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RE: Revision of manuscript # JoVE60360

Dear Drs. Berard and DSouza,

Enclosed, please find a copy with the requested editorial changes made to the manuscript JoVE60360 entitled "**Inducing post-traumatic epilepsy in a mouse model of repetitive diffuse traumatic brain injury**" authored by Oleksii Shandra and Stefanie Robel.

We'd like to sincerely thank you and the reviewers for your positive feedback on the manuscript and have made the requested changes to include all comments regarding the text structure, language, additional technical details and clarification of protocol steps.

We are confident that you will now find the manuscript suitable for publication in The JoVE and are looking forward to your final decision.

With our best regards,

Oleksii Shandra and Stefanie Robel

Point by point response to the comments by the reviewers and editor

Changes in the text are indicated using red font.

First we would like to address changes made with regards to Editorial Comments:

1. Please reduce the summary to 50 words.

We reduced the length of the summary to 49 words.

2. Please rewrite lines 246-264, 277-281, using original text.

We understand that the phrasing used in these steps are identical to phrases in the methods of our previous publications and made an attempt to paraphrase. However, these are basic technical steps, which can only be described in so many different ways. If the editors have suggestions of how to further change the wording, we will be happy to include those.

3. Please ensure that ALL text in the protocol section is written as steps in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

The text was revised and now is in imperative voice/tense in protocol steps. Text that either addresses a specific recommendation or is describing our personal experience is marked as a 'NOTE' or was moved to the discussion section.

4. Section 7 needs to be rewritten in its entirety.

We rewrote this section and it now includes only specific steps and settings as requested.

5. Protocol details:

1) 3.1, 4.1, 4.5: Mention oxygen flow rate.

This information was added.

2) 5.3.1, 5.5.1: Mention drill bit size and drill speed.

This information was added.

6. Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary

and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

The numbering in each section was fixed as per author instructions and editorial comments.

7. Protocol highlight and length

The overall length of the protocol was revised and reduced to fit the 10-page limit. Sections highlighted for filming were written to form a cohesive story and include all necessary steps crucial for reproducibility of the approach. Notes were excluded from the highlighted narrative.

8. Discussion

We revised the structure of this section to address 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. The numbered listing style was removed and transformed into organized paragraphs. Given the complexity of the crucial background information for the first-time users of the TBI, EEG surgery or video-EEG acquisition and description of different surgical approaches and acquisition system configuration in this protocol, it is challenging to limit the text to 6 paragraphs without losing context. Hence, we re-organized it in a way to give a reader sufficient information about advantages, limitations and troubleshooting of each of the technical sections.

9. References. Please spell out journal names.

We used the Endnote style for JoVE with journal names spelled out.

10. Avoid using commercial language

All commercial names for systems, hardware, components and reagents used in this protocol were replaced with either generic or neutral term and the reader is referred to see the table of materials for clarification.

11. Table of materials

All listed materials and reagents were sorted alphabetically; names and information on company and catalog number were included.

To address the comments, suggestions and concerns from the reviewers we revised the manuscript as follows:

Reviewer 1

Minor Concerns:

1. Inflammation plays a major role in the development of post-traumatic epilepsy. It is understandable to prevent pain and distress. However, the inclusion of carprophen (an NSAID) would potentially prevent the development of epilepsy in some animals, which is what the model is designed to study.

We agree that the inflammation plays crucial role in seizures and epilepsy. However, inflammation can be a cause and a consequence of seizures. We administer carprofen acutely on the day of TBI and EEG surgery in a single injection to prevent surgery-related complications in accordance with our institutional IACUC protocol.

A study by Thau-Zuchman et al. 2012 reported potential neuroprotective effect of carprofen after TBI when administered repeatedly for 7 days. Epilepsy in our TBI animals occurs 3-4 weeks after TBI in the absence of pronounced astroglial or microglial activation (see Shandra et al., J Neurosci 2019). While we cannot exclude that the single dose of carprofen as a variable, it does not appear to prevent epileptogenesis given that one third of the animals present with recurrent seizures.

2. Administration of buprenorphine prior to injury would significantly alter the righting reflex of rats and would render this measurement completely untrustworthy. If righting reflex is to be used, it would be more appropriate to administer buprenorphine after the righting response is observed.

In accordance with our institutional IACUC guidelines we are required to provide analgesia to animals undergoing a procedure which may inflict pain such as weight drop traumatic brain injury. Hence it is not acceptable to administer buprenorphine after all impacts are delivered. Animals undergo three impacts (with righting times taken after each) separated by 45 minute intervals. If buprenorphine was administered after the last righting time animals would be in pain for 3 hours before administration of the analgesic.

According to the reference by Gades et al. 2000 (also included in section 3, the first peak analgesic effect of buprenorphine is reached 10 minutes after a subcutaneous injection. The first impact occurs seconds after buprenorphine is administered suggesting that the first measurement of the righting time is unlikely to be affected.

To address the reviewer's concern we added a statement in lines 712-715 making the reader aware that buprenorphine might affect righting times.

3. Spraying surgical gloves with 70% ethanol is not an acceptable means of disinfecting or maintaining sterility.

We agree that 70% ethanol is not creating sterility, which is why we used the term "to disinfect". Areas for rodent surgeries are typically not sterile. For example, the stereotactic apparatus is not sterile. Manipulations of the apparatus will render sterile surgical gloves non-sterile. We use 70% ethanol to disinfect gloves after manipulating non-sterile equipment, which is appropriate to reduce the risk of contamination of the close-by sterile field. In contrast, the incision site is not touched with gloves or any instruments or equipment that were not autoclaved or sterilized using a bead sterilizer.

4. The procedure indicates to weigh the animal then remove it from the stereotaxic frame (sec. 5.3 steps 10 & 11; Sec 5.4 steps 12 & 13; sec 5.5 steps 14 & 15). this order should probably be reversed.

Sections 5.3, 5.4, 5.5 were modified as suggested by the reviewer.

5. The discussion identifies CCI and FPI as a models of penetrating injury. I would disagree that FPI represents a penetrating injury. FPI produces diffuse injury as well as focal injury at a site distant from the pressure pulse.

This is a very valid point and we revised the sentence about the CCI and FPI as follows: "Until recently, only two animal models of post-traumatic epilepsy existed: controlled cortical impact (CCI, focal) or fluid percussion injury (FPI, focal and diffuse. Both induce large focal lesions alongside tissue loss, hemorrhage and gliosis in rodents".

6. There has some confusion in the literature regarding the use of craniotomy and craniotomy. The word craniotomy is used in the discussion referring to CCI and FPI models. The bone flap is typically not replaced in these models. Therefore, the correct term is craniotomy.

We now refer to technique used for CCI as 'craniectomy' since the bone flap is not replaced in experimental animals.

Reviewer 2

Major Concerns:

1. In the Discussion section, the authors emphasize the necessity of video/EEG monitoring to ensure that the electrographic events detected in the EEG are actually

seizures. Since video monitoring is such an important component of the data collection, this should be fully described in the Methods section and not just mentioned in the Discussion.

Section 8 was added addressing specific video acquisition parameters for both EEG systems described in the protocol. We also commented on the significance and critical steps for video data acquisition in the discussion section.

2. The authors should comment further on the clinical relevance of this model to TBI in humans, given that the model involves repeated injuries within a fairly short time frame. According to Section 3, animals are subjected to three weight-drops within a 90 min period, which would correspond to multiple TBIs within the acute (if not hyper-acute) injury/post-injury period. In what types of injury scenarios would this occur in human TBI patients?

We understand the reviewers concern, however it is beyond the scope of this protocol paper to discuss the challenges of aligning timelines of pathology in humans and rodents or the complexity of inter-injury interval modeling. The timeline in humans is certainly different but so is the lifespan, which appears to affect timelines of pathology. Our goal for this manuscript was to provide a systematic protocol for the procedures used in Shandra et al., J Neurosci 2019, which demonstrates that diffuse closed-head TBI without focal injury is sufficient to induce post-traumatic epilepsy.

3. In Figure 5 (panel B), the authors should highlight the fact (i.e., in the figure labels) that the EEG of the convulsive seizure was taken from a non-weight drop experiment. The recording in 5B seems to be saturated, and the duration of ictal/seizure events recorded from weight-drop TBI animals (Fig. 5A) appear to be significantly shorter than the spontaneous convulsive seizure in Fig. 5B. It is somewhat misleading to include the Fig. 5B convulsive seizure traces in this manuscript, since these did not occur in an animal from the repeated weight-drop model. The figure legend already specifies this word to word.

The example in Fig. 5B is indeed recorded in a different TBI model, which is mentioned in the figure legend. The point of this figure is to compare different EEG configurations

and to show a typical convulsive seizure using multi-channel recordings. While we recorded weight drop TBI animals using the Pinnacle Technology system, it so happened that we did not catch a convulsive seizure in the 3-EEG channel configuration in this model.

Minor Concerns:

1. In the Introduction (line 87), there is a typographic error: "ASD" should be "AED."

The typo was corrected.

2. Section 3.1 (Weight Drop Procedure, line 191): What method is used to verify that mice have reached a sufficient plane of anesthesia with "5 minutes" of isoflurane exposure, in order to ensure that they are unresponsive to pain (e.g., toe-pinch withdrawal)? This information should be included.

In this section step 2 was corrected as follows: "Remove the mouse from an induction chamber and place it on the foam pad. Test for the absence of a response to toe or tail pinch."

3. Section 4.7 (Surgical Field Preparation, lines 263 - 269): What are the routes of administration for Buprenorphine and NSAIDS? These should be indicated.

This sentence was corrected as follows: "Administer a mixture of analgesics (0.1 mg/kg buprenorphine) and the non-steroidal anti-inflammatory (5 mg/kg carprofen) in a single injection subcutaneously unless the TBI was performed earlier during the day, in which case the animal already received analgesics and anti-inflammatories."

4. Section 5.3 (Electrode Placement, lines 312, 318): (a) How are the burr holes created?; (b) What is the drill bit size that is used?; (c) Please define Vin+ and Vin-.

a-b) This information was added: "Create 6 burr holes (3 for stability screws and 3 for electrodes) with a steel drill bit (0.5 mm, round, ¼) using the following stereotactic coordinates".

c) This information was added: “NOTE: Vin+ is an active electrode and Vin- is its reference electrode”.

5. Section 5.3.8 (Single EEG, line 356): This section states to "repeat the injection every 2 hours..." What is the agent or solution that is injected? This should be indicated.

This comment was addressed as follows: “If the animal is under anesthesia for longer than 2 hours after the previous injection of sodium lactate solution (given during the TBI induction), administer 3µl per gram of body weight subcutaneously. To maintain proper hydration of the animal, repeat the injection every 2 hours that the animal spends under anesthesia. After the surgery, give a final injection 2 hours from the previous injection. If surgery is less than 2 hours long, administer the final “recovery” dose of sodium lactate solution 2 hours from the first injection”.

6. Figure 3: (a) Legend refers to A, B and C. These letters are not labeled on figure. (b) Images of Biopac pedestal are not visible. These should be taken against a black background. (c) Images of screws and head mounts are too small to see.

Figure was corrected as suggested.

7. What is the average duration of seizures in this model, and are they mostly non-convulsive? This information should be included.

The majority of seizures in our model are convulsive and occasionally non-convulsive seizures were observed as presented in the example. The average seizure duration is 12-15 seconds but is occasionally 30 seconds or longer. The average duration information was added as requested. More detailed data on the seizure phenotype is presented in Shandra et al., J Neurosci. 2019.

Reviewer 3

Two suggestions:

1. Repeated weight drop was used and is mentioned, but what this entailed is a bit hidden (Line 227-228). When mention repeated weight drop was used, why not

indicate it was done three times in other portions of the paper such as the Discussion section?

The information was added throughout the text where it is referred as "repeated diffuse" injury. It now appears as "repeated (3x) diffuse injury".

2. For Figure 4, I found myself having to search which figure was being described. While not difficult to do, please include mention in the legend that the figures on the upper right are similar to those on the left; just with a different time scale. When the power spectrum data are discussed, mention the location of the figure (left, middle), etc.

Figure was revised as suggested.

Minor Concerns:

3. The "body condition score" (Line 724) was not clear. Are the three items of point values of one each; i.e., "mouse under-conditioned, segmentation...."? This should be explicit. Should the mentioned humane endpoint then mean 2 or more and not 2 or less (Line 724)?

This section was revised as follows: "Use a five stage body condition score (BCS) for animal monitoring after experimental procedures: Stage 1 - mouse is emaciated (skeletal structures extremely prominent, vertebrae extremely segmented); Stage (2) - mouse is underconditioned (segmentation of vertebral column is evident, dorsal pelvic bones are readily palpable); (3) - mouse is well-conditioned (vertebrae and dorsal pelvis not prominent palpable with slight pressure); (4) - mouse is over-conditioned (spine is a continuous column, vertebrae palpable only with firm pressure); (5) - mouse is obese (mouse is smooth and bulky, bone structure disappears under flesh and subcutaneous fat). The humane endpoint is reached when the following is observed: BCS 1-2, 20% or more weight loss in an adult mouse compared to its pre-TBI weight, symptoms of pain or distress are not alleviated by analgesics, signs of self-mutilation, symptoms of dehydration, hypothermia, presence of neurologic deficits (abnormal gait or motor paresis)".

4. Line 776: What does passive behavior mean? How is this evaluated?

This and similar sentences were corrected as follows: "Signs of pain or distress include weight loss, poor grooming, dehydration, increased anxiety, low or absent exploratory activity (hydrogel/recovery, chow and/or nestlet remain untouched)".

5. Line 832-833: Please give a bit more description for how the videos are used. Are they referring to using the videos for the Racine scoring mentioned in Lines 910-918?

A modified Racine scale is used to characterize the behavioral seizures. The scoring details and the reference were added.