The pathophysiology underlying repetitive mild traumatic brain injury in a novel mouse model of chronic traumatic encephalopathy

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Abstract

Background: An animal model of chronic traumatic encephalopathy (CTE) is essential for further understanding the pathophysiological link between repetitive head injury and the development of chronic neurodegenerative disease. We previously described a model of repetitive mild traumatic brain injury (mTBI) in mice that encapsulates the neurobehavioral spectrum characteristic of patients with CTE. We aimed to study the pathophysiological mechanisms underlying this animal model.

Methods: Our previously described model allows for controlled, closed head impacts to unanesthetized mice. Briefly, 12-week-old mice were divided into three groups: Control, single, and repetitive mTBI. Repetitive mTBI mice received six concussive impacts daily, for 7 days. Mice were then subsequently sacrificed for macro- and micro-histopathologic analysis at 7 days, 1 month, and 6 months after the last TBI received. Brain sections were immunostained for glial fibrillary acidic protein (GFAP) for astrocytes, CD68 for activated microglia, and AT8 for phosphorylated tau protein.

Results: Brains from single and repetitive mTBI mice lacked macroscopic tissue damage at all time-points. Single mTBI resulted in an acute reactive astrocytosis at 7 days and increased phospho-tau immunoreactivity that was present acutely and at 1 month, but was not persistent at 6 months. Repetitive mTBI resulted in a more marked neuroinflammatory response, with persistent and widespread astrogliosis and microglial activation, as well as significantly elevated phospho-tau immunoreactivity to 6-months.
Conclusions: The neuropathological findings in this new model of repetitive mTBI resemble some of the histopathological hallmarks of CTE, including increased astrogliosis, microglial activation, and hyperphosphorylated tau protein accumulation.

Key Words: Animal model, chronic traumatic encephalopathy, concussion, pathophysiology, repetitive

INTRODUCTION
Concussive head injuries have become a silent epidemic of increasing importance, with millions of sports- and recreation-related concussions occurring each year.[26] Recent evidence garnered from biophysics studies, advanced neuroimaging findings, forensic analyses, and laboratory data has highlighted the ubiquity of concussion and subconcussion in contact sports and explosive blasts, as well as their potential to contribute to the development of sub-acute and chronic post-traumatic sequelae.[32,41,54,55] Chronic traumatic encephalopathy (CTE) has become a popular topic due to its close association with a wide spectrum of sporting activities, including American football, hockey, soccer, boxing, and professional wrestling, as well as with military personnel.[32,38-41,55]

Most of our knowledge regarding this disease has come from post-mortem analyses and retrospective, demographic data. The precise incidence and prevalence of CTE is unknown and difficult to glean from sporadic case series. The risk factors for the development of CTE are not clear; however, the phenomenon seems to be caused by episodic and repetitive blunt force impacts to the head and transfer of acceleration–deceleration forces to the brain. Clinically, the disease presents as a composite syndrome of mood disorders, neuropsychiatric disturbance, and cognitive impairment.[12,38,41,54,55] Behavioral abnormalities such as irritability, judgment issues, increased risk-taking, and depression are characteristic and prominent early in the disease course. Additional symptoms may include difficulty sleeping, poor concentration, or memory impairment.[12,38-40] Definitive and confirmatory diagnosis of CTE is still based on direct tissue histochemical and immunohistochemical analyses, which reveal topographically multifocal or diffuse cortical and subcortical hyperphosphorylated tauopathy, which is accompanied by isomorphic fibrillary astrogliosis, and microglial activation.

The underlying pathophysiological mechanisms in CTE have yet to be clearly elucidated. Some authors have cited a lack of prospective clinical evidence in associating repetitive mild traumatic brain injury (mTBI) with CTE.[11] Others have stated that the symptoms of CTE could be due to confounding factors such as co-morbid disease, medications, or normal aging. Generally, it takes years before the onset of symptoms of neurodegenerative disorders after an individual has experienced a TBI, requiring significant lengths of time to gather this type of epidemiological data from human populations.[39] Ascertainment bias is a major limitation in establishing causation, and not just association, between mild repetitive TBI and CTE. Thus, an experimental animal model could help to decipher the mechanisms by which CTE, as well as other neurological sequelae such as post-concussion syndrome (PCS), post-traumatic stress disorder (PTSD), mild cognitive impairment (MCI), or dementia pugilistica (DP) may be triggered by repetitive brain injury. To address this, we developed a novel model of closed head injury in unanesthetized mice that, as we have previously reported, results in some of the neurobehavioral spectrum observed in CTE patients, including cognitive deficits, increased risk-taking, depression-like behavior, and sleep disturbances. We hypothesized that repetitive mTBI in this model would result in the histopathological hallmarks of CTE, including increased astrogliosis, microglial activation, and phosphorylated tau immunoreactivity.

MATERIALS AND METHODS

Animal care and maintenance
All animals used in this study were treated in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all procedures were performed under approval of the Institutional Animal Care and Use Committee at the University of Rochester. Adult male, C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were purchased and housed with five mice per cage under standard laboratory conditions (automatically controlled temperature, humidity, ventilation, and 12 h light/dark cycle) with unlimited access to food and water throughout the study. Mice were allowed to adapt to the vivarium for at least 1 week prior to the experimental procedures. Following the injury, animals were returned to their home cages.

Mouse controlled, closed head, acceleration–deceleration model of injury
At 12 weeks of age, mice undergoing injury were subjected to mTBI, as described previously.[41] Briefly, mice were placed head first into a small, clear plastic rodent restraint bag/cone (Thermo Fisher Scientific, Waltham, MA). Slits were made at the narrow end of the cone to allow for...
increased head mobility and ventilation space. A twist tie was placed behind the mouse to immobilize the animal within the bag and the ends of the twist ties were positioned to stabilize the body on the foam bed. In this manner, the animal was comfortably restrained, obviating the need for anesthesia. A helmet, designed from 304 stainless steel (Millennium Machinery, Rochester, NY) and measuring 3 mm thickness × 6 mm diameter, was secured to the head with an elastic band. This ultimately allows for accurate delivery of injury and diffuse spread of the force of the impacts. The under-surface of the helmet was lined with 1.0-mm double-sided gel tape (Scotch® Restickable Tabs, 3M, St. Paul, MN). The helmet was engineered to fit the curvature of the mouse skull and cover the left hemisphere between lambda and bregma, up to, but not crossing the midline. Placing the anterior most part of the helmet 1-2 mm behind the left eye places the epicenter of the helmet over the left parieto-temporal cortex. The mice were then positioned on Type E foam padding (Foam to Size, Inc., Ashland, VA) and positioned below the injury device. The mouse was placed on a foam base to allow for the acceleration-deceleration component of injury key to human concussive injury. The foam was changed regularly after each cycle of hits to ensure a similar spring constant for all impacts. A controlled cortical impact (CCI) device (Pittsburgh Precision Instruments, Pittsburgh, PA) was used as previously described to deliver the head impacts. The impounder was rigidly mounted at a 20° angle from the vertical plane. The impactor had a 6-mm-diameter tip and was constructed of rubber (Millennium Machinery). To obtain a zero point, the impactor tip was carefully lowered until it touched the helmet surface. The impactor was angled and zeroed, so that the impacts occurred perpendicular to the helmet surface and were thus orthogonal to the skull of the mouse. During impact, the tip was driven pneumatically to a depth of 1 cm farther than the zero point. The duration of impact was 100 ms and was delivered at a velocity of 5 m/s. Following the impact, the animals were removed from the restraint bag and returned to their cage. Animals in the single mTBI group received a single head impact, whereas mice in the repetitive mTBI group received six head impacts per day (each hit separated by 2 h) for 7 days straight, i.e. for a total of 42 head impacts. There were no post-traumatic apneic episodes or seizure activity observed, and no mortalities secondary to any of the impacts. Sham-injured/control animals underwent the identical procedure as the trauma groups; however, no injury was delivered. All control mice were age-matched to account for any age-related differences in pathology. Mice were then assessed at 7 days, 1 month, and 6 months from the last impact, in a blinded fashion as described below.

### Immunohistochemistry: Sample preparation

Age-matched controls and mice at 7 days, 1 month, or 6 months following single or repetitive mTBI underwent transcardial perfusion with ice-cold heparinized 0.01 M phosphate-buffered saline (PBS) (pH 7.4, Sigma-Aldrich), followed by fixation with 4% para-formaldehyde (PFA) (Sigma-Aldrich) in PBS. Cerebral tissue from all animals was dissected from the calvarium and post-fixed in 4% PFA for 24 h. Following fixation, the cerebral tissue underwent graduated dehydration first in 15% and then in 30% sucrose (Sigma-Aldrich, St. Louis, MO, USA) for 24 h each. Dehydrated tissue was placed in optimal cutting temperature (OCT) compound (Tissue-Tek) and was sliced on a calibrated cryostat (Leica CM1900) into 30 µm coronal sections. Tissue sections were then floated in PBS. Four mice were included in each of the above groups. For each mouse, four representative coronal sections were selected for staining by collecting a single section every 900 µm along a rostral-caudal axis beginning 1.1 mm anterior to and ending 2.5 mm posterior to bregma.

### Imaging protocol

Antibodies were detected at the appropriate wavelength at a magnification of 40× on confocal microscopy (Olympus IX 81, Fluoview v. 4.3), using a standard laser power, image photomultiplier tube (PMT), and
gain. In each of the four coronal sections collected from each brain, one image each at a uniform depth of one high-powered field (1 high-powered field = 1024 pixels = 317 µm) from the surface of the dorsal, dorsolateral, and ventral cortex, as well as one CA1, CA3, dentate gyrus, and amygdala image were obtained in each cerebral hemisphere. The dorsal, dorsolateral, and ventral cortical images were each acquired at a distance of one (317 µm), four (1268 µm), and seven (2219 µm) high-powered fields lateral of the midline, respectively. All images were acquired at a resolution of 1024 × 1024 pixels. All image acquisitions were performed blinded to the experimental group.

Image quantification
All image analyses were performed blinded to the experimental group. ImageJ software (http://rsbweb.nih.gov/ij/) was used to apply a standard threshold to the entire 1024 × 1024 pixel field of all 8-bit immunohistochemical images (GFAP, 75; pTau, 100; CD68, 100). The pixel

Supplementary Figure 1: Representative negative controls. Secondary antibodies alone served as negative controls and revealed no evidence of non-specific staining in control, single mTBI, and repetitive mTBI mice for (a–c) GFAP, (d–f) CD68, and (g–i) phosphorylated tau (AT8) immunostaining.
area encompassed by this thresholding scheme was then quantified and normalized relative to control pixel areas, as described previously.[32,58]

**Statistical analysis**

All statistical analyses were performed using SAS statistical software version 9.3. All tests were two-sided and conducted at 5% significance level. Continuous variables were summarized using sample means. All data are presented as means ± standard error of the mean (SEM). Ipsilateral and contralateral sides were compared to the corresponding sides between groups [i.e. repetitive ipsilateral vs. single ipsilateral vs. control ipsilateral (left side)]. Normalized GFAP, pTau, and CD68 immunoreactive areas were evaluated with thresholded pixel areas analyzed using one-way analysis of variance (ANOVA) including injury group (control, single hit, and repeated hits) as the factor. Post-hoc analyses based on Tukey’s method to adjust for multiple comparisons were conducted to compare pairs of injury groups.

**RESULTS**

Gross examination of the brains in the single and repetitive mTBI groups did not reveal any evidence of brain atrophy, tissue loss, hemorrhage (subdural, epidural, subarachnoid), or contusion 24 h from the last impact, as well as at 7 days, 1 month, and 6 months from the injury [Figure 1].

**Repetitive mTBI results in a persistent and progressive neuroinflammatory response**

Astrogliosis and increased microglial activation are characteristic of the brains in patients with CTE.[32,38-40,55] We thus sought to characterize the development of reactive astrogliosis [Figure 2] and microglial activation [Figure 3] in mice sustaining single and repetitive mTBI in this model at 7 days, 1 month, and 6 months post-injury, both ipsilateral and contralateral to the impact site.

At the 7 day time-point, we found that the effect of injury group on GFAP labeling in the dentate gyrus (ipsilateral: \( F_{2,9} = 26.8 \) and contralateral: \( F_{2,9} = 93; \) ANOVA), CA1 (ipsilateral: \( F_{2,9} = 15.8 \) and contralateral: \( F_{2,9} = 9.3; \) ANOVA), and CA3 (ipsilateral: \( F_{2,9} = 7 \) and contralateral: \( F_{2,9} = 25.1; \) ANOVA) fields of the hippocampus. On post-hoc analyses, single mTBI mice exhibited a statistically significant decrease in astrogliosis in the bilateral dentate gyrus, ipsilateral CA1, and contralateral CA3 fields, compared to age-matched control mice (ANOVA + Tukey) [Figure 2r–t]. At the 1-month time-point, repetitive mTBI mice also demonstrated significantly decreased astrogliosis in the bilateral dentate gyrus, ipsilateral CA1, and bilateral CA3 fields, compared to control mice (ANOVA + Tukey) [Figure 2r–t]. Also, repetitive mTBI mice had significantly less GFAP labeling in the contralateral dentate gyrus compared to the contralateral dentate gyrus in single TBI mice (ANOVA + Tukey).

At the 6-month time-point, the effect of injury on astrogliosis was found to be significant in the cortex (ipsilateral: \( P < 0.01, \) \( F_{1.6} = 6.11 \) and contralateral: \( P < 0.01, \) \( F_{1.6} = 3.21; \) ANOVA) and amygdala (ipsilateral: \( P < 0.001, \) \( F_{1.6} = 23.2 \) and contralateral: \( P < 0.001, \) \( F_{1.6} = 19.4; \) ANOVA). Post-hoc analyses revealed that compared to control mice, single mTBI mice exhibited a significant increase in astrogliosis at the 7-day time-point in the contralateral cortex and bilateral amygdala (ANOVA + Tukey) [Figure 2p and q]. At the 7-day time-point, repetitive mTBI mice demonstrated significantly increased astrogliosis in the bilateral cortices and bilateral amygdalae, compared to control mice (ANOVA + Tukey) [Figure 2p and q].

By 1 month post-injury, the reactive astrocytosis subsided. We did find that there was a significant effect of injury on GFAP labeling in the dentate gyrus (ipsilateral: \( P < 0.001, \) \( F_{2,9} = 26.8 \) and contralateral: \( P < 0.001, \) \( F_{2,9} = 93; \) ANOVA), CA1 (ipsilateral: \( P < 0.01, \) \( F_{2,9} = 15.8 \) and contralateral: \( P < 0.01, \) \( F_{2,9} = 9.3; \) ANOVA), and CA3 (ipsilateral: \( P < 0.05, \) \( F_{2,9} = 7 \) and contralateral: \( P < 0.001, \) \( F_{2,9} = 25.1; \) ANOVA) fields of the hippocampus. On post-hoc analyses, the single mTBI mice exhibited a statistically significant increase in GFAP labeling in the contralateral dentate gyrus, contralateral CA1, and bilateral CA3 fields of the hippocampus, compared to age-matched control mice (ANOVA + Tukey) [Figure 2r–t]. Six months post-injury, repetitive mTBI mice demonstrated significantly increased astrogliosis
in the bilateral cortices, ipsilateral amygdala, bilateral dentate gyri, as well as the bilateral CA1 and bilateral CA3 fields, compared to control mice (ANOVA + Tukey) [Figure 2r–t]. Also, repetitive mTBI mice had significantly more GFAP labeling in the ipsilateral amygdala and the contralateral CA1 regions compared to the single TBI mice (ANOVA + Tukey).

Mice receiving a single mTBI did not exhibit a significant elevation in activated microglia at any of the time-points. On the contrary, the mice in the repetitive mTBI

![Figure 2: Mild TBI results in a dynamic astrocytic response. Representative GFAP immunostaining for astrogliosis in the cortex, amygdala, dentate gyrus, CA1, and CA3 fields of (a–e) age-matched uninjured control, (f–j) 6-month single mild TBI, and (k–o) 6-month repetitive mild TBI mice. (p–t) Single and repetitive mild TBI mice exhibited an acute elevation in GFAP staining in the cortex and amygdala at 7 days which resolved to control levels by 1 month (Tukey). Interestingly, GFAP was significantly decreased in the hippocampus of mild TBI mice compared to controls at 1 month (Tukey). Repetitive mild TBI resulted in a second significant, diffuse astrocytic response in all regions examined at the 6-month time-point, compared to control mice (Tukey) (*P < 0.05 vs. corresponding side in control mice, #P < 0.05 vs. corresponding side in single TBI mice). Values are mean ± SEM.](image-url)
group had microglial activation that increased over time [Figure 3p–t]. At 7 days post-injury, there was a significant effect of injury on microgliosis in the cortex (ipsilateral: $P < 0.01$, $F(2,9) = 15.05$ and contralateral: $P < 0.01$, $F(2,9) = 15.7$; ANOVA), contralateral amygdala ($P < 0.05$, $F(2,9) = 6.1$; ANOVA), and CA3 (ipsilateral: $P < 0.01$, $F(2,9) = 8.2$ and contralateral: $P < 0.05$, $F(2,9) = 5.6$; ANOVA) regions. Post-hoc analyses revealed that repetitive mTBI mice exhibited a significant increase in microgliosis in the bilateral cortices, contralateral amygdala, and bilateral CA3 regions, compared to both single mild TBI and control mice (ANOVA + Tukey) [Figure 3p, q, t].

| Control | Single | Repeat |
|---------|--------|--------|
| Cortex  |        |        |
| Amygdala|        |        |
| Dentate Gyrus | | |
| CA1     |        |        |
| CA3     |        |        |

Figure 3: Repetitive mild TBI results in a persistent diffuse increase in activated microglia. Representative CD68 immunostaining for activated microglia in the cortex, amygdala, dentate gyrus, CA1, and CA3 fields of (a–e) age-matched uninjured control, (f–j) 6-month single mTBI, and (k–o) 6-month repetitive mTBI mice. (p–t) While mice receiving a single mTBI did not exhibit a significant elevation in activated microglia at any of the time-points, mice in the repetitive mTBI group had significantly increased microgliosis compared to single mTBI and control mice at 7 days, as well as at the 1- and 6-month time-points (Tukey) (*$P < 0.05$ vs. corresponding side in control mice, #P < 0.05 vs. corresponding side in single TBI mice). Values are mean ± SEM.
At 1 month, the microgliosis persisted and there was a significant effect of injury in the ipsilateral cortex (P < 0.001, F (2,9) =17.6; ANOVA), ipsilateral amygdala (P < 0.001, F (2,9) =25.9; ANOVA), and dentate gyrus (ipsilateral: P < 0.05, F (2,9) =7.1 and contralateral: P < 0.05, F (2,9) =7.6; ANOVA). Post-hoc analyses revealed that at 1 month post-injury, repetitive mTBI mice exhibited a significant increase in microglial activation in the ipsilateral cortex and amygdala, as well as in the bilateral dentate gyri, compared to both single mTBI and control mice (ANOVA + Tukey) [Figure 3p–t].

At the 6-month time-point, the effect of injury on microgliosis was found to be significant in the cortex (ipsilateral: P < 0.001, F (2,9) = 29.2 and contralateral: P <0.001, F (2,9) = 30.6; ANOVA), amygdala (ipsilateral: P < 0.001, F (2,9) = 47 and contralateral: P < 0.01, F (2,9) = 16.1; ANOVA), dentate gyrus (ipsilateral: P < 0.01, F (2,9) = 9.7 and contralateral: P < 0.01, F (2,9) = 9.02; ANOVA), CA1 (ipsilateral: P < 0.01, F (2,9) =57.2 and contralateral: P < 0.001, F (2,9) =44.3; ANOVA), and CA3 (ipsilateral: F (2,9) = 55.6 and contralateral: P < 0.001, F (2,9) =9.98; ANOVA) regions. On subsequent post-hoc analysis at 6 months post-injury, repetitive mTBI mice had a significant increase in microglial activation in all the regions evaluated, compared to both single mTBI and control mice (ANOVA + Tukey) [Figure 3p–t].

**Repetitive mTBI results in a persistent increase in hyperphosphorylated tau**

Phosphorylated tau is a histopathological hallmark of CTE. We explored whether repetitive mTBI would lead to increased phosphorylated tau immunoreactivity in this model at 7 days, 1 month, and 6 months following the last head impact. At 7 days post-injury, the effect of injury on phospho-tau immunoreactivity was found to be significant in the cortex (ipsilateral: P < 0.001, F (2,9) =16.5 and contralateral: P <0.001, F (2,9) =17.7; ANOVA), amygdala (ipsilateral: P < 0.01, F (2,9) =15.7 and contralateral: P < 0.05, F (2,9) = 6.1; ANOVA), contralateral dentate gyrus (P < 0.05, F (2,9) =5.5; ANOVA), ipsilateral CA1 (P < 0.01, F (2,9) =9.47; ANOVA), and ipsilateral CA3 fields (P < 0.001, F (2,9) =19.5; ANOVA). Post-hoc analyses revealed that in single mTBI mice, there was a significant increase in phosphorylated tau staining in the contralateral cortex, bilateral amygdala, ipsilateral CA1, and ipsilateral CA3 fields, compared to controls (ANOVA + Tukey) [Figure 4p, q, s, t]. Phosphorylated tau immunoreactivity was significantly increased in the repetitive mTBI mice in the bilateral cortices, ipsilateral amygdala, and the contralateral dentate gyrus, as well as in the ipsilateral CA1 and CA3 fields, compared to control mice (ANOVA + Tukey) [Figure 4p–t].

At 1 month post-injury, the effect of injury on phospho-tau immunoreactivity was found to be significant in the cortex (ipsilateral: P < 0.01, F (2,9) =12.75 and contralateral: P < 0.001, F (2,9) =16.4; ANOVA), amygdala (ipsilateral: P < 0.001, F (2,9) =21.6 and contralateral: P < 0.01, F (2,9) =10.15; ANOVA), dentate gyrus (ipsilateral: P <0.01, F (2,9) =8.97 and contralateral: P < 0.001, F (2,9) =23.3; ANOVA), CA1 (ipsilateral: P < 0.01, F (2,9) =8.9 and contralateral: P < 0.001, F (2,9) =30.1; ANOVA), and CA3 (ipsilateral: P < 0.01, F (2,9) =10.5 and contralateral: P < 0.01, F (2,9) =11.2; ANOVA) regions. Post-hoc analyses revealed significantly increased phosphorylated tau staining in both the repetitive and single mTBI groups in all the regions examined, compared to age-matched controls (ANOVA + Tukey) [Figure 4p–t].

At the 6-month time-point [Figure 4a–o], we found a significant effect of injury on phosphorylated tau immunoreactivity in the cortex (ipsilateral: P <0.001, F (2,9) =83.1 and contralateral: P < 0.001, F (2,9) =60.6; ANOVA), amygdala (ipsilateral: P < 0.001, F (2,9) =36 and contralateral: P < 0.001, F (2,9) =90.5; ANOVA), and dentate gyrus (ipsilateral: P < 0.01, F (2,9) =8.5; ANOVA), and contralateral: P < 0.001, F (2,9) =26.9; ANOVA). On post-hoc analyses, no significant elevation in phosphorylated tau staining was appreciated in the single mTBI mice compared to age-matched controls (ANOVA + Tukey) [Figure 4p–t]. The repetitive mTBI group, however, did demonstrate a significant persistent increase in phosphorylated tau immunoreactivity at 6 months post-injury in the bilateral cortices, bilateral amygdalae, and the bilateral dentate gyri, compared to controls (ANOVA + Tukey) [Figure 4p–r]. This persistent phosho-tau immunoreactivity was also significantly elevated in the bilateral cortices, bilateral amygdalae, and contralateral dentate gyrus, compared to single mTBI mice (ANOVA + Tukey) [Figure 4p–r].

**DISCUSSION**

In the present study, we have developed and characterized a novel model of closed head injury to investigate the spectrum of behavioral and neuropathological sequelae following repetitive mTBI. This model is simple, practical, and has been developed with several features similar to our experience in the clinical setting, including occurring in unanesthetized animals. As we have reported in our previous companion article,[43] with this model, the animals exposed to a single mTBI have short-term “post-concussive” behavioral abnormalities. Histopathological analysis showed that this was accompanied by a limited acute reactive astrocytosis (except for hippocampus at 6 months) and increased phosphorylated tau staining that was present at 7 days and 1 month, but was not persistent at 6 months. By contrast, the observed cumulative effects of 42 head impacts delivered over the course of 7 days recapitulate the neurobehavioral...
syndrome and several characteristic pathological features of CTE. As described in our previous manuscript, these mice exhibit depression-like behavior, risk-taking behavior, sleep disturbances, and cognitive deficits. Pathologically, repetitive mTBI resulted in a more marked neuroinflammatory response, with persistent and widespread astrogliosis and microglial activation, as well as a progressive increase in phosphorylated tau immunoreactivity. There has been an increased interest in laboratory research focusing on repetitive mTBI. Most studies have
utilized rodent models, although some have investigated repetitive injury in pigs. Studies of repetitive mTBI have reported abnormalities in glucose utilization, cellular metabolism, cerebral blood flow, cell membrane fluidity, synaptic function, and overall structural integrity. While many of these studies have demonstrated behavioral deficits as well as neuropathological changes, these changes typically improve or even resolve with time,\textsuperscript{[14]} though a few studies have reported chronic deficits following experimental repetitive mTBI.\textsuperscript{[30,33]} Contrary to much of the previous work in this area, the changes we observed in the repetitive mTBI mice progressed and even dynamically changed over a significant length of time. The number of impacts used in our study for the repetitive mTBI group was greater than that typically used in other studies. Also, the 2-h inter-injury interval was shorter than that generally employed in most studies of repetitive mTBI. These aspects of our protocol, in addition to the biomechanics of our model, may have contributed to the persistent pathological and neurobehavioral changes we demonstrated in the current study.

Persistent increased phosphorylated tau immunostaining was observed in the cortex, amygdala, and the hippocampus of repetitive mTBI mice. We also noted that there was there was some co-localization of the phosphorylated tau staining with GFAP-positive astrocytes [Figure 5]. The morphology of the tau was similar and did not appear to be distinct across time-points in the different regions analyzed. This is congruent with the distribution of tau pathology in reported cases of CTE and in those with a history of chronic repetitive mild head trauma.\textsuperscript{[16,32,38,40]} The spatial distribution of astrogliosis and microglial activation that we observed may have numerous causes. While it is possible that the microglial activation and reactive astrocytosis at 6 months is in response to the primary injury, what is more likely is that repetitive mTBI initiates a progressive, chronic neuroinflammatory process that contributes to secondary and tertiary injury. It is not entirely clear what triggers entry into this chronic cycle of inflammation. With repetitive brain injury, microglia may enter a constitutively activated state and become neurodestructive, which may translate to the risk for developing CTE.\textsuperscript{[7,12]} Blaylock and Maroon coined this process as immunoexcitotoxicity.\textsuperscript{[7]} We observed a resolution of the acute astrocytic response by 1 month in the mice of both injured groups in our study, but by 6 months, the repetitive mTBI mice exhibited an additional diffuse re-activation of astrocytes not seen in the single mTBI mice (except for a small focal increase in the hippocampus region). Whether this second wave of astrogliosis and persistent microglial activation is due to the persistent presence of phosphorylated tau, or vice versa, is not known [Figure 6].

There was an acute increase in phosphorylated tau immunoreactivity following a single mTBI that was persistent at 1 month. This parallels the findings of hyperphosphorylated tau accumulation acutely following a single experimental rotational\textsuperscript{[52]} or percussion injury,\textsuperscript{[19,20]} 2 weeks following a single experimental blast injury,\textsuperscript{[17,21]} and acutely following TBI in humans.\textsuperscript{[51]} Post-traumatic phosphorylated tau accumulation has been reported at 1 month after a single mild blast injury\textsuperscript{[25]} and even up to 6 months following a single fluid percussion injury in rats.\textsuperscript{[20]} The increased phospho-tau immunoreactivity following a single injury in our model seemed to clear over time, when the animals were analyzed at 6 months. One interesting observation from the current study was that repetitive mTBI resulted in a similar increase in phosphorylated tau staining; however, this immunoreactivity persisted over time – all in the absence of any further mechanical trauma or outside stress to the animals. This pathological progression has not been previously described in a controlled fashion and lays the foundation for additional analyses elucidating the trigger for delayed tau accumulation and persistent gliosis. It is intriguing to speculate that chronically activated microglia may enhance the hyperphosphorylation of tau and increase the total tau burden, as it has been suggested in other neurodegenerative diseases.\textsuperscript{[45,20]}

The distribution of tau abnormalities in CTE suggests distinctive core pathology within the amygdalo-hippocampal-septo-hypothalamic-mesencephalic continuum, in addition to the cortical and subcortical regions. A recent study performed positron emission tomography (PET) scans after intravenous injections of 2-((1-6-[2-[F-18]fluoroethyl] (methyl) amino)-2-naphthyl) ethyldiene) malononitrile (FDDNP) to explore whether brain tau deposits could be detected in a small group of retired National Football League (NFL) players with cognitive and mood symptoms and compared them with a group of matched controls.\textsuperscript{[90]} The study demonstrated amygdala and subcortical FDDNP binding patterns in former players, consistent with the fibrillary tau deposition patterns observed at autopsy in CTE cases. While only patchy cortical tau deposits have been reported in mild CTE cases, the amygdala has been one region where such deposits are still quite dense.\textsuperscript{[18,32,50]} Another clinical study reported abnormalities of the amygdalo-hippocampal complex, as measured by magnetic resonance spectroscopy, in individuals with aggressive behavior and antisocial activities.\textsuperscript{[11]} Sub-regions of the amygdala play a pivotal role in decision-making as part of the cortico-mesolimbic circuit involving the orbitofrontal cortex and the nucleus accumbens.\textsuperscript{[44,46,71]} The basolateral amygdala is proposed to subserve calculated instrumental behavior and play a significant role in modulating emotionally charged memory formation; and both the basolateral and lateral

Contrary to sub-regions of the amygdala play a pivotal role in decision-making as part of the cortico-mesolimbic circuit involving the orbitofrontal cortex and the nucleus accumbens.\textsuperscript{[44,46,71]} The basolateral amygdala is proposed to subserve calculated instrumental behavior and play a significant role in modulating emotionally charged memory formation; and both the basolateral and lateral
nuclei reciprocally connect with the hippocampus to process emotionally salient sensory information and guide anxiety-like behavioral response.44 The central-medial amygdala subserves affective-impulsive behaviors and plays a different, but certainly important role in fear conditioning by coordinating fear-related behavioral and physiological responses, including freezing behavior, increased respiration, and stress hormone release.4,44

Thus, pathological involvement of the amygdala may underlie many of the early behavioral symptoms observed in CTE, including the tendency toward emotional lability, decreased fear avoidance/risk-taking, impaired social interactions/relationships, aggression, anger, and violent outbursts. The repetitive mTBI mice in our study did have a significant increase in astrocytosis, microglial activation, and phospho-tau immunoreactivity compared to control mice, which could possibly be related to the observed depression-like behavior in the forced swim and tail suspension testing, as well as to the increased risk-taking behavior noted in the elevated plus maze.
Much of the focus in CTE has been on the accumulation of phosphorylated tau protein. While the neurotoxicity of phosphorylated tau may contribute to the CTE phenotype, in this model it does not solely account for the behavioral abnormalities observed. If that were the case, the single mTBI mice would likely have demonstrated more behavioral deficits at 1 month given the elevated phosphorylated tau. Rather, it was only the repetitive mTBI mice in which there was a persistent microglial response. Chronic neuroinflammation and immunoexcitotoxicity may be the principle factor behind the observed behavioral abnormalities and symptoms following repetitive mTBI. Biochemical, cellular, and animal disease models, as well as clinical studies have elucidated the role of neuroinflammation, particularly microglia, in psychiatric disorders such as depression, PTSD, and bipolar disorder, which prominently overlap with the behavior observed in CTE and the repetitive mTBI mice in this model. A recent study demonstrated that the number of TBIs in military personnel was associated with greater suicide risk when controlling for the effects of depression, PTSD, and TBI symptom severity. ‘Those subjects who sustained two or more concussions had a significantly increased incidence of suicidal ideation. While difficult to capture suicidal behavior in any animal model, clinical studies have implicated microgliosis and neuroinflammation in the urobiology of suicide.

With regards to neuroinflammation, we also observed an interesting finding at 1 month with the GFAP labeling in mild TBI mice. At 1 month, we found a significant decrease in GFAP in all regions of bilateral hippocampi in repetitive TBI mice compared to age-matched control mice. GFAP is a cytoskeletal protein involved in processes related to cell movement and structure and has been proposed to play a role in cell communication such as astrocyte–neuron interactions. Down-regulation of GFAP in the hippocampus has been observed in rodents exposed to chronic social stress. In this pre-clinical model of depression, the authors found that rats in a depressive-like state exhibit reduced GFAP levels in the hippocampal formation. It has been hypothesized that glial dysfunction may play a key role in the pathophysiology of psychiatric disorders, including major depression. Post-mortem clinical studies have shown significantly reduced GFAP immunoreactivity (in cell bodies and fibers) in the hippocampal regions CA1 and CA2 of patients with major depression. Our repetitive mTBI mice demonstrated depressed behavior at the 1-month time-point, and it is intriguing to think that similar to these other studies, the diffuse reduced hippocampal GFAP we observed might be implicated in this phenotypic behavior. However, it remains unknown which presumptive glial changes are causal for depression. Our single mTBI mice also demonstrated reduced GFAP in some parts of the hippocampus; however, they did not exhibit depression-like behavior on testing at 1 month. It is more likely that glial dysfunction or reduced glial activity is one component contributing to the overall picture and that there are other mechanisms in play that we have yet to fully understand.

This study has several limitations that should be taken into account. The sample sizes in terms of number of mice and number of fields per mouse could have been greater in order to be fully quantitative. The study was reasonably powered, however, to allow for an analysis and the observation of the reported pathological sequelae. Also, we did not study axonal injury in the present study. Acute and delayed axonal injury as well as progressive axonal transport disruption is believed to play a role in the pathogenesis of post-traumatic neurodegeneration. It will be important for future studies to explore the role axonal injury plays in this new model of TBI. Another limitation was that we chose to explore just one tau antibody (AT8). The AT8 antibody is a sensitive marker in detecting abnormally phosphorylated tau protein without showing cross-reactivity with normal tau epitopes. Future studies should aim to study phosphorylated tau with additional antibodies also, including AT180 and PHF-1. Another area that could have been more thoroughly explored is the localization of the phospho-tau immunoreactivity, specifically relating to its location within the layers of the cortex and additionally with emphasis on the presence of any perivascular tau. The literature suggests that a portion of the pathogenesis of cytoskeletal abnormalities may involve damage to blood vessels or perivascular elements. We did observe that some of the phospho-tau immunostaining appeared to be perivascular in location; however, double labeling would be needed in future studies to determine this more definitively. Undoubtedly, further work is needed; however, this study serves as a good starting point and it is hoped that with continued investigation, this model can contribute to our understanding of the neurological sequelae of repetitive mild TBI, particularly CTE.

CONCLUSION

We describe the pathophysiology underlying single and repetitive mild TBI in a novel mouse model of closed head injury. Single mTBI mice demonstrated an increase in tau phosphorylation acutely that lasted to 1 month; however, it cleared by 6 months post-injury. There was also a limited astrogial response. Repetitive mTBI mice exhibited an acute increase in phosphorylated tau accompanied by an astrocytic and microglial mediated neuroinflammatory response. This neuroinflammatory response progressed and persisted up to 6 months, as did the phosphorylated tau deposition. The interplay of tau pathology and neuroinflammation needs to be further elucidated and future studies investigating
the effects of repetitive mTBI are required. As we learn more about the interplay between this dynamic neuroinflammatory response and post-traumatic behavior/neuropathology, new avenues for developing improved diagnostic measures as well as translational treatment approaches could open up.[49] Whether treatments should be targeting neuroinflammation, tau processing, or mechanisms either upstream or downstream is currently unknown and will only become elucidated as we learn more about the pathophysiological effects of repetitive mild TBI. This novel model will allow for a controlled, mechanistic analysis of repetitive mTBI and CTE in the future because it is the first to encapsulate the spectrum of this human phenomenon.

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