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Neuronal Circuit Activity during Neonatal Hypoxic–Ischemic Seizures in Mice

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Abstract

Objective: To identify circuits active during neonatal hypoxic–ischemic (HI) seizures and seizure propagation using electroencephalography (EEG), behavior, and whole-brain neuronal activity mapping.

Methods: Mice were exposed to HI on postnatal day 10 using unilateral carotid ligation and global hypoxia. EEG and video were recorded for the duration of the experiment. Using immediate early gene reporter mice, active cells expressing *cfos* were permanently tagged with reporter protein tdTomato during a 90-minute window. After 1 week, allowing maximal expression of the reporter protein, whole brains were processed, lipid cleared, and imaged with confocal microscopy. Whole-brain reconstruction and analysis of active neurons (colocalized tdTomato/NeuN) were performed.

Results: HI resulted in seizure behaviors that were bilateral or unilateral tonic–clonic and nonconvulsive in this model. Mice exhibited characteristic EEG background patterns such as burst suppression and suppression. Neuronal activity mapping revealed bilateral motor cortex and unilateral, ischemic somatosensory cortex, lateral thalamus, and hippocampal circuit activation. Immunohistochemical analysis revealed regional differences in myelination, which coincide with these activity patterns. Astrocytes and blood vessel endothelial cells also expressed *cfos* during HI.

Interpretation: Using a combination of EEG, seizure semiology analysis, and whole-brain neuronal activity mapping, we suggest that this rodent model of neonatal HI results in EEG patterns similar to those observed in human neonates. Activation patterns revealed in this study

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Author Contributions

Conception and design of study: J.B. and J.K. Acquisition and analysis of data: P.K.W., L.I., D.S., and J.B. Drafting a significant portion of the manuscript or figures: J.B., J.K., and D.S.

Potential Conflicts of Interest

Nothing to report.

help explain complex seizure behaviors and EEG patterns observed in neonatal HI injury. This pattern may be, in part, secondary to regional differences in development in the neonatal brain.

The newborn period is the most common time in life to develop seizures,¹ the majority of which are secondary to hypoxic–ischemic encephalopathy (HIE).² Neonatal seizures are most often unifocal or multifocal and manifest as tonic, clonic, myoclonic, subtle, or subclinical behaviors.^{3–6} Identification of the circuits driving neonatal hypoxic–ischemic (HI) seizures and seizure spread will improve understanding of the mechanisms of seizure semiology and characteristic electroencephalography (EEG) patterns in neonates.

Previous studies have observed both convulsive and nonconvulsive seizures during HI in neonatal rodents.^{7–12} However, the specific brain regions generating these seizures were not examined. Immediate early genes (IEGs), such as *cfos*, *Arc*, and *cjun*, can be used to examine neuronal activity during behavior and pathologic conditions such as seizures and epilepsy.¹³ An increase in *cfos* expression following neonatal HI has been described^{14–20}; however, the contribution seizures make to increased *cfos* expression and the specific brain regions involved have not been described. None of the previous studies used a broad, unbiased sampling of IEG activity throughout the brain, only recently afforded by the use of transgenic mice with IEG-linked Cre-recombinases²¹ and sophisticated processing and imaging of intact clarified tissue samples.²² Furthermore, none of these studies examined the relationship between *cfos* activity and seizures using electrographic recordings.

The goal of this study was to examine the circuits involved in neonatal hypoxic-ischemic seizures by integrating IEG (*cfos*) activity mapping, EEG, and seizure semiology. Using these techniques, we have described patterns of neuronal activity and the associated seizure behaviors and EEG patterns.

Materials and Methods

Animals

All animals used for this study were handled according to a University of Virginia Animal Care and Use Committee approved protocol. Housing was in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services 85–23, 2011). C57/Bl6 mice (Charles River Laboratories, Wilmington, MA, USA) were used for the EEG portions of this study. To generate TRAP (targeted recombination in active populations) mice for neuronal activity mapping, mice expressing Cre-ER under regulation of the Fos promoter (B6.129[Cg]-*Fos*^{tm1.1(cre/ERT2)Luo}/J, #021882; Jackson Laboratories, Bar Harbor, ME, USA) were bred, with mice expressing tdTomato on the Rosa locus (B6.Cg-*Gt(ROSA)26Sor*^{tm9(CAG-tdTomato)Hze}/J, #007909; Jackson Laboratories), both on C57/Bl6 background.²¹ Genotyping was performed using a tail sample on postnatal day (p) 2 to 5 (KAPA Biosystems, Wilmington, MA, USA). Mice of both sexes were used in all experiments.

HI or Sham Procedure

HI injury was created in p10 mice as previously described (permanent unilateral carotid ligation +45 minutes of hypoxia at $\text{FiO}_2 = 0.08$).²³ The sham procedure involved neck incision and equivalent anesthesia exposure without carotid ligation or hypoxia. The surgical field and hypoxia/EEG recording chamber were on circulating water warming mats. Body temperature checks were performed throughout the experiment using an infrared detector to ensure consistent temperatures between 36.0°C and 37.5°C.²⁴ Ambient temperature was continuously monitored in the hypoxia/EEG recording chamber. Roughly 1 hour following ligation, mice were exposed to hypoxia.

Electroencephalogram

To characterize seizures in this model, C57/Bl6 mice had continuous video EEG recording throughout the entire experimental period (Fig 1A). p9 mice had unipolar insulated stainless steel depth electrodes (0.005 inches bare diameter, 0.008 inches coated; A-M Systems, Sequim, WA, USA) stereotactically implanted in the bilateral parietal cortex (−1.2 dorsoventral [DV], ±0.5 mediolateral [ML], and −1.0 deep [D] mm), bilateral CA1 region of the hippocampus (−3.5 DV, ±2.0 ML, and −1.75 D mm), and a reference electrode in the cerebellum.²⁵ Following recovery, mice were exposed to HI on p10. A unity gain impedance matching head stage (TLC2274 Quad Low-Noise Rail-to Rail Operational Amplifier; Texas Instruments, Dallas, TX, USA) was used for recordings, which were begun 1 hour prior to carotid ligation and continued through 2 hours after reoxygenation (excluding the brief ligation procedure); pups were then returned to the dam.

TRAP Neuronal Activity

TRAP mice underwent HI or sham procedure on p10. TRAP mice use the activity-dependent IEG, *cfos*, to drive the expression of tdTomato, an easily-imaged fluorescent protein.²¹ The *fos* locus is linked to a tamoxifen-dependent recombinase, CreER. This allows visualization of active neurons, permanently tagged by tdTomato, in the presence the short-acting tamoxifen metabolite, 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich, St. Louis, MO, USA). Subcutaneous injection of 4-OHT (50mg/kg) suspended in sunflower oil, allows tagging of active neurons in a 1- to 2-hour window around the time of injection. Mice were injected with 4-OHT 30 minutes after completion of hypoxia; sham mice were injected at an equivalent time (see Fig 1A). Mice were transcardially perfused 7 days later, at the time of maximal tdTomato expression.²¹ Previous studies examined *cfos* immunohistochemistry (IHC) versus tdTomato-tagged *cfos* expression in the TRAP model, and found that TRAP has a superior signal-to-noise ratio and time specificity.^{21,26} Validation in this experiment used *cfos* IHC 2 hours after HI. As a negative control, mice that did not receive 4-OHT were found to have no tdTomato expression.

Tissue Clarification and Processing

Brains were postfixed and processed using the passive clarity technique (PACT) method,²² which included tissue-hydrogel polymerization, lipid removal, and tissue mounting. Horizontal sections (200µm thick) were incubated with primary antibody for 5 days, then a

secondary antibody, and then were mounted in imaging media (refractory imaging medium solution).²²

A subset of samples was sliced into thin coronal sections to perform further IHC and validate analysis in nonclarified samples. Briefly, IHC was performed as previously described on free-floating 50µm coronal sections.²⁷ The antibodies used were as follows: anti-NeuN (1:200, MAB377, clone A60; EMD Millipore, Darmstadt, Germany) for 200µm sections, anti-NeuN (1:200, 24307S; Cell Signaling, Danvers, MA, USA) for 50µm sections, anti-GFAP (1:1000, Ab7260; Abcam, Cambridge, United Kingdom), anti-MBP (1:200, Ab62631; Abcam), anti-CD31 (1:100, Ab28364; Abcam), anti-*cfos* (1:1000, Ab190289; Abcam). Fluorescent labels were AlexaFluor488 and AlexaFluorPlus 680 (1:200 or 1:500; Invitrogen, Carlsbad, CA, USA).

Imaging

Imaging was performed using a Zeiss 780 confocal/multiphoton microscope system with Zeiss Zen software for image acquisition (Carl Zeiss, Oberkochen, Germany). For large whole-slice images, 10× magnification was used, and in regions of interest, 20× magnification was used. Excitation wavelengths used for AlexaFluor488, 680, and tdTomato were 488nm, 633nm, and 561nm, respectively. Emission filter ranges for green, tdTomato, and red were 502 to 550nm, 570 to 691nm, and 688 to 755nm, respectively. Tiled images with a Z-stack interval of 10µm were stitched (overlap 15% for 20× images) using Zen software.

Data Analysis

Imaris 9.2.1 software (Bitplane Scientific, Zurich, Switzerland) was used for colocalization analysis of 3 consecutive 200µm-thick, clarified horizontal slices per brain (bregma −2.36 to −2.96). The Imaris Coloc (Bitplane Scientific) algorithm relies on the exclusion of intensity pairs that do not correlate after a detection threshold is set for each channel.²⁸ We examined colocalization between tdTomato- and NeuN-positive cells to compare percentage of active neurons between groups and between hemispheres within groups. In this analysis, cerebellum and olfactory bulbs were excluded. Anatomic labeling of whole slices was performed in Adobe Photoshop (Adobe, San Jose, CA) using an atlas.²⁹

OriginPro 7.5 (OriginLab, Northampton, MA, USA) was used for statistical analysis of colocalization data. Colocalization percentages were analyzed using a 2-sample independent t test. Ipsilateral and contralateral colocalization percentages in both sham and HI groups were compared.

LabChart Pro (ADInstruments, Colorado Springs, CO, USA) was used to collect and analyze video EEG. Video EEG was reviewed and marked for seizures by a researcher (J.B.) and validated with randomly excerpted segments marked by a second researcher (P.K.W.). Reviews were compared to confirm agreement. Descriptive statistics were performed in OriginPro 7.5 and reported as mean ± standard deviation. Total number of seizures, total seizure duration, individual seizure duration and length, and behavioral seizure score (BSS)¹² were recorded for each file. Because BSS does not incorporate details on focality of behaviors, each event was also categorized as focal/unilateral, bilateral, or mixed. Criteria

for electrographic seizure and background attenuation were as previously described.²⁵ Power spectrograms were derived using LabChart Pro.

Results

HI Results in Both Generalized and Focal Seizure Activity in the Neonatal Mouse

To correlate EEG, behavior, and neuronal maps in mice exposed to HI, electrodes were implanted in the hippocampus and the cortex ($n = 30$ [15 female, 15 male] C57Bl6 mice). TRAP mice (xC57Bl6 background; $n = 5$ HI [3 female, 2 male], 5 sham [2 female, 3 male]) were used to map neuronal activity. Examination of behavior recorded during HI revealed characteristic seizure semiology. Behavior was classified by using a neonatal rodent BSS¹² and categorized based on whether behavior was focal/unilateral, bilateral, or mixed. Characteristic seizure semiology generally fell into 3 patterns: (1) repetitive circling to the side of ligation with extension of contralateral extremities, (2) loss of posture with subsequent flexion of the body and tail curled to the side of ligation, or (3) loss of posture with unilateral or bilateral paddling of extremities with varying levels of severity. At times, events involved only 1 of these behavioral patterns; other events were mixed and involved a series of several behaviors. Over half of the events were focal/unilateral or mixed, and the remainder were bilateral only (see Fig 1B). Lastly, there were periods where pups were immobile with sustained seizure activity on EEG (nonconvulsive seizures; see Fig 1C).

EEG was recorded starting from the preligation baseline period through 2 hours of posthypoxia monitoring. Preinjury baseline EEG activity was similar to that described previously in neonatal mice²⁵ (Fig 2A). A subset of mice ($n = 13/30$, 43%) experienced seizures following ischemia alone (prior to hypoxia), all of which were convulsive (median BSS = 5, range = 3–6; see Fig 1C), and lasted 39 ± 50 seconds. Seventy-five percent of mice that died during hypoxia began exhibiting seizures in the prehypoxia/postischemia phase.

Following induction of hypoxia, EEG background amplitude was progressively decreased and intermittently interrupted by bursts of generalized spike-wave discharges lasting between 2 and 4 seconds, followed by suppression of the activity (see Fig 2A). All mice had electrographic seizures, recorded via hippocampal and cortical electrodes bilaterally, which emerged from a suppressed background as rhythmic spike-wave discharges. Over time, these discharges became more complex, with polyspike waves, and occurred more frequently. Eventually, rapid polyspike discharges evolved that were associated with behavioral manifestations (see Fig 2B) during the hypoxic period. Forty percent of mice ($n = 12/30$) experienced nonconvulsive seizures (BSS = 0–2), which occurred only during hypoxia (see Fig 1C). Seizures began on average 5.5 ± 8.1 minutes following the start of hypoxia and lasted 56 ± 57 seconds ($n = 30$). Of the subset of mice that exhibited seizures prior to hypoxia (postligation), induction of hypoxia increased the number of seizure events. Four mice died during hypoxia, all following a convulsive (BSS = 5–6) seizure ($n = 4/30$, 13%).

Following reoxygenation, over half of the surviving mice continued to have seizures ($n = 14/26$, 54%) during the remaining 2 hours of recording. All posthypoxia seizures were convulsive, with a mean length of 52 ± 63.6 seconds. The posthypoxia period exhibited decreased amplitude of EEG background compared to baseline (see Fig 2A). In this cohort,

a total of 287 seizure events were recorded (9 ± 5 events/mouse) and each lasted 54 ± 57.7 seconds. Male mice exhibited significantly more seizure events compared to females (mean = 11.56 vs 6.8; $p = 0.02$), but total seizure time between sexes was not significantly different (594.3 vs 373.1 seconds; $p = 0.075$). Males were more likely to have prehypoxia/postligation events than females ($p = 0.03$), but quantity of hypoxia and posthypoxia events did not differ between sexes.

EEG recordings confirmed that peak seizure burden was during hypoxia, and that all mice had seizures during that phase. Therefore, to map *cfos*-expressing neurons during seizure peak in TRAP mice, 4-OHT was injected 30 minutes following the end of hypoxia (see Fig 1A). This allowed tdTomato to be TRAPed in active cells throughout hypoxia, 15 minutes prior and 30 minutes following hypoxia. Groups of HI and sham mice were perfused and tissue was sliced and clarified. The entire brain was sectioned, imaged, and analyzed to allow an unbiased large-scale view of tdTomato-positive cells throughout the brain, and areas of activation were mapped using an atlas.²⁹ Three brains were excluded secondary to large porencephalic cysts, making neuronal activity analysis impossible. A subset of mice ($n = 3$) was exposed to hypoxia only (no ischemia) and did not exhibit seizures on EEG or increased *cfos* activity on neuronal activity mapping.

More tdTomato-expressing cells were observed in sections obtained from HI mice compared to sham mice (Fig 3A). The ischemic hemisphere was more active than the contralateral hemisphere. Asymmetric activation was apparent in several regions: somatosensory cortex, olfactory system, lateral thalamus, and hippocampal–parahippocampal structures; however, in rostral areas, such as the motor cortex, activation was symmetric (see Fig 3A). Low-level basal activity in sham mice was identified in the midline thalamic nuclei, somatosensory cortex, and cerebellum.

Focal Seizures and Hemispheric Asymmetry in *cfos* Expression and the Power Spectrogram

Mice commonly exhibited focal/unilateral or mixed (focal/unilateral and bilateral) motor seizures during hypoxia (see Fig 1B), with circling toward the ischemic hemisphere and extensor limb posturing contralaterally. Focal motor seizures were described previously in neonatal mice following carotid ligation, with circling to the side of ligation being the most common behavior.^{10–12}

In HI mice, the ischemic hemisphere appeared to have more tdTomato-tagged cells compared to the contralateral side (see Fig 3A). To quantify active neurons, we performed colocalization analysis of NeuN (neuronal marker) and tdTomato-tagged (*cfos* expressing) cells. The ischemic hemisphere in HI mice had significantly more active neurons compared to the contralateral hemisphere ($n = 5$, 2 male/3 female, $p = 0.00001$) and sham hemispheres ($n = 5$, 3 male/2 female, $p = 0.00001$). Hemispheric comparison in sham mice found no difference ($p = 0.832$, see Fig 3B).

The unilateral and focal motor seizure behavior and significant increase in active neurons in the ischemic hemisphere prompted further examination of the EEG recordings for asymmetry in background and seizures. The power spectral analysis revealed clear

asymmetry in background amplitude, with increased amplitude in the ischemic hemisphere and suppressed background in the contralateral hemisphere (see Fig 3C). Relative suppression from baseline activity is demonstrated in both hemispheres (see Fig 3D) during hypoxia, in the contralateral more than ischemic hemisphere, with recovery during reoxygenation.

Neuronal activity became more asymmetric moving caudally through the brain, with the ischemic hippocampal–parahippocampal circuit, somatosensory cortex, and lateral thalamus expressing the majority of *cfos*. Robust neuronal activation was observed both in the primary and secondary somatosensory cortex in the ischemic hemisphere (Fig 4A). Neurons in layers II/III and V were active (see Fig 4C), which have cortico–cortico connections (both to the somatosensory and motor cortex), inputs from lateral thalamus,^{30–32} and contain pyramidal neurons. Both posteromedial (Po) and ventroposteromedial nuclei in the thalamus, which were highly active (Fig 5A), provide input into the primary somatosensory cortex via the lemniscal and paralemniscal pathways.³¹ Layer V of the barrel cortex receives input from the primary somatosensory cortex and motor cortex (layers III and V),³⁰ all of which exhibited active neurons. There are numerous cortico–cortico connections between the motor and somatosensory cortex, including inputs from the barrel cortex to the superficial layers of the primary motor cortex. There was low-level neuronal activity in the contralateral hemisphere, similar to sham, in S1/2 (layers II/III), M1/2 (layers II/III), and medial thalamic nuclei (paraventricular, intermediodorsal, and centromedian; see Fig 5B).

Mice Exhibit Bilateral Tonic–Clonic Seizures and Symmetric *cfos* Expression in the Motor Cortex

Not surprisingly, given the bilateral tonic–clonic nature of nearly half the seizures, there were active neurons bilaterally in the primary and secondary motor cortex, primarily in the superficial layers II/III (see Fig 4B, D). In the motor cortex, *cfos* expression was symmetric, and tdTomato protein was present in the axons crossing midline in the rostral corpus callosum (see Fig 4E). The primary motor cortex receives inputs from the barrel cortex and secondary motor cortex, which also contained tdTomato-positive neurons. Sensory thalamus, Po, and ventrolateral nuclei, which also project to layers II/III of the motor cortex, were prominently active in the ischemic hemisphere (see Fig 5A). The striatum, another major input to the motor cortex via the cortico-striatal-thalamic tract, exhibited tdTomato expression. However, this tdTomato activity did not colocalize with NeuN staining, suggesting that these were not active neurons. It was also noted that the striatum, cortex, and hippocampus contained tdTomato-positive cells that appeared to be lining the blood vessels, leading to further IHC analysis (described below).

Bilateral activation of the motor cortex and presence of tdTomato in rostral corpus callosum fibers suggested that seizures spread to the motor cortices via axons in the anterior corpus callosum. In contrast, activation of the somatosensory cortex was unilateral, and axons in the posterior corpus callosum lacked tdTomato in processes crossing midline (see Fig 4E).

Given that myelination of axons makes propagation potentials more efficient, we examined whether regional differences in myelination were associated with observed differences in seizure spread. Immunohistochemical analysis of myelin basic protein (MBP) expression

revealed expression in the anterior corpus callosum, the same area where tdTomato-containing processes crossed midline (see Fig 4F). However, in posterior sections, where tdTomato expression is asymmetric, MBP expression was not observed (see Fig 4F). These findings are in agreement with previous studies demonstrating that myelination at this age is not mature and in the cerebrum begins in the deep motor cortex and corpus callosum during the second postnatal week.^{33–35}

Other Active Cell Types

During the analysis of neuronal activity, we noted that many tdTomato-expressing cells did not colocalize with NeuN in HI mice. Morphologically, many cells appeared to be astrocytes, which were most prominent in the hippocampus. To confirm this observation, we performed IHC on a subset of HI and sham brains ($n = 2/\text{group}$) for the astrocytic marker, glial fibrillary acidic protein (GFAP). This experiment confirmed that many tdTomato-tagged active cells colocalized with GFAP in the HI group (Fig 6A). These active astrocytes were most prominently displayed in the ischemic hippocampus, cortex, and striatum.

We also observed what appeared to be significant tdTomato expression in vasculature, meninges, and ependymal cells, most prominently in the ischemic hemisphere, which was not present in sham mice. To determine whether these were blood vessels or active astrocytes abutting the vessels as part of the neurovascular unit, we performed further IHC using the CD31 antibody, which is expressed in blood vessel endothelial cells. The tdTomato expression in these areas colocalized with CD31 (see Fig 6B), confirming that blood vessel endothelial cells expressed *cfos* in response to HI. Areas with the most prominent expression of colocalized tdTomato and CD31 were the cortex and striatum.

Focal Neuronal Activity in the Limbic System

The trisynaptic circuit had robust neuronal activity in the ipsilateral hemisphere, including layer II/III of entorhinal cortex (EC), dentate gyrus granule cells, and pyramidal cells in CA3 and CA1 (Fig 7A–C). Given the active neurons noted throughout the cingulate cortex and parasubiculum, we examined the mammillary bodies and anterior thalamic nuclei, as those regions make up the hippocampal–diencephalic–cingulate network (Papez circuit). However, the subiculum, mammillary bodies and anterior thalamus exhibited very few active neurons.

The olfactory bulb had ipsilateral activity in the granule layer of the main olfactory bulb and medial anterior olfactory nucleus. The olfactory area receives input from CA1 and entorhinal cortex and has efferent connections to the pyriform cortex, midline thalamic nuclei, and EC. The septum and amygdala exhibited little to no active neurons.

Discussion

Neonatal HI is associated with activation of multiple circuits resulting in focal and multifocal seizures. These circuits include the somatosensory cortex and hippocampal–parahippocampal circuits in the ischemic hemisphere and bilateral primary motor cortex. Activation of these areas produced complex behavioral and electrographic seizures, similar to the focal and multifocal seizure semiology that has been previously described in human neonates.^{3–6} Burst suppression pattern, commonly seen clinically in HIE,³⁶ was observed in

the ischemic hemisphere on power spectrogram and was associated with increased neuronal activity in the associated cortex. The regions driving comparable electrographic findings in neonates following HI injury, such as focal and multifocal seizures and burst suppression, have not been previously described. Here we characterize these areas in a broad, unbiased fashion by integrating EEG, whole brain IEG (*cfos*) mapping, and seizure semiology.

We observed that this rodent model of neonatal HI results in a characteristic pattern of behaviors consisting of both focal and multifocal seizures. Previous studies have also observed both focal and multifocal seizure semiology,^{7,37,38} such as circling toward the ischemic side during neonatal rodent HI.^{10–12} In conjunction, we observed clear asymmetries in the power spectrogram and *cfos* mapping of neuronal activity. A burst suppression pattern was present in the ischemic hemisphere, whereas the contralateral hemisphere only demonstrated a suppressed background. Similarly, we observed significant asymmetry in *cfos* mapping, with increased neuronal activity in the ischemic hemisphere compared to the contralateral hemisphere. The exception to this asymmetry was observed in the forebrain, which demonstrated symmetric neuronal activation in the primary motor cortex that may be manifested as bilateral tonic-clonic behavior seen in some seizures.

Neonatal seizures are complex events that arise focally and have limited capacity for propagation beyond the original focus.⁵ Similar to our observations in this model, these events can be unifocal or multifocal and produce tonic, clonic, myoclonic, subtle, or no clinical behaviors.^{3,4} The complexity of the acute seizures associated with neonatal HI may arise from regional differences in the developing brain, a marker of which is myelination. We observed bilateral propagation across the frontal lobe, in particular the motor cortex, but not across the parietal and temporal lobes. The corpus callosum contains axons crossing midline to connect these regions; however, the frontal lobe was the only area exhibiting bilateral seizure spread. In addition, this was the only region we observed tdTomato-expressing axons crossing midline. Coinciding, MBP expression was restricted to this region specifically, in the anterior corpus callosum in the region of motor cortex. Immaturity, as marked by a lack of myelination in this study, in the majority of other regions at this age may contribute to the limited propagation of neonatal seizures. Myelination in the rodent is known to begin around p7 to p10,^{33–35} first in the cerebellum and spinal cord. Over the next postnatal week, myelination begins deep in the motor cortex and corpus callosum and spreads outward.³⁴ Although regional differences in myelination observed here and in other studies may contribute to the lack of seizure spread in the neonatal brain, there are likely other complex developmental processes that differ by brain region involved such as synaptogenesis, neurotransmitter development, astrocyte development, neurogenesis, and pruning.^{39–41} Further studies are required to confirm this association.

Although several studies have observed increased *cfos* expression in the context of neonatal HI,^{16,20,42} the contribution of HI-associated seizures has not been described. Similar to our observations, others have described increased *cfos* expression in the ischemic hemisphere^{16,20} and some bilateral expression anteriorly.²⁰ However, these studies have not taken an unbiased, single-cell approach to *cfos* mapping in conjunction with EEG as we have here. The use of the TRAP technique and colocalization with NeuN has allowed us to separate *cfos* expression in neurons versus other cell lines such as astrocytes and endothelial

cells. For example, in agreement with our initial analysis, previous studies have described robust *cfos* expression in the striatum.^{20,42} However, upon further examination we determined that *cfos* expression in the striatum is not neuronal in origin but largely confined to blood vessel endothelial cells, a finding which may relate to HI-induced angiogenesis.⁴³

Furthermore, we suggest that neuronal *cfos* expression observed during HI is due to seizures. This is supported by our use of simultaneous EEG, behavior, and EEG spectral analysis. Other neonatal seizure models, such as deep global hypoxia and pentylenetetrazole-induced seizure, have observed increases in *cfos* associated with seizure behavior.^{15,19} Pharmacologic prevention of seizure behavior during HI¹⁸ and isolated mild global hypoxia without seizures that we and others have observed¹⁴ do not result in *cfos* upregulation, supporting the argument that seizures are driving *cfos* expression in this model. We speculate that the unilateral somatosensory cortex activity and asymmetric circling behavior, bilateral motor cortex activity, and bilateral tonic-clonic behavior, and the hippocampal circuit activity and subclinical seizure behavior are associated. Although tdTomato expression is relatively low in the contralateral hemisphere compared to ipsilateral, it is mainly restricted to the motor cortex, which correlates to the bilateral seizure behavior observed.

Lastly, we demonstrated that neuronal activity is increased in the ischemic cortex, which generates burst suppression patterns, whereas the contralateral cortex exhibits minimal neuronal activity and generates suppressed background EEG activity. Previous studies have proposed that increased excitability^{44,45} and activation of an adenosine triphosphate (ATP)-gated potassium channel expressed in the cortex, result in alternating suppression and activation.⁴⁵ Here we have shown burst suppression patterns on EEG and spectral analysis generated by the same hemisphere exhibiting extensive neuronal activity in the cortex. The energy failure/decreased ATP known to occur during HI⁴⁶ may contribute to the 30 to 50% reduction in ATP required to induce bursting and further reduction leading to suppression by opening an ATP-gated potassium channel.⁴⁶

One limitation of this study is the necessary lag between HI injury with TRAPing of tdTomato in active cells and perfusion/tissue processing 7 days later (optimal tdTomato expression). Significant cell death, particularly in the hippocampus (CA1, CA3, and DG), striatum, cortex, and thalamus, is known to occur in the days to a week following HI,^{23,47–50} which we would not be able to quantify a week later when the tissue is processed. Previous work (unpublished data) quantified hemispheric volumes in this tissue and demonstrated that the ipsilateral hemisphere is 9.1% smaller than the contralateral. This may have led us to underestimate neuronal activity in these areas. Hence, our inability to fully evaluate *cfos* expression in the most severely injured brains (~15% of brains with porencephalic cyst). Though this is a limitation of this technique, the TRAP technique offers superior signal-to-noise ratio and time specificity compared to traditional *cfos* IHC, which has been shown in our work (Fig 8) and in previous work.^{21,26} Also, it is important to point out that the absence of tdTomato expression does not necessarily mean the cell is not active. Induction of *cfos* response varies based on the frequency and strength of stimulus.^{13,51} Conversely, we cannot definitely conclude that *cfos* expression in this model is solely secondary to seizure activity, as HI alone likely results in *cfos* expression secondary to

neuronal depolarization. The limitation of spatial localization of depth electrode recordings, particularly in the small neonatal brain, may have affected our ability to distinguish specific seizure foci on EEG recordings. Lastly, similar to clinical cases of HIE, there is significant variability in lesion size and seizure burden in this model. Subtle differences in EEG findings were observed between males and females; however, imaging analysis was not powered to detect differences between the sexes. Despite this variability, we observed consistent patterns in seizure behavior, *cfos* neuronal activity mapping, and EEG.

Future studies will examine chronic changes in neuronal activity following HI and focus on the circuits that we have identified. Early-life seizures result in permanent changes to neuronal circuitry in rodent models.^{52,53} These early modifications to neuronal networks likely have ramifications on cognition. Subsequent studies will focus on the link between chronic circuit changes and neurocognitive outcomes. Additionally, this was a mesoscale approach to neuronal activity, and further work will focus on a detailed/regional quantification and analysis of activity in different cell lines.

Using a combination of EEG, seizure behavior, and neuronal activity mapping analysis we suggest that this rodent model of neonatal HI results in EEG patterns similar to those observed clinically in HIE, such as unifocal and multifocal seizures, burst suppression, and suppression. Furthermore, we suggest that this electrographic activity generates a complex multicircuit pattern of *cfos*-tagged neuronal activity. Lastly, regional differences in neurodevelopment, such as myelination, may contribute to the complex pattern of neuronal activity and behavioral manifestations of seizures in the neonate.

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FIGURE 1:

Seizure characteristics in p10 mice exposed to neonatal hypoxia–ischemia. (A)

Representative power spectrogram from the ischemic parietal cortex electrode through the experimental timeline. Timed injection of 4-hydroxytamoxifen (4OHT; syringe icon), 30 minutes after the end of hypoxia, to capture a 90-minute window of neuronal activity (pink box; amplitude color heat map scale $\times 10^{-6}$). Arrows indicate the time that raw electroencephalogram tracings below the spectrogram represent. (B) Seizure behaviors for the entire experiment, postischemia/prehypoxia, during hypoxia, and posthypoxia. (C) Behavioral seizure score (BSS) and timing for all seizure events ($n = 30$ mice, each mouse has a unique symbol, each point is a discrete seizure event). One hundred percent of mice seized during hypoxia (blue box; time = –60 minutes is the completion of carotid ligation, time = 0 is the start of hypoxia). Thirteen percent died during hypoxia following a convulsive seizure (grade 5–6). TRAP = targeted recombination in active populations.

FIGURE 2:

Characteristic electroencephalography (EEG) patterns during hypoxia ischemia. (A) EEG background from left to right: preinjury baseline, burst suppression during hypoxia, posthypoxia suppression. Recording from ipsilateral parietal cortex depth electrode. (B) Evolution of a seizure during hypoxia. Recording from ipsilateral hippocampal depth electrode.

FIGURE 3:

Increased neuronal activity in ischemic hemisphere of hypoxic-ischemic (HI) mice. (A) Representative transverse slices (200 μ m-thick clarified tissue, 10 $\times$ tiled image from bregma -2.96) from female HI (top), male sham (middle), and male hypoxia (H) only (bottom) with active cells, tagged with tdTomato, and neurons expressing NeuN (green). Hemisphere ipsilateral (IL) to carotid ligation/ischemia or neck incision in sham group on the left in all slices, contralateral (CL) hemisphere on the right. (B) Quantification of active neurons (percent of cells both TRAPed and expressing NeuN) compared by hemisphere. Ischemic hemisphere had significantly more active neurons compared to the HI CL hemisphere ($p = 0.00001$) and sham IL hemisphere ($p = 0.00001$). The HI CL hemisphere contained more active neurons than the sham IL ($p = 0.013$). Sham mice did not exhibit any significant difference between hemispheres ($p = 0.832$). $*p < 0.05$, $**p < 0.001$ ($n = 5$ [2 male/3 female] HI, $n = 5$ [3 male/2 female] sham). (C) Asymmetric power spectrogram in HI mice during hypoxia (45-minute period) in ischemic cortex (left) and contralateral cortex (right; amplitude scale $\times 10^{-6}$). Burst suppression pattern and seizures in ischemic hemisphere, suppression in CL hemisphere. (D) Background suppression during hypoxia and reoxygenation in IL and CL hemispheres. All measurements of mean voltage taken from 10-

second random excerpts of the encephalogram over the experimental time period (baseline, 30 minutes postligation, during hypoxia—15 minutes and 30 minutes after start, after reoxygenation—15 minutes and 60 minutes after start) were compared to baseline. Each animal's baseline served as its own control, and data are reported as a percentage of baseline ($n = 5$ mice). Measurements were taken from cortical electrodes.

FIGURE 4:

Differential asymmetries in neuronal activity in the somatosensory cortex and motor cortex of hypoxic–ischemic (HI) mice. (A) Asymmetric, increased neuronal activity in ischemic somatosensory cortex of a female HI mouse (10× tiled slice at bregma –1.23). Dashed boxes represent area pictured in C. (B) Symmetric neuronal activity in motor cortex (10× tiled slice at bregma 1.69). Dashed boxes represent area pictured in D. (C) Neuronal activity in ipsilateral (IL) and contralateral (CL) somatosensory cortex, most prominent in layers II/III and V (20× tiled slice at bregma –1.23). (D) Neuronal activity in IL and CL motor cortex, most prominent in layers II/III (20× tiled slice at bregma 1.69). (E) More tdTomato containing axons crossing midline in the anterior corpus callosum (right) than posterior corpus callosum (left; 20× magnification). (F) Myelin basic protein (MBP) expression in anterior corpus callosum (right) and minimal expression in the posterior corpus callosum (left; 20× magnification).

FIGURE 5:

Increased neuronal activity in the ischemic lateral thalamus. (A) Active neurons in the lateral thalamic nuclei including ventroposterolateral (VPL), ventroposteromedial (VPM), and posteromedial (Po; 20× magnification, left and 10× magnification tiled slice, right, at bregma -1.91 , female HI mouse). (B) Symmetric activation in the midline thalamic nuclei. Paraventricular (PV), centromedian (CM), and intermediodorsal (IMD) nuclei. Boxed inset in A and B with neurons colocalizing tdTomato and NeuN. CL = contralateral; IL = ipsilateral.

FIGURE 6:

Astrocytes and blood vessel endothelial cells expressing *cfos* in hypoxic–ischemic mice. (A) tdTomato-tagged active cells colocalized with astrocytic marker, glial fibrillary acidic protein in hippocampus (20× magnification, tiled). (B) tdTomato tagged active cells lining blood vessels, colocalized with endothelial cell marker CD31 antibody (20× magnification, tiled). High resolution insets indicated by the white box outline in merged images.

FIGURE 7:

Neuronal activity in ischemic hippocampal-parahippocampal circuit. (A) Active neurons (colocalized tdTomato and NeuN) in dentate granule (DG) cells and CA1 in coronal slice (20× magnification). (B) Transverse section through hippocampal regions displaying active neurons in CA2, CA3, and DG. (C) Active neurons in a transverse section of medial entorhinal cortex (CEnt) and parasubiculum (PaS). Perirhinal cortex (PRh) and presubiculum (PrS) also display active neurons. Boxed inset in A, B, and C with neurons colocalizing tdTomato and NeuN.

FIGURE 8:

Comparison of targeted recombination in active populations (TRAP) technique and *c-fos* immunohistochemistry. (A) TRAPed tdTomato activity (tagging cells expressing *c-fos*) in motor cortex following hypoxic–ischemic (HI), 10× magnification, NeuN expression (green channel). (B) Immunohistochemistry for *c-fos* and neuN in motor cortex from tissue 2 hours following HI.