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Impact of age on acute post-TBI neuropathology in mice expressing humanized tau: A Chronic Effects of Neurotrauma Consortium Study.


*To whom correspondence should be addressed: Benoit C. Mouzon PhD, Roskamp Institute, 2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Email: bmouzon@roskampinstitute.org

Co-author e-mail information:

Nicole Saltiel, (nsaltiel@roskampinstitute.net)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Scott Ferguson, PhD, (sferguson@roskampinstitute.org)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Joseph Ojo, PhD, (bojo@roskampinstitute.net)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Carlyn Lungmus, (clungmus@roskampinstitute.net)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Cillian Lynch, (clync@roskampinstitute.net)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Moustafa Algamal, M.Sc., (malgamal@roskampinstitute.net)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681
Alexander Morin, (amorin@roskampinstitute.net)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Benjamin Carper, M.S. (bcarper@rti.org)
RTI International Research Triangle Park, NC 27709. Tel: 919-597-5131 (x25131) Fax: N/A

Gayle S. Bieler, M.S. (bmac@rti.org)
RTI International Research Triangle Park, NC 27709. Tel: 919-597-5131 (x25131) Fax: N/A

Elliott J. Mufson, MD, PhD, (elliott.mufson@dignityhealth.org)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 602-406-8525 Fax: 602-406-8520

Barrow Neurological Institute, 350 W. Thomas Rd, Phoenix, AZ 85013

William Stewart MBChB, PhD, (William.Stewart@glasgow.ac.uk)
Department of Neuropathology, Laboratory Medicine Building, Queen Elizabeth University Hospital, Glasgow G51 4TF. Tel: +44(0)141 354 9535 Fax: N/A

Michael Mullan, MD, PhD, (mmullan@roskampinstitute.net)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681

Fiona Crawford, PhD, (FCrawford@roskampinstitute.org)
2040 Whitfield Ave, Sarasota, FL 34243. Tel: 941-256-8019 Fax: 941-756-3681
Abstract

**Primary Objective:** We hypothesized that polypathology is more severe in older than younger mice during the acute phase following repetitive mild traumatic brain injury (r-mTBI).

**Research Design:** Young and aged male and female mice transgenic for human tau (hTau) were exposed to r-mTBI or a sham procedure. Twenty-four hours post-last injury, animals were immunostained for alterations in astrogliosis, microgliosis, tau pathology and axonal injury.

**Main Outcomes and Results:** Quantitative analysis revealed a greater percent distribution of GFAP and Iba-1 reactivity in the brains of all mice exposed to r-mTBI compared to sham controls. However, no noticeable difference was observed between the young and aged group as initially hypothesized. With respect to axonal injury, the number of APP-positive profiles was increased in young vs. aged mice post r-mTBI. An increase in tau immunoreactivity was found only in young and aged injured male hTau mice.

**Conclusion:** We report first evidence in our model that repetitive mild TBI precipitates a complex sequelae of events in aged versus young hTau mice at an acute time point, typified by an increase in phosphorylated tau and astrogliosis, and a diminished microgliosis response and axonal injury. These findings suggest differential age-dependent effects in TBI pathobiology.

**Introduction**

Traumatic brain injury (TBI) is the leading cause of mortality and morbidity in the world for individuals under the age of 45\(^1\). Mild TBI (mTBI) accounts for approximately 70%-90% of all TBIs and is a major source of morbidity, with up to 15% of patients experiencing long-term symptoms\(^2\)-\(^5\). Since epidemiological data do not account for the substantial number of individuals who do not seek hospital treatment post injury, and the lack of a clinical consensus on diagnostic
criteria for mTBI remains, the prevalence of mTBI and brain health are likely underestimated from the limited prospective studies conducted to date.

Studies on the epidemiology of mTBI have shown that age is a major factor influencing the clinicopathological outcomes following exposure to mTBI, with a bimodal distribution between young adults (13-20 years old) and older adults (>65 years old) recovering differently from injuries of a similar severity.\textsuperscript{6-8} However, a recent review on the chronic consequences of mild and moderate/severe TBI, reported that the association between mild TBI and the long-term mortality (at least 5 years after mTBI) depended largely on sample population. The role that age plays in the pathological response to mTBI remains controversial.\textsuperscript{10-12} Disparity in these findings may be related to environmental factors such as drug abuse, level of education, rehabilitation length and familial support.\textsuperscript{13}

Nevertheless, it has been accepted that age at injury also has a significant influence on dementia risk in patients > 65 years of age following exposure to mTBI.\textsuperscript{14} Characterization of autopsy-acquired tissue from long-term survivors of repetitive mTBI reveal a complex neuropathology, best described as a “polypathology”, including: abnormal tau and amyloid protein aggregation, neuroinflammation, white matter degradation, and axonal degeneration.\textsuperscript{15} This pathology is masked by normal aging and may augment or accelerate pre-existing age-related pathologies. Therefore, it is important to develop a greater understanding of the age-dependent pathophysiological process following mTBI, to improve diagnostic and therapeutic interventions and recognize age-related risk factors for patients.

The present study investigated the “polypathology” associated with repetitive mTBI in 3 and 12-month-old mice at an acute time point post-injury (24hrs). To our knowledge, only a few pre-clinical studies have investigated the influence of age and injury mechanism after TBI.\textsuperscript{16-19}
Therefore, our study aims to address the impact of TBI on acute neuropathology in the young adult vs aged brain. We hypothesized that polypathology is more severe in older mice than younger mice during the acute phase following repetitive mild traumatic brain injury (r-mTBI). Our second objective is to determine whether sex differences in the animals play a role in their recovery. The focus on pathogenic tau pathology has been well documented chronically after exposures to repetitive mTBI in postmortem human brain tissue of patients\textsuperscript{20; 21}, however the origin of this tau pathology and relationship to the inciting injury are undetermined. Therefore, we have chosen to assess the mouse brain tissue at early timepoints (24hrs) following repetitive injuries. We utilized hTau mice that have been genetically modified to express all six isoforms of non- mutant human MAPT in a Mapt knockout background. These mice start to express membranous tau redistribution at 3 months of age and present tau hyperphosphorylation and aggregation by 12 months of age\textsuperscript{22-24}. Here, we explore the influence of age at injury (young [3months] and aged [12months]) on acute neuropathological sequelae in hTau mice.

Materials and Methods

Animals
Young (3 months old) and aged (12-13 months old) male and female mice, expressing all six isoforms of human tau (hTau) on a C57BL/6 and null murine tau background (Jackson Laboratories, Bar Harbor ME), were housed singly under standard laboratory conditions (23°C ± 1°C, 50 ± 5% humidity, and 12-hour light/dark cycle) with free access to food and water. 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**

Forty-eight young and fifty-two aged hTau mice were randomly assigned to TBI or sham conditions. Unless otherwise noted, histochemical studies were performed with: young male TBI (n = 6), young female TBI (n = 8), young male sham (n = 6), young female sham (n = 7), aged male TBI (n = 11), aged female TBI (n = 8), aged male sham (n = 11), aged female sham (n = 10). The rest of the brains were stored for additional future analyses. Mice assigned to repetitive mild TBI (r-mTBI) conditions received five injuries over 9-days, with an inter-injury interval of 48 hours. Sham (r-sham) animals were anesthetized with the same frequency and exposure time as their r-mTBI counterparts, but without injury. As described in our original publication on this model\textsuperscript{25} the inter-injury interval was chosen to accommodate repeated injuries occurring within an asymptomatic window of vulnerability from the previous injury that had been described in a rat model\textsuperscript{26}. We have extensively characterized this model in wild type mice\textsuperscript{25, 27, 28} and therefore have knowledge of the outcomes observed that can be used as point of reference.

**Injury Protocol**
Mice were subjected to closed head mild TBI as previously described\textsuperscript{25}. Prior to mTBI all mice were anesthetized with 1.5L/min of oxygen and 3% isoflurane, the top of their heads were shaved, and they were transferred to a stereotaxic frame (Just For Mice™ Stereotaxic, Stoelting, Wood Dale, IL) placed on a heating pad to maintain body temperature at 37°C and maintained under anesthesia through a nose cone. A 5mm blunt metal impactor tip was retracted and positioned midway relative to the sagittal suture before each impact. Injury was triggered using the myNeuroLab controller at a strike velocity of 5m/s, strike depth of 1.0mm, and dwell time of 200 milliseconds over the shaved area of the head. To be considered as a concussive injury, our injury paradigm should follow the following criteria: no skull fractures, hematomas, or other gross signs of injury, and the presence of APP immunoreactivity profiles in the corpus callosum as a sign of traumatic axonal injury. No mortality was observed with these mice during these experiments. At the end of the procedure, each animal was removed from the stereotaxic table, allowed to recover in its home cage resting on a heating pad until the animal was ambulatory. To control for the effects of repeated anesthesia sham animals underwent the same procedures and were exposed to anesthesia for the same length of time as the mTBI animals, but were not exposed to head trauma.

**Histology**

All mice were euthanized 24 hours after the last mTBI/sham injury by anesthetization with isoflurane, followed by transcardial perfusion with heparinized PBS (pH 7.4) and PBS containing 4% paraformaldehyde. After perfusion, brains were post-fixed in 4% paraformaldehyde (4°C) for 48 h, embedded in paraffin using Tissue-Tek VIP (Sakura, USA), cut at 6μm on a 2030 Biocut microtome (Reichert/Leica, Germany) and mounted on positively
charged glass slides (Fisher, Superfrost Plus). Sections were deparaffinized in xylene, and rehydrated in an ethanol to water gradient. Slides were analyzed using a bright field microscope (BX60, Leica, Germany) and digital images were visualized and acquired using a MagnaFire SP camera (Olympus, Tokyo, Japan). Sets of adjacent sections were stained for glial fibrillary acid protein (GFAP, 1:20,000; Dako, Glostrup, Denmark, ZO334), ionized calcium binding adaptor molecule 1 (Iba1. 1:5000; Abcam, Cambridge, MA, ab5076), or amyloid precursor protein (APP, 1:20,000; Millipore, Billerica, MA, MAB348). Tau immunohistochemistry was performed using the following monoclonal antibodies at a 1:500 dilution: CP13 [pS202]; PHF1 [pS396/404]; RZ3 [pThr231]. CP13, PHF1, and RZ3 were generously provided by Dr. Peter Davies, The Feinstein Institute for Medical Research, Bronx, NY. As a negative control, for each antibody, a single section was processed for immunostaining without the inclusion of the primary antibody. Tissue sections were subjected to antigen retrieval with either heated trisethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) or citrate buffer (pH 6.0) under pressure for 7 min. Endogenous peroxidase activity was quenched with a 15min H2O2 treatment (3% in water). Each section was rinsed and incubated with the appropriate blocking buffer (ABC Elite kit, MOM kit, Vector Laboratories, CA) for 20min, before applying the appropriate primary antibody overnight at 4°C. Then, the diluted biotinylated secondary antibody from the ABC Elite Kit was applied. Antibodies were detected using the avidin-peroxidase complex, after incubation with the chromogen 3,3-diaminobenzidine (DAB) peroxidase solution (0.05% DAB - 0.015% H2O2 in 0.01M PBS, pH 7.2) for 6–7min and counterstained with hematoxylin. Immunofluorescence was performed with an antibody for p-tau RZ3 (1:500). Prior to immunostaining, samples were deparaffinized in xylene and rehydrated through a gradient of ethanol solutions of decreasing concentrations (2 x 100%, 95%, 70%). Antigen retrieval consisted of heating slides in a citrate
solution (pH-6.0) under pressure, washed with PBS and transferred into a Sudan black solution (EMD Millipore, MA) (15 min) to inhibit autofluorescence. Before primary antibody treatment slides were blocked for 1 h with 10% donkey serum. The primary antibody for RZ3 was applied on the slides and left overnight at 4ºC. The next day, donkey anti-Mouse IgG secondary antibody Alexa Fluor 488, was applied for RZ3. Slides were mounted with ProLong Gold Antifade DAPI Mount.

**Quantitative Immunohistochemistry**

Mice from both age groups (n=4) were euthanized 24h post-injury, and sagittal sections were immunostained and then analyzed by an observer blinded to experimental conditions using ImageJ software (US National Institutes of Health, Bethesda, MD). Images were separated into individual color channels (red hematoxylin counter stain and DAB brown chromogen) using the color deconvolution algorithm\textsuperscript{29}. Three non-overlapping areas of 100 μm\textsuperscript{2} from each of two sagittal sections in the corpus callosum (CC) were randomly selected within which the area of GFAP or Iba-1 immunoreactivity was calculated and expressed as a percentage of the field of view as previously reported. The numbers of APP-positive profiles were manually counted in three non-overlapping areas of 100 μm\textsuperscript{2} within the CC. The immunohistochemical outcomes were expressed as percent area of GFAP, Iba-1, and RZ3. Variables of interest included sex, injury group (mTBI vs. sham), age (young vs. aged), and their interactions. Descriptive statistics, including means and standard errors, were calculated from the percent area of GFAP and Iba-1 measurements for each age, injury group, and sex. Average percent areas were calculated within an animal (across sections), prior to calculating age, injury group, and sex averages and standard errors. Descriptive statistics, including medians and 25th and 75th percentiles, were calculated from the number of APP-positive profiles for each age, injury group, and sex. The raw percent
area data were assessed for normality using the Shapiro-Wilk test as well as four alternative transformations (square-root, base-10 logarithm, logit, and arcsine square-root). The transformation that most closely approached normality was used for all subsequent analysis. The GFAP and Iba-1 data were analyzed using a mixed ANOVA model with age, sex, and injury group, and their interactions, as explanatory variables. In addition to these model terms, a random variance component for mouse was included such that multiple observations on the same mouse were weighted together and not individually. This model was used to estimate the size and significance of the difference in percent area between ages (overall and within injury group and gender), injury groups (overall and within age and gender), and between genders (overall and within age and injury group).

For the APP data, no APP-positive profiles were observed for any of the Sham animals. The APP data were analyzed using a mixed Poisson regression model with age, sex, and their interaction as explanatory variables. In addition to these model terms, a random variance component for mouse was included such that multiple observations on the same mouse were weighted together and not individually. This model was used to estimate the size and significance of the difference in the number of APP-positive profiles between ages (overall and within gender) and between genders (overall and within age). All statistical analyses were performed using SAS (ver. 9.4) and all results are reported using the 0.05 level of significance.

**Results**

*Repetitive mTBI induces a stronger astrogliosis response in the corpus callosum in aged mice*
For all groups, the entire corpus callosum (CC) (splenium, body, and genu) was assessed in GFAP stained sections. Quantitative analysis revealed TBI-dependent differences in GFAP immunopositivity in the body of the corpus callosum among females and males and in the young and aged cohorts (Figures 1. b,d,f,h; p < 0.001). Interactions between age and injury group, age and gender, injury group and gender, and the three-way interaction between age, injury group, and gender were not significant. The quantification of GFAP immunostaining is summarized in Table 1.

**Repetitive mTBI induces a stronger microgliosis response in the corpus callosum in aged mice**

To gain insight into whether age influences the degree of inflammation following mTBI, we investigated Iba-1, a marker of microglia in young and aged animals. Microglial cell structures were similar across comparable groups displaying a primed morphology characteristic of an aged mouse brain in the 12 months cohort (with a more inflammatory microglia phenotype e.g., increased major histocompatibility complex II [MHCII], IL-1β, CD68, complement receptor [CR]3)30. Similar to the GFAP analysis, the entire CC was assessed for Iba-1 immunoreactivity. There were TBI-dependent quantitative difference in the level of Iba-1 immunopositivity detected in the body of the corpus callosum among female and males and in the young and aged cohort (Figures 2. b,d,f,h; p < 0.001). The interaction between age and injury group was significant, but the interactions between age and gender, injury group and gender, and the three-way interaction between age, injury group, and gender were not significant. The quantification of Iba-1 immunostaining is summarized in Table 2.

**APP Immunoreactivity is reduced following r-mTBI in aged compared to young mice**
APP-immunoreactive axonal profiles, a marker of axonal injury, were observed 24h post-injury in the CC (Figure 3 i) of both young and aged r-mTBI groups (Figures 3. b, d, f, h) but not in controls (Figures 3. a,c,e,g). These APP immunoreactive axonal profiles were observed as small, granular immunoreactive profiles within the CC (Figures 3. b, d, f, h). The difference in the number of APP-immunoreactive profiles observed was greatest in the young r-mTBI versus aged r-mTBI comparison (Figures 3. j, k p < 0.001), with no gender effects detected (p >0.05). The quantification of APP immunostaining is summarized in Table 3.

**Repetitive mTBI induces an elevation of hippocampal RZ3 p-tau 24h post-injury**

To investigate the effect of TBI on tau in our model, we performed a quantitative immunohistochemical analysis of RZ3, CP13, and PHF1 (an antibody that recognizes early and late tau pathology). The average percent area of RZ3 immunoreactivity in the hippocampal pyramidal layer (Figures 4. a, c, e, g, I, j) was significantly increased in the mTBI group compared to the sham control group among male (averaged over age, p<0.001), aged (averaged over gender, p=0.002), young (averaged over gender, p=0.015), and overall (averaged over gender and age, p<0.001) mice. Additionally, the average percent area was reduced in young vs. aged male mice (averaged over injury group, p=0.026), the mTBI group (averaged over gender, p=0.034), and overall (averaged over gender and injury group, p=0.009). The average percent area was also reduced in females vs. males among aged mice (averaged over injury group, p=0.038) and for the mTBI group (averaged over age, p=0.002). There was no overall gender effect. The interactions between age and injury group, age and gender, and the three-way interaction between age, injury group, and gender were not significant, but the interaction between injury group and gender was significant (p=0.008). Immunohistochemical assessment of soluble phosphorylated tau pSer-202 (CP13) was similar to RZ3 and none of the brains showed
neurons positive for PHF1 (data not shown). The quantification of RZ3 immunostaining is summarized in Table 4.

Discussion

In the current study, we have examined the acute pathological outcome (24h post-last injury) of repetitive mild TBI in the brains of young and aged hTau mice. Our data support a TBI-dependent difference between young and aged animals, with increased astrogliosis and tau pathology in older animals, whereas an opposite pattern was observed for microgliosis and axonal degeneration. In addition, as we and others have previously reported\cite{17,27,31}, we observed age-dependent changes in astrogliosis, microgliosis and axonal injury within the corpus callosum, an area of the white matter of the brain known to be particularly vulnerable to repetitive brain injuries in our model\cite{32}. This study also revealed a possible sex-dependent link between age at injury and a subsequent acute increase in phosphorylated tau species observed in pre-tangle neurons in both, the hippocampus and cortex.

We previously identified an increase in PHF1 positive hyperphosphorylated tau in male TBI mice compared to females using a similar injury model at 15 days post injury\cite{17}, herein we now demonstrate sex-dependent difference in p-tau pathology appear as early as 24h post-last injury. The present study revealed an increase in RZ3 phosphorylated tau in the hippocampus without any appreciable increase in PHF1 levels, supporting time-dependent changes for different p-tau epitopes specific for pre-tangle structures. While p-tau protein is a key component of the pathology seen in neurodegenerative tauopathies\cite{33-35}, it also plays an important role in neuroplasticity, including dendritic/ synaptic remodeling observed in the brain in response to environmental challenges, such as TBI\cite{36,37} and hypothermia/hibernation\cite{38,39}. Therefore, the physiological changes reported in the brains of these mice may be the emergence of an insidious
pathological process, however they may also be part of an attempt by the brains to repair the structural damage caused by the repeated head trauma. Regardless of the biological repercussions, our observations at 24h, in addition to our previous work at 15 days post-injury, indicate that the levels of RZ3 and PHF1 phosphorylated tau are both increased in male mice, while no significant changes were observed in female mice at 24h or 15 days post-injury. We cannot, however, rule out that these observations are unique to hTau mice, because no clinical studies to date have addressed the role of sex on tau pathology after TBI. It is worth noting that because it has been previously reported that male PS19 mice (mutant tau) develop tau pathology more consistently than females, almost all pre-clinical studies exclusively use male animals to reduce the variability of tau pathology\textsuperscript{40}. Similar to the conditions in pre-clinical models, the autopsied brain samples used for much of the current clinical histopathological reports of tauopathy following TBI are almost exclusively male in origin; and thus, the speculation as to how sex influences the outcome on tau pathology has yet to be determined. Further work is necessary to address the gender difference in tau pathology in both, animals and most especially in clinical studies.

Our study has several limitations. The first limitation is that the hTau mouse line has shown that naïve animals start to express signs of early tau pathology at 3 months of age and neurofibrillary tangles at 9 months of age\textsuperscript{23, 24}. Given the increasing evidence that a disruption in the normal phosphorylation state of tau plays a key role in the pathogenic events that occur in other neurodegenerative conditions\textsuperscript{41}, our results may not reflect the pathology that would have been observed in wild type animals. The second limitation of this study lies in its design as it cannot be determined whether the pathology observed is due to the first or last mTBI. However, the results observed from our previous work in wild type animals suggest that our 5 injury
paradigm exacerbates the pathology that would have been observed after a single injury. Another limitation is that even young mice may have early tau pathology and therefore could affect normal TBI pathology. Because we published and demonstrated a lack of injury effect of tau pathology in wild type mice we decided to use the hTau mice which expressed all six human tau isoforms and demonstrate age-related changes in tau pathology as observed in normal ageing. It is well established that age-related tauopathy is a normal feature of ageing (see progressive age-related tauopathy and Hyperphosphorylated tau in young and middle-aged subjects). Therefore, using this model despite the progressive age-related increase in phosphor-tau pathology between 3 months (approx. 14-21yrs – humans) and 12 months (approx. 30-39years - humans) of age, we consider to be related to the pattern of normal tau pathology observed with humans over time and in individuals exposed to injuries at these age groups. Finally, further studies with the inclusion of additional post injury timepoints throughout the lifespan of the animal is required to understand how tau interacts with the polypathology resulting from the cumulative effects of repetitive mTBI. Multiple lines of evidence in pre-clinical and clinical work suggest that TBI is a chronic, evolving, and perhaps a lifelong disorder. Such cohorts could serve as a platform and aid in the design and implementation of clinical trials of new therapies considering the different types of pathological markers present at acute and chronic time-points post-injury. For example, exploring long-term efficacy of different treatment regimen aimed at reducing potentially pathogenic tau species such as the use of an antibody against cis phospho tau conformation or sodium selenate. Given the prominent changes in glial cells, a second possible treatment at chronic time points could target post traumatic neuroinflammation by minimizing the detrimental neurotoxic effects and create the optimal condition for regeneration.
Despite a growing body of clinical evidence suggesting that repetitive mild TBI is an important risk factor for neurodegenerative diseases\textsuperscript{14,15,21,47}, the causal link and the role of tau as a common pathology remain unclear. Moreover, how the aged brain responds to repetitive mTBI compared to a younger brain remains unknown. Nonetheless, considering that older patients demonstrate worse outcomes despite sustaining less high energy impact\textsuperscript{48,49}, several studies have suggested that aged patients are more vulnerable to TBI\textsuperscript{50-54}. However, the particular relationship between mild TBI and increased risk for dementia or morbidity is less clear (reviewed in Gardner and Yaffe, 2015\textsuperscript{55} and Wilson and colleagues 2017\textsuperscript{9}). To that end, we investigated whether an increased levels of total tau and p-tau in the brains of aged hTau mice (12 months of age\textsuperscript{22-24}) is associated with a stronger neuroinflammatory response after r-mTBI. We observed that increased astrogliosis and p-tau was more pronounced in aged animals when compared to young mice, however this was not observed with respect to traumatic axonal injury and microgliosis, at 24h post injury. While our results highlight that older age at injury produces more pronounced astrogliosis and tau phosphorylation, these changes were relatively mild in nature (< two-fold change), suggesting that the polypathology resulting from the exposure of r-mTBI in mid-age animals is likely the result of normal aging and the primed state of the resident glial cells\textsuperscript{31}. Another potential limitation is that our aged mice are only between 12 and 13 months old, and therefore are not representative of the clinical studies of 65+ year-old human’s mentioned in the introduction. Although mouse and human developmental stages are generally not a linear relationship, middle age is considered to be around 12-15 months in mice. In addition to increased astrogliosis, our results also support a TBI-dependent increase in RZ3 p-tau observed in male hTau mice. Yet, this increase in p-tau pathology at 24h post-injury was not associated with a more robust glial response in males when compared to their females’
counterpart, suggesting a diminished role for p-tau on acute neuroinflammation (24h post-injury). Finally, we found that axonal injury was decreased in the aged injured group at 24h post-injury. Whether this represents a true age-related effect on axonal injury will require future studies; it is noteworthy that APP immunostaining only captures a subpopulation of injured axons, and thus further measure of detecting the full extent of axonal injury is needed.

Nonetheless, our results are consistent with our previous reports showing an attenuation of axonal swelling in old mice\textsuperscript{17}. Although the many differences in TBI models and experimental designs make direct comparison challenging, these results are consistent with the recent work of W.H. Cheng et al that showed a robust decrease in axonal neurofilament pathology after mTBI in aged WT and transgenic mice harboring the APP/PS1 mutations\textsuperscript{56}. Whether this pathology is unique to rodent remains to be determined. Further studies are necessitated in human autopsy cases.

Conclusion

This study shows that r-mTBI in young adult hTau mice induces age-dependent, sex-specific differences on pathological outcome at 24h post-injury. Of particular interest here, we only found a sex-dependent difference for phosphorylated tau stained with RZ3 in young and aged male hTau mice. However, this increase in p-tau was not associated with an increase of Iba-1 and GFAP staining typically seen in this model of r-mTBI, suggesting a diminished role of phosphorylated tau in young and aged hTau mice at 24h post-injury. Altogether, these findings suggest that future studies should incorporate both males and females to provide a greater understanding of injury prognosis and better inform clinical practice.

Declaration of Interest
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