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Repeated Mild Traumatic Brain Injuries Impair Visual Discrimination Learning in Adolescent Mice

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Abstract

Cognitive deficits following a mild traumatic brain injury (mTBI) are common and are associated with learning deficits in school-age children. Some of these deficits include problems with long-term memory, working memory, processing speeds, attention, mental fatigue, and executive function. Processing speed deficits have been associated with alterations in white matter, but the underlying mechanisms of many of the other deficits are unclear. Without a clear understanding of the underlying mechanisms we cannot effectively treat these injuries. The goal of these studies is to validate a translatable touchscreen discrimination/reversal task to identify deficits in executive function following a single or repeated mTBIs. Using a mild closed skull injury model in adolescent mice we were able to identify clear deficits in discrimination learning following repeated injuries that were not present from a single mTBI. The repeated injuries were not associated with any deficits in motor-based behavior but did induce a robust increase in astrocyte activation. These studies provide an essential platform to interrogate the underlying neurological dysfunction associated with these injuries.

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Keywords

Cognition; Touchscreen; CatWalk; Rotarod; Glial Fibrillary Acidic Protein (GFAP)

1.1 Introduction

Concussions (a subset of mild traumatic brain injury (mTBI)) in adolescents result in ~175,000 emergency room visits per year in the US^{1,2}. Approximately 85% of those visits are considered mild and no follow-up treatments are given. However, many individuals suffer from prolonged symptoms lasting weeks to months^{3–5}. The symptoms are typically worse and recovery is longer in adolescents⁶. mTBIs are often described by the symptoms of neurological impairment such as; headaches, vertigo, nausea, disorientation, and/or cognitive deficits that typically resolve in a few hours or days⁷. However, between 14 and 29% of adolescents are still symptomatic at three months post-injury^{8,9}. These short-term symptoms are thought to be due to functional abnormalities in the brain rather than structural damage. Many short-term symptoms can be associated with neurochemical imbalance, hypo-metabolism, and/or altered cerebral blood flow^{10–12}. Collectively these abnormalities are known as the neurometabolic cascade and are associated with a period of vulnerability¹³.

Repeated mTBIs (RmTBIs) are particularly devastating due to the negative long-term outcomes, such as cognitive decline, behavioral deficits, and long-term pathology. Fibrillary tangles and Tau accumulation are used to diagnose chronic traumatic encephalopathy (CTE)^{14–18}. Unfortunately, repeated injuries are common, a recent study revealed that 11% of high school athletes being assessed for a head injury do report having a previous head injury⁹. Individuals that have had a previous mTBI exhibit worse post-injury symptoms and recovery is typically longer^{13,19,20}. In one study, they found that individuals that had two or more mTBIs scored worse on post-injury symptoms, and that score correlated with worse cognitive performance in verbal and visual memory tasks, visual-motor processing, and impulse control task²¹. While the dangers of RmTBI are becoming widely accepted, the underlying mechanisms of the cognitive deficits are complex and are poorly understood. Due to the complexity of these injuries effective treatments are lacking.

Neurocognitive deficits following a single or repeated mTBIs can vary widely but often include deficits in long-term memory, working memory, processing speeds, attention, mental fatigue, and executive function^{22,23}. Not only do the neurocognitive deficits vary among individuals, but the same deficits in memory, psychomotor processing speed, and language are also seen in other injuries that do not include the head. This complexity in the symptoms makes it difficult to diagnose and treat²⁴. Many different rodent models of mTBIs have shown deficits in hippocampal based spatial and fear-based learning^{14,15,25–30}. However, these only represent a subset of the cognitive deficits associated with mTBIs. New sophisticated and translatable assays are critically needed to more accurately identify the underlying mechanisms of cognitive dysfunction that are more related to executive function type processes.

Our previous work has shown that a single closed skull injury induces a propagating wave of complete depolarization known as a spreading depolarization (SDs)³¹. The impact-induced

SDs were also associated with a propagating hemodynamic response and a prolonged reduction in cerebral blood flow lasting ~90 minutes post-injury. However, 24 hours after the injury the cerebral blood flow was relative to pre-injury levels. We were unable to detect any motor deficits 24 hours post-injury with rotarod and CatWalk analysis. These injuries were not associated with gross tissue damage, cell death, or the activation of astrocytes. But the injury was associated with an immediate period of immobility and an overall decrease in mobility in the minutes that followed.

In the current study, we utilize a translatable touchscreen cognitive task to assess for long-term cognitive deficits in adolescent mice following a single injury or repeated injuries (RmTBIs). Next, we investigated whether RmTBIs were associated with alterations in motor behavior that may impact operant learning. Finally, we investigated the correlation of astrocyte activation during the learning phase. Our data indicate that repeated injuries are associated with significant deficits in discrimination learning, but display normal motor behavior. The RmTBIs were also associated with an increase in astrocyte activation that may or may not contribute to cognitive performance. These studies provide a clear model of cognitive dysfunction that can be used to interrogate underlying mechanisms and test therapeutic strategies for treatment.

2.1 Materials and Methods

2.2 Animals

Male and Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were utilized in all studies. Mice underwent either sham, mTBI, or RmTBIs procedures at 8 weeks of age to model late adolescence and were maintained on a reversed light/dark cycle. A total of 80 mice were used with group numbers being reported in the figure legends. No significant main effect of sex was detected on any behavioral measure and mice were subsequently collapsed across sex for analysis of the behavioral tasks. All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

2.3 Mild Traumatic Brain Injury Model

All injuries were done at eight weeks of age to model an adolescent injury. Using an electromagnetic impactor (One controlled impactor (Leica Biosystems, Buffalo Grove, IL)) the animals were hit on top of the intact skin and skull without head restriction. Anesthetized animals were placed on a custom Kiazen foam (FastCap, Ferndale, WA) platform. The platform was made from a 40 mm thick piece of foam with a ~20 mm v-shaped notch cut into the foam to hold the head in orientation. The piston was located approximately one mm anterior to bregma and was set at a speed of 4 m/s with a 5 mm head deflection and dwell time of 20 ms. Sham treated mice were anesthetized and the impactor was manually lowered to touch the head. The impact was strategically localized over the cingulate gyrus, which is part of the limbic system and is involved in executive control^{32,33}. This model has been previously described to produce no skull fractures or gross tissue damage but does induce concussion-like behavior³¹. Mice were anesthetized with 1–3% isoflurane before and during

the hit. For the RmTBI group, mice were impacted four times with 24 hours between each impact. This repeat-injury model was based on previously published studies^{13,28,30,34–36}, and our previous work that showed that there were no behavioral or physiological deficits at 24 hours after a single injury³¹.

2.4 Assessment of cognitive alterations from single or repeated mTBIs

To test for long-lasting cognitive effects, a cohort of animals received a single mTBI and another cohort received four impacts given 24 hours apart. Both cohorts underwent treatment two days prior to the beginning of the touchscreen visual discrimination task as previously described³⁷. A timeline is shown in Figure 1a and 2a. All operant behavior was conducted in a chamber measuring $21.6 \times 17.8 \times 12.7$ cm (model # ENV-307W, Med Associates, St. Albans, VT) housed within a sound- and light-attenuating box (Med Associates, St. Albans, VT). The standard grid floor of the chamber was covered with a solid acrylic plate to facilitate ambulation. A liquid dispenser delivering strawberry milk liquid reward (Nesquik. S.A., Vevey, Switzerland) into a magazine, a house light, and a tone generator was located at one end of the chamber. At the opposite end of the chamber, there was a touch-sensitive screen (Conclusive Solutions, Sawbridgeworth, U.K.) covered by a black acrylic aperture plate framing two 7.5×7.5 cm touch areas separated by 1 cm and at a height of 0.8 cm from the floor of the chamber. Stimulus presentation in the response windows and touches were controlled and recorded by the KLimbic Software Package v1.20.2 (Conclusive Solutions, Sawbridgeworth, U.K.).

To ensure motivation to conduct operant tasks mice were slowly reduced and maintained at a mild food restriction of 85% free-feeding body weight (adjusted for growth). Prior to the injury, mice were acclimated to the strawberry milk reward by providing three drops of reward per mouse on a weigh boat in the home cage for 15 min or until consumed over three days. Mice were then pseudorandomly assigned to the mTBI/sham or RmTBI/sham group. Two days following the final treatment, mice underwent a three-stage pretraining regimen. After habituation to the chamber mice were trained to retrieve reward from the magazine by presenting 30 rewards which had to be retrieved in under 30 minutes.

Next, mice had to initiate reward delivery via lever press and then retrieve 30 rewards in less than 30 minutes. Mice were then moved to touch training during which a lever press led to the presentation of a white (variously shaped) stimulus in one of the two response windows. The images remained on the screen until a response was made. Touches in the blank response window were not paired with a punishment. Touches to the white stimulus resulted in reward delivery and immediately cued by a tone and illumination of the magazine light on the opposite side of the operant chamber from the touchscreen. Mice initiating, touching, and retrieving 30 rewards within 30 min were moved to the final stage of pre-training. This stage was identical to touch training except that responses at the blank window during stimulus presentation produced an immediate 10-sec timeout, signaled by illumination of the house light, to discourage indiscriminate screen responding. Errors on this and all subsequent stages were followed by a correction trial. A correction trial would present the same stimuli in the same left/right position until a correct response was made. Mice making

75% of their responses at a stimulus-containing window (excluding correction trials) over a 30-trial session proceeded to visual discrimination.

During discrimination learning, two approximately equiluminescent novel stimuli were presented in a spatially pseudorandomized manner over 30-trial sessions (with a 5-sec inter-trial interval). Responses at one stimulus resulted in reward; responses at the other stimulus resulted in a 15-sec timeout (signaled by illumination of the house light) and were followed by a correction trial. The designation of the initially rewarded stimulus was randomized across groups. Stimuli would remain on the screen until a response was made. Mice were trained to a criterion of 85% correct responses (excluding correction trials) over two consecutive sessions or 60 total sessions. Reversal training began in the next session after the discrimination criterion was attained. Here, the designation of stimuli as correct versus incorrect was reversed for each mouse. Mice were trained on 30-trial daily sessions (just like discrimination) to a criterion of 85% correct responding (excluding correction trials) over two consecutive sessions. For discrimination and reversal phases the following dependent variables were recorded: Average percent correct trials, percent incorrect trials, percent total correction trials, total trials required to reach criteria, average reaction time from initiation to response, and average latency to retrieve reward after a correct response.

2.5 Assessment of activity and motor coordination following RmTBI

Home Cage Activity: A different cohort of mice was assessed for home-cage activity, activity in a Novel Open Field, and fine gait coordination. Home-cage monitoring was measured utilizing a photobeam activity system (PAS-Homecage; San Diego Instruments, San Diego CA) to monitor general horizontal activity and sleep/wake cycles. Mice were monitored for 24 hours prior to the first treatment and then monitored continuously following each treatment and for 48 hours following the final treatment. Total beam breaks were summed into 5-minute bins and post-hoc summed into 12-hour bins³⁸.

Novel Open Field: 72 hours following home-cage assessment, mice were measured on spontaneous activity and exploration activity in a novel open field. Mice were placed in the center of a brightly lit (~300 center lux; ~200 corner lux) square arena (60 × 60 cm) and allowed to explore for 10 minutes. The animals were tracked from above and vertical behavior could not be assessed. Total time spent in the center, total time in the borders, the number of center entries, and latency to the first border entry were automatically calculated utilizing the Ethovision XT8 motion tracking system (Noldus, Leesburg, VA).

Gait Assessment: 24-hours following open-field testing, mice were tested for fine motor movement and gait using the Catwalk XT system (Noldus Information Technology, Wageningen, Netherlands), as previously described³⁹. The mouse was placed in a start box at one end of a 1.3 m long runway consisting of a glass platform covered by a removable black tunnel and allowed to walk freely across the runway to reach their home-cage at the opposite end. The runway was illuminated from one side by reflected green fluorescent light. A high-speed camera recorded each footprint on the glass from the light being reflected downward by the individual footfalls. Three runway trials were recorded for each mouse during one testing session. Each trial was regarded as compliant by the software if the

mouse crossed the recording area in under 5 sec and did not show a maximum speed variation > 65%. All compliant trials were reviewed manually, if the mouse stopped or turned mid-run the trials were excluded. Each run was classified, and the following characteristics were analyzed to assess for gait abnormalities: paw print area, swing duration, and stride length. The print area was calculated by averaging the area of all the footprints for each paw. Swing duration was considered to be the time between the lifting and replacing of the same paw. The stride length was calculated by the distance from the heel of one footprint to the heel of the next footprint of the same paw.

2.6 Assessment of acute motor learning following RmTBI

Motor learning was assessed with another cohort of mice that were tested on the accelerating rotarod system (PanLab/Harvard Apparatus, Holliston, MA). Mice were given two days of training with three trials per day at a constant speed of 5 rpm for three trials of 1–3 minutes with a 10-minute inter-trial interval. Mice were then assessed for motor learning on each day following a RmTBI or sham treatment by placing them on the rotarod spinning at 4 rpm, slowly set to accelerate with a 300-ramp time to a max at 40 rpm at 5 minutes. For the next three days, they performed baseline testing to measure how long they could remain on the constant accelerating rod. On the subsequent days the mice underwent the RmTBI or sham treatment 60 minutes prior to testing. Mice were given an additional day of testing 24 hours after the final hit to examine the cumulative effects or recovery.

2.7 Histological assessment of RmTBI injury

To assess whether RmTBIs were associated with neuroinflammation, mice were sacrificed 24 hours following the last of four impacts (day 5), following activity and coordination testing (day 11), motor learning testing (day 11) or touchscreen cognitive testing (day ~70) as described above. Briefly, animals were cardiac perfused with PBS and 4% paraformaldehyde at the given time points. Tissue was harvested and submerged in 4% paraformaldehyde for 24 – 48 hours. Tissue was sectioned on a vibratome or sliding knife at 50µm and sections were stored in cryoprotectant solutions at –20 C°. For quantification, three sections were selected approximately 1 mm apart from one another to represent different brain regions underneath, proximal, and distal to the impact site. For immunohistochemistry, sections remained free-floating and washed three times with PBS to remove the cryoprotection. Sections were permeabilized and washed with 1% Tween in PBS and 0.4% Triton in PBS. The sections were blocked with 10% Normal Donkey Serum, 0.5% Bovine Serum Albumin, and 1% Tween in PBS. To fluorescently label blood in the brain we incubated the sections with anti-mouse IgG antibody 1:1,000 (Cy3-IgG (H + L) donkey anti-mouse, catalog #715-167-003, Jackson ImmunoResearch) overnight at 4 C°. The following day the sections were washed with 1% PBS-Tween, stained with NucBlue (ThermoFisher Cat# R37605), washed 3 times in PBS, and mounted on slides with Fluoromount-G (Electron Microscopy Services, Cat#17984–25). Immunohistochemistry was also performed for Glial Fibrillary Acidic Protein (GFAP). Sections were washed, permeabilized, and blocked as previously mentioned. Sections were incubated with the primary antibody for GFAP (catalog #NE1015, EMD Millipore, Merck) diluted 1:500 in the blocking buffer overnight at 4°C. Sections were washed with PBS-T and incubated with the secondary

antibody (Cy3-IgG (H + L) donkey anti-mouse, catalog #715-167-003, Jackson ImmunoResearch) at a dilution of 1:1,000.

2.8 Imaging and Quantification

Images were acquired with an Olympus IX71 inverted microscope equipped with an Olympus DP72 RGB camera using a 4× UPlanFL N 0.13 NA objective, 10× UPlanFL 0.3 NA objective, or 40× LUCPlanFLN 0.60 NA objective. Overview images were acquired and stitched using the Olympus CellSens software system and quantification was done with ImageJ^{40,41}. GFAP was quantified from three to four sections that were separated by approximately 1 mm from each other rostral to caudal. Within each section, six regions of interests (ROIs) were imaged at 10x. Three ROIs spanning the medial and dorsal cortex and three ROIs spanning the medial to later corpus callosum. The entire 10x image representing ~138 mm x 104 mm region was used for quantification. Background fluorescence was measured in all images in each respective region (cortex or corpus callosum). The averaged background fluorescence was set as a threshold for each image and the percent of pixels above the threshold was calculated for each image. The three ROIs per region were averaged together and the sections were averaged together to represent the overall GFAP level per animal.

2.9 Statistics

All statistical analyses were performed in Prism (v.8). All statistical analyses of pooled data were performed using an unpaired t-test or two-way ANOVA test, and the level of significance was considered to be $p < 0.05$. All statistics are provided in the text and data are presented as group means and SEMs or box and whisker plots.

3.1 Results

3.2 Cognitive assessment following a single mTBI

Experimental timeline is shown in Figure 1A. Animals underwent sham or mTBI procedures two days prior to the pretraining phase. During the pretraining, the single mTBI animals performed similarly to sham treated animals in the trials to reach criteria to lever press (Figure 1B; unpaired t-test; $t_{14} = 0.521$, $P = 0.610$) and to touch the screen (Figure 1C; unpaired t-test; $t_{14} = 0.971$, $P = 0.0348$). During discrimination the mTBI animals also performed similarly on the average percent correct responses (Figure 1D; unpaired t-test; $t_{14} = 0.137$, $P = 0.893$), incorrect responses (Figure 1E; unpaired t-test; $t_{14} = 0.614$, $P = 0.549$) and correction trials (Figure 1F; unpaired t-test; $t_{14} = 0.095$, $P = 0.926$). Overall, single mTBI mice performed similarly to the sham-treated animals during the discrimination learning as measured by total trials to criterion (Figure 1G; unpaired t-test; $t_{14} = 0.136$, $P = 0.894$). All mice were able to obtain discrimination criterion. During the discrimination learning the mTBI animals did not differ from sham-treated animals in reaction time (the time between lever press and touchscreen response) (Figure 1H; unpaired t-test; $t_{14} = 1.263$, $P = 0.227$) or in the mean latency (the time between a correct response and retrieval of the reward) (Figure 1I; unpaired t-test; $t_{14} = 0.235$, $P = 0.818$). Once the animals reached the criterion of 85% correct responses for two days the animals underwent reversal learning.

During reversal training, mTBI mice also had a similar number of correct responses (Figure 1J; unpaired *t*-test; $t_{14} = 0.488$, $P=0.633$), incorrect responses (Figure 1K; unpaired *t*-test; $t_{14} = 0.652$, $P=0.525$), and correction trials (Figure 1L; unpaired *t*-test; $t_{14} = 0.982$, $P=0.343$). A single mTBI did not robustly alter performance, as mTBI mice did not require significantly more trials to achieve reversal criterion (Figure 1M; unpaired *t*-test; $t_{14} = 1.087$, $P=0.295$). There was no significant difference between the mTBI and sham animals in their reaction time (Figure 1N; unpaired *t*-test; $t_{14}=0.381$, $P=0.709$) or mean latency (Figure 1O; unpaired *t*-test, $t_{14}=0.013$, $P=0.990$) during the reversal phase.

3.3 Cognitive assessment following repeated mTBIs

In contrast, repeated injuries (RmTBI) animals had significantly altered performance during discrimination training than sham controls. RmTBI animals began touchscreen training two days after the last impact or sham treatment (Figure 2A). RmTBI animals learned to lever press (Figure 2B; unpaired *t*-test; $t_{14}=0.978$, $P=0.344$) and touch the screen (Figure 2C: unpaired *t*-test; $t_{14}=0.077$, $P=0.940$) similar to the sham-treated animals. However, RmTBI animals had significantly fewer correct responses (Figure 2D; unpaired *t*-test; $t_{14} = 3.60$, $P=0.003$), more incorrect responses (Figure 2E; unpaired *t*-test; $t_{14} = 2.834$, $P=0.013$), and more correction trials (Figure 2F; unpaired *t*-test; $t_{14} = 3.60$, $P=0.003$) relative to sham animals. All data is presented as a percent of total trials because the RmTBI animals had significantly more discrimination trials (Figure 2G; unpaired *t*-test; $t_{14} = 4.768$, $P=0.0003$), and the vast majority were unable to reach criteria prior to the 60-day termination. Only one of the eight RmTBI animals was able to obtain discrimination criteria and that mouse performed similarly to sham-treated animals during reversal. These severe deficits in discrimination learning were not associated with altered reaction times (Figure 2H; unpaired *t*-test; $t_{14}=0.724$, $P=0.481$) or mean latency (Figure 2I; unpaired *t*-test; $t_{14}=0.452$, $P=0.658$).

3.4 Activity and motor coordination following RmTBI

Motor performance deficits are commonly associated with rodent models of mTBIs, and to test this in our RmTBI model we used a battery of tasks that included home cage monitoring, the catwalk gait analysis system, open field arena, and an accelerating rotarod task. A timeline is shown in figure 3A that indicated when the injuries were given relative to behavioral assessments. RmTBI animals had significantly less overall movement in their home cage (Figure 3B; two-way repeated measure; main effect of days vs treatment, $F_{5,65}=7.608$, $P<0.0001$; main effect of days, $F_{5,65}=8.349$, $P<0.0001$; a main effect of treatment, $F_{1,13}=8.672$, $P=0.011$; post hoc Sidak's multiple comparisons sham vs RmTBI; Dark cycle (DC) DC2, $P=0.0002$; DC3, $P=0.0033$; DC4, $P=0.0353$). Given the reduced locomotion activity in the home cage, we assessed the overall exploratory activity and anxiety in an open field arena two days after the home cage monitoring. Open field behavior was video captured from above and horizontal behavior was tracked. The analysis showed no significant differences were seen between RmTBI and sham groups in the total distance traveled (Figure 3C; unpaired *t*-test; $t_{13} = 1.424$, $P=0.178$). RmTBI animals had similar duration in the center region (Figure 3D; unpaired *t*-test; $t_{13}=0.375$, $P=0.713$), and similar center entries into the center compared to sham-treated animals (Figure 3E; unpaired *t*-test; $t_{13} = 1.302$, $P=0.215$). RmTBI animals did not take longer to reach the border region for the first time (Figure 3F; unpaired *t*-test; $t_{13}=0.791$, $P=0.443$). Fine gait assessment revealed that

RmTBI animals had an overall reduction in stride length (Figure 3G; two-way ANOVA; no main effect on paw, $F_{3,52}=1.10$, $P=0.358$; no interaction, $F_{3,52}=0.05$, $P=0.985$; main effect of treatment, $F_{3,52}=8.10$, $P=0.006$; Sidak's multiple comparisons, RF $P=0.561$, RH $P=0.311$, LF $P=0.512$, LH $P=0.653$), but similar swing duration (Figure 3H; two-way ANOVA; no main effect of paw, $F_{3,52}=0.489$, $P=0.691$; no main effect of treatment, $F_{3,52}=3.206$, $P=0.079$; no interaction, $F_{3,52}=0.291$, $P=0.831$). RmTBI animals also had a slightly larger paw print area (Figure 3I; two-way ANOVA, no main effect on paw, $F_{3,52}=2.318$, $P=0.086$; main effect of treatment, $F_{1,52}=20.72$, $P<0.0001$; no interaction, $F_{3,52}=0.764$, $P=0.519$; Sidak's multiple comparisons, RF $P=0.006$, RH $P=0.098$, LF $P=0.119$, LH $P=0.642$). Separate analysis determined that there was no significant effect of sex in any of these parameters. Our data is under powered for small differences between the sexes but it does suggest that sex differences in animal size is probably not contributing to these deficits.

3.5 Acute motor learning during RmTBI model

Animals were all pre-trained on the accelerating rotarod for three days prior to the initial impact. Animals were pseudo-randomized into RmTBI or sham groups and underwent treatment 60 minutes prior to rotarod training. Animals were again tested 24 hours following the final treatment. All animals showed significant improvement over time, but RmTBI animals did not perform significantly different than the sham-treated animals (Figure 3J; repeated two-way ANOVA: main effect of time, $F_{7,98}=9.40$, $P<0.0001$; no effect of treatment, $F_{1,14}=0.767$, $P=0.396$; Sidak's multiple comparisons; Day -2 $P=0.996$, Day -1 $P=>0.999$, Day 0 $P=0.972$, Day 1 $P=0.925$, Day 2 $P=0.962$, Day3 $P=0.831$, Day 4 $P>0.999$, Day 5 $P=0.970$). Overall, motor performance was not significantly altered by repeated injuries.

3.6 Histological assessment of RmTBI

To begin to assess for a potential mechanism that could underlie the deficits in discrimination we investigated neuroinflammation or tissue damage following RmTBIs. We used immunohistochemistry to stain for the expression of the glial fibrillary acidic protein (GFAP), and serum albumin that has leaked into the tissue following blood-brain barrier disruption. To examine this in our RmTBI model, tissue was collected on day 5 (24 hours after the final impact), day 11 (following motor tasks), and day ~70 (following the completion of the touchscreen tasks). Representative images at low and high magnification indicate an increase in GFAP intensity in the cortex (Ctx) and the corpus callosum (CC) on day 5, 11, and ~70 (Figure 4A). Quantified data indicate that there was no significant increase in the levels of GFAP on day 5 in the cortex (Figure 4B; two-way ANOVA; no main effect of RmTBIs $F_{1,12}=3.870$, $P=0.0727$; no main effect of sex, $F_{1,12}=2.231$, $P=0.161$; with no interaction $F_{1,12}=0.550$, $P=0.473$), but did show a significant increase in the corpus callosum (CC) in females only (Figure 4E; two-way ANOVA; no main effect of RmTBIs, $F_{1,12}=2.966$, $P=0.111$; no main effect of sex $F_{1,12}=4.01$, $P=0.068$; significant interaction $F_{1,12}=5.05$, $P=0.044$; post hoc Sidak's multiple comparison, female sham vs RmTBI, $P=0.031$). However, at day 11 there was a significant increase in GFAP expression in the cortex (Figure 4C; two-way ANOVA; main effect of RmTBIs, $F_{1,26}=4.707$, $P=0.039$; no main effect of sex, $F_{1,26}=0.997$, $P=0.327$; and no interaction, $F_{1,26}=1.122$, $P=0.299$), and in the corpus callosum (Figure 4F; two-way ANOVA; main effect of RmTBIs, $F_{1,22}=22.92$,

$P < 0.0001$; no main effect of sex, $F_{1,26} = 0.398$, $P = 0.534$; no interaction, $F_{1,25} = 0.002$, $P = 0.963$; post hoc Sidak's multiple comparison, female sham vs female RmTBI, $t_{26} = 3.418$; $P = 0.004$, male sham vs male RmTBI, $t_{26} = 3.353$; $P = 0.005$). The enhanced expression of GFAP was resolved by the completion of the touch screen behavioral tasks (Day ~70) in the cortex (Figure 4D; two-way ANOVA; no main effect of RmTBI, $F_{1,12} = 0.743$, $P = 0.406$; no effect of sex, $F_{1,12} = 0.837$, $P = 0.378$; no interaction, $F_{1,12} = 1.228$, $P = 0.289$), and in the corpus callosum (Figure 4G; two-way ANOVA; no main effect of RmTBI, $F_{1,12} = 2.515$, $P = 0.138$; no main effect of sex, $F_{1,12} = 0.083$, $P = 0.777$; no interaction, $F_{1,12} = 0.075$, $P = 0.789$).

As per our previous studies following a single injury, we did not detect significant hemorrhaging in the majority of our RmTBI animals. In the RmTBI model, we did detect sub-dural bleeding in two of the 32 animals. There was one male that had evidence of bleeding following touchscreen behavior (day ~70) (Figure 4H) and one female at day 11 (data not shown). In both cases, the hemorrhaging was detected below the impact site. Similarly, we detected BBB disruption in two different animals out of the 32 RmTBI animals. One female at day 11 (Figure 4I) and one female at day ~70 (data not shown). Together these data indicate that the RmTBI does not produce significant hemorrhaging or BBB disruption in the majority of mice but does produce a significant increase in GFAP levels 7 days after the final injury.

4.1 Discussion

In this study, we found that RmTBIs, but not a single injury, were associated with major deficits in discrimination learning that persisted weeks beyond the time of injury. RmTBIs were not associated with major motor deficits, either immediately or up to one-week post-injury, indicating that the cognitive deficits were present despite the lack of any gross motor deficits. Further, elevated GFAP levels in the cortex were associated with the RmTBIs. These findings provide a unique platform to more accurately identify cognitive deficits in rodents and interrogate the underlying mechanisms of the cognitive dysfunction in a rodent model of RmTBIs.

The current studies utilized a touchscreen reward-based discrimination and reversal task that measures striatal-mediated associative learning and cortical-mediated behavioral flexibility respectively. Our injury model was centered over the cingulate gyrus and motor cortex regions. This was done to model a front to back type injury in humans, and would accurately model the cognitive deficits associated with these injuries. Reversal learning is typically impaired when areas that are required for stimulus attention, such as the ventral-medial PFC, are disrupted^{42,43}. Therefore, we hypothesized there would be larger deficits in the reversal learning due to the location of the injury. However, discrimination learning was so significantly altered we were unable to test reversal learning. Discrimination learning is thought to be mediated by areas of the dorsal striatum across species⁴⁴⁻⁴⁷. Together, these results suggest that RmTBI may alter the function of underlying deep brain structures involved in the ability to transition from early learning to automatized behavior⁴⁸. We found that a single or RmTBI model prior to training and testing did not adversely affect the learning of task parameters. It is possible that there are short-term cognitive deficits in the two days prior to pretraining. Our rotarod behavior was done 1-hour post-injury during the

repeated injuries suggesting that there are no short-term motor deficits associated with a single injury. In addition, our previous study reported that there were no significant deficits in fear-based learning 24 hours following a single injury. However, RmTBI significantly impaired pairwise discrimination learning. Our results with a single mTBI are in agreement with previous findings using a similar touchscreen-based discrimination task⁴⁹. In a previous study using the 5-choice continuous performance touchscreen-based tasks, they did not find significant deficits in attention and/or cognition⁵⁰. This study also examined the impact of repeated injuries that consisted of three injuries given five days apart. They found no differences between the repeated, single injury, or sham animals in this behavioral task⁵⁰. However, the timing of the injuries may be a critical factor. Their model consisted of three injuries separated by five days, whereas ours was four hits 24 hours apart. Taken together, these data support the hypothesis that the interval between the injuries is critical.

While our results clearly show long-lasting deficits in associative learning following RmTBIs, the underlying mechanisms of long-term cognitive deficits remain unclear. Long-term cognitive deficits may be due to structural damage, neuronal loss, or white matter alterations^{51–53}. There is a clear correlation between the degree of white matter damage and cognitive deficits in humans^{54–60}, and repeated injuries exacerbate these changes^{14,30,34,56,61}. It is assumed that particular deficits in executive function or slow processing are due to alterations in the dorsal lateral prefrontal cortex or white matter, respectively⁶². However, it remains unclear how a particular brain region may be more susceptible to mTBIs than another. Our previous study identified that spreading depolarizations (SDs) were initiated by our closed skull injury model and the SDs were correlated with acute mTBI-like behavior. SDs are propagating waves of complete depolarization and are incredibly metabolically demanding to recover from. In strokes or severe TBIs, SDs are associated with cell death and tissue loss in vulnerable tissue^{63–66}. In healthy tissue, cells have sufficient nutrients and the metabolic capacity to fully recover from an SD^{67–69}. However, individual neurons may not fully recover and undergo cell death. It is possible that the cell death that occurs days after the injury may contribute to cognitive dysfunction. SDs are known to increase levels of GFAP in astrocytes⁷⁰, and astrocyte activation can reduce SD susceptibility⁷¹. However, the role of SDs in mTBI pathology and cognitive dysfunction remains unclear.

The lack of gross motor dysfunction supports that even our RmTBI injury is milder in comparison to other models that typically produce robust motor deficits, as well as gross tissue damage^{72–75}. Our previous study reported no significant motor deficits following a single injury⁷⁶. Even in more mild injuries, motor dysfunction is sometimes detected^{77–83}. McAteer and colleagues showed a similar reduction in overall mobility but very little difference in rotarod performance following three injuries spaced five days apart⁵. Using a similar closed skull injury model, Vu and colleagues also showed a reduction in home cage activity and a reduction in the distance traveled in the center of open field arena⁸⁴. Subtle gait abnormalities have also been reported from other closed skull injury models^{77,85}. However, both injuries were targeted above the motor cortex. Given the overall reduced home-cage activity and clear cognitive effects despite no other changes in gait, motor coordination, and motor learning our model may more closely resemble the mild injuries seen in the clinic.

Our previous study indicated that a single mTBI did not result in gross tissue damage, and did not result in an increase of GFAP expression at 24 hours post-injury³¹. GFAP has been strongly correlated with mTBIs. Other closed skull injury models in rodents have shown similar increases in GFAP^{25,85–90}. Most commonly GFAP expression is elevated at 24 hours post-injury^{25,85,88,89}. However, other studies have found prolonged changes in GFAP lasting days to weeks^{25,79,86,87}. Together, the current results and our previous results suggest that there is a delay in the expression of GFAP of a few days that is followed a robust increase in GFAP on day 11. This delay has been supported by other studies and could be a natural part of the recovery phase^{86,90,91}. GFAP has been a reliable marker of injury and has recently been proposed as a blood biomarker for concussions^{92–95}. Repeated injuries are associated with a progressive increase in GFAP¹⁶. Interestingly, we found a significant increase in GFAP at day 11, and this would correlate to the beginning stages of the discrimination learning. It is possible that this peak in GFAP expression disrupts early learning and/or alters long-term performance. However, since the GFAP quantification for Day 5 and 11 was done in separate cohorts it is impossible to directly compare the expression of GFAP to cognitive performance. This would require animals to be trained to a certain criterion then sacrificed for GFAP analysis.

In summary, our findings indicate that RmTBI injuries are associated with significant deficits in cognitive function in the absence of motor alterations. This highlights the validity of the automated touchscreen-based discrimination tasks to assess cognition following a single or repeated mTBI. Furthermore, our data underscore the importance of preventing multiple injuries and highlights the importance of the recovery period. These findings are in agreement with the clinical reports of increasing symptom severity when patients are cognitively challenged^{4,58,96,97}. The current findings support the combination of a mild but repeated mTBI model with a highly translatable assessment tool that can be readily adapted for both in-vivo measurement of neural activity and circuit manipulation approaches. Together, these findings may have important implications for understanding the underlying mechanisms of the cognitive deficits following repeated injuries and may provide a useful model when testing therapeutic strategies for the treatment of RmTBIs.

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Highlights

- A single mTBI did not disrupt visual discrimination or reversal learning.
- Repeated mTBIs significantly disrupted visual discrimination.
- Repeated mTBIs did not disrupt motor behavior.
- Repeated mTBIs were associated with the increased expression of GFAP.

Figure 1:

A single injury is not associated with deficits in discrimination or reversal learning ($n=7$ shams; $n=9$ mTBI). (A) One cohort of 16 mice were first weight restricted then impacted once on day 1 and began touchscreen pretraining 3 days later. mTBI animals performed similar sham animals during the lever press (B), and touch (C) pre-training phases. Cumulative data are expressed as a percent of the total trials during the discrimination phase. Correct trials (D), incorrect trials (E), correction trials (F), and the total trials to reach criteria (G). mTBI and sham animals also performed similarly in the reaction time (the time between lever press and touchscreen response) (H) and mean latency (the time between a correct response and retrieval of the reward) (I). Cumulative data for the reversal phase expressed as a percent of total trials for correct (J), percent incorrect (K), percent correction trials (L), and the total trials to reach criteria (M). The reaction time (N) and mean latency (O) are also shown for the reversal phase.

Figure 2:

RmTBI does result in significant deficits in discrimination learning (n=8 shams; n=8 RmTBI). (A) Experimental design. Animals were impacted once daily for 4 days and allowed to recover for 2 days prior to pretraining. Impacts are indicated by the grey arrows. Two days following the last injury the animals were first trained to press the lever for a reward (B) and then to touch a stimulus on the screen (C). Cumulative data during the discrimination are shown as a percent of the total trials for correct trials (D), percent incorrect trials (E), percent correction trials (F), and total trials to reach criteria (G). Cumulative data of reaction time (time lever press to touch) (H) and mean latency (time between a correct response and reward retrieval) (I).

Figure 3:

RmTBIs are associated with reduced home cage movement, but do not affect motor performance. (A) Experimental Design: 2 cohorts of animals 15 each were used for these studies. One cohort (n=8 shams; n=7 RmTBI) underwent home cage monitoring during the treatments, then open field, and Catwalk following the treatments. A second cohort (n=8 shams; n=8 RmTBI) underwent rotarod training before, during, and after the RmTBIs. (B) Using the photobeam activity system (PAS) home cage monitoring system (San Diego Instruments, San Diego, CA) we were able to assess the overall ambulatory activity of each animal for 7 continuous days starting 24 hours prior to the first hit. Animals were only removed from the cage to undergo treatment and total beam breaks were summed per dark cycle (DC). Shaded bars indicate the days the animals underwent sham or RmTBI. The open-field task was used to assess overall exploratory activity and general anxiety on Day 9. Quantified data of the total distance traveled (C), center duration (D), center entries (E), and

latency to the border region (F). Animals were also tested for gait abnormalities using the CatWalk system. Cumulative data is shown for stride length (G) swing duration (H), and foot print area (I). (J) Accelerating rotarod was used to assess motor performance on day –2 – 5. Treatments were given 1 hour prior to the task on days 1 – 4. Latency to fall off the rod was measure for each animal and each data point represents the average of 3 trials.

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Figure 4:

RmTBIs are associated with increases in GFAP expression and some minor injuries. Immunohistochemistry was used to assess the expression levels of GFAP (cat# NE1015, EMD Millipore, Merck) at day 5 (n=8 shams; n=8 RmTBI; 4 males and 4 females for each), day 11 (n=16 shams; n=16 RmTBI; 8 males and 8 females for each group), and at day ~70 (n=8 shams; n=8 RmTBI; 4 males and 4 females for each group). GFAP expression was quantified by taking 3–4 sections from each animal that were approximately 1 mm apart to sample multiple brain regions. The sections were imaged at 4x magnification to assess over all express and 3 higher magnification images taken at 10x were collected for each region (cortex (Ctx) or corpus callosum (CC)) for quantification. A) Representative images of GFAP at Day 5, 11, and ~70 days after final impact. For each treatment group/sex/time point there is one representative stain with a 4x image of the medial cortex and corpus callosum and two 10x region of interest images in the cortex (top) and corpus callosum (bottom). The

percent fluorescent above the averaged background threshold was calculated for each image and averaged together to represent each animal per region. Quantified expression levels of GFAP in the cortex (B-D) and corpus collosum (E-G) at day 5, 11, and ~70. Representative images of cortical bleeding at the site of injury (H) and blood barrier disruption (I).

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