The Effect of Injury Severity on Behavior: A Phenotypic Study of Cognitive and Emotional Deficits after Mild, Moderate, and Severe Controlled Cortical Impact Injury in Mice

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Abstract

Traumatic brain injury (TBI) can cause a broad array of behavioral problems including cognitive and emotional deficits. Human studies comparing neurobehavioral outcomes after TBI suggest that cognitive impairments increase with injury severity, but emotional problems such as anxiety and depression do not. To determine whether cognitive and emotional impairments increase as a function of injury severity we exposed mice to sham, mild, moderate, or severe controlled cortical impact (CCI) and evaluated performance on a variety of neurobehavioral tests in the same animals before assessing lesion volume as a histological measure of injury severity. Increasing cortical impact depth successfully produced lesions of increasing severity in our model. We found that cognitive impairments in the Morris water maze increased with injury severity, as did the degree of contralateral torso flexion, a measure of unilateral striatal damage. TBI also caused deficits in emotional behavior as quantified in the forced swim test, elevated-plus maze, and prepulse inhibition of acoustic startle, but these deficits were not dependent on injury severity. Stepwise regression analyses revealed that Morris water maze performance and torso flexion predicted the majority of the variability in lesion volume. In summary, we find that cognitive deficits increase in relation to injury severity, but emotional deficits do not. Our data suggest that the threshold for emotional changes after experimental TBI is low, with no variation in behavioral deficits seen between mild and severe brain injury.

Keywords: elevated-plus maze; forced swim test; Morris water maze; prepulse inhibition; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) can cause a variety of cognitive, emotional, and behavioral problems that can occur either individually or in combination. The heterogeneous nature of TBI has made it difficult to identify predictive factors for the type of long-term effects that may arise in survivors, but studies comparing outcome after mild, moderate, and severe TBI in humans have found that injury severity plays a role in determining the type and intensity of neurobehavioral impairments experienced after injury. Deficits in cognitive domains, such as memory, attention, and information-processing speed, are increased as a function of injury severity, with severe TBI causing greater and longer-lasting cognitive deficits than mild or moderate TBI (Jamora

et al., 2012; Levin et al., 1987; Rapoport et al., 2002; Satz et al., 1998). However, the relationship between injury severity and the development of emotional problems is less clear. Many of the same studies that identified a direct relationship between injury severity and cognitive deficits also evaluated emotional outcome after mild, moderate, and severe TBI, and found that unlike cognitive impairments, emotional problems such as anxiety and depression were not related to injury severity (Jamora et al., 2012; Levin et al., 1987; Rapoport et al., 2002; Satz et al., 1998). In fact, more than one-third of patients in each of the mild, moderate, and severe injury groups showed evidence of anxiety and depression (Rapoport et al., 2002), supporting the finding that suffering a TBI increases the risk of developing depression, regardless of injury severity (Bombardier et al., 2010). One study did report a stepwise

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increase in depressive symptomatology with increasing injury severity, but this failed to reach significance (Satz et al., 1998). Grouped by Glasgow Outcome Score (GOS), this same study found that 90% of those with clinically significant depression were in GOS categories 3 and 4 (severe and moderate disability, respectively), while only one case of depression was found in each of the control and the GOS 5 (good recovery) groups (Satz et al., 1998). However, other studies have found the opposite, with 100% of the mild TBI participants scoring in the clinically significant range for depression, compared to 42% in the severe and 28% in the very severe injury groups (Draper and Ponsford, 2009). Although reported rates of comorbid anxiety in patients exhibiting major depression after TBI are as high as 60% (Bombardier et al., 2010), and 76.7% (Jorge et al., 2004), other groups have found that clinically-significant anxiety was less common overall (25% in the severe and very severe injury groups), and completely absent in the mild group, which had the highest incidence of depression (Draper and Ponsford, 2009).

Attempts to make cross-study comparisons are complicated by several factors: (1) lack of standardization between neurobehavioral tasks administered by different research groups; (2) differences in the criteria used to categorize injury severity; and (3) the timing of post-injury evaluation. Perhaps the largest confounding factor in these studies is the dependency on self-reporting of symptoms by TBI patients, which has been shown to be affected by injury severity, injury-induced impairments in self-awareness, and the presence of post-traumatic stress disorder, anxiety, and depression (Draper and Ponsford, 2009; Jamora et al., 2012). A recent study also found that over half its participants had pre-injury psychiatric disorders, and 75% of those with pre-injury disorders had post-injury disorders (Gould et al., 2011). Thus, psychiatric history could have an invisible effect on study outcome that is often not taken into consideration. These unavoidable problems inherent to human studies make it difficult to determine a direct relationship between injury severity and cognitive and emotional problems. In this study we have turned to a scalable experimental model in an attempt to address this question.

Controlled cortical impact (CCI) injury in mice is a widely-used animal model for preclinical evaluation of therapeutics, and successfully captures many of the key features of human head trauma, including lesion development (Loane et al., 2009), necrotic and apoptotic cell death (Newcomb et al., 1999), and cognitive deficits (Fox et al., 1999; Loane et al., 2009). The Morris water maze is the current gold standard for assessing hippocampal-dependent cognitive function, and has been used to show that cognitive deficits increase as a function of injury severity after CCI (Markgraf et al., 2001), and fluid percussion injury (Smith et al., 1991). Only recently have researchers begun investigating emotional problems such as anxiety (Chauhan et al., 2010; Hogg et al., 1998; Jones et al., 2008; Pandey et al., 2009; Schwarzbald et al., 2010; Zohar et al., 2011), and depression (Jones et al., 2008; Milman et al., 2005, 2008; Schwarzbald et al., 2010; Shapira et al., 2007) after experimental TBI, and they have found mixed results. Inconsistencies between studies may stem from differences in injury type and severity, behavioral tasks, and time between injury and evaluation. There are no studies that evaluate both cognitive function in the Morris water maze and anxiety- and depressive-like behaviors after injury in the same animals, to

determine if there is comorbidity between these conditions, or that simultaneously investigate the impact of injury severity on the appearance and severity of these deficits.

Therefore, the purpose of this study was to investigate the comorbidity of cognitive and emotional problems after experimental TBI, and to determine whether cognitive and emotional problems increase as a function of injury severity. To do this we evaluated a variety of cognitive and emotional behaviors in the same cohort of mice after receiving sham, mild, moderate, or severe CCI.

Methods

Controlled cortical impact injury

All procedures were carried out in accordance with protocols approved by the Georgetown University Animal Care and Use Committee. CCI injury was induced using a Leica Impact One Stereotaxic Impactor device. This device uses electromagnetic force to produce an impact velocity with speed, depth, and dwell time all being individually manipulated to produce injuries of different severities. For the present study we used an impact velocity of 5.25 m/sec, a 3.5-mm-diameter impounder tip, and a dwell time of 0.1 sec. We produced injuries of increasing severity by changing the impact depth: 1.5 mm (mild), 2.0 mm (moderate), and 2.5 mm (severe). Adult 3-month-old male C57BL/6J mice were anesthetized with isoflurane (induction at 4% and maintenance at 2%) evaporated in oxygen and administered through a nose mask. Anesthesia depth was monitored by assessing respiration rate and pedal withdrawal reflexes. The mice were placed on a custom-made stereotaxic frame with a built-in heating bed that maintains body temperature at 37°C. The head was mounted in the stereotaxic frame, and the surgical site clipped and cleaned with Nolvasan® and ethanol scrubs. A 10-mm midline incision was made over the skull, and the skin and fascia were reflected to make a 4-mm craniotomy on the central aspect of the left parietal bone. The impactor tip of the injury device was lowered to the surface of the exposed dura until contact was made, and the impactor tip was retracted and lowered by the desired injury depth (1.5, 2.0, or 2.5 mm) before inducing impact. After injury, the incision was closed with staples, anesthesia was terminated, and the animal was placed in a heated cage to maintain normal core temperature for 45 min post-injury. Sham injury consisted of exposure to anesthesia, stereotaxic mounting, skin and fascia reflection, and incision closing with staples. We used 10 mice in each group; however, one mouse in the 1.5-mm (mild) injury group had severe seizures starting 25 min after injury and was euthanized, reducing the number of the mild injury group to 9 mice. Staples were removed 10 days post-injury under isoflurane anesthesia.

Behavioral testing

The mice were housed in a temperature-controlled room with a 12-h light/dark cycle and fed *ad libitum*. All behavioral testing was conducted during the light cycle phase and in enclosed behavior rooms within the housing room. The mice were placed in behavior rooms 30 min for acclimation prior to the onset of behavioral testing. As the design of this experiment was intended to test for comorbid cognitive, motor, and neurobehavioral deficits, the same mice had to be used for all

FIG. 1. Description of experimental procedures. Detailed timeline outlining the timing of all behavioral tests and experimental procedures, beginning day of surgery (day 0). (TF, torsoflexion; MWM, Morris water maze; PPI, prepulse inhibition; OF, open field; EPM, elevated-plus maze; FST, forced swim test; TBI, traumatic brain injury).

of the behavioral tests, and so underwent multiple behavioral tests (Fig. 1 shows a timeline of experimental procedures). Mice were exposed to contralateral torsoflexion testing on days 1, 3, 7, 14, and 21 post-injury (duration 10–30 sec). Morris water maze testing was done from days 15–19 post-injury (maximum swim time was 4 trials of 90 seconds each, with at least 1.5 h between tests). On day 20 they underwent acoustic startle (40-min test), and on day 21 the open-field (5 min), elevated-plus maze (5 min), and forced swim test (6 min) were all performed in the order listed, and each mouse had at least a 2-h break between tasks. On days 22–26 the mice were returned to the Morris water maze for the reversal trials with maximum trial duration as before. Investigators that were blinded to injury severity performed all behavioral testing.

Morris water maze

Spatial learning and memory deficits were evaluated on days 15–19 and 22–26 after CCI using the Morris water maze paradigm as we have described previously (Loane et al., 2009), with additional modifications. Briefly, the water maze apparatus consisted of a 4-foot-diameter pool (San Diego Instruments, San Diego, CA) filled with water maintained at 25°C and made opaque with white paint. Extra-maze visual cues were hung on the walls surrounding the pool and a hidden platform (4 inches in diameter) was submerged 1 cm below the surface of the water. Initial training was conducted on days 15–18 after injury and consisted of four trials per day. The mice were introduced into the pool at one of four entry points, with every entry point used over the course of the day. The location of the platform remained constant throughout the entire training period. The mice were given 90 sec to locate the platform, and remained on the platform for 10 sec before being removed. Mice that did not locate the platform within 90 sec were placed on the platform for 10 sec before removal from the maze. Tracking software (ANYMaze; San Diego Instruments) was used to record latency to find the platform, swim speed, and swim path. The average latency and swim speed was recorded for every trial. On the fifth day of testing (day 19 after injury), a probe trial was conducted in which the platform was removed and the time spent in each quadrant was recorded over one 90-sec trial. This training and probe trial paradigm was repeated 3 days later (days 22–26 after injury), with the platform moved to a new location in the quadrant opposite that of the original location (platform reversal).

Acoustic startle response and prepulse inhibition

We evaluated acoustic startle response (ASR) and prepulse inhibition (PPI) of the acoustic startle response 20 days fol-

lowing injury or sham-injury. ASR was measured as the whole-body response to a startling (i.e., 120-dB) auditory stimulus. PPI is defined as a reduced magnitude of the startle response; this occurs in subjects of different species whenever a weak prepulse precedes a startling stimulus (pulse). ASR and PPI were assessed using the SR-LAB startle response system (San Diego Instruments), as we have previously described (Bhardwaj et al., 2012; Forcelli et al., 2012a, 2012b; Olmos-Serrano et al., 2011). The mice were placed in a clear, non-restrictive acrylic glass cylinder (3.8 cm in diameter) resting on a platform inside a ventilated and illuminated chamber. A high-frequency loudspeaker inside the chamber produced both a continuous background noise of 70 dB and the various acoustic stimuli. The whole-body startle response of the mouse causes vibrations of the acrylic glass cylinder, which are converted into signals by a piezoelectric accelerometer attached to the platform. The signals were digitized, rectified, stored, and analyzed using SR-LAB software.

The test session consisted of a background noise (70 dB) that was presented alone for 5 min (acclimation period), and then continued throughout the session. All sound pressure levels (SPL) were calibrated using a standard SPL meter (model 33-2050; Radio Shack, Ft. Worth, TX) set to the dB(A) scale. After the acclimation period, there were five presentations of a startle-inducing 120-dB broadband noise pulse lasting 30 msec (“pulse alone”) to habituate the animals to testing in order to achieve stable baseline startle responses. These trials were excluded from data analysis. After habituation, the mice were presented with “pulse alone” trials and “prepulse + pulse” trials, with prepulses of 3, 6, 9, and 15 dB above background noise. In the “prepulse + pulse” trials, the prepulse (30 msec) and the pulse (30 msec) were separated by one of the three inter-stimulus interval (ISI) lengths: 50, 100, and 200 msec (onset to onset). Animals were tested on a total of 65 trials (5 pulse alone trials and 5 of each of the prepulse trials) in the session. Trials were presented in a pseudorandom order; in no case did two trials of the same type occur sequentially. An average of 15 sec (with a range of 5–30 sec) separated the trials. Prepulse inhibition was defined as $[1 - (\text{startle amplitude on prepulse trials} / \text{startle amplitude on pulse alone trials})] \times 100$.

Elevated-plus maze

The elevated-plus maze was used to assay anxiety-like behavior 21 days after injury, as previously described (Forcelli and Heinrichs, 2008; Forcelli et al., 2012b), with modifications. The apparatus (San Diego Instruments) consists of a 26-inch-long cross-shaped platform made of non-porous white plastic elevated 15 inches above the ground. One set of arms is

enclosed with walls (closed arms), and one set is exposed (open arms). A spotlight was positioned overhead to provide 750-lux illumination over the center of the maze and the exposed open arms. Mice have a natural preference for dark enclosed spaces, so changes in behavior are detected by quantifying the time spent in and the number of entries into open arms (Pellow et al., 1985; Walf and Frye, 2007). Mice were placed at the center junction of the maze facing an open arm and given 5 min to explore. The number of entries into each arm, the time spent in each arm, and the total distance traveled in the maze (and in each individual arm) was tracked using the video camera positioned over the maze and analyzed using ANYMaze software. The software tracked the center point of the mouse body, and mice were considered to have entered an arm when their center point crossed into the arm.

Forced swim test

Depression-like behavior was assayed 21 days after injury using the Porsolt mouse forced swim test (FST), as we have described previously (Harkin et al., 2004). In the FST a mouse is placed in a 2000-mL glass beaker filled with 7–8 cm of water (25°C) for 6 min with no means of escape. The absence of escape-oriented behaviors such as swimming, jumping, rearing, sniffing, or diving is indicative of behavioral despair, and the time spent immobile was recorded during the last 4 min of the test by an investigator blinded to group status.

Body curl test for contralateral torso flexion

Neurological function was assessed after graded CCI using a modified version of the elevated body swing test. This test was found to be particularly sensitive to detecting unilateral lesions of the substantia nigra, and assessing hemiparkinsonism following unilateral injection of 6-hydroxydopamine into this region (Borlongan and Sanberg, 1995). Contralateral torso flexion is normally one component of multi-component point-based neurological examinations. Here we used it as a stand-alone test, during which mice were hand-suspended by the tail by a blinded investigator and rated for degree of torso flexion from vertical towards the contralateral injury side. A numerical scoring system was developed based on the degree of contralateral body curl: absent (1), mild (2), moderate (3), and severe (4). Normal mice hang vertically without flexion and thus deviate 0° from vertical (rating of 1). Flexion of the torso 22.5° from vertical was rated a 2 (mild); flexion between 22.5° and 45° from vertical was rated 3 (moderate); and flexion of the torso 45° or more and with/or without grasping of the hindlimbs by forelimbs was given a rating of 4 (severe). Contralateral torso flexion was assessed 1, 5, 7, 14, and 21 days after injury.

Open-field testing

Gross motor ability and exploratory behavior was assessed 21 days after injury using the open-field test as we have previously described (Loane et al., 2009). Briefly, the mice were placed in the center of a white novel arena (3 feet in diameter) with 750-lux illumination from bright lights positioned overhead and given 5 min to explore. Mice with reduced levels of anxiety have been shown to spend significantly more time in the center of a novel arena. Mice were tracked with

ANYMaze software and the speed and total distance traveled were measured for each group, as was the time spent in the inner area of the maze compared to time spent in the outer area of the maze. The boundary of the inner area of maze was defined as being 6 inches away from the outer wall.

Lesion volume analysis

Mice were anesthetized and transcardially perfused with ice-cold saline and 4% paraformaldehyde at the completion of behavioral testing (30 days after injury). Upon extraction, the brains were stored in fresh 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose. The brains were frozen and sliced into 40- μ m-thick floating coronal sections using a microtome, and every fifth section was mounted on glass slides and stained with Gill's hematoxylin and 2.5% eosin and cover-slipped. Lesion volume was assessed using the Cavalieri method of unbiased stereology and Stereologer software, as previously described (Loane et al., 2009). Briefly, the hippocampus and cortex on the ipsilateral and contralateral sides were traced on each section to obtain the area, and this was multiplied by the known distance between sections to obtain the volume. Then the volume of the ipsilateral hippocampus and cortex was subtracted from that of the contralateral side to obtain the total lesion volume. The volume of spared ipsilateral hippocampal tissue was also determined using the same stereological techniques, tracing the hippocampus on every fifth section from bregma -0.94 mm to bregma -2.54 mm.

Statistical analysis

For statistical analysis, data obtained from independent measurements are presented as the mean $\pm$ standard error of the mean (SEM), and they were analyzed using an analysis of variance (ANOVA), followed by *post-hoc* analysis with the Newman-Keuls multiple comparison test, with the following exceptions. Learning in the Morris water maze (days 15–18 and 22–25) was analyzed using a two-way ANOVA with repeated measures, followed by the Bonferroni *post-hoc* test. For the non-parametric contralateral torso flexion data, data were analyzed using a rank-transformed ANOVA with test day as a within-subjects factor, and injury severity as a between-subjects factor, followed by a Holm-Sidak multiple comparisons test. Regression analysis was performed on focused predictors to assess which behavioral tests could best predict the variability in lesion volume. A stepwise regression analysis using the backward method with removal criteria set at an F probability of >0.10 was used. All statistical tests were performed using GraphPad Prism software, version 5.0d (GraphPad Software, Inc., San Diego, CA), or SPSS Version 20.0. *p* values of less than 0.05 were considered statistically significant.

Results

CCI causes impairments in cognitive ability assessed by Morris water maze performance

Spatial learning was evaluated on days 15–18 after TBI surgery, and a probe trial for spatial memory was given on day 19. A two-way repeated-measures ANOVA with test day and injury severity as the dependent variables was used to determine efficacy. There was a significant main effect of

injury severity ($F_{3,35}=8.90$, $p=0.0002$), a significant main effect of test day ($F_{3,105}=20.86$, $p<0.0001$), and a significant injury $\times$ test day interaction ($F_{9,105}=2.32$, $p=0.0203$). Bonferroni *post-hoc* testing revealed significant differences between injury groups, as outlined below.

Sham mice learned the task very quickly, and had an average escape latency of 12.6 ± 2.32 sec on day 18 (Fig. 2a). Mild TBI mice were not significantly worse than sham animals on any of days 15–18, even though latency on day 18 was more than double that of sham-injured mice (31.5 ± 5.95 sec; Fig. 2a).

Moderate TBI mice were significantly worse than sham animals on day 15 ($p<0.01$), and day 17 ($p<0.05$), but their latency on day 18 was identical to mild TBI mice (28.3 ± 4.93 sec; Fig. 2a), and was not significantly worse than sham-injured mice. There was no significant difference between moderately-injured and mildly-injured mice from days 15–18. Severely-injured TBI mice were significantly worse than sham-injured mice at locating the platform on day 15 ($p<0.05$), day 16 ($p<0.01$), day 17 ($p<0.001$), and day 18 ($p<0.001$), with their average latency on day 18 being

FIG. 2. TBI mice had learning and memory deficits in the Morris water maze. (A) Spatial learning was assessed in training trials on days 15–18 post-injury. The mice were exposed to four 90-second trials a day for 4 days, with the platform location unchanged. The average latency to the platform for each day is shown on the graphs ($\pm$ SEM). Asterisks indicate comparisons with sham-injured mice on the same day. (B) On day 19 the platform was removed and the mice were placed back in the maze for 90 sec. The percent of total time spent in the correct quadrant was recorded as an indicator of spatial memory. (C) The spatial learning paradigm was repeated on days 22–25, with the platform moved to a new location in the opposite quadrant (platform reversal). The average latency to the new platform for each day is shown on the graphs ($\pm$ SEM). Asterisks indicate comparisons with sham-injured mice on the same day, and plus signs indicate comparisons with mildly-injured mice on the same day. (D and E) On day 26 the mice were exposed to another probe trial, and the percent time spent in the correct quadrant (D) and the number of platform crossings (E) were recorded as indicators of spatial memory. (F) The number of crossings of the original platform location was also assessed in the reversal probe trial, as an indicator of perseverance and adaptability. (G) The average speed of each group of mice was determined for both the original training trials and the reversal trials. (H) Heat maps generated from the tracking software that show the swim paths and search strategies of all mice in each group ($n=9-10$ for each group; TBI, traumatic brain injury; SEM, standard error of the mean).

58.0 ± 8.89 sec (Fig. 2a). Severely-injured mice were also significantly worse than mildly-injured mice on days 17 and 18 ($p < 0.05$), and worse than moderately-injury mice on day 18 ($p < 0.01$). Twenty-four hours later a probe trial was held, during which the platform was removed and the percentage of time spent in the correct quadrant was measured. Although there was a downward trend in the percent of time spent in the correct quadrant, there was no significant difference between sham animals and any of the TBI-severity groups (Fig. 2b).

The mice were returned to the maze and repeated the training and testing paradigm on days 2–26 after injury—but with the platform located in a new position. This time the platform was located in the quadrant opposite its original location (platform reversal).

Two-way repeated-measures ANOVA was again used and there was a significant main effect of injury severity ($F_{3,35} = 11.58$, $p < 0.0001$), a significant main effect of test day ($F_{3,105} = 10.58$, $p < 0.0001$), and a significant injury × test day interaction ($F_{9,105} = 2.32$, $p = 0.0253$). Bonferroni *post-hoc* testing revealed significant differences, as outlined below. Once again, the sham-injured mice demonstrated quick learning of the platform location and the ability to adapt to a new platform location. Similarly to the first round of testing, the three injured groups again all had deficits in locating the platform. However, this time the separation between injury groups was much clearer, with obvious differences on day 25 in latency to locate the platform between sham (9.9 ± 2.53 sec), mild (26.1 ± 2.96 sec; n.s.), moderate (37.7 ± 6.9 sec; $p < 0.05$), and severe (57.3 ± 8.38 sec; $p < 0.001$) injury groups (Fig. 2c).

A probe trial for the reversal platform location was administered on day 26 after training and a number of parameters were assessed. An analysis of the percent time spent in each quadrant demonstrated an injury-dependent decrease in the time spent in the correct quadrant, with the mild ($p < 0.05$), moderate ($p < 0.05$), and severe ($p < 0.01$) injury groups spending less time in the correct quadrant compared to sham-injured mice (Fig. 2d). Further analysis showed that all three injury groups had a significant reduction in the number of crossings over the reversal platform location ($p < 0.001$, Fig. 2e), but there was not much separation between the individual injury groups. Interestingly, the mice seemed to return to the original platform location as part of their search strategy, and when we analyzed the number of crossings over this location during the reversal platform probe trial, we found an injury-dependent effect on crossings over the original platform location, with the moderate ($p < 0.01$) and severe ($p < 0.001$) groups having significantly fewer crossings over this location than sham-injured mice (Fig. 2f). Computer-generated heat maps of the reversal probe trial clearly indicate the effect of injury severity on exploration strategy in the water maze (Fig. 2h; the paths of all animals in a group are collapsed into a single graphic). The greatest area of travel for sham-injured mice was the location of the reversal platform

(platform B on the schematic diagram), followed by the location of the original platform location (platform A on the schematic diagram), with the majority of swimming occurring between these two areas. While mildly-injured mice also visited the reversal platform a number of times (B), they had a much higher concentration of crossings over the original platform location (A). Moderately-injured mice had a much stronger tendency to circle the outer walls of the maze, but again spent a significant amount of their time in the original platform quadrant as opposed to the reversal platform quadrant. Finally, severely-injured mice showed the least amount of exploration activity, hardly leaving the quadrant in which they were placed (Fig. 2h).

An analysis of average swim speed in the learning and reversal trials revealed no effect of injury on average speed over the learning trials; however, the average speed of the severe injury group during the reversal trials was significantly reduced compared to the sham ($p < 0.01$), mild ($p < 0.05$), and moderate ($p < 0.05$) injury groups (Fig. 2g). Testing of gross motor function in the open field showed that this swim speed deficit was not due to actual motor problems (see later sections), and may be due to behavioral despair. This was confirmed by examination of the trial videos, which clearly showed that a number of severely-injured mice essentially gave up on the test and spent significant portions of the test floating.

CCI causes deficits in acoustic startle response and sensorimotor gating

We tested acoustic startle response (ASR) and prepulse inhibition (PPI) 20 days after injury using four different prepulse tones and three different inter-stimulus intervals. Comparison of baseline ASR by ANOVA revealed a significant effect of injury on startle response ($F_{3,35} = 3.428$, $p < 0.05$). *Post-hoc* analysis revealed a significant effect of severe injury compared to sham-injured mice ($p < 0.05$; Fig. 3a).

Unfortunately, a computer error occurred during one of our runs, affecting the data collection in three animals (two sham mice and one severely-injured mouse). As we did not have the full data available for the prepulse tones and inter-stimulus intervals, we excluded these mice from our final PPI analysis. We performed a three-way ANOVA with prepulse intensity and ISI as repeated measures, and injury group as a between-subjects variable. Analysis (Greenhaus-Geisser corrected) revealed a significant main effect of PPI ($F_{3,96} = 127.1$, $p < 0.0001$), a significant main effect of ISI ($F_{2,64} = 8.33$, $p < 0.001$), and a significant prepulse intensity × interstimulus interval interaction ($F_{6,192} = 16.91$, $p < 0.0001$). Despite a trend towards reduced PPI in TBI animals, there was no significant effect of injury group, no significant three-way interaction, and no significant interaction between injury group and PPI or ISI (Fig. 3b–d).

FIG. 3. Traumatic brain injury (TBI) affects the acoustic startle response (ASR) and prepulse inhibition in mice. (A) The whole-body ASR was measured by a piezoelectric accelerometer, with the signal digitized and analyzed using SR-LAB software (* $p < 0.05$ versus sham-injured mice; $n = 9$ –10 per group). (B–D) Prepulse inhibition (PPI) was determined by presenting a prepulse of 3, 6, 9, or 15 dB above background noise before the startle pulse, separated by one of three inter-stimulus intervals (50 msec, B; 100 msec, C; 200 msec, D). The quenching of ASR by the prepulse was measured and presented as the average ± standard error of the mean; ($n = 8$ –10 per group).

FIG. 4. Traumatic brain injury (TBI) causes reduced anxiety in the elevated-plus maze. Anxiety phenotype was determined 21 days after injury using the elevated-plus maze. (A) Total distance travelled in the entire maze over the duration of the test (5 min). (B) Total number of entries into both open and closed arms over the duration of the test. (C) The amount of time spent in open arms. (D) The distance travelled in the open arms. (E) The number of open-arm entries. (F) The percentage of open-arm entries as a percent of total (open + closed) arm entries. (G) Heat maps generated by the tracking software that show the search paths and strategies of the each group of mice (* $p < 0.05$ versus sham mice; $n = 9-10$ for each group).

CCI mice have reduced anxiety in the elevated-plus maze

We tested CCI injured mice in the elevated-plus maze 21 days post-injury and analyzed their behavior. There were three mice (two with mild injury and one with severe injury) that did not explore the maze at all, travelling less than one full length of the maze in 5 min. These mice made no entries into the open arms of the maze; however, we did include these mice in our final analysis. We found that all four groups of mice travelled similar distances in the maze (Fig. 4a), and had a similar number of total arm entries in the maze (Fig. 4b),

showing that overall the mice displayed the same amount of total exploratory behavior regardless of the presence of TBI, or the severity of that TBI. ANOVA revealed a significant effect of injury on the time spent in open arms ($F_{3,35} = 3.23$, $p < 0.05$), and *post-hoc* analysis showed that the time spent in the open arms was significantly different in the mild, moderate, and severe groups, compared to sham mice ($p < 0.05$; Fig. 4c). Further analysis revealed that sham mice only spent an average of 9 sec in the open arms, with mild TBI (97 sec), moderate TBI (102 sec), and severe TBI (107 sec) animals all significantly increasing the time spent in the open arms, indicating a reduced anxiety phenotype at this time point after injury.

We also determined the distance travelled in the open arms (Fig. 4d), the number of open-arm entries (Fig. 3e), and the percentage of open-arm entries (Fig. 4f). While all three measures do clearly show a strong trend towards showing a reduced anxiety phenotype, ANOVA failed to detect any significant effect of injury severity. The difference in behavior could also be seen in the heat maps generated from all mice in each group (Fig. 4g), which clearly show increased exploration of the open arms in TBI mice.

CCI mice display depressive-like behavior in the forced swim test

We tested our CCI-injured mice 21 days after injury and found a significant effect of injury on immobility time (Fig. 5a), as revealed by ANOVA ($F_{3,35}=4.65$, $p<0.01$). Immobility time was significantly increased, by 18% in mild TBI mice ($p<0.05$), 16% in moderate TBI mice ($p<0.05$), and 23% in severe TBI mice ($p<0.01$). While all injury groups were significantly higher than sham animals, there was no difference between the three injury groups. These data demonstrate that while the forced swim test is sensitive enough to detect TBI-

induced depression in mice, it cannot differentiate between differing severities of CCI.

CCI produces severity-dependent flexion of the torso to the contralateral side

Contralateral torso flexion was evaluated 1, 3, 7, 14, and 21 days after injury and the data were analyzed using an aligned, rank-transformed ANOVA, with test day as a within-subjects factor, and injury severity as a between-subjects factor (Wobbrock et al., 2011). There was a significant main effect of injury severity ($F_{3,35}=52.96$, $p<0.0001$), a significant main effect of test day ($F_{4,140}=31.23$, $p<0.0001$), and a significant injury $\times$ test day interaction ($F_{12,140}=5.44$, $p<0.0001$; Fig. 5b). Holm-Sidak multiple comparison tests revealed significant differences between the mild, moderate, and severe injury groups within each time point. We found significant differences between sham, mild, moderate, and severe CCI mice through day 14 (Fig. 5b), with clear separation between the groups. The best resolution between groups was found at 7 days, when the sham, mild, moderate, and severe groups, were all statistically different from one another. There was recovery in all injury groups over the duration of the experiment, with a sharp recovery of function seen between days 14 and 21, but on day 21 severe injury was still significantly worse than sham or mild injury (Fig. 5b).

CCI mice do not show any behavioral changes in the open-field test

On day 21 after injury the mice were individually placed in the center of the novel arena and their behavior was recorded over a 5-min period. We found no difference between groups in overall distance travelled in the maze ($F_{3,35}=0.7338$, n.s.; Fig. 6a), or speed in the maze ($F_{3,35}=0.7270$, n.s.; Fig. 6b). There was a trend towards less time spent in the outer zone ($F_{3,35}=2.007$, n.s.; Fig. 6c), and increased time spent in the inner zone among the injured groups ($F_{3,35}=2.007$, n.s.; Fig. 6d), but this did not reach statistical significance. Heat maps show that the mice spent a large portion of each test in the center of the maze (where the mice were positioned at the start of the test), but most exploration occurred along the perimeter of the maze wall (Fig. 6e).

CCI produces severity-dependent differences in lesion volume

Histological assessment of lesion volume on day 28 following completion of behavioral tasks showed that increased depth of cortical impact and severity of injury produced correspondingly larger lesion volumes. ANOVA revealed a significant effect of TBI ($F_{3,35}=185.02$, $p<0.00001$). *Post-hoc* analysis revealed that animals in the mild group had a significant loss of brain tissue compared to the sham ($p<0.001$) and moderate groups, and that the severe group had greater tissue loss than the sham or mild groups ($p<0.001$; Fig. 7a). We also measured the amount of spared hippocampal volume in each injury group and found an injury severity-dependent reduction in spared tissue ($F_{3,35}=51.45$, $p<0.00001$ by ANOVA), with mice receiving mild injury losing 22% ($p<0.05$), those receiving moderate injury losing 80% ($p<0.001$), and those receiving severe injury losing 94% ($p<0.001$) of their ipsilateral hippocampal volume (Fig. 7b).

FIG. 5. Traumatic brain injury (TBI) causes depression-like behavior in the forced swim test, and abnormal posturing in the contralateral torso flexion test. (A) Behavioral despair (depression) was determined by the forced swim test 21 days after injury by quantifying the amount of time the mice spent immobile in the final 4 min of the test. (B) Abnormal posturing, indicative of striatal damage, was quantified using the contralateral torso flexion test on multiple days after injury ($n=9-10$ for each group; * $p<0.05$, ** $p<0.01$, *** $p<0.0001$ versus sham-injured mice; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$ versus mildly-injured mice).

FIG. 6. Traumatic brain injury (TBI) does not affect gross locomotor function in the open-field test. Gross motor function was measured in the open-field test 21 days after injury. (A) Total distance travelled. (B) Average speed. (C) Time spent in the outer zone. (D) Time spent in the inner zone. (E) Heat maps generated by the tracking software that show the search paths and strategies of the each group of mice ($n=9-10$ for each group).

Multiple regression analysis of behavior with tissue loss

To generate focused predictors for regression analyses (rather than include all behavioral variables), the following summary variables were produced: improvement in the

Morris water maze over 4 days of reversal training (i.e., escape latency on day 25 minus escape latency on day 21); overall PPI (collapsed across PPI and ISI); and maximum swing score (the maximum score selected from swing measurements on days 1, 3, 7, 14, and 21). Additionally, startle amplitude, time spent in the correct quadrant during the water maze reversal probe

FIG. 7. Quantification of traumatic brain injury (TBI)-induced tissue loss. Total lesion volume and the spared hippocampal tissue were determined 28 days post-injury by unbiased stereology using the Cavalieri method. (A) Total lesion volume was determined by subtracting the ipsilateral cortical and hippocampal volume from the contralateral cortical and hippocampal volume. (B) The remaining ipsilateral hippocampus was quantified by measuring hippocampal tissue remaining between bregma -0.94 mm and bregma 2.54 mm ($*p<0.05$, $***p<0.001$ versus sham-injured mice; $n=9-10$ for each group).

test, time spent immobile in the forced swim test, and time spent in the open arms of the elevated-plus maze, were included as predictors. Stepwise regression analyses were then performed (using the "backward" method, with removal criteria set at an *F* probability of >0.10). Lesion volume or spared hippocampus were the dependent variables.

The correlation of behavior with lesion volume showed that improvement in the Morris water maze ($\beta=0.225$), acoustic startle amplitude ($\beta=-0.224$), and maximum body swing score ($\beta=0.736$), together predict 72.3% (69.3% adjusted) of the variability in lesion volume (adjusted $R^2=0.693$).

Correlation of behavior with hippocampal volume showed that improvement in the Morris water maze ($\beta=-0.345$), acoustic startle amplitude ($\beta=0.319$), maximum body swing score ($\beta=-0.712$), PPI ($\beta=-0.212$), and time spent in the correct quadrant during the water maze probe test ($\beta=-0.209$), together predict 72.1% (66.5% adjusted) of the variability in hippocampal volume (adjusted $R^2=0.665$).

These data indicate that while the cognitive and movement behaviors correlate well with injury severity, the emotional behaviors do not.

Discussion

Injury severity plays a major role in determining neurobehavioral outcome after head trauma. While a direct relationship between injury severity and the development of cognitive deficits has been established in humans, it has proven difficult to determine the relationship between injury severity and changes in emotional behavior. The results of this study show that cognitive deficits increase as a function of injury severity, but emotional problems like depressive- and anxiety-like behaviors do not, suggesting that while there is an injury threshold for producing cognitive dysfunction, it is a low threshold, and emotional disturbances can develop after mild, moderate, or severe brain injury.

A particular advantage of the CCI mouse model of TBI is that injuries of increasing severity can be produced in a controlled and repeatable manner by manipulating the depth of impact and the resulting tissue deformation. Here we exposed mice to mild, moderate, or severe CCI, and evaluated their behavior in a battery of tasks to determine whether TBI causes a broad range of behavioral sequelae in mice similarly to those seen in humans, and also to determine whether behavioral deficits increase with injury severity. We found Morris water maze performance and degree of contralateral torso flexion to be the most sensitive to injury severity, with deficits increasing in relation to severity of injury. Alternatively, we investigated sensorimotor gating, and found that all levels of injury caused similar disruptions in PPI. The same was true for behavior on the elevated-plus maze and forced swim test; we found that all levels of CCI cause similar depressive- and anxiety-like behaviors 21 days after trauma. From this we conclude that in addition to causing impairments in the Morris water maze, CCI also causes changes in behavior in the elevated-plus maze, forced swim test, and PPI. Deficits in these tasks, however, were similar in the mild, moderate, and severe CCI groups, so these tasks cannot be used to discriminate between injuries of differing severity like the Morris water maze or contralateral torso flexion.

The Morris water maze has been used in the study of TBI to detect impairments in hippocampal-dependent spatial learn-

ing and memory after injury for the past 20 years (Smith et al., 1991). Over time it has become the gold standard for detecting cognitive deficits after experimental TBI, as well for the pre-clinical evaluation of potential therapeutic interventions. This is due to the versatile nature of the task, with modifications to the training and testing times allowing the study of individual components of memory, such as formation and retrieval (Scheff et al., 1997), and the ability to clearly delineate cognitive changes produced by injuries of differing severity (Markgraf et al., 2001; Smith et al., 1991). In the present study we further confirm the status of the Morris water maze as an excellent tool for determining injury severity in mice after CCI. We found that our standard 4-day training protocol beginning 15 days after surgery differentiated sham and mild mice from severe TBI mice, but the performance of moderate TBI mice fluctuated between that of the mild and severe injury groups, an observation that has also been noted in humans (Satz et al., 1998). The data from the probe trial also showed no significant differences between sham animals and any of the injury groups. Previous researchers who used the water maze for testing after TBI have reported using an additional platform reversal set-up in the weeks following initial training, where training and testing were repeated with the platform in a new location (Hamm et al., 1992). We extended our water maze paradigm to include reversal training and probe trials and found that the reversal probe training resulted in much cleaner data with clear separation between the performance of our four experimental groups. We also obtained injury-dependent changes in the reversal probe trial. Analysis of heat maps from the reversal probe trial showed that TBI mice appear to perseverate on the original platform location, searching for the original platform location as opposed to the location of the reversal platform. Overall, our data confirm the status of the Morris water maze as a test that is extremely sensitive to injury severity, and also show that extending the water maze paradigm to include platform reversal results in reduced variability and improved separation between groups.

Prepulse inhibition occurs when presentation of a weak pre-stimulus dampens the startle response to a stronger subsequent stimulus. Deficits in this response are due to impaired sensorimotor gating. Deficits in sensorimotor gating have been reported in humans after TBI (Arciniegas et al., 1999,2000). To our knowledge, this is the second assessment of either startle or prepulse inhibition after experimental TBI, and the first time that these responses have been measured after CCI or compared between injury severities. The first study found decreased startle and sensorimotor reactivity after midline fluid percussion in rats (Wiley et al., 1996). Our results broadly agree with this study; we found a severity-dependent decrease in acoustic startle after CCI, with severe injury significantly reducing the physical response to auditory stimuli. We designed our experiments to examine a full spectrum of prepulse tones, with an auditory range of 3–15 dB above background, and 50- to 200-msec inter-stimulus intervals. Overall we found that PPI trended lower in almost all conditions, and was most effective when the loudest prepulse was used with lag times greater than 50 msec.

Depression is a common problem after TBI, with a recent study in one cohort of TBI patients finding that 53.1% had at least one episode of major depressive disorder in the year following injury—a rate more than eight times higher than in

the general population (Bombardier et al., 2010). Our data show that mild, moderate, and severe TBI, all increased depression-like behavior in the FST 21 days after trauma. These findings are similar to previously reported results showing increased immobility 1, 7, 60, and 90 days after mild weight drop injury in mice (Milman et al., 2008; Shapira et al., 2007; Zohar et al., 2011). Only one of the referenced studies examined injuries of graded severity (Zohar et al., 2011), but all the animals were collapsed into a single injury group in their FST analysis, so data for individual injury severities were not presented. When we did look at differences between mild, moderate, and severe injury, we did not find significant differences between groups, indicating that while this test can be used for screening depressive-like behaviors after CCI, it cannot be used to differentiate between severity groups.

Anxiety is another common emotional problem that occurs in the chronic phase following TBI (Gould et al., 2011). Anxiolytic and anxiogenic behaviors in rodents are probed using the elevated-plus maze, during which an animal's natural preference for dark, hidden spaces is used to detect changes in behavior. In our studies we found that mild, moderate, and severe TBI mice all exhibited reduced anxiety, spending more time and making more entries into the open arms of the maze. This is the opposite of what is commonly reported in humans, and is also different from behavioral reports in rats and mice after TBI. Another group studying behavior in the elevated-plus maze in C57/BL6 mice following mild CCI found a significant decrease in open-arm entries 48 h after injury, indicative of increased anxiety (Chauhan et al., 2010). Other groups have also demonstrated anxiogenic behavior at times ranging from 11 days (Schwarzbald et al., 2010) to 3 months (Jones et al., 2008; Liu et al., 2010) after weight-drop injury in rodents. The one other study that found similar anxiolytic effects after experimental TBI as ours showed that injury in rats caused a large increase in the time and number of entries into open arms at 25 days post-trauma; however, they appear to incorrectly conclude that this behavior reflects an anxiety-prone phenotype (Pandey et al., 2009). Finally, other studies have reported no difference in behavior in the elevated-plus maze after experimental TBI (Zohar et al., 2011). In conclusion, we do not know why there is so much variability in anxiety measures after TBI. Our data appear to contradict the majority of the published work; however, differences in the injury paradigms, the post-injury duration to plus maze testing, and the number of tests that our mice were exposed, to may explain these differences.

Assessment of contralateral flexion of the torso after experimental injury is normally one of several tests included in point-based neurological examinations (Petullo et al., 1999). It is not generally used as a stand-alone measure as we used it here in our experiments. Flexion of the torso away from the side of brain injury when suspended by the tail is thought to be a direct result of unilateral striatal damage, and has been assessed after 6-hydroxydopamine injection into the substantia nigra using the elevated body swing test (Borlongan and Sanberg, 1995). Upwards twisting of the torso has also been noted after experimental middle cerebral artery occlusion specifically producing striatal infarcts, but again just the presence or absence of flexion was noted as part of a neurological exam (Petullo et al., 1999). As contralateral torso flexion has only been rated qualitatively, and scored based solely on its presence or absence in previous studies, it was not

known if this task could discriminate between injuries of graded severity. In our study we found that torso flexion was an excellent indicator of injury severity, distinguishing between sham, mild, moderate, and severe CCI as quickly as 1 day after injury, and up to 14 days later. Markgraf and colleagues looked at posture reflex, defined as asymmetry of the forelimbs when suspended by the tail after CCI of different severities, but did not find a difference between injury groups (Markgraf et al., 2001), suggesting that our test is more sensitive to injury severity.

While TBI affected behavioral outcomes in the majority of the emotional, motor, and cognitive tests performed, a stepwise regression analysis revealed that only some of our behavioral tests could predict lesion volume, with a bias towards the cognitive and motor paradigms. Comparisons of behavior with tissue loss showed that behavior in the Morris water maze, contralateral torso flexion, and acoustic startle closely correlated with tissue loss after injury. Deficits in emotional paradigms were excluded early in the regression, and were not useful for predicting tissue loss in mice.

In conclusion, we find that deficits in hippocampal-dependent spatial learning in the Morris water maze increase as a function of injury severity, but TBI-induced changes in emotional behavior such as anxiety and depression do not. These results are broadly consistent with what has been reported in humans after TBI. We also report the increased sensitivity of the Morris water maze paradigm by including platform reversal training and probe trials, and the identification of contralateral torso flexion as a simple task that can be used to accurately discriminate sham, mild, moderate, and severe injuries up to 2 weeks post-trauma. The elevated-plus maze, forced swim test, and prepulse inhibition revealed similar TBI-induced deficits across all levels of injury severity. As such, these emotional paradigms can be used to detect impairments after injury, but should not be used for discrimination of injury severity or preclinical evaluation of therapeutic efficacy, because clear separation between mild, moderate, and severe injury groups does not occur.

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