ANNALS of Clinical and Translational Neurology
Open Access
ANA American Neurological Association since 1875

RESEARCH PAPER

Lifelong behavioral and neuropathological consequences of repetitive mild traumatic brain injury

Benoit C. Mouzon[1,2,3], Corbin Bachmeier[1,2,3], Joseph O. Ojo[1,2], Christopher M. Acker[4], Scott Ferguson[1,2], Daniel Paris[1,2,3], Ghania Ait-Ghezala[1,2,3], Gogce Crynen[1,2,3], Peter Davies[4], Michael Mullan[1], William Stewart[5,6] & Fiona Crawford[1,2,3]

[1]Roskamp Institute, Sarasota, Florida
[2]James A. Haley Veterans' Hospital, Tampa, Florida
[3]The Open University, Milton Keynes, United Kingdom
[4]Feinstein Institute for Medical Research, Manhasset, New York
[5]Queen Elizabeth Glasgow University Hospital, Glasgow, United Kingdom
[6]University of Glasgow, Glasgow, United Kingdom

Correspondence
Benoit C. Mouzon, Roskamp Institute, 2040 Whitfield Avenue, Sarasota, FL 34243.
Tel: 941-752-2949; Fax: 941-752-2949;
E-mail: bmouzon@roskampinstitute.org

Funding Information
This research was funded by a Department of Defense award (W81XWH-10-1-0759) to Dr. Fiona Crawford, and by the Roskamp Foundation. Dr. Crawford is a VA Research Career Scientist. Dr. Stewart is supported by NIH grants NS038104 & NS094003, DOD grant PT110785 and an NHS Research Scotland Career Researched Fellowship. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the U.S. Government, or the U.S. Department of Veterans Affairs, and no official endorsement should be inferred.

Received: 7 November 2017; Accepted: 10 November 2017

Annals of Clinical and Translational Neurology **2018; 5(1): 64–80**

doi: 10.1002/acn3.510

Abstract

Objective: Exposure to repetitive concussion, or mild traumatic brain injury (mTBI), has been linked with increased risk of long-term neurodegenerative changes, specifically chronic traumatic encephalopathy (CTE). To date, preclinical studies largely have focused on the immediate aftermath of mTBI, with no literature on the lifelong consequences of mTBI in these models. This study provides the first account of lifelong neurobehavioral and histological consequences of repetitive mTBI providing unique insight into the constellation of evolving and ongoing pathologies with late survival. **Methods**: Male C57BL/6J mice (aged 2–3 months) were exposed to either single or repetitive mild TBI or sham procedure. Thereafter, animals were monitored and assessed at 24 months post last injury for measures of motor coordination, learning deficits, cognitive function, and anxiety-like behavior prior to euthanasia and preparation of the brains for detailed neuropathological and protein biochemical studies. **Results**: At 24 months survival animals exposed to r-mTBI showed clear evidence of learning and working memory impairment with a lack of spatial memory and vestibulemotor vestibulomotor deficits compared to sham animals. Associated with these late behavioral deficits there was evidence of ongoing axonal degeneration and neuroinflammation in subcortical white matter tracts. Notably, these changes were also observed after a single mTBI, albeit to a lesser degree than repetitive mTBI. **Interpretation**: In this context, our current data demonstrate, for the first time, that rather than an acute, time limited event, mild TBI can precipitate a lifelong degenerative process. These data therefore suggest that successful treatment strategies should consider both the acute and chronic nature of mTBI.

Introduction

Mild traumatic brain injury (mTBI) has been called a "silent epidemic" as the initial symptoms can be so subtle that the majority of people who have experienced a concussion do not seek medical attention.[1,2] As a result, the consequences of repeated concussions/mTBI (r-mTBI) have long been underestimated and viewed as a transient injury without long-term consequences. However, a subset of this population demonstrates persisting neurocognitive dysfunction and non-neurological disorders that cannot be considered mild.[3] Notably, it is now well documented that the adverse effects of r-mTBI in participants of contact sports and in former military personnel may continue for many years after the original event,[4–8] with brain trauma being a risk factor for the development of

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

neurodegenerative disease,[9] in particular chronic traumatic encephalopathy (CTE); for review see Smith et al.[10]

Despite increasing evidence that mTBI is associated with late neurodegenerative pathologies,[11,12] there is limited understanding of the processes linking an acute phase biomechanical injury to late and progressive neurodegenerative pathologies. In particular, current models of TBI are largely limited to acute phase studies or, where data with survival are reported, this rarely extends beyond 12 months. Nevertheless, in those few models exploring "late" survival there is notable evidence that early injury can have persisting and evolving consequences, even at a year post -injury including: chronic neuroinflammation,[13–15] ongoing axonal degeneration and white matter atrophy,[13] cerebrovascular abnormality,[16] deposition of abnormal tau in transgenic animals,[15,17,18] and neurobehavioral impairments.[13] However, despite growing evidence that the consequences of r-mTBI might be regarded as a "lifetime illness," to date, existing animal models arguably have failed to provide an accurate surrogate of events in human TBI survival across a lifetime. To address this, we hereby present behavioral, neuropathological and biochemical analyses 24 months after a single mTBI (s-mTBI) or r-mTBI in a validated rodent model of closed head mild TBI.

Materials and Methods

Animals

Male, C57BL/6J mice (aged 2–3 months old, 32–42 g, Jackson Laboratories, Bar Harbor ME) were housed singly under standard laboratory conditions (23 ± 1°C, 50 ± 5% humidity, and 12-h light/dark cycle) with free access to food and water throughout the study until their euthanasia at age 27 months. All procedures were carried out under Institutional Animal Care and Use Committee approval and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Injury groups and schedule

Animals were subjected to closed head mild TBI or anesthetic as sham as previously described.[13,19] For the behavioral analyses, a total of 32 mice were randomly assigned to one of four groups: single injury, single sham (anesthesia alone), repetitive injury (total of five hits with an interconcussion interval of 48 h), and repetitive sham (five anesthesias, 48 h apart). The behavior analysis began 2 years after the sole/last mTBI/anesthesia for each group. By the time of testing, one mouse assigned to r-sham, and one assigned to s-mTBI had died of natural causes, and further, a r-mTBI mouse was excluded from behavioral analysis due to severe dermatitis; thus the final numbers were s-sham (8), r-sham (7), s-mTBI (7), and r-mTBI (7). Of these, tissue from three mice per group were assigned for biochemical analyses, whereas the remaining (4–5 per group) were processed for histological examination.

Injury protocol

The mTBI was administered to mice as previously described.[19] Mice were anesthetized with 1.5 L/min of oxygen and 3% isoflurane prior to anesthesia or mTBI. The animals were placed on a heating pad to maintain their body temperature at 37°C. A 5 mm blunt metal impactor tip was retracted and positioned midway along the sagittal suture before each impact. The injury was triggered using the myNeuroLab controller at a strike velocity of 5 m/sec, strike depth of 1.0 mm, and dwell time of 200 msec. Our criteria for a mild injury were as follows:

(1) a short period of posttraumatic apnea <30 sec (recognized as an animal analog to human loss of consciousness,[20] (2) a short period of righting reflex <6 min, (3) no sign of skull fractures and (4) no history of major hemorrhage(s) at the time of euthanasia. Moreover, our interinjury interval was 48 h, which is similar to our previous work,[13,19] a temporal window during which the mouse brain is known to be vulnerable to subsequent injuries[21] in order to mimic human situations (combat or sports) in which additional injuries are sustained prior to full recovery from the previous injury. At the end of the procedure, each animal was removed from the stereotaxic table and allowed to recover on a heating pad and, upon becoming ambulatory, was returned to its home cage. R-mTBI mice received a total of five hits, with a 48 h interinjury interval. Sham-injured animals underwent the same procedures and were exposed to anesthesia for the same length of time as the mTBI animals, but did not receive a hit, in order to control for the effects of single or repeated anesthesia.

Assessment of motor function

The rotarod apparatus (Ugo Basile, Varese, Italy) was used to assess motor performance by measuring the latency to remain on an elevated accelerating rod as previously described.[19] The device was set to accelerate from 5 to 50 rotations per minute during a period of 5 min. For each trial, latency to fall was timed by an experimenter blinded to group assignment. The trial was also terminated if the animal spun around the rod through three complete revolutions. This trial was repeated three times per day for a period of 5 days, with an intertrial interval of 5 min, and the researchers conducting the experiments were blinded to the group assignment of the mice.

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

Assessment of learning and cognitive function

Cognitive function was evaluated at 24 months post-injury/sham using the Barnes maze (BM) in the same manner we described previously.[13,19] Researchers conducting the experiments were blind to mouse grouping, and the Ethovision XT system (Noldus, Wageningen, NL) was used to track and record the movement of each animal. Mice were given 90 sec to locate and enter the target box, and they were required to remain in the target box for 30 sec prior to their retrieval, regardless of success. Escape latency measured the time taken for the mouse to enter the box in order to escape the brightly lit, open surface of the maze. For a period of 6 days, four trials were given per day, with mice starting from one of four cardinal points on each trial. On the 7th day of testing, a probe trial was conducted in which the target box was removed and the time spent to reach the zone previously containing the box was recorded over one 60 sec trial.

Assessment of anxiety

Anxiety-like behavior was assessed using an elevated plus maze, which relies on the animal's preference for dark enclosed arms rather than brightly lit, open arms. This task assesses the willingness of the mouse to explore the open arms of the maze which are fully exposed and at an elevated height. Time spent in the open arms is decreased in mice that exhibit anxiety-like behaviors. The maze consisted of a polyvinyl chloride plus-shaped platform elevated 50 cm from the floor with four arms intersecting at a 90° angle, creating four individual arms each 55 cm long and 5 cm wide. Closed and open arms were orthogonal to each other; the two closed arms were shielded by 25 cm high side and end walls, whereas the two open arms had no walls. The experimental procedure was initiated by the placement of the mouse into the center zone (intersection point) of the maze, facing one of the open arms. The mouse was allowed to explore the maze for a 5-min period while an overhead video camera recorded the movements of each mouse. Ethovision XT was used to automatically score, in an unbiased manner, the number of entries in each of the arms. All four paws of the mouse had to enter an arm for it to be considered an entry, and the percentage score for the time spent in the open arm was calculated as follows: (time spent in the open arms/[time spent in the open arms + time spent in closed arms]) × 100.

Histology

At 24 months post-mTBI/sham injury, mice assigned to histological studies were anesthetized with isoflurane and perfused transcardially with phosphate-buffered saline (PBS), pH 7.4 followed by PBS containing 4% paraformaldehyde. After perfusion, the brains were post-fixed in a solution of 4% paraformaldehyde at 4°C for 48 h. The intact brains were then blocked and processed as previously described.[19] For each group, sets of sagittal sections (lateral 0.2–0.4 mm) were cut. Sections were stained with Luxol fast blue/cresyl violet (LFB/CV) using standard histological protocols. Sets of adjacent sections were stained with a panel of antibodies (Table 1) for immunohistochemical analyses. Each slide was visualized with a bright field microscope (BX60), and digital images were visualized and acquired using an Olympus MagnaFire SP camera.

Immunohistochemical quantification

For each animal, sagittal sections were stained and analyzed by an observer blinded to experimental conditions using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). Using this software, images were separated into individual color channels (hematoxylin

Table 1. Summary of antibodies used in this study.

Protein target	Antibody	Epitope	IHC-Dilution	Source	Assay
Amyloid precursor protein	22C11	aa 66–81	1:40,000	Millipore	IHC
Glial fibrillary acidic protein	GFAP	GFAP	1:20,000	Dako	IHC
Iba-1	Iba-1	Iba-1	1:5000	Abcam	IHC
Leukocyte common antigen	CD45	CD45 (mca1388)	1:1000	Biorad	IHC
Total-tau (mouse)	DA9	aa 102–140	1:1000	Dr. P. Davies	Dot blot
Total-tau (mouse)	DA31	aa 150–190	N/A	Dr. P. Davies	ELISA/WB
Phospho-tau (mouse)	CP13	pS202	1:400	Dr. P. Davies	ELISA/IHC/WB
Phospho-tau (mouse)	PHF1	pS396/404	1:400	Dr. P. Davies	ELISA/IHC/WB
Phospho-tau (mouse)	RZ3	pT231	1:400	Dr. P. Davies	ELISA/IHC/WB
Phospho-tau (mouse)	MC1	Conformational	1:200	Dr. P. Davies	IHC
Beta amyloid (Aβ)	4G8	aa 17–24	1:750	Covance	IHC

aa, amino acid; IHC, Immunohistochemistry; ELISA, enzyme-linked immuno-sorbent assay; WB, western blot.

 © 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

counter stain and DAB) using the color deconvolution algorithm.[22] Three nonoverlapping areas of 100 μm^2 for the body of the corpus callosum (CC) were randomly selected, within which the area of glial fibrillary acidic protein (GFAP) immunoreactivity was calculated and expressed as a percentage of the field of view (lateral 0.2–0.4 mm). Four nonoverlapping areas of 150 μm^2 between layer III and IV in the primary somatosensory cortex, and three nonoverlapping areas of 100 μm^2 in the CC were randomly selected within which the area of anti-Iba1 immunoreactivity was calculated and expressed as a percentage of the field of view (lateral 0.2–0.4 mm). The extent of axonal injury was determined in APP-stained sections. APP immunoreactive axonal swellings were quantified from the caudal to the dorsal area of the body of the CC. Using ImageJ software, the average thickness of the corpus callosum was calculated as previously described.[13] A total of five slides per animal were averaged for each immunohistochemical analysis.

Quantitative assessment of soluble Aβ_{40}, phosphorylated tau, and total tau protein

Analyses of soluble Aβ_{40}, phosphorylated tau and total tau protein were carried out on cortical and hippocampal brain regions obtained from PBS-perfused mice at 24 months post-s/r-mTBI (n = 3/group) or s/r-sham exposure (n = 3/group). Snap-frozen dissected cortices and hippocampi were sonicated in 0.5 mL and 0.3 mL of chilled M-PER buffer solution (Thermo Fisher Scientific, Waltham, MA) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). Soluble Aβ40 was assessed using a sandwich ELISA (WAKO, Richmond, VA) as per the manufacturer's directions. For tau biochemistry, tissue homogenates were coded and sent to Dr. Peter Davies' group at the Feinstein Institute for Medical Research (Manhasset, NY), for blinded quantitative assessments. Sample preparation for the low-tau sandwich ELISA and quantitation of murine-specific tau protein was performed as previously described.[23] Antibodies to detect total and phospho tau (Table 1) were used as capture antibodies in the low-tau, Sandwich ELISA. Total protein content was determined by the bicinchonic acid (BCA) method (Thermo Scientific, Waltham, MA) and data were expressed as pmol/g protein (Aβ_{40}) and ng tau/mg protein (tau and phosphorylated tau).

Dot blot analyses of total tau in plasma samples

At euthanasia, anesthetized mice were exsanguinated via aortic puncture using a wide-bore needle to prevent hemolysis of red blood cells. Blood samples were collected into a 1.6-mL Eppendorf tube containing EDTA and supplemented with a protease inhibitor cocktail (PIC) (Roche Diagnostics Corporation, Indianapolis, IN) to a final concentration of 1X. Samples were cooled on ice for up to 1 min prior to centrifugation at room temperature at 3000 g for 3 min to minimize platelet coagulation. Following separation, clarified plasma was transferred to a clean Eppendorf tube and snap-frozen in liquid nitrogen. Following a brief vortexing, 3 μL of each sample was spotted directly onto nitrocellulose membranes (Biorad, CA, USA). Membranes were blocked for 1 h at room temperature in 10% nonfat dry milk dissolved in Tris-buffered saline (Biorad, CA, USA) and incubated with the primary antibody total tau DA9 overnight at 4°C. After the hybridization, membranes were rinsed in distilled water for 30 min and incubated for 2 h at room temperature with secondary anti-mouse antibody conjugated with peroxidase (Cell Signaling technology, MA, USA). Signal detection and quantification were performed with a two-fold dilution of the SuperSignal west femto maximum sensitivity chemiluminescent substrate (Thermo Scientific, IL, USA) by chemiluminescence imaging with the Chemi-DocTM XRS (Bio-Rad, CA, USA). After hybridization with the primary antibodies, all membranes were stained for 30 min with a Fast Green solution which was used to adjust for variation in protein amounts spotted onto nitrocellulose membranes. All results were expressed as a ratio of DA9/Fast Green signals.

Statistical analysis

All behavioral and pathological data were analyzed using JMP 8.0 (SAS, Cary, NC). Scores were analyzed separately for groups of animals assigned to survival groups at 24 months post-injury. Data were tested for normality using the Shapiro–Wilk W test; when not normally distributed, the data were transformed using square root or natural log transformation. If the data were still not normal after transformation, nonparametric methods were used for analysis. Repeated-measures ANOVA was used to compare performance during the 6 days of acquisition of the Barnes maze between the matching injury groups when the data were normally distributed. Repeated-measures ANOVA was also used to compare the pathological quantifications and behavior performances during the five time points of acquisition during the life of the animal between the matching injury groups. Potential sphericity violations were corrected by adjusting degrees of freedom for all repeated-measures effects using the Greenhouse-Geisser estimate for epsilon. The Barnes Maze Probe dataset and quantitative histologic parameters were analyzed with one-way ANOVA, with a Tukey's post hoc

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

correction for multiple comparisons, unless otherwise indicated. Behavior, pathological, and ELISA data were plotted and analyzed using Graph-Pad Prism (Prism 6.01, GraphPad Software Inc, La Jolla, CA). One-way ANOVA followed by Tukey's post hoc test was used for comparison of soluble tau and $A\beta_{40}$ levels between the four groups. Only P values <0.05 were considered to be statistically significant and are indicated by an asterisk in the figures. Error bars represent the standard error of the mean (SEM).

Results

mTBI is associated with persistent and long-term learning deficits

At 24 months post-last injury, both s-mTBI and r-mTBI mice travelled a longer distance to reach the target box in the Barnes maze when compared with their respective shams, (r-mTBI vs. r-sham, $P < 0.0001$; s-mTBI vs. s-sham, $P < 0.05$; repeated-measures ANOVA) (Fig. 1A)

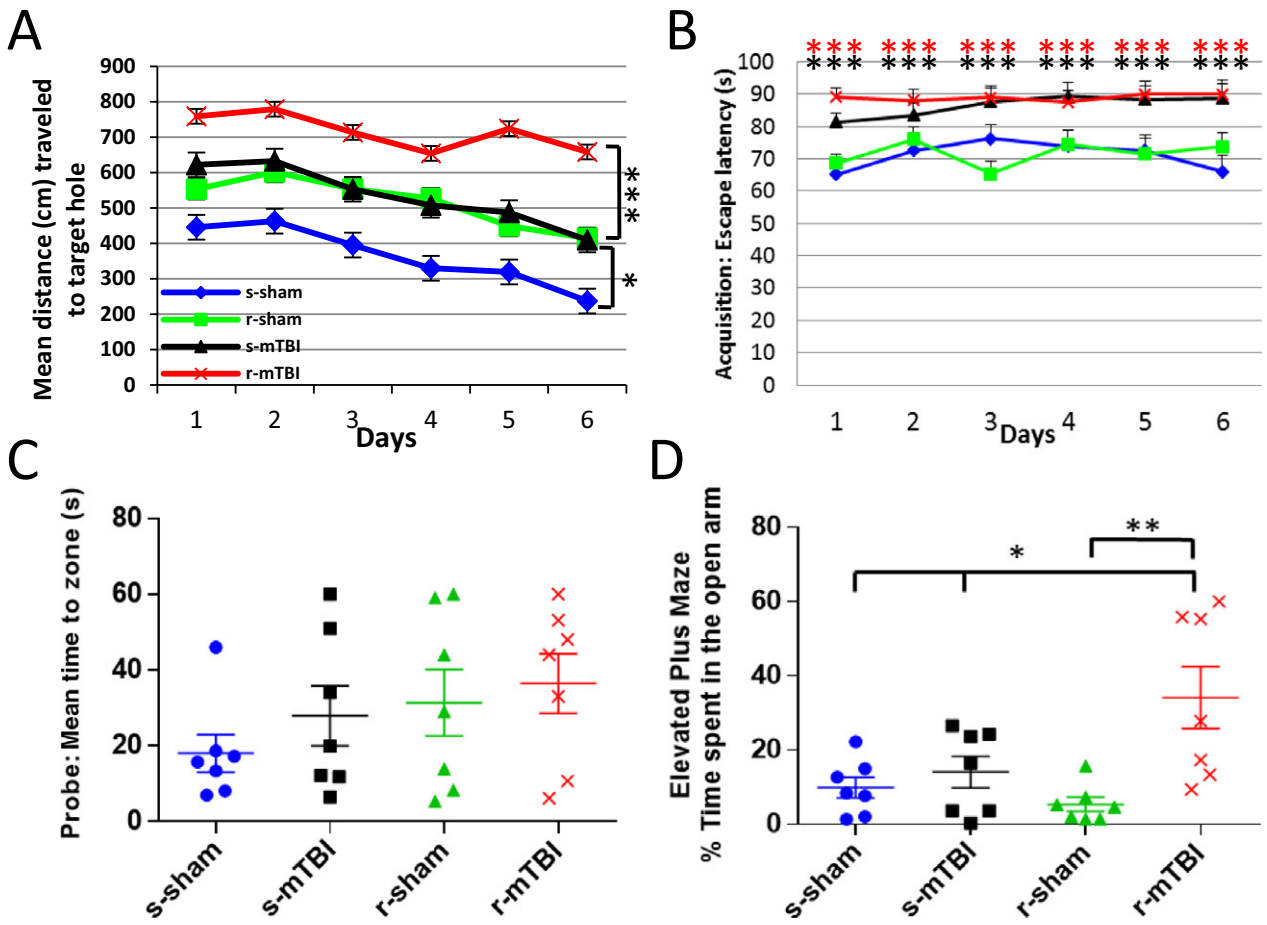

Figure 1. Assessment of neurobehavioral change at 24 months post-mTBI. (A) Learning deficits were evaluated during the 6 days of acquisition. Both injured groups traveled a greater distance before escaping to the target hole compared to their respective anesthesia controls (s-sham vs. s-mTBI, $*P < 0.05$; r-sham vs. r-mTBI and s-mTBI vs. r-mTBI, $***P < 0.0001$; repeated-measures ANOVA). At all-time points, the performance of the r-mTBI group was worse than that of the s-mTBI group (s-mTBI vs. r-mTBI, $***P < 0.0001$; repeated-measures ANOVA). (B) Both injured groups fail escaping to the target hole when compared with their sham controls at every single day of training; Wilcoxon signed rank test, $***P < 0.0001$. (C) The spatial memory test (probe) showed no difference across each group. However, there was a trend for the r-mTBI mice to take longer to reach the target zone indicating a possible deficit of spatial memory. Data are presented as mean ± SEM, one-way analysis of variance with Tukey's post hoc test; $P > 0.05$; each symbol represents 1 mouse. (D) Risk-like behavior measuring the willingness of the animal to explore exposed arm at a specific height was assessed using an elevated plus maze. The r-mTBI mice developed an increased risk taking behavior by spending more time in the open arms of the elevated plus maze when compared with control mice, one-way analysis of variance with Tukey's post hoc test; $*P < 0.01$; $**P < 0.001$. Values represent mean ± SEM ($n = 7$ per group).

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

suggesting an impairment of visuospatial learning. A deficit in performance was also apparent comparing the r-mTBI group to the s-mTBI group (r-mTBI vs. s-mTBI, $P < 0.0001$; repeated-measures ANOVA). Performance of the r-sham group was similar to the s-mTBI (r-sham vs. s-mTBI, $P > 0.05$; repeated-measures ANOVA). Consequently, the average distances travelled by the r-mTBI and the s-mTBI mice over the 6 days acquisition period were 38%, and 45% longer than their respective sham controls. The performance of these animals was directly related to the number of injuries they received.

The dataset for latency to escape was not normally distributed and thus did not satisfy the assumptions required for a repeated-measure ANOVA. The Wilcoxon signed rank test was used to test for the daily correlation between each group. The mean escape latency of the s-mTBI and the r-mTBI did not improve between their first and last acquisition day (s-mTBI 81.4 ± 2.6 sec on day 1 to 88.7 ± 4.4 sec on day 6; r-mTBI 89.1 ± 2.8 sec on day 1 to 89.9 ± 4.1 sec on day 6). Over the 90 sec trial period all the animals found the target hole but almost none of them made the decision to enter into the escape box. Consequently, no difference in escape latency performance was observed between the s-sham vs. r-sham and s-mTBI vs. r-mTBI (day 1–6 of acquisition; $P > 0.05$) (Fig. 1B). Overall, both sham groups could find the hidden box with similar latencies (on average 74 sec) to escape the maze while both injured groups did not reach the hidden box on the table at every single day of training (Wilcoxon signed rank test, $***P < 0.0001$).

No TBI-dependent spatial memory deficits were observed at 24 months post-mTBI

Probe test performance (defined as the latency to reach either the target hole or the holes immediately adjacent to the target hole) revealed that the r-mTBI mice performed the worst, requiring on average 36 sec to reach the target zone, followed by the r-sham (33 sec), the s-mTBI (31 sec), and the s-sham (18 sec) ($P > 0.05$ as determined by one-way ANOVA followed by Tukey's post hoc test) (Fig. 1C). The mean velocity for the probe trial was similar across all groups ($P > 0.05$; data not shown). The performance of each group had declined progressively since they were last tested at 21 months of age (18 months post-injury/anesthesia), however, early life r-mTBI did not significantly worsen spatial memory deficits in these aged animals.

mTBI was not associated with late vestibulo-motor deficits

Following evaluation in the Barnes maze at 24 months post-injury/anesthesia, motor performance for each group was tested with the rotarod. Overall, the performance of singly or repetitively injured animals was not different from controls, supporting the transient nature of the motor deficits detected acutely post-injury, as previously reported.[13,19] The results from day 1 of pretraining compared to the last day of rotarod were as follows: s-sham 99.2 ± 8.5 sec vs. 136.0 ± 6.1 sec; s-mTBI 82.1 ± 6.9 sec vs. 128.2 ± 10.8 sec; r-sham 78.0 ± 6.6 sec vs. 125.8 ± 11.1 sec; r-mTBI 82.7 ± 6.8 sec vs. 137.1 ± 12.5 sec.

r-mTBI causes increased risk behavior/ disinhibition at 24 months post-mTBI

To characterize the long-term effects of single or repetitive mild TBI on anxiety-related behaviors, the exploratory activity of each animal was tested in the elevated plus maze (Fig. 1D). While there was no difference in the percentage of time spent in the open arms between the s-mTBI and either of the control groups (s-sham, 9.9 ±2.7%; r-sham, 6.1 ± 2.1; s-mTBI, 14.1 ± 4.3%; $P > 0.05$ one-way ANOVA followed by Tukey's post hoc test), the r-mTBI group spent on average 30.1±8.3% of their time within the open arm (s-sham or s-mTBI, vs. r-mTBI $P < 0.05$; r-sham vs. r-mTBI, $P < 0.001$; one way ANOVA followed by Tukey's post hoc test). Such an increased amount of time spent in the open arms is suggestive of disinhibition or increased risk-taking behavior which has been previously observed in other mouse models of repetitive closed head injury.[14,24]

mTBI is associated with ongoing axonal degeneration and thinning of the corpus callosum

Changes in white matter integrity and thickness of the body of the corpus callosum were assessed in sections stained for LFB/CV. At 24 months following s-mTBI, there was a 26% reduction in thickness of the body of the CC compared to s-sham ($P < 0.001$; one way ANOVA followed by Tukey's post hoc test) (Fig. 2G). Similarly, the r-mTBI animals showed thinning of the body of the CC, with a decrease of 29% ($P < 0.001$; one-way ANOVA followed by Tukey's post hoc test) when compared with the r-sham group.

At 24 months post-injury, APP-immunoreactive profiles were scattered diffusely throughout the corpus callosum in all groups, injured and sham. Typically, these were observed as sparse, rounded to granular immunoreactive axonal profiles (Fig. 3D). The difference in the number of APP-immunoreactive profiles observed was greatest in the r-mTBI versus r-sham comparison (r-mTBI 8.5 ± 1.4 vs. r-sham 3.6 ± 1.2 axonal

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

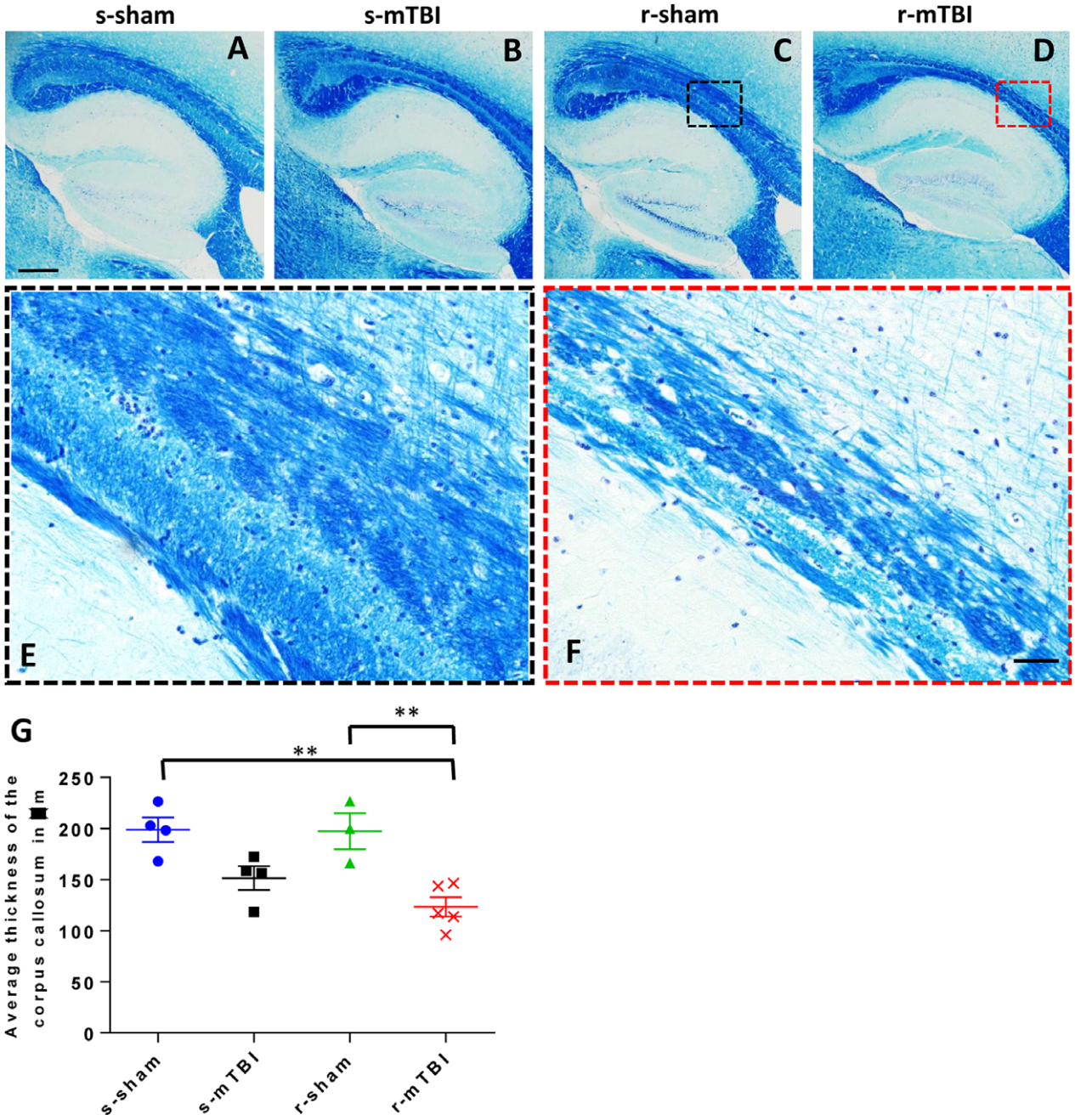

Figure 2. LFB/CV staining indicated changes in white matter integrity in the injured animals at 24 months post-injury. (A-D) Reduction in the CC thickness at 24 months post r-mTBI. Scale bar, 200 μm. (G) At 24 months, the thickness of the CC was decreased on average by 26% after s-mTBI, and by 29% in the r-mTBI. (E, F) Higher power view of the r-sham and r-mTBI. Scale bar, 20 μm The CC thickness was measured with ImageJ by averaging 6 measurements for each hemisphere per mouse. Each symbol represents the average of both hemispheres. Data are presented as mean ± SEM, one-way ANOVA with Tukey's post hoc test was used for comparison of each group; $^{**}P < 0.001$.

profiles/body of CC; $P < 0.001$), with the s-mTBI/sham comparison not reaching significance (s-mTBI 4.6 ± 1.1, s-sham 2.8 ± 0.2 axonal profiles/body of CC).

mTBI induces persisting astrogliosis in the corpus callosum

For all groups, the cortical region underlying the impact site (somatosensory and primary motor cortices) and the CC

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

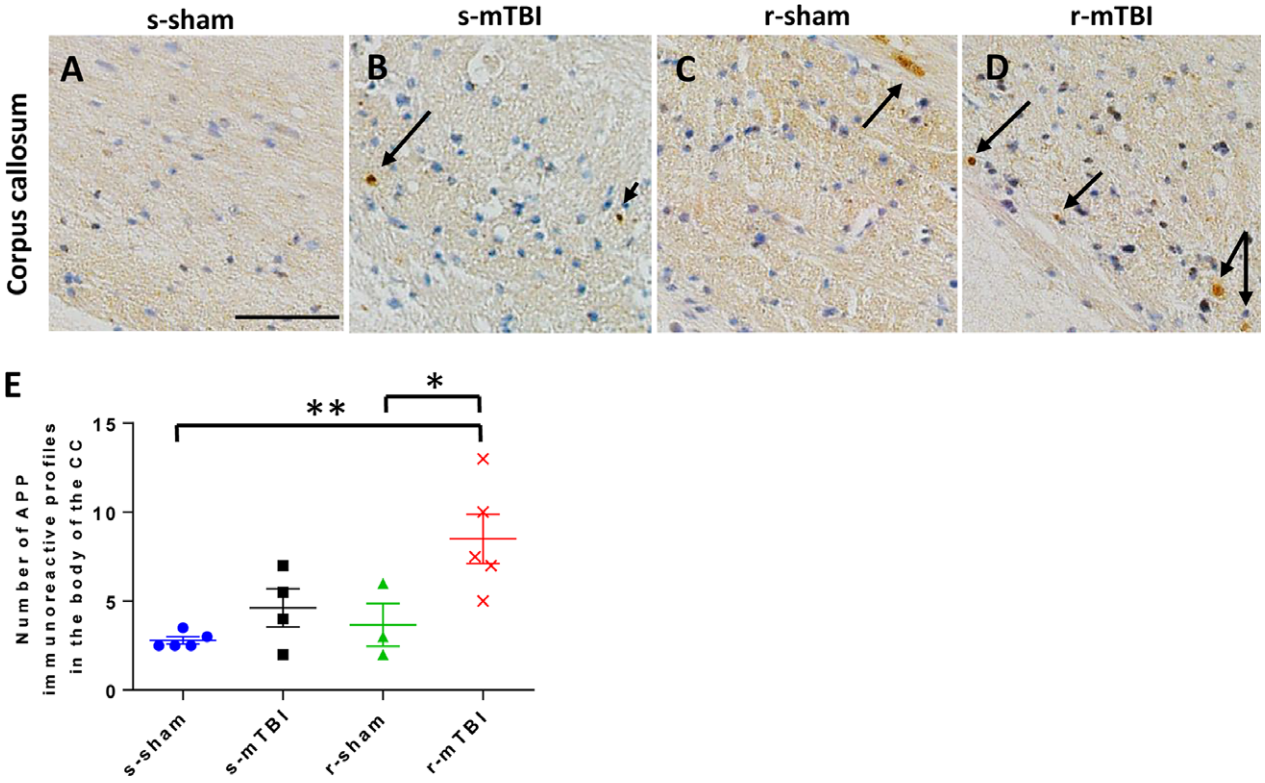

Figure 3. Immunohistochemical labeling for amyloid precursor protein at 24 months post-injury. (A-D) Sagittal sections of the mouse brain approximately 0.2 mm lateral to midline in the corpus callosum. A few axonal profiles were observed in the CC of the sham groups and the s-mTBI. Scale bar, 50 μm. (D) There was an increase in accumulation of discrete axonal profiles in the CC in the r-mTBI compared to their sham counterpart; *$P < 0.01$. Tissue sections were counterstained with hematoxylin. (E) Quantitative analysis of APP staining within the body of the CC; each symbol represents 1 mouse. Data are presented as mean ± SEM, one-way analysis of variance with Tukey's post hoc test was used for comparison of each group; *$P < 0.01$, **$P < 0.001$.

were assessed in sections stained for GFAP. No TBI-dependent quantitative difference in the level of GFAP immunopositivity was detected in the cortices (s-sham 1.4 ± 0.3%; s-mTBI 1.7 ± 0.1%; r-sham 1.1 ± 0.3%; r-mTBI 1.6 ± 0.4%; $P > 0.05$; Fig. 4). However, in the CC, the r-mTBI (Fig. 4) and s-mTBI groups showed a notable increase in the area of GFAP immunoreactivity compared with their respective sham controls (Fig. 4E–H), with the magnitude of this increase being greater in the r-mTBI than in the s-mTBI group (s-mTBI 24.2 ± 1.6% vs. s-sham 15.3 ± 2.1%; $P > 0.05$; r-mTBI 27.4 ± 2.7% vs. r-sham 15.8 ± 2.5%; $P < 0.01$; Fig. 4I).

mTBI induces persisting neuroinflammation in the corpus callosum

To gain insight into whether a single mTBI or r-mTBI could accelerate or sustain the degree of inflammation in aged animals, we investigated two different markers of brain inflammation, Iba-1, a marker of microglia and CD-45, a cell surface marker of monocytes/lymphocytes. In the retrosplenial, sensorifrontal, and motor cortex, there was no appreciable evidence of microglial activation for any group at 24 months post-injury (Fig. 5E–H, J). The morphological characteristics of the microglial cells were relatively similar across each group, with a primed morphology characteristic of an aged mouse brain.

In contrast, in the body of the corpus callosum, the r-mTBI showed a notable increase in anti-Iba1 reactivity (Iba1: r-mTBI 14.7 ± 1.3% vs. r-sham 10.7 ± 1.5% $P < 0.05$) (Fig. 5A–D, I). A slight but noticeable increase in both Iba-1 and CD45+ immunoreactivity was observed in the s-mTBI group compared to controls; however, this was not significant ($P > 0.05$ for either marker).

mTBI is not associated with increased Aβ40 or tau phosphorylation

Immunohistochemical assessment revealed no injury effect on the localization or accumulation of the p-tau epitopes CP13, (Fig. 6A), PHF1 and RZ3 (data not shown). All experimental groups exhibited somatodendritic accumulation of CP13 immunoreactivity in neurons of the superficial layer of the cerebral cortex, and in the CA1 and CA3 regions of the hippocampus. The same brain regions were devoid of PHF1-

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

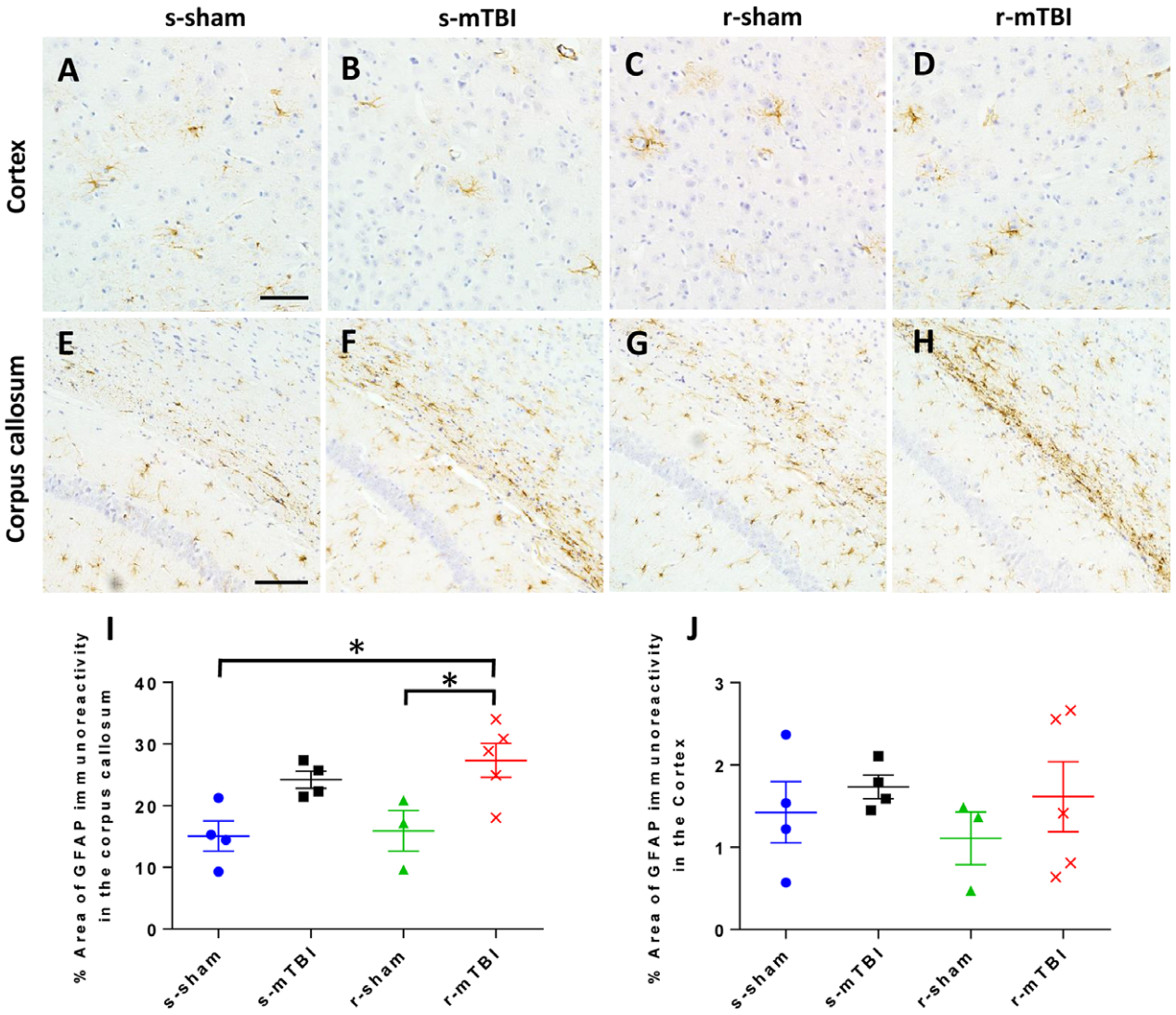

Figure 4. Repetitive mTBI increases astrogliosis in the corpus callosum at 24 months post-injury. Sagittal sections of the mouse brain approximately 0.2 mm lateral to midline in (A–D) the layer III/IV of the cortex and (E–H) in CC with GFAP. Scale bar, 50 μm and 100 μm, respectively. (I) Quantitative analysis of GFAP staining revealed extensive GFAP immunoreactivity within the CC of the r-mTBI group compared to both sham groups; *$P < 0.05$. (J) There was no difference in the expression of GFAP within the cortex of the injured animals compared to their sham counterparts. Tissue sections were counterstained with hematoxylin. (I, J) Quantitative analysis of GFAP staining in three 100 μm^2 fields within the body of the CC and four 200 μm^2 fields between layer III and *V* of the cortex at 24 months post-injury; each symbol represents 1 mouse. Data are presented as mean ± SEM, one-way analysis of variance with Tukey's post hoc test was used for comparison of each group; *$P < 0.05$.

and MC1-positive neurons regardless of the injury status (data not shown). Consistent with our previous observation of lack of increased Aβ40 or tau phosphorylation at 12 months post single and r-mTBI/anesthesia,[13] the analysis of Low-Tau ELISAs at the 24 month time point showed no TBI-dependent increase in cortical or hippocampal soluble p-tau pSer-202 (CP13), pThr-231 (RZ3), and pSer-396/404 (PHF1) ($P > 0.05$) (Fig. 6D). Phospho-tau was also quantitated as a ratio of phosphorylated tau protein to total tau protein; however, there was no injury effect on these ratios in either brain region (cortex; hippocampus) (Fig. 6D).

Changes in the plasma level of total tau have been reported in other neurodegenerative diseases. Therefore we employed a dotblot approach established by Paris et al.[25] to quantify total tau (DA9) concentration in plasma samples at the time of euthanasia. However, no association was revealed between r-mTBI and an accumulation of tau protein in plasma samples of aged animals (Fig. 6B and C).

ELISA analysis of soluble murine Aβ_{40} showed no TBI or age-dependent increases (data not shown). Aβ deposition assessed by the 4G8 antibody or Congo Red staining (data not shown) was not detected in the cerebral cortex

 © 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

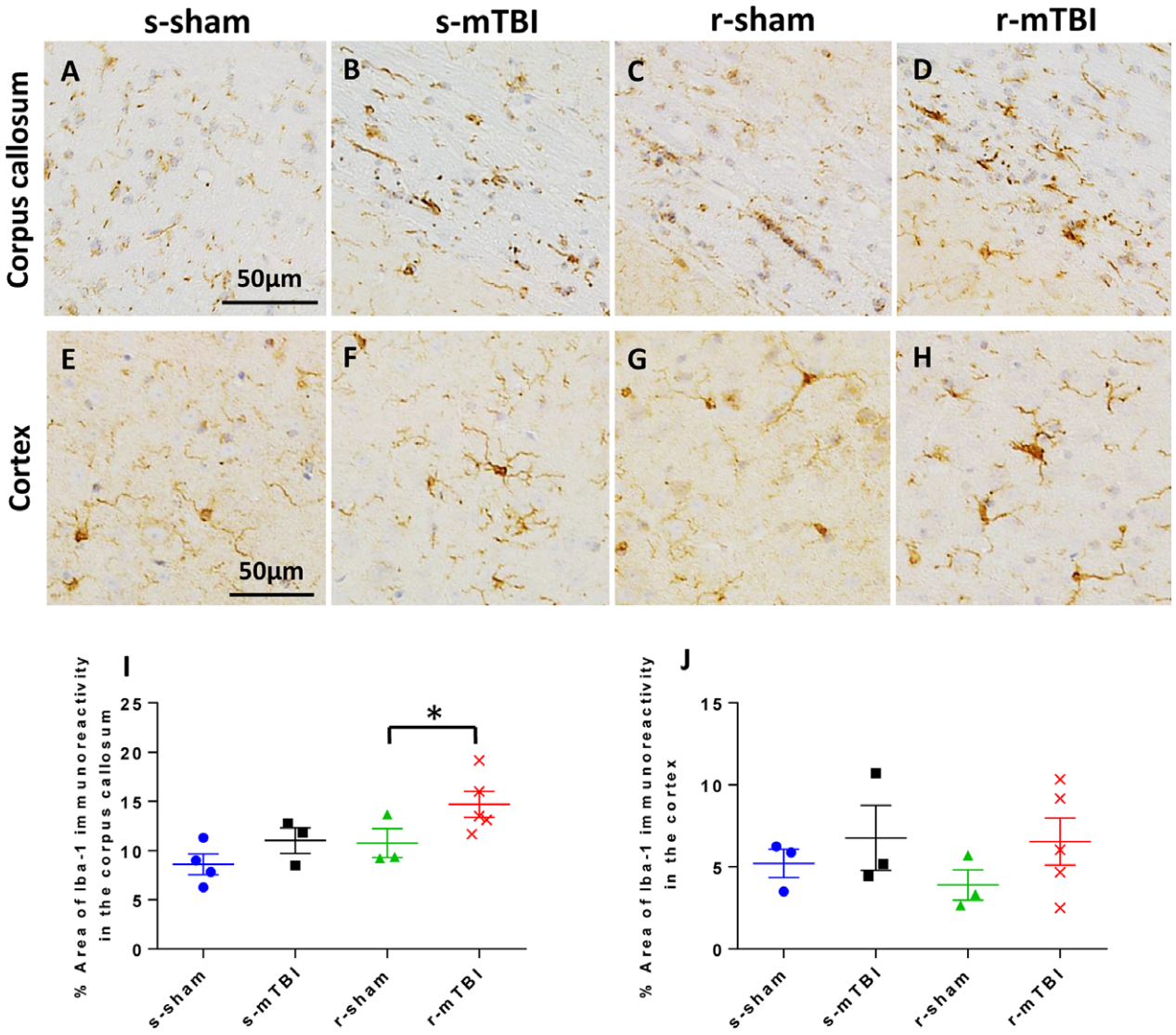

Figure 5. Repetitive mTBI induces persisting neuroinflammation in the corpus callosum at 24 months post-injury. (A-D) Sagittal sections of the mouse brain approximately 0.2 mm lateral to midline in the layer III/IV of the cortex labeled with the microglia marker Iba-1. (E–H) Sagittal sections of the mouse brain approximately 0.2 mm lateral to midline in the body of the CC with the microglia marker Iba-1. (I) There was no difference in the expression of Iba-1 within the cortex of the injured animals compared to their sham counterparts. Quantitative analysis of Iba-1 staining in four 150 μm^2 fields between layer III and *V* of the cortex. (J) Quantitative analysis of Iba-1 staining in the CC was increased in the r-mTBI compared to their sham counterpart; $*P < 0.05$. Quantitative analysis of Iba-1 staining in three 100 μm^2 fields within the body of the CC. Each symbol represents 1 mouse. Data are presented as mean ± SEM, one-way analysis of variance with Tukey's post hoc test was used for comparison of each group; $*P < 0.05$.

or in the hippocampus of any group at 24 months post-injury.

Lifelong consequence of mTBI

To evaluate the changes presented in this study in the context of lifelong exposure to mTBI, data common to both our two previous manuscripts (detailing outcomes in mice 24 h to 18 months post injury) were re-examined along with the 24 months post injury data presented herein (Fig. 7). Collectively, our datasets demonstrate that a history of single or repetitive mTBI experienced early in life alters the neurobehavioral and neuropathological phenotype of animals across the lifespan.

Regarding behavioral outcomes, we observed that single or repetitive mTBI produced irreversible impairment in performance as early as 21 days post-injury. For all groups, the overall performance was steady and improved

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

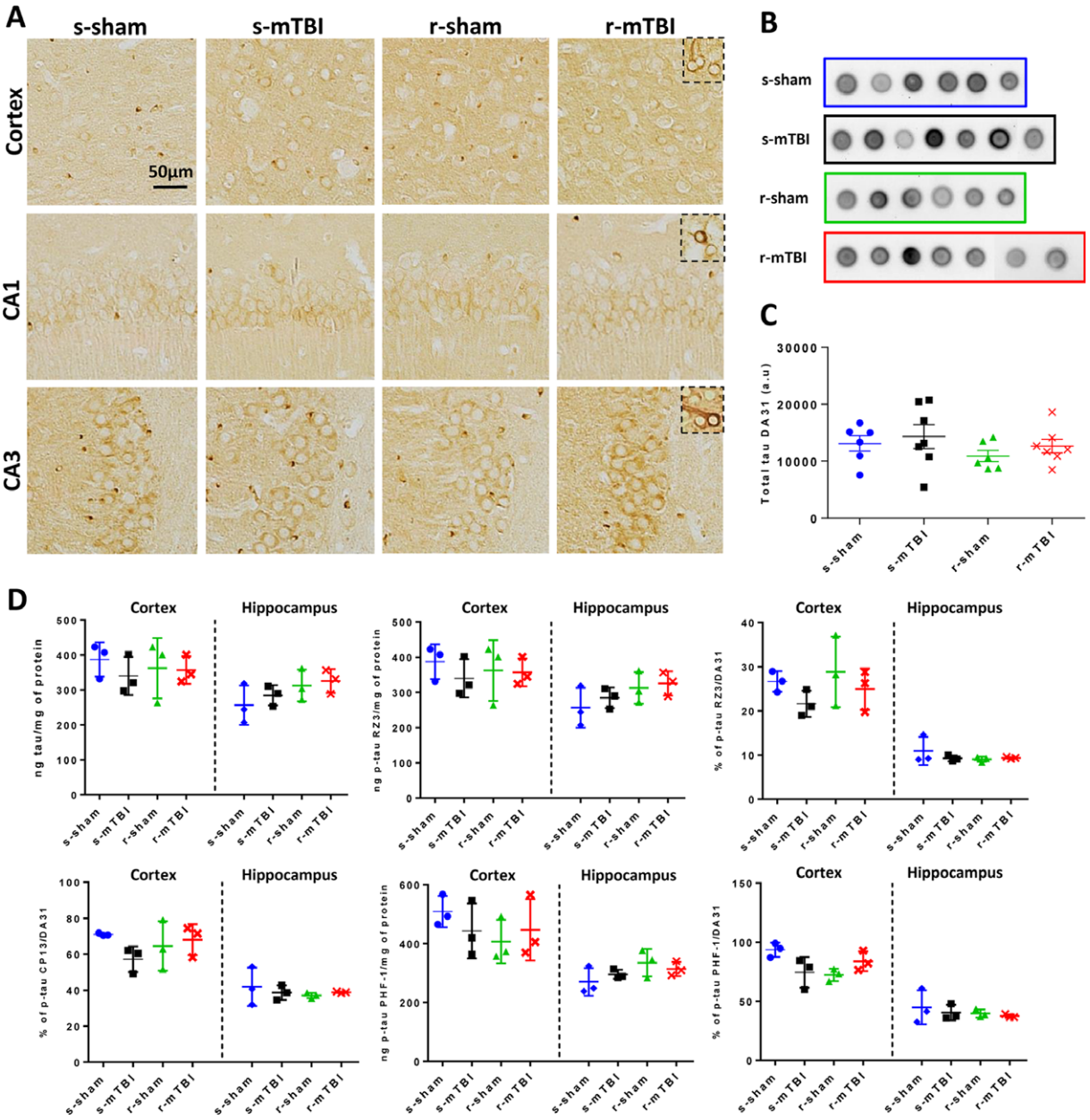

Figure 6. Biochemical and immunohistochemical assessment of p-tau pSer-202 (CP13) in the neocortex and hippocampus at 24 months after injury. (A) There was no TBI-dependent increase in cortical and hippocampal soluble CP13. All experimental groups exhibited somatodendritic localized accumulation of CP13 immunoreactivity in neurons of the superficial layer of the cerebral cortex. Top right inset represents a positive control animal (10 month-old P301S for CP13). (B) Representative dot-blots revealing the amount of DA31 (aa 150–190) in mouse plasma samples at 24 months post-injury. (C) Protein content in each sample was normalized using fast green (FG) quantification. The scatter plot represents the average DA31/FG, chemiluminescent signals quantified in mouse plasma samples. No statistical difference was observed between any of the groups ($P > 0.05$). (D) Quantitative assessment of tau immunohistochemistry revealed no change between sham and injured animals in all groups. Similar to the quantitative analysis of each p-tau epitope, there was no increase in pThr-231 (RZ3), and pSer-396/404 (PHF1) when quantitated as a ratio of p-tau protein to total tau protein. Each symbol represents 1 mouse. Data are presented as mean ± SEM, one-way ANOVA with Tukey's post hoc test was used for comparison of each group; $P > 0.05$.

for the first 15 months post-mTBI before gradually declining at the final timepoint of our behavioral assessment (24 months after last injury). The performance of these animals was directly related to the number of injuries administered. When gathering evidence from all time points examined, the s-mTBI and s-sham groups

 © 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

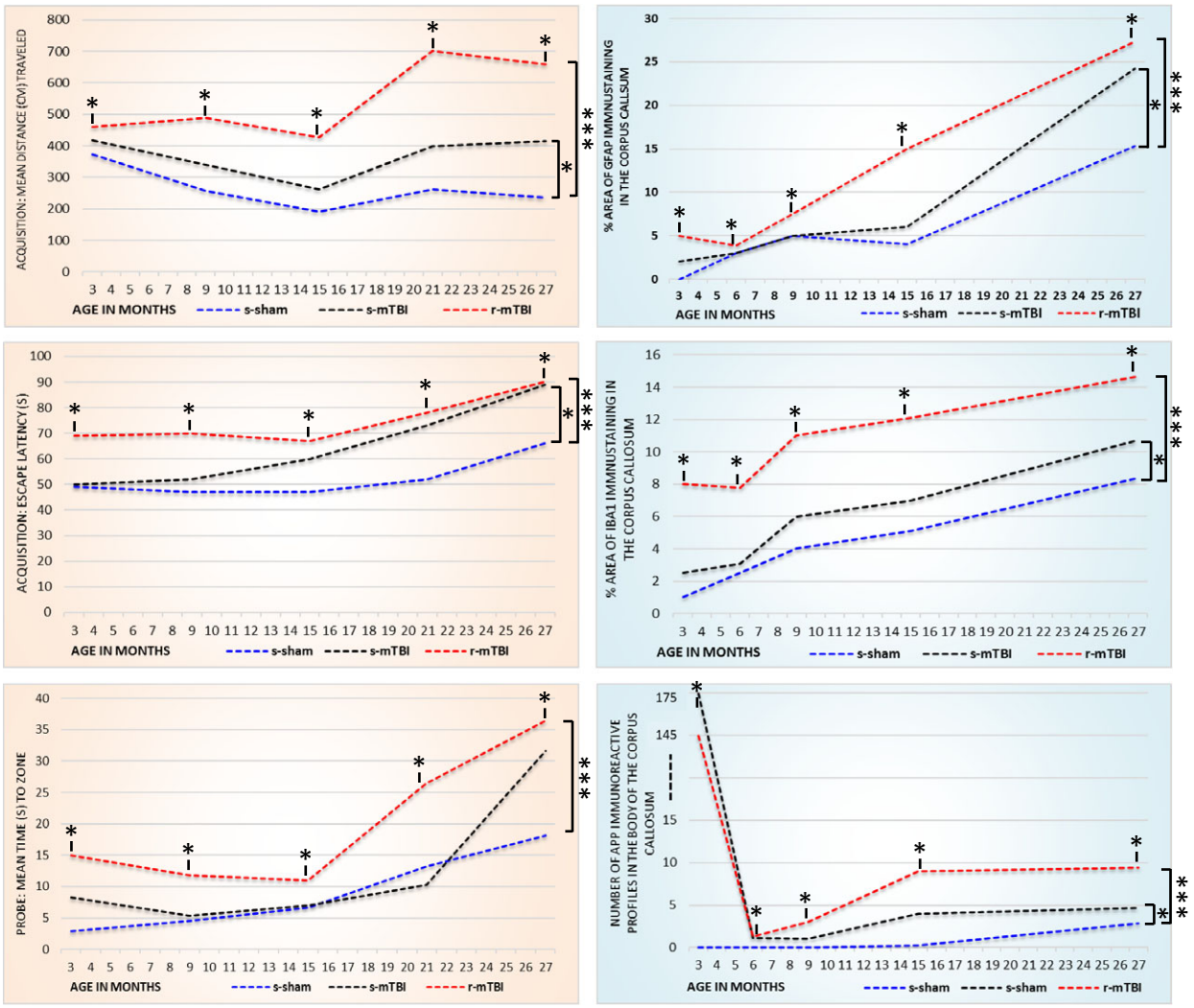

Figure 7. Association of the chronic effects of single mTBI (black line), r-mTBI (red line) and normal aging (sham animal, blue line) on neurobehavioral and neuropathological abnormalities in the body of the corpus callosum: a hypothesis based on different times the animals were sacrificed. The temporal time course of Iba-1, GFAP and APP were plotted based on the values collected at 24 h, 3, 6, 12, and 24 months post injury (stars). The neurobehavioral effects of mTBI were plotted based on data collected at 24 h, 6, 12, 18, and 24 months post injury. The course of pathology between each time point is purely speculative and may not be representative of the actual behavioral/pathology relationship. Similarly, single and r-mTBI triggered diffuse white matter pathology, with respect to axons showing immunopositivity for APP, astrocytic gliosis, and microgliosis. The phenotype of these features was influenced by the frequency of the injuries. Compared to sham-injured mice, brain-injured mice exhibited lifelong learning deficits and cellular abnormalities characterized by ongoing microgliosis and a low level of axonal damage in the corpus callosum (s-mTBI vs. s-sham, $*P < 0.05$; repeated-measures ANOVA). In contrast, animals exposed to r-mTBI displayed a slower rate of learning and exacerbated deficits in behavioral performance (r-mTBI vs. s-sham, $***P < 0.0001$; repeated-measures ANOVA). These deficits arise in parallel with a number of neuropathological abnormalities, including progressive neuroinflammation and axonal damage in the corpus callosum (r-mTBI vs. s-sham, $***P < 0.0001$; repeated-measures ANOVA). In this mouse model, neither a single nor a r-mTBI support an accelerated accumulation of pathology as the animal aged, but rather a shift in increased pathology which progressed at the same rate as the sham animals as they aged. It is interesting to speculate that additional head injuries at later time points in the life of the animal may potentially trigger a stronger pathological response as the pathology has increased by both normal aging and a history of mTBI.

performed similarly, with an exception at the acute timepoint, suggesting that all the animals recovered to their baseline within a few weeks. However, when tested at 18 months or 24 months post-injury, their performances were found to deteriorate at a faster rate than their sham counterparts, suggesting that the consequences of a single mild TBI may reappear in the context of normal aging at late timepoints.

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

Single and repetitive mTBI resulted in increased lifelong microgliosis and astrocytosis in the corpus callosum, and the extent of the neuropathological lesion was also dependent on the frequency of the injuries (s-mTBI vs. s-sham, $*P < 0.05$; r-mTBI vs. s-sham, $***P < 0.0001$; repeated-measures ANOVA). Our results suggest that mild TBI initiates a neuroinflammatory milieu in brain regions susceptible to the type of injury received; with our model being vulnerable to subcortical white matter lesions. When compared with the previously reported earlier time points, there was a noticeable age-related increase in astrogliosis and microgliosis in the CC regardless of the group assignment. Interestingly our study also suggests that while repetitive mTBI induced a more robust microglial and astroglial response in the corpus callosum but the pathology was not exacerbated in the last few months of their lifespan. However, mice who received a single injury experienced an accelerated decline between 15 and 27 months that could possibly correlate with the decrease in cognitive performance observed during the same period.

Although diffuse traumatic axonal injury has been increasingly documented in studies acquired at autopsy from patients in all severities of TBI, the question of whether white mater pathology is a lifelong condition remains to be established in humans. In our animal model of mTBI, we previously reported ongoing axonal injury that was accompanied by a thinning of the corpus callosum at 12 months post-mTBI. We now can confirm that white matter degradation and axonal degeneration is a lifelong event in this model of r-mTBI.

Table 2. Composite neurobehavioral and immunohistochemical scores at 24 months post-mTBI/sham injury with sham group taken as a baseline.

	s-sham	s-mTBI	r-sham	r-mTBI
Behavior tests				
Vestibulo-motor deficit	0	0	0	0
Anxiety	0	x	0	xx
Spatial learning	0	x	x	xx
Spatial memory	0	x	x	xx
Pathological marker in the CC				
Iba1-microgliosis	0	x	0	xx
GFAP-astrocytosis	0	x	0	xx
APP-axonal injury	0	x	0	xx
LFB-thinning of the CC	0	x	0	xx
Tau/amyloid	0	0	0	0
Total score	0	7	2	14

Mild (0), moderate (X), severe staining/impairment (XX). s-mTBI, single mild traumatic brain injury; r-mTBI, repetitive mild traumatic brain injury, s-sham, single sham; r-sham, repetitive sham; CC, Corpus Callosum; LFB, luxol fast blue.

Discussion

In this model of closed head injury, we have established the first comprehensive profile of the chronic behavioral and neuropathological consequences of single and repetitive mild traumatic brain injury over the life span of the animal. We found that both single and repetitive mTBI can have deleterious effects on the neurobehavior of a mouse, albeit at varying degrees. Animals demonstrated significant improvement following injury, however, they never returned to their control counterparts. Additionally, the incipient hallmarks of injury typified by subtle to moderate changes in spatial learning and memory, chronic neuroinflammation and ongoing axonal injury were still noticeable even at 24 months after injury (see Summary in Table 2). This study is one of the first to demonstrate that the effects of mTBI do not simply disappear following injury, but rather manifest subtly and progressively, with diminished brain function over the lifetime of the animal.

Whereas most patients recover within a few days or weeks after a concussion, a few individuals may develop long-lasting or progressively worsening symptoms because of the repeated blows. Depending on the part of the brain affected, the result can vary greatly across individuals. Repetitive mTBI is clinically associated with symptoms of irritability, impulsivity, aggression, depression, short-term memory loss, heightened suicidality, concentration difficulties, and sleep disturbance.[6] A recent series of studies have demonstrated that cognitive deficits are more common in former athletes who sustained sport concussions when compared with age-matched control patients.[26–30] This spectrum of behavioral changes falls in line with our preclinical data implicating the consequences of repetitive mTBI in the development of chronic neurological sequelae. Collectively, with the results of our previously published work,[13,19] spatial learning performance and executive function as measured by escape latency and distance travelled for both s-mTBI and r-mTBI groups was poorer than their respective controls, despite the age-related decrease in performance observed across all groups. These findings are consistent with observations in the human population in which those suffering from repetitive mTBI appear to also have cognitive deficits when compared with healthy controls.[28] Although there is no clinical study to support this observation, our data also suggest that the neurobehavior of the singly injured animals was normal for most of their lives, but the last 6 months of their lifespan saw rapidly diminishing cognitive performance. This study brings a unique insight into the dynamics of behavioral outcomes from a single mTBI in the context of the life of an animal. Nonetheless further research is required to validate our observation.

In addition to learning and executive function impairment, patients sustaining mTBI can develop behavioral disinhibition or exhibit increased risk taking behavior.[6,31]

 © 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

The mice sustaining repetitive mTBI in this model demonstrated behavioral disinhibition, as manifested in the elevated plus maze at 24 months post injury. This behavior was not observed in control mice and to a lesser extent in the s-mTBI animals, suggesting the potential deleterious, cumulative effect of repetitive mTBI. Together, these observations fall in line with emerging clinical data implicating repetitive mTBI and the development of chronic neurological sequelae.

Interestingly, in the probe trial for the Barnes Maze, which tested long-term retention of the learned target box location, the performance of all groups declined sharply when compared with the previous measurement as they reach a more advance aged with the r-mTBI performing the worst of all but without being significantly different from their respective sham. One limitation of this study is the low number of animals at the end of the study. This lack of an effect may be the result of low numbers of mice per group attributed partly to an aging effect and attrition over the 2-year period of study. Some animals were omitted because of death, frailty, or inactivity due to advanced aging. Such challenges are typically encountered when advanced aging behavioral studies are conducted. Like in the acquisition trials, while we observed differences in cognitive performance between injured and sham animals at 24 months post injury, their performance was not exacerbated by a history of a single or repetitive mTBI at 24 months post injury.

It remains controversial whether the hippocampus plays a role in recognition memory during the probe trial, with some studies showing that damage to the hippocampus has little effect,[32–34] whereas others show a correlation with impaired performance.[35–37] Because our model does not show major neuropathological abnormalities in the hippocampus apart from mild neuroinflammation, we suggest that major alterations to the brain network caused by axonal shearing in the corpus callosum (which contains nerve fibers connecting one hemisphere to the other) may contribute to disruptions in neuronal communication, impaired cognitive processing and spatial learning, thus driving the poor performance of the r-mTBI animals. The effects of TBI on the normal synchrony of neuronal networks and how it leads to cognitive impairments are likely to be complex,[38,39] and the exact nature and timing of this network dysfunction in our model remains to be elucidated. Nevertheless, our observations are consistent with the findings from clinical studies suggesting that r-mTBI associated with white matter changes has long-term implications for brain communication[40] and cognitive functioning.[41–44]

From a neuropathological perspective, the 24-month post injury data were consistent with our observations at earlier timepoints.[13,19] Mice exposed to repetitive injury displayed evidence of ongoing microgliosis localized to the CC. This was accompanied by white matter thinning together with evidence of axonal degeneration, which continued to progress from 12 to 24 months post injury. In contrast, there was no evidence of ongoing white matter pathology in mice exposed to s-mTBI except for in one animal. However, it must be pointed out that further work is required to appreciate the full extent of axonal injury as APP identifies only a population of axons. Our paradigm is the first longitudinal animal study to show a relationship between chronic neuroinflammation and white matter degeneration in the corpus callosum without focal TBI pathology (i.e., contusion, hematoma, etc). This parallels the clinical findings that ongoing axonal injury is a neuropathological feature after all severities of TBI.[45–47] With evidence now coming from clinical studies and preclinical models of mTBI, the potential for in vivo biomarkers of neuroinflammation or white matter degradation may be particularly important in providing insights into mild TBI outcome and strategies for early therapeutic intervention.

In the past decade, there have been several reports on the effects of r-mTBI on amyloid β deposition[6] and the accumulation of the tau protein in very specific and recognizable patterns following human brain trauma.[4] In our rodent mTBI model, we found no association between r-mTBI and abnormal tau or Aβ accumulation at 24 months post injury, mirroring our observations in earlier timepoints. This is common to preclinical mTBI models, as mTBI-dependent tau pathology has been difficult to model.[48] This could be due to differences in injury models,[49] injury severity,[50] injury frequency,[15] age[51] at exposure, species used (pig, rat, mice, gerbil), and genetic background (e.g., whether the animal is expressing wild type or human (or mutant) tau or amyloid). In a recent review, we discussed the current literature and the difficulties and challenges of developing in vivo TBI experimental paradigms to explore the link between repetitive head trauma with a focus on tau-dependent changes.[48] No agreement has yet been reached about the severity and threshold of multiple traumatic brain injuries required to initiate incipient tau or amyloid pathology in controlled preclinical studies. More importantly, this study demonstrates that neurodegenerative changes can occur in the absence of tau or amyloid pathology, therefore suggesting that Tau and amyloid aggregates are not the main driver for the observed neurodegenerative process in this model of mTBI. It also implies that treatments that target amyloid or Tau aggregates might not be the most effective in rodents to treat the consequences of r-mTBI.

Taken together, the findings of our studies support the hypothesis that TBI should be studied and treated, not only as an acute event, but as a chronic health condition.

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

In terms of treatment and rehabilitation, this also suggests that the opportunities for therapeutic intervention in those exposed to single or repetitive mTBI might not be limited to acute time points but could extend throughout the lifespan. The development and lifelong characterization of this model, and the relevance of the neurobehavioral and neuropathological outcomes to human TBI patients provides a platform for therapeutic discovery and exploration of treatment paradigms, including the efficacy of late-stage and chronic treatment approaches such as those recently demonstrated by Ferguson et al. 2016,[52] which may be of relevance to the human mTBI population.

Summary

In this model of closed head injury, we have established the first comprehensive profile of the chronic behavioral and neuropathological consequences of single and repetitive mild traumatic brain injury over the life span of an animal. Undoubtedly, the neurobehavioral and neuropathology of survival from mTBI is complex and multifaceted. It is crucial that TBI researchers and health organizations recognize mTBI as a chronic health condition. In addition, new public policies that affect access to rehabilitation services and treatments over the life span must be promoted to address the future management of mTBI. Overall, our current data demonstrate for the first time that mild TBI can precipitate a lifelong degenerative process.

Author Contributions

B.C.M., F.C., and C.B. contributed to conception and design; B.C.M., J.-O.O., C.M.A., and D.P. contributed to acquisition of data; B.C.M., W.S., G.C., P.D., F.C., C.B., C.M.A., J.-O.O., G.-A.G., and M.M. contributed to analysis and interpretation of data; B.C.M., F.C., C.B., and W.S. drafted and/or revised the article; B.C.M., F.C., C.B., W.S., P.D., C.M.A., J.-O.O., A.F., and M.M. approved the submitted version for publication.

Conflicts of Interest

Nothing to report.

References

1. Goldstein M. Traumatic brain injury: a silent epidemic. Ann Neurol 1990;27:327.
2. Langlois JA, Marr A, Mitchko J, Johnson RL. Tracking the silent epidemic and educating the public: CDC's traumatic brain injury-associated activities under the TBI Act of 1996 and the Children's Health Act of 2000. J Head Trauma Rehabil 2005;20:196–204.
3. Karr JE, Areshenkoff CN, Garcia-Barrera MA. The neuropsychological outcomes of concussion: a systematic review of meta-analyses on the cognitive sequelae of mild traumatic brain injury. Neuropsychology 2014;28:321–336.
4. McKee AC, Cantu RC, Nowinski CJ, et al. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol 2009;68:709–735.
5. McKee AC, Gavett BE, Stern RA, et al. TDP-43 proteinopathy and motor neuron disease in chronic traumatic encephalopathy. J Neuropathol Exp Neurol 2010;69:918–929.
6. McKee AC, Stein TD, Nowinski CJ, et al. The spectrum of disease in chronic traumatic encephalopathy. Brain 2013;136(Pt 1):43–64.
7. Roberts, G. W. (1969). Brain Damage in Boxers: A study of the prevalence of Traumatic Encephalopathy Among Ex-Professional Boxers. London: Pitman.
8. Stewart, W., et al. (2016). Chronic traumatic encephalopathy: a potential late and under recognized consequence of rugby union? QJM., 109, 11–15.
9. Lehman EJ, Hein MJ, Baron SL, Gersic CM. Neurodegenerative causes of death among retired National Football League players. Neurology 2012;79:1970–1974.
10. Smith DH, Johnson VE, Stewart W. Chronic neuropathologies of single and repetitive TBI: substrates of dementia? Nat Rev Neurol 2013;9:211–221.
11. Hay J, Johnson VE, Smith DH, Stewart W. Chronic traumatic encephalopathy: the neuropathological legacy of traumatic brain injury. Ann Rev Pathol 2016;23:21–45.
12. McKee AC, Stein TD, Kiernan PT, Alvarez VE. The neuropathology of chronic traumatic encephalopathy. Brain Pathol 2015;25:350–364.
13. Mouzon BC, Bachmeier C, Ferro A, et al. Chronic neuropathological and neurobehavioral changes in a repetitive mild traumatic brain injury model. Ann Neurol 2014;75:241–254.
14. Winston CN, Noel A, Neustadtl A, et al. Dendritic spine loss and chronic white matter inflammation in a mouse model of highly repetitive head trauma. Am J Pathol 2016;186:552–567.
15. Petraglia AL, Plog BA, Dayawansa S, et al. The pathophysiology underlying repetitive mild traumatic brain injury in a novel mouse model of chronic traumatic encephalopathy. Surg Neurol Int 2014;5:184.
16. Lynch CE, Crynen G, Ferguson S, et al. Chronic cerebrovascular abnormalities in a mouse model of repetitive mild traumatic brain injury. Brain Inj 2016;30:1414–1427.
17. Ojo JO, Mouzon B, Algamal M, et al. Chronic repetitive mild traumatic brain injury results in reduced cerebral blood flow, axonal injury, gliosis, and increased t-tau and tau oligomers. J Neuropathol Exp Neurol 2016;75:636–655.

 © 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

18. Yoshiyama Y, Uryu K, Higuchi M, et al. Enhanced neurofibrillary tangle formation, cerebral atrophy, and cognitive deficits induced by repetitive mild brain injury in a transgenic tauopathy mouse model. J Neurotrauma 2005;22:1134–1141.
19. Mouzon B, Chaytow H, Crynen G, et al. Repetitive mild traumatic brain injury in a mouse model produces learning and memory deficits accompanied by histological changes. J Neurotrauma 2012;29:2761–2773.
20. Dewitt DS, Perez-Polo R, Hulsebosch CE, et al. Challenges in the development of rodent models of mild traumatic brain injury. J Neurotrauma 2013;30:688–701.
21. Longhi L, Saatman KE, Fujimoto S, et al. Temporal window of vulnerability to repetitive experimental concussive brain injury. Neurosurgery 2005;56:364–374. discussion -74.
22. Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol 2001;23:291–299.
23. Acker CM, Forest SK, Zinkowski R, et al. Sensitive quantitative assays for tau and phospho-tau in transgenic mouse models. Neurobiol Aging 2013;34:338–350.
24. Petraglia AL, Plog BA, Dayawansa S, et al. The spectrum of neurobehavioral sequelae after repetitive mild traumatic brain injury: a novel mouse model of chronic traumatic encephalopathy. J Neurotrauma 2014;31:1211–1224.
25. Paris D, Ait-Ghezala G, Bachmeier C, et al. The spleen tyrosine kinase (Syk) regulates Alzheimer amyloid-beta production and Tau hyperphosphorylation. J Biol Chem 2014;289:33927–33944.
26. Amen DG, Newberg A, Thatcher R, et al. Impact of playing American professional football on long-term brain function. J Neuropsychiatry Clin Neurosci 2011;23:98–106.
27. De Beaumont L, Theoret H, Mongeon D, et al. Brain function decline in healthy retired athletes who sustained their last sports concussion in early adulthood. Brain 2009;132(Pt 3):695–708.
28. Hart J Jr, Kraut MA, Womack KB, et al. Neuroimaging of cognitive dysfunction and depression in aging retired National Football League players: a cross-sectional study. JAMA Neurol 2013;70:326–335.
29. Koerte IK, Mayinger M, Muehlmann M, et al. Cortical thinning in former professional soccer players. Brain Imaging Behav 2015;10:792–798.
30. Ling H, Morris HR, Neal JW, et al. Mixed pathologies including chronic traumatic encephalopathy account for dementia in retired association football (soccer) players. Acta Neuropathol 2017;133:337–352.
31. Omalu B, Bailes J, Hamilton RL, et al. Emerging histomorphologic phenotypes of chronic traumatic encephalopathy in American athletes. Neurosurgery 2011;69:173–183; discussion 83.
32. Forwood, S. E., Winters, B. D., & Bussey, T. J. (2005). Hippocampal lesions that abolish spatial maze performance spare object recognition memory at delays of up to 48 hours. Hippocampus., 15, 347–355.
33. Good, M. A., et al. (2007). Context- but not familiarity-dependent forms of object recognition are impaired following excitotoxic hippocampal lesions in rats. Behav Neurosci., 121, 218–223.
34. Langston, R. F., & Wood, E. R. (2010). Associative recognition and the hippocampus: differential effects of hippocampal lesions on object-place, object-context and object-place-context memory. Hippocampus., 20, 1139–1153.
35. Clark, R. E., Zola, S. M., & Squire, L. R. (2000). Impaired recognition memory in rats after damage to the hippocampus. J Neurosci., 20, 8853–8860.
36. Barker GR, Warburton EC. When is the hippocampus involved in recognition memory? J Neurosci 2011;31:10721–10731.
37. Sharp, D. J., Scott, G., & Leech, R. (2014). Network dysfunction after traumatic brain injury. Nat Rev Neurol., 10, 156–166.
38. Wolf, J. A., & Koch, P. F. (2016). Disruption of Network Synchrony and Cognitive Dysfunction After Traumatic Brain Injury. Front Syst Neurosci., 10, 43.
39. Blumbergs PC, Scott G, Manavis J, et al. Staining of amyloid precursor protein to study axonal damage in mild head injury. Lancet 1994;344:1055–1056.
40. Capruso, D. X., & Levin, H. S. (1992). Cognitive impairment following closed head injury. Neurol Clin., 10, 879–893.
41. Monti, J. M., et al. (2013). History of mild traumatic brain injury is associated with deficits in relational memory, reduced hippocampal volume, and less neural activity later in life. Front Aging Neurosci., 5, 41.
42. Ryan, L. M., & Warden, D. L. (2003). Post concussion syndrome. Int Rev Psychiatry., 15, 310–316.
43. Strain JF, Didehbani N, Spence J, et al. White matter changes and confrontation naming in retired aging national football league athletes. J Neurotrauma 2016;34:372–379.
44. Johnson VE, Stewart W, Weber MT, et al. SNTF immunostaining reveals previously undetected axonal pathology in traumatic brain injury. Acta Neuropathol 2016;131:115–135.
45. Siman R, Giovannone N, Hanten G, et al. Evidence that the blood biomarker SNTF predicts brain imaging changes and persistent cognitive dysfunction in Mild TBI patients. Front Neurol 2013;4:190.
46. Siman R, Shahim P, Tegner Y, et al. Serum SNTF increases in concussed professional ice hockey players and relates to the severity of postconcussion symptoms. J Neurotrauma 2015;32:1294–1300.
47. Ojo JO, Mouzon BC, Crawford F. Repetitive head trauma, chronic traumatic encephalopathy and tau: challenges in translating from mice to men. Exp Neurol 2016;275(Pt 3):389–404.

© 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.

48. Goldstein LE, McKee AC, Stanton PK. Considerations for animal models of blast-related traumatic brain injury and chronic traumatic encephalopathy. Alzheimer's Res Ther 2014;6:64.
49. Tran HT, LaFerla FM, Holtzman DM, Brody DL. Controlled cortical impact traumatic brain injury in 3xTg-AD mice causes acute intra-axonal amyloid-beta accumulation and independently accelerates the development of tau abnormalities. J Neurosci 2011;31:9513–9525.
50. Ojo JO, Mouzon B, Greenberg MB, et al. Repetitive mild traumatic brain injury augments tau pathology and glial activation in aged hTau mice. J Neuropathol Exp Neurol 2013;72:137–151.
51. Ferguson S, Mouzon BC, Paris D, et al. Acute or delayed treatment with anatabine improves spatial memory and reduces pathological sequelae at chronic timepoints after repetitive mild TBI. J Neurotrauma 2017;37:1676–1691.

 © 2017 The Authors. *Annals of Clinical and Translational Neurology* published by Wiley Periodicals, Inc on behalf of American Neurological Association.