Journal of Visualized Experiments
Detecting Behavioral Deficits in Rats after Traumatic Brain Injury
--Manuscript Draft--

Manuscript Number:	JoVE56044R1
Full Title:	Detecting Behavioral Deficits in Rats after Traumatic Brain Injury
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	behavior; Neurological score; Beam-balance; Beam-walk; Working memory; brain injury; Fluid-percussion injury; Morris water maze; rat
Manuscript Classifications:	3.10.597: Neurologic Manifestations; 3.10.597.606: Neurobehavioral Manifestations; 3.10.900: Trauma, Nervous System; 3.23.888.592.604: Neurobehavioral Manifestations; 3.26.915: Trauma, Nervous System; 6.1.145.113: Behavior, Animal; 6.1.145.126: Behavioral Symptoms; 6.1.700: Neurobehavioral Manifestations; 6.4.96.144: Behavioral Research; 95.53.4: behavioral sciences
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Abstract:	With the increasing incidence of traumatic brain injury (TBI) in both civilian and military populations, TBI is now considered a chronic disease; however, few studies have investigated the long-term effects of injury in rodent models of TBI. Shown here are behavioral measures that are well-established in TBI research for times early after injury, such as two weeks, until two months. Some of these methods have previously been used at later times after injury, up to one year, but by very few laboratories. The methods demonstrated here are a short neurological assessment to test reflexes, a Beam-Balance to test balance, a Beam-Walk to test balance and motor coordination, and a working memory version of the Morris water maze that can be sensitive to deficits in reference memory. Male rats were handled and pre-trained to neurological, balance, and motor coordination tests prior to receiving parasagittal fluid-percussion-injury (FPI) or sham-injury. Rats can be tested on the short neurological assessment (Neuroscore), the Beam-Balance, and the Beam-Walk multiple times, while testing on the water maze can only be done once. This difference is because rats can remember the task, thus confounding the results if repeated testing is attempted in the same animal. When testing from one to three days after injury, significant differences are detected in all three non-cognitive tasks. However, differences in the Beam-Walk task were not detectable at later time points (after three months). Deficits were detected at three months in the Beam-Balance and at six months in the Neuroscore. Deficits in

	working memory were detected out to 12 months after injury, and a deficit in a reference memory first appeared at 12 months. Thus, standard behavioral tests can be useful measures of persistent behavioral deficits after FPI.
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DEPARTMENT OF ANESTHESIOLOGY

Friday, May 26, 2017

From: Dr. Stacy L. Sell
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To: Dr. Mala Mani
Review Editor, JoVE

Subject: Manuscript Re-Submission JoVE 56044

Body: Dear Dr. Mani

Thank you for the opportunity to revise our manuscript entitled, "Detecting Behavioral Deficits in Rats after Traumatic Brain Injury" to the *Journal of Visualized Experiments*. We have extensively revised the manuscript according to the reviewers'' suggestions. We appreciate the efforts they made on our behalf to improve the quality of our submission.

Also, we are grateful for your patience in extending our re-submission deadline.

Please let me know if there is anything else we can do to facilitate this process.

Sincerely,

Stacy L. Sell, Ph.D.
Assistant Professor

TITLE:

Detecting Behavioral Deficits in Rats after Traumatic Brain Injury

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SHORT ABSTRACT:

The goal of the behavioral tests presented here is to detect functional deficits in rats after traumatic brain injury. Four specific tests are presented that detect deficits in behaviors to reflect the damage to specific brain areas at times extending to one year after injury.

LONG ABSTRACT:

With the increasing incidence of traumatic brain injury (TBI) in both civilian and military populations, TBI is now considered a chronic disease; however, few studies have investigated the long-term effects of injury in rodent models of TBI. Shown here are behavioral measures that are well-established in TBI research for times early after injury, such as two weeks, until two months. Some of these methods have previously been used at later times after injury, up to one year, but by very few laboratories. The methods demonstrated here are a short neurological assessment to test reflexes, a Beam-Balance to test balance, a Beam-Walk to test balance and motor coordination, and a working memory version of the Morris water maze that can be sensitive to deficits in reference memory. Male rats were handled and pre-trained to neurological, balance, and motor coordination tests prior to receiving parasagittal fluid percussion injury (FPI) or sham injury. Rats can be tested on the short neurological assessment (neuroscore), the beam-balance, and the Beam-Walk multiple times, while testing on the water

maze can only be done once. This difference is because rats can remember the task, thus confounding the results if repeated testing is attempted in the same animal. When testing from one to three days after injury, significant differences are detected in all three non-cognitive tasks. However, differences in the Beam-Walk task were not detectable at later time points (after 3 months). Deficits were detected at 3 months in the Beam-Balance and at 6 months in the neuroscore. Deficits in working memory were detected out to 12 months after injury, and a deficit in a reference memory first appeared at 12 months. Thus, standard behavioral tests can be useful measures of persistent behavioral deficits after FPI.

INTRODUCTION:
The methods presented here are designed to detect functional deficits in specific brain areas induced by an experimental model of TBI in the rat. Four different behavior tests will be described. First, the short neurological assessment, referred to as the neuroscore, can be performed without requiring any specialized equipment but does require practice; this test detects deficits in reflexes. Second, the Beam-Balance test detects deficits in the ability to balance. This task requires the handler to score the rat based on an ordinal scale and requires some training of the handler. The Beam-Balance test requires a narrow beam and is sensitive to deficits in the vestibular system. The third test assesses vestibulomotor coordination. This test is known as the Beam-Walk task, and although some pre-training of the rat is required, this procedure is more objective than the previous two as the latency to traverse the beam is an objective measure not dependent on subjective scoring. This difference is because the time to traverse a narrow beam to reach a safe box is measured. The Beam-Walk test requires a longer beam than the Beam-Balance as well as an escape box. This test measures deficits in both motor coordination and balance and thus is sensitive to damage to the cerebellum and motor related brain areas. The working memory version of the Morris water maze (MWM-WM) primarily tests hippocampal function and integration with the prefrontal cortex or executive function. This version of the Morris water maze shown can also be used to detect deficits in reference memory[1].

These methods were chosen based on their well-established track record in the literature. Each one has been effective in many hands from different laboratories with multiple strains of rats over numerous years of research. However, in the past, post-injury measures up to two weeks after injury were considered "chronic" time points. Thus, to establish behavioral techniques for the study of chronic effects of TBI in rodents, these well-known methods needed to be evaluated for sensitivity to detect TBI induced deficits at longer time points after injury. While there are now several rodent models of TBI, the FPI model is one of the most widely used, and is applied in this study. This model was first published in the 1950's[2], and since then, more than 1,000 papers have employed FPI in rats[3]. The neuropathology of this type of injury has been well-described by us[4] and others[5-7]. Briefly, neuronal injury in the hippocampus has been shown to be dose-dependent using Fluoro-Jade staining at short times after injury, *i.e.*, 24–48 h (Hellmich *et al.*[4]); while gross atrophy and cavitation including thinning of the internal capsule and cortex has been reported at one year after injury[5,6].

The most meaningful representation of brain function is assessed by using behavioral outcome

measures after an experimental brain injury. However, the vast majority of FPI experiments that use behavioral outcomes make measures relatively early, typically from 1 to 14 days after injury. Using the methods demonstrated here, some behavioral deficits can be detected out to 12 months after injury[1]. Neurological function, gross vestibulomotor function, and fine motor coordination were assessed on post-injury days (PIDs) 1–3 and at 3, 6, and 12 months after surgery, using a short neurological assessment (neuroscore; modified from Schallert[8]), the Beam-Balance task, and the Beam-Walk task[9-11]. Reference and working memory were assessed using a working memory version of the Morris water maze[1,12,13].

PROTOCOL:

All animal experiments are first approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas as directed by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th Edition, National Research Council).

1.   **Surgical Procedures and Fluid Percussion TBI**

1.1.   Obtain adult male 300 g Sprague-Dawley rats from a vendor and house two per cage with food and water *ad libitum* in a vivarium with constant conditions: light cycle (600 h to 1,800 h), temperature (21 °C to 23 °C), and humidity (40–60%).

1.2.   Prior to surgery, handle the rats for three to five days, and then train the rats for the neuroscore, Beam-Balance, and Beam-Walk procedures from one to three days prior to baseline assessment. Conduct the baseline assessment either the day or morning prior to the surgery.

Note: Always prepare control rats, such as sham-operated or surgically naïve rats in the same manner as those that will receive an injury and, either randomly or in a balanced manner, group the rats into the treatment groups.

1.3.   Anesthetize rats using isoflurane at 4% for induction and 1.5–2% for maintenance. Intubate and mechanically ventilate the rats (using isoflurane in air:oxygen (70:30) and prepare for parasagittal fluid-percussion injury as previously described[3,10,11].

2.   **Neuroscore Training and Testing**

2.1.   **Neuroscore training**

2.1.1. For training on rats known to be experimentally naïve, run through the tests in the following order (steps 2.2.1–2.2.5) from start to finish, return to home cage for 1 min, then repeat until a score of zero is achieved.

2.1.2. Mark scores on the score sheet for a record of the training trials for each rat. After

training, perform one test session (see below) on the same or following day.

Note: If the test session does not produce a zero score for the baseline, training and testing may be repeated or the rat may be diverted to a non-behavior experiment.

2.2.   Neuroscore testing

Note: Run through the tests in the following order, return to home cage for 1 min, then repeat twice for a total of three times.

2.2.1.   Forelimb flexion test

2.2.1.1. Lift the rat by the tail and hold about 12 inches above the table surface.

2.2.1.2. Observe whether the rat extends or flexes forelimbs. Score the presence of flexion (1) or absence (0).

Note: Flexion is abnormal. Possible score of 1 x 3 = 3 (Total possible = 3).

2.2.2.   Hindlimb flexion test

2.2.2.1. Lift the rat by the tail and hold about 12 inches above the table surface.

2.2.2.2. Observe whether the rat extends or flexes hindlimbs. Score the presence of flexion (1) or absence (0).

Note: Flexion is abnormal. Possible score of 1 x 3 = 3 (Cumulative Total possible = 6).

2.2.3. Visually triggered placing test

2.2.3.1. Lift the rat by the tail.

2.2.3.2. Slowly lower the rat toward the edge of the table until the nose is about 10 cm from the edge.

2.2.3.3. Move the rat slowly toward the edge (do not allow the whiskers to touch the edge).

2.2.3.4. Observe whether the rat reaches and extends forepaws towards the table. Score the presence (0) or absence (1) of extending forepaws.

Note: Reaching for the table in response to visual cues is normal. Possible score of 1 x 3 = 3 (Cumulative Total possible = 9).

2.2.4.   Contact triggered placing test

2.2.4.1. Hold the rat, with body in hand, parallel to table edge and forelegs free.

2.2.4.2. Slowly lower the rat toward the edge of table until the whiskers on one side touch the edge of the table.

2.2.4.3. Observe whether the rat extends the forelimb on the same side as the whiskers that are touching the table toward the table edge as soon as the whiskers touch.

Note: Elicitation of this response takes considerable practice and researchers need to be well-trained to perform this test consistently.

2.2.4.4. Score the presence (0) or absence (1) of reaching toward the table.

Note: Reaching in response to tactile stimulation is normal. Possible score of 1 x 3 = 3 (Total possible = 12).

2.2.4.5. Repeat steps 2.2.4.1–2.2.4.4 for the opposite side. Possible score of 1 x 3 = 3 (Cumulative Total possible = 15).

2.2.5. Hindpaw grasping reflex test

2.2.5.1. Hold the rat in the left hand with thumb and index finger around the chest under the forelimbs.

2.2.5.2. Gently touch the palm of the each hindpaw with the right forefinger.

2.2.5.3. Observe whether the rat grasps the forefinger. Score the presence (0) or absence (1) of grasping.

Note: Grasping is normal. Possible score of 1 x 3 = 3 (Total possible = 18).

2.2.5.4. Repeat steps 2.2.5.1–2.2.5.4 for the opposite side. Possible score of 1 x 3 = 3 (Total possible = 21).

2.2.6. Scoring

2.2.6.1. Sum the scores for a possible cumulative total of 7 x 3 = 21. A score of zero is normal.

3.   Beam-Balance Training and Testing

3.1.   Equipment

3.1.1.   Use a beam 60 cm in length, 1.75 cm in width, 4.0 cm in height, set 90 cm off the floor,

with a barrier 30 cm in height, 30 cm in width. Secure the beam to a table with the barrier attached so that 50 cm of the beam protrudes from the barrier, away from the table.

3.1.2.   Place a cushioned safety box under the beam to soften the impact of rats that fall.

3.2.   Beam-Balance training

3.2.1.   At 24–48 h before surgery, place the rat on the beam for a 60 s trial.

3.2.2.   If the rat fails to balance on its own, allow the rat to fall into the safety box.

3.2.3.   Begin timing when the rat is securely positioned on the beam.

3.2.4.   Observe the rat for the 60 s period and rate its performance based on the following scale: **1** = Shows stable balance (grooms, walks, attempts to climb the barrier), **2** = Shows shaky balance (grasps sides of beam and/or has unsteady movements), **3** = Tries to balance but slips or spins on the beam, hangs on by hugging the beam, **4** = Tries to balance, but falls after 10 s, **5** = Hangs over or from the beam and falls off in under 10 s, **6** = Falls off, making no effort to balance or hang onto the beam.

3.2.5.   Record the score on the worksheet.

3.2.6.   Allow the rat to rest for 15 s in home cage, then repeat steps 3.2.1–3.2.5 until the rat achieves three scores of 1 or 2. The rat is then considered trained.

3.2.7.   Perform one pre-assessment at 24 h or on the day of surgery prior to surgery.

3.3.   Beam-Balance testing

3.3.1.   Starting 24 h after surgery and continuing for up to 4 days, test the rats daily.

3.3.1.1. Place the rat on the beam and start the timer. Observe the rat closely for 60 s. Record the score on the worksheet.

3.3.1.2 Return the rat to the home cage for a brief rest period (3–5 min).

3.3.1.3 Repeat steps 3.3.1.1–3.3.1.2 for a total of three tests.

4.   Beam-Walk Training and Testing

4.1.   Equipment

4.1.1.   Use a wooden beam 100 cm in length, 2.5 cm in width, and 4.0 cm in height.

4.1.2. Prepare an adjustable stand, an adjustable table, and four pegs, 2 cm in height, and a black goal box 28 cm in length, 18 cm in height, and 18 cm in width, with an opening on one

end large enough for the rat to pass through.

4.1.3.   Attach the target end of the beam to the open side of the goal box that is placed on the adjustable table. Place the bright light and white noise generator near the starting end of the beam. The starting end of the beam is fixed to the adjustable stand so the beam and box are at the same level, about 1 m above the floor.

4.2.   Beam-Walk training

4.2.1.   Start training 24–48 h before surgery.

4.2.2.   Place the rat in the goal box for 2 min. After 2 min, remove the rat and start the trial.

4.2.3.   To start the trial, turn on the light and white noise and place the rat on the beam at the location of the peg hole closest to the goal box and allow the rat to enter the goal box.

4.2.4.   When the rat's front feet cross the threshold of the goal box, immediately turn off the light and noise sources (this is the end of one trial).

4.2.5.   Allow the rat to rest in the goal box for 30 s between each trial.

4.2.6.   Repeat the procedure in step 4.2.3–4.2.5 twice at each peg location and from the starting position. Insert the pegs in the holes and run one complete Beam-Walk with the pegs in place.

4.2.7.   Run three timed Beam-Walk trials.

4.2.7.1. Remove the rat from the goal box. Turn on the light and white noise and start the stopwatch when placing the rat on the beam. Stop the stop watch immediately when the rat's front feet cross the threshold of the goal box, and then immediately turn off the light and noise.

4.2.7.2. Record the time on the worksheet.

4.2.7.3. Repeat steps 4.2.7.1–4.2.7.2 until the rat has achieved three times of 5 s or less. The rat is now considered trained.

4.3.   Beam-Walk baseline assessment

4.3.1.   On the day or morning prior to surgery, do three timed trials with pegs in place.

4.3.2.   Start by placing the rat in the goal box for 30 s. Remove the rat from the goal box and turn on the white noise and light. Place the rat on the start end of the beam and simultaneously start the stop watch. When the rat's front feet cross the threshold of the goal box, immediately turn off the light and noise sources and stop the timer.

4.3.3. Record the time on the worksheet. Allow the rat to remain in the goal box for 30 s.

4.3.4. Repeat steps 4.3.2–4.3.3 until three latencies are recorded on the worksheet. Return the rat to the home cage after three timed trials are completed.

4.4. Beam-Walk testing

4.4.1. Test the rats daily starting 24 h after surgery and continuing for up to 4 days. Perform three timed trials as in steps 4.3.

5. Working Memory Water Maze

5.1 Equipment

5.1.1. Use a tank filled with water to a height of 28 cm and maintained at 26 ± 1 °C.

5.1.2. Use a clear acrylic glass platform that is 10 cm in diameter on a stand 26 cm in height.

Note: The top surface of the platform should be coated with silicon in the shape of a circle with an X across it. This allows the rats to climb up onto the platform and gives them traction so they do not slip off.

5.1.3. Gather a stopwatch, a heat lamp, disposable towels, absorbent pads, extra cages, and a small, long-handled aquarium fish net. Use a computerized video tracking system that is connected to a video camera to record the rat swimming and send the data to the computer. Save the video and data on the computer for later analysis.

5.2 Working memory water maze testing

5.2.1. Give the rats four pairs of trials each day for five consecutive days, place the platform in each of the four quadrants and start the rats from each of the four starting points (N, S, E, W) as described below.

5.2.2. First define the starting location-platform pairs to be used throughout the experiment.

Note: The order of the quadrants where the platform is located and the starting point used needs to be in a different order for each of the five days of swimming, but the same for each rat.

5.2.2.1. Use four starting points (N, S, E, or W) and four platform locations (Quadrants 1, 2, 3, or 4; **Figure 1**). For example, (N, 2; E, 4; S, 1; W, 3; see **Figure 1**). Plan a balanced order (not random) to avoid starting points too close to the platforms (No starting point is the same quadrant as the platform location). Set up a data sheet using the platform quadrants and four starting points.

5.2.2.2. Write a protocol for the video tracking software to use in order to video the rats swimming and to collect specified data (*e.g.*, duration of swim, speed, distance traveled before finding the platform).

Note: The tracking software will automatically stop recording after the specified duration. The protocol should allow for specifying where the platform is placed, how many trials to run per animal, and how many animals will be tested per session, and also the maximum duration allowed (*e.g.*, 120 s).

5.2.2.3. Test 4–6 rats per session.

Note: More than 6 rats create an issue in the timing between rats and can lead to error by the handler. The warming boxes also become crowded.

5.2.3. Trial 1

5.2.3.1. Open the video tracking software and load the correct protocol including the water maze map.

5.2.3.2. Place the platform in the assigned location (*e.g.*, 2; **Figure 1**) and check that it matches the map in the software. Prepare the tracking software to start when the rat enters the field of view of the video camera.

5.2.3.3. Place the rat in the tank facing the wall at the assigned location (*e.g.*, N; **Figure 1**) and immediately start the timer.

5.2.3.4. Allow the rat 120 s to find the platform. When the rat finds the platform, stop the timer and record the time on the worksheet. If the rat fails to find the platform, lead it to the platform by hand and record 120 s. Allow the rat 15 s to remain on the platform.

5.2.4. Trial 2

5.2.4.1. Check that the software is ready for Trial 2. Place the rat back in the tank at the same starting position (N). Repeat step 5.2.3.4.

5.2.5. After Trial 2, place the rat in the heated enclosure for 4 min. Move the platform to the second location (4; **Figure 1**), and check that it matches the map in the software.

5.2.6. Repeat Trial 1 and 2 procedures (steps 5.2.3–5.2.4) until all four starting location/platform pairings are completed.

6. Data Analysis

6.1   Neuroscore

6.1.1.   Manually transfer the handwritten results to a computer spreadsheet.

6.1.2.   Sum the results for each trial to obtain three scores per rat on each day.

6.1.3.   Format the data for statistical analysis (either in the long or wide format depending on the software preference).

Note: The long format has a single column for treatment (in this case, populated with either "Naïve", "Sham", or "TBI"), a single column for day (in this case "0", "1", "2" or "3"), and a single column for trial (either "1", "2" or "3"). The wide format has a single column for each combination of factor levels (so a single column for Naïve, day 0, trial 1, another column for Naïve, day 0, trial 2, *etc.*)

6.1.4. Average the score for each animal on each day. Since there were three trials done on each day there will be three values for each animal per day.

6.1.5. Assess whether the data are normally distributed. Use a nonparametric statistical (*e.g.*, the Kruskal-Wallis) test to analyze whether the score on each day is different between groups. In this case, since these data are not continuous, they are not normally distributed.

6.1.6. To determine where the differences lie, do *post-hoc* tests, such as the Tukey's *post-hoc* analysis.

Note: Here, the R statistical software package, and the kruskal.test() function and the posthoc.kruskal.nemenyi.test function within the Pairwise Multiple Comparison of Mean Ranks Package (PMCMR)[18] were used.

6.1.7. Additionally, test to see if there are any differences between days within each group.

Note: For example, to see if the SHAM animals behave differently on Day 0 compared to Day 1, Day 2, or Day 3. To do this, run a one-way repeated measures ANOVA. This can be accomplished in R using the ezANOVA function within the ez package.

6.1.8. To run a repeated measures ANOVA, first check the assumption about sphericity.

Note: Here, the data indicate that the within factor (Day) does meet the sphericity assumption for NAÏVE and SHAM, but not for TBI. Thus, a correction is not necessary for NAÏVE or SHAM. For the TBI data, use the Greenhouse-Geisser correction.

6.1.9. If significant differences are found, run a *post-hoc* test to determine where the differences lie. This is achieved in R using the pairwise.t.test function. Plot the results as a box plot, as shown in the representative results (**Figure 2**).

6.2   Beam-Balance

6.2.1. Manually transfer the handwritten scores to a computer spreadsheet. Format the data for statistical analysis (either in long or wide format depending on the software preference). See Note in step 6.1.3.

6.2.2. Average the scores for each rat on each day so that each rat will have one score per day. To test whether the score on each day is different between NAÏVE, SHAM, and TBI, assess whether the data are normally distributed.

Note: In this case, since the data are not continuous, these data are not normally distributed. Therefore, use a nonparametric statistical test (*e.g.*, the Kruskal-Wallis test).

6.2.3. To determine where the differences lie, do *post-hoc* tests, *e.g.*, Tukey's *post-hoc* analysis. To test for differences between days within each treatment group, run a one-way repeated measures ANOVA (see step 6.1.7). Check the assumption about sphericity.

Note: In this study, the data indicate that the within factor (Day) does not meet the sphericity assumption for any of the groups, so used continuity corrections. Use the Greenhouse-Grier continuity correction.

6.2.4. Plot the results on a box plot as shown in the representative results (**Figure 3**).

6.3   Beam-walk

6.3.1. Manually transfer handwritten results to a computer spreadsheet. Average the three Beam-Walk latencies for each animal for each day. Format the data for statistical analysis (see step 6.1.3).

6.3.2. Assess whether the data is normally distributed.

Note: In this case, the data is continuous, and is normally distributed. Therefore, use a one-way ANOVA to determine if the latency on each day is different between Naïve, SHAM, and TBI.

6.3.3. To see if there is any difference between days within a treatment group, run a one-way repeated measures ANOVA. First check the assumption about sphericity.

Note: In this study, the data indicate that the within factor (Day) does not meet the sphericity assumption for any of our groups, so continuity corrections are used. Use the Greenhouse-Grier correction.

6.3.4. Plot the results on a box plot as shown in the representative results (**Figure 4**).

6.4.   Working memory water maze

6.4.1. Transfer the data from the worksheet or computer tracking program to a spreadsheet. Select the outcomes to be analyzed.

Note: Many possible outcomes are available for analysis from computer tracking programs. Examples of outcomes selected for analysis may include: latency, path length, thigmotaxia, and swim speed. The most commonly reported outcome is latency, as used in the provided example.

6.4.2. Format the data for statistical analysis (either in long or wide format depending on the software preference).

Note: The long format has a single column for treatment (in this case, populated with either "Naïve", "Sham", or "TBI"), a single column for day (in this case "1", "2", "3", "4", or "5") and a single column for trial (either "1", "2", "3", "4", "5", "6", "7", or "8"). We also need an additional column to identify the attempt (either "1" or "2"). Wide format has a single column for each combination of factor levels (so for example, a single column for Naïve, day 1, trial 1, attempt 1, another column for Naïve, day 1, trial 2, attempt 2). Also, the difference between Trial 1 and Trial 2 can be calculated for each session and analyzed as a difference score.

6.4.3. To find if there is an overall difference between the injury groups, perform the following steps.

6.4.3.1. First, average the water maze latency for each animal for Day 1.

Note: There were four sessions on each day, so average the four values per animal for each of Trial 1 and Trial 2. Do this calculation for the remaining days as well.

6.4.3.2. To check for injury differences overall, run a two-way repeated measures ANOVA. There are two factors, injury and day. Injury is a between group factor and day is a within group factor. Note: Here R was used.

6.4.3.3. If the results indicate a significant difference due to Injury, then run a Tukey's *post-hoc* test to see where the differences lie.

6.4.4. To find out whether there are differences between the injury groups on specific days perform the following steps.

Note: Day 1 is used as an example, and the same analysis needs to be done for all the following days. Also, this analysis is done in several ways, first for Trial 1 only, second for Trial 2 only, and third for the difference between Trial 1 and Trial 2. Trial 1 is used as an example; the same steps need to be used for the other analyses.

6.4.4.1. First, average the water maze latency for each animal for Day 1. Since there were four

repetitions of "Trial 1" on each day, average the four values for each animal.

6.4.4.2. Assess whether the data is normally distributed.

Note: In this case, the data is continuous, and is normally distributed. Therefore, use the one-way ANOVA to determine if the water maze latency on Day 1 is different between NAÏVE, SHAM, and TBI. R statistical software package and the aov() function were used here.

6.4.5.3. Use a 5% level of significance. If the resulting p-value is less than 0.05, then there are significant differences between the groups.

6.4.4.3. To determine where those differences lie, use the Tukey's *post-hoc* test. This is the TukeyHSD() function in R.

6.4.5. To find out if there are differences between days within treatment groups, follow these steps.

6.4.5.1. First, run a one-way repeated measures ANOVA. This can be accomplished in R using the ezANOVA function within the ez package.

6.4.5.2. Before running a repeated measures ANOVA, first check the assumption about sphericity.

Note: The within factor (Day) meets the sphericity assumption for all of the groups, thus there is no need to use continuity corrections.

6.4.5.3. If differences between days are found (p-values less than 0.05), then run a *post-hoc* test to determine exactly where the differences lie. This step is achieved in R using the pairwise.t.test function.

6.4.6. Graph the results using line graphs (**Figure 5**). Also, Trial 1–Trial 2 can be graphed.

REPRESENTATIVE RESULTS:
Results of the neuroscore procedure (**Figure 2**) demonstrate both the potential for false positive (SHAM and TBI groups at day 0) and the sensitivity of this test to detect small differences. False positives can occur when the rat is not well habituated to the procedure, so it is not fully relaxed. Day 0 is prior to surgery, so ideally all rats should reach the criterion of a score of 0 prior to entering a study. Days 1–3 demonstrate the sensitivity of this test to detect small changes in the score. While there is a potential for a score as high as 21, scores higher than 3 are unusual in this model. In this example, repeated measures ANOVA revealed no differences between days for NAÏVE ($p = 0.78$) or SHAM ($p = 0.09$); however, for the TBI group there were differences between days ($p < 0.05$). *Post-hoc* pairwise comparison indicated that Day 0 is significantly different from Days 1, 2, and 3. This result demonstrates that the injury produced small yet significant changes in the neurological assessment.

Further analysis using the Kruskal-Wallis test compared NAÏVE, SHAM, and TBI on each day, followed by the Tukey's *post-hoc* test to determine exactly where the differences lie. For Day 0, the test statistic was 13.37, p = 0.001, and SHAM was significantly different from NAÏVE (p = 0.008). Ideally, there should be no differences between groups on Day 0, as no treatments or procedures have been administered. In this case, the rats should be further habituated to the procedure, or transferred to a non-behavior study. For Day 1, the test statistic was 32.39, p = 9.75e-8, with the *post-hoc* test, indicating that SHAM and TBI were significantly different from NAÏVE (p = 0.002, p = 5.9e-7, respectively). For Day 2, the test statistic was 23.39, p = 8.34e-6, and SHAM and TBI were different from NAÏVE (p = 0.002, p = 6.8e-5). For Day 3, the test statistic was 38.4, p = 4.59e-9, and again, SHAM and TBI were significantly different from NAÏVE (p = 0.001, p = 2.1e-8, respectively). These results point to the fact that the SHAM preparation also produces some deficits in neurological assessment at times early after injury.

Representative Beam-Balance results (**Figure 3**) demonstrate the sensitivity of the Beam-Balance test to deficits shortly after injury (**Figure 3**, left) and at a time point longer after injury (**Figure 3**, right). The sensitivity of the Beam-Balance test to the effects of brain injury diminishes over time, because as the uninjured rats age and gain weight, they have increased difficulty balancing on the beam. At later time points, the beam is turned so the rats are balancing on the wider side of the beam. Nevertheless, by 6 months after injury, this test is no longer sensitive to the effects of injury as age and/or weight confound the ability to perform the task (**Figure 3**, right). Alternatively, healing may have occurred in the vestibular system, and these data accurately reflect that the rats' ability to balance reaches the same level as the control groups.

In comparing Naïve, SHAM, and TBI on each day, we used the Kruskal-Wallis test. The results for time points early after injury are shown in **Figure 3**, left. On Day 0, the Kruskal-Wallis test found the value of the test statistic to be 6.81, p = 0.033. There was a significant difference between the groups, with the Tukey's *post-hoc* test showing that the Naïve group was different than SHAM (p = 0.038); however, all three groups had means well below 2.0, indicating that all rats had met the criteria to continue. It would be preferable to have no differences between groups on Day 0, but since all groups are below 2, they can continue in the study. On PID 1, the Kruskal-Wallis test statistic was 69.72, p = 7.25e-16. The Tukey's *post-hoc* test showed that the TBI group was significantly different from both the Naïve and Sham groups (p = 4.9e-14, p = 9.1e-08, respectively). On Day 2, the Kruskal-Wallis test statistic was 62.84 and p = 2.26e-14, with the *post-hoc* test showing TBI different from NAÏVE and SHAM (p = 1.0e-10, p = 2.1e-10 respectively). On Day 3, the Kruskal-Wallis test statistic was 62.69 and p = 2.44e-14. The *post-hoc* test showed TBI different from Naïve and SHAM, (p = 9.6e-12, p = 1.7e-08, respectively). We additionally looked to see if there were any differences between days within each group. Using a repeated measures ANOVA, for NAÏVE, there were no differences between days (p = 0.367). For SHAM and TBI there were differences between days (p = 0.002, p = 3.90e-29, respectively). *Post-hoc* pairwise comparisons revealed for SHAM Day 1 is significantly different from Day 2 and Day 3 (p = 0.001, p = 0.01, respectively), and for TBI, Day 0 is significantly different form Days 1, 2, and 3 (p < 2e-16, p = 5.5e-16, and p = 2.7e-13, respectively). Day 1 is

also significantly different from Day 3 (p = 0.036).

At 6 months after injury, comparisons between NAÏVE, SHAM, and TBI were made on each day using the Kruskal-Wallis test (**Figure 3**, right). On Day 0, the value of the test statistic was 3.36 and p = 0.187, so there were no differences on Day 0. All means were below 2, indicating that all rats and groups met the criteria to continue in the study. On PID 1, the test statistic was 6.11, p = 0.047; however, *post-hoc* analysis using Tukey's *post-hoc* test showed that none of the groups were significantly different when accounting for multiple hypothesis testing. On Day 2, the test statistic was 4.09, p = 0.13, ns, and on Day 3, the test statistic was 2.91, p = 0.23, ns. Thus, there were no differences between the injury groups on any given day.

Additionally, looking at differences between days within treatment groups, a repeated measures ANOVA revealed significant differences between days for NAÏVE, SHAM, and TBI (p = 0.0003, p = 2.61e-5, p = 5.59e-7, respectively; **Figure 3**, right). *Post-hoc* tests demonstrated the following differences. For NAÏVE, Day 0 was significantly different from Days 1, 2, and 3 (p = 0.002, p = 0.044, p = 0.004, respectively). For SHAM, all days were significantly different from each other: Day 0 was significantly different from Days 1, 2, and 3 (p = 0.0006, p = 0.001, p = 0.0006, respectively); Day 1 was significantly different from Days 2 and 3 (p = 0.031, p = 0.0006, respectively); and Day 2 was significantly different from Day 3 (p = 0.044). For TBI, Day 0 is significantly different from Days 1, 2, and 3 (p = 0.0005, p = 0.0008, p = 0.0005, respectively).

The results of the Beam-Walk test are shown at two time points (**Figure 4**). Similar to the Beam-Balance, this test detects deficits early after injury (**Figure 4**, left). However, by 6 months after injury, there are no significant differences between the groups (**Figure 4**, right), suggesting healing occurred in the injured group. This result may reflect effects of more advanced age and increased weight.

To compare NAÏVE, SHAM, and TBI on each day early after injury, a one-way ANOVA was used. There were no differences on Day 0 (F = 0.859, p = 0.426) and all latencies were below 5 s, indicating that all rats met the criteria to continue in the study. On PID 1, there was a significant test statistic of 15.36, p = 1.18e-6. Tukey's *post hoc* test indicated a significant difference between TBI and NAÏVE (p = 0.000004) and TBI and SHAM (p = 0.0001). On Day 2, there was a significant difference between groups (F = 9.49, p = 0.0002). *Post-hoc* testing revealed differences between TBI and NAÏVE (p = 0.0002) and TBI and SHAM (p = 0.005). On Day 3, the overall test statistic equals 6.27, p = 0.0025, indicating there are differences between the groups. Tukey's *post-hoc* test showed that again, TBI was different from NAÏVE and SHAM (p = 0.003, p = 0.035, respectively).

Using a one-way repeated measure ANOVA, differences between days within treatment groups were explored. First the assumption of sphericity was checked for each group. The within factor (Day) did not meet the sphericity assumption for the NAÏVE or SHAM groups, thus the continuity correction, Greenhouse-Grier was applied to those groups. For SHAM, there were no differences between days (p = 0.066), for NAÏVE and TBI there were (p = 0.006, p = 2.89E-7, respectively). *Post-hoc* comparisons showed for NAÏVE, the difference was between Day 0 and

Day 1 (p = 0.003). For TBI, the differences were between Day 0 and Days 1, 2, and 3 (p = 9.2e-6, p = 0.0005, p = 0.002, respectively), and there was a difference between Day 1 and Day 3 (p = 0.018).

At 6 months after injury, there were no significant differences between NAÏVE, SHAM, or TBI on any day (Day 0, F = 0.315, p = 0.732; Day 1, F = 0.336, p = 0.717; Day 2, F = 0.5, p = 0.61; Day 3, F = 1.17, p = 0.322; **Figure 4**, right). When comparing differences between days within each group, there was a significant difference in the TBI group (p = 0.026), with Day 0 being different from Days 1, 2, and 3 (p = 0.026, p = 0.002, p = 0.002). There were no differences between any days for NAÏVE or SHAM (p =0.104, p = 0.063, respectively).

Data from the working memory version of the Morris water maze can be graphed in a variety of ways. Here we demonstrate the results for 3 months (**Figure 5**, left) and 12 months (**Figure 5**, right) after injury using both line graphs to represent the time course, and box plots to provide an overall summary of the data (**Figure 5**, bottom). We can then visualize Trial 1 comparisons and Trial 2 comparisons independently on each day as well as overall differences due to injury. Trial 1 latencies represent reference memory and Trial 2 latencies depict working memory.

The data from rats 3 months after injury are shown in **Figure 5**, left. For Trial 1 (**Figure 5**, upper left), when comparing NAÏVE, SHAM, and TBI, only Day 4 showed a significant difference between groups (F = 4.12, p = 0.025), with the *post-hoc* Tukey's test indicating that TBI was different from NAÏVE (p = 0.019). For Trial 2 (**Figure 5**, middle left), there was a significant difference on Day 1 (F = 5.93, p = 0.006), with *post-hoc* analysis indicating that TBI was different from SHAM (p = 0.005). The repeated measures ANOVA did not find an overall difference between injury groups at 3 months (p = 0.56). These results suggest that these rats have small yet significant deficits in reference as well as working memory at 3 months after injury.

 At 12 months after injury, comparing Trial 1 NAÏVE, SHAM, and TBI (**Figure 5**, right), repeated measures ANOVA demonstrated a significant overall effect of injury (F = 3.94, p = 0.03). Pairwise comparisons revealed that TBI was significantly different from both NAÏVE and SHAM (p = 0.043 and p = 0.006., respectively) (**Figure 5**, bottom right). In addition, by comparing injury groups on each day, using a one-way ANOVA, a significant difference was detected on Day 3 (F = 7.28, p = 0.003). *Post-hoc* comparison revealed that TBI was different from SHAM (p = 0.0018) (**Figure 5**, upper right). For Trial 2, the repeated measures ANOVA found a significant difference due to injury (F = 3.97, p = 0.029), with *post-hoc* pairwise comparisons detecting the difference between TBI and SHAM (p = 0.017) (**Figure 5**, bottom right). One-way ANOVA on each day found significant differences on Days 2 and 4. On Day 2 (F = 4.02, p = 0.028), Tukey's *post-hoc* test found that TBI was different from SHAM (p = 0.023). On Day 4 (F = 4.12, p = 0.026), *post-hoc* analysis found a difference between TBI and SHAM (p = 0.025) (**Figure 5**, middle right).

FIGURE AND TABLE LEGENDS:
Figure 1. Diagram of the water maze. This diagram demonstrates the possible platform locations (1, 2, 3, 4) and starting points (N, S, E, W) for the working memory Morris water maze. Rats are allowed two trials from each starting location/platform pairing. There is a 15 s inter-

trial interval and 4 min rest in a warming chamber between pairs of trials for a total of four pairs of trials for each daily session.

Figure 2. Results of the neuroscore test. All rats were trained to simple reflex testing tasks prior to Day 0 (see text for details on training, testing, and scoring). Results are shown as median (black line), first and third quartiles (boundaries of box), and 10th and 90th percentiles (error bars). The mean is also shown by the red lines and outlying points as black dots. Data are presented for the Day 0 baseline and post-injury days 1–3. The results of the *post-hoc t*-test for each time point are shown on the graphs: * $p < 0.001$ vs TBI Day 0; ^ $p < 0.001$ vs same day NAÏVE.

Figure 3. Results of the Beam-Balance test. All rats were trained to balance on the beam until they could balance for 60 s for three consecutive trials (see text for details on training, testing and scoring). On subsequent tests, rats were scored on a scale from 1–6 with 1 signifying normal balance and 6 signifying no attempt to stay on the beam. Results are shown as median (black line), first and third quartiles (boundaries of box), and 10th and 90th percentiles (error bars). The mean is also shown by the red lines and outlying points as black dots. Data are presented for the Day 0 baseline score, post-injury days 1–3 (left), and 6 months after injury (right). The results of the *post-hoc t*-test for each time point are shown on the graphs. For Days 0–3: * $p < 0.001$ vs TBI Day 0; ^ $p < 0.001$ vs same day NAÏVE; @ $p < 0.001$ vs same day SHAM. For 6 months: * $p < 0.001$ vs TBI Day 0; # $p < 0.001$ vs NAÏVE Day 0; & $p < 0.001$ vs SHAM Day 0.

Figure 4. Results of the Beam-Walk test. All rats were trained to traverse the beam while weaving between posts to escape into a safety box. They were trained until they met criteria of ≤ 5 s on three consecutive trials (see text for details on training, testing and scoring). Baseline testing was completed on Day 0 and rats were subsequently tested on days 1–3 after injury (left). A subset of rats was also retested at 6 months after injury (right). The results are graphed as median (black line), first and third quartiles (boundaries of box), and 10th and 90th percentiles (error bars). The mean is also indicated by the red lines and outlying points as black dots. The outcomes of the *post-hoc* tests for each time point are shown on the graphs. For Days 0–3: * $p < 0.001$ vs TBI Day 0; ^ $p < 0.001$ vs same day NAÏVE; @ $p < 0.001$ vs same day SHAM; For 6 months: * $p < 0.001$ vs TBI Day 0.

Figure 5. Results of the working memory Morris water maze. Results are shown for separate groups of rats at 3 months (left column) and 12 months (right column). **The upper panels** show the average latencies (time it took the rats to find the hidden platform) on the first trials of the two-trial pairing for each of the five testing days. **The middle panels** show the average latencies of the second trials on each day. Results of the *post-hoc* analysis are shown on the graphs (* $p < 0.05$ vs same day SHAM; ^ $p < 0.05$ vs same day NAÏVE). **The lower panels** summarize the results showing the median (black line), 25th and 75th percentiles (boundaries of box), and 10th and 90th percentiles (error bars). The mean is also shown by the red lines and outlying points as black dots. Results of the *post-hoc* analysis are shown on the graphs (*$p < 0.05$ vs same Trial SHAM, ^ $p < 0.05$ vs same Trial NAÏVE).

DISCUSSION:

When conducting any type of behavioral testing, it is critical to be consistent. This detail includes many considerations that seem insignificant but have a major impact on the response of the animal. An important step that cannot be overlooked is acclimation of animals to their home-cage/housing situation prior to any experiment. This preparation reduces the effects of the animals' physiological stress response, which can alter behavioral outcomes[16]. Similarly, it is absolutely essential that every effort is made to handle all animals in the same manner. This consistency includes, as previously mentioned, acclimation to housing and also acclimation to handling and transportation between rooms prior to training or testing. This concept cannot be overstated. Sloppy animal handling is disastrous to any behavioral testing[17]. Likewise, every effort needs to be made to test animals at the same time of day, whether during their dark or light cycle. For the tests discussed here, testing during light or dark stage is acceptable, as long as the tests are performed consistently. Testing done at different times during the circadian cycle has been shown to alter behavioral outcomes[16,18]. Additionally, the handler as well as the animal must be in a stress free, calm state in order to maximize the accuracy of results.

Particularly in the case of the neuroscore, false positives and negatives are common. False positives typically occur when an animal is not fully habituated to handling and testing. The animal must be completely relaxed so the observed response is reflexive and not due to muscles tightening from reacting out of stress or fear. A tense handler can influence the results by transmitting stress to the animal. Therefore, holding the rat too tight or too loose can both be problematic. Additionally, if the handler is nervous, this can confound the reaction of the rats. There is also the risk that an inexperienced observer will misinterpret the rat's response. Good training and a lot of practice are essential to the success and consistency of the neuroscore.

In general, the chief concern with these tests is the lack of a large difference, and sometimes no difference, between treatment groups. Since animals can react differently to different handlers, noises, times of day, and potentially, seasons[19], every effort must be made to reduce all possible confounding factors.

The results of the Beam-Balance and Beam-Walk tasks shown here demonstrate that these tests are useful early after injury to detect deficits in vestibulomotor function. These deficits typically resolve over time[1,14]. In this model, by 6 months after injury, the injury-induced deficits have resolved. The results of the 6 month time point indicate that there are no differences between NAIVE, SHAM, or injured rats; however, all the rats have been relaxing in their home cages for 6 months, aging and gaining weight. Thus, by the time they are re-tested at 6 months post-surgery (or equivalent in the case of NAÏVE), they are essentially becoming old and fat, and therefore all the groups do not perform as well as they did compared to their baseline Day 0 results.

Another important consideration is that the behavior test used is the correct test. For example, the tests employed here are thought to represent the function of specific brain areas. One example is the vestibular system, which is important for balance. Brain areas involved in

sensorimotor function, such as the cortex including sensorimotor cortex, the thalamus, corticospinal neurons, basal ganglia, nigro-striatum, to name a few, are all involved in vestibulomotor coordination. Thus, deficits in the Beam-Balance or Beam-Walk indicate potential deficits in these areas. Furthermore, the hippocampus and prefrontal cortex are involved in the learning and memory functions tested by the working memory water maze. Even when the correct test is chosen, the limitations of the tests that are employed must be kept in mind. For example, none of the tests presented here are sensitive to deficits in mood, such as depression, anxiety, or social interactions such as aggression, decision making, or impulsivity. To reiterate, it is imperative to choose the appropriate test for the behavior and brain area to be evaluated.

Interpretation and analysis of behavioral data must be approached with caution. It is highly recommended to include power analyses of each type of test separately, because, using a behavioral outcome as a measure of a neural deficit, is by its nature, a crude measure of a subtle effect. Furthermore, different tests require different types of statistical analyses. For example, the neuroscore and Beam-Balance tests described are dependent on the interpretation of a trained observer to score the behavior using an ordinal scale. These types of data are not continuous and not normally distributed, so nonparametric statistics should be used, such as the Kruskal-Wallis test, as demonstrated in Sections 6.1 and 6.2. Alternatively, the Beam-Walk and working memory water maze tests produce data that are continuous and normally distributed, so parametric statistics can be used, such as one-way ANOVA or repeated-measures two-way ANOVA, as demonstrated in Sections 6.3 and 6.4.

The behavioral tasks presented here have stood the test of time and give reproducible results, particularly when paired with the FPI model in rats, although many other methods of behavioral testing for brain injury do exist. The neuroscore is a short assessment performed with a minimum of equipment. Other tests of reflexes and strength are available and could be incorporated into a neurological assessment, such as the lateral pulsion task, the akinesia test, the inclined plane test, and grip strength (see Fujimoto *et al.*[16] and Gold *et al.*[17]). The Beam-Balance and Beam-Walk tasks described are measures of vestibulomotor deficits after injury. Vestibulomotor coordination can be considered one measure of gross locomotor behavior, while other measures of gross locomotor deficits include the Rotarod, the rotating pole, and open field activity. The ability to swim, measured as swim speed during the water maze, is also an indication of gross motor coordination[16,17]. The working memory water maze task completes this set of tests by detecting both reference memory deficits (indicated by Trial 1) and working memory deficits (indicated by Trial 2 or the difference between Trial 1 and Trial 2). Other measures of cognitive function include the eight arm radial maze, the Barnes maze, the novel object recognition test, and different variations of the water maze. These variations include the original Morris water maze and the Lashley III maze (again see Fujimoto *et al.*[16] and Gold *et al.*[17]). This battery of tests has proven to be useful early after injury and, in varying degrees, out to 12 months after injury[1].

Additionally, the tasks demonstrated here can be used with different strains, sex, and ages of rats; however, accommodations may need to be made for differing sizes and in cases of greater

fragility. For example, older, heavier rats need a wider beam for the Beam-Balance task and aged, frail rats, may need shorter duration of swim times in the water maze. Thus, there is room for flexibility in these tests and potential for development of new tests to accommodate different situations and hypotheses.
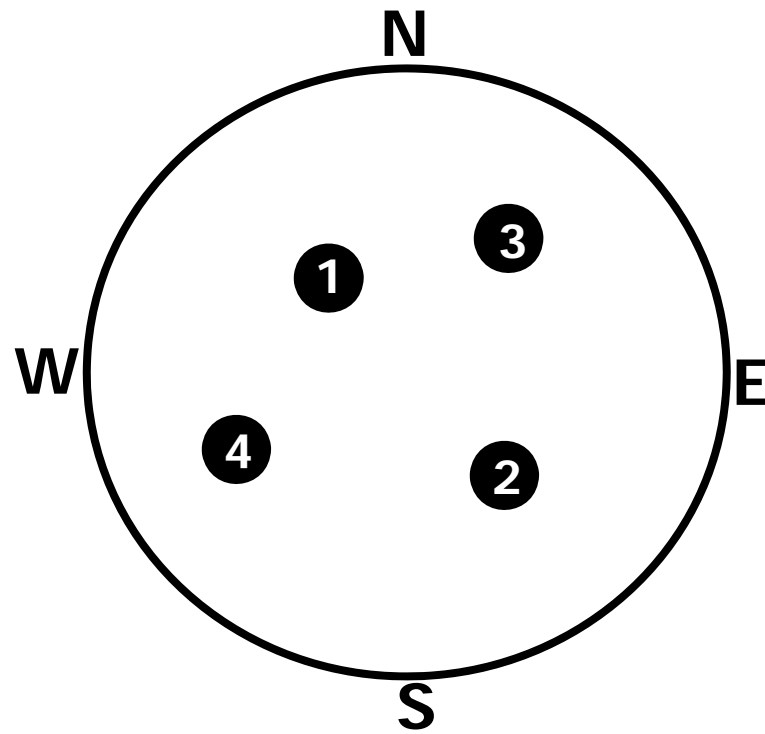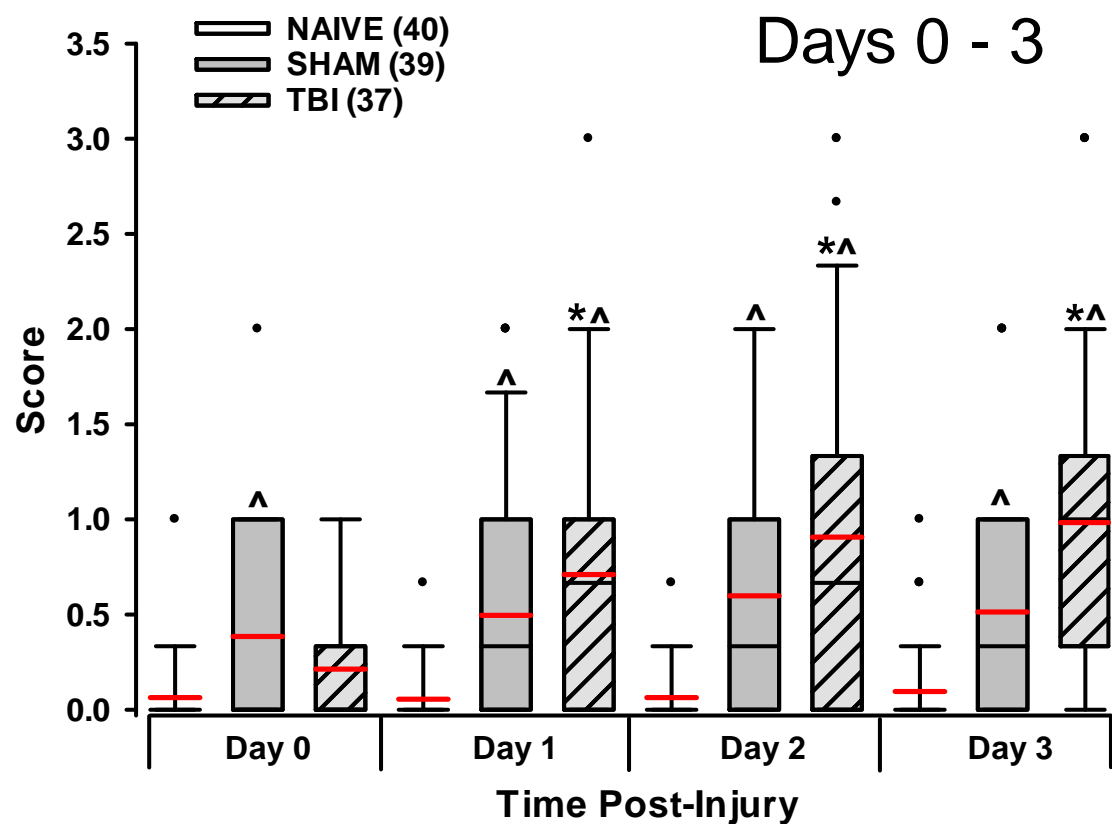
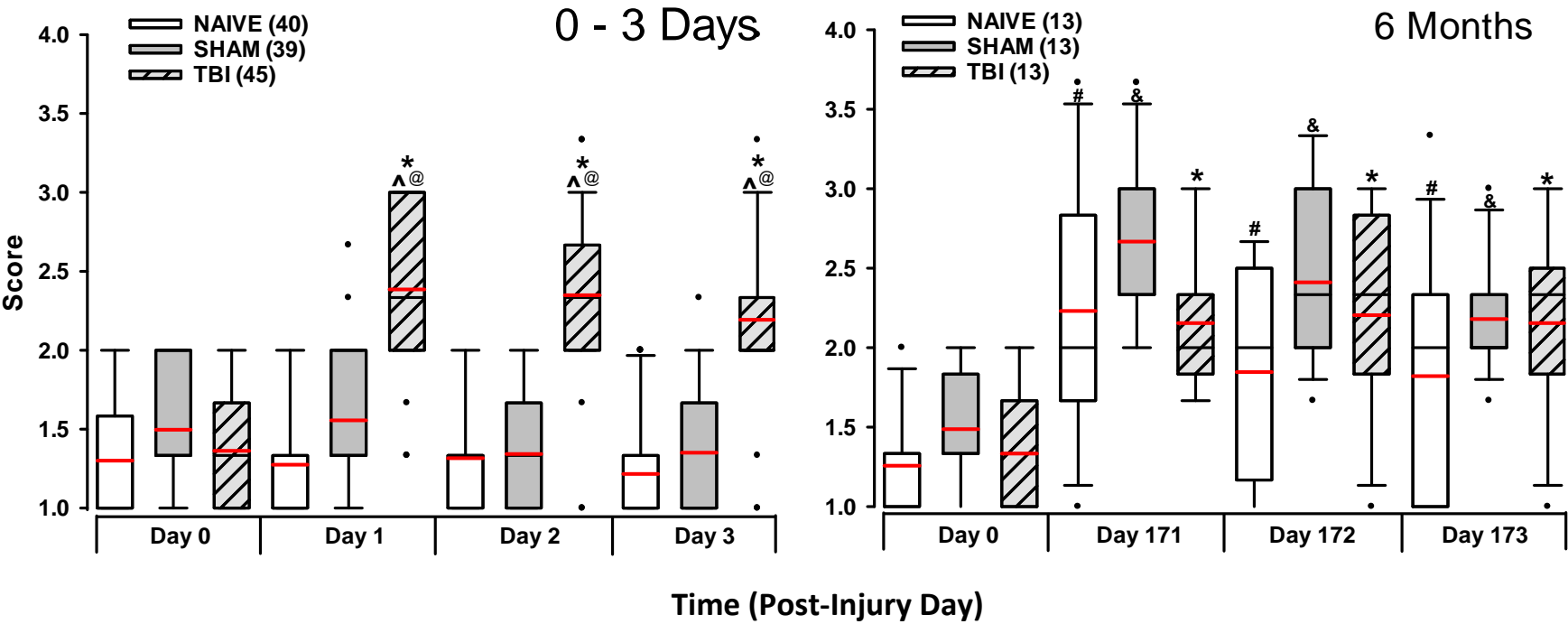**DISCLOSURES:**
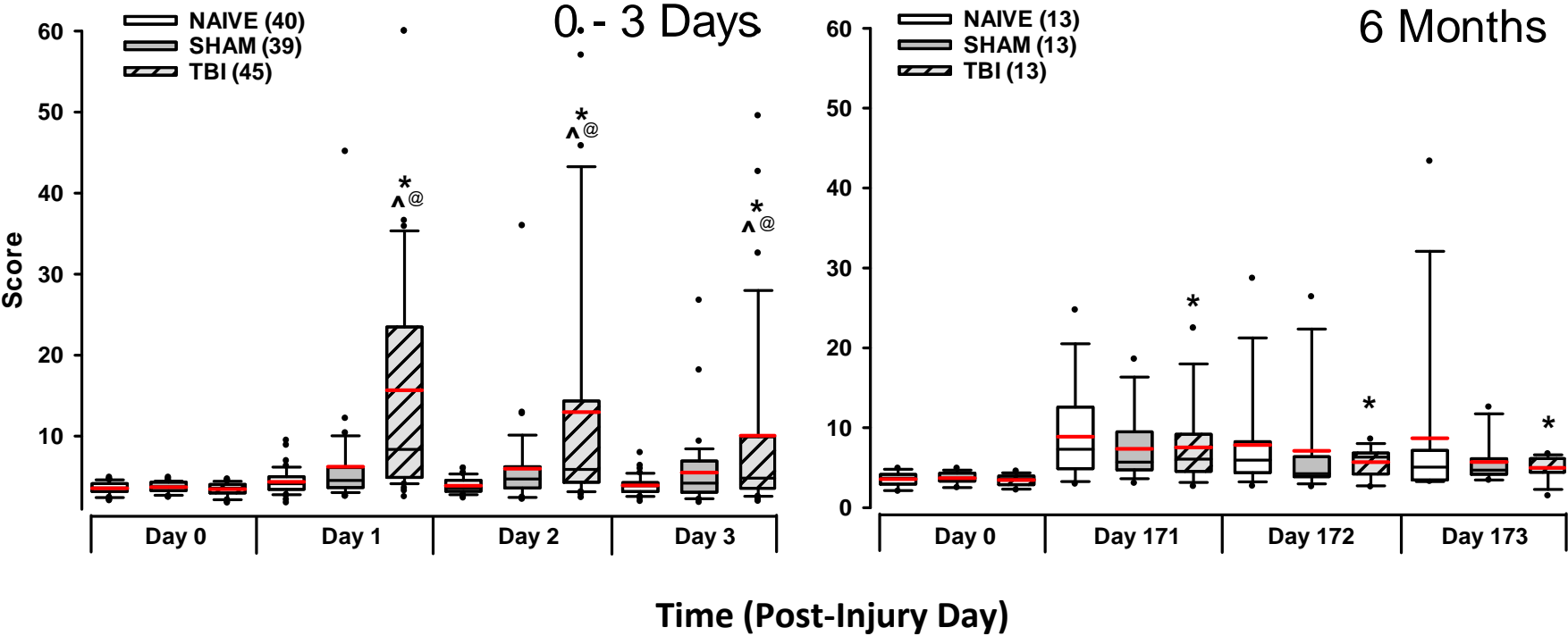The authors have nothing to disclose.

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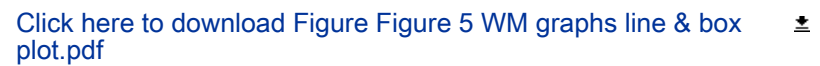
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Sprague-Dawley rats	Charles Rivers Laboratories 251 Ballardvale St Wilmington, MA 01887-1096 Phone: 800-522-7287	CD-IGS rats, strain code 001	male, albino, 300-350g at arrival
Beam-Balance			
Beam	home built		wood, 25" l x 1" h x 3/4" w sealed with polyurethane varnish
C-clamp	Home Depot	1422-C	2 1/2"
barrier	Home Depot		styrofoam, 18" x 17 1/2"
table (for both BB & BW)	generic office supply		37" h x 30" w x 60" l
Beam-Walk			
Beam	home built		wood 38-1/2" l x 1-3/4" h x 1" w sealed with polyurethane varnish (~ 37" off floor)
escape box nails (pegs) hinges clamps	home built		woodpainted black 12 1/2 " l x 9" h x 7-1/4" w 2"
white noise machine	San Diego Instruments 9155 Brown Deer Rd, Suite 8 San Diego, CA 92121 Phone: (858)530-2600		http://www.sandiegoinstruments.com/libraries/misc/datasheets/whitenoise.pdf
light	Home Depot		
Morris Water Maze			

fiberglass pool	manufacturer unknown		
(similar to one made by SDI)	San Diego Instruments	7000-0723	72" diameter x 30" deep (~ 500 gal)
plexiglass platform	hand-made by Maggie Parsley		10 cm diameter, 26" tall with silicone applied to the surface of the platform to provide a gripping surface
(similar to one made by SDI)	SDI	7500-0272	
plexiglass animal boxes w/ lids	UTMB Machine Shop		2 boxes, 10" w x 16" L x 9" h
spot lights/ heat lamps	Home Depot		3 around pool, 2 over boxes to dry animals
AnyMaze	San Diego Instruments	/9001	
	9155 Brown Deer Rd, Suite 8		http://www.sandiegoinstruments.com/any-maze-video-tracking/
	San Diego, CA 92121		
	Phone: (858)530-2600		

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Author(s): Nicole Hausser, Kathia Johnson, Margaret A. Parsley, Jutatip Guptarak, Stacy L. Sell

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Response to Reviewer #1:

Major Concerns:

1. The authors present a battery of tests. It would be beneficial in the text and subsequent video to mention other behaviors that can be substituted into this battery. Fujimoto, 2004 (Neurosci Biobehav Rev) and/or Gold, 2013 (Regen Med) provide a nice breakdown of various tasks for different aspects of function (e.g. Fine motor, Gross motor, somatosensory, etc).

The last paragraph of the Discussion has been expanded to include mention of other possible tests that can be substituted into the battery presented in this paper and the two references mentioned above have been cited. There is some concern that this is beyond the scope of this paper, as there is only room to mention each alternative example by name and not detailed description as this is not meant to be a comprehensive review.

2. It would be good to note the behaviors that this battery will not address: depression/emotionality-related deficits, social interactions, decision making, impulsivity. This is hinted at in the discussion, but not particularly emphasized.

A statement to this effect has been added to the discussion.

3. TBI models have many different manifestations. The current paper utilizes FPI, which is typically regarded as a moderate to severe injury. Would this battery be as effective in milder or concussive TBI?

Considering this is a methods paper describing the method that we use, it is beyond the scope of this paper to speculate on whether or not this battery of tests would be as effective in other forms of TBI. These are experiments that could be done in the future.

4. The authors should clarify in the neuroscore section where repeating by side adds to the score (e.g. Repeat on opposite side and score (0) or (1)). A quick read-through suggests 5 total points.

Possible scores have been added to each section of the neuroscore for clarification.

5. The description of the Beam Walk apparatus is not clear. The video may clarify this, but I am not clear on what purpose the 'pegs' serve.

The pegs are inserted at equally spaced intervals along the beam to create obstacles for the rats to navigate as they walk the length of the beam. The purpose of the pegs is to increase the difficulty of the Beam-Walk task.

6. The figures do not match the methods of analysis. Since continuous data (Group x Day) is being presented, they should be line graphs. When a bar graph is presented, it implies that the data are independent between days. This is especially egregious for the MWM data which appears to be completely collapsed across multiple days of testing.

All the figures and associated statistical analysis has been revised. Please see Section 6.0 Data Analysis and the Representative Results section.

7. For the MWM analysis, please clarify if you are using a 3x5x2 (Injury x Day x Trial) ANOVA. A simpler approach might be to do an ANOVA on the difference scores, which would also make the graphing clearer by providing a single data point.

We have revised our analysis. We now use a two-way repeated measures ANOVA with Injury as a between group factor and Day as a within group factor. When a significant difference was found, a Tukey's post-hoc test was used to find which groups were different.

A one-way ANOVA was used to determine if there were differences between NAÏVE, SHAM and TBI on each day. If a significant difference was found, a Tukey's post-hoc test was used to determine where the differences lay.

Figure 5 now shows the line graph time course of each Trial of the working memory water maze test as well as the summarized data I a box and whisker plot.

8. The posthoc analyses suggested would be inappropriate for anything beyond 3 groups, and should be noted as researchers who are unfamiliar are likely to follow the protocol very closely.

Please see Section 6, Data Analysis for our revised statistical analyses. For post-hoc testing we used either the Tukey's post-hoc test or pairwise t-test comparisons as necessary.

9. Figures 3 & 4 are missing a y-axis label. The y-axis of Figure 5 is confusing - it indicates that the authors have summed the average latencies, when it appears to more likely just be the average.

Thank you for pointing out the missing labels. Figures 3 and 4 have been corrected. The label for the Y-axis of Figure 5 has been changed to say "overall average".

10. Some discussion of the inherent problems of using ordinal scored tests (NeuroScore, Beam balance), particularly as it applies to domain-specific impairments as opposed to general impairments as well as statistical challenges would improve the discussion.

A paragraph has been added to the discussion to address these issues.

Minor Concerns:
1. It should be noted that while the beam balance is sensitive to vestibular dysfunction, gross motor dyscoordination will also generate deficits. A similar statement could be mentioned about cerebellar deficits on the beam walk task. This is brought up again in the discussion - do the authors think it is more likely to be gross motor deficits that mimic vestibular dysfunction, or actual vestibular dysfunction?

This is a good question and possibly not one that we can prove one way or the other with these data. However, these rats do not look grossly impaired and perform quite well on all these tasks with only small differences detected compared to sham or naïve rats. Thus we speculate that the small deficits we detect are due to vestibular dysfunction more than gross motor dysfunction.

2. A comment on counterbalancing design such that injured and sham animals are interspersed in the testing period would be beneficial. Sadly, some researchers do not consider the effects of variables such as these.

A statement mentioning our use of a balanced design has been added as a note in the surgical procedures section.

3. The authors mention the MWM-WM can be used to test reference memory. Please clarify what is meant by this as there are usually separate reference and working paradigms for this task.

It is true that there are separate tests designed to evaluate reference memory or working memory. We suggest that the Trial 1 latencies are representative of reference memory. Considering that there are four possible platform locations, even though these locations change randomly over the course of the 5 day testing period, the rats demonstrate that they learn the four platform locations. This is shown by the decrease in the latency of Trial 1 over the 5 day period. This indicates that the rats retain the platform locations over the 5 days, indicating reference memory.

The Trial 2 measurement is a measure of working memory because the location of the platform for trial 2 is only relevant for that one trial and then it is no longer useful in the next session.

4. The original reference you provide for the FPI model describes an air blast, which eliminates the fluid component. A qualification ("a similar model was first published…") or an update to the 1976 paper describing the modern iteration of the device would be preferred.

I apologize if there has been a misunderstanding. We are not aware of the 1976 paper that is mentioned here. The articles we referenced describing the FPI model were:

1. (reference #10 in the manuscript) Dixon, et al., J Neurosurg 67:110-119, 1987 entitled, "A fluid percussion model of experimental brain injury in the rat", in which Figure 1 is a diagram of the exact device we currently employ in our laboratory.

2. (reference #11 in the manuscript) DeWitt, et al., J Neurotrauma 14(4):223-233, 1997 entitled, "L-Arginine and superoxide dismutase prevent or reverse cerebral hypoperfusion after fluid-percussion traumatic brain injury". This paper is an early paper from our laboratory.

Both of these papers describe the fluid-percussion injury model. Additionally, we have added our recent paper Sell et al., J Neurotrauma 33:1-11, 2016, which also describes the injury procedure.

5. The authors make the claim that FPI is the most common method of injury. CCI is arguably as common or more common at 1200 references [PubMed search string: ("controlled cortical impact" OR "cortical contusion injury")]. The referenced search does not link properly in the references. Further, the search string should be [("fluid percussion injury" rat)] rather than terms without the quotes. When searching

for the term without the quotes, it returns any abstract that contains all of the words, while the quoted term returns all abstracts with the words in that specific order. This search returns 427, a number which is much lower.

This statement has been altered to reflect this point and now reads, "…the fluid-percussion injury model is one of the most widely used…"

6. A note should be added to 1.2.4 that this takes considerable practice to reliably elicit a vibrissae reflex, and that researchers should be well trained on this aspect.

A note to this effect has been added to what is now 2.2.4.

7. Spelling error in 3.2.13 'noice'

Corrected – thank you

8. Section 4.1 should note the size of tank necessary for rat water maze

The dimensions of the tank are in the Materials Table as specified.

9. In your MWM materials, the authors describe everything as conducted by hand, but then reference AnyMaze in the analysis.

Section 5.1.8 has been added to mention the use of a video tracking system.

Reviewer #2:
Major Concerns:
1. While the tasks chosen for motor and cognitive function are commonly used among traumatic brain injury researchers, it is a limited list. More complex exams are being used in conjunction as MWM along is not a complete story. Novel object task, rotorod, gait, social interaction, Y-maze, Barnes maze are all techniques that are often preferred by reviewers and researchers in the field. There should be mention of the other exams.

Yes, there are many other useful behavioral measures of brain function. Describing these is beyond the scope of this paper. The discussion has been expanded to include mention of other paradigms.

2. The surgical procedure for FP involves intubation, which is not always used by researchers who utilize the injury model. How might this affect behavior? It is also important to mention whether or not a stereotaxic frame was used and the type of ear bars. Puncturing of the ear drums will directly affect animal behavior. This is not mentioned at all. There is no description as to the type of TBI pathology produced with the FP in this study. Is there cell loss? Contusion? The severity of pathology will dictate the duration of detectable impairments. This must be clarified.

Intubation and a stereotaxic frame have been the standard procedure for our laboratory since at least 1997 (see DeWitt et al., 1997 mentioned above and referenced in the manuscript). We have no evidence that intubation alters the results of behavioral testing, but the reason that SHAM animals are included is to take into consideration all of the effects of the surgical preparation which includes intubation and stereotaxic mounting, minus the injury. Our stereotaxic technique does not puncture the ear drums. Since this manuscript is focused on behavioral methods, the readers are referred to two previous publications for details on the FPI surgical technique.

Several previous publications have addressed the type of brain pathology that is produced by FPI. We expanded the introduction to address the issue of neuronal injury and added some references.

3. The beam balance and beam walking beams are wood, which is great for gripping, but they also absorb urine…there is no mention of wood treatment. This can especially affect the walking.

The wood is sealed with polyurethane varnish; this has been specified in the Materials Table.

4. There is no mention of a video camera for beam walking. This is often used for analysis of the limb slips as they go across the beam. A mirror can be used for the contralateral limb. Analysis of only time is limited.

We do not use a video camera for our beam walking task as we do not measure limb slips.

5. In the MWM task description, there is no mention of extramaze cues (type, size or location relative to the maze). This varies a lot between labs and can be a major issue. Also any intramaze cues can alter results. The use of a plexiglass platform can also be problematic since it is really slippery. Others have made multiple holes in the platform for the animals to grip. There is also no mention of the color of the tank, which is essential for video tracking.

Thank you for bringing up this important point. The walls surrounding the pool have large shapes including three dimensional objects hung on them and one side has a curtain that the computer and researcher stay behind while the rat is swimming. There are no intra-maze cues. The plexiglass platform has silicone applied to the surface that has hardened and provides a surface that the rats can grip. These details have been specified in the Materials Table.

The tank is white, but with the proper settings on the video-tracking system this is not a problem.

6. There is also no mention of the temperature maintained in the tank. The water temperature will affect performance.

This is a good point. We maintain the water temperature at 26+/- 1o C. A statement to this effect has been added to the protocol.

7. The duration of time allowed to find the platform is also something that varies between groups. 120s is a long time and can add to fatigue and poor performance. No mention of this.

It is true that the time allowed to the rats to locate the platform varies between laboratories and experimental designs. In fact, one of the early Morris papers (Morris, 1981) allows 180 seconds. However, in our search of the literature 120 seconds seems to be quite common and we have not had any problems with fatigue in our rats.

8. The analysis for MWM is also rather simplistic. Most researchers now utilize tracking programs for latency, distance, pathway pattern analysis. There is much more data that can be acquired than latency alone. Also the 2 day testing is not always used. Many people use the MWM for working memory without pre training. This is not mentioned.

The explanation of the use of AnyMaze has been addressed previously. (see item #5). Item 6.4.2 in the protocol mentions that other outcomes can be measured using tracking software:

"6.4.2. Many possible outcomes are available for analysis from computer tracking programs so outcomes to be analyzed must be selected. Examples of outcomes selected for analysis may include: latency, path length, thigmotaxia and swim speed."

We choose latency to use as an example because it has proven to be the most reliable and significant outcome measure for this version of the water maze.

We are confused about the mention of "2 day testing" and "pre training". Our paradigm includes 5 days of testing and no pre-training. On each day there are 4 sessions which are distinguished by 4 different platform locations. The rats are given two trials per platform location. The first trial is a learning trial and the second trial is the memory trial. Please see section 5.2 of the protocol.

9. These tasks can be used with other age groups and gender, for which accommodations need to be made in the tasks. Including pretraining duration, beam size and length, durations of swimming in the maze. These needs to be mentioned.

A statement mentioning these issues has been included in the discussion.

Reviewer #3:
Major Concerns:
1. Authors should re-write the whole section of discussion to describe more clearly about the interactions between the results from different neuromotor tests after brain injury. For example, why the score is increased in naïve group but decreased in TBI group in beam walk and beam balance tests after 6 months later? Why the behavioral situation is getting worse in the control group but getting better in the brain injury rats?

A section has been added to the discussion to explain the results of the 6 month beam balance and beam walk test:

"The results of the Beam-Balance and Beam-Walk tasks shown here demonstrate that these tests are useful early after injury to detect deficits in vestibulomotor function. These deficits

typically resolve over time. In this model, by six months after injury, the injury-induced deficits have resolved (Sell et al., 2016; Dixon et al., 1987) The results of the six month time point indicate that there are no differences between naïve, sham or injured rats. However, all the rats have been relaxing in their home cages for 6 months, aging and gaining weight. Thus, by the time they are re-tested at six months post-surgery (or equivalent in the case of NAÏVE), they are essentially becoming old and fat and therefore all the groups do not perform as well as they did compared to their baseline Day 0 results."

Minor Concerns:
1. Authors should explain the meaning of score of beam walk and beam balance tests in the section of protocol.

The Beam-Balance scores are defined in the protocol section 3.2 as follows:

> **1** = Shows stable balance (grooms, walks, attempts to climb barrier)
> **2** = Shows shaky balance (grasps sides of beam and/or has unsteady movements)
> **3** = Tries to balance but slips or spins on the beam, hangs on by hugging the beam
> **4** = Tries to balance, but falls after 10 s
> **5** = Hangs over or from the beam and falls off in under 10 s
> **6** = Falls off, making no effort to balance or hang onto the beam

The Beam-Walk is a timed assessment, so there is no score as such. The time it takes the rat to traverse the beam and enter the escape box is recorded in seconds. Three timed trials are conducted and the average of these trials is the outcome for that rat for that day. These processes are described in the protocol Section 4,

2. Some citations are inappropriate; please check them clearly.

The citations have been carefully checked. It would have been helpful if the reviewer would specify which citations s/he felt were inappropriate. We did find that the wrong Morris paper (Morris et al., 1982, *Nature*) was listed, so this has been replaced with Morris & Hagan, 1986 *Q J of Exp Psych*). We hope this is satisfactory.

Reviewer #4:
Major Concerns:
-The limitations of the FPI model should be discussed. The variability in this model is high for example. Further I believe the CCI model is more common in the present times.

We have altered the wording to reflect that the FPI model is one of the popular models, not the most popular model.

-A description of the location of the injury and how that affects behavioral outcomes should also be included.

The details on the site of the injury are available in the references cited. The focus of this paper is how to do behavior testing. A statement mentioning that the site of the injury could alter the outcome of the tests has been added.

-A description of pre training should be included.

The pre-training is described in the protocol section for each of the behaviors that requires pretraining.

-How do you get a total score for each round of 7? Each test (5 in total) only with 0 and 1 as possible scores suggests the formula should be 5*3=15 as the high score.

Possible scores have been added to each section of the neuroscore for clarification.

-For the Beam-balancing test you should specify how long to wait between each trial during training and during experimentation.

The words "allow the rat to rest for 15 s in the home cage between trials" has been added to the Beam Balance section.

Minor Concerns:
There are some typos in the article that should be corrected.
Thank you for pointing these out. We have attempted to rid our manuscript of all typos.