Comparing the Predictive Value of Multiple Cognitive, Affective, and Motor Tasks after Rodent Traumatic Brain Injury

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Abstract

Controlled cortical impact injury (CCI) is a widely-used, clinically-relevant model of traumatic brain injury (TBI). Although functional outcomes have been used for years in this model, little work has been done to compare the predictive value of various cognitive and sensorimotor assessment tests, singly or in combination. Such information would be particularly useful for assessing mechanisms of injury or therapeutic interventions. Following isoflurane anesthesia, C57BL/6 mice were subjected to sham, mild (5.0 m/sec), moderate (6.0 m/sec), or severe (7.5 m/sec) CCI. A battery of behavioral tests were evaluated and compared, including the standard Morris water maze (sMWM), reversal Morris water maze (rMWM), novel object recognition (NOR), passive avoidance (PA), tail-suspension (TS), beam walk (BW), and open-field locomotor activity. The BW task, performed at post-injury days (PID) 0, 1, 3, 7, 14, 21, and 28, showed good discrimination as a function of injury severity. The sMWM and rMWM tests (PID 14–23), as well as NOR (PID 24 and 25), effectively discriminated spatial and novel object learning and memory across injury severity levels. Notably, the rMWM showed the greatest separation between mild and moderate/severe injury. PA (PID 27 and 28) and TS (PID 24) also reflected differences across injury levels, but to a lesser degree. We also compared individual functional measures with histological outcomes such as lesion volume and neuronal cell loss across anatomical regions. In addition, we created a novel composite behavioral score index from individual complementary behavioral scores, and it provided superior discrimination across injury severities compared to individual tests. In summary, this study demonstrates the feasibility of using a larger number of complementary functional outcome behavioral tests than those traditionally employed to follow post-traumatic recovery after TBI, and suggests that the composite score may be a helpful tool for screening new neuroprotective agents or for addressing injury mechanisms.

Key words: behavior; composite score; controlled cortical impact; correlation; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a major public health problem, with a median annual incidence of 101 per 100,000 in the United States, and an estimated 2% of this population suffering TBI-related disabilities, according to the Centers for Disease Control and Prevention (CDC). The incidence data likely represent underestimates, as these figures do not adequately reflect concussion, which appears to be under-reported and is often not seen in emergency departments. There is increasing recognition of the consequences of concussion and repeated mild TBI in both contact sports,^{1–3} and in the military setting.⁴

TBI causes tissue damage and associated neurological dysfunction through both primary and secondary injury mechanisms. Primary injury reflects mechanical damage that occurs at the time of trauma as

a result of shearing, tearing, or stretching. Secondary injury evolves over a period from minutes to weeks or months after TBI, and results from delayed biochemical, metabolic, and cellular changes. These secondary alterations are believed to account for the development of much of the neurological dysfunction observed after TBI. Considerable research efforts have sought to delineate secondary injury mechanisms in order to develop effective neuroprotective treatments. Although numerous pre-clinical studies have suggested many promising pharmacological agents, more than 30 Phase III prospective clinical trials have failed to show significance for their primary end-point.⁵ Better-characterized and clinically-relevant animal models are required to define critical injury mechanisms and the effectiveness of new neuroprotective agents. Moreover, better-delineated functional evaluations of experimental TBI are needed that reflect the functional disabilities observed in human head injury.

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Controlled cortical impact (CCI) is a widely-used and clinically-relevant model of TBI. It induces a focal contusion injury with secondary progression at moderate to severe levels. It results in consistent histological damage and sustained behavioral deficits.^{6–9} CCI can cause intracerebral hemorrhage, cytotoxic and vasogenic brain edema, neuronal loss, and motor and cognitive deficits.^{10–16} Both behavioral and histological outcomes are injury-severity dependent, as demonstrated by varying impact depth and/or velocity in rats,^{17,18} or mice.^{11,12,19} In the present study we compared multiple behavioral outcomes as a function of injury severity in the mouse CCI model to assess which tests or test combinations best reflect changes of lesion volume and cell loss in specific anatomical regions. These included the standard Morris water maze, reversal Morris water maze, novel object recognition, passive avoidance, tail suspension, beam walk, and open-field locomotor activity tests. The goal was to better delineate functional assessments in the CCI model that can be utilized to study the role of secondary injury mechanisms that contribute to tissue damage after TBI, and to screen potential neuroprotective agents.

Methods

Animals

Ten-week-old male C57BL/6 mice (20–25 g) were obtained from Charles River Laboratories Inc. and housed in shoebox cages (4–5 mice in each cage) at least 1 week prior to any procedures in a room (22–23°C) with a 12-h/12-h light-dark cycle. Food and water were provided *ad libitum*. The mice were handled briefly before use. Procedures were conducted from 10:00 to 17:00 in a quiet room. All protocols involving the use of animals complied with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) (DHEW publication NIH 85-23-2985), and were approved by the University of Maryland Animal Use Committee.

Controlled cortical impact injury

CCI TBI was performed as previously described¹² with some modifications. The injury device consists of a microprocessor-controlled pneumatic impactor, driven by compressed air, with a 3.5-mm-diameter tip. Surgical anesthesia was induced and maintained with 3% and 2% isoflurane, respectively, evaporated in a gas mixture containing 70% N₂O and 30% O₂ and administered through a nose mask. Depth of anesthesia was monitored by respiration rate and depth, as well as palpebral and pedal withdrawal reflexes. The mouse was placed on a heated pad, and core body temperature was maintained at 37°C. The head was mounted in a stereotaxic frame, and the surgical site was clipped and cleaned with chlorhexidine diacetate and ethanol scrubs. A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone. The impounder tip of the injury device was then extended to its full stroke distance (44 mm), positioned to the surface of the exposed dura, and reset to impact the cortical surface. Mild ($n = 20$), moderate ($n = 14$), and severe ($n = 16$) injury levels were induced by impact velocities of 5, 6, and 7.5 m/sec, respectively, and a deformation depth of 2 mm. After injury, the incision was closed and anesthesia was terminated, and the animal was placed on a heated pad to maintain normal core temperature for 30–60 min post-injury. All animals were monitored carefully for at least 4 h after surgery and then daily. Sham animals ($n = 15$) underwent the same procedure as injured mice except for the impact. Randomization was performed in each study cohort, and equal numbers of sham, mild, moderate, and severe injuries were performed on each day of surgery. There were three cohorts of animals that underwent CCI and behavioral testing, all within a 1-year time

period. Cohorts 1 and 2 had 25 mice each, and cohort 3 had 15 mice. All conditions were kept consistent throughout the three cohort experiments. The C57BL/6 mice were obtained from the same vendor and were the same age at the time of injury. All of the procedures were performed by the same investigator using the same CCI device. In addition, all behavioral tests were performed by the same investigator using the same equipment and room. All behavioral tests were performed by an independent experimenter blinded to injury severity group.

Motor function evaluation

Chronic motor recovery was evaluated using a beam-walking task, a test which is particularly good at discriminating fine motor coordination differences between injured and sham-operated animals.^{9,20,21} The device consists of a narrow wooden beam 5 mm wide and 120 cm in length, which is suspended 1 m above the ground. The mouse was placed on the beam and the number of foot-faults for the right hindlimb was recorded over 50 steps. A basal level of performance was achieved following 3 days of training prior to surgery, with an acceptance level of fewer than 10 foot-faults per 50 steps. The test was performed at 0 (immediately before CCI), 1, 3, 7, 14, and 28 days after injury.

Morris water maze and reversal Morris water maze tests

Spatial learning and memory was assessed using the acquisition phase of the standard Morris water maze (sMWM), and reversal MWM (rMWM) tests, as previously described, with some modifications.^{12,22,23} The Morris water maze protocol included four phases: (1) standard hidden platform training (acquisition); (2) standard probe test; (3) reversal hidden platform training (reversal acquisition); and (4) reversal probe test. A circular tank (100 cm in diameter) was filled with water (23 ± 2°C) that was made opaque with white non-toxic paint. The maze was surrounded by various extra-maze cues on the wall of the room. A transparent platform (10 cm in diameter) was submerged 0.5 cm below the surface of the opaque water. Starting on post-injury day (PID) 14, the mice were trained to find the hidden submerged platform located in the northeast (NE) quadrant of tank for 4 consecutive days (PID 14–17). The mice underwent four trials per day, starting from a randomly-selected release point (east, south, west, and north). Each mouse was allowed a maximum of 90 sec to find the hidden submerged platform, and mice that failed to find the platform within this time were placed onto the platform and allowed to remain there for 25 sec on the first day of training, and for 10 sec on subsequent training days. The swim path, latency to platform, time spent in each zone, and swim velocity were recorded by the computer-based Any-Maze automated video tracking system (Stoelting Co., Wood Dale, IL). Reference memory was assessed by a probe test on PID 18. The platform was removed and the mice were released from the southwest (SW) position, and the time spent in each quadrant was recorded out of a maximum of 60 sec. For the reversal MWM training, the hidden platform was moved from the NE to the SW quadrant without changing any visual cues. The mice were trained to find the new platform position for the following 4 days (PID 19–22; 4 trials/day). A reversal probe test was performed on PID 23. The platform was removed and the mice were released from the NE position, and the time spent in each quadrant was recorded. Water maze search strategy analysis was performed as previously described.^{24,25} Three strategies were identified using the following categorization scheme: spatial strategies was defined as swimming directly to the platform after no more than 1 loop, or swimming directly to the correct target quadrant and searching; systematic strategies was defined as searching the interior portion of or the entire tank without spatial bias, and searching an incorrect target quadrant; and looping

strategies was defined as circular swimming around the tank, swimming in tight circles, and swimming around the wall of the tank. The search strategies were analyzed for each of the four trials on day 4 of the MWM and reversal MWM. The percentage of each strategy in each group was calculated. Mice that failed to swim and remained immobile throughout the 90-sec trial in the MWM test were excluded from the analysis.

Novel object recognition test

Novel object recognition (NOR), conducted as previously reported,^{26,27} evaluated non-spatial hippocampal-mediated memory,²⁸ on PID 24 and 25. The apparatus consists of an open field (22.5 × 22.5 cm) with two adjacently-located imaginary circular zones. Briefly, the mice were habituated to the open field and were allowed to freely explore the area for 5 min (no data were collected). After 24 h, the mice were placed in the chamber where two identical objects were placed near the left and right corners of the open field for training (sample phase), and allowed to freely explore until they spent a total of 30 sec exploring the objects (exploration was recorded when the front paws or nose contacted the object). The mice were then removed and returned to their home cage. After 1 h, object recognition was tested by substituting a novel object for a familiar training object (the novel object location was counterbalanced across mice). The time spent with each object was recorded; because mice inherently prefer to explore novel objects, a preference for the novel object (more time than chance [15 sec] spent with the novel object) indicates intact memory for the familiar object. Mice that failed to explore and remained immobile throughout the NOR test were excluded from the analysis.

Passive avoidance test

The passive avoidance (PA) test was used to evaluate non-spatial, hippocampus-mediated contextual memory and fear-related amygdala-dependent emotional memory²⁹ on PID 26–28. The one-trial step-through PACS-30 Passive Avoidance apparatus (Columbus Instruments, Columbus, OH) consisted of two adjoining compartments (24 × 23 × 27 cm), one lighted and one darkened, divided by a guillotine door. The floor of each compartment consists of steel rods that deliver an electric foot shock to the mice. On day 1 (PID 26), the mice were habituated in the passive avoidance apparatus for 5 minutes. Twenty-four hours later (PID 27), the mice were placed in the lighted compartment, and when the mice entered the dark compartment the guillotine door was closed manually by the experimenter and an electric foot shock (0.3 mA for 5 sec) was delivered. The mice were confined in the dark compartment for 20 sec after the electric shock, and were then removed from the apparatus and returned to their home cages. Twenty-four hours later (PID 28), the mice were tested for the retention of the passive avoidance response by placing them in the lighted compartment and recording the latency to enter into the dark, previously shocked compartment. No shock was given during the test phase. If the mice did not enter into the dark compartment within 5 min it was assigned a latency of 300 sec.

Tail-suspension test

The tail-suspension (TS) test assesses depression-like behavior in mice, and is based on the observation that mice develop an immobile posture when placed in the inescapable hemodynamic stress of being hung by their tail.^{30,31} The TS was performed on PID 24 as described previously,³² with small modifications. Each mouse was suspended at a height of 50 cm using adhesive tape placed approximately 1 cm from the tip of its tail. The duration of immobility was recorded throughout the 5-min test period. The definition of immobility was passive hanging and complete motionlessness.

Open-field test

The open-field test was used to measure locomotor activity³² on PID 24. The mice were individually placed in a corner facing the wall of the open-field chamber (22.5 × 22.5 cm), and allowed to freely explore the chamber for 5 min. The distance traveled was recorded by Any-Maze software.

Composite score

To establish our composite score (CS) we did not perform a systematic examination of all possible permutations of individual behavioral tests. Instead, we empirically restricted our analysis to four distinct composite scores, which included individual behavioral indexes that tested complementary aspects of neurological function. The composite score that resulted in the most significant separation between groups of different injury severities included the following tests: sMWM probe trial, rMWM probe trial, tail-suspension test, and beam-walk test. These tests were each converted to an ordinal scale ranging from 0 to 5. Two indices from the MWM were used to calculate CS, because the sMWM measures spatial memory acquisition, whereas the rMWM measures spatial retention memory after memory extinguishing and reacquisition. Further, the TS test represents affective behavior, while the BW test represents sensorimotor function. Therefore, the composite score provides an overall assessment of neurological function in injured mice. These four individual scores were combined, without weighting, to yield a 20-point CS ranging from 0 (severe) to 20 (sham).

Lesion volume assessment

The mice were anesthetized and transcardially perfused with saline and 10% buffered formalin phosphate solution (containing 4% paraformaldehyde; Fisher Scientific, Pittsburgh, PA) on PID 28. The brains were removed, post-fixed in paraformaldehyde for 24 h and protected in 30% sucrose. Frozen brain sections (60 μm and 20 μm) were cut on a cryostat and mounted onto glass slides. Every fourth 60-μm section was processed for immunohistochemical analysis beginning from a random start point. Sixty-micron sections were stained with cresyl violet (FD NeuroTechnologies, Baltimore, MD), dehydrated, and mounted for analysis. Lesion volume was determined based on the Cavalieri method as previously described.³³ The lesion area, including both the cavity and surrounding damaged tissue, was outlined using the Stereologer 2000 program (Systems Planning and Analysis, Alexandria, VA) to obtain the final volume measurements.

Assessment of neuronal cell loss in the hippocampal sub-regions

Stereoinvestigator software (MBF Biosciences, Williston, VT) was used to count the total number of surviving neurons in CA1, CA2/3, and dentate gyrus (DG) sub-regions of the hippocampus using the optical fractionator method of un-biased stereology.³³ Our analysis was focused on the dorsal region of the hippocampus, in the area directly underneath the impact site. The sampled region for each subfield was demarcated in the injured hemisphere and cresyl violet neuronal cell bodies were counted. The volume of the hippocampal subfield was measured using the Cavalieri estimator method. The estimated number of surviving neurons in each field was obtained based on the absolute cell number, and was expressed as the number of cells.

Statistical analysis

For the beam walk, acquisition, and reversal acquisition trials of the MWM test, repeated-measures two-way analyses of variance (ANOVAs) were conducted, followed by the Student's

Newman-Keuls *post-hoc* test, to compare the differences between each group. One-way ANOVA analysis followed by the Student's Newman-Keuls *post-hoc* test was performed for the other behavioral tests, lesion volume, and neuronal cell counts. For the search strategy analysis chi-square analysis was performed. Spearman's rho correlation analysis was performed to correlate neuronal cell loss in the sub-regions of the hippocampus with cognitive function using the variety of behavioral tests described. For the composite score a nonparametric Kruskal-Wallis test was used. Q-Q plots demonstrated that the composite score data approached normality and showed equal variance; therefore these data were subsequently analyzed using parametric statistics, and one-way ANOVA followed by the Student's Newman-Keuls *post-hoc* test was performed. Statistical analysis was performed using SigmaPlot, version 12 (Systat Software, San Jose, CA) or GraphPad Prism software, version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean $\pm$ standard error of the mean (SEM), and significance was determined at $p < 0.05$.

Results

In these studies a battery of behavioral tests were evaluated and compared following sham, mild (5.0 m/sec), moderate (6.0 m/sec), or severe (7.5 m/sec) CCI. These behavioral tests included the sMWM, rMWM, NOR, PA, TS, BW, and open-field locomotor activity, and were performed according to the schedule outlined in Figure 1.

The beam-walk task showed good discrimination across severities of injury

All mice underwent beam-walk training for 3 consecutive days prior to sham surgery or CCI. A total of 50 steps were counted and all mice had a baseline competence of less than 10 foot-faults on the final day of training. The mice were tested on the beam walk immediately prior to sham surgery or CCI, and again on PID 1, 3, 7, 14, 21, and 28. On PID 1, mildly-, moderately-, and severely-injured mice had significant impairments in fine motor coordination, resulting in approximately 50 foot-faults on the beam-walk test (Fig. 2). There was a clear improvement in motor function recovery, and a separation between the mildly-, moderately- and severely-injured groups starting from PID 3 through to PID 28. Sham-injured mice showed no significant impairment in fine-motor coordination. Repeated-measures two-way ANOVA showed a significant effect of injury severity [$F_{(3,388)} = 227.4$; $p < 0.001$], and day [$F_{(6,388)} = 95.67$, $p < 0.001$], and Student-Newman-Keuls *post-hoc* analysis demonstrated significant differences between the injured and sham groups across all time points except day 0 ($p < 0.001$). In particular, significant differences were observed between the mildly- and moderately-injured groups on day 3

FIG. 2. Assessment of sensorimotor function using the beam-walk test. All animals had fewer than 10 foot-faults prior to cortical impact injury (CCI). Significant differences were observed between the mildly-, moderately-, and severely-injured groups and the sham-injured group ($^{aaa}p < 0.001$) across each of the time points. Significant differences were also observed between the mildly- and moderately-injured groups on day 3 ($^b p < 0.05$), day 7 ($^{bb} p < 0.01$), day 14 ($^b p < 0.05$), day 21 ($^b p < 0.05$), and day 28 ($^{bb} p < 0.01$); and between the moderately- and severely-injured groups on day 14 ($^c p < 0.05$), day 21 ($^c p < 0.05$), and day 28 ($^{cc} p < 0.01$). Analysis by repeated-measures two-way analysis of variance (injury severity $\times$ day; $n = 12-20$).

($p < 0.05$), day 7 ($p < 0.01$), day 14 ($p < 0.05$), day 21 ($p < 0.05$), and day 28 ($p < 0.01$); and between the moderate- and severely-injured groups on day 14 ($p < 0.05$), day 21 ($p < 0.05$), and day 28 ($p < 0.0001$).

The reversal MWM showed the greatest separation between mild and moderate-to-severe injury

To investigate the effect of injury severity on spatial learning and memory impairments after CCI, we performed the sMWM and rMWM tests from day 14 to day 23 after injury. From PID 14 to PID 17 (acquisition trials), the mice were trained to find the hidden submerged platform (4 trials per day), and the mean latency time for each of the 4 trials was calculated. The mean escape latency on the last day of training was 43.74 ± 5.54 sec for the sham-injured group, and 58.58 ± 5.96 for the mildly-, 67.44 ± 4.57 moderately-, and 82.63 ± 3.50 severely-injured groups (Fig. 3A). Repeated-measures two-way ANOVA showed a significant effect of injury severity [$F_{(3,236)} = 24.59$; $p < 0.0001$] and day [$F_{(3,236)} = 14.42$, $p < 0.0001$], but the injury severity $\times$ day interaction failed to show significance [$F_{(9,236)} = 1.38$, $p > 0.05$]. Student's Newman-Keuls *post-hoc* analysis revealed a significant difference between the moderately- and severely-injured groups on day 3 ($p < 0.01$) and day 4 ($p < 0.05$), and the mildly- and severely-injured groups on day 3 ($p < 0.0001$) and day 4 ($p < 0.001$). There were no significant

FIG. 1. Behavioral test schedule. The beam walk (B) task was performed at 0, 1, 3, 7, 14, 21, and 28 days post-injury (PID). The standard Morris water maze (sMWM) test was performed on PID 14 to 18, followed by the reversal Morris water maze (rMWM) test on PID 19 to 23. The tail-suspension (TS) test was performed on PID 24. The novel object recognition (NOR) test was conducted on PID 24 and 25. The passive avoidance (PA) test was performed on PID 27 and 28. On PID 28, all of the mice were euthanized and perfused for histological (H) analysis (CCI, cortical impact injury).

FIG. 3. Assessment of cognitive function using the standard Morris water maze (sMWM) and reverse Morris water maze (rMWM) tests. The MWM procedure consisted of four phases: (1) sMWM, PID 14–17, 4 trials each day; (2) standard probe and cue tests, PID 18; (3) rMWM, PID 19–22, 4 trials each day; and (4) reversed probe and visible cue tests, PID 23. (A) Acquisition trials. A significant difference was detected between the sham- and mildly-injured groups at day 1 ($^ap < 0.05$) and day 4 ($^ap < 0.05$); the moderately- and severely-injured groups on day 3 ($^ccp < 0.01$) and day 4 ($^cp < 0.05$); the sham- and moderately-injured groups on day 1 ($^dap < 0.05$) and day 4 ($^ddp < 0.001$); the sham- and severely-injured groups on day 1 ($^ep < 0.05$), day 3 ($^ecp < 0.001$), and day 4 ($^eep < 0.001$); and the mildly- and severely-injured groups on day 3 ($^ffp < 0.001$) and day 4 ($^ffp < 0.0001$). (B) Reversal acquisition trials. Significant differences were observed between the sham- and moderately-injured groups on day 7 ($^dp < 0.05$) and day 9 ($^ddp < 0.01$); the sham- and severely-injured groups on day 7 ($^cep < 0.01$), day 8 ($^ecp < 0.001$), and day 9 ($^eep < 0.001$); and the mildly- and severely-injured groups on day 8 ($^ffp < 0.001$), and day 9 ($^ffp < 0.001$). A significant difference was also observed between the mildly- and moderately-injured groups on day 9 ($^bp < 0.05$); the moderately- and severely-injured groups on day 8 ($^cp < 0.05$). (C and D) The reversal probe trial showed better separation of the moderately- and mildly-injured groups compared to the sham-injured group. In the sMWM probe test, severely-injured mice spent significantly less time in the target quadrant compared to sham-injured mice ($^aap < 0.01$). Significant differences were also observed between the moderately- and severely-injured groups ($^bp < 0.05$), and between the mildly- and severely-injured groups ($^bp < 0.05$). In the rMWM probe test, severely-injured mice spent significantly less time in the target quadrant than sham-injured mice ($^aaa < 0.001$). Furthermore, in contrast to the sMWM probe test, there were significant differences between the sham- and moderately-injured groups ($^ap < 0.05$), as well as between the mildly- and severely-injured groups ($^bp < 0.01$). (E and F) Search strategy was examined for each of the 4 trials on acquisition day 4 and reversal acquisition day 9. Search strategy in the sMWM and rMWM showed good injury severity separation (E and F, chi-square = 214.7, $p < 0.001$; and chi-square = 50.91, $p < 0.001$, respectively). Analysis was by repeated-measures two-way analysis of variance (ANOVA; injury severity $\times$ day) in A and B, by one-way ANOVA in C and D, and by chi-square analysis in E and F ($n = 14$ –18).

differences between the mildly- and moderately-injured groups across each of the days. In addition, there was a significant difference between the sham- and mildly-injured groups at day 1 ($p < 0.05$) and day 4 ($p < 0.05$); the sham- and moderately-injured groups at day 1 ($p < 0.05$) and day 4 ($p < 0.001$); and the sham- and severely-injured groups at the day 1 ($p < 0.05$), day 3 ($p < 0.001$), and day 4 ($p < 0.001$).

The rMWM test was performed on PID 19 to PID 22, in which the hidden platform was moved to the opposite (southwest) quadrant (Fig. 3B). Repeated-measures two-way ANOVA showed a

significant effect of injury severity [$F_{(3,236)} = 22.82$; $p < 0.0001$], and day [$F_{(3,236)} = 4.79$, $p < 0.01$], but the injury severity $\times$ day interaction failed to show significance ($F_{(9,236)} = 0.766$, $p > 0.05$). Student's Newman-Keuls *post-hoc* analysis revealed significant differences between the moderately- and severely-injured groups on day 8 ($p < 0.05$), between the mildly- and severely-injured groups on day 8 ($p < 0.001$) and day 9 ($p < 0.001$), and between the mildly- and moderately- injured groups on day 9 ($p < 0.05$). In addition there were significant differences between the sham- and moderately-injured groups at day 7 ($p < 0.05$) and day 9 ($p < 0.01$),

and between the sham- and severely-injured groups at day 7 ($p < 0.01$), day 8 ($p < 0.001$), and day 9 ($p < 0.001$).

Probe tests were performed on PID 18 (sMWM) and PID 23 (rMWM). The hidden submerged platform was removed and the time sham and injured mice spent in the target quadrant where the platform had originally been placed was recorded. Reduced time spent in the target quadrant indicated impaired reference memory. For the sMWM probe test (Fig. 3C), the time spent in the target quadrant was injury severity-dependent [$F_{(3,60)} = 4.535$, $p < 0.0001$], and Student's Newman-Keuls *post-hoc* analysis indicated that severely-injured mice spent significantly less time in the target quadrant than sham-injured mice ($p < 0.01$). Significant differences were also observed between the moderately- and severely-injured groups ($p < 0.05$), and between the mildly- and severely-injured groups ($p < 0.05$). Notably, in the rMWM probe test, the effects of injury severity on this task were more pronounced [$F_{(3,60)} = 7.627$, $p < 0.00001$; Fig. 3D]. Student's Newman-Keuls *post-hoc* analysis demonstrated a significant difference between the sham- and severely-injured groups ($p < 0.001$). Furthermore, in contrast to the sMWM probe test, there were significant differences between the sham- and moderately-injured groups ($p < 0.05$), as well as between the mildly- and severely-injured groups ($p < 0.01$). These data suggest that the rMWM probe test is a superior test for separating injury severity-related deficits in spatial/retention memory than the sMWM probe test.

In addition, visible cue tests were performed on PID 18 (sMWM) and on PID 23 (rMWM), and no significant differences in latency to locate the visible platform were found between the sham-, mildly-, and moderately-injured groups in both tests [visible cue-sMWM: 36.38 ± 6.60 (sham), 39.58 ± 7.11 (mild), and 43.57 ± 7.99 sec (moderate); visible cue-rMWM: 43.01 ± 6.55 (sham), 45.19 ± 6.77 (mild), and 49.75 ± 7.13 sec (moderate)]. However, there was a significant increase in latency time in the severely-injured group compared to the sham-injured group on the rMWM visible cue test (68.77 ± 6.03 sec; $p < 0.05$), and a trend toward increased latency time in the severely-injured group on the sMWM visible cue test (59.05 ± 7.36 sec). Swim speeds did not differ between the sham-, mildly- and moderately-injured groups, on both the sMWM and the rMWM [sMWM day 4: 0.063 ± 0.005 (sham), 0.061 ± 0.003 (mild), and 0.063 ± 0.004 m/sec (moderate); rMWM day 4: 0.046 ± 0.004 (sham), 0.048 ± 0.004 (mild), and 0.049 ± 0.005 m/sec (moderate)]. There were non-significant reductions in swim speed in the severely-injured group compared to the sham-injured group for both tests [sMWM day 4: 0.047 ± 0.004 m/sec (severe); rMWM day 4: 0.032 ± 0.003 m/sec (severe)].

We then performed search strategy analysis to evaluate the efficiency of locating the platform. Based on previously published criteria,^{24,25} three search strategies were evaluated for each of the 4 trials on PID 17 for the sMWM and PID 22 for the rMWM. Occasionally mice changed search strategies during a trial. When this happened, the strategy that best described the major swimming path was assigned. Search strategy in the sMWM and rMWM showed good injury severity separation (chi-square=214.7, $p < 0.001$, and chi-square=50.91, $p < 0.001$, respectively; Fig. 3E and F). Severely-injured mice exhibited significantly higher reliance on looping search strategies than spatial and systematic search strategies than moderately-, mildly-, and sham-injured mice in both the sMWM and rMWM. Specifically, the use of spatial search strategies in the rMWM ranged from 60.0% in the sham-injured group to 11.1% in the severely-injured group, whereas in the sMWM it ranged from 48.3% in the sham-injured group to 8.3% in the severely-injured group.

FIG. 4. Assessment of non-spatial memory using the novel object recognition (NOR) test. All groups were subjected to an open-field trial 1 day prior (PID 24) to the choice phase (PID 25) of the NOR test. Sham and injured mice spent equal time with the two identical objects during the sample phase on PID 25 (dashed line at 15 sec). One hour after the sample phase, the time spent with the novel and familiar objects during the choice phase was recorded. The moderately- and severely-injured groups spent significantly less time with the novel object than the sham-injured group (^a $p < 0.05$ and ^{aa} $p < 0.01$, respectively). The sham- and mildly-injured groups spent similar times with the novel object (analysis by one-way analysis of variance; $n = 9-16$).

The novel object recognition test effectively discriminated non-spatial learning and memory across injury severities

In addition to spatial learning and memory measured by the MWM test, hippocampal-mediated non-spatial learning and memory after CCI injury were assessed by the NOR test. As shown in Figure 4, the sham-injured group spent more time than chance (15 sec) with the novel object 1 h after training (sample phase), indicating intact memory. Similarly, the mildly-injured group spent more time than chance with the novel object 1 h after training, whereas the moderately-injured group spent less time with the novel object, but significantly greater time than chance. Notably, the severely-injured group spent similar time periods with the novel and familiar objects. Furthermore, one-way ANOVA showed a significant effect of injury severity on time with the novel object [$F_{(3,51)} = 9.19$; $p < 0.001$], and Student's Newman-Keuls *post-hoc* analysis revealed that the moderately- and severely-injured groups spent significantly reduced time with the novel object compared to the sham-injured group ($p < 0.05$ and $p < 0.01$, respectively). No significant differences were observed between the sham and mildly-injured groups. These data indicate that moderately- and

severely-injured mice performed poorly in the NOR test, indicating that non-spatial learning and memory were impaired in these groups.

The passive avoidance test delineated injury-severity-dependent differences across groups

To determine the long-term effect of injury severity on what is believed to be amygdala-dependent non-spatial learning ability,²⁹ the passive avoidance (PA) test was conducted on PID 26–28. There were no significant differences in the latency to enter the dark compartment in the training phase between the sham and the injured groups (Fig. 5). Twenty-four hours after training, the latency to enter to the dark compartment was decreased in an injury-severity-dependent manner. One-way ANOVA showed a significant effect of injury severity on transfer times to the dark compartment [$F_{(3,62)} = 6.171$; $p < 0.001$], and Student's Newman-Keuls *post-hoc* analysis revealed that the mildly-, moderately-, and severely-injured groups had significantly reduced transfer latency compared with the sham-injured group ($p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively). No significant differences were observed between

FIG. 5. Assessment of contextual and emotional memory using the passive avoidance (PA) test. Sham and injured mice performed the passive avoidance test on PID 27 and 28. On PID 27, the mice were placed in a transfer chamber and a foot shock was delivered upon entry into the dark compartment from the bright compartment. The mice from each group showed similar transfer times. Twenty-four hours later, the mice were placed in the transfer chamber again and the transfer time was recorded without foot shock. The mildly-, moderately-, and severely-injured mice had significantly reduced transfer latency compared with sham-injured mice (^a $p < 0.05$, ^{aa} $p < 0.01$, and ^{aaa} $p < 0.01$, respectively; analysis by one-way analysis of variance; $n = 12$ –20).

FIG. 6. Assessment of depression-like behavior using the tail-suspension (TS) test. The TS test was performed on post-injury day 24. Significantly increased immobility times were observed in moderately- (^a $p < 0.05$) and severely-injured (^{aaa} $p < 0.001$) mice, compared to sham-injured mice. Significant differences were also observed between the mildly- and moderately-injured group (^b $p < 0.05$), and between the mildly- and severely-injured groups (^{ccc} $p < 0.001$; analysis by one-way analysis of variance ($n = 6$ –10).

the mildly- and moderately-injured groups, and between the moderately- and severely-injured groups.

The tail-suspension test demonstrated injury-severity-dependent effects

To determine the long-term effects on depression-like behavior³² following TBI, sham-, mildly-, moderately-, and severely-injured mice were tested in a tail suspension test on PID 24. As shown in Figure 6, the sham-injured group showed an immobility time of approximately 80 sec, with injury-severity-dependent increases in immobility times in the moderately- and severely-injured groups. One-way ANOVA showed a significant effect of injury severity in the tail suspension test [$F_{(3, 30)} = 10.84$; $p < 0.001$], and Student's Newman-Keuls *post-hoc* analysis showed significantly increased immobility times in the moderately- ($p < 0.05$) and severely-injured groups ($p < 0.001$) compared to the sham-injured group. Significant differences were also observed between the mildly- and moderately-injured groups ($p < 0.05$), and between the mildly- and severely-injured groups ($p < 0.001$). In addition, we performed the elevated plus maze test to evaluate anxiety-like effects^{34,35} in the same cohort of mice on PID 26, but this test failed to show TBI-induced anxiety-like behavior in these mice (data was not shown).

Open-field test and locomotor activity

The open-field test was performed on PID 24 to examine spontaneous locomotor activity following TBI. As shown in Figure 7, sham- (4.98 ± 0.43 m), mildly- (4.91 ± 0.37 m), and moderately-injured mice (5.83 ± 0.77 m) displayed similar locomotor activity (distance traveled) during the 5 min of testing. Severely-injured mice showed reduced locomotor activity (3.55 ± 0.42 m), and one-way ANOVA demonstrated a significant effect of injury severity on locomotor activity [$F_{(3,56)} = 3.14$; $p < 0.05$]. Although differences across injury level did not reach significance using the Student's Newman-Keuls *post-hoc* analysis, significantly decreased locomotor activity was detected in the severely-injured group compared with the sham-injured group using a paired *t*-test with Welch correction ($t = 2.301$, $p < 0.05$). Decreased locomotor activity in the

FIG. 7. Determination of locomotor activity using the open-field test. The distance traveled in an open field over 5 min was recorded in sham and injured mice. There was a significant effect of injury severity on locomotor activity (one-way analysis of variance; $n = 11-19$). Although differences across injury level did not reach statistical significance using the Student's Newman-Keuls *post-hoc* analysis, a paired *t*-test with Welch correction demonstrated significantly decreased locomotor activity in the severely-injured group compared with the sham-injured group ($t = 2.301$, ^a $p < 0.05$).

severely-injured group was paralleled by reduced speed (sham 0.018 ± 0.0020 m/sec versus severe 0.013 ± 0.0013 m/sec) in the open-field test using Student's *t*-test ($t = 2.376$, $p < 0.05$).

Injury-severity-dependent increases in lesion volume correlated with impairments in sensorimotor performance

TBI-induced lesion volume was quantified in cresyl violet-stained brain sections from sham-, mildly-, moderately-, and severely-injured mice at 28 days post-injury by the Cavalieri method (Fig. 8A and B). One-way ANOVA demonstrated a significant injury-severity-dependent increase in lesion size [$F_{(3,29)} = 9.01$, $p < 0.001$], and Student's Newman-Keuls *post-hoc* analysis demonstrated significant differences in the mildly- ($p < 0.05$), moderately- ($p < 0.01$), and severely-injured ($p < 0.001$) group compared to the sham-injured group. Significant differences were also observed between the mildly- and severely-injured groups ($p < 0.05$). TBI-induced lesion size was strongly correlated with impairments in sensorimotor performance, as demonstrated by a positive correlation by linear regression analysis ($r^2 = 0.88$, $p < 0.0001$; Fig. 8C).

Hippocampal neuronal loss was injury-severity-dependent and correlated with impairments in cognitive performance

TBI-induced neuronal loss in the CA1, CA2/3, and DG subregions of the hippocampus was quantified in cresyl violet-stained

FIG. 8. Assessment of lesion volume at 28 days post-injury and its correlation with the beam-walk test. Lesion volume was quantified using the Cavalieri method. (A) Representative images show the extent of TBI-induced lesion volumes in sham-, mildly-, moderately-, and severely-injured mice. (B) Significant differences were observed in the mildly- (^a $p < 0.05$), moderately- (^{aa} $p < 0.01$), and severely-injured (^{aaa} $p < 0.001$) groups compared to the sham-injured group. Significant differences were also observed between the mildly- and severely-injured groups (^b $p < 0.05$). (C) Linear regression analysis was performed to assess the correlation between lesion volume and motor function using the beam-walk test. A strong correlation was demonstrated between injury-severity-dependent lesion volumes and motor function performance ($r^2 = 0.88$, $p < 0.0001$; analysis by one-way analysis of variance; $n = 5-10$).

FIG. 9. Stereological assessment of traumatic brain injury (TBI)-induced neuronal loss in the hippocampus. Stereological assessment of absolute neuronal cell numbers on post-injury day (PID) 28 was performed on cresyl violet-stained sections in the CA1, CA2/3, and dentate gyrus (DG) sub-regions of the hippocampus. (A) Severely-injured mice had significantly reduced neuronal cell numbers in the CA1 region (^{aa} $p < 0.01$). Significant differences were also observed between the mildly- and severely-injured groups (^b $p < 0.05$). (B) Significant differences in the CA2/3 region were observed between the sham- and severely-injured groups (^{aa} $p < 0.01$), and between the mildly- and severely-injured groups (^{bb} $p < 0.01$). (C) A significant difference in the DG region was observed between the sham- and severely-injured groups (^{aaa} $p < 0.001$). Significant differences were also observed between the moderately- and severely-injured groups (^b $p < 0.05$), and between the moderately- and severely-injured groups (^c $p < 0.05$). (D) Cell counts were summed from the CA1, CA2/3, and DG regions, and significant reductions were observed in total neuronal cell numbers in the mildly- (^a $p < 0.05$), moderately- (^a $p < 0.05$), and severely-injured (^{aaa} $p < 0.001$) groups, compared to the sham-injured group. Significant differences were also observed between the moderately- and severely-injured groups (^b $p < 0.05$; analysis by one-way analysis of variance; $n = 5-10$).

brain sections from sham-, mildly-, moderately-, and severely-injured mice at 28 days post injury by un-biased stereological techniques (Fig. 9). One-way ANOVA found that TBI induced a significant injury-severity-dependent loss of neurons in the CA1 [$F_{(3,27)} = 6.062$, $p < 0.01$; Fig. 9A], CA2/3 [$F_{(3,27)} = 6.960$, $p < 0.01$; Fig. 9B], and the DG [$F_{(3,27)} = 7.334$, $p < 0.01$; Fig. 9C] sub-regions of the hippocampus. Student's Newman-Keuls *post-hoc* analysis demonstrated that the severely-injured group had significantly reduced neuronal cell numbers in the CA1 ($p < 0.01$), CA2/3 ($p < 0.01$), and DG ($p < 0.001$) sub-regions compared to the sham-injured group ($p < 0.01$). Significant differences were also observed between the mildly- and severely-injured groups in the CA1 ($p < 0.05$) and CA2/3 ($p < 0.01$) sub-regions, between the moderately- and severely-injured groups in the DG sub-region ($p < 0.05$), and between the mildly- and severely-injured groups in the DG sub-region ($p < 0.05$).

We randomly selected five samples from each group and performed correlation analysis between selected individual cognitive tests and neuronal loss in selected hippocampal sub-regions and present the data in Table 1. The analysis demonstrates that neuronal loss in the CA1, CA2/3, and DG sub-regions, and the combined hippocampal cell loss (total cells), were strongly correlated with the sMWM day 4 acquisition trial and rMWM day 4 acquisition trial ($\rho = -0.93$, $p = 4.2E-09$ [sMWM]; $\rho = -0.94$, $p = 1.62E-09$ [rMWM]). The reversal probe test showed better correlation with regard to neuronal loss in the CA1 and CA2/3 sub-regions than the sMWM probe test. In contrast, the correlations between hippocampal DG sub-region neuron cell loss and the PA and NOR tests were less significant ($\rho = 0.89$, $p = 1.19E-07$ [PA]; $\rho = 0.9$, $p = 4.79E-08$ [NOR]).

The composite behavioral score provides greater separation between injury groups than individual tests at lower injury levels

In order to determine whether a combination of test scores can provide better discrimination than individual scores, we combined

TABLE 1. COGNITIVE FUNCTION IMPAIRMENTS AND NEURONAL LOSS IN SUB-REGIONS OF THE HIPPOCAMPUS

	CA1		CA2/3	
	Spearman rho	p	Spearman rho	p
sMWM day 4	-0.92	1.12E-08	-0.92	9.43E-09
rMWM day 4	-0.95	1.91E-10	-0.94	1.05E-09
Probe	0.93	2.89E-09	0.90	5.65E-08
Reverse probe	0.94	1.41E-09	0.97	2.78E-12
NOR	0.91	4.98E-08	0.91	2.02E-08
PA	0.93	3.27E-09	0.94	1.03E-09
	DG		Total cells	
	Spearman rho	p	Spearman rho	p
sMWM day 4	-0.93	1.99E-09	-0.93	4.20E-09
rMWM day 4	-0.91	2.18E-08	-0.94	1.62E-09
Probe	0.94	1.14E-09	0.92	4.14E-10
Reverse probe	0.92	5.53E-09	0.94	6.36E-09
NOR	0.80	1.92E-05	0.90	4.79E-08
PA	0.88	3.68E-07	0.89	1.19E-07

sMWM, standard Morris water maze; rMWM, reversal Morris water maze; NOR, novel object recognition; PA, passive avoidance; DG, dentate gyrus.

TABLE 2. CONVERSION TABLE FOR THE COMPOSITE SCORE (CS)

CS	sMWM probe	rMWM probe	TS	BW
Severe 0	0–4	0–4	> 165	42–50
1	4–8	4–8	150–165	34–42
2	8–12	8–12	135–150	26–34
3	12–16	12–16	120–135	18–26
4	16–20	16–20	105–120	10–18
Sham 5	> 20	> 20	< 105	0–10

sMWM, standard Morris water maze; rMWM, reversal Morris water maze; TS, tail suspension; BW, beam walk.

and converted the motor, cognitive, and affective test results into a composite score (CS). We empirically restricted our analysis to four distinct composite scores that included individual behavioral indexes: (1) six behavioral tests (sMWM probe, rMWM probe, NOR, PA, BW, and TS); (2) five tests excluding NOR; (3) five tests excluding PA; and (4) four tests excluding NOR and PA. Because we evaluated four different composite scores we applied a conservative Bonferroni correction and set the significance level at 0.0125 to ensure a total false-positive rate of 5%. It is important to note that three out of four of the combined scores described above resulted in significant separation between different injury severities ($p < 0.0125$). The selected composite score (sMWM probe, rMWM probe, BW, and TS) resulted in the most significant separation between groups and the lowest p values ($p < 0.001$) compared to the other combinations (6 tests, 5 tests minus NOR, and 5 tests minus PA). The score for each test was then converted to an ordinal scale from 0 (most severe) to 5 for each behavioral test. The summation of these individual scores resulted in a CS ranging from 0 to 20 (Table 2). Sham-injured mice had a CS score of 19.43. As shown in Figure 10A, the CS showed great separation between the mildly-, moderately- and severely-injured groups. Non-parametric Kruskal-Wallis analysis demonstrated a significant difference among these groups (Kruskal-Wallis statistic=27.10, $p < 0.0001$). Q-Q plot analysis demonstrated that the data displayed normality and equal variance; therefore we analyzed these interval data using parametric statistics [one-way ANOVA; $F_{(3,27)} = 79.89$, $p < 0.001$]. Student's Newman-Keuls *post-hoc* analysis demonstrated a significant difference between sham and mild injury ($p < 0.001$), between mild and moderate injury ($p < 0.001$), and between moderate and severe injury ($p < 0.001$). The separation of behavioral outcomes after different levels of injury severity was greater using the CS than any behavioral task alone. We compared the separation between injury severity levels by evaluating the percentage difference and *post-hoc* p values for each behavioral test and the CS between sham and mild, mild and moderate, and moderate and

severe levels of injury (Table 3). The CS better discriminated between mild and moderate injury levels than each of the behavioral tasks (sMWM probe/rMWM probe/TS/BW) alone, and was also superior in discriminating sham from mild, and moderate from severe, injury levels in these tasks. Linear regression was performed to compare CS to lesion volume and total hippocampal neurons. As shown in Figure 10B, a strong positive correlation was found between the CS and lesion volume ($r^2 = 0.86$, $p < 0.0001$), and between the CS and the total number of neurons in the CA1, CA2/3, and DG sub-regions of the hippocampus ($r^2 = 0.92$, $p < 0.0001$; Fig. 10C).

Discussion

We compared the predictive value of a series of behavioral tests, either singly or in combination, as a function of injury severity following CCI in mice. These included the classical or standard Morris water maze (sMWM) and reversal Morris water maze (rMWM) tests, novel object recognition (NOR), passive avoidance (PA), tail suspension (TS), beam walk (BW), and open-field locomotor activity. The goals were to examine the following questions: (1) whether more detailed behavioral assessment than is usually employed is feasible in experimental TBI; (2) to what degree the various behavioral tasks reflect lesion changes and loss of cells in specific anatomical regions; (3) whether the use of multiple behavioral outcomes enhances the ability to discriminate across levels of injury severity; and (4) whether a combined assessment “neuroscore” could be developed that increases predictive value in distinguishing injury severity.

The sMWM, considered to be a test of anterograde spatial learning and memory,³⁶ has been commonly used to examine learning and memory deficits in rodents after TBI.^{10,37} It was reported that the time to locate the platform in the MWM was TBI severity-dependent in a rat CCI model.¹⁵ The rMWM paradigm has been far less studied following TBI.^{23,38,39} One day after the sMWM probe test, the platform was moved to the opposite quadrant (southwest), and the mice were trained for 4 consecutive days in the rMWM followed by another probe trial. In the rMWM task the animal must develop a new escape strategy to locate a submerged platform no longer placed in the quadrant where the animal had previously found it.^{39,40} Our data showed that the rMWM paradigm was superior to the sMWM in distinguishing mild from moderate and severe injury in the training phase. In addition, moderately- and severely-injured mice displayed more deficits in spatial memory in the rMWM probe test than the sMWM probe test. As the rMWM evaluates inhibitory learning, in which mice must learn to suppress a previously learned response,^{41,42} the present results suggest that the extinction of previously acquired

TABLE 3. PERCENTAGE DIFFERENCES AND P VALUE BETWEEN INJURY SEVERITY GROUPS FOR THE COMPOSITE SCORE (CS) AND INDIVIDUAL BEHAVIORAL TESTS

	Sham versus mild		Mild versus moderate		Moderate versus severe	
	% difference	p	% difference	p	% difference	p
CS	20.2	<0.001	57.3	<0.001	23.3	<0.001
sMWM probe	9.1	0.565	10.4	0.521	35.4	0.036
rMWM probe	14.1	0.298	22.9	0.101	24.8	0.088
TS	12.9	0.345	34.6	0.020	22.8	0.133
BW	26.0	0.002	18.8	0.039	19.2	0.020

sMWM, standard Morris water maze; rMWM, reversal Morris water maze; TS, tail suspension; BW, beam walk.

FIG. 10. A composite score that integrates multiple behavioral outcomes after cortical impact injury. (A) Four behavioral indexes were converted to a composite score, which ranged from 0 (severe) to 5 (sham). The composite score was obtained by summing the scores for the four indexes. Significant differences were observed between the sham- and mildly-injured groups (^{aaa} $p < 0.001$); the mildly- and moderately-injured groups (^{bbb} $p < 0.001$); and the moderately- and severely-injured groups (^{ccc} $p < 0.001$). (B and C) A correlation of composite score to lesion volume (B) and total numbers of hippocampus neurons (C) was performed by linear regression analysis and positive correlations were found between lesion volume and composite score (B; $r^2 = 0.86$), and between total hippocampal cell counts and composite score (C; $r^2 = 0.92$; by one-way analysis of variance; $n = 6-10$).

memories and the reacquisition process may provide a more sensitive measure than the traditional MWM test for identifying cognitive deficits after experimental TBI. However, despite the fact that the rMWM may be a more sensitive test for discriminating injury-severity-related deficits in cognition, it requires 10 days of testing. This longer duration of testing reduces the ability of the rMWM to identify injury-related changes on a short time scale,

which may somewhat diminish its usability in experimental TBI studies.

As the MWM task is particularly sensitive to hippocampal dysfunction,⁴³ neuronal cell losses in sub-regions of the hippocampus were quantified to examine the correlation between MWM and neuronal cell loss. In the present study we found that neuronal cell loss in the CA1, CA2/3, and DG sub-regions of the

hippocampus was injury-severity-dependent, and correlated with cognitive dysfunction as demonstrated by both the sMWM and rMWM tests. However, the Spearman rho correlation coefficients between latency of rMWM and sMWM on day 4 and cell loss in these respective hippocampal sub-regions differed: for CA1, this was -0.95 and -0.92 , respectively; for CA2/3, the correlations were -0.94 and -0.92 ; and for DG the correlations were -0.91 and -0.93 . These data suggest that the latency on day 4 in the rMWM has a better correlation with CA1 and CA2/3 neuronal cell loss than the sMWM. Similarly, the reversal probe test showed higher correlations to CA2/3 neuronal cell counts compared to the standard probe test (0.97 versus 0.90 , respectively), whereas correlations to CA1 (0.94 [reverse probe] versus 0.93 [probe]), and DG (0.92 [reverse probe] versus 0.94 [probe]) neuronal cell counts were similar. These data further suggest that the sMWM and rMWM reflect cell loss to different degrees in different anatomical regions in the hippocampus.

In the present study, we observed that some severely-injured mice were somewhat reluctant to swim, tending at times to float rather than swim; this may in part reflect motor deficits, fatigue, and/or depressive-like behavior in severely-injured mice, as suggested by a trend toward a reduced swim speed in the MWM, significantly decreased activity in the open-field test, and increased immobility time in the TS, respectively. Thus the MWM results in severely-injured animals may not reflect pure cognitive deficits, but may be confounded by motor and affective impairments. Therefore, we also examined the search strategy used by the injured mice to locate the hidden platform.²⁴ This parallels the type of search strategy analysis proposed for the Barnes maze,^{44,45} which was effectively used previously in this TBI model.¹² Sham-injured mice primarily employed a spatial strategy to locate the platform; use of this strategy decreased with injury severity. In contrast, sham-injured mice rarely used a looping strategy to locate the platform, and this strategy was more prominently used with increased injury severity. Moreover, whereas sham animals swam in the center of the maze to find the hidden platform, injured animals spent most of their time swimming around the perimeter of the maze.

The NOR task evaluates non-spatial hippocampal memory,^{26–28} and is therefore complementary to spatial memory assessment by the MWM test. NOR has been widely used to measure cognitive function after experimental TBI in rodents.^{46–48} In the present study, mildly-injured mice spent a similar length of time with the novel object as sham-injured mice, whereas moderately- and severely-injured mice spent significantly less time. Correlation coefficients between NOR and CA1, CA2/3, and DG neuronal cell counts, were 0.91 , 0.91 , and 0.80 , respectively.

The hippocampus plays an important role in contextual memory; but PA learning involves both contextual memory and amygdala-dependent emotional memory.^{49,50} Thus, performance on tests of PA learning decreases as a result of defects in either contextual or emotional memory. Generally, the dorsal hippocampus is specifically involved in memory function, and the ventral hippocampus modulates emotional and affective processes.⁵¹ Our histological analysis was focused on the dorsal region of the hippocampus, in the area directly underneath the impact site. The mildly-, moderately-, and severely-injured groups had significantly reduced transfer latency in the PA test compared with the sham-injured group, but this test did not effectively discriminate between injury severity levels. Correlation coefficients between PA and CA1, CA2/3, and DG neuronal cell counts were 0.93 , 0.94 , and 0.88 , respectively.

Stereological methods were used to examine total lesion volume and hippocampal cell loss at 28 days post-injury for each group of

mice. Both approaches showed strong correlations between histological changes and injury severity. Conclusions about which sub-regions of the hippocampus are most involved in the mediation of spatial learning have been conflicting. It was reported that dorsal hippocampal lesions have more profound effects than ventral hippocampal lesions.^{52,53} Morris and colleagues⁵⁴ showed that intraventricular infusion of amino-phosphonovaleric acid (AP5) impairs hidden-platform MWM performance while blocking long-term potentiation (LTP) in the hippocampal dentate gyrus. Hicks and colleagues⁵⁵ reported a significant correlation between post-traumatic memory scores using the rMWM after mild lateral fluid percussion and neuronal loss in the hilus of the dentate gyrus. However, another study using genetically-engineered mice showed that blunted LTP in hippocampal CA1 area coincided with impaired MWM learning.⁵⁶ Our results were more nuanced. We found significant correlations between cognitive dysfunction and neuronal cell loss in the CA1, CA2/3, and DG sub-regions, but these differed as a function of each specific cognitive task (Table 1).

The hippocampus is important for tasks such as the MWM, which depends on relating or combining information from multiple sources.⁵⁷ However, a task that does not have such requirements, such as NOR, may reflect involvement of larger areas, including both the hippocampus and the cortex adjacent to the hippocampus.⁵⁸ Broadbent and colleagues suggested that larger hippocampal lesions may be needed to impair recognition memory than are needed to impair spatial memory.⁵⁹ This is consistent with our finding that mildly-injured mice showed cognitive impairments in the MWM, but not in NOR. Few studies have examined the correlation between NOR and neuronal cell loss in hippocampal sub-regions. By measuring differential neuronal activation produced by novel and familiar pictures, it was found that novel pictures produce greater activation in subfield CA1 and less activation in the DG.⁶⁰ Consistent with these observations, the present study demonstrated that the correlation between NOR and CA1 ($r=0.91$) and CA2/3 ($r=0.91$) is greater than that between NOR and DG ($r=0.80$), suggesting a more critical role for CA1 and CA2/3 in the mediation of novel recognition memory. In addition, we compared cognitive dysfunction assessed by each behavioral test with neuronal cell loss in the CA1, CA2/3, and DG sub-regions of the hippocampus, and determined the Spearman rho correlation coefficient and the p value for each regression analysis. This analysis demonstrated that the correlation between the hippocampal neuronal number and the NOR and PA tests were less significant than hippocampal neuronal number and the sMWM, rMWM, probe, and reverse probe tests (Table 1).

Numerous groups have examined sensorimotor or vestibulomotor changes after rodent TBI.^{61–65} A previous report from our laboratory showed that moderately-injured mice had significantly more foot-faults than sham-injured mice in the beam-walk test.²¹ Flierl and colleagues evaluated sensorimotor function and cortical tissue damage after TBI as a function of injury severity in mice,⁶⁵ but the correlation between sensorimotor function and lesion volume was not studied.³⁵ In the present study, we found that changes in sensorimotor function using the beam-walk test reflected injury severity. Furthermore, in the present experimental conditions, injury-severity-dependent impairments in sensorimotor function were significantly correlated with lesion volumes in the injured cortex ($r^2=0.88$).

Combinations of behavioral tests have long been used in acute experimental brain and spinal cord injury models to increase sensitivity for discriminating both injury mechanism and the response to therapeutic interventions.^{66–71} Faden and colleagues developed several versions of a composite score for fluid percussion-induced

TBI in the rat,^{72–74} adapted from prior work in stroke and spinal cord injury. Saatman and colleagues¹⁹ developed a composite score that included the MWM test and a motor functional behavioral test. A previous study by Hamm⁷⁵ used multiple neurobehavioral tasks to evaluate the outcomes of TBI, including reflex suppression (pinna, corneal, and righting reflex), vestibulomotor function (beam balance, beam walking, and rotarod), and cognitive function. Here we sought to build upon and extend these earlier approaches by examining multiple cognitive and motor functional behavioral outcomes. We evaluated and compared various combinations of cognitive, affective, and motor function behavioral tests, to determine if such combined scores could better discriminate injury severity than individual scores. Results from the sMWM probe trial, the rMWM probe trial, the TS test, and the BW test were each converted to an ordinal scale ranging from 0 to 5. Two indices from the MWM were used to calculate CS, because the sMWM measures spatial memory acquisition, whereas the rMWM measures spatial retention memory after memory extinguishing and reacquisition. Further, the TS test represents affective behavior function, while the BW test represents sensorimotor function. Therefore, the CS provides an overall assessment of neurological function in injured mice. These four individual scores were combined, without weighting, to yield a 20-point CS ranging from 0 (severe) to 20 (sham). This CS served to increase differences between the sham- and mildly-injured group from 1–2 points to 4–7 points, enhancing the ability to observe significant differences between these groups, often a problem for studies examining mild or concussive-like insults. Here we found an increased discriminative ability for the CS compared to individual behavioral test scores (Table 3), which may allow better delineation between groups for future genetic or pharmacological studies. Notably, in a more recent pharmacological neuroprotection study (manuscript in preparation), we show that the CS provides superior discrimination of functional recovery than individual behavioral tests.

Despite the apparent benefits of using batteries of behavioral tasks to evaluate injury-severity-dependent outcomes after TBI, the present study also revealed some limitations. For example, the severely-injured, but not mildly- or moderately-injured mice, demonstrated less movement in the MWM and reduced locomotor activity in the open-field task. Fatigue, anxiety, depression, or some combination may contribute to reduced swimming ability and locomotor activity in severely-injured mice. To overcome such potential confounding issues, several modifications of the MWM should be considered: (1) reducing the number of daily trials in the test to three; (2) reducing the number of training days to 3; and (3) restricting the mice to a smaller area around the target hidden platform to facilitate locating the platform on the first day of training. In the present study, the severely-injured mice also demonstrated significantly longer times to locate the visible platform, which may have been related to fatigue. The performance of a visible cue test at an earlier time point should be considered to circumvent this issue.

Taken together, the data from this study demonstrate that multiple cognitive, affective, and motor tasks are able to delineate injury severity after CCI in mice. Both cortical lesion volume and neuronal cell loss in sub-regions of the hippocampus were well correlated with sensorimotor and cognitive dysfunction. Further, the composite score developed in the present study was able to better discriminate injury severity levels than traditional individual outcome tests. The CS may therefore be helpful in screening new neuroprotective agents or in investigating secondary injury mechanisms using transgenic mouse models.

Acknowledgments

We thank Rainier Cabatbat for expert technical support and Dr. Shruti Kabadi and Dr. Ming Tan for helpful discussion. This work was supported by NIH grants R01 (NS052568) and R01 (NS061839) to A.I.F.

Author Disclosure Statement

No competing financial interests exist.

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