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Fast Ripple-Delta Coupling as Early Biomarker for Post-Traumatic Epileptogenesis in Repetitive Brain Injury

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Authors

Oleksii Shandra
Corresponding Author
Submitting Author

ORCiD
https://orcid.org/0000-0003-4447-3312

Affiliations
- Department of Biomedical Engineering, Florida International University, Miami, FL, 33174

Dzenis Mahmutovic

ORCiD
https://orcid.org/0000-0002-2180-2147

Affiliations
- Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, 35294

Biswajit Maharathi

Affiliations
- Department of Neurology and Rehabilitation, University of Illinois, Chicago, IL 60612

Md Adil Arman

ORCiD
https://orcid.org/0009-0002-5937-8027

Affiliations
- Department of Biomedical Engineering, Florida International University, Miami, FL, 33174

Michael J Benko

Affiliations
- Division of Neurosurgery, David Grant Medical Center, Travis AFB, CA 94535

Owen Leitzel

Affiliations

- Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, 35294

Pritom Kumar Saha

Affiliations

- Department of Biomedical Engineering, Florida International University, Miami, FL, 33174

Stefanie Robel

Affiliations

- Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, 35294
- Department of Physical Medicine and Rehabilitation, University of Alabama at Birmingham, Birmingham, AL, 35294

ORCiD
https://orcid.org/0000-0001-6716-3670

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Fast Ripple-Delta Coupling as Early Biomarker for Post-Traumatic Epileptogenesis in Repetitive Brain Injury

Oleksii Shandra[1#], Dzenis Mahmutovic[2], Biswajit Maharathi[3], Md Adil Arman[1], Michael J Benko[4], Owen Leitzel[2], Pritom Kumar Saha[1], Stefanie Robel[2,5]

[1]Department of Biomedical Engineering, Florida International University, Miami, FL, 33174

[2]Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, 35294

[3]Department of Neurology and Rehabilitation, University of Illinois, Chicago, IL 60612

[4]Division of Neurosurgery, David Grant Medical Center, Travis AFB, CA 94535

[5]Department of Physical Medicine and Rehabilitation, University of Alabama at Birmingham, Birmingham, AL, 35294

Corresponding author: Oleksii Shandra, MD, PhD

Department of Biomedical Engineering

Florida International University

10555 W Flagler St, EC-2671, Miami, FL, 33174

Email: oshandra@fiu.edu

Phone: 305-348-0240

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Abbreviations

ADR - Alpha-Delta Ratio

CDMRP - Congressionally Directed Medical Research Programs

CCI - Controlled Cortical Impact

CURE - Citizens United for Research in Epilepsy

DoD - Department of Defense

EEG - Electroencephalography

FPI - Fluid Percussion Injury

HFO - High-Frequency Oscillations

IIR - Infinite Impulse Response

NIH - National Institutes of Health

NINDS - National Institute of Neurological Disorders and Stroke

PTE - Post-Traumatic Epilepsy

PTS - Post-Traumatic Seizure

RAV - Relative Alpha Variability

rdTBI - Repetitive Diffuse Traumatic Brain Injury

SEM - Standard Error of the Mean

STE - Short Time Energy

TBI - Traumatic Brain Injury

ZT - Zeitgeber Time

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Conflict of Interest Statement

None.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Oleksii Shandra https://orcid.org/0000-0003-4447-3312

Stefanie Robel https://orcid.org/0000-0001-6716-3670

Dzenis Mahmutovic https://orcid.org/0000-0002-2180-2147

Biswajit Maharathi https://orcid.org/0000-0002-9869-1801

Md Adil Arman https://orcid.org/0009-0002-5937-8027

Michael Benko https://orcid.org/0000-0002-0741-0788

Owen Leitzel https://orcid.org/0000-0002-2924-9366

Pritom Kumar Saha https://orcid.org/0009-0003-7211-826X

Abstract

Traumatic brain injury (TBI) can induce post-traumatic epilepsy (PTE), but early biomarkers for epileptogenesis are lacking. We used a repetitive diffuse TBI (rdTBI) model in mice with continuous video-EEG monitoring up to 4½ months post-injury to investigate electrographic biomarkers before and during post-traumatic seizure development. 25% of mice developed post-traumatic seizures with highly variable latency (5-126 days post-injury). Most significantly, we identified fast ripple-delta DOWN state coupling as an early biomarker that was detectable at 4

days post-TBI and appeared before seizure onset in all seizure-experiencing mice. This EEG signature distinguished seizure-experiencing from seizure-free TBI mice with high specificity. Power spectrum analysis revealed elevated delta and theta power, reduced physiological fast oscillations (alpha, beta, gamma) and increased pathological high-frequency oscillations (fast ripples) in seizure-experiencing animals, indicating network hyperexcitability. Spike analysis showed that while TBI itself increased cortical excitability, seizure onset triggered a dramatic further escalation in interictal activity. These electrographic signatures were remarkably consistent across all seizure-experiencing animals regardless of single or recurrent seizure pattern. Our results demonstrate that fast ripple-delta coupling represents a promising early biomarker detectable at 4 days post-TBI, before seizure onset, offering potential for early identification of post-traumatic seizure susceptibility. Importantly, this biomarker identified all seizure-prone animals regardless of whether they developed single or recurrent seizures, suggesting shared underlying mechanisms and clinical relevance for any post-traumatic seizure occurrence. These findings emphasize the utility of temporal EEG analysis for detecting early electrographic changes in post-traumatic epileptogenesis and may inform future intervention strategies.

Key Points

- Fast ripple-delta DOWN state coupling was detectable as early as 4 days post-TBI and appeared before seizure onset in seizure-experiencing mice, representing the first early biomarker that can stratify animals for epileptogenesis risk during the critical latent period.
- Delta and theta power increased while alpha, beta and gamma power decreased in all seizure-experiencing mice post-TBI, creating a consistent electrographic signature regardless of whether animals developed single or recurrent seizures.
- Fast ripples were elevated and gamma-to-HFO ratios were reduced in seizure-experiencing mice, reflecting network hyperexcitability shift and potential inhibitory dysfunction that preceded seizure onset.
- Seizure onset triggered a 3-fold escalation in spike activity, while baseline spike differences between TBI and pre-seizure mice were not significant, highlighting the limitation of spike counts alone as predictive biomarkers during the latent period.
- Electrographic signatures were almost similar across all seizure patterns (single and recurrent), suggesting shared underlying mechanisms of network dysfunction, though larger studies are needed to determine if biomarkers can predict seizure frequency in addition to seizure susceptibility.

Introduction

Post-traumatic epilepsy (PTE) is a particularly debilitating chronic disorder induced by traumatic brain injury (TBI) characterized by unprovoked, recurrent seizures[1, 2]. Forty percent of patients who develop PTE experience their first late seizure within the first 6 months after TBI and 80% of PTE patients will have had their first late seizure within 2 years after injury[3]. Currently, it is impossible to determine early after TBI which patients will ultimately develop PTE.

The emergence of spontaneous, recurrent seizures has been confirmed in rodent models of fluid percussion injury (FPI)[4-6], controlled cortical impact (CCI)[6-8], weight drop[9, 10] and blast injury[11-13], which mimic different types of TBI including focal and diffuse TBI or a combination thereof. While no animal is perfect in resembling the complexities of human TBIs, specific pathologies including primary cell death[14], blood-brain barrier breakdown[15] and glial cell activation[7] are recapitulated and can be examined for their role in driving PTE development. However, a significant challenge in utilizing these models arises from the variable and sometimes low incidence of PTE. For example, lateral FPI in mice has an incidence of ~3%[6], CCI models range from 36% to 58%[8, 16], blast models show 46%-50% of PTE incidence[13] and a weight drop model reports an incidence of 50%[9, 10].

Since not all animals that undergo TBI develop PTE, it is critical to distinguish animals that develop seizures from those that do not before identifying molecular and cellular mechanisms that cause PTE. We previously found that mice that developed PTE had larger areas of atypical astrocytes compared to mice that did not develop PTE[10]. Atypical astrocytes rapidly lose homeostatic proteins involved in maintaining neuronal function including the potassium channel

Kir4.1 and the glutamate transporter Glt1, which may contribute to the development of PTE[10]. However, to identify this difference in PTE mice, we had to first record EEGs for several months after mice underwent TBI. Tissue changes were examined after PTE onset, making it impossible to determine if the detected changes are the cause or consequence of PTE. It is essential to establish methods to determine which mice will develop PTE earlier following TBI and ideally before seizure onset, since repeated seizures also drive neuroinflammation and other pathological changes.

In humans, electroencephalography (EEG) has formed the basis for epilepsy diagnosis and management[17]. The latency period following TBI, during which patients do not show overt seizures but are likely undergoing subtle neurophysiological changes that ultimately lead to PTE, is thought to be a critical window for early intervention. Yet identifying reliable biomarkers of the epileptogenic process before PTE onset is crucial before preventative treatment becomes an option. EEG, particularly through analysis of high-frequency oscillations (HFOs), especially those in the 250-1000 Hz range called fast ripples, have recently drawn attention as one of the most promising biomarkers of the epileptogenic brain[18-21]. Fast ripples are believed to represent pathological synchronization of cellular assemblies and have been related to the seizure onset zone in epileptic patients[18]. Furthermore, fast ripples synchronized with delta peaks may be predictive biomarkers for epilepsy[18] because such synchronization suggests coordinated neural activity, which typically precedes epileptic events. Yet, whether fast ripples synchronized with delta waves are useful to predict PTE in animal models has yet to be assessed.

Here, we used a model of repeated mild, diffuse TBI (rdTBI) caused by weight-drop[9]. We previously reported the development of PTE in a subset of mice in this model in the absence of

primary tissue loss, pronounced glial activation and hemorrhage[9, 10]. The primary goal of the present study was to identify reliable electrographic biomarkers for early post-traumatic seizure detection. We aimed to identify EEG biomarker profiles that precede and predict seizure onset, with particular focus on fast ripples and their coupling with delta waves before seizure onset in TBI mice that developed post-traumatic seizures.

Materials and Methods

Experimental design and exclusion criteria

45 mice were randomly assigned to either the TBI or Sham groups. 4 naïve mice were additionally included. Continuous 24/7 recordings were conducted for up to 5 months after TBI induction, though a few mice (6 TBI, 3 sham and 1 naïve) were monitored for a shorter period of 2 months starting 2 months post-TBI. Exclusion criteria were removal of mice that experienced hemorrhage at the electrode implantation site during surgery, that lost their EEG cap, or whose EEG signal was lost before the end of monitoring. Data were excluded if noise corrupted more than half of the EEG recording. One naïve mouse and one sham mouse were excluded from analysis due to significant hemorrhage from the reference electrode site in the nasal bone.

Animals

10- to 16-week-old male C57BL/6 mice (bred in-house or purchased from *Jackson Laboratory*) were used. All animal procedures were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University (*Virginia Tech*) and were conducted in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Weight-drop injury model

Mice received repetitive diffuse TBI based on an established protocol[9, 10]. Briefly, mice were anesthetized and placed on a foam pad, with a metal disc on their head. Buprenorphine was administered at a dose of 0.05-0.1 mg/kg subcutaneously before the procedure. TBI group mice received three impacts from a 50 cm height with 45-minute intervals between impacts. This injury modality induced mild/concussive injury as previously validated[10]. Sham mice underwent isoflurane anesthesia, administration of buprenorphine and placement in the weight drop device but not the impact. Naïve mice were excluded from these procedures.

Stereotactic surgery

After one hour recovery period, mice were anesthetized with isoflurane and placed in a stereotactic apparatus for implantation of intracranial stainless steel screw electrodes with wire leads (00-96 x 1.6 mm, Plastics1) for either single-channel or multi-channel tethered recording. Depending on electrode configuration and data acquisition system configuration, monopolar (referential) or bipolar montage were used. A step-by-step protocol of the TBI and EEG implantation procedure was previously reported[9].

Video-EEG data acquisition

Mice were housed individually in monitoring cages. Two EEG systems were used: Pinnacle Technology and Biopac Systems. Continuous, 24/7 video-EEG data acquisition was performed from day one post-TBI for up to 19 weeks. In the Pinnacle Technology system, video-synchronized EEG data were collected at a sampling rate of 2000 Hz using Sirenia Acquisition software. In the

Biopac Systems, video-synchronized EEG data were collected at a sampling rate of 500 Hz for single channel EEG and 2000 Hz for six-channel EEG using AcqKnowledge 4.1 software.

Seizure detection and characteristics

EEG data and video analysis was conducted by an investigator blinded to the experimental groups. Electrographic seizures were defined using the following inclusion criteria: 1) duration of at least five seconds; 2) signal evolution in amplitude (at least three times compared to background activity) and frequency (3-12 Hz ictal spikes 20-70 milliseconds). These are separated by prolonged "interictal" periods during which the EEG trace is relatively stable. For each mouse, seizure incidence, frequency and duration were documented. All mice with at least one seizure, early or late, were included in the PTE group. To determine whether seizures were electroclinical or exclusively electrographic, a blinded investigator scored video traces corresponding to the electrographic seizures according to the modified Racine scale.[22] The EEG data was initially imported and subjected to pre-processing. First, the powerline interference at 60 Hz was removed using an infinite impulse response (IIR) filter. Further, the data was filtered into two different frequency bands, 2-10 Hz and 20-50Hz with a 4th order IIR bandpass zero-phase digital filter. The sum of the signal after two filters was used for further analysis. The data was then passed through an artifact detection logic. The artifact algorithm divided the data into 10-minute segments. If, for any segment of EEG data, the median and standard deviation were significantly higher than a set threshold, the entire data was considered noise. Once the data was filtered and error segments were removed, the data was further divided into 0.5 seconds, and the Shannon entropy was calculated. Entropy values that exceed one standard deviation from the median were processed for potential seizure segments. If multiple potential seizure segments appeared

next to each other with a maximum gap of 2.5 seconds, the segments were consolidated into one seizure. After consolidation, seizure edge detection was done for each detected seizure segment. For each seizure candidate, the upper and lower envelope was calculated. The first data point that goes lower than the 25th percentile of the gap between the upper and lower envelope was further considered as the start and end point of the seizure. The finalized segments were further consolidated when they appeared within 2.5 seconds of the gap. Cross-verification was done for the detected event using surrounding time points. For any event, if the power content of the signal is at least 50% higher than the power in either 10 min before or after the candidate signal, then that detected event is considered a seizure. Reviewers blinded to experimental conditions cross-verified the seizure annotations.

Spike Detection

Interictal spikes were detected and quantified using NeuroScore software with dynamic thresholding method. EEG data were preprocessed by applying a 60 Hz notch filter to remove powerline interference, followed by bandpass filtering (3-70 Hz). Automated spike detection was performed using dynamic thresholding parameters (minimum 4× baseline RMS; maximum 15× RMS), with an minimum value of 100 µV and spike width 30–70 ms)[23, 24] optimized for interictal epileptiform discharges. All automatically detected spike events were subsequently verified through manual visual inspection by a trained investigator to ensure accuracy and eliminate false positives.

Power Spectrum Analysis

The average spectral power within specific frequency bands, including a wide range (1-500 Hz), delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), gamma (30-50 Hz), high frequency oscillation (HFO, 80-250 Hz) and fast ripples (250-500 Hz) was calculated in MATLAB 2022b (MathWorks Inc., Natick, MA). The signal was segmented into continuous, non-overlapping 10-minute intervals. Each segment underwent further processing to determine the average power content within the designated frequency bands by employing the modified periodogram technique with a Hamming window. Additionally, alpha-delta ratio (ADR) and relative alpha variability (RAV) were assessed, with ADR quantifying the ratio of alpha to delta power within the selected EEG segment.

To quantify the number of fast ripples, data were filtered by removing noise using an automated MATLAB algorithm. Next, fast ripples were identified using the Short Time Energy (STE) method[25]. After identifying potential fast ripples, these detections were cross-checked using RIPPLELAB and then manually validated. Fast ripple analysis was performed using 24-hour continuous EEG recordings at three time points: 4 days post-injury in TBI mice (seizure-free), 4 days post-injury in seizure-experiencing mice (pre-seizure latent period) and at seizure onset in seizure-experiencing mice.

Phase-Amplitude Coupling Analysis of Delta Waves and Fast Ripples

The timing of each fast ripple was analyzed to determine its coupling with delta waves. We extracted a 0.8 second time window around each FR and filtered the data to isolate delta (1-4 Hz) and fast ripples (250-500 Hz) components. We assessed if fast ripples occurred during a peak-

to-trough (DOWN state) or trough-to-peak (UP state) transition of delta using Hilbert transform at each fast ripple time.

Statistical Analysis

To assess seizure occurrences during light or dark periods, we applied two-tailed Wilcoxon and binomial tests. We used one way ANOVA to compare spike counts among SHAM, TBI, and mice with seizures during the latent period. Spike counts between the latent period, and the seizure onset phase were compared with a two tailed t test. For fast ripples, we compared counts and their coupling to delta during up and down states across three groups: TBI, mice with seizures before onset (latent), and mice with seizures after onset using one way ANOVA.

Results

Characteristics of seizures after rdTBI.

On day 0, mice underwent both rdTBI and EEG surgery. From day 1, we recorded video-EEG for up to 4½ months to track brain activity and seizures (**Fig. 1A**). EEGs used a monopolar setup with two recording electrodes plus ground and reference (**Fig. 1B**). **Fig. 1C** summarizes the distribution of animals across experimental groups. In total, 29 mice received rdTBI. Among these, 8 developed seizures during monitoring. 6 of those mice experienced recurrent seizures, and we labeled them as post-traumatic epilepsy (PTE). The remaining 2 mice had only a single seizure event and were labeled as post-traumatic seizure (PTS). 21 rdTBI mice, did not develop seizures. For comparison, 13 mice underwent sham procedures and served as controls (**Fig. 1C**). To characterize post-traumatic seizures after rdTBI, we measured the latency, onset, number, frequency, and incidence of seizures following established PTE classification criteria (**Fig. 1D**).

Seizures were classified as early (≤7 days post-TBI) or late (>7 days post-TBI), with PTE defined as ≥2 unprovoked seizures occurring on separate occasions AND at least one seizure occurring >7 days after injury. Two mice experienced seizures within 7 days post-TBI. One mouse had a single early seizure (onset day 5) with no subsequent seizure activity during 115 days of monitoring and was classified as having an early single seizure. The second mouse had seizure onset on day 5 but developed 11 total seizures over 115 days of recording, meeting PTE criteria due to seizure recurrence. Five mice developed PTE with late seizure onset on days 8, 40, 80, 81, and 100, respectively, all exhibiting recurrent seizures during the monitoring period. One additional mouse experienced a single late seizure on day 126 with no subsequent seizure activity and was classified as having a single late seizure rather than PTE. Including the mouse with early seizure onset that progressed to recurrent seizures, the overall incidence of PTE was 20.6.% of injured mice (6/29 total TBI mice). For mice with late-onset PTE, seizures occurred with a mean latency period of 75.25 ± 12.7 days (mean ± SEM) from TBI. All PTE mice exhibited 6.8 ± 4.4 seizures (mean ± SEM) during their monitoring periods. **Fig. 1E** illustrates a representative seizure event recorded from one of the affected mice.

Diurnal seizure patterns after rdTBI.

We next investigated seizures during different phases of the day. We found no significant difference in the seizure numbers' median value between the light and dark phases (two-tailed Wilcoxon test, which does not *a priori* assume the direction of the difference, $p = 0.75$, **Fig. 1F**). Some mice had more seizures in the dark while others had more during the light phase. Yet, we observed that all mice experienced at least one seizure during the light phase and 3/8 (37.5%) mice had seizures exclusively during light hours, indicating a potential non-random pattern. We

hypothesized that if there was no preference of seizure occurrence in either phase, then p will be 0.5 or the probability that an animal would experience one seizure at least in each phase is 50%. We determined that during the light phase the statistical probability of a seizure was significantly higher (two-tail binomial test, $p = 0.0156$). In contrast, no deviation from chance was determined for the dark phase (two-tail binomial test, $p = 1.0$). This suggests that individual mice are more likely to experience seizures during light hours.

Seizure Onset Drives Further Spike Elevation in PTE

To characterize electrographic changes associated with PTE development, we analyzed spike activity patterns across experimental groups and disease progression phases. An example of typical epileptic spike in our mice is shown in **Fig. 2A.** One-way ANOVA revealed significant differences in 24-hour spike counts among experimental groups (Welch's ANOVA: $W (2, 9.913) = 15.45$, $p = 0.0009$). Post-hoc analysis using Dunnett's T3 multiple comparisons test demonstrated that TBI groups exhibited significantly elevated spike activity compared to SHAM controls (**Fig. 2B**)**.** SHAM mice showed minimal baseline spike activity (1.0 ± 0.2 spikes per 24 hours, $n = 4$). TBI mice that did not develop PTE exhibited a significant 5.4-fold increase in spike frequency compared to SHAM (6.4 ± 0.8 spikes, mean difference = -5.364, 95% CI [-9.185 to -1.542], $p = 0.0067$), indicating that traumatic brain injury itself induces cortical hyperexcitability independent of seizure development. PTE mice during the pre-onset latent phase showed the highest spike activity (8.5 ± 1.0 spikes, $n = 6$), representing a significant 7.5-fold increase compared to SHAM controls (mean difference = -7.500, 95% CI [-12.79 to -2.206], $p = 0.0110$). However, the difference between TBI and PTE pre-onset groups did not reach statistical significance (mean difference = -2.136, 95% CI [-7.944 to 3.671], $p = 0.6753$), suggesting that spike

frequency alone during the latent period cannot reliably distinguish mice that will develop PTE from those that will not.

A more pronounced change occurred when comparing spike activity before and after seizure onset within the same PTE mice (**Fig. 2C**). A paired t-test comparing spike counts in PTE mice pre- and post-seizure onset revealed a significant increase in spike frequency (mean increase = +12.33 spikes; 95% CI [6.996, 17.67]; $t_5 = 5.94$, $p = 0.0019$, $\eta^2 = 0.876$). This represents an exceptionally large effect size (Cohen's d = 2.43), with seizure onset accounting for approximately 88% of the variance in spike frequency changes. Spike activity increased from 7.3 ± 1.2 spikes during the pre-onset phase to 20.2 ± 1.7 spikes following seizure development, representing a nearly 3-fold escalation in interictal hyperexcitability. This dramatic increase suggests that the emergence of spontaneous seizures triggers a cascade of network instability that extends well beyond ictal periods.

These findings reveal a biphasic pattern of spike activity in post-traumatic epileptogenesis: (1) an initial TBI-induced elevation in cortical excitability that affects all injured animals and (2) a dramatic secondary escalation specifically following seizure onset in PTE mice. While baseline spike elevation reflects general post-traumatic network dysfunction, the pronounced post-seizure spike increase represents a distinct pathophysiological transition marking the establishment of chronic epileptic networks. This progression supports the concept of epileptogenesis as a dynamic process where initial seizures facilitate further network destabilization and increased seizure susceptibility. However, given the lack of significant difference between TBI and PTE mice during the critical pre-seizure latent period ($p = 0.6753$),

spike counts alone do not constitute a reliable predictive biomarker for identifying which animals will develop PTE following traumatic brain injury.

EEG Biomarker Analysis in PTE Through Power Spectrum Changes

For EEG biomarker analysis, we aimed to determine whether power spectrum differences could distinguish mice that developed post-traumatic seizures (regardless of seizure pattern - single or recurrent) from TBI mice that remained seizure-free throughout the observation period. We analyzed EEG power spectrum changes across multiple frequency bands over 17 weeks using an automated MATLAB algorithm to identify potential electrographic signatures that precede or accompany seizure development (**Fig. 3**). EEG recordings from SHAM, TBI and seizure-experiencing groups were analyzed across delta (0.5-4 Hz), theta (4.5-7.5 Hz), alpha (8-13 Hz), beta (13-30 Hz), gamma (30-80 Hz) and high-frequency oscillations (HFO; 100-200 Hz) bands, along with derived metrics including alpha-delta ratio (ADR), relative alpha variability (RAV) and total power.

Mice that developed post-traumatic seizures exhibited distinctly elevated delta power compared to both SHAM and seizure-free TBI mice, particularly during the early post-injury weeks (**Fig. 3A**). This elevation in slow-wave activity was sustained throughout the monitoring period, with prominent episodic spikes occurring around weeks 8 and 12, likely corresponding to periods of increased seizure activity. Similarly, theta power showed consistent elevations in seizure-experienced mice compared to seizure-free TBI mice (**Fig. 3B**), suggesting that increased slow-wave activity may represent an early electrographic marker of seizure susceptibility.

In contrast to slow-wave elevations, seizure-experienced mice demonstrated consistently reduced power in faster physiological frequency bands. Alpha, beta and gamma powers were all diminished in seizure-experiencing mice relative to both SHAM and TBI groups throughout the 17-week observation period (**Fig. 3C, D, E)**. This reduction in physiological fast oscillations suggests progressive cortical network dysfunction in mice predisposed to seizure development.

Paradoxically, while physiological gamma activity was reduced, HFO were markedly elevated in seizure-experiencing mice, with prominent peaks occurring around weeks 2, 4 and 6 (**Fig. 3F**). This shift toward HFO activity, combined with reduced physiological gamma oscillations, indicates a fundamental alteration in network excitability patterns that distinguishes seizure-prone animals from seizure-resistant TBI mice. The ADR and RAV both showed sustained reductions in seizure-experiencing mice compared to controls (**Fig. 3G, H**). These composite measures effectively captured the dual pattern of increased slow-wave activity and decreased fast-wave activity that characterized mice destined to develop post-traumatic seizures, suggesting their potential utility as integrated biomarkers of seizure risk. Total power showed a decreased trend in seizure experiencing mice compared to TBI and SHAM (**Fig. 3I**).

Fast Ripple Dynamics and Delta Coupling in Post-Traumatic Epilepsy

Fast ripples (250-500 Hz) are pathological HFO distinct from physiological HFO and interictal spikes, representing synchronized activity of small neuronal populations that has been associated with epileptogenic tissue in both human patients[18, 26] and animal models[27]. Unlike spikes, which are brief transient discharges, fast ripples are sustained oscillatory events that may reflect underlying network hyperexcitability. A representative trace of fast ripples in RIPPLELAB has been

shown in **Fig. 4A**. We quantified fast ripples at three time points: 4 days post-TBI (latent period) and at seizure onset in seizure-experiencing mice. Fast ripple frequency was significantly elevated in seizure-experiencing mice compared to seizure-free TBI mice both during the latent period (**Fig. 4B,** p=0.0018) and at seizure onset (**Fig. 4B,** p=0.0038), establishing fast ripples as a consistent biomarker across the epileptogenic process. To examine the temporal relationship between fast ripples and slow-wave activity, we analyzed phase-amplitude coupling between fast ripples and delta waves (1-4 Hz). Delta oscillations alternate between UP states (depolarized, high-amplitude) and DOWN states (hyperpolarized, low-amplitude), representing different phases of cortical excitability. Examples of coupling in TBI and PTE mice have been shown in **Fig. 4C**. While fast ripple coupling to delta UP states showed no significant differences between groups (**Fig. 4D**), fast ripple coupling to delta DOWN states was significantly increased in seizure-experiencing mice both during the latent period (**Fig. 4E,** p=0.0082) and after seizure onset (**Fig. 4E**, p=0.0392) compared to seizure-free TBI mice.

This preferential coupling of fast ripples to delta DOWN states suggests that pathological high-frequency activity emerges during periods of cortical inhibition, potentially reflecting a breakdown in normal inhibitory control mechanisms. The presence of this coupling signature during the latent period, before any seizures occur, establishes fast ripple-delta DOWN state coupling as a promising early biomarker for post-traumatic seizure susceptibility.

Discussion

Here, we assessed differences in EEG signatures between TBI animals that do or do not develop seizures for their usefulness as early biomarkers. Our findings, in concert with those of

recent investigations in focal epilepsy[18], indicate that fast ripples, especially delta-coupled fast ripples, may be early biomarkers for epileptogenesis. In human patients with epilepsy, HFOs, especially fast ripples, were suggested as biomarkers of the seizure onset zone[28]. They were also recorded in various rodent models of epilepsy[18].

Delta coupling analysis revealed distinct patterns between TBI and seizure-experiencing mice, with a preference for coupling to the delta DOWN state rather than the UP state before seizure onset. This is particularly interesting because delta DOWN-state coupling of fast ripples emerged before seizure onset, suggesting early network instability specific to the latent phase of post-traumatic seizure development. After seizure onset, coupling shifted further toward the delta DOWN state, indicating a dynamic interaction between these oscillations and the progression of epileptogenesis. Disruptions in delta wave coupling may reflect changes in thalamocortical network dynamics that may contribute to the persistence of hyperexcitability. This aligns with prior reports that these states are critical for initiating epileptiform activity, as they represent periods of synchronized cortical inhibition that can facilitate seizure propagation[29].

We found significant elevations in delta power during the early post-injury weeks in seizure-experiencing animals, supporting its role as a biomarker for epileptogenesis[30]. The sustained increase in delta activity provides evidence of its association with chronic network dysfunction[31]. Additionally, the decreased power observed in alpha and beta frequency bands aligns with findings of cortical network disruptions that are frequently observed in post-TBI studies[32].

Gamma-to-HFO ratio reductions and consistent gamma band deficits may reflect disrupted GABAergic inhibition within cortical networks, as gamma oscillations are typically associated with

inhibitory interneuron activity[33]. A reduction in gamma power alongside increased HFO suggests an imbalance between excitatory and inhibitory signaling, potentially driven by a loss of GABA-mediated control, leading to increased network excitability and seizure susceptibility. These findings extend the predictive capability of EEG analysis by highlighting shifts in physiological oscillations toward pathological activity, an observation also reported in human qEEG studies of post-traumatic seizure disorders[31].

Critically, our power spectrum analysis demonstrated that mice experiencing any post-traumatic seizure, whether single early, single late or recurrent, exhibited similar patterns of brain network dysfunction compared to seizure-free TBI animals. These seizure-experiencing mice showed elevated delta and theta power, reduced physiological fast oscillations and increased pathological HFO activity, regardless of seizure frequency or timing. This electrographic similarity challenges the assumption that single seizures represent fundamentally different pathophysiological processes from recurrent seizures.

Our spike analysis exemplified the limitation of individual biomarkers: while seizure-experiencing mice showed dramatic increases in spike activity after seizure onset, the overall difference between TBI (seizure-free) and pre-seizure PTE groups was non-significant, suggesting that spikes count alone during the latent period cannot reliably distinguish which animals will develop seizures from those that will not. This highlights a fundamental challenge in PTE biomarker development, individual electrographic measures may lack sufficient sensitivity during the critical pre-seizure window when intervention would be most valuable. The fast ripple-delta coupling we identified as an early biomarker emerged precisely because we examined more

sophisticated temporal relationships rather than relying on simple power measurements or event counts during arbitrary time windows.

While our study included animals with single seizures (n=2), the small sample size precludes meaningful statistical comparison with the PTE group (n=6). However, both single-seizure animals exhibited the same general pattern of electrographic abnormalities (elevated fast ripples and delta-coupling) observed in PTE mice, though larger studies are needed to determine whether single and recurrent post-traumatic seizures truly represent the same pathophysiological spectrum. The current binary classification system may warrant re-examination once sufficient data from adequately powered studies becomes available. If animals with single post-traumatic seizures demonstrate the same brain network alterations as those with multiple seizures, the strict recurrence criterion may be excluding cases that have fundamentally similar pathophysiology but simply haven't yet progressed to multiple seizures.

The considerable heterogeneity in seizure development we observed, ranging from 5 days to 126 days post-injury, with diverse seizure frequency and diurnal patterns reflects the complexity of post-traumatic epileptogenesis as also seen in patients where seizure onset and frequency can also widely vary (ref) This suggests that effective biomarker strategies must account for individual trajectories rather than assuming uniform pathophysiology.

Our current findings build upon our previous research[9, 10], where continuous EEG monitoring was limited to a two-month period post-injury. The extension of monitoring up to 19 weeks in the present study has allowed for a more comprehensive analysis of fast ripples during the latent and chronic phases of seizure development. The variability in seizure onset times we observed

aligns with the variability seen in human TBI cases[34-39], highlighting the challenge of using rigid diagnostic criteria for these heterogeneous disorders.

In conclusion, we identified several EEG biomarkers that distinguished rdTBI mice experiencing any form of post-traumatic seizure from those that remained seizure-free, with fast ripple-delta coupling emerging as the most promising early biomarker. Our findings demonstrate that this biomarker can identify seizure-prone animals during the critical latent period when intervention would be most beneficial. Future research should validate these findings in larger cohorts with adequate sample sizes to determine whether single and recurrent post-traumatic seizures represent similar pathophysiological processes and whether the binary recurrence threshold for PTE diagnosis adequately captures the biological reality of post-traumatic network dysfunction.

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Figure Legends

Figure 1. Seizure Incidence and Diurnal Patterns After rdTBI.

(A) Mice underwent repetitive diffuse traumatic brain injury (rdTBI) and EEG surgery on the same day, followed by continuous 24/7 EEG-video monitoring for 130 days. (B) EEG electrode implantation was performed using monopolar montage with 2 recording electrodes (red), one ground (blue), and one reference (green) electrode. (C) In total, 29 mice underwent rdTBI. 8 developed seizures, with 6 progressing to post-traumatic epilepsy (PTE) and 2 showing only a single seizure, classified as post-traumatic seizure (PTS). The remaining 21 rdTBI mice did not develop seizures, and 13 additional mice served as sham controls. (D) Average number of seizures per mouse per week (mean ± SEM). (E) EEG trace displaying seizure in mice. (F) Comparison of seizure occurrences during the light (zeitgeber [ZT] 0-11) and dark (ZT 12-23) phases. No significant difference in median seizure numbers between light and dark phases was detected (two-tailed Wilcoxon test, $p = 0.75$).

Figure 2. Spike comparison across animal groups

(A) Representative trace of typical epileptic spikes in PTE mice. (B) At 4 days post injury (dpi), SHAM mice had significantly fewer spikes than TBI mice (one-way ANOVA, $p = 0.0067$; $n = 4$ SHAM, $n = 11$ TBI) and mice with seizures in the latent phase (one-way ANOVA, $p = 0.0110$; $n = 4$ SHAM, $n = 6$ latent). No difference was found between TBI mice and latent phase mice (one-way ANOVA, $p = 0.6753$; $n = 11$ TBI, $n = 6$ latent). (C) The number of spikes was significantly higher in

mice with seizures after seizure onset compared to the latent phase (two-tailed t-test, t=5.940, df=5, p=0.0019, n=6 per group).

Figure 3. EEG Power Spectrum Trends in PTE mice

Average power changes over the 17-week period post-rdTBI for (A) delta (0.5 - 4 Hz), (B) theta (4.5 - 7.5 Hz), (C) alpha (8 - 13 Hz), (D) beta (13 - 30 Hz), (E) gamma (30 - 80 Hz), (F) high-frequency oscillations (HFO; 100 - 200 Hz), (G) ADR (Alpha-Delta Ratio), (H) RAV (Relative Alpha Variability), and (I) total power. Power is presented as mean (lines) with standard deviation (cloud) for each group: SHAM (n=4, green), TBI (n=11, blue), PTE+PTS (n=6, orange) and PTE (n=4, red). When the two post traumatic seizure (PTS) mice were excluded from the post traumatic epilepsy (PTE) group, the overall trend remained unchanged, suggesting PTS and PTE share similar features.

Figure 4: Fast ripple dynamics and delta coupling in PTE

(A) Representative EEG trace showing fast ripple events in PTE mice. (B) Fast ripples were significantly higher in seizure experiencing mice compared to TBI during the latent phase (p = 0.0018, n = 3 per group) and remained elevated after seizure onset (p = 0.0038), with no difference between latent and post onset phases (p = 0.0963) which suggests sustained fast ripples activity during both periods. (C) Normalized delta amplitude waveforms coupled with fast ripples, showing differences between TBI and PTE mice. (D) Fast ripples coupling to delta UP states showed no significant difference between seizure and TBI mice during the latent phase (p

= 0.7093) or after seizure onset (p = 0.1686). (E) Fast ripples coupling to delta DOWN states were significantly increased in seizure mice during the latent phase (p = 0.0082) and remained higher after onset (*p* = 0.0390), with no difference between pre and post onset phases (p = 0.8733).

Figure 1

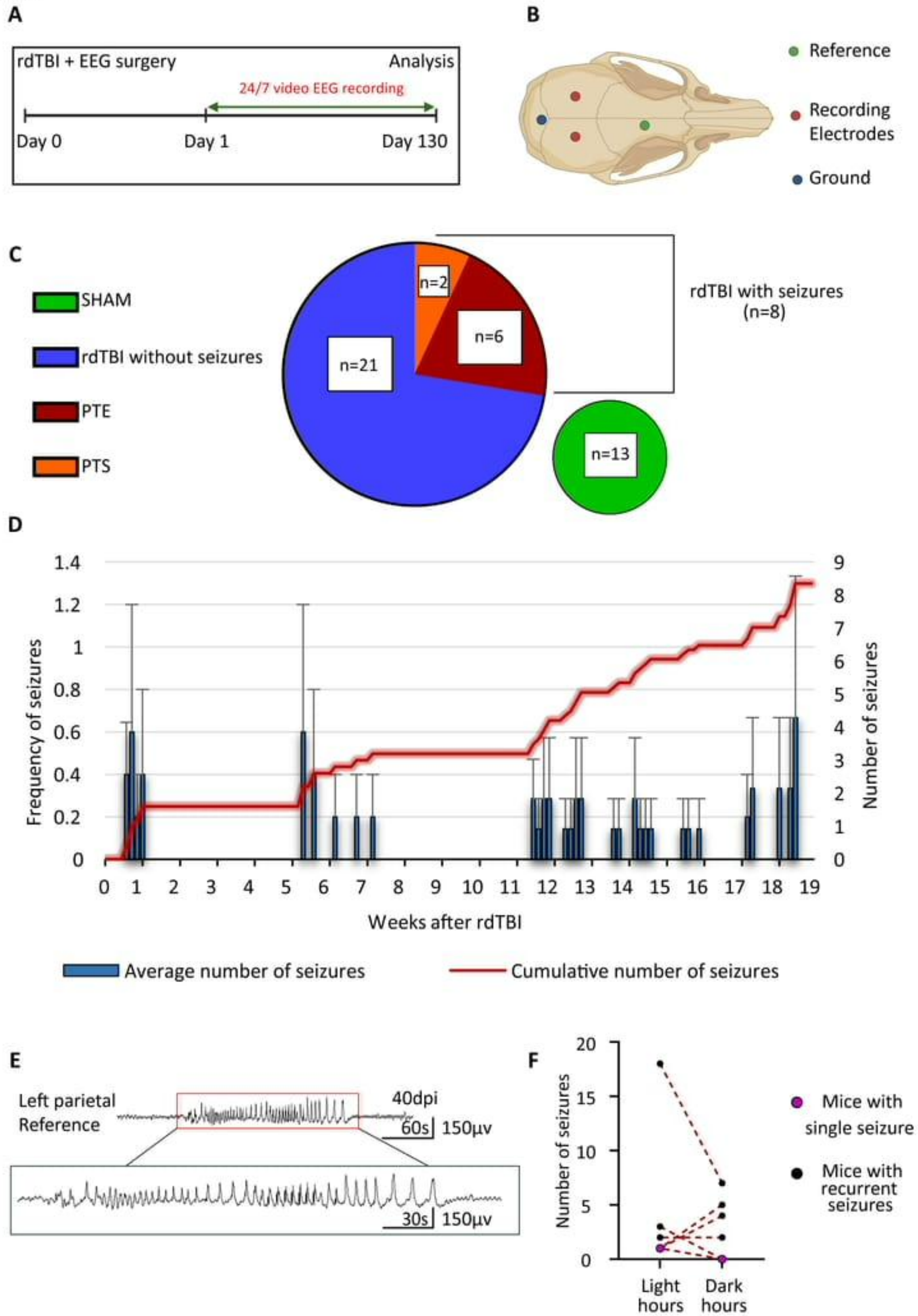

A
rdTBI + EEG surgery
Analysis
24/7 video EEG recording
Day 0
Day 1
Day 130
B
Reference
Recording Electrodes
Ground
C
SHAM
rdTBI without seizures
PTE
PTS
n=2
n=6
n=21
n=13
rdTBI with seizures (n=8)
D
Frequency of seizures
Number of seizures
Weeks after rdTBI
Average number of seizures
Cumulative number of seizures
E
Left parietal
Reference
40dpi
60s
150µv
30s
150µv
F
Number of seizures
Light hours
Dark hours
Mice with single seizure
Mice with recurrent seizures

Figure 2

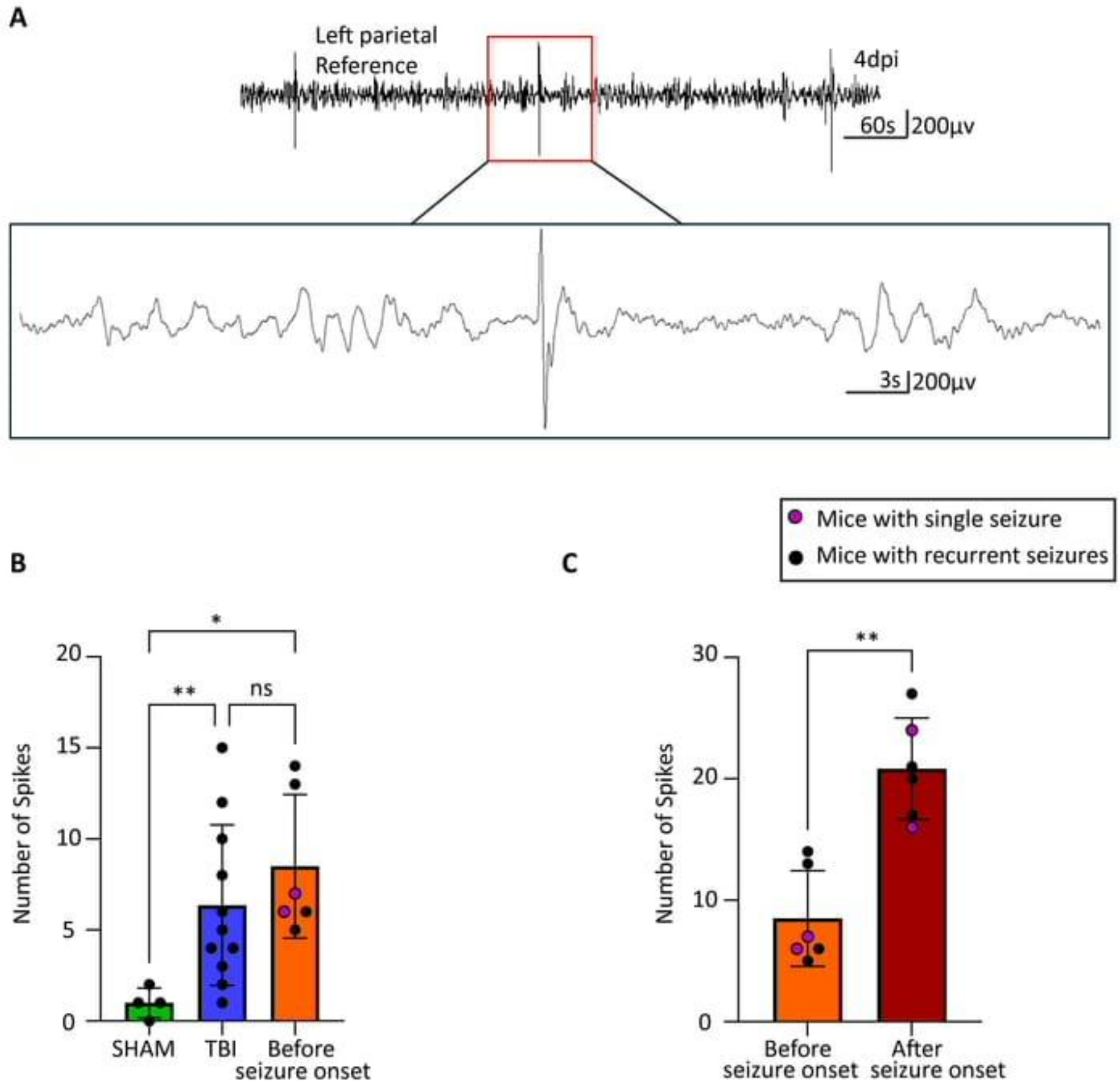

A
Left parietal
Reference
4dpi
60s
200µv
3s
200µv
B
Number of Spikes
20
15
10
5
0
*
**
ns
SHAM
TBI
Before
seizure onset
C
Mice with single seizure
Mice with recurrent seizures
Number of Spikes
30
20
10
0
**
Before
seizure onset
After
seizure onset

Figure 3

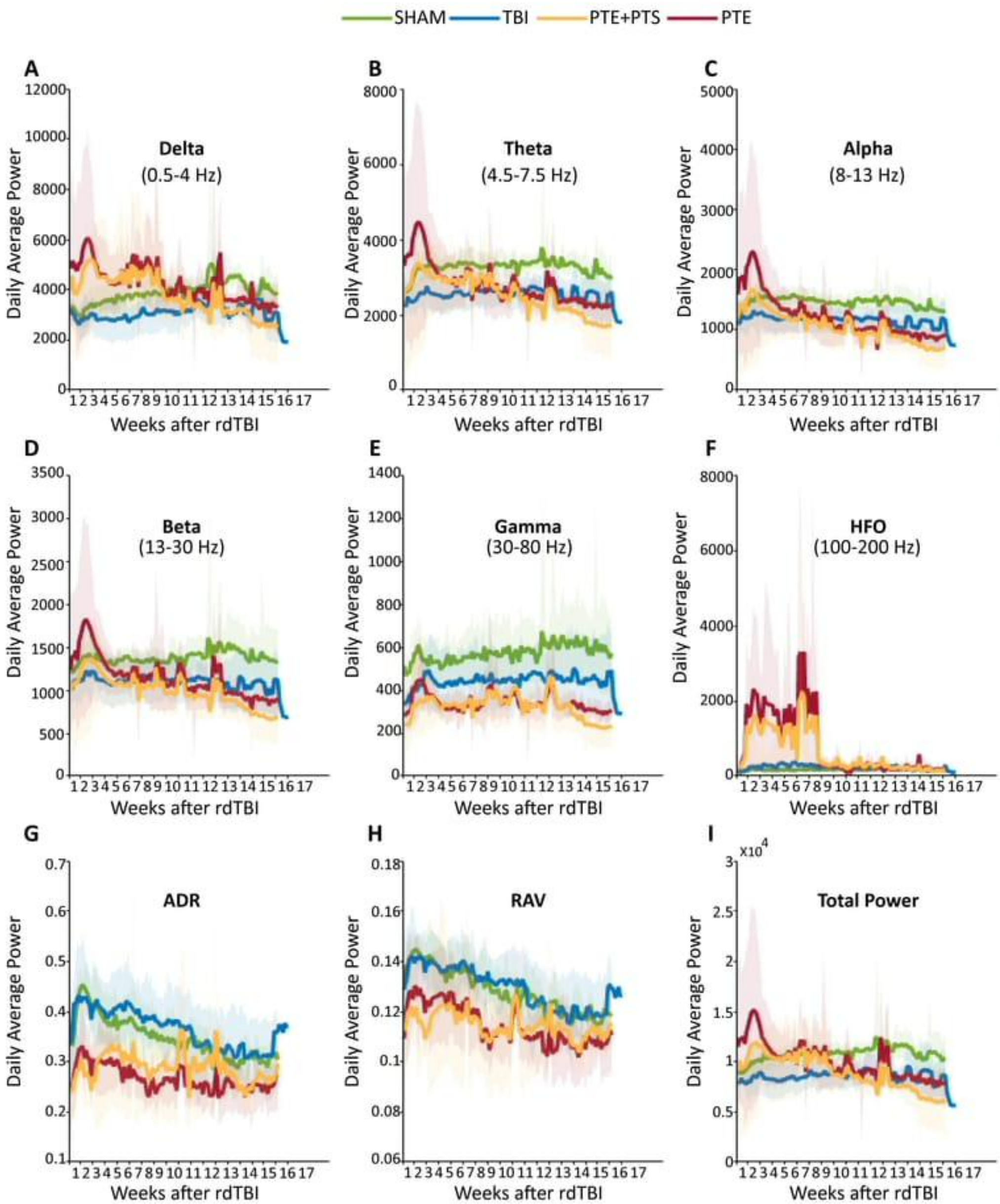
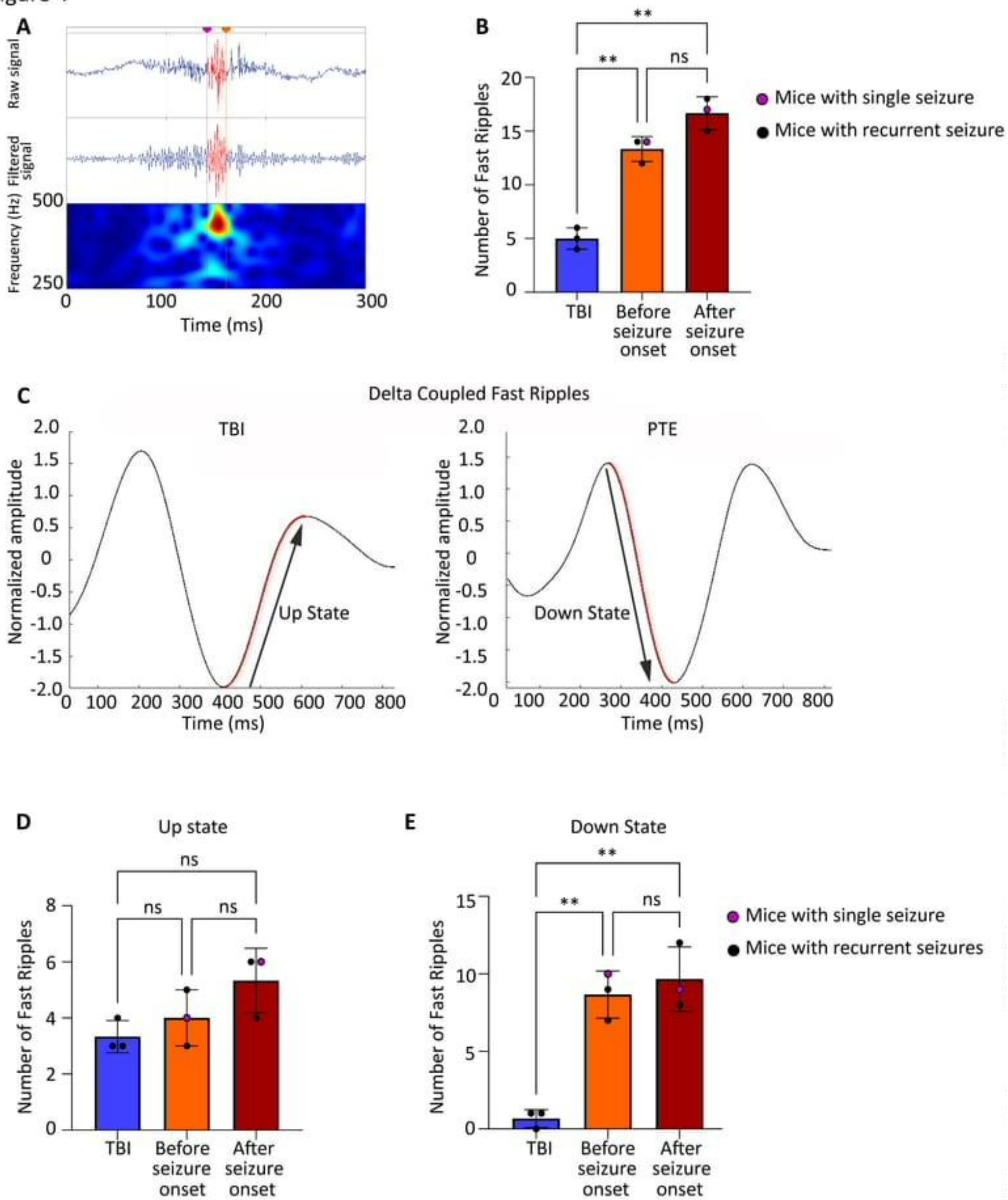

SHAM
TBI
PTE+PTS
PTE
A
Delta
(0.5-4 Hz)
B
Theta
(4.5-7.5 Hz)
C
Alpha
(8-13 Hz)
D
Beta
(13-30 Hz)
E
Gamma
(30-80 Hz)
F
HFO
(100-200 Hz)
G
ADR
H
RAV
I
Total Power
Daily Average Power
Weeks after rdTBI

Figure 4


A
Raw signal
Filtered signal
Frequency (Hz)
500
250
0
100
200
300
Time (ms)
B
**
**
ns
Number of Fast Ripples
20
15
10
5
0
TBI
Before seizure onset
After seizure onset
Mice with single seizure
Mice with recurrent seizure
C
Delta Coupled Fast Ripples
TBI
PTE
Normalized amplitude
2.0
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
Up State
Down State
0 100 200 300 400 500 600 700 800
Time (ms)
D
Up state
ns
ns
ns
Number of Fast Ripples
8
6
4
2
0
TBI
Before seizure onset
After seizure onset
E
Down State
**
**
ns
Number of Fast Ripples
15
10
5
0
TBI
Before seizure onset
After seizure onset
Mice with single seizure
Mice with recurrent seizures