An active avoidance behavioral paradigm for use in a mild closed head model of traumatic brain injury in mice

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GRAPHICAL ABSTRACT

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ABSTRACT

Background: A mild traumatic brain injury (TBI) occurs to millions of people each year. Translational approaches to understanding the pathogenesis of neurological diseases and the testing of the effectiveness of interventions typically require cognitive function assays in rodents.

New methods: Our goal was to validate the active avoidance task using the GEMINI avoidance system in a mouse model of mild closed head injury (CHI).

Results: We found that shock intensity had only a marginal effect on the test. We found that sex was an important biological variable, as female mice learned the task better than male mice. We demonstrate that a single mild CHI in mice caused deficits in the task at four weeks post-injury.

Comparison with existing methods: Active avoidance is a classical conditioning test in which mice must pair the presence of a conditioned stimulus with moving between two chambers to avoid an electric shock. External conditions (i.e., apparatus), as well as inherent differences in the mice, which may not be directly linked to the model of the disease (i.e., sensory differences), can affect the reproducibility of a behavioral assay. Before our study, there was a lack of standard operating procedures and validated methods for the active avoidance behavior for phenotyping mouse models of injury and disease.

Abbreviations: CHI, closed head injury; CS, conditioned stimulus; NOR, novel object recognition; MWM, Morris water maze; TBI, traumatic brain injury; US, unconditioned stimulus

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Conclusion: We offer a method for validating the active avoidance test, and a standard operating procedure, which will be useful in other models of neurological injury and disease.

1. Introduction

Animal models of traumatic brain injury (TBI) are essential for studying mechanisms and testing therapeutic interventions, and a critical step is often demonstrating an effect on an animal’s behavior. This is particularly true in models of mild traumatic brain injury (mTBI) where pathological lesions, such as loss of brain tissue, are, by definition, not part of the model. A recent systematic review of rodent models of mTBI found that the most common assays used to assess cognitive function were the novel object recognition (NOR) task, and the Morris water maze (MWM) (Bodnar et al., 2019). However, NOR and MWM have limitations. The NOR task is difficult to validate, scoring can be subjective, and the discriminative potential is often low because of high variability between subjects. The MWM is time-consuming, and mice are not particularly well-suited for the task (Harrison et al., 2009).

In search of additional behavioral assays, we identified the active avoidance task. First studied in animals in the 1930s (Mowrer, 1939), avoidance behavior is mediated by at least three neuronal systems: cortex, amygdala, and thalamus (Cain, 2019; Diehl et al., 2019; Hormigo et al., 2019; LeDoux et al., 2017). In the active avoidance behavior, a subject is trained to avoid an aversive unconditioned stimulus (US), by associating the conditioned stimulus (CS: light and/or auditory tone) with the foot-shock (US). Over repeated trials, the subject learns to avoid the foot-shock (US) by shuttling between chambers when the warning cue (CS) is presented.

Validation of behavioral protocols is imperative before conducting experiments. Behavioral assays are intrinsically variable. However, proper training, optimization, and consideration of other important biological variables can increase the ability to detect subtle deficits in learning and memory. It is often assumed that testing animals in a behavioral assay should be the easiest part of the experiment. Unfortunately, it is not as easy as it seems on the surface. Many factors influence the sensitivity and reproducibility of a behavioral assay. Standardization of behavioral tests alone does not guarantee the same results across laboratories (Wahlsen, 2001; Wahlsen et al., 2003a, b), as even the sex of experimenters performing the behavioral assay alters outcomes (Sorge et al., 2014). Steps have been defined to ensure the reproducibility and validity of a behavioral assay (Mandillo et al., 2008). These steps include: (1) defining the common data elements of the assay; (2) establishing and following a standard operating procedure; (3) establishing methods to train inexperienced investigators and ensure that those new to the assay can replicate prior results; (4) establish automatic scoring software and criteria to reduce human error and qualitative metrics; and (5) defining methods to validate that the animal can complete the task. We believe that following these steps can improve the reproducibility of TBI studies and beyond. Within, we report the results of our attempts to standardize and validate the active avoidance behavior for use in mouse models of TBI.

2. Materials and methods

2.1. Chemicals

Scopolamine hydrobromide (Sigma, cat. no.6533–68-2), a competitive non-selective muscarinic acetylcholine antagonist, was used to induce memory impairments. It was dissolved in double-distilled water, sterile filtered (VWR North America, cat. # 76012 – 774) and intraperitoneal (IP) injections were done at a dose of 1 mg/kg/ 10 mL. 30 min before trial-1 each day during the 5-day test.

2.2. Animals

This study used male and female C57BL/6J mice (3–5 months old, Jackson laboratory, Bar Harbor, ME, stock number: 000,664). The Institutional Animal Care and Use Committee (IACUC, protocol # 2015–2290) approved all procedures. Experiments were conducted in accordance with the guide for the care and use of laboratory animals, and reporting follows the ARRIVE guidelines. Animals were group-housed (up to 5 per cage) in controlled humidity and temperature environment (T = 22 – 23 °C, Humidity = 43–47 %) and 12/12-h (7 am-7 pm) light/dark cycle with free access to food and water. The experiments were conducted between 7.30 a.m. and 3.30 pm. A total of 114 (50/64 ♀/♂) mice were used in this project. The number of mice used for each experiment is listed in the figures. No mouse was tested in more than one behavioral assay that used a shock stimulus.

The person performing the test handled the mice for three days before the beginning of the experiment. Mice were habituated to handling by allowing the mouse to explore the experimenter’s hands for 1 – 2 min, before being returned to their home cage. Mice were acclimated to the testing room for at least 30 min before the start of the experiment. Mice were assigned randomly to groups before the start of the experiment, and group order of testing was also randomized. Male and female mice were tested in separate shuttle boxes if tested on the same session or in independent cohorts. Each cage contained more than one experimental group. The people conducting the experiments were blinded to the experimental/treatment conditions. The light levels within the testing room were kept between 40 and 70 lx.

2.3. Visual acuity test

A visual reflex test was used to evaluate a mouse’s ability to respond to visual cues. The trial began by suspending a mouse by the base of the tail 25 cm above the edge of a solid surface, then slowly lowering the mouse toward the table without any contact with the vibrissae. The mouse had three seconds to extend its forelimbs and place them onto the table. Each mouse was tested in three consecutive trials. The behavior was scored using a scale between 0 and 2 (Fox, 1965; Metz and Schwab, 2004). An example of the scoring criteria is shown in Fig. 1. A mouse that raised its head, arched its back, and reached to grasp the edge of the table scored a two. A score of one was obtained if the mouse attempted but failed to reach the table. A mouse that showed no response scored a zero on the task. A mouse with an average score over the three trials of 1 or more was considered visually competent and could proceed with the study. A subset of mice that received a CHI were screened in the visual acuity test before surgery and at two weeks post-surgery to evaluate if surgery had an effect on their visual acuity.

2.4. Two-way shuttle box apparatus

The two-way shuttle box (Gemini, San Diego Instruments, USA) consists of two adjacent compartment enclosures of identical dimensions (24 cm-W x20.3 cm-D x20.3 cm-H) with a grid floor made of stainless steel bars spaced 1 cm apart and a removable tray below the grid floor (Fig. 2). Bedding (~2-cm depth; sani-chips, P.J. Murphy forest products, Montville, NJ) was placed in a removable tray below the grid floor of the chamber. Each day prior to testing the first mouse and between individual testing sessions, the apparatus was cleaned with MB-10 solution (100 ppm Chlorine dioxide, C.A.S. # 10049 –04-4) to eliminate smell and urine and decrease the likelihood that the mice would detect the scent of other mice. Also, bedding was mixed up
between animals to reduce odor cues, and accumulation of feces and urine. The grid floor was wiped dry with paper towels. Following each day of testing, the bedding was removed, the tray was cleaned with MB-10 solution and allowed to air dry. The chamber uses 16 photobeams (8 per side) to determine the location of the animal. Non-heating LED lights were located in each chamber. An automatic sliding door separated the two compartments. Gemini’s software (San Diego Instruments, USA) automated control of the two-way shuttle box and scoring of the test.

2.5. Shock sensitivity threshold evaluation test

The sensitivity to shock was tested in the Gemini two-way shuttle box. The mouse was placed in one compartment with the sliding door kept down during the entirety of the test. After 120 s of acclimation, the mouse was exposed to a series of 2 s foot shocks with increasing amperage at 30 s inter-shock intervals. A total of 11 shock intensities were delivered in ascending order (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.20, 0.25 and 0.30 mA). The Gemini’s software controlled the 30 s inter-shock interval and delivery of the shock. Shock intensity was manually changed between trials. A 5-point scale was created based on previous research (Barrot, 2012; Chang et al., 1999; El-Ghundi et al., 2001; Kazdoba et al., 2007) and modified after pilot studies to score the mouse’s response to the foot shock (Fig. 3). After the testing, the mice were returned to their home cage and housing room and were not used in any other behavioral assays.

2.6. Active avoidance

The Gemini shuttle box was used to perform the active avoidance test. Before establishing the protocol used in this publication, several pilot experiments were completed to evaluate the optimal conditioned stimulus and the number of trials. For the conditioned stimulus, we evaluated house light alone, or an auditory tone paired with the cue light (n = 3–4 mice per group). The visual cue was the house light (1864 lx at the center of the compartment) presented for 10 s, in the empty chamber. The auditory signal (72db, 2000 Hz) paired with cue light (18 lx) was presented for 10 s. We found the auditory cue evoked a startle response, in which the mouse shuttled between chambers without receiving the foot-shock. As a result, we decided to use the house light as the CS in our experiments. The second series of pilot studies were conducted to determine the total number of trials per day and the number of testing days. We used three different conditions: 1) 100 trials per day for 2 days; 2) 50 trials per day for 5 days; 3) 25 trials per day for 5 days (n = 3–10 mice per group), all at a shock intensity of 0.2 mA. Mice receiving 100 trials/day avoided 40 % of trials on day 1 and reached 90 % by day 2. Mice receiving 50 trials per day reached 80–90 % of avoided trials by day 4 and plateau on day 5. Mice receiving 25 trials per day avoided 20 % of trials on day 1 and reached 80 % avoided trials by day 5, with no plateau in the learning curve. From this pilot study, we selected 50 trials per day for 5 days.

What follows is the five-day active avoidance protocol used in our studies. We have also provided a step-by-step standard operating procedure available on www.protocols.io (dx.doi.org/10.17504/protocols.io.bbu6inze). Following the 30 min acclimation to the testing room, a day of testing began with the mouse being gently placed in one of the compartments of the shuttle box, free to explore both sides of the dark chamber for 300 s (Fig. 4A). After the 300 s acclimation period, the house light (CS) was presented for 10 s in the unoccupied chamber (Fig. 4B). A foot shock (0.1, 0.2 or 0.3 mA, 2 s) (US) was then delivered.
through an electronic scrambler. The result of the trial period has three possibilities; 1) avoided if the mouse crossed to the opposite compartment before the onset shock, 2) escaped when the animal crossed to the other side of the chamber during the delivery of the shock, 3) no response when the mouse remained in the original compartment receiving 2 s of shock (Fig. 4C). If the animal crosses into the opposite compartment, or after a total of 12 s, both the light and the shock go off and the inter-trial interval (ITI) begins (Fig. 4D). During the ITI, the mouse can move freely between compartments for 30 ± 0.5 s, defined at random by the Gemini software. After the ITI elapses, a new trial will automatically begin (i.e., Fig. 4B), for a total of 50 trials a day for 5 consecutive days. On each day of testing, the start chamber was alternated.

The Gemini software recorded the following data points that we evaluated in our analysis. During the 300 s habituation, the number of times the animal crossed between chambers was recorded. In the testing phase, the following behaviors are recorded: if the mouse avoided the shock, escaped the shock, or failed to shuttle between boxes to avoid the shock. For our analysis, we used the percentage per day of successfully avoided trials where the mouse did not receive a shock as our endpoint measure. We also recorded latency to escape, i.e. how long it took the mouse to shuttle between the boxes after the cue light (CS) was presented. Finally, during the inter-trial intervals we evaluated the number of times the mouse crossed between the two chambers.

2.7. Closed head injury (CHI) procedure

Mice were anesthetized with 5% isoflurane before the surgery, and the mouse was kept under anesthesia with continuous inhalation of isoflurane (3–5%, 1 L/min) through a nose cone during surgery.

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Fig. 3. Illustration of the shock sensitivity scale. A five-point scale was used to evaluate a mouse’s response to an electric shock. No change in behavior during the 2 s shock was scored as a 0. A small movement or flinch, (i.e., a shudder; shaking of shoulders, feet, or tail; movement of tail to point upwards) received a score of 1. If the mouse hopped or ran in any direction, it was given a score of 2. An audible squeak along with a hop or run received a score of 3. A vocalization along with a jump that lifts all four paws from the surface of the bars was scored as a 4.

Fig. 4. Step-by-step illustration of the active avoidance procedure. (A) During the 300 s habituation period the mouse is free to explore both sides of the dark chamber. (B) A cue is then presented (house light) for 10 s in the unoccupied chamber. (C) There are three possible outcomes. 1) The mouse steps into the safe compartment before the shock is delivered; 2) the mouse crosses into the opposite side of the chamber during the delivery of the shock; 3) the mouse does not cross into the other chamber. Light and shock go off as soon as the mouse crosses into the opposite compartment, or after a total of 12 s (10 s of CS + 2 s of US) and the inter-trial interval (ITI) begins. (D) During the ITI, the mouse can move freely between compartments for 30 ± 5 s, defined at random by the Gemini software. After the ITI elapses, a new trial will automatically begin (i.e., back to step B), for a total of 50 trials a day for 5 consecutive days.
Bodyweight was recorded, the head was shaved, sterilized with 70 % ethanol and 4% lidocaine cream was applied before surgery. Ear bars were used to secure the animal in a digital mouse stereotaxic instrument (Stoelting; Wood Dale, IL, USA), and a midline sagittal scalp incision was made, exposing the coronal and sagittal sutures of the skull. To diffuse the force of the impact away from the ear bars, a 1 mL latex pipette bulb was placed under the head of the mouse and filled with water. The stereotaxic electromagnetic impactor (Impact one, Leica; Buffalo Grove, IL, USA) was equipped with a 5.0 mm flat steel tip and able to deliver a single controlled midline impact (coordinates: mediolateral, 0.0 mm; anteroposterior, 1.5 mm), at a 1.0 mm depth, with a velocity of 5.0 ± 0.2 m/s, and a dwell time of 100 ms. Sham-injured mice received identical surgical procedures as the CHI group, but no injury, for example, can cause hyperalgesia (Rowe et al., 2016). Therefore, a first step in validating the active avoidance behavior was to develop a standardized method to calculate group differences in response to a foot shock. Following prior published examples (Barrot, 2012; Chang et al., 1999; El-Ghundi et al., 2001; Kaczuba et al., 2007), and refinement through pilot experiments, we developed a five-point ordinal scale that we used to calculate a shock response score (Fig. 3).

To test our shock response score, we first compared male and female C57BL/6 J (B.6) mice to test sex-based differences in shock response score. As shown in Fig. 5A, we found the response curves between the male and female B.6 mice almost entirely overlap, with no statistical difference between groups. At the 0.25 mA shock intensity, there was a difference between the male and female B.6 mice (t = 3.03; p = 0.009), but we have not attempted to determine the reproducibility of this finding. We next wanted to define three discreet shock intensity levels, to see if they had an effect on learning. We chose 0.1, 0.2, and 0.3 mA because a similar response was seen in both the male and female mice, and there was nearly complete discrimination in shock response score for the three different intensities of shock (Fig. 5B).

3. Results

3.1. Shock sensitivity threshold evaluation

Active avoidance behavior requires a subject to pair a conditioned stimulus (CS; light or auditory tone) – also called a warning signal (WS) – with the unconditioned stimulus (US; foot shock) to avoid future adverse stimuli. Just as it is important to assess the ability of mice to swim or be able to use spatial cues to navigate the Morris water maze, we hypothesized that it would be important to determine if there are inherent differences between animals in how they perceive the aversive stimuli. This is particularly true in the case of a foot shock where a brain injury, for example, can cause hyperalgesia (Rowe et al., 2016). Therefore, a first step in validating the active avoidance behavior was to develop a standardized method to calculate group differences in response to a foot shock. Following prior published examples (Barrot, 2012; Chang et al., 1999; El-Ghundi et al., 2001; Kaczuba et al., 2007), and refinement through pilot experiments, we developed a five-point ordinal scale that we used to calculate a shock response score (Fig. 3).

At the start, mice were excluded if they failed a general health assessment (Sukoff Rizzo et al., 2018). Additional exclusion criteria for the CHI model included skull fractures or prominent vestibular disturbances, including tilting of the head, or slight spin when lowered (Sukoff Rizzo et al., 2018). No mice were excluded from the study following the CHI. In the current study, the visual acuity was tested only in a small subset of the mice, with no mice failing the test. Young adult C57BL/6 J mice are known to have intact vision compared to other common inbred mouse strains (Wong and Brown, 2006). Images shown in Fig. 1 were from an example mouse with known visual impairments that were not part of the study. In our experience, the visual acuity test can be done repeatedly, and thus may be useful during different points in the study. No mice were excluded for visual impairments. For the active avoidance behavior, a mouse that failed to be shocked on the first day should be excluded from the study. Also, hyperactivity should be evaluated as this could impact the interpretation of active avoidance behavior. A mouse with hyperactivity that was found to be an outlier from the experimental group should be considered for exclusion. We did not exclude any mice from the active avoidance task.

2.9. Statistical analysis

JMP Pro software version 14.0 (SAS institute, Cary, NC, USA) was used for statistical analysis. A standard least square repeated-measures model was used to consider day of testing, the independent variable, and the interaction of day of testing the independent variable. Differences between mean were considered significant at α = 0.05. Graphs were generated using GraphPad Prism version 8.0. Values are expressed as mean ± SEM, unless otherwise noted. Scatter plots represent individual mice. Mouse numbers used are indicated in the figure or figure legend.
use a shock scale to adjust the level of shock between groups, so that responses between groups would be equivalent.

To compare differences between groups we took two approaches using either the percent of the 50 trials each day where the mouse successfully avoided the shock (Fig. 5C) or the average latency to escape per day (Fig. 5D). With both measures, we found that the 0.1 mA and 0.2 mA appeared to learn the task better than the 0.3 mA groups; however, the difference between the groups for latency to avoid shock or the percent of avoided trials was not statistically different. These results suggest that the intensity of the shock, within the range tested, did not contribute significantly to the ability of the mice to learn the task.

When we included the sex of the mice in the statistical model, we found an effect of sex (p < 0.001) and an interaction of sex and shock intensity (p = 0.01) for the percent avoided trials. However, for latency to escape we found an effect of sex (p < 0.0001), but not an interaction of sex and shock intensity (p = 0.1233) (Fig. 5C-D).

To understand the effect of sex on active avoidance behavior, we disaggregated the data by sex and compared the learning curves for the three different shock intensities (Fig. 6). We found with sex a slightly different pattern in the ability to learn the active avoidance task in response to different shock intensities. While the male mice learned best at a 0.1 mA shock intensity, the females learned the worst at the 0.1 mA shock intensity. Overall, for both the percentage of avoided trials (Fig. 6A, C) or latency to escape (Fig. 6B, D) no statistical difference was found between shock intensities for either the female or male mice. These results again suggest that shock intensity had only a marginal effect on the ability of the mice to learn the task.

Comparing the male versus the female mice at each of the three different shock sensitivities allowed us to discern the sex by shock intensity interaction (Fig. 7). At 0.1 mA, we found a complete overlap in the learning curves for the male and female mice for the percent of avoided trials (p = 0.0005, Fig. 7C) and the latency to escape the shock (p = 0.0007, Fig. 7D). The separation between males and females became greater with the 0.3 mA shock intensity, with females again outperforming the male mice, as determined by the percentage of avoided trials (p < 0.0001, Fig. 7E) and the latency to escape the shock (p < 0.0001, Fig. 7F). Results in Figs. 6 and 7 show that sex must be considered as an important biological variable in the active avoidance task as female mice learn the active avoidance task better than male mice regardless of shock intensity.

While shock intensities ranging from 0.1 mA–0.3 mA resulted in similar learning curves, we selected a shock intensity of 0.2 mA as our standard shock intensity level for the remainder of the studies. Our results demonstrate that a shock response score of 1–4 results in a similar learning curve. A shock intensity of 0.2 mA was in the middle of the shock response score range; thus, allowing for inter-mouse variability in the perceived shock intensity while staying in our known effective range of shock levels.

3.3. Validation of active avoidance behavior using scopolamine

Our next step in validating the active avoidance behavior was to use a pharmacological approach to impair memory and confirm that we could detect a cognitive impairment if one were present. Scopolamine is known to cause cognitive impairment and is recommended as a positive control agent for many different behavioral assays of learning and memory (Sukoff Rizzo and Silverman, 2016). Scopolamine-treated mice have been shown to be impaired in the active avoidance task compared to vehicle-treated mice (Vinader-Caerols et al., 1996). Scopolamine (1 mg/kg, i.p.) or vehicle, was given 30 min prior to the start of testing for each mouse on each day. As previously reported (Rosis et al., 1980), we observed hyperactivity in the scopolamine mice, as can be seen by the high number of times the mice on scopolamine shuttle between the two boxes during the one-minute delay between trials (Fig. 8A, p < 0.0001). The scopolamine-induced hyperactivity resulted in a significantly higher number of avoided trials as a result of the shuttling between the two compartments not paired to the cue-light on day 1 (Fig. 8B, p < 0.0001, one-way ANOVA for day 1). As previously reported, the mice treated with scopolamine developed a behavioral tolerance to the hyperactivity effect with the average number of inter-trial crossings converging with those of vehicle-treated mice by day 5 (Fig. 8A) (Rosis et al., 1980). Because of the artificially high avoided trials on day 1, we statistically compared the vehicle and scopolamine treated mice from day 3–5, and we found an effect of treatment (p < 0.0001). While scopolamine may be a suboptimal positive-control drug for active avoidance behavior, because of the hyperactivity, we still think that it is a good candidate to use for training inexperienced investigators as the effects seen with the drug are robust.

3.4. A single CHI caused impairments in the active avoidance behavior at 4-weeks post-injury

A single mild CHI has been shown to cause impairments in cognitive behavioral assays, but no study has reported deficits in the active avoidance task following a single mild CHI (Bodnar et al., 2019). We found no difference between sham or CHI mice at 4 weeks post–CHI in the shock sensitivity test. At the 0.2 mA shock intensity, all mice, regardless of sex or injury status, scored a 3 on the shock sensitivity test (n = 3 per group). Male mice that received a CHI were found to have worse performance on the active avoidance task, both in terms of % avoided trials (Fig. 9A, p = 0.0005) or in the latency to escape the foot-shock (Fig. 9B, p = 0.0035). In female mice, a CHI also was found to reduce the % avoided trials (Fig. 9C, p < 0.0001) and in the latency to escape the foot-shock (Fig. 9D, p < 0.0001). As found in naïve mice (Fig. 7), female mice overall had better performance in the avoidance task compared to the male mice (p < 0.0001, % avoided). Tukey’s
posthoc analysis showed sham-female mice did better on the task than sham-male mice ($p < 0.0001$, %avoided), but there was no statistical difference between CHI-female-mice, and CHI-male-mice ($p = 0.089$, %avoided). No injury effect was seen in male or female mice for the number of crossing during habituation on day 1 or inter-trial crossing. These results support that hyperactivity, or other motor impairments were not the cause of the CHI-induced deficits in the avoidance task.

### 4. Discussion

Within, we sought to expand the behavioral tests used in models of mild CHI to include associative learning-based tests (Bodnar et al., 2019). We identified the active avoidance behavior as a novel behavioral assay for use in models of mild closed head TBI, which had the potential to be standardized and automated. Female mice were found to perform the test better than male mice. Differences in the intensity of foot shock were not predictive of performance on the active avoidance task. Finally, the active avoidance task was found to be sensitive to detect cognitive deficits associated with a mild CHI at one-month post-injury. Overall, our results provide a needed standardized behavioral assay to measure cognitive deficits in models of mild TBI and will provide a framework for standardizing and validating active avoidance in other disease-relevant animal models.

Behavioral assessment in disease-relevant animal models, following therapeutic interventions or manipulation of proposed biological mechanisms, for instance, can provide essential translational relevance for a study. Behavioral assays are all too often conducted without careful consideration for standardizing an operating procedure and identifying relevant biological variables that may confound the study results. Behavioral assays can also be subjective, labor-intensive, and create a bottleneck in an experimental workflow. Mandillo et al., 2008, previously established five criteria for increasing reproducibility and validity of a behavioral assay. We used these five criteria while designing...
our study and reporting our results. First, we defined the important common data elements for the assay, including the cues, shock intensity, number of trials and testing days, time of day, and testing apparatus. Second, we provide a standard operating procedure, along with a protocol, step-by-step lab procedure. Third, we propose scopolamine as a positive control and provide expected results for scopolamine to ensure that those new to the assay can replicate prior results. Fourth, automated scoring is standard for the commercial two-way shuttle box (Gemini, San Diego Instruments) used in our study, which reduces human error and provides quantitative scoring. Finally, to validate that the animal can complete the task, we provide the general health assessment (Sukoff Rizzo et al., 2018), shock sensitivity test, and visual acuity test.

An advantage of the active avoidance test is that shuttle boxes are commercially available, with automated scoring. We believe that the active avoidance test could easily complement other behavioral assays. A detailed comparison of the active avoidance task to other similar behavioral assays can be found in two recent reviews (Cain, 2019; Diehl et al., 2019). Mice tested in a behavioral battery involving the use of shock should be tested from the least stressful and invasive test to the most stressful, and wash-out time between each test should be considered (Paylor et al., 2006; Voikar et al., 2004). For example, we routinely complete a cognitive-behavioral battery, that would include Y-maze, radial arm water maze, and then active avoidance. Active avoidance could also be a substitute behavior for animals with motor impairments that cannot swim (like in MWM), as the active avoidance task requires the animal only to walk a short distance.

At the onset, we hypothesized that sensitivity to the electric shock would influence the animal’s ability to learn the active avoidance task. That is, if a mouse had a lower sensitivity to the electric shock, the negative reinforcement might not be sufficient to induce a response. Or, if the mouse had increased sensitivity to the electric shock, there would be interference in learning the assay. However, after reviewing the literature, we found only a few studies that tried to test a correlation between relative shock intensity and learning a task. One of the first studies, performed in 1980 in cats, found no relationship between stimulus intensity and learning (Werka, 1980). In agreement with our results, no association was found between learning in a fear conditioning test and reflex to a shock, as measured by a finch-jump or a tail-flick test (Lehner et al., 2010). On the opposite side, rats that are more vulnerable to pain, according to their sensitivity (low-, medium- or high-sensitivity group) to the finch-test, had increased freezing behavior in conditioned fear test (Lehner et al., 2006). Further, in the passive avoidance task better performance was obtained using a high shock intensity (Vinader-Caerols et al., 1996). Despite the lack of a direct correlation between shock intensity and performance on the active avoidance task, we still believe that differences seen on the shock sensitivity test could be a relevant confounding biological variable at the group level. Given the wide range of shock intensities that produced overlapping learning curves, it appears that the active avoidance assay remains a reliable cognitive test across a range of shock intensities. We would not recommend using the active avoidance behavior if profound group differences were seen on the shock sensitivity test (for instance, a difference of 3 or more points on the shock sensitivity scale).

A major finding of our study was that female mice outperformed the male mice in the active avoidance task, which is in agreement with several prior studies in rats (Aguilar et al., 2003; Dalla and Shors, 2009; Denti and Epstein, 1972; Saavedra et al., 1990; van Haaren et al., 1990), and mice (Sprovitz et al., 2013). Interestingly, previous studies report in a passive avoidance test that female rats performed poorer than males because of their higher exploratory activity (Denti and Epstein, 1972; van Oyen et al., 1980). The proposed mechanism for the sex-based differences in the active avoidance task includes anxiety (Fernandes et al., 1999), basal activity (Aguilar et al., 2003; Dalla and Shors, 2009; Denti and Epstein, 1972; Fernandes et al., 1999), learning strategy, or involvement of different brain regions and networks (van Haaren et al., 1990). While it is beyond our current scope to determine the causes of the sex-related difference in the active avoidance task, we do believe that it is necessary to consider sex as an important biological variable.

In both male and female mice, we found a CHI reduced performance in the active avoidance task. The effect of the CHI appeared to be greater in the female mice compared to the male mice, which is in agreement with our recent study that found larger CHI-induced deficits in female mice (Lyons et al., 2018). However, as naïve or sham-injured female mice are able to outperform their male counterparts on the avoidance task, it is not possible to contribute the larger CHI-deficits in female mice solely to the CHI inducing more damage to the female brain. We aged-matched and did not weight-match the male and female mice in our study, as age is often a biological variable in our studies. Biomechanical differences associated with the same impact forces on a smaller object could also contribute to the male and female differences observed after CHI in our study. Given the paucity of studies comparing the effects of CHI between males and females, our studies highlight the importance of including both sexes in TBI experiments.

One limitation in our study is that CHI mice were only tested at 0.2 mA in active avoidance test, that shock intensity was chosen according to the results obtained from the characterization of naïve mice tested in active avoidance task. We do not predict that using a lower or higher shock intensity could significantly affect the performance output, as CHI, sham, and naïve mice showed no difference in the shock sensitivity test. An exciting future direction will be evaluating CHI’s effect on the extinction of the learned behavioral and reversal training to evaluate the effects of CHI on cognitive flexibility.

In summary, our study demonstrated that a single mild CHI in mice could cause persistent deficits in cognitive function. We also demonstrate the importance of considering sex as a biological variable. Finally, we believe our most significant contribution is to provide a standard operating procedure and methods to validate the active avoidance behavior for phenotyping other mouse models. TBI is highly heterogeneous, and depending on the TBI model (mild, moderate, severe) adjustments to our protocol may be needed; however, following the steps described within this report should ensure the successful implementation of this behavior in multiple TBI models.

Author contributions

TM, and ADB designed the study. TM, HS, JBW, KNR, and ADB performed the research. TM and ADB wrote the paper.

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CRediT authorship contribution statement

Teresa Macheda: Conceptualization, Methodology, Investigation, Writing - original draft. Henry C. Snider: Investigation, Writing - review & editing. James B. Watson: Investigation, Writing - review & editing. Kelly N. Roberts: Investigation, Writing - review & editing. Adam D. Bachstetter: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing - original draft.

Declaration of Competing Interest

None.
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