Video Article

# A Repetitive Concussive Head Injury Model in Mice

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## Abstract

Despite the concussion/ mild traumatic brain injury (mTBI) being the most frequent occurrence of traumatic brain injury, there is still a lack of knowledge on the injury and its effects. To develop a better understanding of concussions, animals are often used because they provide a controlled, rigorous, and efficient model. Studies have adapted traditional animal models to perform mTBI to stimulate mild injury severity by changing the injury parameters. These models have been used because they can produce morphologically similar brain injuries to the clinical condition and provide a spectrum of injury severities. However, they are limited in their ability to present the identical features of injuries in patients. Using a traditional impact system, a repetitive concussive injury (rCHI) model can induce mild to moderate human-like concussion. The injury degree can be determined by measuring the period of loss of consciousness (LOC) with a sign of a transient termination of breathing. The rCHI model is beneficial to use for its accuracy and simplicity in determining mTBI effects and potential treatments.

## Video Link

The video component of this article can be found at http://www.jove.com/video/54530/

## Introduction

Concussion, also called mild traumatic brain injury (mTBI), is the most frequent occurrence of traumatic brain injury (TBI) and affects millions of people in United States. Concussions can be tricky to diagnose and there is no specific cure for concussion. There is a growing recognition and some evidence that mild mechanical trauma resulting from sports injuries, military combat, and other physically engaging pursuits may have cumulative and chronic neurological consequences[1,2]. However, there is still a lack of knowledge regarding concussions and their effects. Current methodology restricts the studies of pathology and treatment in humans since only neurologic assessment and imaging evaluation are available for clinical diagnosis. Animal models provide a means to study concussions in an efficient, rigorous, and controlled manner with the hope of further diagnosis and treatment of mTBI.

Studies have adapted traditional TBI models such as controlled cortical impact (CCI), fluid-percussion impact (FPI), weight drop injury, and blast injury to perform mTBI and stimulate low injury severities by changing the injury parameters. These models are beneficial to use due to their ability to replicate brain trauma morphologically similar to the clinical condition; however, they also have their own limitations. The severity of injury induced by an acceleration injury (weight drop) is often highly variable. The two results of the mild CCI — subarachnoid hemorrhage and focal contusion — are not comparable with typical human concussions. CCI and FPI require a craniotomy, which is not clinically relevant, while blast injury is a more controversial model in regards to the different exposure position and peak pressure measurements as well as variable secondary injury during the exposure[3-6]. An updated concussive animal model that can translate pre-clinical research into the clinical setting is necessary in research.

The key issue in modeling mild TBI is to define the experimental injury severity, which most closely replicates the injury in a clinical setting. Recently, different research groups developed the closed head injury or concussive head injury (CHI) model[7-10]. CHI is a modification of CCI without a craniotomy, but it still uses a traditional electronic magnetic impact system to generate a head impact. A CHI can induce a concussion ranging from mild to moderate by adjusting the impact parameters. Loss of consciousness (LOC) can be observed immediately after an impact by detecting a decrease in the breathing rate or the transient termination of breathing. The period of LOC is used to determine the severity of injury. This paper includes a slightly improved and updated version of a repetitive CHI (rCHI) model in mice, along with a detailed step-by-step protocol and representative results. The rCHI model research strategies are beneficial in determining mTBI effects and potential treatments, especially since there is no individual animal model capable of imitating all of the concussion-induced pathological changes.

## Protocol

All procedures were performed under protocols #201207692 approved by the Institutional Animal Care and Use Committee of University of Florida and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## 1. Animal Care

1. Use 3–4-month-old male C57BL/6J mice. Provide bedding, nesting material, food, and water *ad libitum*. Keep the mice in ambient temperatures controlled at 20 - 22 °C with constant 12-hr light/12-hr dark cycles.

## 2. Pre-impaction Preparation

1. Attach a custom-made silicone rubber-coated metal tip to an electromagnetic stereotaxic impact device. Make sure the flat bottom of the tip is parallel to the surface of the probe tip (**Figure 1A**).
2. Anesthetize the mouse with 4% isoflurane followed by maintenance anesthesia of 2.5% isoflurane. Check the anesthesia via the flow meter. Monitor the anesthesia level until the animal reaches a surgical level of anesthesia by showing loss of pedal withdrawal reflex.
3. Put the mouse in a prone position on a heating pad. Use a funnel-shaped nose cone to keep the mouse under anesthesia. Completely shave the head using a trimmer. Use petrolatum ophthalmic ointment on the mouse's eyes to prevent dryness while under anesthesia.

## 3. Impact Parameters Setting

NOTE: The impact system includes a control box to set impact parameters, an actuator to perform the impaction, and a digital stereotactic frame with 3-movement axes.

1. Pre-set the velocity of the impact device to 4 m/sec and dwell time to 240 msec on the control box.

## 4. Positioning the Impact Center

1. Put a soft heating pad under the animal's body to keep the body temperature near 39 °C. Mount the mouse in a stereotactic frame in a prone position with the blunt-end ear bars.
2. Lower the impact tip close to the mouse's head by moving the Z-driver. Adjust the flat impact tip (9 mm diameter) by moving the X- and Y-drivers midway to the target coordinates above the sagittal suture.
3. Make sure one edge of the impact tip is vertically parallel to an imaginary horizontal line drawn between the two ears (**Figure 1C**). The center of impact corresponds to the central sagittal suture midway between interfrontal and lambdoid sutures (interaural 9 mm to interaural 0 mm, lateral 4.5 mm).

## 5. Impact Depth Setting

1. To correctly set the impact depth, use additional probe tip to replace the insulated silicone rubber-coated impact tip.
2. To make sure there is no shift of the impact center after switching tips, set the X and Y channel on the digital stereotaxic control panel to zero before switching the tips.
3. Move the probe tip to the center of the impact area by manually moving the X-and Y-drives.
4. Clip contact sensor to the mouse's tail.
5. Move the impactor (Z drive) down until the probe tip touches the surface of the impact site.
6. Set the Z channel on the stereotaxic control panel to zero.
7. Move the impact tip back to the impact area by manually adjusting X- and Y-drivers (NOT the zero buttons on the digital stereotaxic control panel) until X- and Y-drivers are zero (where the impact tip was previously positioned).
8. Retract the actuator by moving the retract switch on the control box. Manually move the impactor down (Z driver) by 4 mm.

## 6. Impact

1. Trigger the impact by clicking the impact switch on the control box and achieve a deformation depth of 4 mm.

## 7. Post-impaction

1. Measure the time from the impact until the mouse's first breath using a timer.
2. Remove the animal from the instrument and place them on a warm pad to maintain their body temperature. Do not leave the animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.
3. Allow for recovery before returning the animal back into a clean cage. Do not return an animal to the company of other animals until fully recovered.
4. Observe and weigh the mice daily. If the mice show signs of pain, intraperitoneally inject them with Meloxicam at 1 - 2 mg/kg every 12 - 24 hr.

## 8. Repetitive Impaction

1. Give the mice additional injuries on days 4, 7, and 10 after the initial injury (72 hr interval between impacts).

## 9. Immunohistochemistry (IHC)

1. **Transcardial perfusion**
    1. Anesthetize the mice via intraperitoneal injection with 200 m/kg pentobarbital.
    2. Assess and assure surgical-plane anesthesia by a toe pinch. Secure the mouse in the supine position by gently taping the forepaws and hind paws to a Styrofoam work surface inside a chemical fume hood.
    3. Make an incision through the skin along the thoracic midline from just beneath the xiphoid process to the clavicle. Make two additional skin incisions at the xiphoid process and proceed along the base of the ventral ribcage laterally.
    4. Open the thoracic cavity and expose the heart by cutting through the thoracic musculature and ribcage.
    5. Secure the beating heart with blunt forceps and make a 1 - 2 mm incision in the left ventricle.
    6. Immediately insert a butterfly needle into the right atrium. Begin the infusion of 20ml saline by pushing the syringe slowly.
    7. Switch from saline to 4% paraformaldehyde. Continue perfusion with 20ml of paraformaldehyde.
    8. Decapitate the mouse and remove the skin with scissors. Isolate the brain from the skull using a bone cutter.

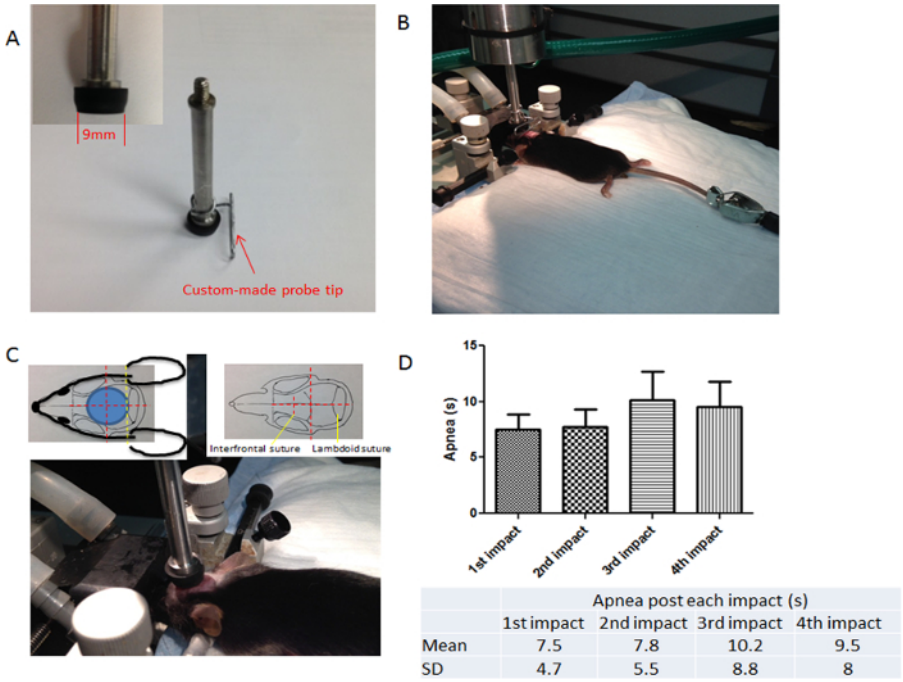
2. **Cryostat sectioning**
    1. Embed brain tissues in optimum cutting temperature (O.C.T.) formulation and freeze at -80 °C. Place the brain in the cryostat in a sagittal orientation. Cut brain sections 5 µm thick.

3. **Staining**
    1. Dry the frozen sections at room temperature for 1 hr.
    2. Incubate the slides with 100 µl of 2% goat serum and 0.1% Triton X-100 in phosphate buffered saline (PBS) for 1 hr at RT.
    3. Wash the slides 3 times with 300 µl of PBS. Then incubate the slides with anti-GFAP (1:200) or anti-ferritin antibody (1:200) separately over night at 4 °C.
    4. Wash the slides 3 times with 300 µl of PBS. Then incubate the slides for 2 hr at room temperature with biotin-conjugated secondary antibody.
    5. Wash the slides 3 times with 300 µl of PBS. Then incubate the slides with avidin-biotin complex (ABC) solution (1:50) at room temperature for 30 min.
    6. Wash the slides 3 times with 300 µl of PBS. Then incubate in 3,3′-diaminobenzidine (DAB) substrate solution (50 ml PBS, 10 µl $H_2O_2$, 10 mg DAB pill, filter before using) for 5 - 8 min. Observe the slides under the microscope until the positive cells appear.
    7. Rinse the slides in slow running tap water for 5 min. Clean slides with a lab-wipe. Then mount the sections with mounting medium and coverslip.

## Representative Results

In this model (**Figure 1 A-C**), there were brief periods of gasping and shallow respirations. A loss of consciousness (unconscious) is defined as a decrease in the breathing rate or transient termination of breathing before resuming a normal respiration. An impact on the center of the head caused short-term unconsciousness (7.5 ± 4.7, 7.8 ± 5.5, 10.2 ± 8.8, 9.5 ± 8.0 sec at each impact separately, **Figure 1D**). Mouse brains showed normal morphology by H&E histological staining, which indicated no obvious structural lesions or tissue damage resulting from the impact (**Figure 2A**). In response to TBI, astrocytes are known to undergo certain changes including activation, proliferation, or reactive gliosis[11,12]. Increased glial fibrillary acidic protein (GFAP) positive cells with large cell bodies and thick synapses are the activated astrocytes. The corpus callosum from rCHI mouse brains showed obvious signs of astrocytes activation at 7 days after the last impact (**Figure 2B**).

Microbleeds in the tissue are common in mTBI and may lead to the release of iron from hemoglobin[13]. Iron overload in the serum can be detected by ferritin tests in clinical settings[13]. The ferritin immunopositive cells in the mouse cortex were found one day after the last impact and lasted at least seven days, suggesting that multiple impactions may result in cortical microbleeds (**Figure 2C**).




| | Apnea post each impact (s) | | | |
|---|---|---|---|---|
| | 1st impact | 2nd impact | 3rd impact | 4th impact |
| Mean | 7.5 | 7.8 | 10.2 | 9.5 |
| SD | 4.7 | 5.5 | 8.8 | 8 |

**Figure 1. A Mouse Model of Repetitive Concussive Head Injury. (A)** Custom-made 1 mm thick silicone rubber-coated tip measuring 9 mm in diameter with a probe tip. **(B)** A mouse is mounted in a stereotactic frame in a prone position with a soft heating pad under the body. **(C)** The impact center positioning. The edge of the impact tip is vertically parallel to an imaginary horizontal line drawn between the two ears. The impact center corresponds to midway between interfrontal and lambdoid sutures (interaural 9 mm to interaural 0 mm, lateral 4.5 mm). **(D)** Apnea is defined as brief periods of transient termination of breathing. Mean and SD are shown in the lower panel. Please click here to view a larger version of this figure.

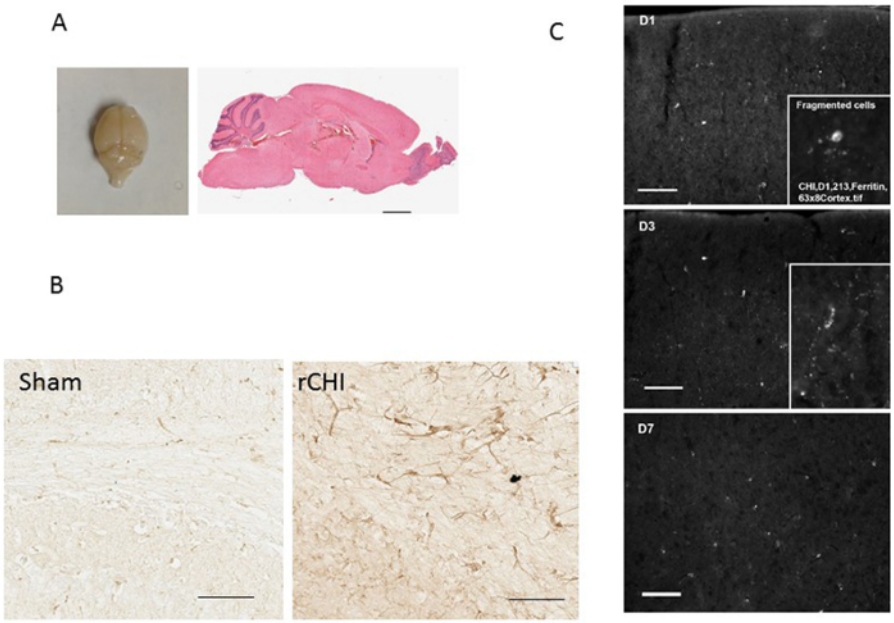



**Figure 2. Histology for Repetitive Concussive Head Injury. (A, left)** A mouse brain was removed after perfusion with 4% paraformaldehyde. No tissue damage was found. **(A, right)** H&E staining was performed on a mouse one day after the last injury. Scale bar = 1.5 mm. **(B)** Increase of the biochemical marker for gliosis (GFAP) in the corpus callosum 7 days after the last injury. Scale bar = 200 µm. **(C)** By immunohistochemistry, the ferritin-H-chain was found to be expressed in brain cortex after injury. The insert pictures represent magnified positive cells. Scale bar = 200 µm. Please click here to view a larger version of this figure.

## Discussion

To mimic brain injuries morphologically similar to the clinical condition, post-concussion symptoms are expected. Post-concussion symptoms generally include headaches, dizziness, vertigo, fatigue, memory and sleeping problems, trouble concentrating as well as anxiety, and depressed mood. Since somatic symptoms may not yet be measurable in animal models, the changes of motor and cognitive function and emotional

behavior are used as criteria for rationally evaluating concussion in animal models. In a previous reported study, it was shown that the rCHI mouse model induces deficits in spatial learning, memory, and anxiety[8]. More importantly, the rCHI model used in this protocol represents the clinical setting without invasive brain injury or brain structure fracture, both of which may result in bleeding, hemorrhage, edema, or acute cell death/tissue loss.

The following are key tips for successfully modeling consistent concussion/mTBI using an electronic magnet impact system:

Avoid a second brain injury directly following the first brain injury that can be caused by movement during the impact. The mouse head may move slightly down during the impact. To avoid a brain contusion caused by a quick movement against hard ground or head stretching, a soft heating pad must be put under the mouse body. The head and body must also be kept horizontal. In addition, use blunt-end ear bars to fix the mouse head in the stereotactic frame, and do not insert them inside the ear canal. This protects the mouse from injury caused by the sharp ends during the movement.

Correctly position the impact center and establish the zero. Unlike open head injury, the impact tip positioning is relatively difficult. The size of the impact tip and the impact center affect the injury severity and lesions. Based on mouse brain anatomy, the impact center is designed to correspond to midway between interfrontal and lambdoid sutures (interaural 9 mm to interaural 0 mm, lateral 4.5 mm). Thus, an optimized 9 mm tip is required. The impact tip must be adjusted to the target coordinates above the sagittal suture midway, and one edge of the impact tip must be vertically parallel to an imaginary horizontal line drawn between the two ears (**Figure 1C**). The insulated impact tip with a silicone rubber coating blocks the contact sensor and prevents the impact depth setting. A probe tip is needed and should be parallel to the surface of the button on the impact tip. The center of the impact is adjusted to the probe tip touching site by operating the stereotaxic instrument. Scrubbing the head with saline increases the electro-sensitivity. In addition, the probe is removable or designed not to hurt the brain during the impact. An alternative way is to build two tips with the same length; one tip coated with silicone rubber and the other tip would be metal, which will be used as a probe tip. The two tips should be switched between positioning and impacting.

Monitor the mouse's brief unconscious symptoms immediately after an impact. As discussed above, most post-concussion symptoms are difficult to observe immediately in a laboratory mouse animal model. mTBI patients may experience a brief loss of consciousness after the injury. To establish the visible injury parameters, a brief loss of consciousness was a symptom used to evaluate the validity of this concussive TBI model. Loss of consciousness (LOC) is normally used as criteria to classify the injury severity in TBI patients. In most sports-related concussions, the duration of LOC is less than one minute[14]. By optimizing the experimental conditions, such as impact speed and dwell time, the LOC is less than 10 sec after an impact. The optimum impact condition is a 4 mm impact depth, 240 msec dwell time, and 4 m/s impact speed. Increased impact speed and dwell time may cause acute increased intracranial pressure over a large amount of time, which may result in severe brain injury or death immediately from respiratory depression. Mice will lose body weight after each impact, but will regain weight after 72 hr of recovery. 72 hr repetitive intervals are chosen to mimic a recovery period for injured athletes before returning to their sport.

Beside loss of consciousness and respiratory issues, the clinic symptoms of a concussion can include convulsions, headache, dizziness, nausea and vomiting. In model, brain pain may be the majority uncomfortable symptom to the animals. Body condition score and pain category description should be used as humane endpoints. In addition, other specific neurological endpoints such as uncontrolled seizure, spontaneous circling behavior, loss of balance and unable to walk or stand should be considered as rCHI-specific humane endpoints. Since this is a mild injury model, normally no significant signs of pain are observed post each impact. Analgesics are typically unnecessary at this level of brain injury. This protocol provides detailed key steps for modeling a repetitive concussive mild TBI. The velocity and depth of each impact may be adjusted depending on the desired severity of injury. This model uses an electronic magnet impact system to deliver impacts. It is stable with a precisely controlled velocity, dwell time, and deformation depth. However, because it is a closed head injury without a craniotomy, it is impossible to precisely position the mouse brain impact using stereotaxic coordinates. Also switching the impact/probe tips may result in an impact site shift, which is the major cause of inconsistent injuries. Considering the diffuse injury and concussion turned out as expected, this model remains precise and easy to control.

This model is beneficial to use for its accuracy and simplicity in determining the effects of impact-related mild brain injury, especially sports-related concussion. It serves as a platform for preclinical studies such as exploring diagnostic and prognostic biomarkers as well as testing medical devices, drugs and gene therapy solution. This model can also be used for the studies of chronic traumatic encephalopathy (CTE), which currently is only diagnosable through the post-mortem neuropathological exam.

## Disclosures

The authors have no financial interest to disclose.

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