Demonstration of Greater Post-Injury Deficits in Rats Exposed to Repeated Concussion Using Behavioral Testing

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Abstract Mild traumatic brain injuries occur frequently and are generally associated with a lack of pathological evidence, although chronic behavioral disturbances can result. Repeated injuries are common and cumulative brain damage may exacerbate behavioral disturbances. The MCW rotational mTBI model was used to investigate cognitive, motor, and emotional disturbances following single and repeated injury in rats. The interval between injuries was seven days. All rats survived rotational acceleration without skull fracture, cervical spine injury, or evidence of focal trauma or brain hemorrhage. Motor and cognitive deficits were not apparent at seven days post-injury. However, rats with repeated injuries demonstrated statistically significant hyperactive and decreased defensive behaviors during Open Field and Elevated Plus Maze testing. Present results from cognitive and motor testing agree with previous studies of repeated mTBI. However, this is the first study to investigate emotional-type disturbances in rats following repeated injuries. Behavioral differences following repeated injuries were similar to previously reported findings of an olfactory bulbectomized rat model of depression, which has been reported in patients following mTBI. The present experimental model is ideal for the study of repeated mTBI as the injury mechanism and lack of pathological evidence mimic the human condition, and it produces clinically relevant outcomes.

Keywords: mild traumatic brain injury, biomechanics, cognitive deficits, post-injury behavior.

I. INTRODUCTION

Traumatic brain injury (TBI) affects approximately 1.6 million Americans annually [1]. Lower severity injuries, graded as mild to moderate TBI, are common and account for approximately 300,000 hospital admissions annually [2]. Additionally, many mild TBIs (i.e., mTBI) go unreported due to the low level of initial symptoms. The classical description of mTBI as advanced by Ommaya and Gennarelli was a reversible syndrome without detectable pathology [3]. While physical evidence of pathology is often elusive, patients with mTBI suffer from cognitive deficits, emotional difficulties, and behavioral disturbances [4, 5]. Cumulative damage from repeated injuries may exacerbate symptoms of mTBI. Clinical studies have reported impaired neuropsychological performance for athletes sustaining 2-5 mTBI compared to a single incident [6]. Increased deficits for repeated injuries are compounded by the possibility that patients with a single mTBI may be more susceptible to additional injuries. Recent literature has suggested that patients sustaining an initial TBI are likely to incur a second TBI, particularly those engaged in contact or collision sports such as American football and boxing [7-10]. A recent study of American college football players reported that athletes with a history of previous concussions are significantly more likely to sustain a second concussion within the initial seven to ten days following injury [11].

Experimental studies have investigated effects of repeated TBI using different animal models [12-17]. One of the more clinically useful outcomes of experimentally-based mTBI research is the quantification of behavioral deficits associated with specific injury types/levels. This is due to the fact that a majority of symptoms in mTBI patients are behaviorally-based, and results of well-characterized behavioral tests (e.g., Morris Water Maze)
may be translatable to the human. Previously conducted repeated TBI studies have generally focused on quantifying cognitive and motor deficits. Although emotional difficulties are a primary outcome of mTBI, to our knowledge, no experimental study has reported changes in anxiety, aggressivity, or depression following repeated injuries. Results of cognitive testing using the Morris Water Maze have not been consistent between studies, with some studies identifying some statistically significant deficits [15, 17] and others reporting statistically equivalent responses between animals with repeated injury and those sustaining a single or no injury [12, 13, 17, 18]. Differences in cognitive behavioral outcomes between these studies generally broke down according to the injury model employed, with the closed skull controlled cortical impact model resulting in non-significant cognitive deficits for repeated injuries in most cases.

These findings highlight the importance of injury model in generating clinically useful behaviorally-based data using experimental animal models of repeated TBI. Our group has developed and previously reported at IRCOBI meetings an experimental rodent model that induces mTBI through the biomechanically-correct mechanism of head angular acceleration [19]. Similar rotationally-based models have since been reported at IRCOBI, although focusing on sagittal rotations and more severe injury levels [20]. Because of greater biomechanical similarity to human injury mechanisms, outcomes from these models are expected to be more relevant to the human condition than models that do not incorporate angular acceleration (e.g., weight-drop, controlled cortical impact, fluid percussion). The purpose of this study was to quantify differences in cognitive, neuromotor, and emotional outcomes between groups of rats with repeated injury, single injury, and no injury.

II. METHODS

The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at our institution. Adult female Sprague Dawley rats (200 to 250 g) were stored in separate cages with twelve hour light/dark cycles and provided with food and water as needed. The experimental protocol consisted of administration of general anesthesia using ketamine (90 mg/kg) and xylazine (10 mg/kg). Once rats became unresponsive after approximately ten minutes, monitoring equipment (ADInstruments, Inc, Colorado Spring, CO) was used to obtain basal levels for physiological biosignals including blood pressure, heart rate, blood oxygen saturation levels, and body temperature for five minutes or until 15 minutes after initial administration of anesthesia. Rats were then placed in the injury device and exposed to rotation-induced injury (details provided below). Following injury, at 25 minutes following administration of anesthesia, rats were given a reversal agent to counteract effects of anesthesia and physiological biosignals were again monitored. As the rats became responsive, full recovery was assessed as the return of corneal, escape, pinch, pinna, whisker, and righting reflexes. Following recovery, rats were again stored in separate cages with twelve hour light/dark cycles until behavioral testing and/or sacrifice. Food and water was provided as needed. Post-injury assessments were conducted approximately one week following injury as described below.

INJURY INDUCTION: Anesthetized rats received mechanical insult resulting in mTBI using our existing rotational brain injury device [21]. The helmet was attached to the head of the rat and mounted to the anterior support, with the body of the rat placed in its support. The helmet was aligned in a level position, with moment arm horizontal, and the weight was raised to the desired height and released. Gravity accelerated the weight down the drop tube to strike the moment arm and rotate the helmet to the desired rotational acceleration level. Characteristics of the coronal plane rotational acceleration versus time pulse were modulated to induce mTBI (i.e., concussion) [22]. The rotational acceleration event consisted of 90 deg angular rotation with a maximum angular acceleration of 400 krad/s² and 2.0 msec duration of the positive angular acceleration pulse (Fig. 1). To achieve this, compressive properties of the elastomer, and drop height and mass of the impactor were used to control the duration and peak magnitude of helmet angular acceleration. Following insult, the helmet was detached from the anterior support and removed from the rat head. The rat was then placed on a warming blanket and the protocol continued as described above. Rats were divided into three groups. The single injury received one exposure to head rotational acceleration followed by behavioral testing approximately one week later. The repeated injury group, a completely separate group of animals, received one exposure to head rotational acceleration, followed by a second exposure seven days later. The control group was subjected to the entire experimental protocol, including anesthesia and placement in the helmet,
without exposure to head rotational acceleration. Head rotational accelerations for the single injury group, the first injury for the repeated injury group, and the second injury for the repeated injury group were not statistically different.

MORRIS WATER MAZE PROTOCOL: The Morris Water Maze Visuo-Spatial Learning Paradigm has been used to grade post-traumatic anterograde amnesia and spatial learning following TBI in rats [23, 24]. Unlike assessments for retrograde amnesia that require pre-training to locate and remember the location of the hidden platform, the current learning paradigm required a novel environment post-injury. The testing paradigm consisted of four sets of trials conducted over two days. Sets were conducted in the morning and afternoon on post-injury days five and six. For each set, four trials were conducted with rats initially placed at each of the four cardinal locations within the 183-cm diameter maze (N, E, S, W), facing the outer wall. The sequence of initial placement was randomized for each trial. Rats were allowed to swim in the 25-cm deep water until finding and mounting a hidden platform, or until 60 sec had passed. The platform surface was located approximately 2 cm below the surface of the water for all trials. Water temperature was maintained within one degree of 24 deg C, as water temperature can affect swim speed in rats. The plexiglass platform location was identical (e.g., SE) for all four trials of set, but was moved to a different position for each set (NE, NW, SW). The sequence of platform location was randomized between sets. The maze was placed in a room with numerous visual cues external to the maze and oriented identically for each test. The inner walls and floor of the maze were painted black, along with the plexiglass platform. Latency to find the submerged platform was measured using a digital video camera mounted above the center of the platform. Latency was averaged over the final three trials of each set because the ability to find the platform in a novel position on the first trial was not dependent upon spatial memory. An unsuccessful trial was determined as a trial in which the rat did not find the submerged platform within 60 s. The number of unsuccessful trials across the last three trials of each set (n=12) was counted for each rat. The ‘slope of learning’ metric was computed as the slope of the latency versus set number plot. Greater anterograde amnesia was associated with longer mean latency to find the submerged platform and a greater number of unsuccessful trials. Latency and the percentage of unsuccessful trials were statistically compared (p<0.05) using two-factor repeated measures ANOVA, with post-injury time (sets 1-4) as the within-group (repeated) factor and experimental group as the between-group factor.

COMPOSITE NEUROSCORE PROTOCOL: The Composite Neuroscore Motor Assessment Task (CN) has been used to grade post-traumatic gross neurological dysfunction [25]. Rats were exposed to the Composite Neuroscore on the seventh post-injury day. The task assessed nine neurologically-related reflexes with scores of 0 (severely impaired), 1 (minor impairment), and 2 (normal). The composite score from all tests provided an overall assessment of neurological function. The investigator scoring each reflex was blinded to the experimental group of each rat (i.e., controls, single injury, or repeated injury). The nine tasks included: (1) left and (2) right forelimb flexion during suspension by the tail; (3) left and (4) right hind limb flexion with forelimbs remaining on a flat surface as hind limbs are lifted up and down by the tail; (5) ability to resist lateral pulsion to the left and (6) right; (7) ability to stand on an inclined plane in the left, (8) right, and (9) vertical position.
Lower Composite Neuroscore was associated with higher levels of neuromotor dysfunction.

![Diagrams of Morris Water Maze (left), Elevated Plus Maze (middle), and Open Field Test (right).](image)

**ELEVATED PLUS MAZE PROTOCOL:** The Elevated Plus Maze has been used to describe anxiety-related behavior following TBI [26-28] and has been widely used to assess anxiety in rats for other applications [29]. The assessments rely on monitoring behavior of the rats when exposed to an unknown (e.g., novel) environment from which escape is not possible [30]. Rats were exposed to the Open Field Test on the seventh post-injury day. The test was conducted in a flat, square shaped arena (40 by 40 cm) with 20 cm walls around the periphery. The arena was illuminated from above using ceiling lights. Rats were initially placed in the center and allowed to explore the arena for five minutes. The entire test was recorded from above using a digital video recorder suspended above the center of the arena. The periphery of the arena was determined to be the area within 10 cm of the outer walls. Metrics quantified during the test included distance traveled (ambulatory distance, cm), ambulatory time (sec), the number of rearing events, the number of center entries, distance traveled along the periphery (margin distance, cm), and time spent in the periphery (margin time, sec). These metrics were scored by an observer blinded to the injury status of the rat. Two-factor repeated measures ANOVA was used for statistical comparisons (p<0.05), with post-injury time (minutes 1-5) as the within-group (repeated) factor and experimental group as the between-group factor. Behaviors associated with increased anxiety included a greater percentage of time spent in the periphery, decreased total distance traveled, fewer center entries, and an increased number of rearing events.

**OPEN FIELD TEST:** The Open Field test has been used to generate data to describe anxiety-related behavior following TBI [27, 28] and has been widely used to assess anxiety in rats for other applications [29]. The assessments rely on monitoring behavior of the rats when exposed to an unknown (e.g., novel) environment from which escape is not possible [30]. Rats were exposed to the Open Field Test on the seventh post-injury day. The test was conducted in a flat, square shaped arena (40 by 40 cm) with 20 cm walls around the periphery. The arena was illuminated from above using ceiling lights. Rats were initially placed in the center and allowed to explore the arena for five minutes. The entire test was recorded from above using a digital video recorder suspended above the center of the arena. The periphery of the arena was determined to be the area within 10 cm of the outer walls. Metrics quantified during the test included distance traveled (ambulatory distance, cm), ambulatory time (sec), the number of rearing events, the number of center entries, distance traveled along the periphery (margin distance, cm), and time spent in the periphery (margin time, sec). These metrics were scored by an observer blinded to the injury status of the rat. Two-factor repeated measures ANOVA was used for statistical comparisons (p<0.05), with post-injury time (minutes 1-5) as the within-group (repeated) factor and experimental group as the between-group factor. Behaviors associated with increased anxiety included a greater percentage of time spent in the periphery, decreased total distance traveled, fewer center entries, and an increased number of rearing events.

**III. RESULTS**

Twelve control rats were subjected to the sham procedure, ten rats were subjected to single injury, and five
rats were subjected to repeated injury. All rats survived the injury event without skull fracture or cervical spine injury and fully recovered all six reflexes. Physiological signals obtained following single or repeated injury were not significantly different from basal levels. Although histological analysis was not complete at the time of this report, previous experimental reports for this rotational model incorporated H&E staining to reveal perineuronal vacuolation dorsal to the cingulum and corpus callosum [21]. That study also reported an absence of axonal swellings identifiable using β-APP immunohistochemistry in all experimental rats. Following sacrifice, there was no evidence of subarachnoid or subdural hemorrhage in any rats.

COMPOSITE NEUROSCORE: The composite neuroscore was conducted on the seventh day following sham procedure (control group), injury (single injury group), or second injury (repeated injury group). Overall post-injury locomotor ability as determined using the composite neuroscore was not significantly different (p>0.05) between experimental groups. Mean and standard deviation composite neuroscore values for the control rats was 9.7 ± 2.6. Compared to controls, composite neuroscore was slightly higher for the single injury group (11.1 ± 1.3) and approximately equal for the repeated injury group (9.5 ± 1.0).

MORRIS WATER MAZE: The morris water maze protocol was conducted on days five and six following sham procedure (control group), injury (single injury group), or second injury (repeated injury group). Measures of spatial learning performance were not statistically significantly different between experimental groups (p>0.05). Across the entire sample, rats were unable to find the submerged platform in approximately 30% of all trials. The percentage of unsuccessful trials increased from controls (27.1%) to rats receiving a single injury (36.2%), but decreased for rats with repeated injury (19.3%). The slope of learning metric demonstrated decreased set-to-set latency for all groups, with average latencies decreasing by 6 sec per set. Differences between groups were not statistically significant and trends were not evident between controls (-4.8 sec/set), single injury (-8.0 sec/set), and repeated injury (-5.3 sec/set). Therefore, while all groups demonstrated spatial learning, as evidenced by decreasing set-to-set latencies, there were no remarkable differences based on injury type.

ELEVATED PLUS MAZE TESTING: The elevated plus maze protocol was conducted on day six following the sham procedure (control group), injury (single injury group), or second injury (repeated injury group). Time spent in closed arms of the elevated plus maze per minute was significantly different (p<0.005) between the three experimental groups. Post-hoc analysis revealed that rats in the repeated injury group spent significantly less time in the closed arms than controls or the single injury group (p<0.01). Accordingly, rats in that group spent more time in the two open arms and center of the maze. Minute-by-minute analysis demonstrated that all groups spent progressively more time in the closed arms across the five-minute duration (Figure 3). After an initial period of exploration in the open arms during the first minute, rats in the control and single injury groups spent greater than 83% of the time in the closed arms starting at the second minute and continuing through the fifth minute. However, rats in the repeated injury group spent considerably less time in the closed arms following the first minute of exploration, accounting for only 64 to 75% of their time in the closed arms during the second through fifth minutes. Minute-by-minute group differences were statistically significantly different during the third minute (p<0.05), and approached statistical significance for the fourth minute (p=0.14).

The number of arm changes per minute, another measure of activity in the maze, significantly decreased from minute to minute for all groups (p=0.01), although between-group differences were not significant (Figure 3). Nonetheless, the mean response of the repeated injury group was different from controls and the single injury group. Control rats and rats receiving single injury demonstrated considerable early activity, steeply decreasing to the third minute, and remaining below two arm changes per minute from the third through the fifth minutes. However, the slope of decreasing arm changes for the repeated injury group was spread across all five minutes and was considerably less steep than the other groups.
Fig. 3. Time spent in closed arms and number of arm changes evaluated using the Elevated Plus Maze (mean ± standard error).

The number of rearing events per minute in the elevated plus maze assessment was not significantly different between experimental groups or based on time in the maze (Fig. 4). The number of head dips per minute significantly decreased with time in the elevated plus maze (p<0.001), although between-group differences were not significant (Fig. 4). However, as with the number of arm changes per minute (Fig. 3), the number of head dips per minute for the repeated injury group decreased with time at a more gradual rate than the controls or the single injury group. This resulted in the repeated injury group demonstrating a considerably higher number of head dips during the second, third, and fourth minutes than the other groups.

Fig. 4. Number of rearing events and head dips on a minute-by-minute basis evaluated using the Elevated Plus Maze (mean ± standard error).

OPEN FIELD TEST: The open field test protocol was conducted on day seven following the sham procedure (control group), injury (single injury group), or second injury (repeated injury group). Ambulatory time was not significantly different (p>0.05) between experimental groups, although the repeated injury group averaged greater ambulatory time per minute (15.5 ± 4.8 sec) than the single injury group (13.1 ± 5.2 sec) and controls (12.9 ± 5.6 sec) (Fig. 5). Ambulatory time significantly decreased (p<0.0005) during the duration of the open field test for all groups, although the slope of decreasing ambulation was lower in the repeated injury group than the other groups. Ambulatory distance was not significantly different between experimental groups (p>0.05), but significantly decreased during the duration of the open field test (p<0.05) for all groups (Fig. 5). Once again, the slope of decreasing ambulatory distance per minute was steeper for the controls and single injury group than for the repeated injury group. A trend was evident during minutes 1, 4, and 5, wherein the repeated injury group spent more time ambulating and ambulated a greater distance than the other two groups.
Fig. 5. Minute-by-minute time spent ambulating (left) and ambulatory distance (right) for each experimental group during the Open Field Test (mean ± standard error).

The number of rearing events was significantly different between experimental groups (p<0.05), although it did not change significantly with time during the open field test (Fig. 6). The repeated injury group demonstrated a significantly greater number of rearing events per minute (5.9 ± 2.6 entries/min) than the single injury group (5.1 ± 3.7 entries/min) and controls (3.9 ± 2.8 entries/min) across the entire duration. In particular, the repeated injury group demonstrated a considerably greater number of rearing events during the second, third, and fourth minutes than the other groups. With regard to time, it should be noted that controls and the single injury group demonstrated a general trend of decreasing rearing events with increasing time in the open field. However, the number of rearing events was greater in the second, third, and fourth minute than the first. This discrepancy in rearing events during the duration of the test may explain the lack of statistical significance for the time variable across the entire sample. The number of center entries did not significantly vary by group, although the number of entries per minute significantly decreased across the duration of the test (p<0.05) for all groups (Fig. 6). The slope of decreasing center entries from minute to minute was lower in both injury groups than in the controls. The number of center entries per minute for control rats was 16.0 ± 1.3 (mean ± standard error), for the single injury group was 14.7 ± 1.4, and for the repeated injury group was 17.1 ± 2.3.

Fig. 6. Minute-by-minute rearing events and center entries during the Open Field Test (mean ± standard error).

Margin time and margin distance were not significantly different between experimental groups or based on time in the open field. In general, rats spent more time near the outer wall with each successive minute in the field. This trend was most evident for controls and the single injury group, although not statistically significant. Across the entire test, the repeated injury group spent less time near the outer walls (69%) than the single injury group (73%) or controls (76%). A summary of experimental results is provided below.
Table 1: Summary of experimental results (NA: not applicable, NSD: not significantly different, RIG: repeated injury group, SIG: single injury group).

<table>
<thead>
<tr>
<th>Test</th>
<th>p-value</th>
<th>Values (mean ± stderror)</th>
<th>Trends/Interpretation</th>
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<tbody>
<tr>
<td></td>
<td>Time</td>
<td>Group</td>
<td>Controls</td>
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<td>Composite Neuroscore</td>
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<td>NSD</td>
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<td>Morris Water Maze</td>
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<td>• Unsuccessful trials</td>
<td>NA</td>
<td>NSD</td>
<td>-4.8 s/set</td>
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<tr>
<td>• Slope of learning</td>
<td>NA</td>
<td>NSD</td>
<td></td>
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<tr>
<td>Elevated Plus Maze</td>
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<td></td>
<td>52±3 s/min</td>
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<tr>
<td>• Closed arm time</td>
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<td>• Arm changes/min</td>
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<td>• Rearing events/min</td>
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<td>2.8±0.3</td>
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<tr>
<td>• Head dips/min</td>
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<td>Open Field Test</td>
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<td>• Center entries</td>
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IV. DISCUSSION

The present study focused on identifying graded post-injury behavioral outcome differences between rats sustaining single and repeated concussive injuries. Behavioral testing following mTBI serves two primary purposes. Firstly, deficits identified following injury can be used to grade the relative level of severity in the absence of macroscopic brain pathologies that are traditionally associated with higher severity injuries [31-34]. Macroscopic investigation of rat brains following single or repeated injury in the present study failed to identify the presence of focal contusions or evidence of vascular injury including subdural or subarachnoid hemorrhage. Therefore, behavioral testing was required as an indirect method of determining the presence or absence of injury and qualifying injury severity between controls, rats sustaining single injury, and rats with repeated injury. Secondly, behavioral deficits identified using well-characterized assessments, such as the Morris Water Maze, Elevated Plus Maze, and Open Field Test are relatable to human abnormalities following mTBI. Specifically, patients suffering from this condition commonly display evidence of cognitive disorder, behavioral disturbances, and emotional difficulties [4, 5]. Behavioral assessments performed in this study have been used in the assessment of outcomes following TBI to determine spatial memory/learning deficits and anterograde amnesia [35, 36], and altered emotionality including anxiety, defensive behavior, depression, and post-traumatic stress [37-41]. Identification of statistically significant differences and non-significant trends in the present preliminary study validates the utility of the experimental model to produce mTBI in rats with behavioral deficits matching the human condition and warrants continued investigation of the phenomena described in this manuscript.

Results of the behavioral assessments incorporated in this study demonstrated some remarkable differences between experimental groups. Rats in the repeated injury group demonstrated statistically significantly more time in the center and open arms of the elevated plus maze than the other groups. Although not statistically significant, the repeated injury group also performed a greater number of head dips, particularly during minutes two, three, and four in the maze. Additionally, although the repeated injury group demonstrated fewer total rearing events than the other groups, the relative frequency of rearing events changes when normalized to time spent in the closed arms. Rearing events only occurred in the closed arms with the rat bracing itself against the wall to stand on its rear legs. Because the repeated injury group spent less time in the closed arms, those rats had less opportunity for rearing events. Therefore, by dividing the total number of rearing events by time spent in the closed arms, it was determined that the repeated injury group actually performed slightly more rearing events per 60 sec in the closed arms (3.5 events) than the controls (3.3 events) and single injury group (3.3 events). This finding is consistent with results of the Open Field Test, wherein rats with repeated injury...
Findings from the present study are consistent with other previously reported behavioral deficits identified using the elevated plus maze [26, 28, 42]. Specifically, Pandey et al. [28] reported that rats with TBI exhibited increased open arm activity. The authors posited that this resulted from decreased defensive behavior in an unfamiliar environment, which had been attributed to depression-like symptoms of the olfactory bulbectomy model [43, 44]. Other studies have attributed increased locomotor activity in these behavioral assessments to spatial learning deficits that reflected impairment of habituation [45], although that does not seem to be the case in the present study as the Morris Water Maze assessment did not identify significant differences between groups. Another study subjecting mice to varying injury levels found similar results to those presented in this manuscript in that animals with more severe injuries spent a greater percentage of time in open arms of the Elevated Plus Maze [42].

Behavioral deficits during the present study were most apparent in the open field test. Rats in the repeated injury group demonstrated a statistically significantly greater number of rearing events, along with 20% greater ambulation time and 10% greater ambulation distance than controls. Compared to the single injury group, rats with repeated injury had 18% greater ambulation time and 10% greater ambulation distance. Increased ambulation and rearing behaviors in rats sustaining TBI were previously identified using other injury models [28, 42, 46], although effects of TBI on behavioral outcomes in the open field test have not been completely uniform. Other studies have identified decreased activity in the open field test following TBI [27, 40]. Differences in behavioral outcomes may highlight the variability in injury type and severity between different injury models. In general, behavioral deficits resulting from head acceleration-based injury models have been consistent with present findings [28, 42]. However, behavioral deficits resulting from other injury models, including the fluid percussion [47] and controlled cortical impact [48, 49] models, have produced dissimilar results [27, 46]. Those models require invasive craniotomies and often induce focal trauma in addition to histological evidence of diffuse brain trauma, although injury levels have been qualitatively graded as mild, moderate, and severe [36, 50, 51].

Acceleration-based models, specifically angular acceleration-based, reproduce the biomechanical mechanism of mTBI in the human more accurately, with injury severity dependent upon characteristics of the acceleration versus time pulse. Because of this, acceleration-based injury thresholds are scalable from the rat to the human. Present results from the single injury group highlighted the ability of the present model to induce mild-level brain injuries (i.e., concussion). In most cases, behavioral response of the single injury group was not remarkably different from controls. In particular, there were no metrics that were statistically significantly different between the single injury group and controls. For most metrics, mean response of the single injury group mirrored the controls very closely. The only notable differences between the groups were for ambulatory activity during the open field test and rearing events in the open field test and elevated plus maze. It is remarkable, then, that such an apparently mild injury as was imparted in the current study, producing minimal behavioral deficits when delivered once, can result in statistically significantly different and varied behavioral alterations when delivered twice. Although cognitive and motor response of the repeated injury group were not different from the single injury group or controls, significant emotional deficits were apparent using multiple metrics and non-significant trends occurred for a majority of emotionality-based metrics.

The Morris Water Maze is a robust and commonly used behavioral model to assess cognitive deficits, which are a common outcome for patients with mTBI. The lack of identifiable cognitive deficits following repeated mTBI in the present study is not unique in literature. Other studies incorporating mice and rats with repeated injuries have reported similar results for repeated injuries occurring 24 hours to seven days apart [12, 13, 17]. Those studies incorporated the closed skull controlled cortical impact injury model and had only one incidence of significant cognitive deficits. Longhi et al. identified significantly increased latency for the repeated injury group only when injuries were separated by three days, with no deficits for five- and seven-day intervals [17]. Cognitive deficits may have been apparent in that study due to the shorter inter-injury interval or possibly a more severe injury level as 32% of rats sustained post-concussive seizures. Another study reporting significant
cognitive deficits for the repeated injury group subjected rats to four successive injuries, each separated by 24-hour intervals [15]. These studies highlight the complexity of the repeated mTBI mechanism by demonstrating variable outcomes based on injury severity and interval between injuries. Continued research along this line will focus on quantification of the complex interaction between injury severity, inter-injury interval, and number of injuries for defining repeated mTBI thresholds. Each of these factors has been hypothesized to influence outcomes following repeated mTBI [15].

V. CONCLUSIONS

Acutely considered a low severity injury, mTBI can lead to chronic cognitive, neuromotor, and emotional deficits. Multiple injuries are likely to accumulate brain damage and lead to higher levels of patient morbidity. Although previous literature has investigated cognitive and motor deficits, this is the first study to investigate emotional abnormalities following repeated mTBI using the Elevated Plus Maze and Open Field Test assessments. Significant emotional deficits were apparent for the repeated injury group although response of the single injury group was not remarkably different from controls. These findings contribute to the overall understanding of outcomes following repeated mTBI and demonstrate a need to include these types of assessments in future investigations. Identification of statistically significant differences and non-significant trends in the present preliminary study validates the utility of the experimental model to produce mTBI in rats with behavioral deficits matching the human condition and warrants continued investigation of the phenomena described in this manuscript.

VI. ACKNOWLEDGEMENT

The research reported here was supported by the Department of Veterans Affairs, Veterans Health Administration, Rehabilitation Research and Development Service and the Department of Neurosurgery, Medical College of Wisconsin.

VII. REFERENCES