maturation of the GABAergic inhibitory and glutamatergic excitatory synaptic transmission (Brooks-Kayal, 2005; Jensen, 2006). NR2B subunit containing NMDA receptors with long current decay times and low magnesium sensitivity are present in multiple brain regions, including the hippocampus, in immature animals when compared to adult animals (Cathala et al., 2000; Hestrin, 1992; Morrisett et al., 1990). Similarly, AMPA receptors in the immature hippocampus are deficient in GluA2 subunit resulting in Ca^{2+} permeable AMPA receptors (Pickard et al., 2000; Sanchez et al., 2001). These NMDA and AMPA receptors characteristics lead to greater Ca^{2+} influx in response to hypoxia-ischemia, which may lower seizure threshold. Other mechanisms of hyperexcitability in the immature brain include postnatal synaptic density overshoot (Rakic et al., 1986). All of these developmentally regulated characteristics contribute to an excitation/inhibition imbalance with overall increased excitability.

Early life seizures in humans often involve the neocortex, however, autopsy studies in infants with seizures have consistently showed concomitant involvement of the hippocampus (Aso et al., 1989; Rodríguez et al., 2001). Further, in the setting of hypoxia-ischemia, hippocampal injury has been demonstrated on brain MRI studies of newborns with hypoxic-ischemic encephalopathy (Kasdorf et al., 2014; Okerefor et al., 2008). The CA1 region of the hippocampus particularly has been shown to be a region of high vulnerability after hypoxia-ischemia (Gee et al., 2006) including in the postnatal period (Kumral et al., 2006).

CA1 pyramidal cells are interconnected by local recurrent collaterals of their axons (Crépel et al., 1997; Thomson and Radpour, 1991). In adult rats, low levels of interconnectivity have been reported in CA1 pyramidal neurons, but those result in large excitatory postsynaptic potentials (Deuchars and Thomson, 1996). Further, under pathological conditions such as ischemia the CA1 local excitatory network may be activated, leading to increased

excitability(Fan et al., 2008). Interestingly, in culture, CA1 neurons have high recurrent connectivity, and can generate recurrent synchronous bursts in response to blocking GABA receptors or lowering extracellular magnesium(Johnson SE, Hudson JL, 2015). Therefore, alterations in CA1 neuronal excitability following hypoxia-ischemia may be important in the pathophysiology of hypoxia-induced seizures.

The effects of hypoxia on membrane properties in CA1 pyramidal neurons from immature animals are incompletely understood. In this study, we propose to investigate the effects of *in vitro* ischemia on CA1 pyramidal neuron membrane properties as well as excitatory synaptic transmission using the oxygen-glucose deprivation (OGD) model(Tanaka et al., 1999). This *in vitro* design was selected to allow for the evaluation of hypoxia and reoxygenation separately. We hypothesize that exposure to *in vitro* hypoxia-ischemia alters membrane properties and excitability as well as excitatory synaptic transmission of CA1 pyramidal neurons in the neonatal mouse.

2. Experimental Procedure

2.1- Animals

All procedures were approved by the Animal Care and Use Committee of the University of Virginia and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines. Time-pregnant C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and housed with free access to food and water until pup delivery. Following birth, pups were reared with the dam until the experimental day.

Hippocampal slice preparation—All chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise specified. Following anesthesia with isoflurane, P7 to P9 C57BL/6J were decapitated and brains placed in an 2-4°C slicing solution (in mM) NaCl 65.5, KCl 2, MgSO₄ 5, KH₂PO₄ 1.1, CaCl₂ 1, glucose 10, sucrose 113) saturated with 95% O₂ / 5% CO₂. Coronal slices (300 µm) were prepared using a vibratome (Leica VT 1200S, Germany) and kept in aCSF containing (in mM): NaCl 127, KCl 2, CaCl₂ 1.5, MgSO₄ 1.5, KH₂PO₄ 1, NaHCO₃ 25.7, D-glucose 10 (pH 7.4, 305-320 mOsm) and continuously oxygenated with 95% O₂ / 5% CO₂ for a minimum of 30 min prior to use.

2.2- Oxygen-glucose deprivation protocol

In vitro hypoxic-ischemic conditions were created as previously described(Tanaka et al., 1997) by perfusing hippocampal slices with aCSF equilibrated with 95% N₂ / 5% CO₂ and deprived of glucose (oxygen glucose deprivation, OGD), a method shown to lead to a rapid and reproducible response. OGD was maintained for 10 min, after which oxygenated and glucose containing ACSF was superfused.

2.3- Whole-cell patch-clamp electrophysiology

Recordings were obtained from visually identified CA1 pyramidal hippocampal neurons using a Nikon Eclipse E600FN microscope equipped with a 40X water-immersion objective and a high performance CCD camera. Each neuron was recorded for 10 min epochs before, during and after OGD. Thick-walled borosilicate patch electrodes (1.5 mmOD, 0.86 mmID)

were pulled on a P-97 Flaming-Brown horizontal puller (Sutter Instruments, Novato, CA), using a three-stage pull to a final resistance of 3-8 m Ω .

Active and passive neuronal membrane properties were assessed using standard current clamp patch-clamp electrophysiology techniques (Staff et al., 2000). Glass microelectrodes were filled with an internal solution containing (in mM): potassium gluconate 120, NaCl 10, MgCl₂ 2, EGTA 0.5, HEPES 10, Na ATP 4, NaGTP 0.3 (pH 7.2, 280-295 mOsm). Resting membrane potential (RMP) was recorded immediately upon seal rupture and only cells with RMP of at least -55 mV were used. Once stable access was obtained input resistance (R_{in}), action potential (AP) threshold, amplitude and $\frac{1}{2}$ width were assessed by applying 300 msec current pulses (from -60 to +80 pA, 5 pA increments) at the end of each 10 min epoch (baseline, OGD and reoxygenation) under current clamp. Input resistance was calculated as the slope of the linear fit of the voltage-current plot. AP threshold were determined as the most negative membrane potential at which an AP was evoked. Time constant was assessed by applying a 50 msec, 10 pA pre-pulse. In some experiments, tetrodotoxin, (TTX, 1 μ M, Alomone Laboratory, Jerusalem) was added to the external solution to block sodium channels. To investigate the role hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels hyperpolarizing current steps were applied as previously described (Zhang et al., 2006). The hyperpolarization activated depolarizing current (I_h)-mediated component was assessed by measuring the depolarizing response (sag – steady state).

Excitatory post-synaptic currents (EPSCs) were recorded using standard voltage clamp patch-clamp electrophysiology techniques at 30°C (Mangan and Kapur, 2004). In brief, glass microelectrodes were filled with an internal solution containing (in mM): CsMeSO₄ 117.5, MgCl₂ 1, HEPES 10, EGTA 0.3, TEACl 10, CsCl 15.5, NaCl 8, ATP magnesium salt 4, lidocaine N-ethyl-bromide 5 (pH 7.3, 285-295 mOsm). Slices were perfused in aCSF containing picrotoxin (50 μ M) to block GABA receptor-mediated currents. For AP-independent miniature EPSCs (mEPSCs) recording, TTX (1 μ M) was added to block APs. Recordings were made at a holding potential of -60mV. Series resistance and capacitance were compensated for each neuron.

Currents were recorded after input resistance stabilization with a 200A Axopatch amplifier (Axon Instruments, Union City, CA) and low-pass filtered at 2-3 kHz with an 8-pole Bessel filter prior to digitization (Digidata 1322 digitizer; Molecular Devices, Sunnyvale, CA) storage and display (Clampex 10.1 software; Molecular Devices).

Off-line analysis of current-clamp experiments was performed using Clampfit 10.1. EPSCs analysis was performed using MiniAnalysis software (Synaptosoft, Decatur, GA). Amplitude, frequency, and decay times were analyzed using a threshold for current detection at 3 times the root mean square of baseline noise. All currents detected were visually confirmed. Decay was analyzed by fitting individual post-synaptic currents with a 10-90% rise time < 3 msec to a 2-exponential curve characterized by 2 time constants (τ_1 and τ_2) and accepted if $r^2 > 0.70$. To directly compare decay times between experimental conditions, τ_1 and τ_2 were combined into a weighted time constant (τ_w) using the equation: $\tau_w = [(\tau_1 \times A_1) + (\tau_2 \times A_2)] / [A_1 + A_2]$, where τ_1 and τ_2 represent the fast and slow decay

times, respectively; and A_1 and A_2 represent the amplitude of the fast and slow components, respectively (Rumbaugh and Vicini, 1999).

2.4- Statistics

All current clamp data as well as EPSC frequency values are reported as means while EPSC amplitude and kinetics are reported as mean of the medians. EPSC amplitude and kinetics are described by median values because they represent skewed populations and are not normally distributed. To compare the frequency of occurrence of mEPSCs between baseline, OGD and reoxygenation, cumulative inter-event interval frequency histograms were constructed, and a Kolmogorov–Smirnov (K-S) test was used to detect statistically significant differences. All other statistical analyses were performed using Sigma Stat 3.0 software (Systat Software Inc., Point Richmond, CA). Conditions outcomes were compared using One-Way ANOVA for multiple groups and Tukey's multiple comparison test for post-hoc analysis. A value of $P < 0.05$ was considered statistically significant. Of note number of animals and number of slices (n, cells) is specified for each experiments in the results section.

3. Results

2.1- Effects of OGD on passive and active CA1 neurons membrane properties in the neonatal mouse

To examine how OGD affects CA1 neurons intrinsic membrane properties, we evaluated changes in membrane voltage (V_m) at baseline and during OGD by recording in current-clamp mode (**Table 1**). The mean RMP at membrane rupture was -62.5 ± 0.6 mV. Recordings were started after initial stabilization of baseline was achieved (5-10 min). During the 10 min baseline recording, V_m remained stable at -66.4 ± 0.9 mV (n=20, 12 animals) and as seen in **Figure 1A**, progressive membrane depolarization was observed with onset of OGD. By the end of the 10 min OGD period, we observed a significant depolarization with a V_m reaching a peak of -63.0 ± 1.2 mV ($p=0.02$ vs. baseline, ANOVA, **Figure 1B**).

Baseline input resistance, time constant as well as active membrane properties (AP firing properties) were measured at the end of the 10 min baseline epoch just prior to OGD. Interestingly, no changes in the membrane input resistance (R_{in}) were detected at the end of OGD: 41.6 ± 5 m Ω vs. 43.7 ± 4.1 m Ω at baseline (**Figure 1C**). However, the membrane time constant decreased significantly from 32.6 ± 3.4 msec at baseline to 23.4 ± 2.5 msec at the end of OGD ($p=0.02$ vs. baseline; ANOVA) indicating significant changes in membrane capacitance during OGD which may be reflecting reversible changes in cell size during OGD exposure.

Exposure to OGD also resulted in significant changes in active membrane properties in CA1 pyramidal neurons (**Table 1 and Figure 1D**) including decrease in AP threshold ($p<0.001$ vs. baseline, ANOVA) as well as an increase in AP amplitude ($p=0.006$ vs. baseline; ANOVA). A combination of depolarization and reduced AP threshold likely increases the

excitability of CA1 pyramidal neurons from neonatal mice. Of note most of the neurons recorded (15/20) were bursting neurons (**Figure 1D**).

2.2- Effects of reoxygenation on passive and active CA1 neurons membrane properties in the neonatal mouse

After a 10 min exposure to OGD, slices were perfused with oxygenated aCSF and progressive membrane hyperpolarization was observed (**Figure 1A**) with V_m reaching a trough of -70.6 ± 0.9 mV within 6.2 ± 0.2 min of reoxygenation ($p < 0.001$ vs. baseline and OGD, ANOVA). Subsequently, the V_m remained stable throughout the remainder of the recording (15.5 ± 0.9 min) with no return to pre-OGD baseline observed. During reoxygenation, R_{in} and time constant decreased to 37.0 ± 5.2 m Ω ($p = 0.008$ vs. baseline, ANOVA) and 18 ± 2.8 msec ($p < 0.01$ vs. baseline, **Figure 1C**), respectively. AP threshold and amplitude returned to baseline values during that stage (**Table 1** and **Figure 1D**). These data indicate decreased membrane excitability in the early reoxygenation period following exposure to OGD. We did not observe a recovery of the membrane potential back to baseline over the 10 min recording during reoxygenation.

2.3- Mechanisms of OGD-mediated depolarization

Opening of voltage-gated sodium channels during hypoxia has been shown to lead to depolarization (Eijkelkamp et al., 2012) and represent a likely mechanism of the depolarization we observed during OGD. To confirm this, TTX was applied to the bath to block sodium channels. As shown in **Figure 2**, in CA1 neurons exposed to OGD in the presence of TTX in the perfusing ACSF, membrane depolarization during OGD was prevented ($V_m -65.2 \pm 1.0$ mV, $p = 0.438$ vs. baseline, $n = 6$; 4 animals). In contrast, TTX did not block the membrane potential hyperpolarization during reoxygenation ($V_m -68.6 \pm 1.6$, $p = 0.022$ vs. baseline). These results indicate that hypoxic depolarization during OGD is at least in part mediated by activation of sodium channels.

2.4- Mechanisms of reoxygenation-mediated hyperpolarization

To investigate the type of channels involved in the observed hyperpolarization during reoxygenation, we studied the membrane potential reversal in voltage clamp mode in the presence of TTX, D-APV, DNQX and bicuculline ($n = 4$). Results from these experiments indicate a reversal potential around -84.7 ± 2.1 mV. Based on the Nernst equation, the potassium equilibrium potential (E_K) of our solutions is -94.2 mV versus -57.8 mV and $+61.6$ mV for chloride and sodium, respectively. This suggests that potassium channels are involved in the membrane potential hyperpolarization observed during reoxygenation.

Because of the hyperpolarization observed during reoxygenation, we wanted to explore the effects of OGD and reoxygenation on hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels as they have been shown to play a critical role in intrinsic excitability regulation and are responsible for a hyperpolarization activated depolarizing current (I_h) (Zhang et al., 2006). Therefore, additional experiments were performed to investigate the effects of OGD and reoxygenation on HCN channels. In control CA1 pyramidal neurons, hyperpolarizing current steps evoked a slowly developing depolarizing voltage sag typical of I_h -mediated depolarization followed by late depolarization (**Figure 3**). Although the sag

amplitude was not changed during OGD or reoxygenation, the late hyperpolarization was significantly decreased after reoxygenation suggesting that OGD followed by reoxygenation may result in decrease I_h . These findings are in line with previously published evidence indicating that I_h is persistently decreased after hypoxic seizures in CA1 pyramidal neurons from immature rats (Zhang et al., 2006). The mechanisms of hyperpolarization during reoxygenation were not investigated further in our system.

2.5- Effects of OGD on excitatory synaptic transmission

To investigate the effects of OGD on excitatory synaptic transmission, we measured the effects of OGD and reoxygenation on spontaneous excitatory postsynaptic currents (sEPSCs) using voltage-clamp electrophysiology in P7-9 mice (**Figure 4**). We found that OGD results in a 75% increase in sEPSC frequency from 0.40 ± 0.11 Hz to 0.70 ± 0.17 Hz ($p=0.01$ vs. baseline, ANOVA, $n=7$, 5 animals). During reoxygenation, sEPSC frequency remained elevated at 0.80 ± 0.21 Hz ($p=0.01$ vs. baseline, ANOVA). sEPSCs amplitude did not change: 17.9 ± 1.3 pA at baseline vs. 21.9 ± 1.9 pA during OGD and 16.3 ± 2.1 pA during reoxygenation (median, $p=0.14$, ANOVA).

To determine the relative contribution of presynaptic mechanisms underlying increased sEPSC frequency, TTX was added to study AP-independent release (**Figure 5**). Under these condition, we again noted a significant increase in mEPSC frequency during OGD; from 0.21 ± 0.04 Hz at baseline to 0.32 ± 0.04 Hz at the end of the OGD period ($p=0.007$ vs. baseline, ANOVA [$n=10$, 9 animals]). Similarly, mEPSCs frequency remained elevated during reoxygenation at 0.45 ± 0.09 Hz ($p=0.007$ vs. baseline, ANOVA). These results indicate that the increase in EPSC frequency during OGD and reoxygenation is AP-independent. There was no change in mEPSCs amplitude (16.2 ± 1.5 , 17.9 ± 3.5 and 17.8 ± 3.2 pA at baseline, OGD and reoxygenation, respectively, median, $p=0.52$, ANOVA). However, decay times were prolonged during OGD and reoxygenation. Tau1 was 6.1 ± 0.2 msec at baseline and increased to 7.1 ± 0.4 msec during OGD and 7.5 ± 0.4 msec during reoxygenation ($p=0.002$ vs. baseline, ANOVA). Tau2 also increased to 12.3 ± 0.6 msec during OGD and 12.3 ± 0.6 msec during reoxygenation from 10.9 ± 0.7 msec at baseline ($p=0.03$ vs. baseline, ANOVA). To more accurately report decay times, we also calculated a weighted Tau (Tw) as described in the method section. Tw increased from 8.2 ± 0.4 msec at baseline to 9.3 ± 0.4 msec and 9.5 ± 0.4 msec during OGD and reoxygenation, respectively, $p=0.001$ vs. baseline, ANOVA. These results indicate that both pre-synaptic and post-synaptic mechanisms are likely to underlie the effects of OGD and reoxygenation on excitatory synaptic transmission.

4. Discussion

In this study we investigated the effects of OGD and reoxygenation on CA1 pyramidal neurons passive and active membrane properties as well as excitatory synaptic transmission in the neonatal mouse. Our results demonstrate that OGD causes depolarization of CA1 pyramidal neurons and decreases AP threshold. During reoxygenation, there is hyperpolarization of CA1 pyramidal neurons with normalization of AP threshold. Given the noted hyperpolarization during reoxygenation, it is unlikely that extrasynaptic mechanisms

related to changes in membrane properties play a significant role in the progression from hypoxic insult to seizures in the neonatal brain. We also found that *in vitro* ischemia leads to increased excitatory synaptic transmission in immature CA1 neurons both during OGD and during reoxygenation. Our data indicate that OGD and reoxygenation result in increased sEPSC and mEPSC frequency without changes in EPSC amplitude. An increase in the frequency of currents indicates increased release, a presynaptic mechanism. These presynaptic mechanisms are likely to represent a critical step in the generation of hypoxic seizures in the neonatal brain. We also found a small but significant change in decay time kinetics suggesting that postsynaptic mechanisms also play a role in the pathophysiology of seizures after hypoxia in the neonatal brain.

Both hypoxia and substrate depletion are necessary to produce brain injury in neonates (du Plessis and Volpe, 2002). OGD is a well-described and widely-used method to produce hypoxic-ischemic conditions *in vitro* (Tasca CI, Dal-Cim T, 2015) including in the immature brain (Fernández-López et al., 2005). It reproduces findings of *in vivo* brain ischemia including increase in glutamate release, inhibition of mitochondrial complexes, decrease in ATP production as well as NO production (Fernández-López et al., 2005). For the purpose of this study, it had the added advantage of allowing an independent assessment of the effects of *in vitro* ischemia versus reoxygenation.

Studies using intracellular recording techniques and sharp electrodes have shown that hippocampal pyramidal neurons from adult rats respond to *in vitro* hypoxia-reoxygenation in a triphasic manner. After a brief period of hypoxic depolarization, a period of hypoxic hyperpolarization associated with a 50% decrease in R_{in} is seen. During reoxygenation a more pronounced hyperpolarization phase with increased R_{in} is seen with recovery to baseline over 10 to 20 min (Fujiwara et al., 1987; Hsu and Huang, 1997; Leblond and Krnjević, 1989). The transient hypoxic hyperpolarization is not seen in a glucose-free environment (Shimizu et al., 1996) and can be blocked by the ryanodine receptor inhibitor dantrolene, suggesting a role of intracellular calcium stores (Krnjević and Xu, 1989). Different results were reported in a study using the whole-cell patch clamp technique in young adult rats (Zhang and Krnjević, 1993). In this study, brief anoxia (3-6 min) resulted in minor depolarization instead of hyperpolarization and small reduction in input resistance (12%).

Previous studies have shown that neonatal rats are significantly more resistant to anoxia than adult rats. In P5-8 rats, significantly longer exposures to OGD were required to observe anoxic depolarization (Luhmann and Kral, 1997). Further, in P1-4 rats, excitatory postsynaptic potentials (EPSPs) amplitude were not abolished even after prolongation of anoxia to 7 min in contrast to adult rats where EPSPs were reduced by 90% after a 2 min exposure (Cherubini et al., 1989). Nevertheless, prolonged OGD (50 min) in somatosensory slices from P0-4 rats, causes profound dysfunction in subplate neurons and pyramidal cortical neurons with long-lasting ischemic hyperpolarization followed by ischemic depolarization and post-ischemic hyperpolarization (Albrecht et al., 2005). Additionally in P4-5 rats, shorter anoxic episodes (4-6 min) lead to rapid and reversible depression of neuronal activity as indicated by decrease in giant depolarizing potentials as well as EPSP amplitude both in whole-cell and extracellular field recordings in the stratum radiatum of

CA3/CA1(Dzhala et al., 1999). The same investigators also demonstrated that hippocampal neurons from P5 rats respond to OGD in a biphasic manner with initial slight hyperpolarization followed by anoxic depolarization with prolongation of OGD. In this study, epileptiform discharges were observed just prior to the start of anoxic depolarization. Of note, adenosine inhibitor increased the incidence of epileptiform discharges and precipitated the start of anoxic depolarization suggesting that seizure aggravate anoxic brain injury(Dzhala et al., 2001, 2000).

Our results are in line with these previous studies and demonstrate that OGD also significantly alters neuronal membrane properties in the neonatal mouse. We did not observed a phase of ischemic hyperpolarization in CA1 neurons but found that immature CA1 neurons respond to OGD with significant depolarization and decreased AP threshold followed by significant hyperpolarization during reoxygenation. These changes would lead to increased excitability via extrasynaptic mechanisms in immature animals. However, hypoxic seizures are typically observed in the reoxygenation period in neonates. During the reoxygenation period, we observed significant membrane hyperpolarization, which leads to decreased excitability. Based on these findings, we speculate that ischemia-mediated changes in membrane properties do not play a critical role in seizure generation after a hypoxic insult in the immature brain.

Our results also suggest that following *in vitro* ischemia, there is a decrease in *I_h* during the reoxygenation phase in CA1 pyramidal neurons from neonatal mice. These results are in concordance with a previous study from Zhang et al, demonstrating persistent downregulation of *I_h* following hypoxic seizures in immature rats(Zhang et al., 2006). HCN channels are continuously active at resting Vm and are activated by Vm hyperpolarization resulting in stabilization of the resting membrane potential(Benarroch EE, 2013). HCN channels have been implicated in regulation of network excitability and their role in epileptogenesis has been extensively studied(Noam et al., 2011). Loss of expression and function of HCN1 has been shown to occur in various epilepsy models including pediatric seizure models such as febrile seizures(Brewster et al., 2002). In addition, rapid loss of dendritic input has been shown to occur following status epilepticus(Jung et al., 2011). Because HCN input is mainly dendritic in CA1 pyramidal neurons(Magee, 1999), a loss of *I_h* may in fact result in increased synaptic input and overall increased excitability.

Although we found changes in decay time during the OGD and reoxygenation exposure, we did not see significant changes in events amplitude. These findings are in contrast to previous data demonstrating increased sEPSC frequency and amplitude 1h after hypoxic seizures in neonatal rats(Rakhade et al., 2012, 2008). In these studies, posttranslational modification, specifically phosphorylation of AMPA receptor GluR1 subunit at S831 and S845 was found to occur rapidly after hypoxic seizure. This step appears to be critically important in the initiation of seizures as demonstrated by delayed latency to PTZ-induced seizures in transgenic knock-in mice with deficits in GluR1 S831 and S845 phosphorylation(Rakhade et al., 2012). These studies elegantly emphasized a key role of postsynaptic mechanisms in the pathophysiology on ongoing hypoxic seizures in immature rodents. As detailed above, our study indicates a predominant role for presynaptic mechanisms of glutamate release. Some of the differences in the findings may be attributed

to the different models (*in vivo* versus *in vitro*) as well as differences in timing. Because we are trying to resolve how hypoxia causes seizures acutely, we elected to use the OGD model and measure changes during the actual insult. We found that during OGD exposure and immediately after, excitatory synaptic transmission in CA1 neurons does increase, likely representing one of the major mechanisms of hypoxia-induced seizures. Based on our finding of increased EPSC frequency without changes in amplitude, we conclude that presynaptic mechanisms of glutamate release play a critical role. This is a novel finding that may represent a critical step in the progression from a hypoxic insult to seizures in the developing brain.

In summary, *in vitro* ischemia alters CA1 pyramidal neurons membrane properties with depolarization during *in vitro* asphyxia followed by hyperpolarization. In addition, excitatory synaptic transmission increases both during OGD and reoxygenation and presynaptic mechanisms appear to play an important role in this observation. Further studies are underway to investigate the mechanisms underlying these observations.

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HIGHLIGHTS

- CA1 pyramidal neurons membrane properties are altered during oxygen-glucose deprivation leading to increased excitability.
- Excitatory synaptic transmission is increased during both oxygen-glucose deprivation and reoxygenation.
- Excitatory postsynaptic current amplitude is unchanged indicating increased release and presynaptic mechanisms.
- These mechanisms may represent key steps in the progression from ischemic insult to acute seizure in the neonatal brain.

Figure 1. Effects of OGD and reoxygenation on passive membrane properties in CA1 pyramidal neurons from P7-9 mice. Panels A-C: Passive membrane properties

(A): Representative recording showing significant depolarization of membrane potential during OGD followed by membrane potential hyperpolarization during reoxygenation. Inset demonstrates changes in Vm just prior to OGD onset, at the end of OGD and at the end of reoxygenation (area depicted are highlighted by the rectangles). **(B):** Representative IV curves showing no change in Rin during OGD, and decrease in Rin following reoxygenation. **(C):** Summary of Vm data for the entire group (n=20 cells, 12 animals). * p=0.02 vs. baseline and ** p<0.001 vs. baseline (ANOVA with Tukey's multiple comparison test for post-hoc analysis). **Panel D: Active membrane properties. A**

representative bursting CA1 pyramidal neuron demonstrating decreased AP threshold during OGD followed by return to baseline with reoxygenation.

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Figure 2. Effects of TTX on membrane properties during OGD and reoxygenation in CA1 pyramidal neurons from P7-9 mice

To investigate mechanisms of hypoxic depolarization, experiments were performed in the presence of 1 μ M TTX. **Panel A:** The presence of TTX in the superfusing aCSF solution blocked membrane potential depolarization during OGD. The hyperpolarization during reoxygenation was preserved. Recording sections outlined by rectangle are shown in the inset and demonstrate the absence of Vm change during OGD in the presence of TTX.

Panel B: Summary of Vm data for the entire group (n=6 cells, 4 animals). * $p=0.022$ vs. baseline (ANOVA with Tukey's multiple comparison test for post-hoc analysis). **Panel C:** Similar to recordings in the absence of TTX, no change in Rin was observed during OGD, however a decrease in Rin was observed following reoxygenation.

Figure 3. Effect of OGD and reoxygenation on HCN channels in the neonatal mouse

Hyperpolarizing steps were applied to CA1 neurons in current clamp mode to evaluate the effects of OGD on HCN channels. **Panel A:** Representative recording from CA1 neuron showing decrease in I_h amplitude in OGD and reoxygenation vs. baseline (similar initial hyperpolarization sag but smaller late depolarization). **Panel B:** Pooled amplitudes of hyperpolarization-induced depolarization responses demonstrating reduction in I_h current in both OGD and reoxygenation. The amplitudes of the subtracted depolarizing responses were significantly after exposure to OGD followed by reoxygenation (* $p < 0.05$, reoxygenation vs. baseline, ANOVA with Tukey's multiple comparison test for post-hoc analysis).

Figure 4. Effects of OGD and reoxygenation on sEPSCs

Panel A: Representative voltage-clamp traces of sEPSCs recorded at baseline, during and after OGD, demonstrating increased sEPSCs frequency during both OGD and reoxygenation.

Panel B: A representative inter-event cumulative histogram is shown.

* $p < 0.0001$ vs. baseline (K-S test). Inset showing significant increase in sEPSC frequency during OGD and reoxygenation for the entire group ($n=7$, 5 animals, ** $p=0.01$ vs. baseline, ANOVA with Tukey's multiple comparison test for post-hoc analysis). **Panel C:** A representative amplitude cumulative histogram is shown illustrating that sEPSC amplitude remains unchanged during OGD and reoxygenation. K-S test = NS vs. baseline.

Figure 5. Effects of OGD and reoxygenation on mEPSCs

Panel A: Representative voltage-clamp traces of mEPSCs recorded at baseline, during and after OGD, demonstrating increased mEPSCs frequency. **Panel B:** A representative inter-event cumulative histogram is shown. * $p < 0.0001$ vs. baseline (K-S test). A frequency histogram for 1 representative CA1 pyramidal neuron is shown in the inset and demonstrates that the increase in sEPSC frequency remained stable during the OGD and reoxygenation periods. Arrows indicate start of OGD and start of reoxygenation. **Panel C:** A representative average curve is shown, demonstrating that OGD and reoxygenation did not lead to mEPSC amplitude change. Representative example of the goodness of fit is shown in the inset.

Table 1

Effects of OGD on passive and active membrane properties

	Baseline Mean $\pm$ SEM (n)	OGD Mean $\pm$ SEM (n)	Reoxygenation Mean $\pm$ SEM (n)
Passive membrane properties			
V _m (mV)	-66.45 ± 0.91 (20)	-63.03 ± 1.25 (20) *	-70.64 ± 0.94 (18) [#]
R _{in} (m Ω)	43.69 ± 4.09 (19)	41.56 ± 5.04 (18)	37.02 ± 5.23 (15) **
Membrane time constant (msec)	32.6 ± 3.4 (18)	23.4 ± 2.5 (18) *	18 ± 2.8 (16) [#]
Active membrane properties			
AP threshold (mV)	-35.96 ± 1.58	-40.07 ± 1.34 [#]	-31.76 ± 5.37
AP amplitude (mV)	96.78 ± 2.67	102.28 ± 2.84 **	92.55 ± 4.41 ^{β}
AP 1/2 width (msec)	1.52 ± 0.09	1.54 ± 0.09	1.75 ± 0.22
Conductance velocity (V/s)	15.2 ± 0.76	14.96 ± 0.95	13.2 ± 1.28

n=number of cells, 12 animals.

Comparisons between groups were assessed by One-Way ANOVA and Tukey's post-hoc test for multiple comparisons.

*
p<0.05**
p<0.01#
p<0.001 vs. baseline ^{β}
p<0.05 vs. OGD.