Adolescent Mice Demonstrate a Distinct Pattern of Injury after Repetitive Mild Traumatic Brain Injury

Rebekah Mannix,^{1,2*} Justin Berkner,^{1*} Zhengrong Mei,³ Sasha Alcon,¹ Jumana Hashim,¹ Shenandoah Robinson,^{2,4} Lauren Jantzie,⁵ William P. Meehan III,^{1,2,6,7} and Jianhua Qiu^{1,2}

Abstract

Recently, there has been increasing interest in outcomes after repetitive mild traumatic brain injury (rmTBI) (e.g., sports concussions). Although most of the scientific attention has focused on elite athlete populations, the sequelae of rmTBI in children and young adults have not been well studied. Prior TBI studies have suggested that developmental differences in response to injury, including differences in excitotoxicity and inflammation, could result in differences in functional and histopathological outcomes after injury. The purpose of this study is to compare outcomes in adolescent (5-week-old) versus adult (4-month-old) mice in a clinically relevant model of rmTBI. We hypothesized that functional and histopathological outcomes after rmTBI would differ in developing adolescent brains compared with mature adult brains. Male adolescent and adult (C57Bl/6) mice were subjected to a weight drop model of rmTBI ($n=10$ – 16 /group). Loss of consciousness (LOC) after each injury was measured. Functional outcomes were assessed including tests of balance (rotorod), spatial memory (Morris water maze), and impulsivity (elevated plus maze). After behavioral testing, brains were assessed for histopathological outcomes including microglial immunolabeling and *N*-methyl-D-aspartate (NMDA) receptor subunit expression. Injured adolescent mice had longer LOC than injured adult mice compared with their respective sham controls. Compared with sham mice, adolescent and adult mice subjected to rmTBI had impaired balance, increased impulsivity, and worse spatial memory that persisted up to 3 months after injury, and the effect of injury was worse in adolescent than in adult mice in terms of spatial memory. Three months after injury, adolescent and adult mice demonstrated increased ionized calcium binding adaptor 1 (IbA1) immunolabeling compared with sham controls. Compared with sham controls, NMDA receptor subtype 2B (NR2B) expression in the hippocampus was reduced by ~20% in both adolescent and adult injured mice. The data suggest that injured adolescent mice may show a distinct pattern of functional deficits after injury that warrants further mechanistic studies.

Keywords: age; pediatric brain injury; TBI

Introduction

RECENTLY, THERE HAS BEEN increasing scientific attention paid to the sequelae of repetitive mild traumatic brain injury (rmTBI). Clinically, rmTBI has been associated with long-term neurological impairment including memory disturbances, Parkinsonism, behavioral changes, speech irregularities, and gait abnormalities.^{1–5} Case series of subjects exposed to rmTBI have described gross pathological changes such as brain volume loss and histopathological features of neurodegenerative disease.^{6–9}

However, the majority of these clinical and pathological reports have occurred in retrospective case series of adults, including professional athletes and military veterans. Developmental considerations of the effect of rmTBI have not been well studied. One recent study using magnetic resonance imaging suggested that former National Football League players exposed to American football before the age of 12 years had differences in the microstructure of the corpus callosum than those starting at a later age.¹⁰ mTBI, or concussion, is a common injury sustained by high school athletes; as many as 11.5–13.2% of sport-related concussions are

¹Division of Emergency Medicine, ⁴Department of Neurosurgery, and ⁶The Micheli Center for Sports Injury Prevention, and ⁷Sports Concussion Clinic, Division of Sports Medicine, Boston Children's Hospital, Boston, Massachusetts.

²Harvard Medical School, Boston, Massachusetts.

³Department of Pharmacy, The Third Affiliated Hospital, Guangzhou Medical University, Guangzhou, Guangdong Province, People's Republic of China.

⁵Department of Pediatrics and Neuroscience, Office of Pediatric Research, University of New Mexico, Albuquerque, New Mexico.

*The first two authors contributed equally.

recurrent injuries.^{11,12} A recent study demonstrated worse neurocognitive outcomes in adolescents who sustained concussion when compared with adults.¹³ Whether or not the pathophysiology and sequelae of adolescent rmTBI differs from that described in adults is unknown.

Pre-clinical models of rmTBI offer the opportunity to investigate age-dependent outcomes. We have previously reported an rmTBI model in adult (2–3-month-old) mice that resulted in persistent behavioral deficits in exploratory behavior, balance, and spatial memory, associated with chronic increased astrogliosis and microgliosis.¹⁴ Extending our findings to an adolescent model (5-week-old mice) of rmTBI is important to understanding whether developmental factors contribute to outcomes after rmTBI. Maturation differences and regulation of the inflammatory response and excitotoxicity after various experimental models of brain injury^{15,16} have previously been reported, but whether these are relevant to rmTBI is unknown. Additionally, the impact of frequency of injury on these pathophysiological variables is unknown.

Understanding whether or not outcomes after rmTBI differ between adolescent and adult mice is a vital first step in evaluating age-specific mechanistic questions, prevention strategies, and therapeutic interventions. In the present study, we compared the performance of adolescent and adult mice subjected to rmTBI on a variety of behavioral tasks assessing learning, memory, balance, behavior, and sensorimotor function. Additionally, we evaluated microgliosis and *N*-methyl-D-aspartate (NMDA) receptor subunit expression. For this study, we hypothesized that functional and histopathological outcomes after rmTBI would differ in developing adolescent brains compared with mature adult brains.

Methods

All experiments were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee, and complied with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*. Adult (age 16 weeks) and adolescent (age 5 weeks) male C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were housed in a temperature- and humidity- controlled room with a 12 h light/dark cycle, and fed *ad libitum*.

rmTBI

Mice were randomized to either rmTBI or sham injury. The rmTBI was performed as previously described.^{14,17} Briefly, mice were anesthetized for 45 sec using 4% isoflurane in a 70:30 mixture of oxygen. Anesthetized mice were placed on a delicate task wiper (Kimwipe, Kimberly-Clark, Irving, TX) and the head was placed directly under a hollow guide tube. Mice were held by the tail as an impact was delivered to the dorsal aspect of the skull. The impact was delivered by dropping a 54 g metal bolt from a height of 71 cm via a guide pipe, resulting in rotational acceleration of the head through the delicate task wiper. Sham injured mice underwent anesthesia but without injury. Mice were randomized to the following groups: seven injuries in 9 days ($n=12$ adult mice, $n=17$ adolescent mice), four injuries in 4 days ($n=10$ adult mice, $n=10$ adolescent mice), seven sham exposures in 9 days ($n=11$ adult mice, $n=16$ adolescent mice), and four sham exposures in 4 days ($n=10$ adult mice, $n=10$ adolescent mice) (Fig. 1). For the seven injuries in 9 days protocol, mice were injured daily for 5 days, followed by 2 days without injury, and then by two additional daily injuries. All mice recovered in room air after injury or sham injury. Following injury or sham injury, all behavioral testing and histological and biochemical assays were conducted by investigators

blinded to injury status, using color coding stored in a password-protected computer.

Assessments of loss of consciousness, (LOC), motor function, spatial memory and impulsivity-like phenotypes

LOC, defined as the time from removal of anesthesia to spontaneous righting, was evaluated after each injury or sham injury.

Motor ability and function was assessed on days 1–3 and again at 3 months after the last injury or sham injury using a rotarod as we previously reported.¹⁴ In brief, the rotarod consists of a 4 cm diameter rotating drum, on which mice were placed. The time (sec) between placement on the rotarod and falling off of the rotarod was recorded as a measure of motor function. Rotarod testing was conducted over 3 days with 1 day of habituation followed by 2 days of testing. On testing days, mice were placed on the rod at 4 rpm for 10 sec, to acclimate to the rod speed, after which the rod was accelerated at 0.1 rpm/sec. Each mouse completed four trials on the testing days, with a minimum of 15 min rest between trials.

Spatial learning and memory were assessed using the Morris water maze (MWM) on days 6–9 following injury and again at 3 months after injury. MWM testing was conducted as previously described.^{14,18} Briefly, a white pool (83 cm diameter, 60 cm deep) was filled with water to 29 cm depth. Water temperature was maintained at $\sim 24^{\circ}\text{C}$, and a target platform (a round, clear, plastic platform 10 cm in diameter) was positioned 1 cm below the surface of the water. Several highly visible intra- and extra-maze cues were located in and around the pool. During hidden and visible platform trials, mice were randomized to one of four starting quadrants. Mice were placed in the tank facing the wall and given 90 sec to find the platform, mount the platform, and remain on it for 5 sec. Mice were then placed under a heat lamp to dry before their next run. Time until the mouse mounted the platform (escape latency) was measured and recorded. Mice that failed to mount the platform within the allotted time (90 sec) were guided to the platform by the experimenter and allowed 10 sec to become acquainted with its location. Each mouse was subjected to a maximum of two trials per day, each consisting of four runs, with a 45 min break between trials. For visible platform trials, a red reflector was used to mark the top of the target platform. For probe trials, mice were placed in the tank with the platform removed, and given 60 sec to explore the tank. Noldus Ethovision 9 software tracked swim speed, total distance moved, and time spent in the target quadrant where the platform had been previously located.

Impulsivity behaviors were assessed in the elevated plus maze on day 21 and again at 3 months after injury as we previously described.¹⁴ The elevated plus maze apparatus (Lafayette Instruments, Lafayette, IN) consisted of two open and two closed arms (30 $\times$ 5 cm) extended out opposite from each other from a central platform (decision zone). Mice were placed on the center platform of the maze, facing a closed arm, and allowed to explore the apparatus for 5 min. A computer-assisted video-tracking system (Noldus Ethovision) recorded the total time spent in the open center (decision zone) and the open and closed compartments. The percent of time spent in the open arms is used as a surrogate measure of impulsivity behaviors; mice with greater impulsivity spend more time in the open arms. The maze was cleaned between tests with a weak ethanol solution and dried. Data were expressed as percentage of (time in open arms)/(time in open + closed arms).

Synaptosome preparation and immunoblot for NMDA receptors (NMDARs)

The separation of synaptic fractions was defined as previously described with slight modifications.¹⁹ Briefly, the hippocampi and cortices from injured ($n=6$) and sham ($n=6$) mice from adolescent and adult mice 3 months post-injury were homogenized with a

FIG. 1. Study flow diagram.

tissue grinder (Thermo Fisher Scientific, MA) in buffer I (320 mM of sucrose, 10 mM of Tris, pH 7.4, 1 mM of sodium orthovanadate [Na₃VO₄], 5 mM of NaF, 1 mM EDTA, 1 mM of EGTA, 0.1 mM of phenylmethanesulfonylfluoride [PMSF], and protease inhibitor cocktail [Roch Life Science, IN]). The homogenates were centrifuged for 10 min at 800g at 4°C to remove uncompletely homogenized tissues and nuclei (p1). The supernatants were further spun at 10,000g for 10 min. The pellets (p2) were separated from the supernatant (s1). The p2 pellets were washed once in buffer I and resuspended in 70 μ L (for hippocampus) or 140 μ L (for cortex) of buffer II (10 mM of Tris, pH 7.4, 1 mM of Na₃VO₄, 5 mM of NaF, 1 mM EDTA, 1 mM of EGTA, 0.1 mM of PMSF and 0.5% of triton-X100). The suspensions were incubated on ice for additional 20 min with pipetting every 5 min, and then the suspensions were spun at 100,000g for an additional 1 h at 4°C. The supernatants (s2, containing synaptic vesicle fraction) were transferred to new tubes. The pellets (p3, containing synaptosome fraction) were then resuspended in 50 μ L (for hippocampus) or 100 μ L (for cortex) of buffer I with 1% of sodium dodecyl sulfate (SDS). Protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad, CA). The p3 fractions were boiled in sample buffer. Twenty μ g of protein was then loaded into 4–15% TGX[™] gradient gel (Bio-rad, Hercules, CA) for electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes, and blocked with 5% of milk. Subsequently, anti-NR2B antibodies (Millipore) were applied at

a 1:500 dilution and incubated overnight at 4°C. The following day, membranes were incubated with species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and washed, and electrochemiluminescent substrate was applied (SuperSignal[™] West Femto Maximum Sensitivity Substrate, Life Technologies). An LAS 4000 Imager (GE) was used to detect signal. Actin was used to confirm equal loading between lanes. Blots were quantified using NIH ImageJ software (<http://rsb.info.nih.gov/ij>). The expression levels were normalized to actin and compared with the sham group.

Immunohistochemistry

Mice (both ages) were perfused 3 months after injury, and brains were collected for histopathological analysis. Serial 20 μ m coronal frozen sections from sham and injured adolescent ($n = 5$ /group) and adult ($n = 7$ /group) brains were cut on a cryostat (Leica) from the anterior frontal lobes through the posterior extent of the dorsal hippocampus. Every 10th section was collected sequentially and mounted on slides consistent with stereological techniques. After hydrogen peroxide treatment and incubation in a blocking solution containing 10% normal goat serum, sections were incubated overnight at 4°C in anti-ionized calcium binding adaptor 1 (IbA1) for microglia (WAKO, 1:250) antibodies. The following day, sections were washed and incubated sequentially with appropriate secondary antibodies, Vectastain ABC kit, and diaminobenzidine

(DAB) (Vector), and mounted with Permount (Thermo-Fisher Scientific).

Stereological estimates

As described previously, Stereologer software (Stereology Resource Center) was used for analyzing the microglia cell immunolabeling after TBI.¹⁴ Briefly, estimates of the number of microglia were obtained in the CA1 of sham and injured mice using a thin section modification of the optical fractionator method to determine cell immunolabeling using object area fraction and region point counting.²⁰ An investigator blinded to age and injury status quantified the total volume of the CA1, using the Cavalieri-point counting method, and the total volume of Iba1 immunolabelling. The CA1 was defined as the most dorsal peak of the hippocampus. The medial border of the CA1 was congruent with the dorsal peak of the cingulum, the lateral border was the lateral border of the molecular layer of the dentate gyrus, and the anterior margin was concomitant with the first appearance of a closed loop between the pyramidal cell layer and the dentate granule cell layer in the hippocampal midline and the posterior margin with the anterior margin of the fasciola cinereum in the mesial hippocampus. On every 10th section, the software superimposed a lattice of regularly spaced plus signs over the region of interest (ROI), and the ROI was outlined. Then, under high magnification, the number of cells within each systematically spaced unbiased sampling frame was counted. At the completion of the stereological analyses, the samples were decoded, and mean and SEM of the CA1 volumes and immunolabeling were calculated. All coefficients of error (CE) values for the stereological estimates were <10%.

White matter quantification

An investigator blinded to age and injury status quantified white matter area using ImageJ software (Bethesda, MD) for adolescent ($n=7$ /group) and adult ($n=8$ /group) brains. After stereological estimates, sectioned brains were scanned into the computer with a ruler for scale calibration. The color and brightness of the pictures were adjusted to obtain the best visibility of the white matter through all the sections. White matter was measured on two sections from each animal: the first section closest to bregma 0.38 mm and the second section 400 μ m following the first. Visible white matter from these sections was traced, and the number of pixels was calculated by the software, then converted to area. At the completion of measuring two sections per slide, the samples were decoded, and the average and SEM were calculated from each group.

Statistical analysis

Data are presented as mean $\pm$ SEM. Continuous variables were inspected for normality using the Shapiro–Wilk test and compared between injured and sham-injured mice, and between adolescent and adult mice at single time points using a Student's t test or Wilcoxon rank sum test, as appropriate. Sample size calculations were based on the t test, a moderate effect size of 0.5, power of 0.8, and an α of 0.05, estimating 10 mice/group needed for these studies. MWM and rotorod latencies were analyzed by linear regression, using clustered standard errors to account for repeated measures and time as a covariate when appropriate. The regression models were inspected with residual versus predictor plots to ensure that the residuals were normally distributed. Finally, to evaluate whether or not the effect of injury was modified by age, we created an interaction term for age and injury status, and re-ran the linear regression models with the interaction term. Statistical significance was considered $p < 0.05$. All analyses were performed using Stata 11.2 (StataCorp, College Station, TX).

Results

Repetitive concussive brain injury causes longer LOC in adolescent mice than in adult mice

Across all cohorts and ages, there were no convulsions after injury and all mice survived. After seven hits in 9 days, both adolescent ($n=17$) and adult injured mice ($n=12$) had prolonged LOC compared with their respective sham controls on day 1 and day 2 ($p < 0.01$); however, injured adolescent mice had persistent prolonged LOC compared with sham ($p < 0.01$) for all time points. There were no significant differences in LOC times between adult mice and sham on days 3–7 (Fig. 2A).

Similar results were found after four hits in 4 days: both adolescent ($n=10$) and adult injured mice ($n=10$) had prolonged LOC compared with their respective sham controls ($n=10$ adolescent and $n=10$ adult, respectively) on days 1 and 2 ($p < 0.05$); however, only injured adolescent mice had persistent prolonged LOC compared with sham on day 3 ($p = 0.021$; Fig. 2B).

Combining all injury groups, the interaction term for age and injury number was significant ($p = 0.045$), suggesting that the effect of injury number on LOC was different between adolescent and adult mice.

Repetitive concussive brain injury results in motor, spatial memory, and impulsivity abnormality at subacute time points after injury

One to three days after final injury, both adolescent ($n=12$) and adult mice ($n=12$) subjected to seven injuries over 9 days had impaired performance on rotorod testing compared with their respective sham controls ($n=17$ injured adolescents and $n=12$ injured adults, $p < 0.001$ and $p = 0.02$ respectively, Fig. 3A). The effect of injury was not significantly different between adolescent and adult mice ($p = 0.9$ for interaction term).

On days 13–16 after seven hits in 9 days, both adolescent ($n=14$) and adult injured mice ($n=12$) had impaired performance on MWM testing compared with their respective sham controls ($n=14$ adolescent and $n=11$ adult, $p < 0.001$, Fig. 3B). Adolescent sham ($p < 0.001$ for the effect of time) and adult sham mice demonstrated time-dependent learning ($p < 0.001$ for the effect of time), but injured adolescent ($p = 0.1$) and injured adult ($p = 0.7$) mice did not. The effect of injury was worse in adolescent mice than in adult mice ($p = 0.03$ for interaction term).

On day 21 after seven hits in 9 days, adult injured mice ($n=12$) spent a similar percentage of time in open and closed arms on the elevated plus maze as their sham controls ($n=11$, $p = 0.3$). In contrast, injured adolescent mice ($n=14$) spent a greater proportion of time in the open arm than did their sham controls ($n=14$, $p = 0.012$, Fig. 3C). The effect of injury was similar in adolescent and adult mice ($p = 0.8$).

Reduction in injury number diminishes neurological and cognitive deficits in adolescent and adult mice

To evaluate the effect of injury number between adolescent and adult mice, we subjected mice to a modified injury protocol of four hits in 4 days. Compared with adolescent mice who underwent the more intense injury regimen (seven hits in 9 days), injured adolescent mice on the modified regimen ($n=10$) had improved performance on rotorod testing ($p = 0.03$) and hidden trial of the MWM ($p = 0.02$) but no difference in the proportion of time in the open arm of the elevated plus maze ($p = 0.8$, Fig. 4), although deficits in the MWM persisted when compared with sham-injured

FIG. 2. Loss of consciousness (LOC) after injury. (A) After seven injuries in 9 days, both adolescent (5 weeks old, $n=17$) and adult injured mice (4 months old, $n=12$) had prolonged LOC compared with their respective sham controls (16 adolescent and 12 adult, respectively) on days 1 and 2 ($p<0.01$), but injured adolescent mice had persistent prolonged LOC compared with sham ($p<0.01$ for all time points); there were no significant differences in adult mice on days 3–7 after injury. (B) After four injuries in 4 days, both adolescent ($n=10$) and adult injured mice ($n=10$) had prolonged LOC compared with their respective sham controls (10 adolescent and 10 adult, respectively) on days 1 and 2 ($p<0.05$), but injured adolescent mice had persistent prolonged LOC compared with sham on day 3 ($p=0.021$) as well. The data are expressed as mean $\pm$ SEM. # (for 4-month-old injured mice) and * (for 5-week-old injured mice) indicate that there is a significant difference ($p<0.05$ or $p<0.01$) compared to their age-matched sham littermates.

FIG. 3. Functional outcomes in the first month after seven injuries in 9 days. (A) Balance performance after injury. One to three days after injury (seven injuries in 9 days), both adolescent (5 weeks old $n=12$) and adult injured mice (4 months old, $n=12$) had impaired performance on rotarod testing compared with their respective sham controls (12 adolescent and 12 adult, $p<0.001$ and $p=0.02$ respectively). * $p<0.05$. (B) Spatial memory. On days 13–16 after seven injuries in 9 days, both adolescent ($n=14$) and adult injured mice ($n=12$) had impaired performance on Morris water maze (MWM) testing compared with their respective sham controls (14 adolescent and 11 adult, $p<0.001$). Adolescent sham-injured ($p<0.001$ for the effect of time) and adult sham-injured mice ($p<0.001$ for the effect of time) but not injured adolescent ($p=0.1$) or injured adult ($p=0.7$) mice demonstrated time-dependent learning. (C) Anxiety measures. On day 21 after seven injuries in 9 days, adult injured mice ($n=12$) spent a similar percentage of time in open arms as sham controls ($n=11$), 9.4 ± 2.1 versus 6.7 ± 1.7 , $p=0.3$. In contrast, injured adolescent mice ($n=14$) spent a greater percentage of time in the open arms than did sham-injured controls ($n=14$), 12.3 ± 1.8 versus 7.0 ± 1.2 , $p=0.012$. # (for 4-month-old injured mice) and * (for 5-week-old injured mice) indicate there is a significant difference ($p<0.05$ or $p<0.01$) compared with their age-matched sham controls. # (for 4-month-old injured mice) and * (for 5-week-old injured mice) indicate there is a significant difference ($p<0.05$ or $p<0.01$) compared with their age-matched sham controls. All data in these figures are expressed as mean $\pm$ SEM.

FIG. 4. Functional outcomes in the first month after four injuries in 4 days. **(A)** After four injuries in 4 days, there were no significant differences in rotorod performance between adolescent (5 weeks old) injured and sham mice or between adult (4 months old) injured and sham mice. **(B)** Both adolescent (5 weeks old) injured mice and adult (4 months old) injured mice had significant spatial memory deficits after injury compared with age-matched sham controls. **(C)** Elevated plus maze task. No significant differences in time spent in the open arms were observed between injured adolescent mice and sham controls (13.3 ± 2.7 vs. 6.9 ± 1.9 , $p=0.07$), although injured adult mice demonstrated increased time in the open arms compared with adult sham controls (12.3 ± 2.3 vs. 3.5 ± 1.1 , $p=0.002$). # (for 4-month-old injured mice) and * (for 5-week-old injured mice) indicate there is a significant difference ($p<0.05$ or $p<0.01$) compared with their age-matched sham controls. All the data are expressed as mean $\pm$ SEM.

mice. Compared with the more intense seven hits in 9 days regimen, adult mice subjected to the modified four hits in 4 days regimen ($n=10$) had better rotorod performance ($p=0.004$), without significant difference in hidden platform MWM performance ($p=0.9$) or percent of time in the open arm of the elevated plus maze ($p=0.4$, Fig. 4), demonstrating persistent deficits in MWM and elevated plus performance when compared with sham controls.

Long-term behavioral deficits persist in both injured adolescent and adult mice

Three months after seven hits in 9 days, adolescent injured ($n=14$), but not adult injured ($n=12$), mice demonstrated persistent impairment in rotorod performance ($p<0.001$ and $p=0.1$, respectively; $p<0.001$ for the interaction term testing the effect of age Fig. 5A). In contrast, no deficits in rotorod performance between injured and sham mice were found 3 months after four hits in 4 days in adolescent ($p=0.8$) or adult mice ($p=0.8$) (data not shown). Adolescent and adult injured mice had persistently impaired MWM performance compared with sham mice 3 months after sustaining seven injuries in 9 days ($p<0.0001$, Fig. 5B), as well as after the modified injury regimen of four hits in 4 days ($p<0.001$, data not shown). Adult but not adolescent mice demonstrated persistent impairment in elevated plus maze performance 3 months after seven hits in 9 days ($p=0.02$ for adult injured versus adult sham, Fig. 5C); however, neither adult nor adolescent injured mice had

deficits in elevated plus maze performance compared with their respective shams 3 months after four hits in 4 days ($p=0.8$ and $p=0.9$, respectively; data not shown).

Stereological estimates of microglia

Compared with their respective shams, Iba1 immunolabeling in the injured brain was significantly increased 3 months after seven hits in 9 days in both adolescent and adult mice (Fig. 6), although the effect was not different in injured adolescent mice than in injured adult mice ($p=0.3$).

White matter area

Compared with their respective shams, white matter area in the injured brain was significantly decreased 3 months after seven hits in 9 days in adult mice (0.32 ± 0.01 mm² for injured vs 0.38 ± 0.02 mm² for sham, $p=0.02$), but not in adolescent mice (0.34 ± 0.01 mm² for injured vs 0.36 ± 0.02 mm² for sham, $p=0.3$).

NR2B receptor subunit expression

Three months after injury (seven hits in 9 days), NR2B expression in the hippocampus was reduced by $\sim 20\%$ in both adolescent and adult mice compared with shams (Fig. 7) ($p=0.04$ for adolescent mice and 0.03 for adult mice), although there was no

FIG. 5. Long-term functional outcomes 3 months after seven injuries in 9 days. **(A)** Balance. Injured adolescent mice ($n = 14$), but not injured adult mice ($n = 12$), demonstrated persistent impairment in rotorod performance ($p < 0.001$ and $p = 0.1$, respectively). **(B)** Spatial memory. Adolescent and adult injured mice had persistently impaired Morris water maze (MWM) performance compared with sham mice ($p < 0.0001$). # (for 4-month-old CHI) and * (for 5-week-old CHI) indicate $p < 0.0001$. **(C)** Adult, but not adolescent, mice demonstrated persistent impairment in the elevated plus maze ($p = 0.02$ for adult injured vs. adult sham).

difference in NR2B expression in the cortex between injured and sham adolescent or adult mice (data not shown).

Discussion

To our knowledge, these data represent the first comparison of outcomes after rmTBI in adolescent versus adult mice. Understanding age-dependent differences in outcomes after mTBI is important because of the large public health burden of mTBI in adolescent populations. These data extend our prior study detailing behavioral and histopathological outcomes after rmTBIs sustained by adult mice.¹⁴ Whereas most pre-clinical models of mTBI to date, including our prior studies, have focused on the pathophysiology of mTBI in adult populations, investigation of developmental considerations using less mature animals is warranted. Injury during childhood and adolescence may interrupt critical processes such as the rapid increase in gray and white matter, as well as the acquisition of functional skills such as motor dexterity, problem solving, memory, language, abstract thinking, social behavior, and emotional skills.²¹ Prior studies of more severe TBI have suggested age-specific differences in response to injury.^{22–24} We found that adolescent mice had longer LOC and age-dependent deficits in subacute MWM performance and long-term rotorod performance. It is notable that the distinct functional sequelae of rmTBI in adolescent mice were associated with a similar magnitude of gliosis as that found in adult mice, which, if progressive, could have even greater long-term significance in a young population.

Our findings may be in part explained by physics alone. Based on Newton's second law, the 5-week-old mice, exposed to the same amount of force, likely experienced greater acceleration of the head than did the older animals. In our model, exposed to the same forces, we observed that LOC in 5-week-old mice was significantly longer than in 4-month-old mice. Given the heterogeneous nature of adolescent growth patterns, we believe that this is a clinically relevant scenario in which smaller adolescents could sustain adult-magnitude injuries from their larger peers, especially in the context of collision sports.²⁵ Although we did not find histopathological correlates of the distinct functional outcomes observed between adolescent and adult mice in this study, we studied a limited number of histopathological outcomes, and could not, therefore, assess whether age-dependent differences in outcomes are purely explained by biomechanics.

In both adolescent and adult mice, we found that deficits after injury are both dose dependent, with an increasing number of injuries associated with worse functional outcomes, and persistent, with continued functional deficits 3 months after injuries. We have previously reported the effect of increased number and frequency of injuries in adult mice, and extend these data now to adolescent populations.¹⁷ Our studies suggest distinct vulnerabilities of adolescent mice when compared with adult mice. Although we did not ascertain in this study the structural or biochemical substrate of these developmental differences, our findings suggest that the adolescent population might warrant further pre-clinical and clinical investigation to evaluate its unique vulnerability to rmTBI.

FIG. 6. Representative photomicrographs of ionized calcium binding adaptor 1 (IbA1) positive cells 3 months after seven injuries in 9 days in adolescent sham (A) versus adolescent injured (B) and adult sham (C) versus injured (D) mice. IBA1 immunolabeling is significantly different between injured mice and their respective sham controls in both adolescent ($n=5/\text{group}$) and adult mice ($n=7/\text{group}$) (E). The data are expressed as mean $\pm$ SEM. $*p < 0.05$.

Although the pathophysiology of rmTBI is complex, one of the major pathophysiological mechanisms underlying the regional, time-dependent, and maturational vulnerability to injury is excitotoxicity via the overactivation of ionotropic glutamate receptors.²⁶ Ionotropic glutamate receptors include the α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid glutamate receptors

(AMPA), NMDARs, and kainate receptors, each of which is known to play a central role in the evolution of TBI. Glutamate-mediated neurotransmission is required for normal brain development and injury response, and glutamate receptor function is tightly regulated under normal conditions for maintenance of homeostasis.²⁷ NMDAR subunits are developmentally regulated.²⁷ NR2B

FIG. 7. N-methyl-D-aspartate receptor subtype 2B (NR2B) expression and semiquantification. Three months after injury, NR2B expression in the hippocampus was reduced in both adolescent and adult mice compared with shams. $*p < 0.05$.

plays a central role in learning, memory, and pain.^{19,28,29} Previously, it has been reported increases in cyclin dependent kinase 5 (CDK5) levels reduce synaptic NR2B levels, leading to significant impairment of learning and memory.¹⁹ We demonstrate persistently reduced synaptic NR2B expression in both adolescent and adult mice with seven closed head injuries in 9 days compared with sham animals. Specifically, synaptic NR2B protein expression is reduced at 3 months compared with sham, regardless of whether the rmTBI occurred at 5 weeks or 4 months of age. These data are consistent with our data demonstrating altered escape latency in the MWM and deficits in hippocampal dependent memory and spatial learning in both adolescent and adult injured mice. Notably, decreases in hippocampal NR2B has been reported in TBI and in the postmortem hippocampus of patients with Alzheimer's disease.³⁰ Given its role as a critical component of molecular signaling pathways regulating calcium flux, long-term potentiation (LTP), long-term depression (LTD), synaptic growth, and plasticity,³¹ our findings suggest abnormal NR2B levels may be associated with loss of in-membrane stability and disruption of glutamateric homeostasis following repetitive injury. Notably, our study expands prior data indicating that NMDARs change in a regional and time-dependent fashion following closed head injury in mice for up to 3 months post-injury.²⁶

Conclusion

In summary, we demonstrate that injured adolescent mice show a distinct pattern of functional deficits after injury when compared with adult mice. Determining whether these findings reflect differences in the biomechanics of injury, or developmental differences in the response to injury, warrants further investigation, particularly given the increasing public health burden associated with TBIs sustained by adolescents.

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No competing financial interests exist.

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Address correspondence to:
 Rebekah Mannix, MD, MPH
 Division of Emergency Medicine
 Boston Children's Hospital
 Boston, MA 02115

E-mail: Rebekah.Mannix@childrens.harvard.edu