Ethosuximide and Phenytoin Dose-Dependently Attenuate Acute Nonconvulsive Seizures after Traumatic Brain Injury in Rats

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Abstract

Acute seizures frequently occur following severe traumatic brain injury (TBI) and have been associated with poor patient prognosis. Silent or nonconvulsive seizures (NCS) manifest in the absence of motor convulsion, can only be detected via continuous electroencephalographic (EEG) recordings, and are often unidentified and untreated. Identification of effective anti-epileptic drugs (AED) against post-traumatic NCS remains crucial to improve neurological outcome. Here, we assessed the anti-seizure profile of ethosuximide (ETX, 12.5–187.5 mg/kg) and phenytoin (PHT, 5–30 mg/kg) in a spontaneously occurring NCS model associated with penetrating ballistic-like brain injury (PBBI). Rats were divided between two drug cohorts, PHT or ETX, and randomly assigned to one of four doses or vehicle within each cohort. Following PBBI, NCS were detected by continuous EEG monitoring for 72 h post-injury. Drug efficacy was evaluated on NCS parameters of incidence, frequency, episode duration, total duration, and onset latency. Both PHT and ETX attenuated NCS in a dose-dependent manner. In vehicle-treated animals, 69–73% experienced NCS (averaging 9–10 episodes/rat) with average onset of NCS occurring at 30 h post-injury. Compared with control treatment, the two highest PHT and ETX doses significantly reduced NCS incidence to 13–40%, reduced NCS frequency (1.8–6.2 episodes/rat), and delayed seizure onset: <20% of treated animals exhibited NCS within the first 48 h. NCS durations were also dose-dependently mitigated. For the first time, we demonstrate that ETX and PHT are effective against spontaneously occurring NCS following PBBI, and suggest that these AEDs may be effective at treating post-traumatic NCS.

Key words: EEG; ETX; NCS; PBBI; PHT; Rats; TBI

Introduction

POST-TRAUMATIC SEIZURES (PTS), convulsive or nonconvulsive, are a common occurrence in patients with severe traumatic brain injury (TBI), particularly in penetrating brain injury (PBI), during the acute post-injury period.¹ Whereas convulsive seizures are readily recognizable and aggressively treated with anti-epileptic drugs (AED), nonconvulsive seizures (NCS) occur without motor manifestation and are only detected definitively by continuous electroencephalogram (cEEG) in the neurointensive care unit (NICU). Depending upon the type of brain injury, it is estimated that 6–37% of patients display NCS.^{1–3} Seizures can exacerbate secondary injury, which may result in additional injuries, including aneurysmal rupture, intermittent and sustained intracranial pressure (ICP), hypoxia, and death.⁴ A clinical study compared two TBI patient cohorts (those who did or did not display seizures). The seizure group showed NCS that occurred in a bimodal pattern, demonstrated higher ICP, and showed increased brain lactate/pyruvate ratios. These

metrics remained elevated for a longer post-injury time period compared to the nonseizure group.^{4,5} Finally, it has been suggested that early PTS are associated with long-term hippocampal atrophy, and may be predictive of subsequent epilepsy development.^{6–9}

Pharmacoprophylaxis using AEDs against early seizures in severe TBI patients is currently recommended in the Trauma Brain Foundation Guidelines, and endorsed by the American Association of Neurologic Surgeons Joint Section on Neurotrauma and Critical Care, The World Health Organization's Committee on Neurotrauma, and the Congress of Neurologic Surgeons.¹⁰ AEDs may reduce brain metabolic demands, attenuate ICP and neurotransmitter release, and minimize secondary injury.¹¹ Although several AEDs are available for treating post-traumatic seizures after severe TBI, phenytoin (PHT) or its prodrug, fosphenytoin (FOS), are most commonly used.¹⁰ An international survey of neurologists that focused on cEEG and NCS management found that 73% of respondents would use PHT or FOS as a first or second line medication to attenuate NCS in patients.¹² Despite the common practice

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of PHT prophylaxis, the drug does not completely eliminate all seizures. Studies have shown a 22% seizure prevalence (of which half were NCS) in severe TBI patients and a pervasiveness of late-onset seizures.^{1,11} The persistence of refractory NCS and the need for close drug monitoring to maintain a narrow PHT therapeutic window have underscored the need for alternative AED treatments.

Given the harmful role that NCS play in the injured brain, increased attention has focused on early diagnosis and identification of additional promising anti-epileptic interventions to improve neurological outcome. To this concern, we previously compared the anti-seizure efficacy of seven clinically available AEDs (ethosuximide, FOS, gabapentin, midazolam, phenobarbital, and valproate) with distinct biochemical targets in a rat middle cerebral artery occlusion (MCAo) model in order to counteract these general pathophysiological mechanisms.¹³ We further profiled the dose-response effects of ethosuximide (ETX) and gabapentin in the same rat MCAo model.^{13,14} We have since extended those studies to investigate the AED efficacy against spontaneously occurring NCS following TBI.

Both ETX (Zarontin) and PHT (Dilantin) are United States Food and Drug Administration (FDA)-approved AEDs available for treatment of distinct types of epileptic conditions. ETX is commonly used to treat generalized absence (*petit mal*) seizures, and is less effective against other forms of generalized or partial seizures.^{15,16} ETX, a T-type low-voltage-activated calcium channel blocker, has been shown to effectively attenuate three cycle/sec spike-and-wave discharges associated with lapses of consciousness that occur during absence seizures. In contrast, PHT inhibits voltage-gated sodium channels, and is currently prescribed as an anticonvulsant to prevent or treat generalized tonic-clonic (*grand mal*) seizures, complex partial seizures, or seizures that occur during or following neurosurgery. Although PHT has been used in TBI patients, ETX is not commonly administered prophylactically for acute brain injury.^{11,17} The goal of the present study was to evaluate and compare the full dose-response efficacy of these two classic AEDs, ETX and PHT, to attenuate spontaneously occurring NCS following penetrating ballistic-like brain injury (PBBI) in rats.

Methods

Chemicals and drugs

ETX (2-ethyl-2-methylsuccinimide), PHT (5,5-Diphenyl-2,4-imidazolidinedione), and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Mefloquine (WR142490) was obtained from the Walter Reed Army Institute of Research chemical repository (Silver Spring, MD). For drug administration, ETX was dissolved in 0.9% physiological saline and PHT was formulated in an ethanol/1, 2-propanediol/water mixture (10:40:50%, v/v). Drugs were delivered over 60 sec intervals as a single bolus intravenous (i.v.) injection to awake, freely behaving animals.

General surgical procedures

Male Sprague-Dawley rats (275–350 g; Charles River laboratories, Raleigh, NC) were used in all studies. Food and water were provided *ad libitum* pre- and post-injury, and animals were individually housed under a normal 12 h light/dark cycle. For all surgical procedures, anesthesia was induced by 5% isoflurane and maintained at 2% isoflurane delivered in oxygen with a body temperature maintained at 37.0°C using a heating blanket (Harvard Apparatus, Holliston, MA).

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Walter Reed Army Institute of Research. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adhered to the principles stated in the "Guide for the Care and Use of Laboratory Animals." The animals' housing facility was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

EEG electrode implantation was conducted as previously described.¹⁸ Briefly, 1 week prior to brain injury, four skull EEG electrodes composed of insulated Nichrome wire (0.2 mm in diameter, uninsulated at each end) were soldered to a 0.3175 cm stainless steel screw (Component Supply Company, Fort Mead, FL) and implanted through bilateral burr holes in the frontal and parietal skull (1 mm anterior and 4 mm posterior to bregma, $\pm$ 3.5 mm lateral to midline) of anesthetized rats (Fig 1A). A fifth reference electrode was implanted posterior to lambda over the transverse sinus. The free end of each electrode wire was soldered to a Dale multi-pin connector (March Electronics, West Hempstead, NY) in the sequence of A, B, C, and D, which corresponded to the left frontal, left parietal, right frontal, and right parietal mono-referential recording montage, respectively. The connector was firmly secured on the rat head by dental acrylate. For drug delivery, indwelling intravenous cannulas (polyethylene-50) were placed into the right jugular vein of all animals 2–3 days after EEG implantation.

Penetrating brain injury model

After 2–3 days of recovery from the abovementioned procedures, animals were subjected to unilateral frontal PBBI as previously described.¹⁸ Right frontal PBBI was performed using a computer-controlled simulated ballistic injury device (Mitre Corp., McLean, VA) attached to a specially designed stainless steel probe (Popper & Sons Inc., New Hyde Park, NY) with fixed perforations along one side that were sealed by airtight elastic tubing. This model of penetrating brain injury has been previously described.^{19–21} The anesthetized rat was positioned in a stereotaxic frame (Kopf, Tujunga, CA) for probe insertion. After midline incision, a craniotomy was performed to expose the right frontal pole of the brain (+4.5 mm anteroposterior, +2 mm mediolateral from bregma). The PBBI probe was mounted to a stereotaxic arm at a fixed angle of 50 degrees from the vertical axis and 25 degrees counterclockwise from the anterior-posterior axis. The probe was then manually advanced through the cranial window, penetrating into the right frontal lobe to a distance of 1.2 cm (from dura). The ballistic component of the injury causing the temporary cavity was induced by a rapid (36 ms in duration) water pressure pulse (63–65 psi) designed to fill the elastic tubing and form an elliptical-shaped balloon calibrated to equal 10% of total rat brain volume.

Continuous EEG recording

On the day of PBBI, all animals were individually housed in custom-designed Plexiglass EEG recording chambers equipped with multichannel gold contact swivel communicators (Dragonfly Inc., Ridgeley, WV) for cEEG monitoring. A flexible shielded cable connected the swivel to the multipin connector on the rat skull to allow free movement of the animal for the duration of the experiment. The swivel commutator was interfaced with an EEG amplifier, and the EEG signals were recorded and digitized by a computerized data acquisition system (Harmonie software, Natus Medical Incorporated, San Carlos, CA). EEG and video recordings were collected for 30 min prior to PBBI, and continuously after injury for the 72 h duration of the experiment.

AED treatment of seizures induced by PBBI

The dose-response anti-seizure efficacies of both PHT and ETX were assessed following PBBI. Rats were divided between two

FIG. 1. Pathological continuous electroencephalogram (cEEG) signatures following penetrating ballistic-like brain injury (PBBI). (A) Schematic of EEG electrode configuration. (B) Representative cEEG tracing showing pre-injury baseline recordings and post-injury changes, including contralateral EEG signal attenuation (channels 1 and 2) and ipsilateral EEG signal slowing (channels 3 and 4) at 2 h post-injury following PBBI. (C) Typical post-PBBI generalized nonconvulsive seizures (NCS). (D) Expansion of EEG signal inset in (C). Representative EEG tracings of (E) generalized rhythmic spike and (F) sharp-wave discharges of >1 Hz.

drug cohorts and randomly assigned to one of five groups ($n=10$ – 12 /group) within each cohort. Four doses of each AED were tested. Each group received an initial loading dose of ETX (25, 125, 150, or 375 mg/kg, i.v.), PHT (10, 20, 40, or 60 mg/kg, i.v.) followed by a maintenance dose of ETX (12.5, 62.5, 125, and 187.5 mg/kg, i.v.) or PHT (5, 10, 20, and 30 mg/kg, i.v.). The initial loading dose was given 30 min post-PBBI, and the first maintenance dose was given 8 h later and repeated twice daily at 09:00 and 17:00. Two separate control groups received respective vehicle treatments with matched volumes and time intervals.

Seizure detection

The focus of this study was to treat post-traumatic NCS; therefore, the EEG recording was accompanied with synchronized digital video monitoring. Digital video clips coinciding with each EEG seizure event were reviewed to confirm whether the seizure was convulsive or nonconvulsive. If a convulsion (e.g., tonic-clonic seizure) occurred during the EEG seizure episode, the animal was excluded from the study. However, subtle behavioral expressions, including jaw movement, nodding, or wet dog shakes,

that coincided with EEG seizure episodes, were considered non-convulsive behavioral components of the NCS.²² NCS were identified and quantified by manual off-line review of all EEG recordings at a display resolution of 10 mm/s, and subsequently verified at a recoding speed of 30 mm/s by experimenters blind to treatment groups. NCS were defined and identified as previously described,²² based on the following criteria: 1) repetitive rhythmic spike discharges occurring at a frequency of 1–4 Hz, 2) spike amplitude greater than background activity, and 3) duration of continuous seizure activity for >5 sec. Consecutive seizures separated by <5 sec were considered a single event. Seizures were classified as generalized (bihemispheric) or focal/partial (unihemispheric). Based on the abovementioned seizure criteria, several descriptive parameters were evaluated for all groups: 1) NCS incidence, number of animals that experienced at least one NCS event; 2) NCS frequency, number of NCS events per animal; 3) NCS episode duration (sec), the duration of one NCS event; 4) NCS total duration (sec), the sum duration of all NCS events per animal; and 5) NCS onset latency (h), the time interval between PBBI on the occurrence of the first NCS event per animal. To control for “floor” effects, vehicle-treated animals that displayed no incidence of post-

traumatic NCS (and corresponding ranked pairs for each drug/dose) were excluded from statistical analyses of other NCS outcome metrics. All NCS events were confirmed with video recordings. Any convulsive seizures were excluded from analysis.

Histopathology

At 72 h post-injury, animals were deeply anesthetized with ketamine/xylazine (70 and 6 mg/kg, i.m., respectively) and were transcardially perfused with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA). Brains were removed and fixed for 6 h in 4% PFA-PB then placed in 20% sucrose solution. Following cryoprotection, coronal sections (40 μ m) were cut from +4.0 to -7.0 mm anterior-posterior (AP) from bregma. For analysis of lesion volume, serial sections of brain tissue were collected at 960- μ m intervals processed using hematoxylin and eosin (H&E) staining (FD Neurotechnologies, Baltimore, MD). Brightfield images were collected on an Olympus BX61 Microscope, and the lesion volume was quantified by observers blind to treatment groups. Sequential lesion areas were defined by tracing the perimeter of the lesion cavity (including injured penumbra) across 12 serial sections (spaced 960 μ m; +4 to -7 mm AP from bregma) and lesion volume was calculated using the Cavalieri formula (Inquiry Digital Analysis System; Loats Assoc., Westminster, MD). Mean lesion volumes between groups within each cohort were used for statistical comparison.

Pharmacokinetic profile

In a separate group of animals, rats were implanted with an indwelling jugular cannula and randomly assigned to one of five groups within the two drug cohorts ($n \geq 4$ rats/group). Animals received PBBI and were dosed with EXT, PHT, or vehicle as described. Blood samples were collected from the jugular vein at pre-injury, 1, 8, 24, 32, 48, 56, and 72 h post-injury time points. Blood samples (200 μ L/sample) were collected in serum separation tubes (Sarstedt, Newton, NC) and immediately centrifuged at 3000 rpm for 10 min and stored at -80°C until analysis. Daily body temperature and weight measurements were recorded for each animal for the 72 h duration.

Determination of ETX and PHT levels in serum

For each compound a calibration curve and quality control (QC) samples were prepared by spiking blank rat serum obtained from naïve animals to the desired concentrations (ETX, 0-50,000 ng/mL and PHT, 0-1000 ng/mL). The standard curve, QC, and serum samples were prepared for analysis by extraction with 1:2 ratios of sample to acetonitrile containing 250 ng/mL of mefloquine as internal standard, followed by brief vortexing and centrifugation at 13,000 rpm for 10 min at 4°C. Samples (7.5 μ L) were analyzed by high-performance liquid chromatography using a Waters Acquity UPLC system (Milford, MA) coupled to an AB-Sciex 4000 Q Trap mass spectrometer (Framingham, MA) equipped with standard electrospray ionization source. Chromatographic separation was achieved on a Waters Xterra MS C18 column (2.1 $\times$ 50 mm, 3.5 μ m) by using a gradient elution of mobile phases consisting of water/methanol mixtures: mobile phase A (95/5, v/v) and mobile phase B (10/90, v/v), both containing 0.05% formic acid. The gradient starting with mobile phase B was increased linearly from 5% to 98% over 2.5 min, held constant at 98% B from 2.5 to 4.5 min, and then decreased to 5% B over a 1 min period prior to column re-equilibration (1 min). The retention times and flow rates for PHT and ETX were 2.62 min at 400 μ L/min and 2.29 min at 300 μ L/min, respectively. PHT and ETX detection was achieved using multiple reaction monitoring (MRM) in positive electrospray ionization mode. MS conditions were optimized for each analyte and the corresponding instrumental parameters were: ion spray

voltage 5.5 kV, source temperature 300°C, curtain gas 20 psi, declustering potential 96 V, collision energy 25 V for PHT, and ion spray voltage 5.5 kV, source temperature 100°C, curtain gas 10 psi, declustering potential 51 V, collision energy 19 V for ETX. The monitored ion transitions were 253.20 m/z $\rightarrow$ 182.20 m/z for PHT, 142.20 m/z $\rightarrow$ 72.10 m/z for ETX, and 379.10 m/z $\rightarrow$ 361.10 m/z for the mefloquine internal standard. Data processing and analyses were conducted using AB-Sciex Analyst[®] software. PHT and ETX serum drug concentrations were interpolated from each corresponding standard curve. The lower limit of quantification (LLOQ) for PHT and ETX were 5 and 25 ng/mL, respectively.

Statistical analysis

Statistical significance for seizure criteria was evaluated using nonparametric analysis (Kruskal-Wallis test) followed by Dunn's multiple comparisons where appropriate. Lesion volumes, body temperature, body weight, and serum AED concentration were compared between groups and time points by one-way or two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test where appropriate. Chi square analysis was used for evaluating a trend in proportions of quantal data (NCS incidence) and Kaplan-Meier curves and the log-rank test were used to analyze univariate distributions for NCS latency. Log-nonlinear regression analysis was used to calculate the intravenous ETX and PHT ED₅₀ values with 95% confidence limits. All analyses were conducted using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego CA) and Systat 13 software (Systat Software Inc., San Jose, CA). Group differences were considered statistically significant at $p < 0.05$. All data were expressed as mean $\pm$ S.E.M.

Results

NCS in the PBBI model

In all rats, PBBI induced an immediate attenuation of EEG amplitude in the contralateral hemisphere accompanied by a concomitant slowing of EEG signals (≤ 1 Hz) in the ipsilateral hemisphere when compared with pre-injury baseline recordings (Fig. 1B). Individual spontaneous NCS events occurred as repetitive spike and/or sharp wave discharges with amplitudes significantly higher than background (Fig. 1C and D). NCS occurred in a randomized fashion as individual events or in a series of double and triple seizures (Fig. 1C). ETX and PHT vehicle-treated groups did not significantly differ in NCS metrics ($p > 0.05$). Spontaneous NCS were detected in ~71% of all vehicle-treated animals (i.e., ETX = 69%; PHT = 73%). ETX and PHT vehicle-treated rats that experienced NCS had an average frequency of either 9 ± 2 or 10 ± 3 episodes/rat with an average duration of 32 ± 4 sec or 37 ± 4 sec, respectively. The majority of NCS events (for both vehicle groups) occurred between 24 and 48 h post-PBBI, with a mean total duration of NCS activity ranging from 267 ± 63 (ETX vehicle) and 350 ± 100 sec (PHT vehicle) (Fig. 2). NCS was confirmed via video recording using the criteria described in the Methods section. In rare situations (<1% of all EEG-detected seizures), animals displayed convulsive seizure activity detected by the video recordings. These convulsive seizures were excluded from analysis. The majority of NCS were generalized seizures occurring in both hemispheres, whereas only a small number of NCS were focal seizures limited to either hemisphere. Most NCS EEG signals were dominated by the repetitive rhythmic spike discharges (1-4 Hz), although some seizures exhibited rhythmic sharp waves or arrhythmic spike clusters. (Fig. 1 E and F). The analyses of the AED treatment effects included all seizure types, despite of their EEG signal variations.

Dose-response effects of ETX or PHT on PBBI-induced NCS

Effects of ETX treatment on NCS incidence, frequency, and duration. The anti-seizure effects of ETX were dose-related across all NCS parameters, with the highest two dosing regimens (125 and 187.5 mg/kg) affording the most significant effects (Fig 2 A, C, E, and G). Within these doses, NCS incidence was significantly attenuated from 69% in vehicle-treated animals to 27% and 13% in the higher ETX-dosed groups (125 and 187.5 mg/kg, respectively, $p < 0.05$, Fig. 2A). Lower doses (12.5 and 62.5 mg/kg) failed to significantly reduce the number of rats that displayed NCS and were ineffective in other NCS assessment metrics ($ED_{50} = 89.6 \text{ mg/kg}$ – 95% confidence level [CL]: 38.7–208.3). Similarly, NCS frequency was significantly reduced from 9.2 NCS/rat, (veh-

icle) to < 1.5 NCS/rat in the highest ETX doses (125 and 187.5 mg/kg, $p < 0.05$). Additionally these two doses resulted in a $> 87\%$ reduction in total time spent in NCS, decreasing total NCS duration to 37 and 30 sec compared with vehicle (267 sec, $p < 0.01$). In contrast, only the highest ETX dose significantly reduced NCS episode duration (~ 5 sec) compared with vehicle ($p < 0.05$; Fig. 2E)

Effects of PHT treatment on NCS incidence, frequency and duration. PHT showed a similar dose-response anti-seizure profile to that of ETX (Fig. 2B, D, F, and H). The highest PHT dose (30 mg/kg) had the greatest effect on NCS parameters, reducing NCS incidence from 73% (vehicle) to 33% ($ED_{50} = 20.4 \text{ mg/kg}$ [95% CL: 12.8–34.9 mg/kg] $p < 0.05$, Fig. 2) whereas the two lower doses were ineffective at reducing the number of NCS-positive

FIG. 2. Dose-response effects of ethosuximide (ETX) and phenytoin (PHT) treatment on spontaneous nonconvulsive seizures (NCS). Following penetrating ballistic-like brain injury (PBBI), animals were intermittently dosed with ETX (12.5, 62.5, 125, 187.5 mg/kg), PHT (5, 10, 20, 30 mg/kg), or respective vehicle initiated 30 min post-injury. (A, B) Incidence of NCS activity. (C, D) NCS frequency. (E, F) Duration of individual NCS episodes. (G, H) Total time spent in NCS (summation of all NCS); drug doses are graphically displayed as maintenance dose concentrations (mg/kg), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with respective vehicle.

animals. The two highest PHT doses appeared equally effective in mitigating NCS frequency and total NCS duration. PHT-treated rats (20 and 30 mg/kg) showed, on average, fewer than two NCS per animal than those treated with vehicle (10.5 NCS/rat), and total time spent in NCS was reduced $\sim 80\%$ compared with those treated with vehicle alone (PHT: 57–73 sec, vehicle: 361 sec, $p < 0.05$, Fig. 2). For individual NCS episodes, PHT treatment showed a U-shaped dose-response curve: the second highest dose (20 mg/kg) displayed the greatest reduction in NCS (13 sec) compared with vehicle (37 sec), whereas the highest PHT dose appeared less effective (19 sec) (Fig. 2F, $p < 0.05$).

NCS temporal profile following PHT and ETX treatment

The majority of NCS events following PBBI occurred between 24 and 48 h, with 75% of all vehicle-treated rats experiencing their first NCS event by 48 h post-injury (Fig. 3A and B). ETX and PHT significantly delayed NCS onset, as $< 20\%$ of animals treated with the two highest doses of ETX (125 and 187.5 mg/kg) and PHT (20

and 30 mg/kg) had experienced an initial NCS by 48 h post-PBBI, and $< 40\%$ of all animals had displayed an NCS by 72 h post-injury (Kaplan–Meier curves, $p < 0.01$ compared to respective vehicle, Fig. 3C, D). Among the temporal distribution of all NCS for each treatment group, all four PHT (5–30 mg/kg) doses and the highest ETX (187.5 mg/kg) dose significantly delayed average NCS onset from 30 h post-injury in vehicle animals to > 45 h (Fig. 3A and B).

Attempts to improve anti-seizure drug effects across all NCS parameters by increasing drug doses were unsuccessful; ETX and PHT produced moderate but transient polymorphic delta activity and sedation at the highest doses tested (187.5 mg/kg and 30 mg/kg) and pilot studies using increased ETX concentrations (ETX 250 mg/kg) resulted in severe sedation and were not carried forward (data not shown).

ETX and PHT do not affect lesion volume

In vehicle-treated animals, PBBI induced severe brain injury with intracerebral hemorrhage and extensive tissue damage corresponding to total lesion volume (including injured penumbra) of

FIG. 3. Temporal profile of penetrating ballistic-like brain injury (PBBI)-induced nonconvulsive seizures (NCS) after ethosuximide (ETX) and phenytoin (PHT) treatment. (A, B) Temporal distribution of individual NCS of all PBBI and treated animals. Red line denotes mean time of NCS occurrence within ETX and PHT cohorts. (C, D) Kaplan–Meier curves depicting the percentage of animals that experienced an initial NCS at discrete time points following ETX or PHT treatment. (Right, boxed) p values from log-rank (Mantel–Cox) test between drug cohorts vehicle group; $*p < 0.05$, compared with vehicle.

FIG. 4. Ethosuximide (ETX) and phenytoin (PHT) do not significantly affect lesion volume. Representative bright field images of hematoxylin and eosin stained sections showing histopathological damage at 3 days post-injury for animals treated with (A) vehicle, (B) ETX (187.5 mg/kg), and (C) PHT (30 mg/kg). Volumetric measurements of lesioned brain tissue across all (D) ETX- and (E) PHT-treated groups. Scale bar = 1mm.

84–94 mm³. No dose of ETX and PHT significantly affected lesion volume (Fig. 4), and there were no significant correlations between the lesion volume and NCS frequency or duration within each drug group for ETX or PHT (data not shown).

Physiological parameters and pharmacokinetic profile of ETX and PHT

In a separate cohort of rats subjected to 10% frontal PBBI, we conducted a pharmacokinetic study to measure AED serum levels following ETX or PHT administration. Concomitantly, changes in body weight and temperature were recorded. Although all animals showed a time-dependent reduction in body weight following injury, we detected no significant group differences in weight loss between treatment groups of ETX or PHT and respective vehicle groups ($p > 0.05$, Fig. 5A and B). Similarly, changes in body temperature of ETX-treated rats at all doses tested were not significantly different from vehicle treatment at any time point over the 72 h monitoring period. In contrast, rats receiving the highest two doses of PHT (20 and 30 mg/kg) showed significant reductions in body temperature ($> 2^{\circ}\text{C}$ decrease) at 1 h post-injury ($p < 0.05$, Fig. 5D). Body temperature was indistinguishable from that of vehicle-treated animals at all other time points.

Serum samples were collected 30 min following first bolus injection and then twice daily, immediately prior to AED dosing. The observed ETX and PHT concentrations as a function of time in all doses are displayed in Fig. 5E and F. Thirty minutes following bolus injection of ETX or PHT, AED drug serum levels showed an immediate increase, which was dose-proportional across all ETX and PHT doses. ETX serum levels of the doses with the greatest anti-epileptic effect (125 and 187.5 mg/kg) ranged between 400 and 600 $\mu\text{g/mL}$, whereas serum levels undulated between 5 and 10 $\mu\text{g/mL}$ of the most effective PHT dose (30 mg/mL, Fig. 5F, inset).

Discussion

In this study, we report the dose-response profiles of ETX and PHT to attenuate spontaneous NCS induced by penetrating brain injury. Because it is known that the epileptogenic process starts with the trauma itself, we initiated our prophylactic AED intervention within 1 h post-injury: an administration time in concordance with clinical practice.²³ At the highest doses tested, independent post-injury treatment with ETX or PHT significantly reduced seizure incidence, frequency, and duration, and delayed NCS onset latency.

ETX has been shown to increase seizure threshold in various rodent models of experimentally induced seizures, as well as in animals genetically predisposed to epilepsy, albeit at various effective doses.^{24–27} However, our rodent model of spontaneously occurring NCS is unique in its inherent seizure etiology and real-time clinical profile, and, therefore, is more reflective of clinically relevant NCS following TBI than exogenously induced seizure models. Previously, our laboratory profiled the dose-response anti-NCS efficacy of ETX in a rat model of focal cerebral ischemia. Prophylactic ETX anti-seizure activity following MCAo was mirrored in the current study, as evidenced by reduced seizure incidence and frequency, albeit at a higher dose (250 mg/kg).¹⁴ Notably, the ED₅₀ in our current TBI-induced NCS model (89.6 mg/kg, 95% CL, 38.7–208.3 mg/kg) falls within the range of the 50% effective doses reported in the aforementioned studies (range, 21.5–328 mg/kg). In contrast, ETX has been found ineffective in the maximal electroshock seizure (MES) test (ED₅₀ > 500 mg/kg) in mice and rats, fails to reduce tonic convulsions in spontaneously epileptic rats (SER), and does not exhibit anticonvulsant properties in kindling seizure models, except at neurotoxic concentrations.^{26,28–30}

Similarly, the anticonvulsant activity of PHT has been extensively profiled in pre-clinical studies. At doses comparable to those

FIG 5. Changes in physiologic parameters and pharmacokinetic profile in penetrating ballistic-like brain injured (PBBi) rats following ethosuximide (ETX) or phenytoin (PHT) treatment. Separate groups of PBBi-injured animals were dosed with ETX, PHT, or vehicle. Changes in body weight (**A, B**) and body temperature (ΔT_b) (**C, D**) were recorded over the 72 h post-injury time period. Serum samples were collected and analyzed for anti-epileptic drug (AED) levels. Dose-dependent serum concentration time profiles in PBBi-injured rats after intermittent intravenous administration of (**E**) ETX (12.5–187.5 mg/kg) or (**F**) PHT (5–30 mg/kg); (inset) expansion of boxed region in (**F**), * $p < 0.05$, 30 mg/kg group compared with vehicle; † $p < 0.05$, 20 mg/kg group compared with vehicle.

used in this study, PHT was found to attenuate tonic-clonic seizures and wild running in rodents following global cerebral ischemia, demonstrated anticonvulsant potency by the MES test, and reduced audiogenic seizures (AGS) and significantly decreased the number of tonic convulsions in the SER.^{31–34} Unlike ETX, PHT has proven ineffective in elevating minimal electroshock seizure threshold, did not protect against metrazol-induced seizures, and did not affect the absence-like seizures in SER.^{32,33}

In our model, both ETX and PHT significantly attenuated NCS following TBI. Such results are intriguing considering that both

drugs are clinically approved for treating distinct epileptic conditions. ETX, a T-type calcium channel blocker that has a narrow therapeutic profile, is approved for treatment of *petit mal* seizures, and is a valuable agent in studies of absence epilepsy. PHT, a sodium channel blocker, is mechanistically distinct from ETX, and is a broad spectrum AED effective against convulsive seizures. It is likely that dysregulation of both calcium and sodium channels contribute to NCS initiation and propagation, as NCS are susceptible to AEDs that inhibit either target. Despite clear anti-seizure profiles, neither ETX nor PHT was found to be completely effective

in eliminating NCS; however, increasing AED doses was not feasible. Attempts to increase PHT and ETX levels produced drug-induced polymorphic delta activity and heavy sedation (data not shown), similar to reports in other rodent models.³⁵

Following PBBI, injured rats exhibited a mild decrease in body temperature at 1 h post-injury, and subsequent weight loss over the duration of the experiment (72 h). Although we detected no significant difference in weight loss or body temperature between vehicle and ETX treatment groups, animals treated with the two highest PHT doses exhibited a significant 2–3°C reduction in core body temperature at 30 min post PHT administration compared with those receiving vehicle or lower PHT doses. These results are in concordance with our previous study, in which animals treated with FOS exhibited a reduction in core body temperature from baseline measures at 1 h following focal brain ischemia.¹³ Similar temperature changes have been reported in rodent seizure models at doses comparable to those reported in our current study.³⁶ Although PHT is not known to induce hypothermia, a systemic reduction in body temperature, similar to experimental models of mild hypothermia, may afford neuroprotective benefit and significantly alter the pathological responses in brain injury.³⁷ However, similar to our previous study, PHT, at any of the doses tested, did not significantly reduce lesion volume, a commonly assessed metric of potential neuroprotective agents.¹³ Given the mechanism of action of PHT, it is doubtful that the anti-seizure effect would be mitigated solely via a neuroprotective mechanism, and a reduction in body temperature may simply be an idiosyncratic side effect.

For many AEDs, the clinical effect often better correlates with anticonvulsant blood levels than with individual drug doses. Consequently, we monitored serum ETX and PHT levels over the 72 h post-injury recording period. As expected, dose-dependent AED serum levels were immediately elevated following bolus injection, and decreased to steady-state concentrations. Similar effective PHT plasma levels (range 7–20 µg/mL) in other rodent seizure models and epilepsy patients have been reported.²⁵ In contrast, ED₅₀ ETX plasma concentrations range 40–134 µg/mL for rodent AGS and pentylenetetrazole (PTZ) studies, substantially lower than the serum levels (266 µg/mL) of the ED₅₀ value calculated for this study. In fact, our lowest two ETX doses (12.5 and 62.5 mg/kg, serum levels 20–200 µg/mL) offered no significant therapeutic benefit on any NCS metric assessed. However, it must be noted that the plasma half life of ETX is significantly longer in human adults (30–60 h) compared with rats (10–16 h), and serum drug levels would be more rapidly eliminated in rodent models leading to reduced systemic drug concentrations.³⁸ Nevertheless, the ETX half-life in rats is sufficiently long enough to sustain effective drug levels during the prolonged treatment period. Maintaining effective drug plasma levels, in the case of other AEDs following intermittent drug dosing, has proven difficult, particularly for drugs with half-lives < 5 h in the rat. In contrast, for drugs that exhibit nonlinear kinetics such as PHT, slight increases in dose may lead to considerable elevation of serum levels.³⁸ Despite showing sharp initial declines in serum concentrations, PHT at the highest doses tested was effective against NCS, and serum levels appeared to approach steady state over the 72 h period. Interestingly, studies have shown that PHT concentrations can remain elevated (40–60 min) in the rat hippocampus after crossing the blood–brain barrier despite decreasing serum concentrations.³⁹ Such factors may account for prolonged anti-seizure effects, despite sharp decreases in serum concentrations detected in our study. Attempts to increase PHT doses were not conducted because of sedation

and the known adverse effects at higher PHT plasma levels (> 30 µg/mL).

Identification of optimal anti-epileptic drug compounds and/or combinations may offer a safe and effective way to prophylactically treat brain injury-induced NCS without adversely affecting patients. The clear anti-seizure effects of both ETX and PHT to attenuate NCS following rodent TBI, in this study, present the intriguing possibility for future combination drug studies. Whereas PHT remains a first-line drug for acute TBI, patients showing PHT-resistant or refractory seizures may benefit from the potential additive or synergistic effects of combination ETX/PHT treatment, which would independently target distinct anti-seizure mechanisms.

Conclusion

In summary, penetrating brain injury in rats induced spontaneous NCS activity in the acute post-injury phase. The results of this study provide the first demonstration that either PHT or ETX protects against spontaneously occurring NCS in a dose-dependent manner following TBI in rodents. Treatment with either ETX or PHT attenuated NCS incidence, frequency, duration, and delayed seizure onset. Moreover, because PHT and ETX act via different mechanistic pathways, our data indicate that PBBI-induced NCS are likely mediated by both sodium and calcium channel dysfunctions. These results underscore the need for combination drug therapy development capable of targeting multiple mechanisms that underlie post-traumatic NCS. Subthreshold combinations of these two drugs may provide an improved pharmacological strategy for treating post-traumatic NCS, and improve patient outcome.

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