Ganaxolone versus Phenobarbital for Neonatal Seizure Management

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Objective: Seizures are more common in the neonatal period than at any other stage of life. Phenobarbital is the first-line treatment for neonatal seizures and is at best effective in approximately 50% of babies, but may contribute to neuronal injury. Here, we assessed the efficacy of phenobarbital versus the synthetic neurosteroid, ganaxolone, to moderate seizure activity and neuropathology in neonatal lambs exposed to perinatal asphyxia.
Methods: Asphyxia was induced via umbilical cord occlusion in term lambs at birth. Lambs were treated with ganaxolone (5mg/kg/bolus then 5mg/kg/day for 2 days) or phenobarbital (20mg/kg/bolus then 5mg/kg/day for 2 days) at 6 hours. Abnormal brain activity was classified as stereotypic evolving (SE) seizures, epileptiform discharges (EDs), and epileptiform transients (ETs) using continuous amplitude-integrated electroencephalographic recordings. At 48 hours, lambs were euthanized for brain pathology.
Results: Asphyxia caused abnormal brain activity, including SE seizures that peaked at 18 to 20 hours, EDs, and ETs, and induced neuronal degeneration and neuroinflammation. Ganaxolone treatment was associated with an 86.4% reduction in the number of seizures compared to the asphyxia group. The total seizure duration in the asphyxia+-ganaxolone group was less than the untreated asphyxia group. There was no difference in the number of SE seizures between the asphyxia and asphyxia+phenobarbital groups or duration of SE seizures. Ganaxolone treatment, but not phenobarbital, reduced neuronal degeneration within hippocampal CA1 and CA3 regions, and cortical neurons, and ganaxolone reduced neuroinflammation within the thalamus.
Interpretation: Ganaxolone provided better seizure control than phenobarbital in this perinatal asphyxia model and was neuroprotective for the newborn brain, affording a new therapeutic opportunity for treatment of neonatal seizures.

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Seizures are more common in the neonatal period than at any other stage of life and are the most distinctive indication of neurological abnormalities.[1] Neonatal seizures are strongly linked to infant death or neurological abnormalities in surviving infants; approximately 50% of neonates with seizures will develop defi-

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cits in motor, cognitive, and/or behavioral functions.[2] The reported incidence of seizures in term infants is 1 to 5/1,000[3]; however, this statistic is misleading as, in years past, the diagnosis of seizures was based on clinical observations only, thus under-reporting the actual incidence of seizures. The increasing use of brain activity monitoring via electroencephalogram (EEG) has demonstrated that the true rate of neonatal seizures is likely to be at least double the number reported by clinical observation alone.[4]

Perinatal asphyxia, and subsequent neonatal encephalopathy, are the most readily identifiable causes of neonatal seizures in term infants,[5] but additionally, seizures may occur secondary to stroke, infection, congenital heart disease, and metabolic deficits.[3] Although the underlying etiology is the primary determinant of neurological outcomes in the presence of seizures, there is also strong evidence from human and preclinical animal studies that seizures independently induce, or worsen, brain injury.[1,6,7] In the developing brain, seizures may exacerbate neuronal injury by increasing metabolic demand, altering cerebral oxygenation, inducing further release of excitatory neurotransmitters, and thus affecting neuronal connectivity.[8] Accordingly, neonatal seizures are highly correlated with neurological compromise,[9] and treating seizures effectively is paramount in clinical care.

Phenobarbital was discovered as an antiseizure medication for adults in 1912 and progressively adopted into clinical neonatal practice. Phenobarbital remains the first-line treatment for infants with seizures today. Phenobarbital is a γ-aminobutyric acid type A ($GABA_A$) agonist with actions on the β-subunit,[10] and the efficacy of phenobarbital as a first-line treatment varies according to the cause of neonatal seizures. Overall, up to 65% of infants show a decrease in EEG seizures with phenobarbital therapy,[9,10] whereas for the remainder, electrographic discharges are largely unchanged.[4] The most common second-line treatment for neonatal seizures is phenytoin, another compound that has been in clinical use for decades.[10] Meta-analysis demonstrates that, following perinatal asphyxia, a combination of barbiturates with conventional therapy does not improve risks of death, severe neurodevelopmental disability, or the combined outcome of death or severe neurodevelopmental disability.[11]

Ganaxolone is a positive allosteric modulator of the $GABA_A$ receptor with actions on the δ-subunit that do not correspond with the modulatory sites of benzodiazepines and barbiturates. Ganaxolone treatment has been examined in infants and children with seizures uncontrolled by other therapies and shown to be safe and efficacious. Two studies in children aged 6 months to 15 years have shown that, in ∼50% of patients, seizure frequency was significantly decreased with ganaxolone,[12,13] whereas others have failed to report a uniform response to the drug, but report good safety and tolerability in infants 4 to 24 months old, as reported by the EILAT X group.[14] Ganaxolone is a synthetic neurosteroid with potency and efficacy comparable to its endogenous neurosteroid allopregnanolone. Ganaxolone demonstrates anticonvulsant and neuroprotective actions in preclinical animal studies.[15,16] We have interrogated the neurosteroid synthetic pathway in an effort to identify candidate agents for neuroprotection, specifically following perinatal hypoxia–ischemia. We have shown that in fetal life, allopregnanolone not only promotes brain growth and protects against hypoxic damage, but it also provides a tonic suppression of brain activity.[17] The physiological importance of allopregnanolone for the brain before birth is evidenced by the inhibition of allopregnanolone resulting in an increased incidence of isoelectric and spiking EEG activity in response to brief in utero asphyxia.[17] These effects are ameliorated with administration of the synthetic allopregnanolone analog alfaxalone.[17] Furthermore, inhibiting allopregnanolone synthesis markedly increases asphyxia-induced cell death within the brain,[18] but this is prevented with alphaxalone treatment.[19] Ganaxolone is the only neurosteroid evaluated so far for the treatment of epilepsy in humans, and to our knowledge, ganaxolone has not been tested, preclinically or clinically, for neonatal seizures.

In the current study, we compared the efficacy of phenobarbital and ganaxolone in reducing seizure activity following asphyxia induced at birth in neonatal lambs, and the brain was subsequently assessed for indices of neuropathology. First, we hypothesized that ganaxolone would reduce seizure activity and have neuroprotective effects in neonatal lambs, and to a significantly greater degree than phenobarbital. Second, we hypothesized that phenobarbital itself, but not ganaxolone, would cause neuronal damage.

Materials and Methods

Animals, Ethics, and Surgery

Animal experimental procedures adhered to the National Health and Medical Research Council of Australia guidelines for the care and use of animals for scientific purposes and were approved by the Monash Medical Centre Animal Ethics Committee.

Pregnant Border Leicester-Merino crossbred ewes carrying twins at ∼140 days gestation (term is 147 days) underwent sterile surgery under general anesthesia induced by sodium thiopentone (20mg/kg intravenous [IV] bolus; Pentothal, Boehringer Ingelheim, Macquarie Park, New South Wales, Australia) and maintained with 1 to 2.5% isoflurane (Isoflo, Abbott, Macquarie Park, New South Wales, Australia) in oxygen/room

air. In turn, both fetuses were instrumented and delivered via caesarean section. First, the hindlimbs and rump of the fetus were exteriorized, and femoral artery and vein catheters were inserted (outer diameter = 1.5mm, inner diameter = 0.8mm; Dural Plastics, Silverwater, New South Wales, Australia). Catheters were filled with saline, and the femoral catheter was connected to a pressure transducer for continuous digital recording of heart rate and mean arterial pressure (MAP), calculated as diastolic pressure plus one third of systolic minus diastolic blood pressure (Powerlab SP, ADInstruments, Bella Vista, New South Wales, Australia).

Perinatal Asphyxia, Delivery, and Monitoring

With the fetus remaining in utero, the umbilical cord was completely clamped (umbilical cord occlusion) to cause asphyxia. Cord occlusion was maintained until fetal MAP decreased to ~20mmHg, at which point the umbilical cord was cut (which was already clamped) and the lamb was immediately delivered and resuscitated. Lambs in the control group were delivered without umbilical cord occlusion and resuscitated immediately once the fetal instrumentation had been completed. The lambs (control, asphyxiated) were placed on an infant Resuscitaire, intubated (size = 4.5mm endotracheal tube, Portex; Smiths Medical Australasia, Macquarie Park, NSW, Australia), and dried with towels. Positive pressure ventilation (Neopuff, Fisher & Paykel Healthcare, Panmure, Auckland, New Zealand; 30cmH_2O positive inspiratory pressure, 5cmH_2O positive end expiratory pressure [PEEP], 10 l/min room air, 30 breaths/min) was initiated. If bradycardia or prolonged hypotension was present, lambs were administered IV adrenaline (0.1mg) and fluid (0.9% saline; 10ml/kg). A pulse oximetry cuff (Radical7; Masimo, Irvine, CA) was placed around an area of shaved tail, and oxygen saturation (SaO_2) was continuously monitored. After 10 minutes of resuscitation, lambs were moved to continuous mechanical ventilation (Babylog 8000 Plus, Dräger, Lübeck, Germany; pressure support ventilation on volume guarantee = 5ml/kg, PEEP = 5–7cmH_2O, 30 breaths/min), with SaO_2 targeted at 91 to 95% and $PaCO_2$ targeted at 45 to 55mmHg for 48 hours.

Immediately after stabilization of the lambs after birth, the lambs were weighed, and BrainZ low impedance needle electrodes (BRM3; Natus Medical, Pleasanton, CA) were placed 5cm from the midline and 4cm from bregma on the shaved head of the lamb, with 4 active electrodes and 1 reference (on the neck), which were connected to the BrainZ monitor, allowing continuous monitoring of brain activity via amplitude-integrated EEG (aEEG). The BrainZ monitor is a 2-channel EEG that measures electrical signals from each hemisphere of the brain. The device records the signals in real time and as compressed data over time for later analysis and interpretation. To allow continuous aEEG monitoring, lambs were lightly sedated for the duration of the study using IV infusion of Alfaxan (1.5mg/kg/h; Jurox Animal Health, Rutherford, NSW, Australia). A cohort of control (no asphyxia; termed "sham") lambs did not have aEEG monitoring and were not ventilated or sedated, to allow separate assessment of the effects of continuous 48-hour ventilation and sedation. Temperature was monitored every hour via rectal probe and maintained within the specific range (38.5–39.5°C) for a newborn lamb. Lambs were given 10% glucose IV (to maintain a normal range of blood glucose at 4–8mmol/l over the next 48 hours), and personnel monitored the lambs throughout this time. Blood samples were collected from the arterial catheter for assessment of fetal and neonatal blood gas parameters immediately preasphyxia (fetal sample), at 5 and 8 minutes during asphyxia, and at 10 and 30 minutes and 1, 2, 6, 12, 24, and 48 hours after birth.

Experimental Groups and Treatments

A total of 40 lambs were delivered and studied in 5 experimental groups. Five of these lambs could not be resuscitated or did not survive the full 48-hour experimental period, and therefore were not included in any analyses. The groups studied were sham (no ventilation, sedation, or brain monitoring; n = 6), control (no asphyxia, with ventilation, sedation, and brain monitoring; n = 8), asphyxia alone (n = 7), asphyxia+phenobarbital (asphyxia+pheno; n = 7), and asphyxia+ganaxolone (asphyxia+-ganax; n = 7). The animals were randomly allocated at surgery immediately prior to asphyxia or sham asphyxia.

In animals allocated to receive phenobarbital or ganaxolone, treatment commenced at 6 hours after birth, regardless of the presence or absence of seizures at the time, thereby allowing direct comparison of their neuroprotective potential at 48 hours.

Phenobarbital (Aspen Australia, St Leonards, NSW, Australia) was delivered as an IV loading dose of 20mg/kg over 20 minutes in 20ml saline starting at 6 hours, followed immediately by a maintenance dose of 5mg/kg over 20 minutes in 20ml saline, which was then repeated 24 hours later. This dose followed the Australian Guidelines for Treating Neonatal Seizures.[11] Ganaxolone (Tocris Bioscience, Avon, UK; BS11 9QD) powder was first dissolved in 70% ethanol to make a stock solution, and ~700µl to 1ml of stock solution was further diluted in saline so that the final ethanol concentration was <1%. Ganaxolone was delivered IV as a loading dose of 5mg/kg/20 min in 20ml saline at 6 hours after birth, followed directly by a maintenance dose of 5mg/kg/day in 48ml saline over 2 days (as a continuous infusion, unlike phenobarbital, which was infused over 20 minutes). This dosing regimen was selected to reflect the lowest preclinical dose that demonstrates efficacy for seizure control with no reported side effects.[20]

Ganaxolone Levels

Ganaxolone concentrations in lamb plasma were determined using liquid chromatography–mass spectrometry. Standards were prepared by spiking solutions of ganaxolone in acetonitrile (ACN; 5µl) in blank rat plasma (50µl) from 1 to 200ng/ml final concentration. Standards and samples (50µl) were spiked with alphaxalone (5µl) as an internal standard at 200ng/ml final concentration in ACN and diluted to the same volume (175µl) dropwise with ACN to precipitate proteins. Samples and standards were centrifuged to remove precipitated protein, and 95µl was transferred to a glass insert for injection. A volume of 10µl

of sample was injected onto an Agilent Technologies (Santa Clara, CA) Polaris C18 column (5μm, 50 × 2.1mm) at 40°C and eluted at a rate of 0.4ml/min using a gradient comprising mobile phase A: 0.1% formic acid in H_2O and mobile phase B: methanol. The gradient ran from 20% B to 80% B over 0.6 minutes, then from 80% back to 20% B at 2.5 minutes and held for 3.5 minutes. Ganaxolone and alphaxalone eluted at 2.4 and 1.7 minutes, respectively. Detection was conducted using a triple quadrupole mass spectrometer with a Shimadzu (Kyoto, Japan) Nexera Autosampler (Model SIL-30 AC MP), a Shimadzu Nexera Liquid Chromatograph (Model LC-30 AD), a Shimadzu Prominence Communications Bus Module (Model CBM-20A), a Shimadzu Prominence Degasser (Model DGU-20A5), and Shimadzu LCMS Liquid Chromatograph Mass Spectrometer. The system was controlled and data analyses were performed with Shimadzu LabSolutions software. The instrument was set to detect ganaxolone (mass-to-charge ratio (m/z) 315 → 297) and alphaxalone (m/z 333 → 215). The MS was operated in positive ion mode (electrospray ionization+; detector voltage = 4.5kV, collision energy = −15 for both ganaxolone and alphaxalone, dwell time = 100 milliseconds, nebulizing gas flow = 3 l/min, drying gas flow = 5 l/min, heat block and desolvation line temperature both = 200°C).

Seizure Analysis

In fetal sheep, we have shown that an acute hypoxic–ischemic (HI) insult induces abnormal brain activity, with EEG analysis characterizing the presence of stereotypic evolving (SE) seizures, generally peaking at 24 hours after HI injury and correlating with cortical injury.[21] However, in the latent phase of injury, when EEG activity is suppressed, 2 to 3 hours after HI injury, epileptiform transients (ETs; spikes) are observed, with the maximum frequency of these events associated with cerebral deoxygenation and severity of neural injury. We have thus used previously published EEG criteria[21] in the current study, as described below.

Abnormal electrographic brain activity was assessed offline and classified using continuous EEG recording. We classified

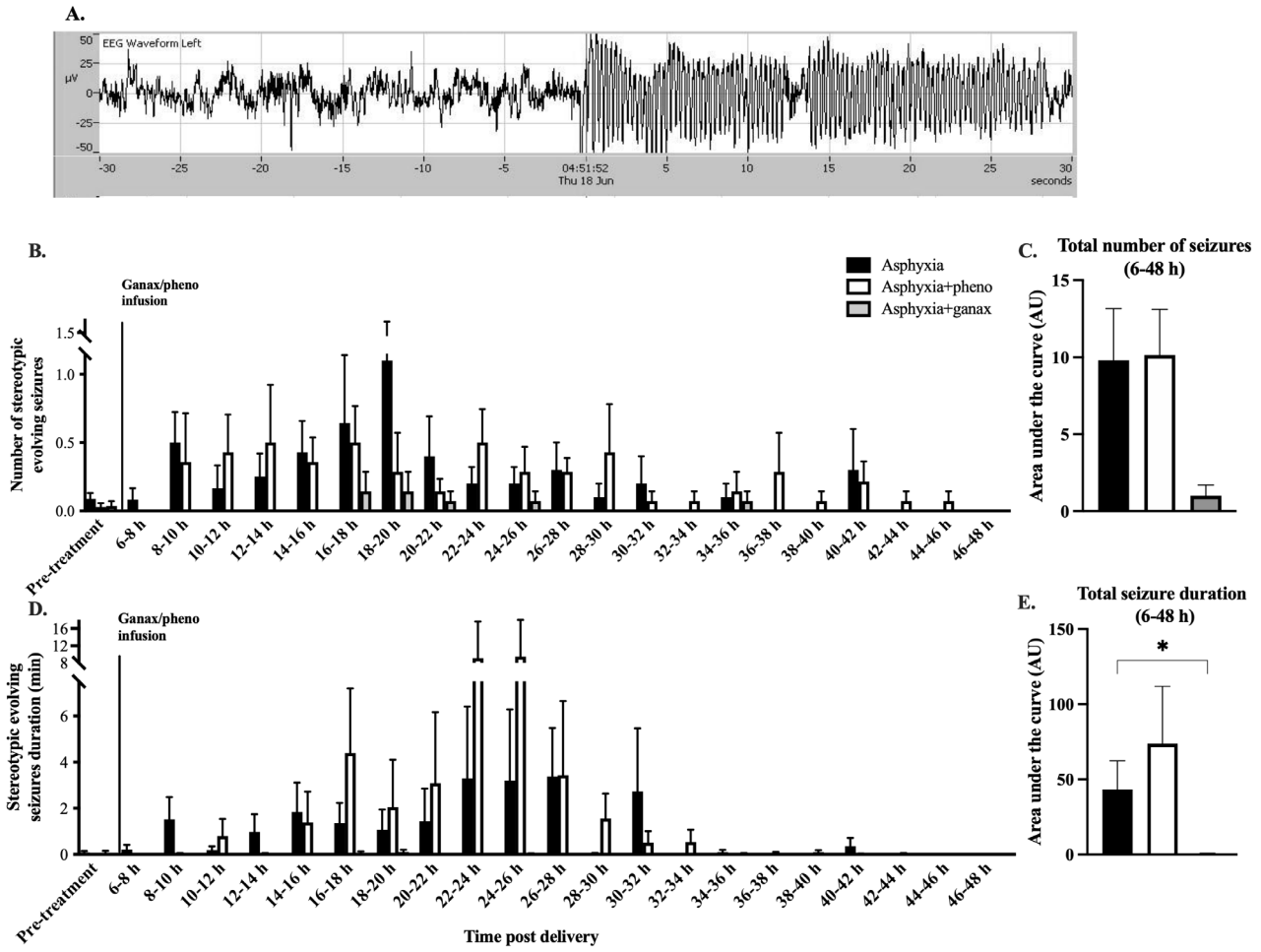

FIGURE 1: Stereotypic evolving (SE) seizures. (A) Real-time amplitude-integrated electroencephalographic (EEG) recording from a term lamb exposed to asphyxia at birth showing brain electrical activity immediately prior to and after SE seizure onset, measured in seconds. (B, C) The number of SE seizures per hour (B) and area under the curve analysis (C). (D, E) The duration of SE seizures per minute over an hour (D) and area under the curve analysis (E). Values are expressed as mean ± standard error of the mean of the incidence of each state per 2-hour epoch, observed before (pretreatment) and after the start of infusion at time 6 hours. Vertical lines indicate the time of ganaxolone (ganax) or phenobarbital (pheno) infusion. *$p < 0.05$.

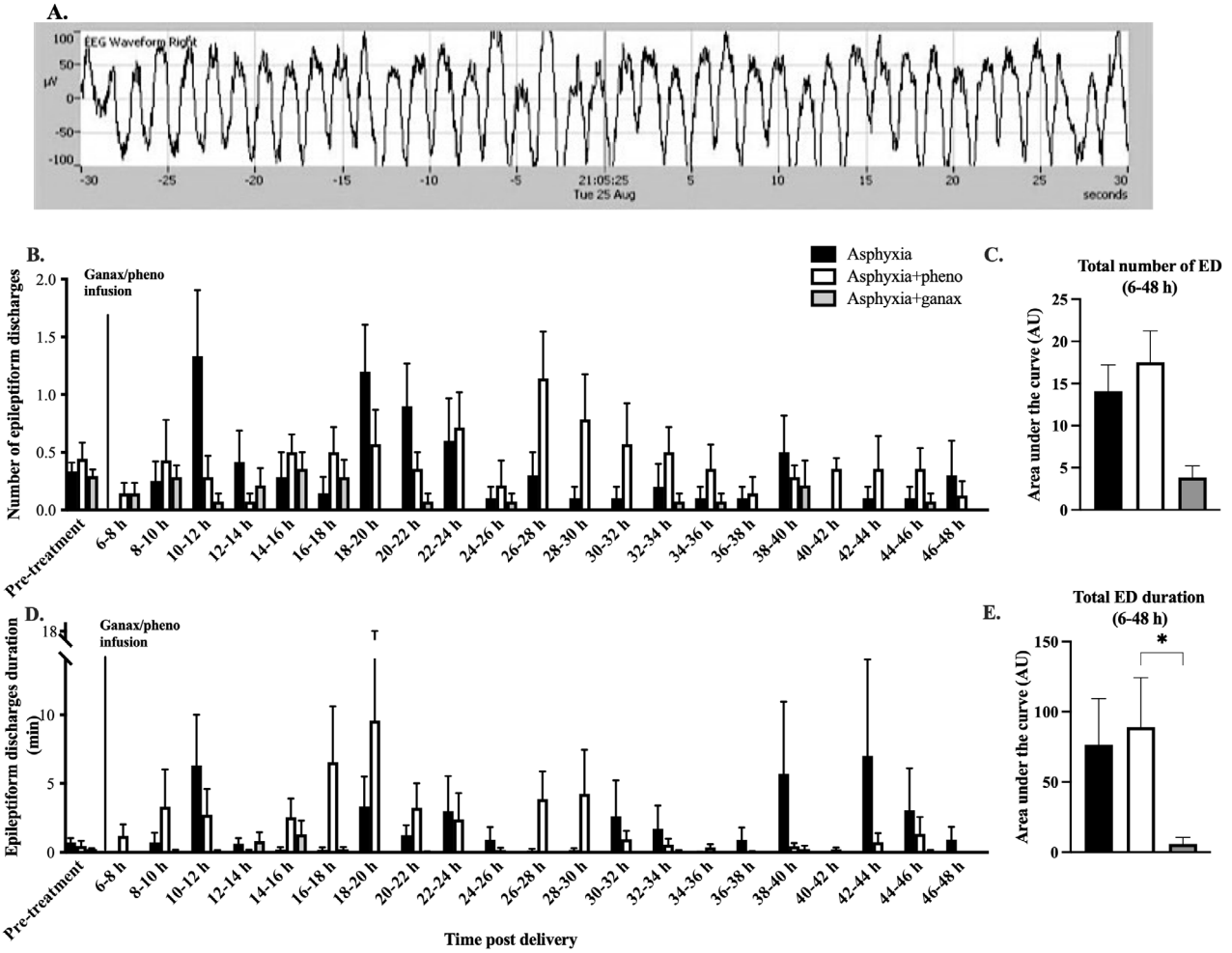

FIGURE 2: Epileptiform discharges (EDs). (A) Real-time amplitude-integrated electroencephalographic (EEG) recording from a term lamb exposed to asphyxia at birth showing brain electrical activity of EDs, measured in seconds. (B, C) The number of EDs per hour (B) and area under the curve analysis (C). (D, E) The duration of EDs per minute over an hour (D) and area under the curve analysis (E). Values are expressed as mean ± standard error of the mean of the incidence of each state per 2-hour epoch, observed before (pretreatment) and after the start of infusion at time 6 hours. Vertical lines indicate the time of ganaxolone (ganax) or phenobarbital infusion (pheno). *$p < 0.05$.

abnormal brain activity in 3 forms: (1) SE seizures, (2) epileptiform discharges (EDs), and (3) ETs. SE seizures were characterized by the appearance of sudden, repetitive, evolving stereotypic waveforms with a definite beginning and end, lasting >10 seconds, and with an EEG amplitude > 20μV (Fig 1A).[22] EDs were either brief rhythmic bursts or large continuous waveforms defined by a period of 200 to 350 milliseconds from trough to peak, with waveforms forming consistent events lasting >10 seconds (Fig 2A).[23] Both the SE seizures and ED activity patterns are indicative of an increased prevalence of brain injury.[24] ETs were characterized by brief waves/spikes (70–350 milliseconds), single or multiple fast spikes, sharp waves, or slow waves (Fig 3A), known to be associated with HI injury.[17] Each minute of the raw aEEG recording was analyzed manually, by a single assessor blinded to groups, for the presence of SE seizures, EDs, or ETs using a program allowing visualization of aEEG records at a resolution of 5 seconds. The number (per hour) and duration (min/h) of SE seizures and EDs were averaged over 2-hour epochs and presented over the entire 48 hours of the experiment. The pretreatment period was the average data between the time the electrodes were placed (~1–3 hours postdelivery) until 6 hours before the treatments commenced. All ET waveforms were combined and presented as the total ET duration in each 2-hour epoch, over 48 hours. Seizure characteristics in Table 3 were generated from an animal that had SE seizures only and not the entire cohort as presented in Figure 1. All coded aEEG files were analyzed by T.Y. and verified by L.B.

Assessment of Neuropathology

At 48 hours after birth, lambs were euthanized with pentobarbitone (100mg/kg; Valabarb, Jurox Animal Health) and weighed before removing the brain. The brain was weighed and divided in half sagittally. The left cerebral hemisphere was separated into anatomical regions, snap frozen in liquid nitrogen, and stored at −80°C for future assessment. The right hemisphere was cut coronally into 5mm blocks, fixed in formalin for 48 hours, and

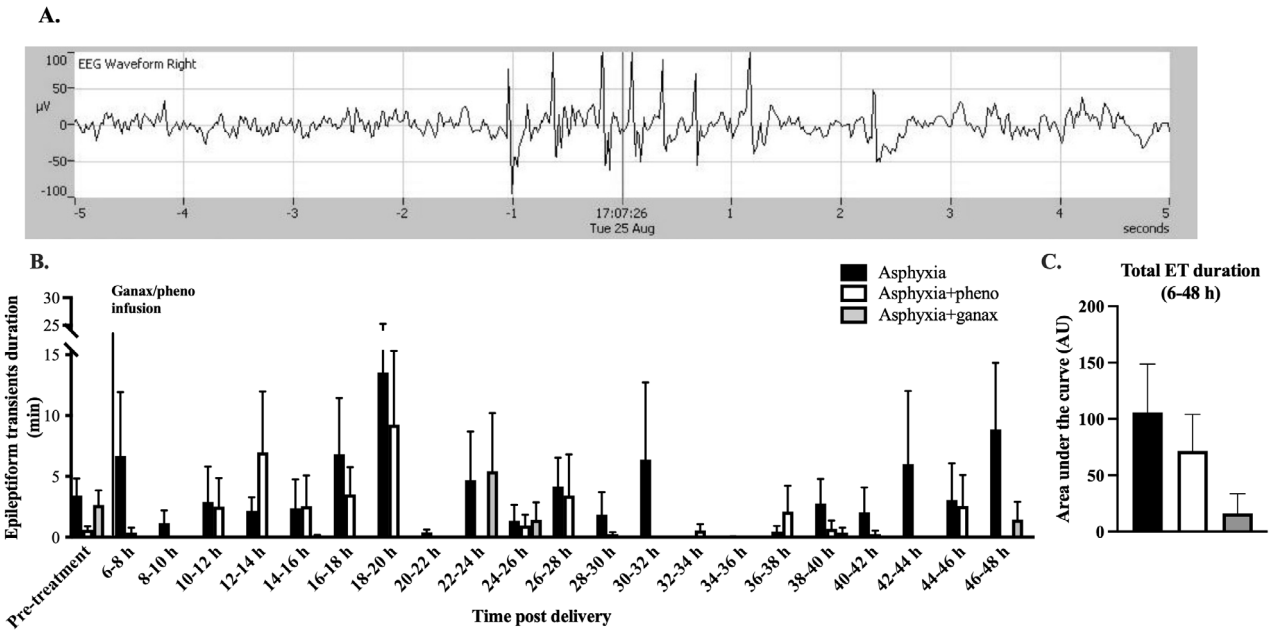

FIGURE 3: Epileptiform transients (ETs). (A) Real-time amplitude-integrated EEG recording from a term lamb exposed to asphyxia at birth showing brain electrical activity of ET events (spike activity), measured in seconds. (B, C) The duration of ET events averaged in minutes over an hour (B) and area under the curve analysis (C). Values are expressed as mean ± standard error of the mean of the duration of the transient event per 2-hour epoch, observed before (pretreatment) and after the start of infusion at time 6 hours. The vertical line indicates the time of ganaxolone (ganax) or phenobarbital (pheno) infusion. *$p < 0.05$.

then embedded in paraffin (ProSci Tech, Thuringowa, QLD, Australia) for histological and immunohistochemistry analysis. Subsequently, 10μm sections were cut for examination of brain pathology.

Neuropathology was first identified by staining sections with cresyl violet–acid fuchsin (CV/AF; Amber Scientific, Midvale, WA, Australia), with manual counts of cells demonstrating necrosis, including organelle swelling, loss of membrane integrity, pyknotic nuclei, bright eosinophilic cytoplasm, or cells with darkened and condensed cytoplasm.[25,26] Neuronal nuclei were identified using NeuN (1:500; Millipore Corporation, Billerica, MA); mouse anti-NeuN antibody was incubated overnight at 4°C, the sections then treated with a secondary antibody (1:200; biotinylated antimouse; Vector Laboratories, Burlingame, CA), and staining was revealed using 3,3-diaminobenzidine (Pierce Biotechnology, Rockford, IL). Inflammatory cells, including activated microglia and macrophages, were identified using rabbit anti–ionized calcium-binding adaptor molecule 1 (Iba-1) antibody (Wako Pure Chemical Industries, Osaka, Japan), raised against a synthetic peptide corresponding to the C-terminal of Iba-1. The antibody was diluted 1:1,000 in phosphate-buffered saline solution (0.1mol/l, pH 7.4) and incubated overnight at 4°C. Sections were then treated with a secondary antibody (1:200; biotinylated antirabbit; Vector Laboratories).[27] The appearance of activated microglia (ameboid with large cell bodies) was morphologically quantified, as done previously.[28,29] Immunopositive and necrotic cells were manually counted under light microscopy (Olympus, Tokyo, Japan) using ImageJ (v1.48, National Institutes of Health). Two sections of each brain region per animal were examined, the number of immunopositive cells per region was calculated using the average of 4 fields of view per section, and the results were averaged across all the animals in each group. All images were coded so the observer was blinded to the treatment group.

Statistics

Data are expressed as mean ± standard error of the mean. A 2-step analysis was performed to examine significance related to gestational age, asphyxia time, lamb body and brain weight, and neuropathology. First, we examined asphyxia versus control via an unpaired t test, followed by 1-way analysis of variance (ANOVA) with phenobarbital and ganaxolone as independent variables to assess outcomes within the asphyxia groups (asphyxia, asphyxia+pheno, asphyxia+ganax). Two-way repeated-measures ANOVA, with treatment and time as independent factors, was used to analyze blood gases and pH between treatment groups and across time. Where the ANOVA indicated significant interaction between treatment and time, a Tukey multiple comparisons test was applied. One-way ANOVA was used to analyze seizure characteristics and area under the curve for total seizure duration, total ED duration, and total ET duration with post hoc Tukey test as required. Poisson regression was used to analyze the number of seizures and EDs in Figures 1 and 2 and Table 3. Multiple linear regression was used to analyze neuropathology with asphyxia, phenobarbitone, and ganaxolone as variables. Least squares multiple linear regression analysis was used to investigate whether there was a relationship between neuropathology and the number or duration of seizures and EDs from 6 to 24 hours after birth. Statistical significance was set at $p \leq 0.05$. Statistical comparisons were carried out using Prism 9 (GraphPad Software, La Jolla, CA).

TABLE 1. Lamb Outcomes

Outcome	Control	Asphyxia	Asphyxia+Pheno	Asphyxia+Ganax
Number (M/F)	8 (4/4)	7 (5/2)	7 (3/4)	7 (1/6)
Gestational age, days [95% CI]	137.6 ± 0.3 [137–138]	138.1 ± 0.3 [138–139]	138.1 ± 0.4 [137–139]	138.3 ± 0.4 [137–139]
Asphyxia time, min [95% CI]	N/A	11.53 ± 0.79 [9.5–13]	10.99 ± 0.61 [9.2–12]	10.34 ± 0.46 [8.9–12]
Body weight, kg [95% CI]	3.66 ± 0.25 [3–4.3]	3.82 ± 0.37 [2.9–4.7]	3.66 ± 0.27 [3–4.3]	3.82 ± 0.21 [3.3–4.3]
Brain weight, g [95% CI]	51.88 ± 1.08 [49–55]	49.09 ± 2.77 [42–56]	52.30 ± 0.85 [50–54]	51.47 ± 0.88 [49–54]

There were no significant differences between any of the treatment groups. Data are mean ± standard error of the mean.
CI = confidence interval; F = female; Ganax = ganaxolone; M = male; N/A = not applicable; Pheno = phenobarbital.

Results

Sham and Control Animals

Two separate baseline groups were studied, sham control (not ventilated or sedated) and control animals (ventilated and sedated). We compared body and brain weight, neuronal survival (NeuN+ cells), neuronal necrosis (CV/AF assessment of morphology), and activated microglia cell counts (Iba-1+ cells) between these cohorts. We did not find any differences across these outcomes for these two baseline groups (data not shown). Henceforth, the sham control animal data have been excluded from analysis, and all treatment groups were compared to the control cohort that received ventilation and sedation as did all the asphyxia animals. Table 1 presents the baseline characteristics for all groups: control (n = 8), asphyxia (n = 7), asphyxia+pheno (n = 7), and asphyxia+ganax (n = 7). There was no difference in lamb body weight (95% confidence interval [CI] = −1.26 to 0.94) or brain weight (95% CI = −3.47 to 9.06) at postmortem between the control and asphyxia groups. Phenobarbitone did not affect body weight (95% CI = −0.94 to 1.26) or brain weight (95% CI = −9.47 to 3.06) at postmortem compared to the asphyxia group, nor did ganaxolone (body weight, 95% CI = −1.10 to 1.10; brain weight, 95% CI = −8.64 to 3.89). Duration of asphyxia was not different between the asphyxia and asphyxia+pheno groups (95% CI = −108 to 173) or the asphyxia and asphyxia+-ganax groups (95% CI = −75 to 218).

Asphyxia and Resuscitation

Blood gas and physiological parameters were assessed preasphyxia in the fetal lambs, and there were no differences between any parameters between groups (Supplementary Table 1; please see Supplementary Table 2 for 95% CIs). Asphyxia following cord clamping caused a severe metabolic acidosis in all groups, consistent with the clinical criteria for a severe acute hypoxic event at birth; pH < 7 and base excess < −14 (Supplementary Table 1).[26] Fetal arterial pH, SaO_2, partial pressure of carbon dioxide, partial pressure of oxygen, lactate, and base excess were all significantly altered from control parameters in response to asphyxia, but the response between asphyxia groups (asphyxia, asphyxia+pheno, asphyxia+ganax) was not different. Parameters returned to control group values within 2 hours of resuscitation. The commencement of phenobarbital or ganaxolone treatment at 6 hours after birth did not affect any of these blood chemistry parameters. There was, however, a decrease in base excess and bicarbonate at 12 hours after birth in the asphyxia and asphyxia+pheno groups compared to control, but not in the ganaxolone treatment group (Supplementary Table 1).

Ganaxolone Levels

Arterial plasma ganaxolone levels were below quantifiable levels (1ng/ml) before ganaxolone infusion but were elevated at 2 hours after the start of the IV infusion (49.7 ± 26.2ng/ml, 95% CI = −8.74 to 108; $p = 0.087$) and were significantly elevated compared to preinfusion values by 6 hours (45.0 ± 18.6ng/ml, 95% CI = 7.06 to 83.02; $p = 0.025$) and remained significantly elevated at 18 hours (58.1 ± 9.8ng/ml, 95% CI = 36.12 to 80.08; $p = 0.0002$) and 42 hours (47.4 ± 5.9ng/ml, 95% CI = 36.55 to 58.35; $p < 0.0001$) after the start of the infusion.

Seizures

There was no evidence of seizure activity in control animals throughout the experiment (data not shown). In the

TABLE 2. Characteristics of Seizures after Umbilical Cord Occlusion

Treatment	Animals with Seizures, n	Seizure Onset, h	Range, h	Seizure End, h	Total Seizures, n	Total Seizure Period, min	Individual Seizure Duration, min
Asphyxia	6/7	11.5 ± 5.94	2.5–40	28 ± 5.03	8.86 ± 3.62	42.83 ± 29.45	5.59 ± 2.54
Asphyxia+pheno	7/7	11.6 ± 2.21	2.5–21	30.71 ± 5.38	10.29 ± 3.56	73.42 ± 48.45	11.84 ± 7.91
Asphyxia+ganax	3/7	17.5 ± 11.18	2.5–34	20.67 ± 11.28	1.14 ± 1.55	1.73 ± 0.71	1.0 ± 0.58

Of the animals that had seizures, total seizure period and individual seizure duration data were generated for the entire experimental period. Data for animals that did not have seizures were not included in this table. Data are mean ± standard error of the mean. Statistical significance was determined by 1-way analysis of variance for seizure onset, range, end, period, and individual duration. Poisson analysis was used for the total number of seizures.

asphyxia groups, SE seizures (example image in Fig 1A) were observed in 6 of 7 animals in the asphyxia alone group, 7 of 7 animals in the asphyxia+pheno group, and 3 of 7 animals in the asphyxia+ganax group (Table 2).

The number of SE seizures per hour and duration of SE seizures in minutes are shown in Figure 1B and D, respectively. There was no significant difference in the number of SE seizures between the asphyxia and

TABLE 3. Seizure–Brain Injury Correlations

Brain Region/ Injury Marker	Number of Seizures			Duration of seizures, min			Number of EDs			Duration of EDs, min		
	r^2	p	95% CI	r^2	p	95% CI	r^2	p	95% CI	r^2	p	95% CI
CA1 (necrosis)	0.02	0.53	−3.55 to 6.71	0.23	0.03	0.10 to 1.6	0.08	0.21	−2.06 to 8.68	0.08	0.2	−0.21 to 0.93
CA3 (necrosis)	0.02	0.53	−23.40 to 12.42	0.002	0.84	−3.26 to 2.67	0.001	0.93	−18.67 to 20.44	0.01	0.69	−2.46 to 1.67
Cortex (necrosis)	0.001	0.92	−2.65 to 2.94	0.01	0.64	−0.55 to 0.37	0.02	0.58	−2.18 to 3.81	0.001	0.91	−0.30 to 0.34
Thalamus (necrosis)	0.11	0.15	−1.05 to 6.58	0.47[a]	<0.001	0.47 to 1.43	0.11	0.15	−1.18 to 7.08	0.2[a]	0.04[a]	0.02 to 0.85
CA1 (inflammation)	0.005	0.77	−4.74 to 3.58	0.03	0.5	−0.88 to 0.45	0.07	0.28	−7.09 to 2.21	0.02	0.61	−0.60 to 0.37
CA3 (inflammation)	0.0002	0.95	−6.79 to 6.40	0.004	0.79	−0.94 to 1.22	0.005	0.76	−8.61 to 6.43	0.01	0.66	−0.60 to 0.91
Cortex (inflammation)	0.19[a]	0.05[a]	−0.05 to 11.93	0.03	0.46	−0.71 to 1.51	0.34[a]	0.007[a]	2.58 to 14.31	0.03	0.46	−0.49 to 1.03
Thalamus (inflammation)	0.33[a]	0.01[a]	2.59 to 15.40	0.002	0.85	−1.15 to 1.39	0.04	0.43	−5.19 to 11.74	0.01	0.62	−0.67 to 1.10

Selected correlation data using multiple linear regression, least squares (r^2) comparing neuronal necrosis and inflammation in the hippocampus (CA1 and CA3), cortex, and thalamus with the number and duration of seizures and EDs from 6 to 24 hours after birth. Total area under the curve data were used for the number and duration of seizures and EDs at 2-hour intervals between 6 and 24 hours after birth.
CI = confidence interval; ED = epileptiform discharge.

asphyxia+pheno groups (estimate = 0.1991, 95% CI = −0.14 to 0.55; $p = 0.26$) or in the total duration of SE seizures (area under the curve analysis, 95% CI = −62.5 to 123.5; $p = 0.49$; see Fig 1). Ganaxolone treatment was associated with an 86.4% (estimate = −1.998, 95% CI = −2.82 to −1.32; $p < 0.0001$) reduction in the number of seizures compared to the asphyxia group. The total seizure duration in the asphyxia+ganax group was also significantly less than the untreated asphyxia group (area under the curve analysis, 95% CI = −84.46 to −0.95; $p = 0.04$).

Of the animals that exhibited SE seizures, seizure characteristics were generated over the 48-hour period (see Table 2). From 6 of 7 animals that exhibited SE seizures in the untreated asphyxia alone group, the time to first seizure was 2.5 hours after umbilical cord occlusion, with a mean seizure onset of 11.5 ± 5.95 hours. This was not statistically different in the phenobarbital (7/7 animals) and ganaxolone (3/7 animals) groups. The total number of SE seizures in the untreated asphyxia group was 8.43 ± 3.15, which was not changed in the asphyxia+pheno group (10.29 ± 3.29; estimate = 0.1991, 95% CI = −0.15 to 0.55; $p = 0.26$) but was significantly reduced in the asphyxia+ganax group (1.14 ± 0.83; estimate = −1.998, 95% CI = −2.82 to −1.32; $p < 0.0001$). There was no difference in the total seizure period (42.83 ± 29.45, 73.41 ± 48.45, 1.73 ± 0.71; $p = 0.5$) and individual seizure duration (5.59 ± 2.54, 11.84 ± 7.91, 1.0 ± 0.58; $p = 0.48$) between the asphyxia, asphyxia+pheno, and asphyxia+ganax groups.

There was no evidence of EDs in control animals throughout the experiment (data not shown). In the asphyxia groups, EDs (example image in Fig 2A) were observed in 7 of 7 animals in the asphyxia alone group, 7 of 7 animals in the asphyxia+pheno group, and 7 of 7 animals in the asphyxia+ganax group. The number of EDs per hour, and duration of EDs in minutes are shown in Figure 2B and D, respectively. There was a significant difference in the total number of EDs between the asphyxia and asphyxia+pheno groups (estimate = 0.4467, 95% CI = 0.18 to 0.72; $p = 0.001$) but no change in the duration of EDs (area under the curve analysis, 95% CI = −113.6 to 88.52; $p = 0.95$; see Fig 2). Ganaxolone treatment significantly reduced the number (estimate = −0.8550, 95% CI = −1.25 to −0.48; $p < 0.0001$) and duration (area under the curve analysis, 95% CI = −160.7 to −5.56; $p = 0.04$) of EDs compared to the asphyxia+pheno group.

There was no evidence of ETs in control animals throughout the experiment (data not shown). In the asphyxia groups, ETs (example image in Fig 3A) were observed in 7 of 7 animals in the asphyxia alone group, 6 of 7 animals in the asphyxia+pheno group, and 7 of 7 animals in the asphyxia+ganax group. In the untreated asphyxia groups, ET activity was highest the first 20 hours after birth. The treatment with phenobarbital or ganaxolone did not change the duration of the ETs over the treatment period compared to the asphyxia group (area under the curve analysis, 95% CI = −151.1 to 82.89 [$p = 0.53$] and 95% CI = −190.6 to 11.21 [$p = 0.08$], respectively; see Fig 3C).

Neuropathology

In untreated asphyxia lambs, there was a significant increase in the number of necrotic (degenerating) neurons in hippocampal CA1 cells (95% CI = 19.34 to 123.1; $p = 0.01$) and CA3 cells (95% CI = 40.6 to 534.4; $p = 0.02$), in addition to the cortex (95% CI = 14.02 to 54.04; $p = 0.002$) and thalamus regions (95% CI = 13.07 to 84.15; $p = 0.01$), compared to control lambs (Fig 4). In the untreated asphyxia lambs, there was a significant increase in the number of inflammatory (Iba-1 positive) cells in the CA3 region of the hippocampus (95% CI = 19.00 to 197.1; $p = 0.02$) and an increase that did not reach statistical significance in the cortex (95% CI = −4.35 to 196.4; $p = 0.059$) compared to control lambs. There was no difference in the number of NeuN-positive neurons between the untreated asphyxia lambs and the control lambs within hippocampal CA1 or CA3 regions, cortex, or thalamus (95% CI = −361.6 to 61.26 [$p = 0.15$], 95% CI = −304.5 to 128.3 [$p = 0.39$], 95% CI = −357.9 to 16.58 [$p = 0.07$], and 95% CI = −255.5 to 82.17 [$p = 0.28$], respectively).

Neuronal necrosis in phenobarbital-treated asphyxia animals remained high and unchanged in hippocampal CA1 and CA3 regions, the cortex, and the thalamus when compared to asphyxia animals (95% CI = −498.6 to 249.4 [$p = 0.67$], 95% CI = −52.35 to 227.0 [$p = 0.27$], 95% CI = −523.7 to 82.45 [$p = 0.18$], and 95% CI = −248.2 to 196.9 [$p = 0.95$], respectively; see Fig 4). The number of inflammatory cells in phenobarbital-treated asphyxia animals remained unchanged in the hippocampal CA1 (95% CI = −73.15 to 81.18; $p = 0.99$) and CA3 regions (95% CI = −82.25 to 134.5; $p = 0.81$), cortex (95% CI = −66.54 to 133.7; $p = 0.67$), and thalamus (95% CI = −150.7 to 79.81; $p = 0.71$) compared to asphyxia alone. Ganaxolone administration reduced the number of necrotic neurons compared to asphyxia animals alone. This reduction was seen in all brain regions examined, including hippocampal CA1 (95% CI = −0.448 to 148.1; $p = 0.05$) and CA3 regions (95% CI = 8.375 to 532.9; $p = 0.04$), cortex (95% CI = −65.19 to −4.65; $p = 0.03$), and thalamus (95% CI = −10.01 to 103.7; $p = 0.11$). Ganaxolone

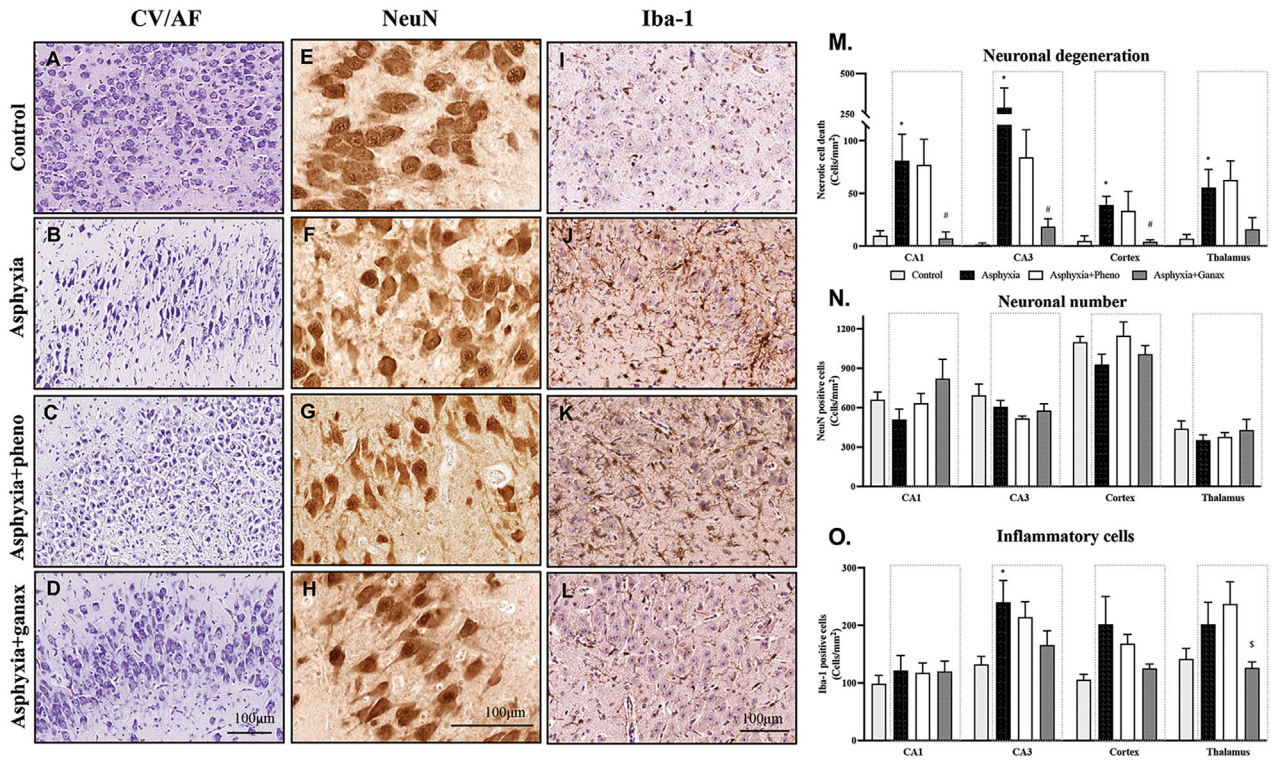

FIGURE 4: Histopathology at 48 hours after birth. (A-L) Representative images are from the CA3 region of the hippocampus for staining with cresyl violet–acid fuchsin (CV/AF; A–D), neuronal nuclei (NeuN; E–H), and ionized calcium-binding adapter molecule (Iba-1; I–L). Scale bar for A–D in D, E–H in H, and I–L in L. (M–O) The number of necrotic neurons stained with CV/AF (M), NeuN-positive neurons (N), and Iba-1–positive cells (O) within the CA1 and CA3 region of the hippocampus, cortex, and thalamus at 48 hours after birth. Values are expressed as mean ± standard error of the mean. The results from 1-way analysis of variance among asphyxia groups are shown in the figure. *$p < 0.05$ versus control, #$p < 0.05$ versus asphyxia, $$p < 0.05$ versus asphyxia+pheno. Comparisons were made within each region and not across different regions. ganax = ganaxolone; pheno = phenobarbital.

administration significantly reduced the number of inflammatory cells following asphyxia within the thalamus (95% CI = 0.1042 to 221.6; $p = 0.04$) and remained low but significantly unchanged within the CA3 region (95% CI = −33.84 to 183.0; $p = 0.2$) and the cortex (95% CI = −23.42 to 176.9; $p = 0.15$) compared to asphyxia alone. Neuronal number in ganaxolone-treated asphyxia animals remained unchanged compared to untreated asphyxia animals within all regions examined; this included the hippocampal CA1 and CA3 regions, cortex, and thalamus (95% CI = −698.6 to 77.63 [$p = 0.13$], 95% CI = −125.2 to 180.8 [$p = 0.88$], 95% CI = −383.8 to 222.4 [$p = 0.77$], and 95% CI = −291.1 to 137.8 [$p = 0.63$], respectively).

Seizure–Neuropathology Correlations

The duration of SE seizures was shown to be a positive predictor for neuronal degeneration within the CA1 region of the hippocampus (95% CI = 0.10 to 1.6; $r^2 = 0.23$; $p = 0.03$) and the thalamus (95% CI = 0.47 to 1.43; $r^2 = 0.47$; $p < 0.001$), but not the cortex or the CA3 region of the hippocampus. The number of SE seizures did not correlate with neuronal degeneration within the hippocampus, cortex, or thalamus (see Table 3). Similarly, the number of EDs did not correlate with neuronal degeneration within the hippocampus, cortex, or thalamus. Finally, the duration of EDs was positively correlated with neuronal necrosis within the thalamus (95% CI = 0.02 to 0.85; $r^2 = 0.2$; $p = 0.04$) but not the cortex or the CA1 and CA3 region of the hippocampus (see Table 3).

Conversely, the number of seizures was a strong predictor for neuronal inflammation within the thalamus (95% CI = 2.59 to 15.40; $r^2 = 0.33$; $p = 0.009$) and cortex (95% CI = −0.05 to 11.93; $r^2 = 0.19$; $p = 0.05$) but not the hippocampus. The number of EDs was also a positive predictor for neuronal inflammation within the cortex (95% CI = 2.58 to 14.31; $r^2 = 0.34$; $p = 0.007$; see Table 3). The duration of SE seizures and EDs did not correlate with neuronal inflammation.

Discussion

Neonatal seizures, predominantly caused by asphyxic insult, are the most identifiable sign of neurological

dysfunction in the newborn period. Currently, phenobarbital is the first-line treatment for neonatal seizures. Here, we present for the first time that the neurosteroid ganaxolone is more effective at reducing birth asphyxia-induced electrographic seizures than standard phenobarbital treatment. Ganaxolone treatment was associated with a significantly greater reduction in both number and duration of stereotypic seizures and EDs compared to phenobarbital treatment. Furthermore, ganaxolone treatment was associated with neuroprotection, preventing neuronal necrosis and a significant region-specific reduction in neuroinflammation, whereas phenobarbital did not demonstrate neuroprotective benefit.

After transient asphyxia, brain injury evolves over time and in phases, the most important of which have been described as the latent and secondary phases of injury.[30] The latent phase describes the period after insult characterized by apparent recovery of cerebral oxidative metabolism, although EEG activity remains depressed (perhaps adaptively), followed by a secondary phase of injury beginning ~6 to 8 hours postinsult, representing a secondary failure of oxidative metabolism, cytotoxic edema, and the onset of delayed seizure activity.[30] Most neurodegeneration occurs in this secondary phase, and consequently until this time there is a window of opportunity during which neuroprotective treatments may be effective. This timeframe provided the basis for commencing our treatments at 6 hours and also explains the requirement to begin therapeutic hypothermia before 6 hours for optimized neuroprotective benefit.[31]

In response to asphyxia, 6 of 7 animals showed aEEG activity indicative of seizures, evident within the first 24 hours after birth asphyxia, and increasing over time, with a peak of stereotypic seizure activity after 18 to 29 hours. The onset of overt stereotypic seizure activity occurred at a mean of 11.5 ± 5.9 hours, in keeping with the clinical evidence of clear seizure activity at 10 to 15 hours in human infants following a likely intrapartum asphyxic event.[32] In the asphyxic animals in this study, the number of seizures and duration spent seizing peaked at 18 to 20 and 22 to 26 hours, respectively, followed by a smaller additional spike in seizure activity at 40 to 42 hours. These data are in good agreement with clinical data showing that seizures peak in the first 24 hours after a severe insult.[33] Furthermore, the total seizure period over the course of this study was >40 minutes. In neonates, a total seizure burden of >40 minutes is correlated with a 9-fold increased risk of abnormal neurodevelopmental outcome at 24 to 48 months of age.[32] It was therefore not surprising that in the untreated asphyxia animals there was also a significant increase in signs of neuroinflammation and neuronal degeneration within the thalamus, cortex, and hippocampus compared to control animals. There is substantial evidence from human and animal studies that seizures independently induce or worsen brain injury.[6,7]

Phenobarbital continues to be the most frequently used first-line treatment for neonatal seizures, despite reservations regarding its efficacy and safety.[34] Phenobarbital is a long-acting GABA receptor agonist, with a half-life of ~140 hours in term infants,[35] and as a first-line treatment, phenobarbital controls clinical seizures in 65% of infants.[36] The effects of phenobarbital are attributed to its sedative and anticonvulsant properties. Primarily, this drug sedates infants and thus reduces the clinical/physical signs of seizures but does not directly act to prevent electrographic seizure discharge.[37] In the current study, we show that treating with phenobarbital at 6 hours did not prevent SE seizure activity between 8 and 18 hours but did prevent the sharp rise in number of seizures that occurred in untreated animals at 18 to 20 hours. However, the duration of stereotypic seizures was high in the phenobarbital group, resulting in a total seizure period of >70 hours, indicative of a high total seizure burden. The use of phenobarbital is associated with motor and cognitive deficits and an increased risk of developing anxiety behaviors later in life,[38] potentially mediated via an increase in apoptosis-mediated cell death and a reduction in synaptic connectivity in the developing brain.[38] Although we did not demonstrate a reduction in seizure activity, ETs, or EDs with phenobarbital treatment, it was reassuring that we did not find that phenobarbital induced additional cell death or neuroinflammation over the 48-hour period of this study.

The key outcome of this study was that ganaxolone treatment, commenced 6 hours after asphyxia, significantly reduced the number of stereotypic seizures compared to both the untreated asphyxia group and the -phenobarbital-treated group. Ganaxolone is a synthetic by-product of the endogenous neurosteroid allopregnanolone that modulates the activity of GABAergic neurons to inhibit neuronal excitability, thereby providing a neurosteroid form of neuroprotection.[39] Neurosteroids are required for normal brain development,[19] mediating cell proliferation and maturation, and are essential for myelination of white matter.[40] Our previous research has shown that neurosteroid administration to normoxic and posthypoxic fetal sheep results in strong EEG suppression,[17] and ganaxolone has extensive anticonvulsant effects in several rodent models of neonatal brain injury (reviewed in Yawno et al[41]). Ganaxolone is the only neurosteroid that has been evaluated in adult epilepsy (>900 subjects) and is well tolerated, with limited side effects, the most common being reversible dose-related

sedation.[42] Ganaxolone has also been used in a pediatric population to treat infantile spasms, providing evidence that it reduces seizures with an acceptable tolerance and safety profile even in those cases that are refractory to other therapies.[12,13] The efficacy of ganaxolone versus phenobarbital for neonatal seizures has not been examined previously. The results of this study demonstrate that ganaxolone prevented stereotypic seizures following birth asphyxia, reflected in a low total seizure burden of <2 hours. Importantly, ganaxolone was significantly more effective than phenobarbital at reducing the number of SE seizures, and the number and duration of EDs evident in the secondary phase of injury after asphyxic insult. These data strongly support that ganaxolone may be more efficacious as an anticonvulsant than phenobarbital for the neonate.

In addition to improved seizure control, ganaxolone significantly reduced neuronal necrosis in the hippocampus and cortex of the neonatal brain compared to the untreated asphyxia lambs, whereas phenobarbital did not. Neurosteroids, like allopregnanolone and its synthetic analogue ganaxolone, have direct actions on the brain to inhibit apoptosis and promote neural stem cell proliferation.[19,43,44] These cellular actions are modulated through binding and activating the membrane progesterone receptor, subtype δ (mPRδ), and mPRδ is highly expressed in the human brain.[44] Our results demonstrate that ganaxolone elicits dual benefits for the neonatal brain following asphyxia at birth, not only by significant prevention of abnormal brain activity and seizures, but also by minimizing cell death and partial neuroinflammation. Furthermore, our data show that the number and the duration of SE seizures and EDs are strong predictors for neuroinflammation and neuronal degeneration, respectively, which indicates that the antiseizure effects of ganaxolone also contribute to its neuroprotective actions. In contrast, in the current study at least, phenobarbital treatment failed to control seizure activity, resulting in a neuropathology profile similar to that observed in the untreated asphyxia animals.

Although the results of this preclinical study are extremely promising, we note the limitations of the study design, in which we used multiple comparisons with primary outcome measures of brain activity and brain histopathology. Phenobarbital or ganaxolone was administered at 6 hours after birth, whether or not seizure activity had commenced. This decision was pragmatic, so that all animals were standardized to the same period of antiepileptic treatment, thereby allowing direct comparison of their neuroprotective potential at 48 hours. However, the rate at which these animals seized in this experimental paradigm was high, with seizures occurring in 86% of asphyxia animals and 100% of asphyxia+phenobarbital animals, reflecting the severity of the insult. Similar rates are seen in human neonates identified as being at high risk of seizures in the perinatal period.[45] In future studies, it will be important to consider commencing treatment only after the onset of neonatal seizures, as occurs clinically. Alternatively, clinical trials of ganaxolone should consider pre-emptive treatment, rather than treatment in reaction to the occurrence of seizures. In this study, phenobarbital did not reduce SE seizures or EDs. Although phenobarbital is still the first-line treatment of choice, having been one of the few anticonvulsants available for parenteral administration, evidence for its efficacy is inconsistent. Painter et al[46] showed, in one of two randomized trials of phenobarbital use, that it was <50% effective when administered as a single agent and only up to 70% effective when administered with phenytoin in their crossover trial. However, more recently, Sharpe and colleagues showed that 80% of infants were seizure-free at 24 hours when treated with phenobarbital (20mg/kg). However, they also noted some nonsignificant adverse events, including respiratory suppression, hypotension, and sedation, when compared to levetiracetam.[47] Here, we provide novel evidence that phenobarbital is less effective than ganaxolone as an anticonvulsant in our animal study, and show that ganaxolone has a direct action on the brain, minimizing cell death and neuroinflammation. These markers of neuropathology also correlated strongly with the number and the duration of SE seizures and EDs. The more striking concern regarding phenobarbital in this age group is reported neurotoxicity.[38,48] Reassuringly, phenobarbital did not impact neuronal well-being in this study, whereas previous preclinical studies have clearly demonstrated that phenobarbital causes neuropathology. This difference in outcome may be because the assessment of brain histology was performed at 48 hours after induction of asphyxia in the current study, which may not have allowed the full spectrum of brain injury to evolve. Finally, we did not include levetiracetam in this study, which is now being used increasingly for the treatment of neonatal seizures, despite a lack of evidence of efficacy or benefit in this patient population. A recent multicenter, randomized, blinded, phase II trial comparing the efficacy and safety of levetiracetam and phenobarbital showed that phenobarbital was more effective at reducing neonatal seizures than levetiracetam.[47]

In the past decade, there has been increasing interest in therapies for neonatal seizures, particularly given the improved capacity to detect neonatal seizures afforded by the clinical use of aEEG and increasing awareness of the potential for seizures to exacerbate brain injury. Despite

this, phenobarbital remains the clinician's drug of choice. For the first time, we have shown that ganaxolone is effective at reducing abnormal brain activity, as shown by significant reduction in SE seizures and EDs. Additionally, we also present evidence that ganaxolone has anti-inflammatory properties and prevents neuronal degeneration. These data lay the foundation for translation of ganaxolone to clinical trials.

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Author Contributions

S.L.M., L.B., J.J.H., D.W.W., G.J., F.W., M.C.F. and T.Y. contributed to the conception and design of the study; S.L.M., L.B., A.M., Y.P., C.M., M.C.-M., B.J.A., J.M., I.N., B.J.B., R.W.H., G.J., A.M., and T.Y. contributed to acquisition and analysis of data; all authors contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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