Chronic repetitive mild traumatic brain injury results in reduced cerebral blood flow, axonal injury, gliosis, and increased T-tau and tau oligomers.

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ABSTRACT

Exposure to repetitive mild traumatic brain injury (mTBI) in athletes is recognized as a risk factor for chronic traumatic encephalopathy (CTE), marked by the deposition of hyperphosphorylated tau aggregates in neurons and astrocytes in a patchy distribution at the depths of cortical sulci. We have developed a new mTBI paradigm to explore chronic effects of repetitive concussive-type injury over several months in a hTau mouse model with human tau genetic background. Mice were exposed totwo injuries weekly, over a period of 3 or 4 months and compared to non-injured, sham animals. Behavioral, in vivo measures and a detailed neuropathological assessment were conducted 6months post-first injury. Our data confirm impairment in cerebral blood flow and white matter damage. This was accompanied by a two-fold increase in total tau levels and mild increases in tau oligomers/conformers and pTau (Thr231) species in the grey matter. There was no evidence of neurofibrillary/astroglial tangles, neuropil threads, or perivascular foci of tau immunoreactivity. Neurobehavioral deficits were observed in mTBI animals (i.e. disinhibition and impaired cognitive performance). These data support the relevance of this new mTBI injury model for studying consequences of chronic repetitive mTBI in humans, and role of tau in TBI.

Key words: Concussion, hTau mice, cerebral blood flow, behavior, phospholipids, cytoskeletal proteins, Tau, glial activation, and axonal injury.

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INTRODUCTION

Exposure to traumatic brain injury (TBI) is recognized as a risk factor for later development of chronic neurodegenerative disorders, in particular chronic traumatic encephalopathy (CTE) (1-13).First described in boxers (14), CTE is now increasingly recognized in autopsy series of non-boxer athletes from a wide range of sports, including American football (11,13,15-16), ice hockey (13), soccer (13,17) and rugby (18). Though the neuropathology of CTE is complex and multi-faceted (9), common to the cases described thus far are abnormal deposits of hyperphosphorylated tau in perivascular neurons and glia and in a patchy distribution towards the depths of cortical sulci (13; NINDS workshop- CTE consensus report). Furthermore, blows to the head during a single boxing bout are associated with increased total tau in the spinal fluid (19,20) and athletes (ice hockey) and military personnel exposed to a history of TBI demonstrate an increase in total tau and tau-C cleaved fragments in the plasma (21-23).

The precise incidence and prevalence of CTE, including the threshold of susceptibility to CTE following exposure to TBI, is unknown. Typically, reports of CTE have come from individuals exposed to repetitive mTBI through contact related sports injuries, though the pathology has also been reported in individuals exposed to single moderate or severe TBI (9,24). Though data on longitudinal follow up is lacking, in the cases reported thus far the mean duration of exposure to mTBI in an athlete's career ranged between 5 and 24 years (11,25). In addition, a majority of these athletes were exposed to mTBI from their high school years to their late twenties, and some during their mid to late thirties (13,25), with the suggestion that extent of pathology at autopsy correlates not only with age at death and number of years after retirement, but also with length of exposure to injury(13,26).

At present there are no published epidemiological, longitudinal, cross-sectional, or prospective studies involving CTE. Thus far, all published autopsy cases have been retrospective studies typically involving small sample sizesand subject to selection bias and incomplete pre-mortem data. Together, these limitations make it difficult to derive a comprehensive understanding of the neuropathological sequelae of exposure to TBI. Further, many current pre-clinical models fail to recapitulate the clinical and neuropathological consequences of mTBI, the cumulative exposures typical of human sports-associated injuries or the pathologies of CTE. Therefore, there is a critical need to develop an animal model that is relevant to lifetime exposure, sports-associated, repetitive mTBI in humans.

To date, different animal models have been developed to explore the effects of repetitive TBI (26-39), most of these experimental models utilize a closed skull mTBI paradigm involving a pneumatic or electromagnetic impactor or weight drop model. However, though these models have described consistent pathologies, such as white matter degradation and gliosis, findings in relation to tau pathologies have been less consistent, with some reporting increases in multiple pathogenic tau species following injury (27,28,32,34,36,39), while others do not (29-31,33,38). Moreover, in most of those studies that report evidence of tau dependent TBI pathology, the authors only examined acute time points after-injury, with only one study showing evidence of persistent tau pathology at chronic timepoints six months after injury (36). Our previous mTBI model in "aged" hTau mice that express the six isoforms of human tau on a null murine background demonstrated augmentation of tau pathology 3weeks after injury, however, this persistent tau pathology was not observed in younger hTau or wild-type mice exposed to mTBI following examinations up to 12 months post-injury (29,33,39, Mouzon et al., 2016 pers comm).It appears therefore that persistent tau pathology is lacking in most TBI models. Additionally, in all the models to date that have depicted increases in intra-axonal or intrasomal phosphorylated-tau (p-tau), other components of CTE-like pathology were absent, such as neurofibrillary or astroglial tangles, neuropil threads and perivascular p-tau; these difficulties, and relatively few successes, in recapitulating tau-associated pathologies after TBI perhaps reflecting failure to adequately model the required variables in human injury exposure, such as chronicity of impacts, age at exposure or duration of exposure to injuries(40).

In this study we set out to develop a new paradigm for our previously established, successful mouse model of mTBI (29,33,35,39), in order to focus primarily on two main aspects: (i) the chronic pathological consequences following cumulative exposure to repeated mTBI over a prolonged exposure period, and (ii) the influence of a human tau genetic background on "tau pathology" following this exposure protocol in mTBI. With regard to the latter, we used the hTau mouse model (41) in which we previously demonstrated TBI-dependent tau pathology in 'aged' animals exposed to mTBI (39). In addition to examining protein biochemistry profiles in our model, we also present supplementary data characterizing brain lipid and peripheral inflammatory profiles and neurobehavioral measures.

In this first report, we confirm a two-fold increase in total tau levels and mild increases in tau oligomers/conformers and pTau (Thr231) species in the grey matter up to three months after cessation of injury, which may be on a continuum of progressive and persistent TBI dependent tau pathology.

METHODS

Animals

Transgenic mice expressing Human Tau on a C57BL/6 and null murine tau background (generated as previously described - 41) were purchased from Jackson Laboratories, Bar Harbor, ME. All mice were12 weeks old at the start of this study (average weight, 18 g). Animals were housed in standard cages under a 12-hour light/12-hour dark schedule at ambient temperature controlled between 22°C and 23°C under specific pathogen free conditions. Animals were given food and water ad libitum and maintained under veterinary supervision throughout the study. There was no evidence of disease among the colony. Mice of both sexes were randomly assigned to experimental groups (n=12 each for sham and injured animals). Two animals in the injury group were euthanized due to development of severe dermatitis of unknown reasons. Experiments were performed in accordance with Office of Laboratory Animal Welfare and National Institutes of Health guidelines under a protocol approved by the Roskamp Institute Institutional Animal Care and Use Committee (IACUC). All analyses were carried out blind to study group assignment.

Experimental mTBI

The experimental TBI methods were performed as previously described (29). Briefly, mice were anesthetized with 1.5 L per minute of oxygen and 3% isoflurane for 3 minutes. After shaving of the injury site, mice were transferred into a stereotaxic frame (Just For Mice Stereotaxic, Stoelting, Wood Dale, IL) mounted with an electromagnetic controlled impact device (Impact One Stereotaxic Motorized Impactor, Richmond, IL). Heads were positioned and fixed in the device, which prevented lateral movements as the impact was delivered. All mice were placed on a heating pad to maintain their body temperature at 37°C. A 5-mm blunt metal impactor tip attached to the electromagnetic motorized device was zeroed on the scalp and positioned above the midsagittal suture before each impact using the NeuroLab controller. On satisfactory positioning, the tip was retracted and the depth was adjusted to the desired level. The scalp was gently stretched by hand to restrict lateralization of the impact and to prevent the rod from delivering an inadequate trauma load at an irregular angle. Injury parameters were 5 m per second strike velocity, 1.0 mm strike depth, 200 milliseconds dwell time, and a force of 72N. This sub-lethal impact does not cause direct tissue damage to the injury site, and there is no development of skull fracture or subdural hemorrhage, even after repetitive injuries. Mice in the r-mTBI group received 2 impacts every week for 3 or 4months (i.e. 24 or 32 impacts), with an inter-injury time

of 72-96hrs. Repetitive sham control mice received anesthesias of the same frequency and duration (~3mins per session) as their r-mTBI counterparts. Animals were grouped as repetitive shams or repetitive injury. This mixed paradigm was chosen to mimic the heterogeneity of cumulative mTBI exposures in the human setting. After each impact was delivered, the mice were allowed to recover on a heating pad set at 37°C to prevent hypothermia. On becoming ambulatory, mice were returned to their cages and carefully monitored for any abnormalities.

Three chamber test for social interaction and novelty recognition test

All neurobehavioral tests were conducted 6 months post first injury. Two social behaviors (social interaction and social memory/novelty recognition) were quantified using a rectangular three-chamber test that includes a middle chamber with two doors leading to two separate (left and right) chambers, each containing a steel cage enclosure. After 5 minutes of habituation in the three chamber compartment, each mouse (experimental subject) was placed in the middle chamber and allowed to freely explore for 10 minutes, with the right chamber empty but an unfamiliar congener (Stranger I) held in the steel cage enclosure in the left chamber. Social interaction was determined by measuring the number of entries by the experimental subject into the chamber holding the unfamiliar congener vs the empty chamber. To measure social memory (or novelty recognition) a new novel stimulus mouse (stranger II) was subsequently placed in the previously empty right chamber. The same parameters as above were measured to determine the preference of the experimental subject for stranger I or stranger II.

Elevated plus maze

The elevated plus maze consists of a plus shaped apparatus with two open and two enclosed arms, each with an open roof, elevated 50-70cm from the floor in a dimly lit room. Each mouse was placed at the junction of the four arms of the maze, facing the open arm. The mouse was allowed to freely maneuver within the maze for 5 mins; number of entries and duration in each arm (open/closed) were recorded with the aid of a video tracking system to evaluate anxiety effects based on a rodent's aversion of open spaces.

Open field test

The open field was conducted in a large circular maze (120cm diameter) setup in a brightly lit room. Animals were placed in the center of the maze and the number of entries/time spent in a predefined center zone and around the walls of the maze was recorded over a 15 minutes trial to evaluate anxiety effects and sensorimotor activity.

Cerebral blood flow measurements

Cerebral blood flow (CBF) was measured prior to euthanasia, six months post-first injurytimepoint. For CBF measurement, mice were anesthetized with a gas mixture of 3% isofluorane, 0.9 l min^{-1} nitrous oxide and 0.5 l min^{-1} oxygen. Animals were then immobilized on a mouse stereotaxic table and maintained under anesthesia with a mouse anesthetic mask (Kopf Instruments, Tunjunga, CA, USA) delivering 3% isofluorane, 0.5 l min^{-1} nitrous oxide, 0.3 l min^{-1} oxygen. Rectal temperature was maintained at 37° C using a mice homeothermic blanket system (Harvard Apparatus, Holliston, MA, USA). An incision was made through the scalp and the skin retracted to expose the skull, which was then cleaned with a sterile cotton swab. Animals were maintained on a mixture of 1.5% isofluorane, 0.5 l min^{-1} nitrous oxide and 0.3 l min-1 oxygen. Cortical perfusion was measured with the Laser-Doppler Perfusion Imager from Moor Instruments (Wilmington, DE, USA) as previously described (42,43). A computer-controlled optical scanner directed a low-power He–Ne laser beam over the exposed cortex. The scanner head was positioned parallel to the cerebral cortex at a distance of 26 cm. The scanning procedure took 1 min and 21 seconds for measurements of 5538 pixels covering an area of 0.64cm^2. At each measuring site, the beam illuminated the tissue to a depth of 0.5 mm. An image color-coded to denote specific relative perfusion levels was displayed on a video monitor. All images were acquired at 2-min intervals for a period of 30min (15 images for each animal). All images were stored in computer memory for subsequent analysis. For each animal, a square area of 0.05cm^2 (360 pixels) equally distributed between the right and left hemispheres was defined and applied to each image of the series in order to measure the CBF in the frontal and occipital cortex. CBF was also measured in the entire cortex by manually delineating for each mouse the cortex area (0.51-0.54cm^2 corresponding to 3504 to 3714 pixels). Relative perfusion values for each area studied were presented as percentage of CBF values in injured compared to sham controls.

ELISA

To obtain blood specimens to measure glucocorticoid and cytokine levels in plasma, animals were lightly anesthetized with isoflurane prior to euthanasia, and approximately 500µl of blood was withdrawn into EDTA capillary tubes by cardiac puncture. Samples were centrifuged at 5000rcf for 3 minutes, and plasma samples (clear supernatant fraction) were flash frozen in liquid nitrogen and stored at -80°C. Plasma glucocorticoid levels were measured using an ELISA kit purchased from Life-sciences-Invitrogen, Grand Island, NY. Cytokine levels (GM-CSF, IL-5, IL-

6, IL-1β, TNFα, IL-17A, IL-10, IFNγ) were determined using Bio-Plex Pro mouse Th17 panel 8-plex ELISA kit (Biorad, Hercules, CA), as instructed by the manufacturer's guide.

Brain Tissue preparation and Western Blotting

Six months post first injury brain tissue was collected following transcardial perfusion by gravity drip with phosphate buffered saline (PBS). One hemisphere was extracted and dissected into two regions (cortex and corpus callosum), flash frozen in liquid nitrogen and kept at -80oC for antibody based/biochemical analyses. The other hemisphere was post-fixed in 4% paraformaldehyde (PFA) for histological/immunohistochemical (IHC) analyses.

For western blotting analyses, the corpus callosum and cortex from each hemisphere were homogenized in 150µl and 750ul (respectively) of PBS (pH 7.4) containing proteinase inhibitor, using a probe sonicator. Homogenized samples were spun in a centrifuge at 15,000rpm for a few minutes and tissue supernatants were collected. Supernatant fractions were either denatured at 95^{o}C by boiling in Laemmli buffer (Bio-Rad, CA, USA) or prepared under non-denaturing conditions in Lammeli buffer without reducing agent. Samples were then subsequently resolved on 4–20% gradient polyacrylamide criterion gels (BioRad, CA, USA) or 4-12% gradient NuPAGE novex Bis-Tris precast polyacrylamide gels (Life Technologies, Grand Island, NY). After electrotransferring, polyvinylidene difluoride (PVDF) membranes were blocked in 5% milk made in tris buffered saline (TBS) and subsequently immunoprobed for different brain specific primary antibodies overnight (see Supplementary Table T1). After a rigorous washing step, membranes were probed with horseradish peroxidase (HRP) linked secondary antibodies (see Supplementary Table T1). For primary antibodies raised in mice, a 20% superblocking buffer was used in the secondary antibody solution. Immunoblots from cortical tissue samples were analyzed by using a housekeeping gene (Beta-actin or GAPDH antibody) to quantify the amount of proteins electrotransferred, and signal intensity ratios were quantified by chemiluminescence imaging with the ChemiDocTM XRS (Bio-Rad, CA, USA). Immunoblots from white matter tissue samples for non-reduced tau conformational antibodies were analyzed by using the most abundant bands from a total protein Coomassie stain as a control reference, and signal intensity ratios were quantified by chemiluminescence imaging with the ChemiDoc TM XRS.

Immunohistochemistry

Half brain hemispheres (left side) were embedded in 4% paraformaldehyde for <48hrs followed by paraffin embedding. Series of 6µm-thick sagittal sections were cut throughout the extent of the cortex and hippocampus guided by known bregma coordinates using a microtome (2030 Biocut, Reichert/ Leica, Buffalo Grove, IL). Cut sections were mounted onto positively charged glass slides (Fisher, Superfrost Plus, Pittsburgh, PA).

Sections were deparaffinized in xylene and rehydrated in a decreasing gradient of ethanol before the immunohistochemical procedure. Sections were rinsed in distilled water and subsequently incubated at room temperature in a solution of endogenous peroxidase blocking solution, containing 3% hydrogen peroxide diluted in PBS for 15 minutes. For primary antibodies requiring antigen retrieval sections were either treated with (i) DAKO target retrieval citrate buffer solution (pH 6) for 8 minutes in the microwave, (ii) Tris-ethylene-diamine-tetraacetic acid –Tris - EDTA buffer (pH 8), or (ii) Proteinase K (IHC world, Woodstock, MD). Following antigen retrieval sections were blocked for 30mins to 1hr in either (i) Dako protein serum free protein block (Dako, Carpinteria, CA), (ii) normal blocking serum to which secondary antibody was raised or (iii) mouse immunoglobulin G blocking reagent mouse on mouse[MOM] Kit (Vectors Laboratories, Burlingame, CA). Sections were stained in batches with primary antibodies made up in supersensitive wash buffer or antibody diluent background-reducing agent. Primary antibodies used can be found in Supplementary Table T1. After overnight incubation, sections were rinsed with PBS and transferred to a solution containing the appropriate secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) for 30mins to 1hr depending on the specific requirement of the antibody protocol. For antibodies raised in a mouse host, secondary antibodies were diluted in 20% superblock blocking buffer (Pierce, Thermo Fisher Scientific, Rockford, IL). After rinsing in water, sections were incubated with avidin-biotin horseradish peroxidase or alkaline phosphatase (AP) enzyme solution (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) solution for 30mins.

Immunoreactivity was visualized with DAB (3,3 - diaminobenzidine) or Vector red AP substrate. Development with the chromogen was timed and applied as a constant across batches to limit technical variability before progressing to quantitative image analysis. The reaction was terminated by rinsing sections in copious amounts of distilled water. Mounted sections were progressed through a graded series of alcohols (dehydrated), cleared in xylene, and coverslipped with permanent mounting medium. The IHC procedure was previously validated for most antibodies used by including negative control sections, whereby the primary mouse monoclonal

antibodies were omitted and replaced, with either blocking agent or biotinylated secondary antibodies alone, to eliminate issues surrounding nonspecific immunoreactivity or detection of mouse immunoglobulin reacting with anti-mouse secondary in the tissue sample. Immunoreacted sections were viewed using a motorized Olympus (BX63) upright microscope, and photographs were taken using the high resolution DP72 color digital camera.

Histology

Series of sections were deparaffinized and processed for different histological stains. The Gallyas silver impregnation method was performed to reveal neurofibrillary tangles (NFTs) using a standardized protocol (see 40). Congo red staining for amyloid plaque was performed using manufacturers instructions (SIGMA, St. Louis, MO). Luxol fast blue (LFB) staining was performed to reveal myelination of axonal tracts within the white matter using a standardized staining procedure as previously described by Mouzon et al. (29). To detect microhemorrhages in the brain tissue a Prussian blue staining was also performed according to the manufacturer's guidelines (ENG Scientific Inc, Clifton, NJ). Gallyas and Prusssian blue stained sections were counterstained with Nuclear fast red, while Luxol fast blue stained sections were counterstained with Cresyl violet. Counterstained sections were subsequently processed through a descending gradient of alcohol, xylene, and coverslipped with Permount mounting medium.

Image Analysis and APP cell counts

Immunoreactivity for cell markers (GFAP, IBA1) was measured by quantitative image analysis (optical segmentation). Rigorous staining protocols were applied to ensure consistency of immunostaining and accuracy of image analysis. Analysis was performed blind to experimental conditions using coded slides to avoid bias in evaluation. Multiple regions of interest were analyzed in standardized fashion for each cell marker/antibody. First, a survey of immunostained tissue sections was performed independently to verify specific immunoreactivity that was subsequently progressed to quantitative image analysis. Briefly, non-overlapping red, green, blue (RGB) images were digitally captured randomly within the defined areas from each section (comprising an average of 5 sections per animal for each marker), providing a systematic survey of each region of interest for each animal within a group. A minimum of 15 microscopic fields (x60 magnification) was analyzed per region per animal. The total microscopic field yielding a total area of 3.5 mm^2 analyzed for each region per animal. Immunoreacted profiles that were optically segmented were analyzed using CellSens morphometric image analysis software (Olympus, Center Valley, PA). A semiautomated RGB histogram-based protocol (specified in the

image analysis program) was used to determine the optimal segmentation (threshold setting) for immunoreactivity for each antibody. Immunoreactive profiles discriminated in this manner were used to determine the specific immunoreactive % area. Data were separately plotted as the mean percentage area of immunoreactivity per field (denoted ‘‘% area’’) ± SEM for each region and grouping.

To assess changes in APP+ profiles in the corpus callosum, axonal profiles were determined from a series of 5 sections. Fifteen separate images spanning a total area of 3.52mm^2 was examined. Only clearly distinguishable profiles with distinct morphological characteristics of axonal bulbs or swellings were included in the counts. Data were separately plotted as density estimates (number per mm^2) ± SEM for the corpus callosum region and grouping.

Volumetric estimates

The volume of the corpus callosum of one brain hemisphere (bound by bregma co-ordinates -0.36 to -0.72mm lateral) was determined by quantitative light microscopy using the Cavalieri method. In brief, sagittal sections containing the corpus callosum regions defined above were stained for Luxol fast blue (LFB) (taking every tenth serial section). An average of 5 sections were collected per animal. Mounted sections were viewed at low magnification and montage images of the entire corpus callosum region were electronically captured using a DP72 digital camera attached to a motorized Olympus BX63 digital photomicroscope (Olympus, Center Valley, PA). Digital images were digitally outlined on each section using the Cell SENS Olympus software package. All analyses were carried out blind to study group assignment. For each animal, the volume of the corpus callosum region (bound by bregma co-ordinates -0.36 to -0.72mm lateral) was subsequently derived by multiplying the calculated total surface area by the mean distance between the series of sections. Data are presented as mean volume (in mm^3) ± SEM per group.

Lipidomic analyses

Lipidomics analyses for different phospholipid species were conducted as follows: Lipids were extracted using the Folch method from the cortex as we have previously described (44) with addition of di-14:0 fatty acid containing internal standards for PE, PI, PG, PC PA, and 14:0 LPG, LPC and LPE, di-17:0 PS, PE, PC, PA, and PG, 14:LPE and LPC and 24:0 LPC. Lipidomic runs were performed in triplicate using SCID LC/MS as described elsewhere (44,45). Identification and calculation of concentrations of specific lipid molecular species within each lipid class [LPC, LPE, PC, PI and SM levels] were calculated from the averaged MS spectrum across the

chromatographic peak for each lipid class using LipidomeDB software. Summed mass spectra for each lipid class in an LC/MS run were analyzed using LipidomeDB onlinehttp://lipidome.bcf.ku.edu:9000/Lipidomics/) to identify and quantify (with reference to added internal standards) each lipid molecular species. Each sample was analyzed in triplicate.The biological significance of changes in lipidomic profiles was evaluated using Lipid map pathway tools (www.lipidmap.org). Moreover, phospholipid classes were grouped according to the degree of unsaturation for the molecular species. For PC, ratios were calculated using docosahexaenoic acid (DHA)-containing species, PC (38:6, 16:0/22:6) and PC (40:7, 18:1/22:6), to arachidonic acid (AA)-containing species, PC (36:4, 16:0/20: 4), PC (38:4, 18:0/20:4), and PC (38:5, 18:1/20:4). For PE, a ratio of DHA-containing species, PE (40:6, 18:0/22:6) and ether PE (ePE; 40:6, o-18:0/22:6), to AA-containing species, PE (38:4, 18:0/20:4) and ePE (38:4, o-18:0/20:4), was calculated. For PI, a ratio of DHA-containing PI (40:6, 18:0/22:6) to AA-containing PI (36:4, 16:0/20:4) and PI (38:4, 18:0/ 20:4) species was examined. Group differences were determined using either ANOVA or the $\chi 2$ test based on the type of variable. When parametric assumptions were not met, values were log transformed for parametric analyses. Non-parametric testing was only used when transformation was found to be unsatisfactory. Principal Component Analysis (PCA) was used to minimize multicollinearity and achieve dimension reduction, as used routinely for the evaluation of lipidomic data (44, 45). The Kaiser-Meyer-Olkin (KMO) and Bartlett's test for sphericity were used to ensure sampling adequacy for PCA (KMO value of > 0.6 and Bartlett $p < 0.05$). Variables with eigenvalues of ≥ 1 were retained and PCA component were extracted using varimax with Kaiser normalization for rotation to simplify and clarify the data structure. In order to perform mixed linear modeling (MLM) regression analysis on each component (the outcome measure), the Anderson-Rubin method was used for exporting uncorrelated scores while adjusting for random (human) factor and assess independent fixed (diagnostic and replication). Following MLM, multiple-test corrections were performed with the Benjamin–Hochberg (B-H) procedure to control for the false discovery rate. Individual lipids were analyzed by MLM to identify lipids specifically altered by the study treatment.

Statistical Analysis

The relationships between mTBI and sham animals for western blotting, IHC, ELISA, neurobehavioral and CBF data were assessed by using either a parametric T-test or non-parametric Mann Whitney U Test with a predefined criterion of $p<0.05$ to assess group differences. Data were assessed by the Kolmogorov-Smirnov test to evaluate if data sets fitted the

normal Gaussian distribution. All analyses were performed with SPSS 21.0 (IBM Corp., Armonk, NY) and Graph pad prism (La Jolla, CA) statistical software.

Results

Neurobehavioral observations

We examined behavioral outcomes (six months post first injury) in our mouse model of chronic repeated TBI using the three-chamber test (social interaction and social novelty recognition or memory), open field (sensorimotor and anxiety) and elevated plus maze (anxiety). No impairment in social interaction was observed in sham and injured animals, both groups of animals twice as frequently entered stranger I chamber compared to empty chamber (Supplementary Figure S1: A). There was a trend ($p=0.058$) for diminished social memory in the injured group, with animals three times more frequently entering Stranger I chamber compared to new novel Stranger II chamber (Supplementary Figure S1: B). Sham animals behaved normally in the social novelty recognition test, frequenting Stranger II chamber >46% more than Stranger I chamber (Supplementary Figure S1: B). In both tests for anxiety, injured animals demonstrated more dis-inhibitory behavior than shams, with a trend towards increased exploration (i.e. time and frequency of entries) in the center zone of the open-field and open arms of the elevated plus maze tests (Supplementary Figure S6: E-F). However, statistical analyses of these outcome measures did not reach significance. Minimal changes were observed in the sensorimotor activity during the open field test; this was typified by a small trend towards decrease in motor activity in injured compared to sham animals (Supplementary Figure S6: D).

Notably, five animals in the injured group presented with dermatitis around the skin of the face (around the eye region) and in some cases around the body, approximately 2-3 months into the study, following the first injury. This was probably due to lack of grooming and restrained use of front limbs following injury in these mice. Two of these animals that had severe dermatitis had to be euthanized, and the other three, which only had a milder form of dermatitis were also subsequently removed from the in vivo imaging and behavioral tests, which reduced our sample sizes.

Cerebral blood flow

Using a laser Doppler imaging system to assess changes in the cerebral blood flow of injured animals compared to their sham counterparts (Figure 1A) a reduction in CBF in the occipital

cortex was identified in injured compared to sham animals ($p<0.01$) (Figure 1B). No change was observed in the frontal cortex of either sham or injured animals; however, a trend towards decrease was observed in the entire cortex readings (Figure 1B).

Neuropathology of chronic repetitive mTBI

Macroscopic pathology

The injury paradigm was consistent with a mild injury, with no evidence of macroscopic pathology in r-TBI exposed mice. Specifically there were no skull fractures or subdural hemorrhages and no gross pathological changes observed in any animals studied (Supplementary Figure S2: A,B).

White matter pathology

Prior examination of acute 24hr post-last injury brain tissue sections from this model depicted an exaggerated rise in APP+ axonal profiles in injured animals (212+ profiles per mm^2) accompanied by an intense GFAP and IBA1 immunostaining in the corpus callosum (data not shown).

At the chronic time point, a persistent increase in APP positive axonal profiles (Figure 2A-F) and extent of GFAP (Figure 3AB, DE, GH) and IBA1 (Figure 4DE, GH, JK) immunoreactivity was observed in the body and splenium of the corpus callosum in injured compared to sham animals. These changes were however, less prominent when compared to the 24hrs time point (data not shown).

Axonal swellings immunopositive for APP (Figure 2 II', JJ', KK') were observed in the corpus callosum of injured animals, including hypertrophic astroglia (Figure 3 FF', II') and activated microglia (Figure 4 FF', II', LL'). These qualitative changes were confirmed by quantitative analyses of segmented profiles showing significant increases in APP+ profile density (5 fold), IBA1 (2 fold) and GFAP (1.85 fold) immunoreactivity levels in the corpus callosum of injured compared to sham animals (Figure 6A-C).

S100ß immunoreactivity (an astroglial marker) was also notably increased in the cell body of astrocytes in the corpus callosum of injured compared to sham animals (Figure 3D, E see inset). Activated microglial cells were devoid of CD45 and MHCII immunostaining in injured animals

within the corpus callosum (data not shown). An increase in cellularity was observed in the corpus callosum of injured animals compared to shams (Figure 5E, F) using cresyl violet staining.

An increase in GFAP immunoreactivity was also observed in the white matter regions of the walls of the lateral ventricles (Figure 3 CC', K). Notably, perivascular regions of the white matter of injured animals were prominently stained with GFAP (Figure 3J).

No changes were observed in luxol fast blue [LFB] (Figure 5A-B) and myelin basic protein (data not shown) staining in the corpus callosum of injured compared to sham animals. However, we did observe a significant thinning of the corpus callosum following stereological analyses of the volume difference between sham and injured animals. A 30% reduction was observed in the corpus callosum volume of injured compared to sham animals (see LFB; Figure 5C,D; Figure 6D).

Glial response (grey matter)

There was a paucity in GFAP and IBA1 staining in the cortex of injured compared to sham animals (See Figure 3 A,B and Figure 4 A,B,CC' respectively). This was confirmed by quantitative analyses (see Figure 6A,B). A notable increase in IBA1 and GFAP immunoreactivity was observed in the molecular cell layer (and white matter region) of the cerebellum of injured animals (Figure 3L). Intracellular APP immunostaining was increased in the cell body of pyramidal neurons in layers II-IV of the parietal cortex (Figure 2 G, H)

Quantification of and immunolocalisation of tau

Grey matter (cerebral cortex)

In a substudy, analysis of brain tissue (grey matter) from mice subjected to this same injury paradigm but euthanized 24hrs after the last injury showed a 1.6 fold increase in total tau expression by immunoblotting in injured vs sham mice. However, immunohistochemistry did not show any changes for all other tau species analyzed (data not shown).

For the chronic timepoint post-injury which is the focus of this manuscript, immunoblotting for total tau (DA9) again showed an increase in injured compared to sham animals in the grey matter (Figure 7A), however the chronic evaluation also revealed increases in pTau Thr231 (RZ3) and tau oligomers (TOC-1) (Figure 7B, E, F). This was confirmed by immunohistochemical analysis

for TOC-1 and RZ3 antibodies on serial sections of sham and injured animals; superficial layers of the cortex showed a mild increase in the immunoreactivity of TOC-1 and RZ3 (Figure 8A-D).

Intriguingly no change was observed by immunoblotting for pTau 396/404 (PHF1) and S202 (CP13) in sham and injured animals (Figure 7C,D). This was also confirmed by immunohistochemistry (see Supplemental Figure S3: A-F; I-K).

Changes in conformational tau species positive for paired helical filaments and for the N terminal region of the phosphatase activating domain (PAD) was detected using the MC1 and TNT antibodies respectively. Immunoblotting showed no changes between sham and injured animals in the grey matter for either MC1 or TNT antibodies (Figure 7A, G, H). Likewise immunohistochemical analysis confirmed no augmentation in the immunoreactivity of MC1 and TNT in injured compared to sham animals (Supplemental Figure S4: A-C; D-G).

Argyrophillic positive neurofibrillary and glial tangles were examined using the Gallyas silver stain. The cortices of both sham and injured animals were devoid of Gallyas staining (Supplemental FigureS3: G,H,L). Caspase cleaved truncated tau fragment (Tau C3 - Asp 421) showed no changes between sham and injured animals by immunohistochemistry (data not shown).

White matter

Changes in total tau (DA9), pTau (PHF1, CP13), PAD+ tau (TNT) and conformational tau species (TOC-1, MC1) were examined by immunoblotting or immunohistochemistry in the white matter of sham vs. injured animals, but no changes were observed (Figure 9A-F). Corresponding immunohistochemistry showed no changes in the white matter region of the corpus callosum.

Neurofilament, synpatophysin, TDP-43, α-synuclein and amyloid

Large caliber myelinated axonal damage in the grey matter was examined using neurofilament L protein marker (46); no changes were observed in sham compared to injured animals (Supplementary Figure S5: A,B). Synaptic integrity was determined using the presynaptic vesicle marker, synaptophsyin; we did not observe any injury-dependent changes in its expression (Supplementary Figure S5: A, C).

No changes were observed in total TDP-43 or α–synuclein levels in the grey matter of sham and injured animals (Supplementary Figure S5: A,D-E). There was no shift in molecular weight (kDa) of these proteins that could indicate an increase in their potentially pathogenic phosphorylated protein levels. Brain tissue from both sham and injured animals was devoid of Congo-red staining and 4G8 immunoreactivity (data not shown).

Vascular response

Levels of Claudin-5 and ZO1 were below limits of detection by western blotting using commercially available antibodies. No changes were observed in laminin or occludin levels in the grey matter between injured compared to sham animals (Supplementary Figure S5: F-G). Immunohistochemistry for laminin and collagen IV positive vessels showed no overt damage to the vasculature and an apparently similar vascular density between both shams and injured animals in different brain regions (Supplemental Figure S6:AA`-DD`).

No evidence of extravasation of endogenous mouse IgG staining was observed in the cortical regions beneath the impact site in injured mice (Supplemental Figure S6: EE) and there was no evidence of microhemorrhages in the brain tissue of injured (or sham) animals examined by Prussian blue staining (Supplemental Figure S6: FF`).

Brain phospholipid species profile

No changes were observed in phosphatidycholine (PC) and phosphatidylinositol (PI) lipid species in injured versus sham animals (Supplementary Figure 7 A-B). However, an increase in total phosphatidylethanolamine (PE) and sphingomyelin (SM) lipid species were observed in injured compared to sham animals (Supplementary Figure 7 C-D). There was an increase in saturated fatty acid (SFA) classes of PE and SM lipid species in injured animals compared to shams (Figure 8C-D). The mono-unsaturated fatty acid (MUFA) class of SM lipid species was also observed to significantly increase in injured compared to sham animals (Supplementary Figure 7D). Only PE lipid species showed an increase in their classes of polyunsaturated fatty acids (PUFA) in injured compared to sham animals (Figure 8C). We also measured arachidonic acid (AA) and docosahexaenoic acid (DHA) containing lipid species (Supplementary Figure 7E-G). A significant increase in AA containing lipid species for PC and PE was observed in injured compared to sham animals (Figure 8E, G). There was no change in DHA containing lipid species for PC and PI (Supplementary Figure 7E, F), but a significant increase in DHA containing PE species was observed in injured compared to sham animals (Supplementary Figure 7G). We

compared the AA:DHA ratio for PC, PE, PI lipid species. A significant increase in the AA:DHA ratio was observed for PE lipid species in injured vs sham animals (Supplementary Figure 7H), no changes were observed in PC and PI (data not shown).

Neuroendocrine and inflammatory cytokine profiles in the plasma

We found a significant decrease in IL-1β and IL-5 inflammatory cytokines in the plasma of injured compared to sham animals (Figure 10 A,B). No statistically significant changes were observed with the other inflammatory cytokines measured (GM-CSF, IFN-γ, TNF-, IL-10), however, a trend towards decrease in the injured group was noted in all cases (Figure 10 C-F). The changes in inflammatory cytokines correlated with an increase in the neuroendocrine marker, corticosterone, in the plasma of injured compared to sham animals (Figure 10 G).

Discussion

We have developed a new model with the intent to explore the chronic effects of repetitive, concussion-like mild TBI over a prolonged period of time in the hTau mouse model expressing all six human tau isoforms. In this first characterization we report persistent increases in total tau, tau oligomers and pTau (Thr231) species a few months after the cessation of injury and this may represent developing tau pathology in our model. Further studies examining extended chronic timepoints will be valuable to confirm progressive TBI dependent tauopathy in our model as this may have relevance to informing studies in CTE in humans exposed to a history of repetitive concussion/ mTBI. In this first characterization we also report behavior (anxiety, cognition, sensorimotor) and neuropathology of exposure to chronic repetitive mTBI, and biochemistry of central and peripheral biomarkers.

Neuropathological changes in grey and white matter after chronic r-mTBI

The most conspicuous histopathological features seen in this animal model were found localized to the corpus callosum, consistent with observations with this same mTBI injury in our previous repetitive injury paradigm where 5 mTBI injuries were delivered with a 48h interval between each injury (29,33).

These features included a pronounced and persistent ongoing axonal degeneration typified by increase APP positive profiles, axonal swellings, bulbs and varicosities that were associated with corpus callosum thinning at chronic timepoints after the last-injury. Previous studies that have examined the effects of APP immunoreactivity in mouse models of repetitive mTBI typically show an initial rapid increase in APP staining (47, 48, 49), which we observed at the acute 24hrs

post-last injury timepoint in this study (a 30-fold increase vs controls). However, in other animal models, at subacute time points post-injury these effects diminish, with only non-existent or minor abnormalities seen between injury and aged-matched controls (47, 48; 49). In contrast, with our animal model we still observed a persistent ongoing increase in APP+ profiles following injury compared to shams, although these effects were significantly less prominent when compared to the acute effects observed 3-4 months earlier. Such inter-laboratory differences could be attributed to variations in experimental models and injury paradigms. For example our experimental device uses a flat tip impounder, whilst other groups mentioned typically use a rubber tip impounder that has been suggested to absolve the force of injury on the site of impact, making the injury more diffuse and less severe (40).We have also focused on chronicity of hits in this study at a level that has not been previously examined by most groups. Considering that mild head injury increases the vulnerability of the brain to a second concussive impact (47), it is plausible that in our current model, by design, there is a limited temporal window of recovery, resulting in the pronounced ongoing axonal degeneration evident months after injury. Other features observed in the corpus callosum include a robust astroglial and microglial activation. These degenerative white matter features observed in our model echo appearances documented in descriptions of human autopsy (12,13,50) and in vivo imaging (51) studies in TBI survivors, and at necropsy examination of brain tissue from a variety of repetitive mTBI animal models (28,31,33,36,39,46).

In contrast to this ongoing white matter damage, there was no evidence of glial activation or ongoing axonal degeneration in grey matter, or of neuronal cell loss or structural damage to neuronal integrity in injured animals. However, immunoblots on material from grey matter did reveal a persistent increase (by two-fold) in total tau levels and this was accompanied by a significant increase in pTau Thr231 (RZ3+), tau oligomers (TOC-1+ [50, 52]), and a trend towards increase in tau conformer species (MC1+). In support of these observations, patchy immunoreactivity to these significantly altered tau species (RZ3, TOC-1) was shown in the superficial cortical layers of r-mTBI exposed animals. PHF1 (Ser394/404) and CP13 (Ser202) levels remained unchanged with injury. There was no evidence of the distinct perivascular accumulation of pTau immunoreactive neurons or glia, typical of human CTE; neither was there evidence of neuropil threads. At this stage we do not consider this model to represent a preclinical model of CTE, however, our findings appear to be among the first to show significant changes in tau oligomer levels at a chronic time point after repetitive mTBI in a mouse model. Small, misfolded tau species or tau oligomers are thought to be the toxic pathogenic species of tau which

are resistant to ubiquitination (see 53-57). They have been demonstrated both in vitro and in vivo to spread in a retrograde and anterograde manner from one neuron to another, within connected anatomical pathways (58). Whether these tau oligomeric species will inevitably form into beta-pleated sheet conformation or fibrillary material is unknown. The specific changes in phospho-tau epitope pThr231 levels in our model, may also implicate the role of cis-trans conformational pTau changes after TBI in vivo. Cis-trans conformational pTau has been demonstrated to play an important role early in mild cognitive impairment, and they further accumulate in neurofibrillary degenerated neurons in Alzheimer's disease and TBI (59, 60). We were not able to analyze these different conformational states in our studies, due to the lack of commercially available antibodies that can distinguish between cis from trans pThr231 pro-conformation in tau, therefore a definitive role of cis versus trans tau forms cannot be made in our study. Further exploration in our model using these antibodies as well as others specific to different tau species at longer timepoints post-injury may be informative in this regard as this would confirm whether the persistent tau changes that we observe are on a continuum towards progressive TBI dependent neurofibrillary tau pathology.

The TBI-dependent changes in tau reported herein are in contrast to our findings with our previous injury paradigm (five mTBI with a 48hr interval) in either C57BL/6 or hTau young mice (8-12 weeks old). In those studies we did not observe any persistent changes in tau species following analyses of brain tissue by ELISA and immunohistochemistry, from 24hrs to 12 months post-injury in either wild type or hTau mice (33, Mouzon et al., 2016 pers comm.).Other groups have also published data reporting absence of tau dependent changes following repeated injuries at acute to chronic timepoints (30,31,38). Our findings suggest factors such as chronicity of impacts, age at exposure and frequency of injuries may play a role in precipitating tau pathologies at chronic timepoints after mTBI. This observation is supported by data from Petraglia and colleagues using a model of repeated mild traumatic brain injury in wild-type mice, said to recapitulate "subconcussive" injuries; the model involved 6 mTBIs per day (2hr intervals) for 7 days totaling 42 hits (36). Following this paradigm, the authors reported an increase in murine pTau levels by IHC at 7days, 1 month and 6 months post-injury. Intriguingly other models of chronic TBI exposure in younger mice involving fewer total injuries (ranging from 2-5 mTBI) (28,32,34,37) and also in a single mouse model of blast related traumatic injury (61,62) have reported significant changes in pTau levels immediately post-injury. However, while these studies have reported acute alterations in tau, data on tau with chronic survival are not available from these models.

Thus far the majority of studies exploring effects of TBI on tau pathology have been conducted in young, wild-type mice which have a strong bias for the 4R murine tau isoform which some investigators have suggested to have a low propensity to form toxic tau aggregates in vivo. Our use of hTau transgenics may shed some light on the factors that influence propagation of (human) tau pathology after injury. hTau mice typically develop aggregated, hyper-phosphorylated tau by 9 months of age, in a spatial temporally relevant distribution localized to regions such as the entorhinal cortex, subiculum and hippocampus, and by 15 months of age develop thioflavin-S-positive neurofibrillary tangles, loss of neurons and apoptotic damage (41). In our previous work on aged hTau mice (>18months) exposed to 5 repetitive hits, we demonstrated augmentation of pTau pathology in superficial layers of the cortex and also in the hippocampus by immunohistochemistry (39). Of note, these effects appeared to be more pronounced than those observed in this current study using younger mice <13weeks at first injury, perhaps suggesting the importance of an underlying tau pathology at the time of injury as a vital trigger for precipitating persistent TBI-dependent tauopathy.

In addition to tau we have also investigated other signature proteins related to neurodegeneration in our model. We found no evidence of amyloid pathology in repetitive mTBI injured animals, as determined by histopathology. To date, although the role of amyloid in CTE remains unknown, retrospective case studies have shown that a majority of CTE patients will have amyloid plaques at autopsy (13, 25). We also did not observe any changes in the levels of total neuronal transactive response DNA-binding protein - TDP43 (or its phosphorylated species) after injury. TDP43 has been identified as the major disease protein in frontal temporal lobar degeneration (FTLD) with ubiquitinated inclusions, and in amyotrophic lateral sclerosis (ALS). TDP43 immunoreactive intraneuronal and intraglial inclusions are commonly described in CTE, and present in a majority of cases studied (13, 63-65). To our knowledge this among one of the first studies to asses TDP-43 levels at a chronic timepoint in a rodent repetitive mTBI model. Although our findings did not show any correlation between repetitive mTBI and TDP43, we are cautious when interpreting our findings regarding the role of TDP43 in r-mTBI, given that there might be differences between murine vs human TDP43. More studies need to be conducted to evaluate the role of this protein following TBI.

Lipid dysfunction after chronic r-mTBI

Lipids are known to play an important role in neurological disorders and CNS injury, and have been implicated in TBI (66). We previously demonstrated TBI-dependent brain lipid changes in a controlled cortical injury (CCI) model using our lipidomic platform (67), and thus sought to evaluate changes in different lipid species in this new model. Our lipidomic analyses revealed an increase in total PE and SM lipid species in the cortex of our chronic repetitive mTBI exposed animals, accompanied by increases in saturated, mono or poly-unsaturated fatty acid classes of PE and SM lipid species. The reason for these lipid changes and their role in late TBI pathologies is not clear at this time, but we hypothesize may reflect: (i) dysregulation in brain lipid metabolism; (ii) failure of abluminal lipid transporters; or (iii) increased luminal transport from the periphery as free fatty acids or in esterified forms bound to lipoproteins. These high lipid levels after TBI could predispose to oxidative damage (i.e. lipid peroxidation), especially under inflammatory conditions. We additionally observed an increase in the ratio of arachidonic acid (AA - 20:4) to docosahexaenoic acid (DHA - 22:6) containing polyunsaturated lipid species in injured animals. Arachidonic Acid is metabolized to pro-inflammatory and vasoactive eicosanoids (such as, prostaglandins, leukotrienes and thromboxane) by lipoxygenase (LOX) and/or cycloxygenase (COX) pathways (66). Docosahexaenoic Acid, the most abundant polyunsaturated fatty acid, is metabolized by phospholipase A2 enzymes to produce metabolites resolvins and protectins, such as neuroprotectin D1 (68). These metabolites serve as an endogenous neuroprotectant that inhibits apoptosis, oxidative stress and pro-inflammatory processes (68-71). The consequences of this increase in AA to DHA ratio could, therefore, indicate an increase in pro-inflammatory versus anti-inflammatory environment at this late time point following exposure to chronic repetitive mTBI.

Cerebral blood flow outcomes after chronic r-mTBI

We observed a reduction in cerebral blood flow (CBF) in the head injured mice, localized primarily to the regions beneath the impact site around the occipital (/parietal) lobes. Intriguingly, in a study utilizing brain perfusion single photon emission computed tomography (SPECT) imaging in a cohort of >100 active and former American football players, the authors reported a decrease in brain perfusion, with a preponderance for the prefrontal lobe, temporal lobe, occipital lobe, and cingulate gyrus and hippocampus regions in their cohort (72). Although we cannot draw direct comparisons between our mouse CBF and human SPECT data, the similarities in the direction of change in brain perfusion highlights vascular dysfunction as another sequelae of TBI. The reason behind this is unknown, given that we did not observe any focal injury or pathological changes in this region. Cerebral glucose metabolism has been linked

to reductions in blood flow after TBI and this could be possibly altered in our model (73). Of note, though we did not observe any impairment in blood brain barrier (BBB) integrity in control or injured animals, we did observe astroglial activation in perivascular regions in injured animals. Though observations in human, autopsy-derived material from patients surviving single moderate to severe TBI demonstrate widespread BBB disruption in a high proportion of both acute and late (>1 year survival) survivors (74), data on vascular pathology in CTE, in particular BBB integrity, are lacking.

Neurobehavior, neuroendocrine and neuroimmune outcomes after chronic r-mTBI

We conducted our behavioral analyses of hTau mice at 9 months of age. hTau mice typically do not show any significant impairment in spatial learning and memory until 12 months of age, although 6 month old mice have been noted by one group to show abnormal spatial learning compared to control mice (75, 76). Locomotor function, anxiety levels and gross motor functions are typically not different from age-matched controls (75). Our observations were conducted prior to the appearance of age-related neurobehavioral dysfunction to eliminate underlying confounding phenotypes. Behavioral evaluations report a pattern towards dis-inhibitory-like behavior and deficits in cognitive performance. The effects we report were more pronounced compared to our previously reported mTBI paradigms in young mice, which employed exactly the same level and nature of injury but investigated the effects of either a single or a different repetitive injury (5 hits over a 9day period) (29,33,39). Behavioral outcomes in our model could be attributed to underlying neuropathological correlates involving white-matter (corpus callosum) degeneration and accumulation of abnormal tau in neurons within the grey matter. Both sites of localized pathologies are relevant given their involvement in controlling dis-inhibitory behavior and cognitive function. Comparable behavioral outcome measures have also been reported in another repetitive mTBI model involving multiple “subconcussive” impacts; the authors observed cognitive deficits, increased risk taking, depression-like behavior, and sleep disturbances at 1 month post exposure (36). However, this model differed from ours in that it was a much more concentrated paradigm with all the impacts occurring within a one week period. Human patients exposed to a history of mTBI in contact sports demonstrate a spectrum of neurobehavioral symptoms including post-concussive syndrome (PCS) and chronic traumatic encephalopathy (CTE); involving alterations in mood, neuropsychiatric behavior, and cognition (77-84).

Correlating with the neurobehavioral deficits seen in our model, we also observed an increase in the glucocorticoid steroid hormone, corticosterone. Abnormalities in neuroendocrine function

and circadian rhythm have been shown after TBI of different severities (85-91). Upregulation in cortisol levels may result in an increased negative feedback to the brain via the HPA axis. This can result in the dysregulation of sympathetic and autonomic nervous system functions implicated in the regulation of the fight or flight response (92). Neuroinflammatory cytokine levels in the periphery were also reduced in our model. Glucocorticoids, such as corticosterone, are known to influence a variety of immune-related functions (93), and can dampen down immune-inflammatory cytokine signaling as seen in our model. What specific effects down-regulation in systemic cytokine profiles may have on the injured brain is unknown and warrants further studies in the context of TBI. Our lipidomic studies did implicate a possible increase in baseline pro-inflammatory response in the cortex based on the modest increase in arachidonic acid containing lipid species we found. A closer assessment of microglial cell markers (IBA1, CD45 and MHCII) showed a paucity of staining in the cortex in sham and injured animals. These findings are intriguing and may be a unique feature of this animal model. Very few studies exploring systemic inflammation and even brain neuroglial responses after repeated mTBI in humans have been conducted to date, it is therefore very difficult to draw a direct comparison between our model and what happens in the human setting.

Conclusion

In this study, we explored the effects of chronic repetitive mTBI in a mouse model with a human tau genetic background, with an injury paradigm designed to mimic the effects of repeated, mTBI injuries sustained over a prolonged timeframe. Our characterization encompasses the chronic histopathological, biochemical and neurobehavioral effects of these injuries after many months survival. In this first characterization of our chronic TBI experiments, we confirm a two-fold increase in total tau levels and mild increases in tau oligomers/conformers and pre-tangle pTau (Thr231) species in the grey matter up to three months after cessation of injury, which we anticipate may be on a continuum of progressive TBI-dependent tauopathy. Our data also confirm impairment in cerebral blood flow (hypo-perfusion), and a prominent feature of white matter damage typified by gliosis, axonal injury and corpus callosum thinning. These changes were also accompanied by increases in lipid species of different fatty acid classes and their secondary messengers. Subtle neurobehavioral deficits typified by dis-inhibition and deficits in cognitive performance were also observed. Together these data support the relevance of this concussive injury model in studying the consequences of repetitive mTBI in humans, and its usefulness in testing potential neuroprotective therapeutic strategies for chronic repeated mTBI.

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Legends

Figure 1: Cerebral blood flow in a hTau mouse model of chronic repeated TBI

Two dimensional color coded cerebral microvascular blood flow maps recorded using the laser Doppler imager, shows flow data representing variation of regional blood cerebral blood flow in the cortex of sham and injured animals (A). Quantitative analysis of mean cerebral blood flow, obtained from 6 measurements per animal, demonstrates a significant reduction in the occipital cortex of injured compared to sham animals, a marginally significant decrease in the entire cortex, with no change observed in the frontal cortex (B). Data presented as percentage of sham. Blood flow velocity was visualized as a two dimensional color-coded map, and expressed in an arbitrary perfusion unit as cerebral blood flow flux. N= 6 (injured) or 8 (shams). Asterisks denote: ** $P<0.01$.

Figure 2: Axonal injury in a hTau mouse model of chronic repeated TBI

Immunohistochemical staining for APP (axonal injury) demonstrated a significant increase in the corpus callosum (see white arrow in A, B) and this was localized within the body (C, D) and the splenium (E, F) of the corpus callosum. High power micrographs show APP accumulation along the length of damaged axons (II'), and in axonal bulbs or swellings (JJ', KK') throughout the entire length of the corpus callosum of injured animals. Increased APP staining was observed in the cell body of pyramidal neurons in layer III of the parietal cortex of injured compared to sham animals (G,H). High power micrograph (LL') shows increase APP staining in the cell body and dendrites of injured axons.Abbreviation: CC (corpus callosum); HP (Hippocampus); SPL (Splenium).

Figure 3: Astrogliosis in a hTau mouse model of chronic repeated TBI

Immunohistochemical staining for GFAP (astrogliosis) demonstrated a dramatic increase along the length of the corpus callosum of injured animals compared to shams (A,B). Hypertrophic astrocytes were seen in the body (D, E) and splenium (G, H) of the corpus callosum of injured compared to sham animals. Hypetrophic astrocytes demonstrated large cell body and prominent thick processes in these regions (FF', II'). A parallel increase in astroglial marker S100ß, was also

observed in the cell body of astroglial cells in the corpus callosum of injured compared to sham animals (see inset of D and E). White matter regions surrounding the ventricles also showed increased GFAP staining in the injured (C') compared to sham animals (C). High power micrograph of the ventricles can be seen in K. Prominent and hypertrophic perivascular astrocyte endfeet (strongly stained with GFAP) were observed along the length of small blood vessels in injured animals (J). Cerebellar injury was also present, and GFAP staining was significantly increased in the molecular cell layer in injured animals (L). Astroglial activation was not present in the cortex of injured animals (A, B). Abbreviation: CC (corpus callosum); VTR (ventricles); CEREB (Cerebellum); Hippo (Hippocampus).

Figure 4: Microgliosis in a hTau mouse model of chronic repeated TBI

Immunohistochemical staining for IBA1 (microgliosis) demonstrated no change in the cortex of sham and injured animals (A, B). Microglial cells in the cortex had a quiescent morphology in both sham and injured animals (CC'). However, the corpus callosum showed strong immunostaining of IBA1 in injured animals (see body [C, D] and splenium [E,F and J,K] of the corpus callosum) compared to their sham counterparts. Higher power micrographs of activated microglial cells demonstrate a prominent cell body and short thick processes in injured animals (FF', II' and LL'). Abbreviation: CC (corpus callosum).

Figure 5: Luxol fast blue (LFB) and cresyl violet immunostaining in a hTau mouse model of chronic repeated TBI

Luxol fast blue (LFB) staining depict a prominent thinning of the corpus callosum region in injured compared to sham animals (A,B and C,D). LFB staining intensity appeared similar in both sham and injured animals. (G-J). Cresyl violet staining showed a dramatic increase in cellularity within this region in injured compared to sham animals (E, F) Abbreviation: SPL (splenium); CC (corpus callosum); Hippo (hippocampus).

Figure 6: Quantitative assessment of GFAP, IBA1 and APP immunostaining and corpus callosum volume in a hTau mouse model of chronic repeated TBI

GFAP and IBA1 immunostaining were significantly upregulated in the corpus callosum of injured compared to sham animals (A, B). No changes in GFAP and IBA1 was observed in other

regions (of grey matter) analyzed (A, B). APP+ spherical profiles in the corpus callosum was dramatically increased by ~6fold in the corpus callosum of injured compared to sham animals (C). The volume of the sub-region of the corpus callosum bound by bregma (-0.36 to -0.72mm lateral) was significantly reduced by ~30% in injured compared to sham animals (D). N=6 (sham/injured) for A-C and N=4 (sham/injured) for D. Asterisks denote: *P<0.05; **P<0.01; ***P<0.001. Abbreviation: CC (corpus callosum), P-Cortex (parietal cortex), DG (dentate gyrus).

Figure 7: Quantitative assessment of total tau, pTau and conformational tau species in the grey matter by western blotting in a hTau mouse model of chronic repeated TBI

Representative immunoblots for different tau antibodies is shown in A. Total tau (DA9), pTau Thr231 (RZ3), and tau oligomer (TOC-1) levels were significantly increased in injured compared to sham animals (B, E, F). The conformational tau antibody MC1 raised against neurofibrillary tangles was marginally increased in injured animals compared to sham animals. No changes were observed in pTau S396/404 (PHF1), pTau S202 (CP13), and tau+ N-terminal phosphatase activated domain (TNT) levels between sham and injured animals (C, D, H). Data are expressed as ratio of GAPDH or ß-actin levels. N=6-8 (sham/injured). Asterisks denote: *P<0.05; **P<0.01; ***P<0.001.

Figure 8: Immunohistochemical assessment of conformational tau antibody TOC1 and phosphorylated tau antibody RZ3 (pThr231) in an hTau mouse model of chronic repeated TBI

A notable increase in TOC-1 immunostaining was observed in the cortex of injured compared to sham animals (A-B). An increase in pTau Thr231 (RZ3) immunostaining in pyramidal neurons was also observed in layers II - IV of the cortex of injured compared to sham animals (C, D). High power micrographs inset B and D were taken from injured animals (see white box).

Figure 9: Quantitative assessment of total tau, pTau, conformational tau species, alpha-synuclein and neurofilament (NFL) in the white matter by western blotting in an hTau mouse model of chronic repeated TBI

Representative immunoblots for different protein markers are shown in A. No changes were observed in total tau (DA9), pTau S202 (CP13), and tau+ N-terminal phosphatase activated

domain (TNT) levels between sham and injured animals (B, C, D, F). A marginally significant increase was observed in tau oligomer (TOC-1) levels in injured compared to sham animals (E). No changes were observed in alpha-synuclein or neurofilament L (NFL) levels between sham and injured animals (G, H). Data are expressed as ratio of ß-actin or total protein levels. N=6-8 (injured/sham).

Figure 10: Inflammatory cytokine profile and corticosterone levels in the plasma of a hTau mouse model of chronic repeated TBI

A significant decrease in IL-1ß and IL-5 was observed in the plasma of injured compared to sham animals (A,B). A trend towards decrease in GM-CSF, IFN-, TNF-, IL-10 was observed in injured compared to sham animals, however these did not reach statistical significance (C-F).
N=10-12 (sham/injured). There was a significant increase in corticosterone in the plasma of injured animals compared to sham counterparts. N=6-8 (sham/injured). Asterisks denote: *P<0.05; **P<0.01.

Supplementary Table T1: List of primary and secondary antibodies used in this study.

Supplementary Figure S1: Neurobehavioral changes in a hTau mouse model of chronic repeated TBI

The Three-chamber test demonstrated normal social interaction behavior in both sham and injured animals, with a notable trend for increased entries into the stranger I chamber compared to the empty chamber (A). In the social novelty recognition test for social memory, injured animals demonstrated a marginally significant decrease in entries into the new novel stranger II chamber compared to stranger I (B). The Open-field test showed that injured mice had a trend towards an increase in center-zone time compared to sham animals (C; see time-bins and total mean times inset), while sensorimotor activity pattern showed a trend towards reduction in injured compared to sham animals (D). None of these changes observed in the open field test were statistically significant. The Elevated plus maze test demonstrated an increase in time (E) and frequency of entries into the open arms (F), however this was also not statistically significant.

Supplementary Figure S2: Macroscopic changes to the mouse brain following chronic repeated TBI

Mouse brains from sham and injured animals post perfusion are shown in A and B respectively. No gross pathological changes were observed in injured brains, both brains appeared normal, with no evidence of hemorrhages or skull fractures (see B inset).

Supplementary Figure S3: Immunohistochemical assessment of phosho-tau antibodies and neurofibrillary tangles in a hTau mouse model of chronic repeated TBI

Control hTau mice showed low expression of CP13 in the cortex (A), however, a strong immunostaining of CP13 was observed in the hippocampus, notably localized to the CA3 (C) and sibiculum (not shown) regions. No change in CP13 was observed between sham and injured animals in both the cortex and hippocampus areas (AB, CD). PHF1 immunoreactivity was low and undetectable in sham mice (E), and no change was observed between injured and sham control mice (E, F). Both sham and injured animals were devoid of Gallyas immunostaining for neurofibrillary tangles (G, H). For comparison, positive immunostaining for CP13 (I,J), PHF1 (K), and Gallyas staining (L) are shown from aged P301L or aged hTau mice.

Supplementary Figure S4: Immunohistochemical assessment of conformational tau antibodies in an hTau mouse model of chronic repeated TBI

hTau mice showed low levels for MC1 and TNT antibodies by immunohistochemistry at 9 months of age in sham mice (A, D). No changes were observed in both MC1 and TNT immmunostaining in the cortex between sham and injured animals (see A, B for MC1; and D, E for TNT). For comparison, positive immunostaining for MC1 is shown from an aged P301L mouse (C); and also for TNT in dystrophic neurites from an aged PSAPP (F) and hTau mouse model (G).

Supplementary Figure S5: Quantitative assessment of synaptic and axonal proteins, potentially pathogenic markers of neurodegeneration, and vascular associated proteins in the grey matter by western blotting in a hTau mouse model of chronic repeated TBI

Representative immunoblots for different markers are shown in A. No changes were observed in levels of neurofilament L (NFL - axonal protein), synaptophysin (presynaptic marker), TDP43 or alpha-synuclein (markers of protein misfolding and neurodegeneration), laminin and occludin

(vascular and blood brain barrier integrity) in sham compared to injured animals. Data are expressed as ratio of GAPDH or ß-actin levels. N=6-8 (sham/injured).

SupplementaryFigure S6: Immunohistochemical assessment of vascular markers in a hTau mouse model of chronic repeated TBI

No change in collagen IV or laminin immunostaining pattern was observed in sham and injured animals in the grey or white matter (AA'-DD`). Images were captured from the midbrain (AA`), cerebellum (BB`), striatum (CC`) and pons-medulla (DD`). There was no evidence of extravasation of IgG molecules into the brain parenchyma in injured or sham animals (EE` from parietal cortex). Positive control from corpus callosum is shown inset in E` from a severe TBI mouse brain with subdural hemorrhage. Evidence of microhemorrhage was examined using Prussian blue staining for hemosiderin in combination with CD45 microglial cell marker in the brain parenchyma. There was no evidence of positive hemosiderin staining or hemosiderin-laden microglia/macrophages in the brain parenchyma (FF` from splenium). Positive staining for hemosiderin-laden macrophage is shown inset in F` from spleen for positive comparison. Abbreviations: COLL4 (Collagen IV); Pr-B (Prussian blue), HE (Haematoxylin); IgG (immunoglobulin).

Supplementary Figure S7: Quantitative assessment of phospholipid species and AA:DHA lipid ratio in the cortex of a hTau mouse model of chronic repeated TBI

Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), sphingomyelin (SM) and phosphatidylcholine (PC/etherPC) were examined in cortical tissue homogenates of sham and injured animals. No changes were observed in total PC and PIin injured compared to sham animals (A, B). However, a significant increase in total PE and SM was observed in injured compared to sham animals (C-D). A significant increase in saturated and poly-unsaturated PEcontaining lipids was observed in injured animals compared to sham animals; no changes were observed in mono-unsaturated PE containing lipids (C). Both saturated and mono-unsaturated SM containing lipids were increased in injured compared to sham animals (D). No changes were observed in saturated, mono and poly-unsaturated PC and PI containing lipid species in injured compared to sham counterparts (A, B).

Arachidonic acid (AA) and docosahexaenoic acid (DHA) containing lipid species were calculated for PC, PI and PE. There was a significant increase in AA containing PC and PE species and a trend towards increase in PI species in injured compared to sham animals (E-G). No change was observed in DHA containing lipid species for PI (F), however, a significant increase was observed in DHA containing lipid species for PC and PE in injured compared to sham animals (E, G). The ratio of AA:DHA containing species for PE was significantly increased in injured compared to sham animals (H); a trend towards increase was also observed for AA:DHA containing species for PC and PI but this was not statistically significant (data not shown). N=5 (sham/injured). Asterisks denote: *P<0.05; **P<0.01; ***P<0.001. Abbreviation: MUFA (monounsaturated lipid), PUFA (polyunsaturated lipid); AA (Arachidonic acid), DHA (docosahexaenoic acid).