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TITLE:

Use of a Wireless Video-EEG System to Monitor Epileptiform Discharges Following Lateral Fluid-Percussion Induced Traumatic Brain Injury

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KEYWORDS:

post-traumatic epilepsy, epileptogenesis, lateral fluid-percussion injury, seizure, video-EEG monitoring, wireless telemetry

SUMMARY:

Here we present a protocol to induce severe TBI with the lateral fluid percussion injury (FPI) model in adult, male Wistar rats. We also demonstrate the use of a wireless telemetry system to collect continuous video-EEG recordings and monitor for epileptiform discharges consistent with post-traumatic epileptogenesis.

LONG ABSTRACT:

The lateral fluid percussion injury (FPI) model is well established and has been used to study TBI and post-traumatic epilepsy (PTE). However, considerable variability has been reported for the specific parameters used in different studies that have employed this model, making it difficult to harmonize and interpret the results between laboratories. For example, variability has been reported regarding the size and location of the craniectomy, how the Luer lock hub is placed relative to the craniectomy, the atmospheric pressure applied to the dura and the duration of the pressure pulse. Each of these parameters can impact injury severity, which directly correlates with the incidence of PTE. This has been manifested as a wide range of mortality rates, righting reflex times and incidence of convulsive seizures reported. Here we provide a detailed protocol for the method we have used to help facilitate harmonization between studies. We used FPI in combination with a wireless EEG telemetry system to continuously monitor for electrographic changes and detect seizure activity. FPI is induced by creating a 5 mm craniectomy over the left

hemisphere, between the Bregma and Lambda and adjacent to the lateral ridge. A Luer lock hub is secured onto the skull over the craniectomy. This hub is connected to the FPI device, and a 20-millisecond pressure pulse is delivered directly to the intact dura through pressure tubing connected to the hub via a twist lock connector. Following recovery, rats are re-anesthetized to remove the hub. Five 0.5 mm, stainless steel EEG electrode screws are placed in contact with the dura through the skull and serve as four recording electrodes and one reference electrode. The electrode wires are collected into a pedestal connector which is secured into place with bone cement. Continuous video/EEG recordings are collected for up to 4 weeks post TBI.

INTRODUCTION:

In a 2015 report to Congress, the Centers for Disease Control reported that approximately 2.5 million people per year suffer traumatic brain injury (TBI) in the US[1]. It is estimated that TBI causes 20% of symptomatic epilepsies and 5% of all epilepsies[2-4]. In addition, about 20% of TBI patients develop post-traumatic epilepsy[5]. Importantly, chronic, recurrent seizures that occur as a consequence of TBI are often pharmacoresistant, increasing the burden of the disease[6]. The exact mechanisms that lead to post-traumatic epilepsy (PTE) remain unclear. However, several key epidemiology studies have examined the incidence and potential risk of developing post-traumatic epilepsy (PTE)[2,4,7-11]. These epidemiology studies each reinforced the correlation of injury severity with the risk of epileptogenesis.

Current methods that have been extensively used to identify novel anti-epilepsy therapies have relied heavily on models that use chemo-convulsants or electrical kindling to induce epilepsy[12]. Given the high incidence of pharmaco-resistance to drugs developed in these models by TBI patients, we hypothesize that TBI-induced seizures may be different from chemoconvulsant or kindling-induced seizures and may involve different pathways or processes of epileptogenesis. Therefore, a TBI model may be better suited for the development of treatments that are more effective to prevent post-traumatic epileptogenesis.

The fluid percussion injury (FPI) model of TBI has been used for decades and is a well-established method to investigate both TBI and PTE[13-18]. However, as we recently reviewed, there is a high degree of variability in the FPI methods reported across laboratories[19,20]. This lack of consistency between laboratories prevents reproducibility of preclinical findings and makes the interpretation of results a challenge. As a consequence, increased interest and efforts have been applied towards establishing a greater harmonization for these types of studies[21-24].

In an effort to further increase the consistency and harmonization between laboratories focused on studying post-traumatic epileptogenesis, we provide here a detailed methodology of our approach. We have previously reported a 60% incidence of convulsive seizures within six weeks after severe TBI[20]. We now use this approach to monitor rats beginning the day of injury and continuously follow them 24 hours a day for up to 4 weeks. We have chosen to use a wireless telemetry system which affords several advantages. First, rats are able to freely move about their cage, and thus reduces stress. Second, rats can be assessed for behavioral function, weighed and receive supplemental care following severe TBI without having to disconnect them from a tethered system. Third, a reduction in signal noise as the rat serves as the ground. In addition,

our current system employs an accelerometer which detects rapid movement in all three planes (X, Y and Z) and can be helpful to identify convulsive seizure events. Finally, the wireless telemetry system allows for easier management of rats such as supplemental saline injections, weighing and conducting neurological severity scores, which is complicated when rats are attached to a tether. However, this approach also has several limitations. First, the initial cost of a system to record from up to eight rats simultaneously can be in the range of $60,000. Second, power is limited by a battery source. This requires daily monitoring and replacement of batteries. The time required between battery changes can be influenced by the sampling rate. However, for a 1000 Hz sampling rate, batteries are typically changed once a week. The limited power supply also restricts the system to recording from only four EEG signals. Finally, signal drop out is limited but does occasionally occur. However, this approach provides a consistent and reliable method to monitor post-traumatic epileptogenesis and can aid in the identification of novel therapeutic treatments.

PROTOCOL:

All procedures were approved by and followed guidelines of the University at Buffalo Institutional Animal Care and Use committee.

1. **Fluid percussion injury**

1.1. Wear a lab coat or surgical gown, surgical mask, surgical gloves, and head covering and sterilize all tools and materials that contact the surgical site.

1.2. Anesthetize a 10-12-week-old, male, Wistar rat (350-400 g) with 3% isoflurane and 1 L/min oxygen in an induction chamber of appropriate size for rats. Remove the rat from the induction chamber and move it to the prep area once it is unconscious. Put the sterile ophthalmic ointment into both eyes.

1.3. Shave the fur on the rat's head with electric clippers with a #40 blade from just above the eyes to the caudal base of the ears to produce enough surgical field. Remove any loose, clipped hair from the site.

1.4. Clean the surgical site by applying 2% chlorohexidine scrub to the shaved scalp followed by 70% ethanol. Start at the center and move outward in concentric circles away from the incision site. Repeat this process 3 times. Apply Betadine solution to the site in the same fashion and allowed to dry.

1.5. Place the anesthetized rat into the stereotaxic frame and maintain anesthesia at 2-3% isoflurane-1 L/min oxygen via nosecone. Check for loss of withdrawal reflex of forelimb and loss of palpebral reflex to ensure the rat is in a surgical plane of anesthesia.

1.6. Monitor the respiratory rate, heart rate, body temperature and oxygen saturation throughout the surgery. Maintain heart rate between 300-400 bpm, and SpO_2 above 90%.

NOTE: A pulse-oximeter attached to a rear foot can be used to provide the constant read out of heart rate and SpO_2. A heart rate above 400 bpm indicates the rat is not sufficiently anesthetized. A self-regulating warming pad, coupled to a rectal thermometer, set at 37 °C, can be positioned under the rat throughout the surgery to maintain body temperature. A stereomicroscope with a light source in combination with an optic fiber lamp are helpful for visualizing the procedure.

1.7. Use a 23 g needle to inject 0.5% bupivacaine hydrochloride intradermally into the scalp at the incision site for local analgesia 10 – 15 minutes prior to making an incision.

1.8. Make a 1.5-2.5 cm midline incision through the skin and muscle of the scalp using a #10 scalpel blade. Retract the skin and muscle to expose the skull and provide a clear surgical field. Reflect the underlying fascia and fatty tissue away from the bone with sterile cotton swabs.

NOTE: An electric cautery unit is useful for achieving a quick hemostasis.

1.9. Shave down the lateral ridge of the left parietal bone using a surgical curette to produce a smooth flat surface so that the base of the female-female Luer lock hub can rest flush with the skull.

1.10. Irrigate the skull surface and surrounding tissues with 0.5 mg/mL gentamicin solution in sterile saline. Blot excess solution with a sterile gauze.

1.11. Apply 3% hydrogen peroxide to the skull to dry the bone.

NOTE: If the bone is not sufficiently dry the dental cement will not adhere properly and form a solid seal.

1.12. Create a 5 mm diameter craniectomy site through the left parietal bone.

NOTE: A trephine bit placed into a power drill attached to the stereotactic frame can be helpful to initiate the craniectomy. Use a hand drill with a 5 mm diameter trephine to slowly finish the craniectomy through the remaining bone. When close to completing the craniectomy, rotate the trephine in reverse to prevent rupture of the underlying dura mater. There will be a thinning of the skull around the perimeter of the disk and the skull flap will feel loose when pressed lightly.

1.13. Remove the bone flap with the surgical curette and smooth tissue forceps.

NOTE: Some bleeding may occur, but hemostasis can be quickly achieved by applying gentle pressure with sterile cotton swabs.

1.14. Use a stereomicroscope and illumination to visually inspect the dura for any signs of rupture. A thin rim of bone will remain around the circumference of the craniectomy site. Gently remove this rim with smooth tissue forceps taking care not to rupture the dura.

1.15. Swab the skull with 70% ethanol to remove any bone dust and to dry the skull.

1.16. Apply a thin layer of cyanoacrylate gel glue around the bottom edge of the Luer lock hub and secure it to the skull over the craniectomy without obstructing the opening. Use caution not to bring the glue in contact with the dura. Further, seal the Luer lock in place with an additional thin layer of glue around the outside base of the hub.

1.17. Prepare a slurry of dental cement. Apply the cement to the surface of the skull around and over the base of the Luer lock hub to secure it in place.

1.18. Fill the Luer lock hub with a sterile preservative free solution containing multiple electrolytes (pH 7.4) using a syringe and needle so that a convex bolus of saline can be seen above the top of the rim.

NOTE: The solution will keep the dura moist as the dental cement dries as well as serves as an indication of the integrity of the seal. If the solution level falls at all, that is an indication of a leak in the system and the Luer lock must be removed and replaced.

1.19. Once the dental cement is completely cured, discontinue gas anesthesia and remove the rat from the stereotaxic frame.

1.20. Place the rat on a platform next to the FPI device.

1.21. The FPI device has a curved metal tip that extends from the pressure transducer at the end of the fluid reservoir. Secure a 12 cm length of pressure tubing to the end of the curved tip with the opposite end terminating in a 2 cm male Luer lock twist connector. Secure the rat to the FPI device by connecting the female end of the hub on the rat's skull to the male connector.

NOTE: Ensure the connection is tightly secured and that all air bubbles have been removed from the system.

1.22. Place the animal in sternal recumbency and repeatedly check for return of withdrawal reflex. As soon as the rat regains withdrawal reflex but is still sedated, release the pendulum of the FPI device to cause a single 20 ms pressure pulse and induce injury.

NOTE: It is important to not induce the injury while the animal is deeply anesthetized as this tends to cause increased mortality due to neurogenic-induced pulmonary edema. All devices show variability. However, on the device used for this experiment, a 17° angle placement of the hammer produces a 2.2 - 2.3 atmospheric pressure pulse. Uninjured, sham animals undergo all of the same procedures with the exception of the actual fluid pulse to the induce injury.

1.23. Immediately disconnect the rat from the FPI device after injury, place it in sternal recumbency, and provide supplemental oxygen (1 L/min) via a nose cone until spontaneous breathing returns. Apnea is an anticipated consequence of the injury. If necessary, provide

periodic manual breaths via a bag valve mask until the rat begins to spontaneously breathe on its own.

NOTE: Typically, apnea lasts less than 2 min. A transient rapid rise in heart rate (>500 bpm) is observed immediately after the administration of the pressure pulse due to a catecholamine burst. This can be monitored with a pulse oximeter attached to the rat's foot and can serve as a possible indicator that a severe injury has occurred.

1.24. Monitor the rat continuously and record the time of return of righting reflex (stable ambulation on all four limbs).

1.25. The magnitude of the atmospheric pressure pulse for each rat should be within ± 0.05 atmospheres of each other. Confirm that each of the pressure pulse produces a smooth signal on the oscilloscope with consistent amplitude and duration.

NOTE: A noisy signal may indicate air bubbles in the system that must be removed prior to delivering the injury pulse. Atmospheric pressure pulses that produce a severe injury, in this experiment, are those that typically result in animal righting times of 30-60 min. This range of righting times are associated with a mortality rate of approximately 40-50%).

1.26. Administer 10 mL of prewarmed saline subcutaneously as a supportive care.

1.27. Return the rat to its home cage and allow it to recover for at least 4 h.

NOTE: Increased mortality has been observed when the rats are placed immediately back under anesthesia.

2. Implantation of cortical EEG electrodes and video-EEG recording

2.1 At 4 h after injury, anesthetize the rat as previously described and place it back into the stereotactic frame to remove the Luer lock hub and dental cement.

NOTE: The hub and cement will easily snap off with moderate pressure. When removing the hub, check carefully for any rupture or damage to the dura. Immediate euthanize any animal with damage to the dura.

2.2 Apply a small drop of 0.5% bupivacaine hydrochloride to the skull in each of the locations where 5 pilot holes are to be drilled (see **Figure 1**).

2.3 Drill pilot holes through the skull with a hand-held 0.1 mm drill bit.

2.4 Secure a stainless-steel electrode screw into each pilot hole at the following locations: a reference screw is placed caudal to the lambda over the cerebellum. Recording electrodes are placed: 1) over the hemisphere ipsilateral and rostral to the craniectomy; 2) over the hemisphere

ipsilateral and caudal to the craniectomy; 3) over the hemisphere contralateral and rostral to the craniectomy; 4) over the hemisphere contralateral and caudal to the craniectomy.

2.5 Swab the skull with 70% ethanol to remove any bone dust.

2.6 Cover the craniectomy site with a thin layer of sterile bone wax to cover the exposed dura.

2.7 Connect an electrode array to the 5 EEG electrodes by wrapping the exposed end of a color-coded electrode wire tightly around its designated stainless-steel electrode screw.

NOTE: The opposite ends of each electrode wire is positioned into a specific, designated location within the pedestal connector.

2.8 Prepare a slurry of bone cement.

2.9 Collect the electrode wires into a coil underneath the pedestal and secure the wires and pedestal into place with bone cement. Hold the pedestal in position until the bone cement has cured.

NOTE: The bone must be particularly dry and void of any residual blood in order to achieve proper adhesion and prevent premature removal of the transmitter.

2.10 Attach the wireless transmitter with fresh batteries to the pedestal before removing the animal from the stereotactic frame.

2.11 Place the animal in its home cage and place the cage in proximity to the receiver and in view of a designated video camera. Initiate video/EEG recording.

3 Collection of video-EEG recordings

3.1 Prior to collecting EEG signals, do a frequency sweep of the room where rats will be housed for EEG collection to identify any potential interfering frequencies to prevent the collection of EEG recording with any frequency that has background noise.

3.2 Set all transmitters to specific frequencies that are free of interference.

3.3 Set the sampling frequency and the input range of each programmable transmitter.

NOTE: This can be done using a smart tool provided by the system manufacturer. Transmitters can sample at a maximum rate of 1000 Hz, and a maximum input range of ±10 mV. In this experiment, EEG recordings between 0.5 Hz to 30 Hz was analyzed. Therefore, the sample rate was set at 250 Hz. We typically see Amplitudes of less than 1 mV was observed. Therefore, the set input range was at ±2 mV.

3.4 Use EEG collection software provided by the manufacturer to continuously record video-EEG beginning on the day of injury linking each wireless transmitter via a unique frequency to a specific receiver.

NOTE: Each transmitter receiver pair is capable of monitoring 4 monopolar EEG channels, and acceleration in the X, Y and Z planes. EEG data can be written to a storage server. The video data should be saved on a NAS device linked to the storage server. The EEG analysis software synchronizes the video and EEG recording based on the time maintained by the storage server.

3.5 Use the video collection software to record video of each rat with its own 2 MP resolution camera (1920 x 1080) configured to record at 30 frames/s.

NOTE: Each camera has its own infrared illumination for video collection at night.

3.6 Configure the system to automatically save all video and EEG recordings to a storage server every 24 h. The videos produce rather large files.

4 Video/EEG analysis

4.1 Synchronize the video with each EEG recording at 1/10 s resolution. Do this by using the system manufacturers video/EEG analysis software that creates a metafile with the stamp of the precise time of both of the EEG and the video.

4.2 Manually screen through EEG recordings to identify index events that define seizure activity.

4.3 Using the video/EEG analysis software and index EEG events, create a configuration file that uses key parameters (i.e., power in specific frequency bands, the ratio of frequency bands to the total power, acceleration threshold, etc.) to define the characteristics of the potential seizure events.

4.4 Run the EEG analysis software to identify potential regions of EEG recording that qualify based on the parameters selected in the configuration file.

NOTE: The EEG analysis software allows for automatic seizure detection and highlights regions of interest in the EEG signals and provides FFT power spectrum analysis across the signal.

4.5 Confirm potential convulsive seizures by using video recordings collected during acquisition, which are synchronized with each rat's respective EEG recordings.

REPRESENTATIVE RESULTS:

With this model, we induced severe TBI into adult, male, Wistar rats. Under the conditions we describe here, we typically observe mortality rates of 40-50%, and righting reflex times of 30 - 60 min as previously described[20]. We were able to collect video/EEG recordings 24 h/day beginning on the day of injury. A diagram showing the location of four monopolar EEG electrodes and a

single reference electrode is shown in **Figure 1A.** Images which demonstrate the location and appearance of the TBI lesions expected with the conditions described here are shown in **Figure 1B-D**. Under the conditions described here, we consistently observe delta slowing within the first three days post TBI. Less severely injured rats exhibit unilateral, intermittent delta slowing (**Figures 2C-D**). In contrast, continuous, bilateral delta slowing is observed after more severe injuries (**Figure 3C-D**). Some degree of delta slowing was consistently observed in all TBI rats but was not detected in any sham operated (craniectomy only) control rats (**Figures 2A-B; 3A-B).** Extensive delta slowing was consistently observed during the first three days after injury in most TBI rats. Interestingly, rats typically show pronounced weight loss during the first three days post injury. Non-convulsive seizures are occasionally observed within the first week following TBI (**Figure 4 C-D**). Clinical seizures, presenting as spike clusters associated with rearing and falling as well as forearm clonus can be observed after 1-week post TBI (**Figure 5C-D**). Finally, **Figure 6** presents representative images of occasional intermittent signal drop out and loss of signal due to battery failure.

FIGURE LEGENDS:

Figure 1. Location of craniectomy, electrode placement, and lesion. (**A**) shows a schematic diagram of the rat skull with the locations of the craniectomy (grey circle in the left hemisphere), four monopolar electrodes (Black dots; 1,2,3,4) located between the Bregma and Lambda and a reference electrode (Black dot, R) placed midline, posterior to the lambda; (**B**) shows coronal post-mortem T2 MRI scans with the location of the lesion identified by a red circle; (**C**) shows a 2-D map of the cortex where the location and size of the lesion is identified (blue region). (**D**) shows a Nissl stained coronal section with the lesion boxed, lesion is 100x magnified in image to the right.

Figure 2. Unilateral, intermittent delta slowing collected on the day of a moderate TBI. (**A**) shows a 90 s EEG trace from a sham operated, uninjured control rat on the day of surgery. All four channels are presented. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the baseline EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**B**) FFT analysis of 2048 ms selected EPOC from the uninjured sham operated animal on the day of surgery. (**C**) shows a 90 s EEG trace, which demonstrates the intermittent, unilateral delta slowing pattern of a moderately injured animal on the day of injury. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the delta slowing EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**D**) FFT analysis of 2048 ms selected EPOC from the moderate TBI animal on the day of injury. 90 s EEG tracings, from top to bottom are biopotentials 1, 2, 3, 4, corresponding to their locations around the craniectomy site as seen in **Figure 1**. Grey vertical marks define 1 s intervals on the EEG traces. All EEG traces are shown on a scale of (±500 μV). Within FFT Analysis graphs, overall analyzed frequency range was 0.5-30 Hz. This was further broken down into 4 separate frequency bands of Delta (Yellow, 0.5-4 Hz), Theta (Purple, 4-8 Hz), Alpha (Red, 8-12 Hz), and Beta (Green, 12-30 Hz). % (Power) graph shown within the FFT analysis tells what percentage of the total power in the analyzed EPOC comes from

each previously specified frequency band, allowing for further mathematical characterization of the EEG waveform patterns.

Figure 3. Bilateral, continuous delta slowing collected on the day of a severe TBI. (**A**) shows a 90 s EEG trace from a sham operated, uninjured control rat on the day of surgery. All four channels are presented. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the baseline EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**B**) FFT analysis of 2048 ms selected EPOC from the uninjured sham operated animal on the day of surgery. (**C**) shows a 90 s EEG trace, which demonstrates the continuous, bilateral delta slowing pattern of a severely injured animal on the day of injury. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the delta slowing EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**D**) FFT analysis of 2048 ms selected EPOC from the severe TBI animal on the day of injury. 90 s EEG tracings, from top to bottom are biopotentials 1, 2, 3, 4, corresponding to their locations around the craniectomy site as seen in **Figure 1**. Grey vertical marks define 1 s intervals on the EEG traces. All EEG traces are shown on a scale of (± 500 µV). Within FFT Analysis graphs, overall analyzed frequency range was 0.5-30 Hz. This was further broken down into 4 separate frequency bands of Delta (Yellow, 0.5-4 Hz), Theta (Purple, 4-8 Hz), Alpha (Red, 8-12 Hz), and Beta (Green, 12-30 Hz). % (Power) graph shown within the FFT analysis tells what percentage of the total power in the analyzed EPOC comes from each previously specified frequency band, allowing for further mathematical characterization of the EEG waveform patterns.

Figure 4. Nonconvulsive electrographic seizure collected 3 days post severe TBI. (**A**) shows a 90 s EEG trace from a sham operated, uninjured control rat 3 days[25] after surgery. All four channels are presented. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the baseline EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**B**) FFT analysis of 2048 ms selected EPOC from the uninjured sham operated animal on the day three[25] after of surgery. (**C**) shows a 90 s EEG trace three [25] days post severe injury. This show building, fast spiking pattern present bilaterally and across all 4 collecting channels. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the spiking EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**D**) FFT analysis of 2048 ms selected EPOC from the severe TBI animal on the day of injury. 90 s EEG tracings, from top to bottom are biopotentials 1, 2, 3, 4, corresponding to their locations around the craniectomy site as seen in **Figure 1**. Grey vertical marks define 1 s intervals on the EEG traces. All EEG traces are shown on a scale of (± 500 µV). Within FFT Analysis graphs, overall analyzed frequency range was 0.5-30 Hz. This was further broken down into 4 separate frequency bands of Delta (Yellow, 0.5-4 Hz), Theta (Purple, 4-8 Hz), Alpha (Red, 8-12 Hz), and Beta (Green, 12-30 Hz). % (Power) graph shown within the FFT analysis tells what percentage of the total power in the analyzed EPOC comes from each previously specified frequency band, allowing for further mathematical characterization of the EEG waveform patterns.

Figure 5. Convulsive electrographic seizure collected 9 days post TBI. (**A**) shows a 90 s EEG trace from a sham operated, uninjured control rat nine (9) days after surgery. All four channels are presented. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the baseline EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**B**) FFT analysis of 2048 ms selected EPOC from the uninjured sham operated animal on the day nine (9) after of surgery. (**C**) shows a 90 s EEG trace nine (9) days post severe injury. This show building, fast spiking pattern present bilaterally and across all 4 collecting channels. A 10 s long trace (taken from the boxed region) was extracted from the 3rd channel to better visualize the spiking EEG pattern. A 2048 ms EPOC section of this was then selected to be analyzed in the corresponding FFT. (**D**) FFT analysis of 2048 ms selected EPOC from the severe TBI animal nine (9) days post injury. 90 s EEG tracings, from top to bottom are biopotentials 1, 2, 3, 4, corresponding to their locations around the craniectomy site as seen in **Figure 1**. Grey vertical marks define 1 s intervals on the EEG traces. All EEG traces are shown on a scale of (± 500 µV). Within FFT Analysis graphs, overall analyzed frequency range was 0.5-30 Hz. This was further broken down into 4 separate frequency bands of Delta (Yellow, 0.5-4 Hz), Theta (Purple, 4-8 Hz), Alpha (Red, 8-12 Hz), and Beta (Green, 12-30 Hz). %(Power) graph shown within the FFT analysis tells what percentage of the total power in the analyzed EPOC comes from each previously specified frequency band, allowing for further mathematical characterization of the EEG waveform patterns.

Figure 6. Signal drop out. These are 3 separate examples of what signal drop out due to transmitter or receiver issues appears as on the EEG recording. (**A**) This is an example of intermittent dropout of the EEG signal on a recording. (**B**) This is an example of drop out due to battery failure during continuous wireless telemetry appears as on an EEG tracing. (**C**) Within the circled region, it can be seen that when the Quality of Signal (QoS) drops from 100 to 0, the EEG tracing becomes flattened and stagnant at 0 µV. Grey vertical marks define 1 s intervals on the EEG traces. All EEG traces are shown on a scale of (± 500 µV).

DISCUSSION:

Considerable variability has been reported between laboratories regarding the specific parameters and methods used for the FPI TBI model [14,26,27,28]. These inconsistencies have resulted in conflicting results and make it difficult to harmonize efforts and outcomes between labs. Here, we have presented a detailed methodology describing our approach to long-term, continuous recording of video/EEG to monitor for post-traumatic epileptiform activity. A number of steps are critical to generating reproducible results with the described method.

First, given that the incidence of post-traumatic epilepsy correlates with injury severity, apply conditions that result in the most severe TBI. Specifically, use a 5 mm craniectomy to ensure that a sufficiently large area of dura is exposed. In addition, secure a female-female Luer lock device onto the surface of the skull, with the opening placed directly over the craniectomy. This differs from other labs that have used a smaller craniectomy (3 mm) and/or placed a modified needle hub inside the craniectomy, which effectively reduces the opening size. By placing the Luer lock outside of the craniectomy, the 5mm opening is maintained. These specific parameters impact the overall force applied to the dura. The atmospheric pressure applied to the dura also has a

major impact on the severity of injury observed. Unfortunately, atmospheric pressure is highly variable and appears to be device dependent. Some labs have reported applying a pressure pulse of 8 - 10 ms[18]. In contrast, the method described here results in a 20 ms pressure pulse. This is consistent with other labs that appear to generate more severe injury [14,28]. It is clear that the injury-inducing pressure pulse is a parameter that shows considerable variability between labs and must be empirically defined. However, injury severity may be determined based on a combination of mortality rates (40-50%), righting reflex times (>30 min)[26]. It is also critical that only animals with an intact dura be included in the study. In addition, if the craniectomy is occluded by any glue or cement such that part of the dura beneath the craniectomy is not exposed to the full force of the fluid pressure pulse, then the animal should be eliminated from the study. Also, excess glue beneath the Luer lock can adhere to the dura and remove it with the cement cap even after a successful injury. Finally, the smooth shape of the pressure pulse curve on the oscilloscope trace gives the indication that there are no air bubbles in the fluid chamber and indicates the plunger is moving without impedance.

Anesthesia is another critical factor that must be controlled. Isoflurane exposure should be kept to the lowest levels possible to maintain a surgical plane of anesthesia. Rats exposed to higher levels of isoflurane or for long durations are more likely to develop neurogenic-induced pulmonary edema. Preparation of the skull represents another critical aspect of the method. Particularly, drying the skull and removing any bone dust helps to prevent the rats from removing the transmitter prematurely.

The placement of screws and the connection of the EEG wires are obviously critical to producing consistently reproducible recordings. It is important that the screws are not placed too deeply as to induce a lesion on the brain. The bone flap recovered from the craniectomy of adult (12 weeks old) male Wistar rats is consistently 2 mm thick. Use EEG electrode screws with a 2.5 mm shaft. It is helpful to use the tips of curved mosquito hemostatic forceps as a spacer to ensure that the screws only extend to the base of the bone and do not protrude into the brain.

The approach presented here does have some limitations. Batteries must be changed on a regular basis. The frequency of battery changes depends on the sampling rate. Batteries are typically changed once a week for a sampling rate of 1000 Hz. This time frame can be extended by reducing the sampling rate. The system is also limited to recording from four monopolar EEG electrodes. However, this provides two channels per hemisphere and can differentiate between focal and generalized events and can differentiate between anterior and posterior changes. Despite these limitations, this approach provides a reasonable method to conduct continuous video/EEG monitoring and detection of epileptiform changes following severe TBI.

The method described here results in both electrographic and convulsive seizures within one month following TBI. Therefore, this approach provides a reasonable time frame in which to study potential therapeutics for preventing epileptogenesis following severe TBI. This approach also provides a method to investigate the molecular mechanisms associated with PTE and may lead to the identification of potential biomarkers that can be used to identify patients who are most at risk of developing PTE.

ACKNOWLEDGMENTS:

We wish to thank Paul Dressel for his invaluable support in graphical design and preparation of figures.

DISCLOSURES:

Chelasea R Richardson is an employee of emka Scientific, the supplier of this wireless telemetry system described.

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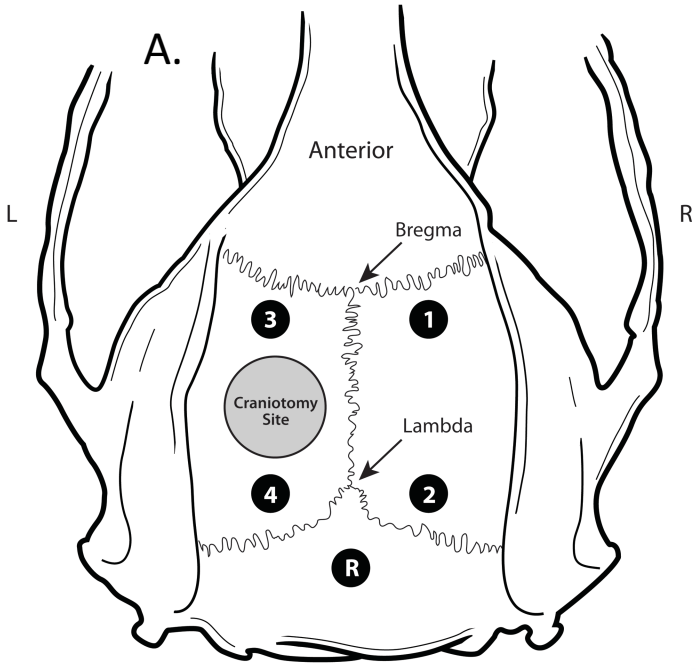
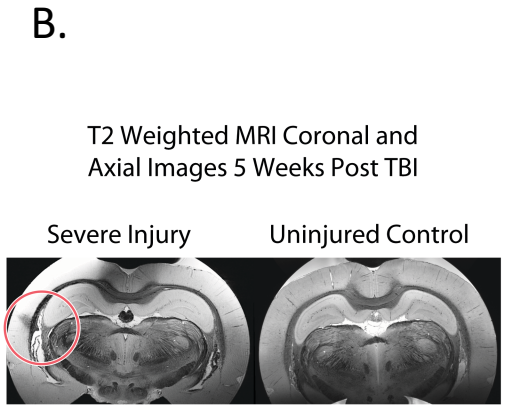
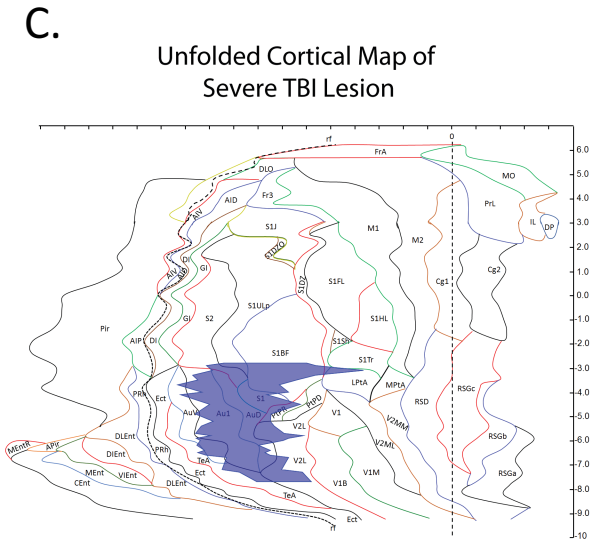
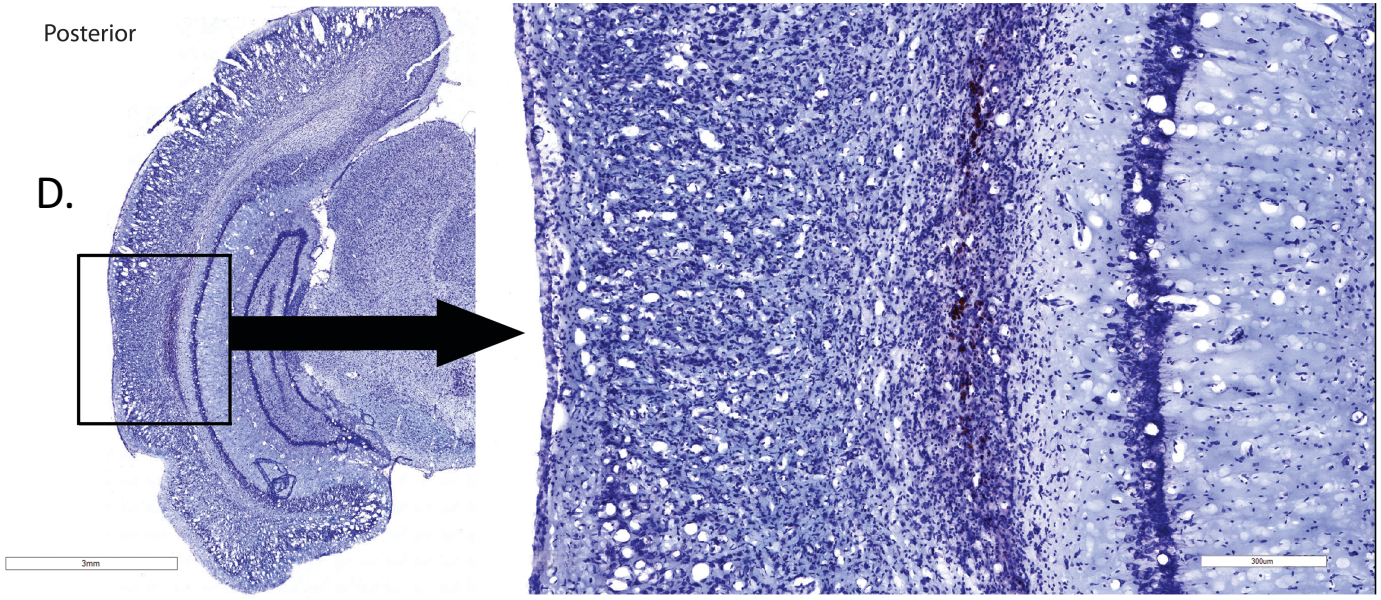
Figure 1









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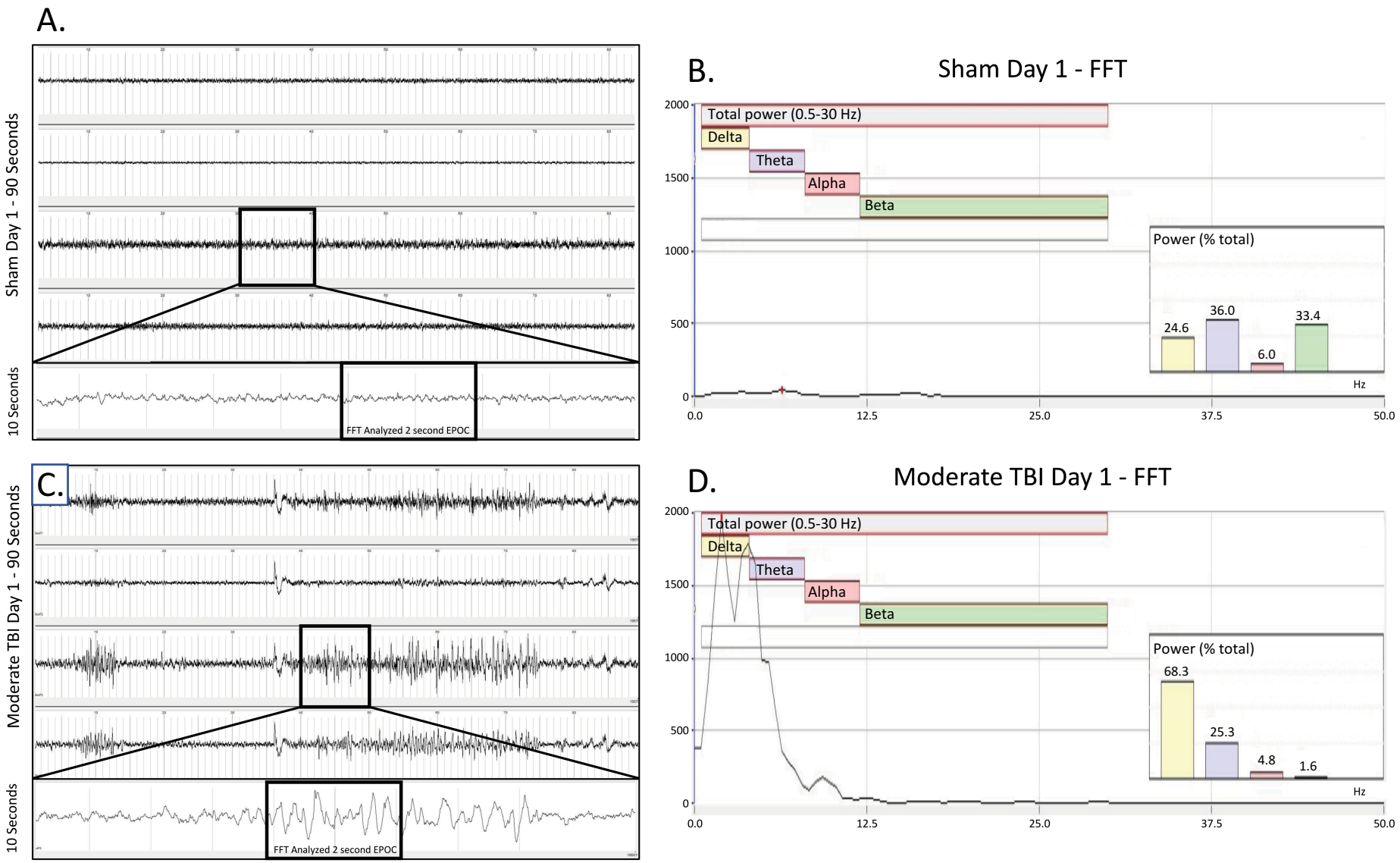

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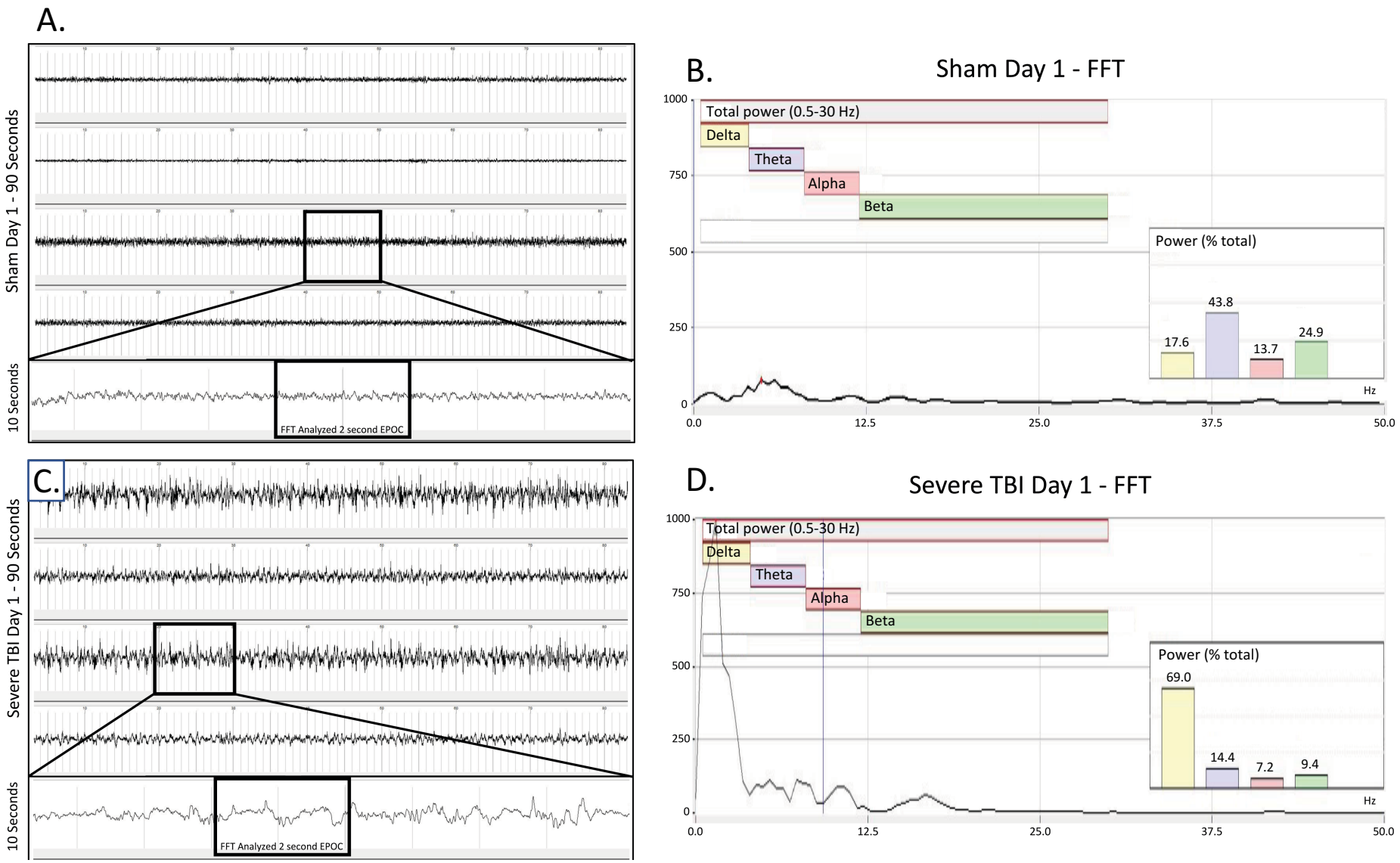

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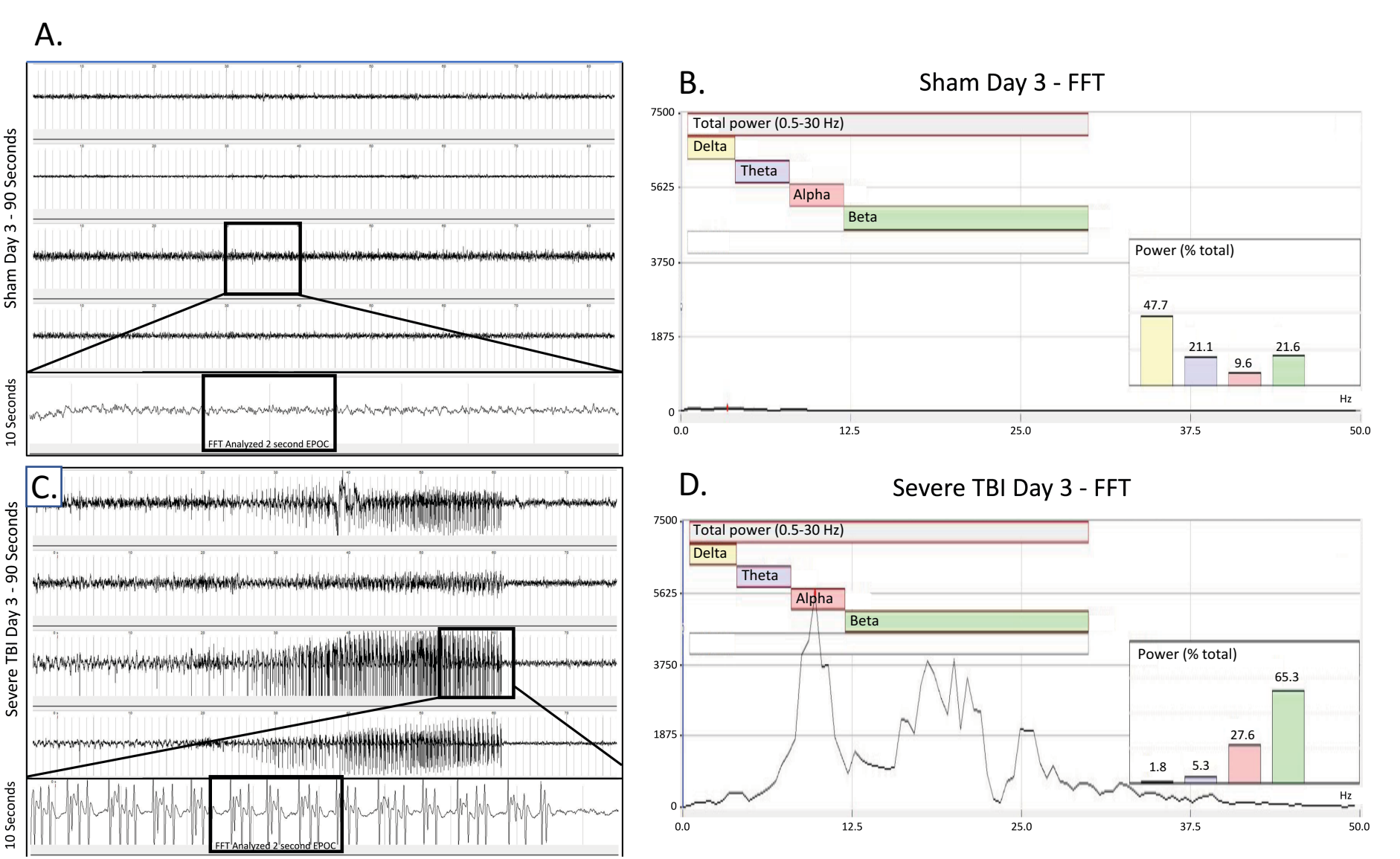

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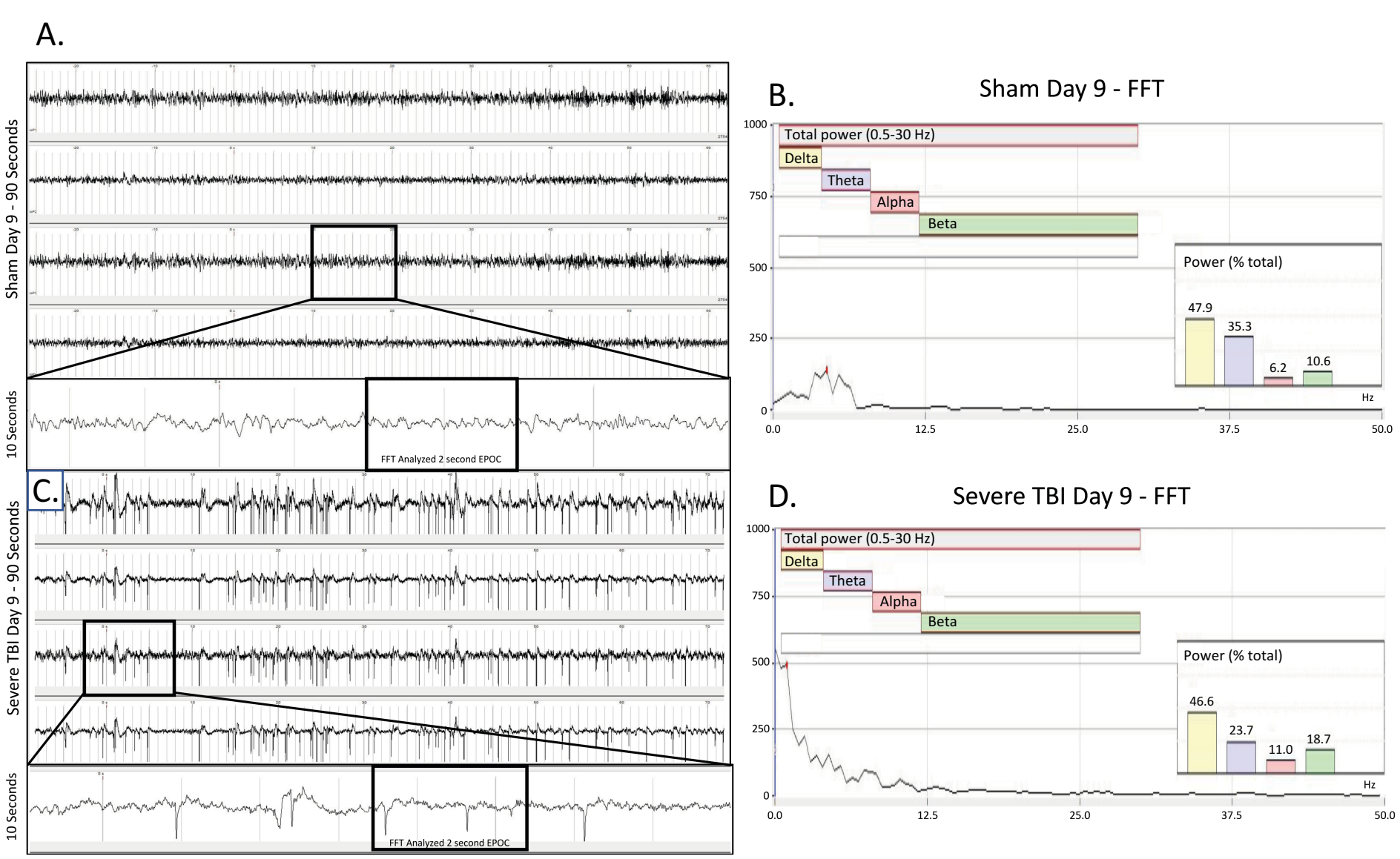

Figure 6

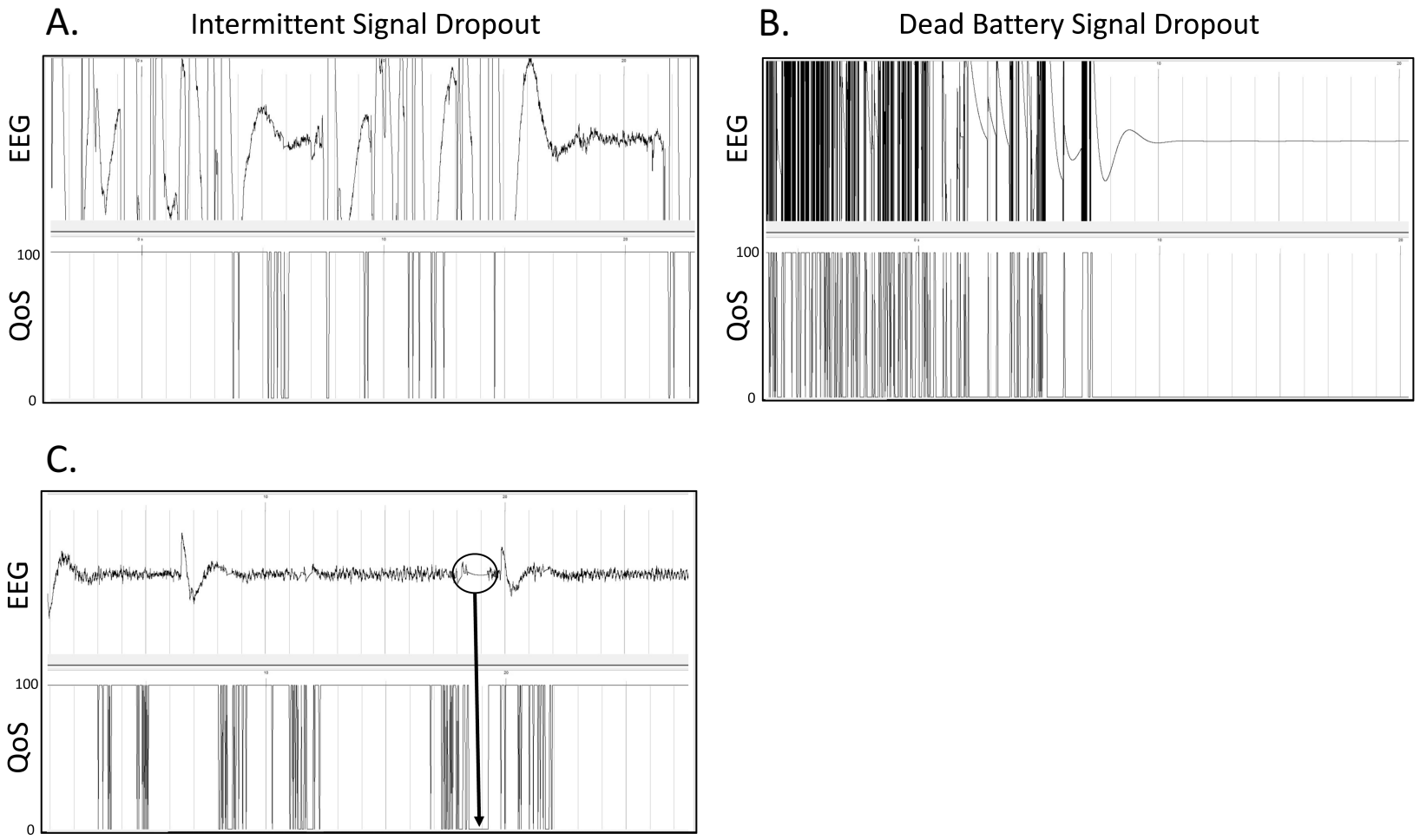

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.00 mm Drill Bits	Drill Bit City: New Carbide Tools	05M200	
3M ESPE Durelon Carboxylate Cement	3M , Neuss Germany	38019	Dental Cement
4-0 Suture	Ethicon, Sommerville, NJ	K831H	4-0 Ethicon Perma-hand Silk, 26mm 1/2c Taperpoint, 30" (75cm), Black Braided non-absorbable suture
5 mm outer diameter trephine	Fine Science Tools	18004-50	
Bonewax	Medline Industries, Mendelcin, IL	REF DYNJBW25	
Buprenorphine HCL, Injection (0.3 mg/mL) 1 mL vial	Par Pharmalogical, Chestnut Ridge NY	3003706	NDC 42023-179-01
Dumont #6 Forceps	Fine Science Tools	11260-20	
Dumont #7b Forceps	Fine Science Tools	11270-20	
ecgAUTO	EMKA Technologies, Falls Church, VA		
Female Luer Thread Style Coupler Clear Polycarbonare	Cole-Palmer instrument	SKO#45501-22	Order lot #214271
Foot Power Drill	Grobet USA, Carlstadt, NJ	Model C-300	
GentaMax 100 (Gentamicin, Sulfate Solution)	Phoenix, Manufactured by Clipper Distributing Company LLC, St. Joseph, MO		NDC 57319-520-05
Hill's Prescription Diet a/d Canine/Feline	Hill's Pet Nutrition, Inc. , Topeka, KS		
IOX2 Software	EMKA Technologies, Falls Church, VA		
Isoflorane, USP	Piramal Enterprise Limited, Andhra, India		NDC 66794-013-25
IsoTech Anesthesia machine	SurgiVet	WWV9000	
Lateral FPI device	AmScien	302	curved tip, with pressure tubing extension. connected via screw lock connector (Cole-Palmer; #4550-22)
Leica A60 Stereomicroscope	Leica Biosystems, Richmond, VA	PN: 10 450 488	
Marcaine (0.5%) Bupivacaine hcl injection usp 5 mg/mL	Hospira, Lake Forest, IL	CA-3627	50mL multiple dose vial; NDC 0409-1610-50
Micro-Adson Forceps	Fine Science Tools	11018-12	
Olsen-Hegar Needle Holders with Suture Cutters	Fine Science Tools	12002-14	
PALACOS R+G bone cement with gentamicin	Heraeus,	REF: 5036964	Radiopaque bone cement containing 1 x 0.5g Gentamicin
Physio Suite	Kent Scientific, Terrington, CT		
Povidone-iodine solution			Betadine
Puralube Vet Ointment	Dechra Veterinary Products, Overland Park KS		NDC 17033-211-38
Scalpel blade (#10) and holder	Integra Miltex, York, PA	REF: 4-110	
Scalpel Handle - #4	Fine Science Tools	10004-13	
Sickle Knife	Bausch + Lomb Storz Instruments	N1705 HM	5mm curved blade. Round handle. Overall length 168mm, 6.6 inches.
Silverstein Micro Mirror	Bausch + Lomb Storz Instruments	N1706 S8	3mm diameter. Angled 45 degrees. Overall length 180mm, 7.2 inches
Storage NAS	Synology Inc.	DS3615xs	
Synology Assistant	Synology Inc.		
Thermal Cautery Unit	Geiger Medical Technology, Delasco Council Bluffs, IA	Model NO: 150	
Vetivex	Dechra Veterinary Products, Overland Park KS		Veterinary pHyLyteTM Injection pH 7.4 (Multiple Electrolytes Injection, Type 1, USP)
Video Cameras	TRENDnet, Torrance, CA	TV-IP314PI	Indoor/Outdoor 4MP H.265 WDR PoE IR Bullet Network Cameral
Video NAS	Synology Inc.	DS916	
Wistar IGS rats	Charles River	strain code 003	12 wk old at the time of injury
Wullstein Retractor	Fine Science Tools	17018-11	

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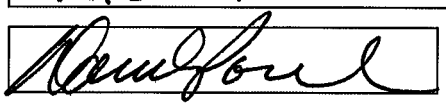
Signature:	
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8. Introduction section lacks citation. For example, line 54-55, 56-57, 59-70, 61-62, 76077,
9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.
10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
12. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
13. The Protocol should contain only action items that direct the reader to do something.
14. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step
15. Please ensure the numbering of the Protocol to follow the JoVE Instructions for

Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
16. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? So someone should be able to replicate the experiment step by step.
17. 1.2: How do you check the depth of anesthesia? Toe pinch, this has been added
18. 1.3: Do you perform the shaving and surgery at the same site? How do you maintain sterility? This was a error. The rats are shaved before they are placed on the stereotaxic frame in a separate area. This has been corrected.
19. 3: For the video EEG and analysis step performed with the software, please provide all the button clicks, graphical user interface if any, Scripts generated if any.
The video/EEG collection and analysis software user manuals represent a combined 600 pages describing key stroke, screen shots and configurations. It is not possible to provide step by step button clicks and screen shots with the 10 page protocol limit of the journal. In addition, this would not be possible without identifying the manufacturer of the system. We have made an attempt at responding to this request but in reality, this really is not possible to provide meaningful instruction of software use in this format.
20. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
21. All figure panels should be uploaded together as one figure. Either combine all the figure panels or number each figure independently (1,2,3 etc.)
22. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the techniques
24. We cannot have less than 10 references in the reference section. Please include more citations in your manuscript.
25. Please alphabetically sort the materials table.

Reviewers' comments:

Reviewer #1:
This paper precisely describes procedures for fluid percussion injury in a rat model of post traumatic epilepsy, and subsequent wireless instrumentation. Given that there is no standardization of these procedures in the field, this should serve the useful purpose of

guiding researchers toward a common method so that data can be compared between labs.

I only had a few minor comments:

1) Figure 1D and 1E are described as spike and wave discharges. It may be more accurate to refer to these as spike clusters since they are in short repeating bursts of 3-5 spikes. The longer trace in 1E also shows a gradual onset with frequency progression, which is more common for complex partial seizures (convulsive or non-convulsive). The term spike-wave discharge (SWD) should be reserved for 7-10 Hz repetitive spikes (and waves) with sudden onset, little or no frequency progression, sensitivity to sensory interruption and to ETX. The distinction is important since SWDs are uniquely associated with models of absence epilepsy and not PTE.
Thank you for this comment. We understand the issue with the term “spike wave discharge” and we agree with the reviewer. We have corrected the text and identified events as either complex partial seizures or spike clusters.

2) In general, the MS would benefit from a final proof-reading. I have spotted some typos etc. but this may not be exhaustive:
Line 41 "dura through." ?
Line 62 "chomoconvulsants"
Line 65 "from chemical induced seizures"
Line 101 "is kept at 37°C is positioned"
Line 163 "Injury pressures delivered to produce a severe injury are those"
Line 133 I think it is usually "Luer Lock" not "Luer Lok", not sure, but both words are always capitalized.
Thank you for the comments. The manuscript has been proofread more thouroughly and changes have been made.

Reviewer #2:
Manuscript Summary:
The manuscript presented by McGuire and colleague describes a detailed protocol of the lateral fluid-percussion traumatic brain injury model in rodents and a wireless telemetry recording of EEG from TBI animals. With the ever-increasing number of reports on TBI and motoring of post-traumatic seizure, this manuscript could not have come at a better time. The authors provide a detailed systematic procedure and addressed some the factors that are responsible for the variability and harmonization difficulties between different labs. However, I have a few concerns that may be helpful in improving the manuscript.

Major Concerns:
I generally agree that the bases of this manuscript was to demonstrate a protocol for FPI as performed by these authors. In addition, the authors justify the publication of their protocol based on the fact that there is significant variability in the FPI method across different laboratories. However, they fail to convince the reader as to why their

protocol or adopting a common protocol will be ideal, especially in studying post-traumatic epileptogenesis. I think this should be addressed in the discussion.
Clearly there are various ways to perform the FPI method, which has been the problem. Each lab performs the method with slight variations, each of which can have a major impact on the outcome and the interpretation of the data. To compare results from two different labs obviously requires harmonization and reproduction of the methods. Our goal was not to define our method as the only or definitive approach. It was simply to provide a detailed method so that others could reproducibly replicate our results to accurately compare outcomes. We also provide here a detailed method on how we perform EEG collection with the specific wireless telemetry system. Text has now been added to the introduction and discussion to address this concern.

Minor Concerns:
1 Can the authors provide a reference to the first paragraph in the introduction?
We extensively revised the introduction and added multiple references.
2 The authors report in the "introduction" that they have previously reported a 60% incidence of convulsive seizure within six weeks after TBI. Can they provide a reference to this?
This reference has been added as well
3 Generally, 5% isoflurane is used for induction. This usually gets the animal anesthetized fast, thereby reducing prolonged period of drowsiness. Can the authors clarify why they use 3%?
We have found that limiting the exposure to isoflurane reduces the incidence of neurogenic-induced pulmonary edema, which can be a major cause of acute and sub-acute mortality. This comment has been added to the method as a note.
4 Can the authors indicate the anesthesia carrier gas?
We use 1L/min oxygen. This information has been added to the text.
5 In this protocol, the luer-lok is place around the craniectomy rather that in the craniectomy as in other laboratories. The authors claim that the saline level in the luer lok is use to check for leakage in this system. However, it is possible that pressure from the fluid (during the impact) can cause this to open and thus reduce the overall impact pressure on the dura. How do the authors verify that this is not the case after the impact?
This is a good question. We have chosen to place the Luer Lock outside of the craniectomy so as not to reduce the inner diameter and thus reduce the total force applied to the brain. We monitor the level of saline in the Luer Lock after injury as well as before. Thus, reduction in the level of saline provides an obvious indication if there is a leak. Alternatively, we have also performed neurological severity score (NSS) assessments on rats within 8 hours after injury to confirm severity based on functional behavior. In addition, mortality rates and righting reflex times can be helpful additional parameters to consider in combination with the NSS. We have added this information to the protocol.
6 The atmosphere displayed in the oscilloscope depends on many factors including the tube size as explained the authors. It will be nice if the authors can give a rough guide on the amount of atm, they normally observe when they induce severe TBI with this protocol. This will be useful for someone setting up the FPI protocol. Righting reflex

above 30 min usually indicates severe injury, but in the FPI model there is usually huge variability. Thus, rough estimates of approximate values of independent variables such as the angle and atm will be useful.
We have most recently observed that atm of 2.2-2.3 and an angle value of 17 produce severe injury. However, atm serves as a poor indicator of severity. Over time, we have observed that the atm required to induce severe injury vary with a device as the O-rings age. The atm also changes depending on the person doing the injury. We have found that heating the plunger end of the reservoir prevents sticking and improves consistency between hits. This information has been added as a note in the procedure.
7 Can the authors improve figure 1 B-E, (EEG tracings)? The green lines make it difficult to appreciate the EEG. add a scale bar when the green line are taken off.
Unfortunately, the software does not allow us to remove them. However, we have changed the vertical lines to a more subtle gray tone to make them les distracting.
8 Can the authors add in point 1.12 that the FPI device used in this protocol had a curved tip?
Thank you for this observation. We agree that this may represent a key element for consistency. This has been added to the protocol. We have also included a description of the pressure tubing and connective device.

Reviewer #3:
Manuscript Summary:
This JOVE manuscript describes two important technical contributions to the study of traumatic brain injury and posttraumatic epilepsy: first, it describes the procedures used by the Poulson laboratory to induce traumatic brain injury using the lateral fluid percussion technique, and then second, it describes how they used radiotelemetry to record four-channel EEG from, with use of an accelerometer to record behavioral changes. The paper builds from the Epilepsy Res. publication by Smith and coworkers from the Poulson laboratory concerning the effects of traumatic brain injury on electrophysiological spike activity and behavioral seizures. Overall, the level of detail and the usefulness of the methods are apparent, and so the concept of Jove paper is solid. There are some concerns, however, that should be addressed.

Major Concerns:
In the long abstract, and elsewhere in the paper, the authors make the point lines 33-34 of page 2) that there is a need to harmonize studies between laboratories in regard to this approach to traumatic brain injury. Although this is likely to be true, the manuscript might be improved if the authors made it clearer where potential mistakes could occur and how their method differs from others. In other words, is this the exact same approach used by other laboratories, such as D'Ambrosio and others, where they have found no evidence of convulsive seizures? Showing pictures of the resulting injury (to help with reproducibility) would seem very useful. How would the reader know if the resulting injury is the same as the authors intended? Whole-mount image of injured brain as well as brain sections would also be beneficial, if not the minimum required. Where on the skull is the craniectomy performed? How does one keep the location consistent? Are there stereotaxic coordinates? If so, what are they?
These are very relevant comments and we thank the reviewer for the suggestions. We

have revised the long abstract and identified critical variables that have been reported which impact outcomes. In addition, we have modified the figures significantly. Figure 1 is now focused on providing the information listed above. Specifically, figure 1 now contains a diagram mapping the location, and size of the craniectomy, and includes coronal and horizontal MRI views of a representative normal brain compared to a severe TBI rat brain with the location of the lesion identified. We have also included as part of figure 1 an image of a Nissl stained brain section from a severe TBI rat along with a 2D unfolded cortex map demonstrating the location and size of the lesion. Finally, we have included the stereotaxic coordinates for the center of the craniectomy (AP -4.0; ML 3.5) into the protocol.

The authors emphasize the significance of the paper in relation to posttraumatic epilepsy, however it is not clear whether the event in figure 1, panel D and E is a "generalized spike and slow wave discharge" or a seizure. The authors say it is the former, but the event actually looks more like an actual seizure, but is it an acute seizure (soon after the TBI) or a "spontaneous" seizure (i.e., weeks after injury)? It is confusing, because the slowing in 1C is specifically labeled as at 24 h. Is the seizure from the same recording time point?
We have significantly revised and expanded the figures within the paper. Figure 1 is already described above. Figure 2 depicts unilateral intermittent slow wave discharge at 1-day post TBI. A time matched, uninjured control is shown for comparison. Figure 3 shows a representative bilateral, continuous delta slowing at day -1. Figure 4 shows a representative seizure recorded at 3 days post TBI. Figure 5 shows a behavioral seizure manifest as spike clusters on day 9 post TBI. Figure 6 depicts examples of signal drop out. Each figure includes example EEG recordings with their respective FFT results which show the percentage power within each frequency and the max power in specific frequencies.

In addition, in the regards to the wireless recording system, it would be useful to the reader to know more about how the wireless system leads to a "significant reduction in noise" and to learn more about the nature of the signal dropouts, since dropouts can be problem with radiotelemetry. Although there are advantages and disadvantages to using a wired or tethered recording system vs wireless, it would be useful if the authors had the data to show more clearly the pros and cons of these two types of recording configurations (page 3, lines 45-50).
The wireless system does not have to contend with the noise typically observed with a tethered system……..
We would argue that providing a direct comparison with data between the wireless and tethered systems is beyond the scope of this methods paper.

In the Introduction, the authors talk about the advantages of using traumatic brain injury and posttraumatic epilepsy to study anti-epileptogenic drugs, compared to status epilepticus models (line 61-64, pg. 3). Without a doubt, it is useful to use traumatic brain injury and posttraumatic epilepsy to study anti-epileptogenesis, and these types of models may have advantages over status epilepticus models, but the authors seem to mix-up the issue in terms of their reference of status epilepticus models. When drugs

are used to block the seizures during status epilepticus, they are purely seizure blocking drugs, but once status epilepticus is complete and epileptogenesis has begun to occur, any potential therapy given after the status epilepticus would be an effect on epileptogenesis. Similarly, any drug/therapy administered during or soon after TBI would not necessarily be antiepileptogenic; it would only be considered an antiepileptogenic therapy for posttraumatic epilepsy if also given well after the injury. Thus, the authors need to revise what they have written to avoid future criticism. In fact, some would argue that status epilepticus models have an important advantage over posttraumatic epilepsy, because very few studies have found convincing evidence of epilepsy after traumatic brain injury and in those cases the seizure frequency is low and the fraction of animals that develp epilepsy is typically low, which are huge problems compared to the models based on status epilepticus. The point is that the authors may want to rewrite this to more appropriately address the real issues, because status epilepticus models can be and have been used effectively to study epileptogenesis.
We agree and this is a reasonable comment. We have extensively revised the introduction to address these concerns.

In the introduction on pg. 3, line 72-74, the authors argue that monitoring of posttraumatic seizures is labor intensive and requires multiple laboratories in order to achieve a large enough number of animals for appropriate statistical power. One can make an argument that any animal model of epilepsy may require more than a single laboratory to generate enough data to have enough statistical power for a rigorous study. A major issue is that the authors should address is the degree to which they actually have genuine evidence for epilepsy with spontaneous recurrent seizures. If those data are available, the authors may want to delay publication of the present paper until that information is published. Furthermore, it is not clear how the data in this paper are going to address the problem of whether one needs one or multiple labs to address this issue. If this paper aims to address the issue of how to generate homogeneous data across laboratories, then the authors should better address the problem of how this method differs from (and is better) than others, which hopefully could be done in an objective manner. If the authors have electrophysiological evidence for both clear convulsive and non-convulsive seizures, that should be included in this paper.
Again, these are reasonable comments and we appreciate the reviewer's suggestions. We have revised the manuscript to focus on the method presented. Researchers will clearly choose whatever method they feel best fits with the question under investigation. We are presenting this JoVE paper as an option with detailed description to improve reproducibility. We have indeed observed both convulsive (figure 5) seizures and non-convulsive seizures (Figure 4). A separate manuscript is currently being prepared that describes the incidence and frequency of these events under the conditions and methods we describe here.

The authors discuss the advantages of wireless recording and attempt to be objective about the pros and cons of the two approaches. It might be useful to better explain what is meant by "a significant reduction in signal noise which reduces the amount of filtering required" pg. 3 lines 82-83. Are the authors referring to 60-Hz noise, high frequency noise, movement artifact, and what type of filtering they are talking about? Since this

deals with optimization of electrophysiological recordings, more evidence along this line is warranted to make this a strong paper.

On page 6, in terms of video analysis, the authors discuss automated sleep scoring and studies of sleep after brain injury. If the authors are going to being up this issue, they may want to provide more data on it, since right now it seems like an ancillary issue.
This is a reasonable comment and sleep scoring is indeed an ancillary issue and not critical to the method. As a consequence, we have removed sleep scoring from the description.

Figures
It is not clear why the authors have grouped everything into a single figure.
We now have 6 figures that address the concerns raised by this reviewer and others.

Figure 1A seems to be completely different topic than the others, which are EEG traces. Furthermore, Figures 1B and C seem to deal with the slowing of the EEG, whereas figure 1D and E relate to spike-type events.
see comment above

For all of these traces, and for the data in the paper in general, the authors show more explicitly define and show figures to illustrate the difference between control and experimental animals.
We completely agree with this comment and thank the reviewer for the suggestion. The figures now include time matched, representative recordings from sham operated, uninjured controls.

It is difficult, if not impossible, to understand or show EEG slowing without a comparison between control and experimental animals. To appropriately portray the EEG slowing the authors should consider both an illustration of the raw data (as they have now), but also a graphical illustration in the form of a spectrogram or some other quantitative illustration of how power has changed in different frequency bands.
We have included FFT results for the representative EEG traces displayed, which provide total power relative individual frequency bands.

For the experimental animals, it would be useful to know whether they have had actual seizures, versus spike wave discharges. In Fig 1Dand E, the authors seem to be saying these are "generalized" spike wave discharges, but they do not appear to be generalized, they look more like actual unilateral seizures.
We agree with the reviewer’s comment, and we have revised the text accordingly.

Finally, for all the figures showing electrophysiological recordings, although the authors do show more than one time base, they are very similar. It would be helpful if they first showed data at a very slow time base, so one can see the beginning and the end of the seizure or spike wave discharge with adequate baseline before and after, and then in

another panel, an expanded view with a substantially different time base, to show the waveform of the events in the middle of the seizure or spike wave discharge.
We agree with the reviewer's comment and have modified the figures accordingly.

To summarize, this could be an important paper, but it needs to address several important tissues (above and below).

1. It is not really clear whether the paper is about TBI or telemetry or both. If it is about TBI, which it appears to be, the authors should define how to make the method and data more reproducible than others, such as showing pictures of the resulting injury, etc.
See comments above

2. The authors mention signal drop-outs, but never show an example of one in a figure. It would be useful for a reader to see dropouts and artifacts, so they could not be confused with actual spikes and seizures. And see how the telemetry is better than wired recordings.
Figure 6 now provides examples of different types of signal drop outs.

3. In relation In regard to posttraumatic epilepsy, it is not clear what is what in the traces in Fig 1. Slowing is not clear, what is TBI vs control, seizure vs SWD, etc
See comments above

Minor Concerns:
see above

Reviewer #4:
Manuscript Summary:
The authors describe a straightforward method to produce a LFPI and implant EEG recording electrodes using a telemetry system. The Manuscript provides an alternative to improve consistency and rigor in preclinical studies of TBI and PTE.

Minor Concerns:
Referencing of relevant literature should be improved throughout the manuscript. Paragraph would benefit form the inclusion of appropriate references
Additional references have been added

Line 61 to 64, the statement s presented there are incorrect. Both models have been described for the development and screening of potentially antiepileptogenic compounds - See McNmara 2012 neuron, Liu 2015 Brain, Pitkanen 2016, Saletti 2019
This is a reasonable comment and is consistent with other reviewers comments as well. We have revised the text in both the abstract abstract and introduction accordingly.

Line 68 and 69, seminal papers of LFPI induced TBI and PTE should be cited here.
Multiple references have been added here.

A very interesting paper that discuss a pipeline for Harmonization of LFPI in the search for biomarkers and potential antiepileptogenic treatment by Ndode-Ekane 2019 should be cited in this paragraph and possibly in the discussion
We agree and a statement along with this reference and other references regarding harmonization have been added.

The statement form line 76-77 should be referenced.
This has been addressed. The reference has been added.

Protocol 1.1 - as this is a methods paper, the carrier of the inhaled Anesthesia should be decried whether is air, oxygen etc) as well as the flow of the gas in l/min.
We used 1L/min oxygen as the carrier. This has been added to the protocol.

Protocol 1.8 -The stereotactic location of the center of the craniotomy must be described. This is crucial for a proper Harmonization of the methodology of LFPI. Rupture of the dura after LFPI is one of the exclusion criteria for studies of TBI and PTE.
The stereotaxic coordinates for the center of the craniectomy are: AP -4.0; ML 3.5. This information has been added to the protocol.

Does this thinning of the skull be a problem for the estabilituy of the skull cap in long term(i.e. > 6months?)
We can't answer this question as we have only recorded up to 6 weeks post TBI. However, we have found that using osteo-bond bone cement in place of dental cement makes it much more difficult for the rats to remove the head gear. We have placed a note in the protocol that includes this information.

Protocol 1.11Would the researches think that use of gentamicin may be a cofounding factor in AEG studies, particularly those that look at infection and/or Neuroinflammation?
It would seem reasonable that the gentamicin would interfere with any model that uses bacterial infection to induce seizures. However, we don't believe it has an impact on the neuroinflammatory response induced by TBI.

Protocol 1.12 Would it be relevant that the authors provide the details of the FPI device?
The journal does not allow the identification or the reference to specific commercial products in the protocol itself. However, this information is provided in the material list section.

Protocol 1.13 the flow rate of oxygen should be described here, apnea and pain reflex (elicited by the toe pinch) have been recorded in many published TBI and PTE liiterature. Would the authors think these a relevant acute injury measures of severity of the TBI?

We use 1L/min oxygen, which has been added to the protocol. In our experience, we have not observed apnea to be a good representation of injury severity. In addition, the use of acute toe pinch after injury is not a reliable measure of injury severity.

Protocol. 2.1 an extra period of anaesthesia may be a cofounding factor in AEG studies
This may be a possibility. However, this is not something that can be avoided in this model. The inclusion of sham operated and untreated injured controls would address this as a possible confounding factor.

Protocol 2.4 is analgesia only given after the EEG implantation? Shouldn't be ethically appropriate to give the analgesia after FPI?
We have actually adjusted our protocol on this matter. Opiates are rarely used, and only with extreme caution, in humans that have suffered from traumatic brain injuries. The nature of this model is to induce traumatic brain injury, which is associated with increased intracranial pressure. All opiates can potentially complicate head injuries because they may actually cause an increase in intracranial pressure and increase the severity of the injury. A documented side effect of the TBI model is transient pulmonary edema and apnea. Respiratory depression from these two conditions causes an increase in P_aCO_2 leading to cerebral vasodilation. Buprenorphine and other opiates also increase respiratory depression compounding the vasodilation and thereby increasing intracranial pressure and injury severity. We do however administer topical analgesics and provide close monitoring and supportive care post operatively.

It may be useful to provide the details (vendor) of the telemetry system. What is the maximum sampling rate of this device? Also, the recording parameters and filters used in the EEG should be described here. For how long the does the EEG cap can stay attached to the rats? Does the system need a ground electrode?

1) The vendor of the telemetry system is emka Technologies. This information is provided in the materials list.
2) The maximum sampling rate of the device is 1000 Hz and the power range is +/- 10mV.
3) As we are monitoring between 0.5-30 Hz we set our transmitters to 250 Hz. The signal amplitude is typically less than 750 µV. Therefore, we have set our transmitters to a range of +/- 2 mV.
4) We have added a note describing information about the filtering and recording parameters.
5) We have only monitored rats up to 6 weeks. We anticipate that the head gear can remain attached longer but have no data to inform us on this question.
6) As this is a wireless telemetry system the rat serves as the ground. Therefore, we do not need to add an additional ground screw.

The details of the filtering in the EEG figures should be described in the legend,
We have added this information into the legends.

At the start of manuscript and the abstract the authors mentioned video-EEG (vEEG)

However, I cannot see the details of the video monitoring done on this animals in the methodology part of the manuscript.

The cameras are mounted to above and to the side of each rat box. Each camera has infrared lighting to allow recording at night during the dark cycle. The video cameras each feed into a video NAS. Each camera is assigned to a specific station and is synched with the respective EEG recordings collected for the rat in a specific bow. The respective time synched video can be recalled and displayed with the respective EEG recordings during analysis. We have added information describing the video monitoring system to the protocol.