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Chronic white matter degeneration, but no tau pathology at 1-year post-repetitive mild traumatic brain injury in tau transgenic model.

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Running Head: Neuropathology in hTau mice at 1-year post-r-mTBI

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Abstract:

Tau pathology associated with chronic traumatic encephalopathy has been documented in the brains of individuals with a history of repetitive mild traumatic brain injury (r-mTBI). At this stage, the pathobiological role of tau in r-mTBI has not been extensively explored in appropriate preclinical models. Herein, we describe the acute and chronic behavioral and histopathological effects of single and repetitive mild TBI (five injuries given at 48 h intervals) in young adult (3 months old) hTau mice that express all six isoforms of human tau on a null murine background. Animals exposed to r-mTBI showed impaired visuospatial learning in the Barnes maze test that progressively worsened from 2 weeks to 12 months post-injury, which was also accompanied by significant deficits in visuospatial memory consolidation at 12 months post-injury. In contrast, only marginal changes were observed in visuospatial learning at 6 and 12 months following single mTBI. Histopathological analyses revealed that hTau mice developed axonal injury, thinning of the corpus callosum, microgliosis and astrogliosis in the white matter at acute and chronic timepoints following injury. However, tau immunohistochemistry and ELISA data suggest only transient, injury-dependent increases in phosphorylated tau in the cerebral cortex beneath the impact site and in the CA1/CA3 sub-region of the hippocampus after single or r-mTBI. This study implicates white matter degeneration as a prominent feature of survival from mTBI, while the role of tau pathology in the neuropathological sequelae of TBI remains elusive.

Introduction:

Traumatic brain injury (TBI) has long been recognized as the strongest environmental risk factor for later development of Dementia[1, 2]. Although epidemiological data are strong, very little is known about the mechanistic links between exposure to a single moderate or severe TBI or repeated mild TBI (r-mTBI) and later development of neurodegenerative disease, in particular, chronic traumatic encephalopathy (CTE)[3-12]. Although the polypathology of CTE is complex and multifaceted, the current preliminary pathological diagnostic criteria for CTE have been defined, with the presence of perivascular accumulation of phosphorylated tau (p-tau) in neurons and glia in a patchy distribution at the depths of cortical sulci regarded as pathognomonic[13]. However, thus far, no published experimental models of single or repetitive mTBI fully replicate the pathological characteristics of this neurodegenerative condition, in particular the spectrum of tau pathologies described, including the pathognomonic lesion.

Although several animal models of repetitive mTBI are described[5, 14-20], few studies report any evidence of sustained tau pathology at time points beyond the acute, post-injury period (for a review see Ojo et al; 2016)[21]. In previous work, we have reported evidence of phosphorylated tau (p-tau) in two different model of r-mTBI: at 21 days following 5 r-mTBIs at 48h apart in "aged" hTau mice and at 3 months post-32 r-mTBI 72h apart in young hTau animals[15, 22]. Persistent tau pathology at 6 months post-injury has also been observed in wild type mice exposed to 42 r-mTBI over 7 days, thus suggesting that a number of impacts may be required to trigger sustained tau pathology[23].

Since the pathological hallmarks of Alzheimer disease (AD) including amyloid plaques and neurofibrillary tangles (NFTs) have been reported in some TBI patients, the use of transgenic models of AD and mutated tau have also been proposed to investigate TBI pathogenesis. In this regard, Brody and colleagues showed that AD (3xTg-AD) and mutated Tau (P301L) mouse models demonstrate an acute increase in tau immunoreactivity and tau phosphorylation up to 2 weeks after controlled cortical impact (CCI) injury[10]. Because the cumulative effects of r-mTBI may cause the development of CTE in humans, in a recent study, Winston et al, 2016 also explored the effects of r-mTBI in the same 3xTg-AD mice[20]. However, there was no evidence of increased phospho-tau at either 1 or 30 days after repeated mild Injury. The caveats of these models are that tau mutations are rare in the general population, tau is not present at physiological levels in mutant mice (p301L) and the models investigated thus far (e.g. WT, Tg44, p301L) do not express each human tau isoform or on a null murine background. Consequently, it is conceivable that none of these models adequately recapitulate the human tau-dependent response to TBI.

To address limitations in previous studies, in this study we address whether single or r-mTBI is associated with an acute and/or chronic p-tau pathologies in hTau transgenic animals expressing 6 isoforms of human, non-mutated tau on a null murine tau background[24, 25], and whether these changes correlate with altered neurobehavioral performance following injury. To this end, we will use our previously characterized closed head injury model[14, 26, 27] and repeat the same experimental procedures (e.g. Injury paradigm, age at time of injury, inter injury interval, etc.) including behavioral, pathological, and biochemical analyses in hTau transgenic mice.

Materials and Methods:

Animals:

Male mice expressing human tau (hTau) on a C57BL/6 and null murine tau background[24, 25] (aged 10-12 weeks, 20-24g, 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 throughout the study. These hTau mice were generated by crossing mice that express a tau transgene derived from a human PAC, H1 haplotype, known as 8c mice[25], with tau knockout (KO) mice in which cDNA for the enhanced green fluorescent protein (EGFP) was inserted into exon one of tau. The resulting hTau mice express all 6 human tau isoforms, but do not express mouse tau[24]. Of note, the physiological tau isoform ratio (3R/4R) in the hTau mice is not 1:1 as in human, rather it is 4:1[24] ~~(Peter Davies, *pers. comm.*)~~, but they are still closer to mimicking physiological occurrence of human tau than wild type mice or other tau transgenic models which express single isoforms of either mutated or wild type tau. All procedures were carried out under Institutional Animal Care and Use Committee approval and in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Injury groups and schedule:

For the behavioral analyses a total of 36 mice were randomly assigned to one of four treatment groups: single injury, single sham, 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 24 h or 12 months after the sole/last mTBI/anesthesia for each group. Behavior outcomes were assessed blinded to group assignment. Following the acute time point

of behavioral analyses, this cohort of mice was then allowed to survive for analyses at 6 and 12 months post-injury. One animal from the r-sham group died from natural cause before the last behavioral measurement. There was no mortality rate among both mTBI groups. Separately from this behavioral cohort, two other cohorts of hTau animals were assigned for pathological (n=20) and biochemical (n=20) examination. As before, the animals were randomly assigned to one of the 4 treatment groups, with 5 mice per group for pathology and biochemistry. All mice for pathological and biochemical analyses were euthanized at 24 h or 12 months post-sole/last mTBI/anesthesia.

Injury protocol:

The mTBI was administered to mice as previously described[14]. Mice were anesthetized with 1.5 L/min of oxygen and 3% isoflurane prior to anesthesia or mTBI. A 5 mm blunt metal impactor tip was retracted and positioned midway relative to the sagittal suture before each impact. The injury was triggered using the myNeuroLab controller at a strike velocity of 5m/s, strike depth of 1.0 mm, and dwell time of 200 ms. Sham injured animals underwent the same procedures and were exposed to anesthesia for the same length of time as the mTBI animals.

Assessment of Cognitive Function:

Learning and memory were evaluated using Barnes maze and the Ethovision XT software (Noldus) by monitoring the distance and time taken to find an escape hole over a period of 6 acquisition days (learning) and a final probe trial (memory). During the acquisition trials, mice were given 90 sec to locate and enter the target box. Mice were led to the target box if they did not locate it within 90 sec, and all mice were required to remain in the target box for 30 sec prior to retrieval, regardless of success. 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, a single probe trial lasting 60 sec was performed with the mouse starting from the center of the maze and the target box removed.

Assessment of Anxiety:

Anxiety-related and risk-taking behavior of mice were evaluated using the elevated plus maze (EPM) test, which relies on the animal's preference for dark enclosed arms rather than brightly lit, open arms at an elevated height. Time spent in the open arm 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 4 arms intersecting at a 90° angle, creating 4 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-minute 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 as well as the time spent in each arm. All four paws of the mouse had to enter an arm for it to be considered an entry, as 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]) x 100.

Histology:

At 24 h after their last injury/anesthesia the 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 in paraffin using Tissue-Tek VIP (Sakura, USA). Sagittal (n = 5 brains/group) and 6 µm sections were cut with a microtome (2030 Biocut, Reichert/Leica, Germany) and mounted on positively charged glass slides (Fisher, Superfrost Plus). Prior to staining, sections were deparaffinized in xylene, and rehydrated in an ethanol to water gradient. For each group, sets of sagittal (lateral 0.2–0.4mm) sections were cut. Each slide was visualized with a bright field microscope (Leica, Germany) and digital images were taken for further analysis and quatification.

Immunohistochemical quantification:

For each animal, (n=5 per group at 24h and 12 months post-mTBI), 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 counter stain and DAB) using the color deconvolution algorithm. Three non-overlapping 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 non-overlapping areas of 150 μm^2 between layer III and IV in the primary somatosensory cortex, and 3 non-overlapping 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. A total of 5 slides per animal were averaged for each immunohistochemical analysis.

For Tau immunohistochemistry sections were stained with the following monoclonal antibodies at a 1:400 dilution: CP13 [pS202]; PHF1 [pS396/404]; RZ3 [pT231] (Fig.1A). MC1 is a conformation-dependent antibody that reacts with both the N terminus (amino acids 7–9), and an amino acid sequence of tau in the third MTB (amino acids 313–322) that is necessary for *in vitro* formation of filamentous aggregates of tau similar to those seen in AD[28] (Fig.1B). These Tau antibodies and protocols were generously provided by Dr. Peter Davies, The Feinstein Institute for Medical Research, Bronx, NY. A summary of antibodies used for these neuropathological analyses is shown in table 1. Changes in CP13 and RZ3 immunoreactivity were calculated and expressed as a percentage of the field of view within the pyramidal cell layer of the CA1 and CA3 sub-regions of the hippocampus.

For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, the in-situ cell death detection kit (Roche Diagnostics, Indianapolis, IN) was used following the manufacturer's guidelines. Labeling was performed with DAB as the chromogen. To avoid bias, positive and negative controls were included to show nonspecific binding/reaction.

Biochemical assessment of phosphorylated and total tau protein:

Mice were exsanguinated via aortic puncture using a wide-bore needle to prevent hemolysis of red blood cells. Immediately after cardiac puncture, mouse brains were perfused with chilled 1X PBS for 1 min to eliminate the confounding effects of blood proteins present in

the brain vasculature. Brains were dissected at 4°C into hemispheres, then cortices, hippocampi and cerebella, and then snap frozen in liquid nitrogen.

Phosphorylated tau and total tau protein were analyzed in the hemisected hippocampi and cortices obtained from all groups (n=5/group). Snap-frozen hemisected 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 protease inhibitor cocktail (Roche, Indianapolis, IN). Biochemical analyses were carried out by our collaborators at the Feinstein Institute for Medical Research, Bronx, NY, to whom we sent coded tissue homogenates for quantitative assessments. Sample preparation for low-tau sandwich ELISA and quantitation of murine-specific tau protein were performed as previously described. Total tau DA31, CP13, PHF1 and RZ3 were used as capture antibodies in the low-tau, Sandwich ELISA. Data were expressed as µg tau/mg protein (tau and phosphorylated tau).

Statistics:

Behavioral data were analyzed using JMP 8.0 (SAS, Cary, NC) as previously published[14]. Quantitative histologic parameters were analyzed with one-way ANOVA, with a Tukey's post-hoc correction for multiple comparisons, unless indicated. 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:

Barnes maze acquisition:

To investigate whether hTau animals are more sensitive to single or r-mTBI than wild type animals, we examined their learning and cognition under the same testing condition as we previously reported[14, 26, 27, 29]: Barnes maze at 2 weeks, 6 and 12 months post-mTBI/anesthesia. Acute acquisition deficits were only observed in the repetitively injured group relative to their sham controls (Fig 2A; r-mTBI vs. r-sham, $p < 0.05$, repeated measures ANOVA). The mean distance travelled over the 6 days acquisition period in the r-mTBI mice was 24% longer than the r-sham mice. There was no difference between the s-mTBI, s-sham and r-sham groups at the acute time point ($p > 0.05$, repeated measures ANOVA). At chronic time-points, both injured groups traveled on average longer distances to reach the target box when compared to their respective sham groups (Fig 2; s-mTBI vs. s-sham, $p < 0.001$; r-mTBI vs. r-sham, $p < 0.0001$ and s-mTBI vs. s-sham, $p < 0.0001$; r-mTBI vs. r-sham, $p < 0.001$, respectively at 6 and 12 months after injury; repeated measures ANOVA). The average distance traveled by the s-mTBI mice over the 6-day acquisition period was 32%, and 48% longer than the s-sham mice at 6 and 12 months after injury, respectively. At the same time points, the r-mTBI had a greater and progressive separation on average distance traveled (62% and 28%, respectively) when compared to the r-sham. Analyses of the Barnes maze tracks on the last day of acquisition revealed that the travel path of the r-mTBI animals was more circuitous, searching in all quadrants when compared to the path of the shams of singly injured animals (data not shown).

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. No difference in escape latency performance was observed across all groups at the acute time point ($p > 0.05$) (Fig.2). However, when tested at 6 and 12 months after injury, both injured group showed a progressive decline in escape latency performance. At 6 months after injury, the mean escape latency of the s-mTBI mice improved from 87.2 seconds on the first day of acquisition to 42.7 seconds on their last day of acquisition. By 12 months after injury, they spent on average 89.8 seconds on the first day of acquisition and 59.9 seconds on their last acquisition day. In contrast, even at the 6-month time point the r-mTBI mice exhibited less improvement over the 6 days than the other groups (see Fig 2D; days 1 and 6 of acquisition; r-mTBI, 89.2 seconds and 69.2 seconds; r-sham, 83.9 seconds and 42.2 seconds; $p<0.0002$); this lack of improvement during acquisition persisted at 12 months after injury (75.2 seconds and 66.6 seconds; $p>0.05$). The average velocity was similar across all groups and all-time points (data not shown).

Barnes maze: probe:

The probe trial analyses determined the average time to reach target zone, (defined by the target escape hole). At 2 weeks and 6 months post-injury, the analyses revealed that the r-mTBI mice performed the worst, followed by the r-sham, the s-mTBI, and the s-sham (Fig.3). While there was a trend for both injured groups to perform worse than their respective sham groups, the time to reach the target or adjacent holes did not reach statistical significance ($p > 0.05$ for both injury groups; ANOVA). Probe test performance was only markedly impaired in the r-mTBI mice compared to both control groups at 12 months post-injury, requiring on average 32 seconds to reach the target zone, as determined by 1-way ANOVA followed by

Tukey *post-hoc* test (p > 0.05). The mean velocity for the probe trial was similar across all groups (p > 0.05).

Elevated plus maze:

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 (**Sup Fig. 1**). There was no difference in the percentage of time spent in the open arms across all groups. (s-sham, 32.72±13.17%; r-sham, 39.77±12.12%; s-mTBI, 36.19±11.76%; r-mTBI, 48.15±12.12%; p>0.05 one-way ANOVA followed by Tukey's *post-hoc* test).

Macroscopic pathology:

Consistent with previous experience, there were no skull fractures, cerebral hemorrhages, or contusions identified using this injury model in hTau mice, save for evidence of focal microhemorrhage (<1 mm^2) in the inferior surface of the cerebellum in all animals subjected to r-mTBI. Otherwise, the brains of the hTau animals had no gross macroscopic differences when compared to the wild type animals.

Glial fibrillary acidic protein immunostaining:

To determine if a similar astroglial response occurs in the brains of the hTau animals as we observed in the brain of the WT mice[14, 26, 27, 30], histological analyses were performed in the brain regions known to be affected in this model of mTBI. For all groups, the cortical region underlying the impact site (somatosensory and primary motor cortices), the corpus callosum, and the hippocampal regions were assessed in sections stained for GFAP. No astrocytes with morphological appearances of reactive glia, manifest as GFAP immunoreactive astrocytes with

thickened cell processes and hypertrophied cell soma, were observed in the cortex or in hippocampal sector CA1 (data not shown) at 24h or 12 months following single or repetitive anesthesia shams. Regarding the corpus callosum, the r-mTBI group showed an increased GFAP immunoreactivity at both 24h and 12 months post-injury (significant main effect of treatment by ANOVA $F(3, 32) = 10.63$ $p < 0.0001$; post hoc comparison shows difference between r-mTBI 11.2 ± 2.5% vs. r-sham 2.3 ± 0.4% at 24h post-injury and a significant main effect of treatment by ANOVA $F(3, 74) = 8.369$ $p < 0.0001$; post hoc comparison shows difference between r-mTBI 10.9 ± 1.1% vs. r-sham 4.8 ± 0.5%; $p < 0.05$ at 12 months post-injury; $p < 0.05$; Fig.4,5). Notably, although there was no measurable increased GFAP immunoreactivity in the corpus callosum at 24h post-injury when compared to their corresponding sham, there was a mild increase in immunoreactivity at 12 months post-injury (s-mTBI 3.0 ± 0.9% vs. s-sham 3.6% ± 0.45% at 24h post-injury and s-mTBI 7.2 ± 1.1% vs. s-sham 4.6 ± 1.0%; $p < 0.05$ at 12 months post-injury; $p < 0.05$; Fig.4,5). In the region of the cortex underlying the impact site a mild reactive astrogliosis was only observed at 24h post-injury in the r-mTBI (data not shown). In the CA1 region, there was no evidence of increased immunoreactivity within the injured groups (data not shown).

Amyloid precursor protein immunostaining:

Numerous APP-immunoreactive axonal profiles were identified in sections from both injury groups (Fig.4,5). These APP-immunoreactive axonal profiles were observed as either granular or more elongated, fusiform swellings in the white matter of the parasagittal cortex, including the splenium, body and genu of the corpus callosum, and the spinal trigeminal tracts of the brain stem (BS). APP-immunoreactive axonal profiles were observed at 24 h and 12 months post-injury in the CC of the s-mTBI (Fig.4,5F) and r-mTBI groups (Fig.4,5H) but not in their

controls (Fig.4,5EG). The numbers of APP-immunoreactive profiles in the CC of the s-mTBI was greater than in the r-mTBI group at 24h post-injury (s-mTBI group 18 ± 2.26 vs. r-mTBI 14 ± 0.8 axonal profiles/100 μm^2; $p < 0.001$), contrasting with higher numbers of positive profiles in the r-mTBI when compared to s-mTBI at 12 months (s-mTBI group 3.4 ± 0.9 vs. r-mTBI 11.5 ± 1.4 axonal profiles/100 μm^2 $p < 0.001$) (Fig. 4,5). At 24h post-injury, axonal damage in the BS was minimal in the s-mTBI, whereas greater numbers of punctate immunoreactive swellings were present in the r-mTBI group. There was no evidence of ongoing axonal injury in the brain stem at 12 months post-injury.

Ionized calcium binding adaptor molecule 1 immunostaining:

No evidence of ongoing, active neuroinflammation was identified in sections stained for ionized calcium binding adaptor molecule 1 (Iba1) in the hTau sham animals at any timepoint (Fig.4,5). In the single injury animals, both resting and activated microglia (with a bushy morphology) were observed at 24h post-injury in the CC (significant main effect of treatment by ANOVA $F(3, 68) = 79.84$ $p < 0.0001$; and post hoc comparisons shows difference between s-mTBI 6.22 ± 0.9% vs. s-sham 3.22 ± 0.9%; $p < 0.05$; Sup Fig. 2) and in the cortex (Fig.4J) (significant main effect of treatment by ANOVA $F(3, 68) = 60.58$ $p < 0.0001$; and post hoc comparisons shows difference between s-mTBI 6.2 ± 0.9% vs. s-sham 4.01 ± 0.3%; $p < 0.001$; Fig.4J). For mice subjected to r-mTBI, immunostaining for Iba-1 revealed clusters of activated microglia in the CC (significant main effect of treatment by ANOVA $F(3, 68) = 79.84$ $p < 0.0001$; and post hoc comparisons shows difference between r-mTBI 13.7 ± 1.3% vs. r-sham 2.21 ± 0.6%; $p < 0.0001$; s-mTBI 6.22 ± 0.9% vs. r-mTBI 13.7 ± 1.3%; $p < 0.05$; Sup Fig. 2), and microglia with a bushy morphology in the region of the cortex underlying the impact site (significant main

effect of treatment by ANOVA $F (3, 68) = 60.58$ $p < 0.0001$; and post hoc comparisons shows difference between r-mTBI 10.4 ± 1.3% vs. r-sham 2.01± 0.5%; $p < 0.0001$; s-mTBI 6.2 ± 0.9% vs. r-mTBI 10.4 ± 1.3%; $p < 0.05$; Fig.4L). By 12 months post-injury, there was no evidence of activated microglia in the cortical region of any groups. However, for the single and r-mTBI, increased microglial activity in the splenium and body of the corpus callosum was observed when compared to corresponding shams (r-mTBI 10.4 ± 1.3% vs. r-sham 3.01± 0.5%; $p < 0.0001$; s-mTBI 6.1 ± 0.9% vs. s-sham 3.4 ± 0.7%; $p < 0.05$; Sup Fig. 2).

Tau immunohistochemistry in the cortex:

No TBI-dependent increase in cortical soluble p-tau CP13 (Fig.6A-C), RZ3 (Fig.6D-F) or PHF1 (Fig.6G-I) was observed at 24h or 12 months post-injury measured by ELISA analyses ($p>0.05$; one-way ANOVA). Nonetheless, an age-dependent increase for each soluble p-tau CP13 and RZ3 immunoreactivity was observed between 24h and 12 months post-mTBI in the cortices of all groups ($p<0.01$; one-way ANOVA followed by Tukey's post-hoc t-test). However, in animals subjected to single and repetitive mTBI, an increase of CP13, RZ3 and PHF1 immunoreactivity was observed in cell bodies and apical dendrites of occasional cortical neurons underlying the impact site at 24h post-injury, while no cortical neurons were labeled in control groups (Fig.6C,F,I). These p-tau neurons was not persistent at the chronic time point of 12 months after injury. There was no increase of p-tau when quantitated as a ratio of p-tau protein to total tau protein with ELISA (Fig.6B,E,H).

Tau immunohistochemistry in the hippocampus:

The low-tau ELISAs that quantitatively assess different epitopes of tau did not reveal any TBI-dependent increase in cortical soluble p-tau CP13 (Fig.7A-C), RZ3 (Fig.7D-F) and PHF1 (Fig.7G-I) at 24h and 12 months post-injury ($p>0.05$; one-way ANOVA). However, qualitative observation of the IHC-stained sections revealed a trend for increased immunostaining for CP13 and RZ3 within the CA1 and CA3 sub-region of the hippocampus in both injured groups when compared to their respective shams at 24h post-injury. Generally, the r-mTBI group showed greater dendritic and membranous staining than the other groups. In addition, p-tau was also quantitated as a ratio of phosphorylated tau protein to total tau protein. However, no change was observed for p-tau CP13, RZ3 and PHF1 in sham and injured animals (Fig.7B,E,H).

Soluble DA31 (Total tau) ELISA and aggregated tau MonoELISA DA9-DA9HRP:

In all experimental groups, at both 24h and 12 months after mTBI/anesthesia, there was no TBI-dependent increase in cortical or hippocampal soluble tau (Sup Fig.3; $p>0.05$) and aggregated tau (Sup Fig.4; $p>0.05$). An age-dependent increase in aggregated tau was observed between 24h and 12 months post-in the cortex (Sup Fig.4; $p<0.001$).

Amyloid Biochemistry/Immunohistochemistry:

No evidence of amyloid pathology was identified in r-mTBI injured animals (diffuse or neuritic Aβ plaques), as determined by histopathology in sections from cortex or hippocampus stained for 4G8 or Congo red at 24h or 12 months after mTBI/anesthesia (data not shown). Further, ELISA analysis of soluble murine Ab40 showed no TBI or age-dependent increases (data not shown).

TUNEL:

Pyramidal neurons of the hippocampus, and neurons that stained positive for Tau in the cortex, were devoid of TUNEL positive staining, indicating that there was no apoptotic programmed cell death in either sham or injured animals (data not shown).

Discussion:

Herein we report observations on acute and chronic effects of single and r-mTBI in a transgenic mouse model to explore the potential role of tau pathobiology after mTBI. Our data show that single and r-mTBI induced a modest increase in the cortical soluble fraction of three different p-tau epitopes: CP13, PHF1 and RZ3 that resorbed by 12 months post-injury. This increase was not associated with worse behavioral performance when compared to our previous reports in wild type animals with the same experimental design[14, 26, 27]. Secondly, we showed that the post-TBI neuropathology in hTau and wild type mice was otherwise comparable, with essentially similar levels of axonal injury and chronic neuroinflammation in both study groups.

Very little is known about the mechanisms involved in the accumulation of tau proteins observed in human cases late after mTBI. Evidence from a preclinical model of TBI demonstrates rapid formation of neurotoxic oligomeric and p-tau aggregates that may be followed by a toxic gain of function[31]. Therefore, we questioned whether r-mTBI could increase soluble p-tau in our model at 24 h post-injury and if these changes were transient or sustained; possibly leading to pre-tangle formation. Our data confirm an increase in CP13 (pS202) and RZ3 (pT231) acutely post-injury, however, this change was transient. Further work is required to address the time course and the presence if any, of other tau species such as soluble small

oligomers, and insoluble tau species. However, the absence of aggregated tau and MC1 immunostaining suggests that our injury paradigm does not trigger the formation of toxic tau conformational species at 24h and 12 months post-single and r-mTBI.

Our immunohistochemical observations showed an increase in somatodendritic phosphorylated tau (CP13, PHF1, RZ3) in the superficial layer of the cortex at 24h, but not at 12 months in injured animals. Supporting these observations, ELISA of the cortical homogenate also revealed a trend toward an elevation of phosphorylated tau epitopes pS202 and pT231 at 24h but not at 12 months post-injury. In the hippocampus, despite subtle changes in p-tau levels as determined by ELISA, we observed a dramatic increase in p-tau immunoreactivity to the pyramidal layer of the hippocampus, but again these changes were transient in nature suggesting that our rodent injury paradigm does not replicate the pathology that as has been described many years after r-mTBI in human CTE cases.

In our model we used hTau mice that expresses all six human tau isoforms on a null murine background. However, unlike humans that express 3R:4R tau isoform ratio at (50:50), they have been reported to express a higher 3R:4R tau isoform ratio (4:1)[24]. Changes in the tau isoform ratio are associated with distinct tauopathies, for example, a shift toward an increase of 4R isoforms is observed in patients with progressive supranuclear palsy or argyrophilic grain disease, whereas a shift toward an increase of 3R is associated with frontotemporal dementia with parkinsonism-17 (FTDP17)[32]. The effects of some of the FTDP-causing mutations in tau (for example; P310L, V337M and G272V) have been extensively studied in transgenic mice that develop widespread NFTs[33, 34], and these studies as well as in vitro cell culture models suggest, that the 4R tau isoform is potentially a more favorable substrate for aberrant

hyperphosphorylation, and also has the propensity to self-aggregate into filaments more readily at a lower phosphorylation stoichiometry[35]. Therefore, the significant imbalance in the 3R:4R tau ratio in our hTau mouse model may be an important factor in the appearance of tau pathology following injury and should be considered in the interpretation of our data.

Based on these results, it remains unclear if the acute increase of cortical tau phosphorylation originates from the endogenous pool of tau from formerly healthy axons damaged by injury, or from tau newly synthesized as a response to cytoskeletal damage. While further work is required to answer this question, TUNEL staining revealed no apoptotic neurons in the cortex or hippocampal regions in sham or injured animals. This suggests that neurons showing enhanced somatodendritic phosphorylated tau staining are not apoptotic. Owing to its known involvement in AD pathogenesis, tau has been considered in general as a disease-causing agent. However, the potential link between a transient surge of tau hyperphosphorylation, as observed after TBI, and neurodegeneration has not yet been clearly defined. In fact, evidence suggests that tau phosphorylation at certain sites (e.g. KXGS motifs) may protect against tau aggregation[36]. In addition, Arendt and colleagues revealed that hyperphosphorylation of tau in hibernating animals reverses rapidly upon arousal from torpor[37]. Similar to these observations from hibernating animals, tau hyperphosphorylation resulting from TBI might potentially involve an intrinsic response to protect the neurons from further damage through mechanisms that stabilize the cytoskeleton and synaptic structure[38, 39].

Other pathological features seen in human CTE cases, such as perivascular tau pathology, neuropil threads and astrocytic tangles[6], were not observed in our mouse model of mTBI. It is possible that these differences between human CTE cases and our mouse model

may be constrained by differences in the severity and frequency of injury, interspecies differences; including the rodent inherent ability in clearing hyperphosphorylated tau, the craniospinal angle, the significantly greater deformability of the murine skull, and the white to grey matter ratio in mice and humans. Overall, these data seem to suggest that tau hyperphosphorylation at acute time points post-injury may be a normal physiological response of the brain following mTBI.

Our findings herein also raise the question concerning the link between anesthesia exposure and increased tau phosphorylation, which have been reported on several occasions in the literature[40-42]. Indeed, a trend for lower spatial memory performance (Barnes maze probe trial) was observed in the r-sham group. The pathological examination also revealed a tendency for increased tau phosphorylation in mice with to multiple anesthesia exposures. This trend was not observed in our previous study in aged hTau mice[22]. This could be explained by the transient increase of tau phosphorylation that resorbs within weeks post-exposure[43] or by the fact that aged tau mice have such high levels of phosphorylated tau that they supersede that which could be induced by anesthesia.

When tested for behavioral performance in the Barnes maze, the r-mTBI group showed impaired learning consistent with our mTBI study in WT mice at both time point tested (24h and 12 months post-injury)[26]. However, in contrast to our previous study, the single injury paradigm failed to demonstrate any significant effect on the behavior of the hTau mice as they performed at the same level as their sham control counterparts at the acute timepoint (in terms of escape latency and distance travelled). Alterations in genetic background of the animals could be the cause of such differences, because mice which have been genetically

manipulated are known to perform differently in certain behavioral tests. It is interesting to note that the overall performance of the young hTau mice was superior to the young WT groups as they were able to find the hidden box with the shortest distance and time travelled when compared to WT animals over the acquisition trial phase, although they performed worse on the probe trial at both time points. We did not observe an increase in the time spent in the open arms of the maze at the chronic time point suggesting that the mice do not express risk-taking behavior at this stage.

To date, many reports on human pathology in CTE have consistently focused on the development of p-tau pathology years following exposure to TBI[6, 44, 45]. However, the majority of these studies have been retrospective in nature and from convenience samples of clinically symptomatic cases with inherent biases in case selection. The experimental data presented here and in previous pre-clinical models continue to suggest neuroinflammation and axonal injury, rather than tau pathology, have a significant role in the neuropathological events following single or repetitive mTBI; these pathologies in turn showing strong correlation with the acute and chronic behavioral changes observed post-injury.

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