Repellent Mild Traumatic Brain Injury Augments Tau Pathology and Glial Activation in Aged hTau Mice

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Abstract

Extensive tau-immunoreactive neurons and glial cells associated with chronic traumatic encephalopathy (CTE) have been documented in the brains of some professional athletes and others with a history of repetitive mild traumatic brain injury (r-mTBI). The neuropathology and tau involvement in mTBI have not been extensively studied in animal models, particularly in aged animals. We investigated the effects of single mTBI (s-mTBI) and r-mTBI in 18-month-old hTau mice, which express wild-type human tau isoforms on a null murine tau background (n = 3–5 per group). At this age, hTau mice already demonstrate tau pathology, but there was a significant increase in phospho-tau immunoreactivity in response to r-mTBI, but not to s-mTBI, as determined using multiple phospho-tau-specific antibodies. Repetitive mTBI also resulted in a marked increase in astrocyte/microglia activation notably in the superficial layer of the motor/somatosensory cortex and the corpus callosum. We did not observe perivascular tau pathology, neuritic threads, or astrocytic tangles that are commonly found in human CTE. The increase in phospho-tau in the r-mTBI mice suggests that this may be a useful model for investigating further the link between mTBI, particularly r-mTBI, and tau pathobiology in CTE and in understanding responses of the aged brain to mTBI.

Key Words: Aging, Chronic traumatic encephalopathy, Glia, Human tau, Mild traumatic brain injury, Phospho-tau.

INTRODUCTION

Repetitive (and/or concussive) closed head injury is most frequently encountered in professional athletes engaged in contact sports (1, 2). Although most athletes recover within a few days or weeks after initial injury, a fraction develop progressive multiple symptoms that may appear months or even years after initial trauma (3). This has been documented in a small number of patients with a history of repetitive concussion who develop chronic traumatic encephalopathy (CTE), previously recognized as dementia pugilistica (4–9). Although careful prospective studies have not been done, in the few cases that have been pathologically characterized, the neurologic deficits of CTE progressively exacerbate and expand in symptomatology, often during several decades, consistent with a progressive neurodegenerative condition. Behavioral abnormalities, such as abrupt mood swings with explosive rage, depression, impulsive acts, and substance abuse, are prominent early in the course. Other symptoms include difficulty sleeping, memory impairment, poor concentration, fatigue, and difficulty with executive functioning and concentration (10–14). In the later stages, slurring of speech, motor signs and symptoms of parkinsonism and ataxia, and dementia are common (12–14). Not infrequently, patients have come to autopsy as a result of suicide (15).

Little data exist regarding the precise incidence, prevalence, and risk factors implicated in the development of CTE after repetitive concussion. A study of retired professional boxers estimated that 17% developed the syndrome (16), but this is likely an underestimation because dementia and neuropsychiatric disorders, in the absence of prominent motor symptoms, were not sufficient for a CTE diagnosis in this study. Chronic traumatic encephalopathy is believed to be uncommon in amateur boxers and in athletes involved in other sports such as football or hockey, but the true prevalence and natural history are unknown. There is a critical need to develop an animal model of CTE to understand the pathogenesis of CTE and to develop effective therapies.

Tau pathology is implicated in repetitive head injury (7, 8, 17–19). Blows to the head during a single boxing bout are associated with increased tau in the spinal fluid (20). Pathologically, CTE has been described as “a slowly progressive ‘tauopathy’ with a clearly distinguishable environmental etiology, characterized by the degeneration of brain tissue, and a unique spectrum of abnormalities in phosphorylated tau accumulation.” (8). The hallmarks of this distinct tauopathy implicated in CTE have been extensively discussed by Omalu et al (6) and McKee et al (8) and is typified by extensive phospho-tau–immunoreactive neurofibrillary tangles (NFTs), marked tau-immunoreactive protoplasmic astrocytes, and large globose and spindle-shaped neuropil neurites (7, 8, 17–19). This pattern of neurofibrillary degeneration is distinguishable from other tauopathies by the preferential involvement of the superficial cortical lamina, accompanied by irregular multifocal patchy distribution in the depths of the...
cortical sulci, and prominent perivascular and periventricular and subpial distribution with marked fibrillar astrocytic tangles (8).

Although these studies and others consistently describe phosho-tau and NFT pathology within hours to years in brains of individuals who have sustained a single severe traumatic brain injury (TBI) or repetitive mild traumatic brain injuries (r-mTBIs) (7, 8, 17–19, 21–24), the role of tau pathology in mTBI is not yet clear. Moreover, limited preclinical research has been performed in appropriate animal models to investigate the pathologic consequences of mTBI/concussion and/or the involvement of tau. One of the major challenges in this field is to characterize mTBI in a relevant model that takes into consideration the differences between the adult profiles of tau isoforms in both humans and mice. Some TBI studies have used transgenic mouse models of neurodegeneration with familial genetic mutations in tau (25–29); most of these report evidence of greater pathologic phospho-tau species (and, in some cases, NFT pathology) in response to injury as compared with aged-matched controls. For example, phospho-tau pathology was evident (24 hours–7 days) post–single controlled cortical impact injury (25–27) but also persisted at a chronic time point 9 months after repetitive (16 times) nonpenetrating concussive head injuries (29). However, all these models lack the normal expression of the human tau gene and so cannot adequately model human tau pathology in response to TBI. To address this, we focus on hTau (human Tau) transgenic mice (30–33), which express the 6 isoforms of human tau on a null murine-tau background. Using our recently developed mTBI model (34), we demonstrate that tau pathology, which is readily observed in aged hTau mice, is remarkably augmented in response to r-mTBI, but not single mTBI (s-mTBI), and that this is evident 3 weeks after injury. We also report other distinct pathologic changes, notably, pronounced and dramatic neuroglial (astrocyte and microglia) activation and neuronal damage. We did not observe any perivascular tau deposition, neuritic threads, or astrocytic tangles, which are commonly identified in human CTE cases.

MATERIALS AND METHODS

hTau mice

Human Tau mice on a C57BL/6 and null murine tau background (generated as previously described by Andorfer et al [30]) were purchased from Jackson Laboratories, Bar Harbor, ME. All hTau mice used in this study were aged 18 months (average weight, 25 g) and 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 different experimental groups (s-mTBI, n = 3; r-mTBI, n = 5; and both shams, n = 4). 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.

Closed Head Injury: Experimental mTBI

The experimental TBI methods were performed as previously described (34). Briefly, mice were anesthetized with 1.5 L per minute of oxygen and 3% isoflurane for 3 minutes. After shaving (injury site/around anterior fontanelle), the 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). The head/skull of the mice was positioned 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 sagittal suture midway 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 sublethal impact does not cause direct tissue damage to the injury site in the motor/somatosensory cortex, and there is no development of skull fracture or subdural hemorrhage.

Mice in the s-mTBI group received 1 hit, whereas those assigned to the r-mTBI received 5 impacts during a 9-day period with an interinjury interval of 48 hours (34). Sham control mice received single (s-sham) or multiple (r-sham) anesthetias of the same duration as their mTBI counterparts (34). After impact was delivered, the mice were allowed to recover on a heating pad set at 37°C to prevent hypothermia-induced hyperphosphorylation of tau (35). On becoming ambulatory (regaining consciousness), mice were returned to their cages and carefully monitored for any abnormalities.

Immunohistochemical Staining

At 3 weeks after the last or s-mTBI or anesthesia control, all animals were deeply anesthetized with isoflurane. They were then intracardially perfused by gravity drip with a heparinized PBS solution, pH 7.4, and 4% paraformaldehyde for 3 minutes. This was followed by an overnight postfixation of entire brain samples in 4% paraformaldehyde followed by paraffin embedding. Separate series of 5- to 6-µm-thick coronal sections were cut throughout the extent of the cortex, hippocampus, and associated areas (guided by known bregma coordinates [36]) 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 then rinsed in water and
subsequently incubated at room temperature in a solution of endogenous peroxidase blocking solution, containing 0.3% H$_2$O$_2$ diluted in PBS (0.1 mol/L, pH 7.4) for 30 minutes. After rinsing, sections were treated with target retrieval solution for 8 minutes in the microwave (if recommended) for antigen retrieval. Further incubation with either protein block ‘‘serum-free’’ solution (Dako, Carpinteria, CA) or mouse immunoglobulin G blocking reagent (mouse on mouse [MOM] Kit, Vector Laboratories, Burlingame, CA) (depending on antibody used) was performed for a period of 1 hour in a humid chamber at room temperature.

Sections were stained in batches with primary antibodies made up in antibody diluent, background-reducing agent. The antibodies were raised against 1) a range of mouse phospho-tau epitopes including CP13 (detects phospho-serine residue 202), PHF-1 (detects paired helical filaments at both ser396 and ser404), and RZ3 (detects phospho-threonine 231); these antibodies and protocols were generously provided by Peter Davies, Bronx, NY; 2) glial fibrillary acidic protein (GFAP) (rabbit anti-GFAP, 1:10,000; Dako) for astrocytosis; and 3) CD45 (mouse anti-rat CD45, 1:1000; Serotec, Raleigh, NC) for microglia.

After overnight incubation with the relevant primary antibodies, sections were rinsed with PBS, transferred to a solution containing the appropriate secondary antibody (from the Vectastain Elite ABC Kit, Vector Laboratories), diluted in 20% superblock (Pierce, Thermo Fisher Scientific, Rockford, IL) for 1 hour and further incubated with avidin–biotin–horseradish peroxidase solution (Vectastain Elite ABC Kit; Vector Laboratories) or streptavidin–biotin–horseradish peroxidase solution (Pierce) for 1 hour.

Immunoreactivity was visualized with DAB (3,3-diaminobenzidine) chromogen and H$_2$O$_2$. Development with H$_2$O$_2$ diluted in PBS (0.1 mol/L, pH 7.4) for 30 minutes. After rinsing, sections were treated with target retrieval solution for 8 minutes in the microwave (if recommended) for antigen retrieval. Further incubation with either protein block ‘‘serum-free’’ solution (Dako, Carpinteria, CA) or mouse immunoglobulin G blocking reagent (mouse on mouse [MOM] Kit, Vector Laboratories, Burlingame, CA) (depending on antibody used) was performed for a period of 1 hour in a humid chamber at room temperature.

Immunoreactivity for cell markers 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 by blind assessment on coded slides to avoid bias in evaluation. Immunoreactivity for each cell marker was assessed within the cortex, hippocampus, and/or associated regions. A survey of immunostained tissue sections was performed independently to verify specific immunoreactivity in each series (~1 in 10) of sections that was subsequently progressed to quantitative image analysis. Briefly, nonoverlapping red, green, blue (RGB) images were digitally captured randomly within the defined areas from each section (comprising an average of 4 to 6 sections per animal for each marker), providing a systematic survey of each region throughout the area of interest for each animal within a group. A minimum of 20 microscopic fields were analyzed per region per animal to yield a sufficient coefficient of error less than 0.05 determined from previous trial runs. The microscopic fields measured 400 µm × 325 µm, yielding a total area of 2.6 mm$^2$ analyzed for each region per animal.

Immunoreacted profiles that were optically segmented were analyzed using Image-Pro Plus morphometric image analysis software (Media Cybernetics, Bethesda, MD). 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 (the mean RGB value obtained by subtracting the total mean RGB value from nonimmunostained value per defined field). 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 PHF-1–positive cells in the hippocampus, cell counts were determined from a series of 4 to 6 sections (the same used above for image analysis) throughout the hippocampus. Cell counts were easily determined at low magnification because PHF-1–positive cells were in relatively small proportions in each section. Data were separately plotted as PHF-positive cells counts/hippocampal section ± SEM for each region and grouping.

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.
differences test. All analyses were performed with SPSS 17.0 (IBM Corp., Armonk, NY).

RESULTS

Augmented Phospho-tau Pathology in Cortex and Hippocampus in Aged hTau Mice After r-mTBI

A marker of the phospho-tau epitope at serine residue 202, CP13, was used to detect pretangles and late-stage tau (intraneuronal) NFTs (30, 39–41). Previous immunoblotting and pathologic studies on aged hTau naive mice (13–15 months) report neuronal cell body accumulation of CP13 immunoreactivity in the hippocampus and the neocortex, resembling that occurring in early-stage NFTs of the human brain with tauopathy (30, 31). Within the cortex and hippocampus, but not the dentate gyrus, more CP13 staining was evident in the r-mTBI group compared with that in r-sham controls (Fig. 1D, H, L, P vs B, F, J, N). CP13 immunostaining in the r-mTBI group was expressed in neuronal cell bodies and apical dendrites (Fig. 1D1, D2, H1, H2, L1, L2, P1, P2). Some CP13-positive neurons showed evidence of darkly stained tortuous-like apical dendrites, resembling NFTs (Fig. 1H1, L2). The s-mTBI mice showed no appreciable effect on CP13 immunoreactivity compared with that in s-sham controls (Fig. 1C, G, K, O vs A, E, I, M). In contrast to the effects of r-mTBI, CP13 immunoreactivity was mainly localized to the cell body of s-mTBI and sham control animals. In the cortex, s-sham, r-sham, and s-mTBI animals were almost devoid of CP13 immunoreactivity (Fig. 1A–C). Quantitation

FIGURE 1. Abnormal phosphorylated tau immunoreactivity (using CP13; phosphoserine 202) in the motor/somatosensory cortex and hippocampus of aged hTau mice after single (s-mTBI) and repetitive (r-mTBI) mild traumatic brain injury and sham controls. (A–P) CP13 immunoreactivity is present in the cell body and apical dendrites of neurons in the cortex and hippocampus of r-mTBI mice ([D, H, L, P] see also high-power micrographs inset) compared with those in sham control (B, F, J, N). CP13 immunostaining is predominantly localized to the cell body of pyramidal neurons in s-mTBI mice (C, G, K, O). Their immunoreactivity levels are greater in the hippocampus than in both sham groups. The cortex shows a paucity of CP13 immunoreactivity. Sham groups show minimal immunoreactivity for CP13. The s-mTBI group (K, O) shows slightly greater intensity of CP13 immunostaining compared with that in the s-sham group in the hippocampus (I, M). In both cases, the cortex is also devoid of CP13 immunoreactivity (A, B). CA1, hippocampal CA1 area; CA3, hippocampal CA3 area; DG, dentate gyrus/hilus of the hippocampus; r-mTBI, repetitive (×5) mild traumatic brain injury; s-mTBI, single mild traumatic brain injury; r-sham, repetitive (×5) anesthesia exposure; s-sham, single anesthesia exposure. Scale bar = 80 μm.
showed that immunoreactivity levels were significantly greater in the cortex of r-mTBI animals than in the other groups (Fig. 2A).

RZ3

RZ3-2E12 immunoreactivity was used to detect abnormally phosphorylated tau protein accumulation (phospho-threonine 231 epitope). Previous studies report that RZ3 levels were evidently increased in immunoblots of heat-stable fractions (enriched for tau) prepared from brain homogenates of aged (15-month-old) hTau mice (30).

We also found that RZ3 staining was evident in neuronal cell bodies and some apical dendrites in our 18-month-old hTau mice (Fig. 3A, B inset). The RZ3 immunoreactivity pattern was relatively similar in all groups (Fig. 3), but in the CA3 region, r-mTBI animals seemed to have greater immunoreactivity (Fig. 3L, P). However, quantitative analysis showed this to be significant only in the DG (Fig. 2B). RZ3-positive NFT-like tangles were observed in the CA3 region (Fig. 3L, inset).

PHF-1

To detect late-stage neurofibrillary-like pathology, we used the PHF-1 antibody, which recognizes phospho-tau epitopes at both Serine 396 and Serine 404. PHF-1 expression in hTau mice has previously been reported to be unchanged at extended time points up to 15 months of age (42–44). We found that PHF-1 immunoreactivity was upregulated in the cortex of 18-month-old r-mTBI mice (Figs. 2D; 4D, H, L, P) versus sham control and s-mTBI mice; there was a paucity of PHF-1 immunostaining in the cortex of these 3 groups (Fig. 4A–C). The hippocampus was also devoid of PHF-1 immunoreactivity in both shams and s-mTBI animals (Figs. 2C; 4C).
4E–G, I–K, M–O), whereas the r-mTBI mice had markedly greater PHF-1 immunoreactivity by 4- to 5-fold (Figs. 2C; 4D, H, L). PHF-1 immunoreactivity was also localized to neuron cell bodies and apical dendrites in the cortex and hippocampus in the r-mTBI mice (Fig. 4D1, H1, H2). Gallyas silver staining also supports the evidence of late-stage tau-like pathology in the hippocampus and cortex after r-mTBI compared with sham controls (Fig. 5).

**Effects of mTBI on Microglia and Astrocyte Responses**

Glial fibrillary acidic protein–positive immunoreactivity is known to be moderately upregulated with aging in rodents (45–47). We found that GFAP-positive cells, especially in the cortex and corpus callosum (Fig. 6D, H), exhibited thicker processes and hypertrophic cell somata after r-mTBI compared with those in controls (Fig. 6). These cells were widely distributed beneath the pia matter of the impact site in the motor/somatosensory cortex (Fig. 6D inset). Glial fibrillary acidic protein immunoreactivity was increased by almost 4-fold in the cortex of r-mTBI animals (Fig. 7A). There was also slightly increased GFAP immunoreactivity in the s-mTBI CA1-CA3 versus s-sham mice (Fig. 7A). Moreover, there were also comparative differences in brain regions after r-mTBI injury, whereby GFAP immunoreactivity was reduced significantly in the hippocampus by approximately 4-fold compared with sham controls (Fig. 7A).

**Augmented Widespread Tau Pathology in the Brains of Aged-hTau Mice After r-mTBI**

There was a similar pattern of augmented phospho-tau pathology in other relevant brain regions in the r-mTBI versus r-sham mice. These areas included the substantia nigra (pars reticular), cerebellum, basolateral amygdala, brainstem, olfactory bulb, and the ventral thalamus (Fig. 5A–F, I–N).

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**FIGURE 3.** Abnormal phosphorylated tau immunoreactivity (using RZ3-2E12 /detects phospho-threonine 231) in the motor/somatosensory cortex and hippocampus of aged hTau mice after single and repetitive mild traumatic brain injury (mTBI). (A–P) RZ3 immunoreactivity is uniformly expressed and similar in both sham controls and s-mTBI (A–C, E–G, I–K, M–O). RZ3 staining is predominantly localized to cell bodies and apical dendrites of neurons (white arrowheads in [A] and [B]; inset). The r-mTBI animals show greater staining intensity only in the CA3 and DG (L, P), r-mTBI, repetitive (‘×5’) mild traumatic brain injury; s-mTBI, single mild traumatic brain injury; r-sham, repetitive (‘×5’) anesthesia exposure; s-sham, single anesthesia exposure. Scale bar = 80 μm.
1.5- to 2-fold compared with that in s-mTBI or either sham group (Figs. 6I; 7A).

De novo CD45-positive immunoreactivity (an indicator of microglial activation [48, 49]) is known to be mildly upregulated with aging (50, 51). We found that CD45-positive microglia in the superficial layer of the motor/somatosensory cortex, below the pia matter of the injury site after r-mTBI, displayed prominent cell bodies with thickened processes, largely adopting a bushy ramified morphology (Fig. 8D, D).

Few CD45-positive microglia were observed in the other groups in any region examined (Fig. 8). A few CD45-positive cells resembling phagocyte-like microglia with prominent cell somata and shortened processes were seen in the r-mTBI group (Fig. 8H, H). Few CD45-positive microglial cells were located around perivascular cells (Fig. 8H, L, P, T). Neither was there evidence in any of the regions investigated of infiltrating cells such as CD45-positive monocytes, macrophages, or natural killer cells that would have suggested significant compromise to the blood-brain barrier nor was there any evidence of cortical contusions and/or visible focal loss of cortical necrosis beneath the impact sites of injury. CD45 immunoreactivity was significantly increased within all the major regions analyzed after r-mTBI (Fig. 7B).

**Effects of mTBI on Pyramidal Neurons**

Using both cresyl violet and TUNEL staining, we identified no distinct abnormalities in pyramidal neurons in either s-sham or s-mTBI animals (Fig. 9A and C, respectively). Pyramidal neuronal nuclei generally seemed normal and were devoid of TUNEL-positive staining. However, in r-mTBI animals, there were notable changes in pyramidal cell morphology in the cortex (Fig. 9D) and CA3 (Fig. 9E); some of these neurons seemed irreversibly damaged with 1) condensed darkly stained perikarya, 2) shrunken basophilic pyknotic nuclei, and 3) short tortuous dendrites. In addition, TUNEL-positive cells were seen in the cortex of r-mTBI animals and to a lesser extent in r-sham animals (Fig. 9B, D).

**DISCUSSION**

We characterized the effects of our recently developed model of single and repetitive closed head injury (34) in aged...
FIGURE 5. Widespread augmented tau pathology in the brains of aged hTau mice after repetitive mild traumatic brain injury (r-mTBI). (A–R) CP13 immunoreactivity was upregulated in the substantia nigra pars reticulata (SNR), olfactory bulb (OLFB), and the cerebellum (CereB) in r-mTBI mice versus repetitive anesthesia exposure (r-sham) controls (A–D, I–J). PHF-1 immunoreactivity was increased in the basolateral amygdala (BLA), brainstem (BS), and ventral thalamus (VT) after r-mTBI compared with those in shams (E–F, K–N). Pyramidal neurons in the DG, CA3, and the cortex were also positively stained with Gallyas silver staining after repetitive injury compared with those in sham control (G–H, O–R). Scale bars = (A, B, E–P) 80 mm; (C, D) 235 μm; (Q, R) 36 μm.
FIGURE 6. Repetitive mild traumatic brain injury (mTBI) modulates astrocyte activation in the corpus callosum (Cc), motor/somatosensory cortex (Cortex), and hippocampus (Hippo) of aged hTau mice. (A-T) In r-mTBI mice, glial fibrillary acidic protein (GFAP) immunoreactivity and GFAP-positive cell density are increased in the superficial layer of the cerebral cortex ([D] and inset at 20× magnification, white arrowheads) and in the overlying white matter tract of the corpus callosum (H, L) versus sham control and s-mTBI mice (A-C, E-G). Glial fibrillary acidic protein–positive stellate astrocytes exhibit thicker intermediate filaments/processes and hypertrophic cell somata in the cortex ([D] inset). In the hippocampus, immunoreactivity seemed to be reduced in the CA3 and DG (P, T) compared with those in other groups (M-O, Q-S). Glial fibrillary acidic protein immunostaining in the s-mTBI group (C-G, K-O, S) and r-sham group (B-F, J-N, R) was upregulated in the CA3 compared with that in the single sham group (A, E-I, M-Q). In the latter 3 groups, the cortex was notably devoid of GFAP immunoreactivity (A-C). r-mTBI, repetitive (’×5’) mild traumatic brain injury; s-mTBI, single mild traumatic brain injury; r-sham, repetitive (’×5’) anesthesia exposure; s-sham, single anesthesia exposure. Scale bars = (A-D) 125 μm; (E-H, M-T) 85 μm; (I-L) 485 μm.
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Pronounced Tau Pathology After Repetitive (but Not Single) TBI in hTau Mice

In pathologic conditions associated with tauopathy, soluble cytosolic tau protein, the major component of NFTs, is hyperphosphorylated at specific epitopes, forming insoluble inclusion bodies in neuronal axons, cell soma, and somatodendritic compartments. The brains of some individuals who experience multiple mTBI and develop CTE display a unique tauopathy characterized by the deposition of tau-immunoreactive NFTs, astrocytic tangles, and globose and spindle-shaped neuropil neurites (6, 8). The distribution in the brain is irregular, involving multifocal patches of dense NFTs that are often concentrated in a perivascular arrangement in superficial cortical laminae, usually at the depths of the cortical sulci, and in other areas such as the olfactory bulb, hippocampus, entorhinal cortex, and amygdala (6, 8). The occurrence of these alterations may vary from within the initial hours postinjury to more prominently after a period of months to years (8, 17–24, 52).

Here, we observed some similarities to human CTE, but with notable limitations. Tau pathology is already present in hTau animals at 18 months of age, and no major alterations were detected 3 weeks after s-mTBI. However, the mice subjected to r-mTBI showed a trend of increased phospho-tau pathology in neuronal cell body and somatodendritic compartments compared with that in sham controls. These data suggest that r-mTBI, rather than s-mTBI, has a more pivotal role in the development of the tau pathology. Most of the pathologic changes were confined to the superficial layer of the cerebral cortex, as in CTE (at basal brain surfaces beneath impact sites), and some in the underlying hippocampal region (especially CA1–CA3). Other regions that demonstrated augmented tau pathology were the substantia nigra (pars reticulata), ventral thalamus, brainstem, olfactory bulb, cerebellum, and basolateral amygdala. This is also consistent with observations in some human CTE/mTBI cases (8). We did not detect the hallmark perivascular and periventricular deposition of tau pathology or neuritic threads that are occasionally seen in human CTE; neither were there fibillary tau immunoreactive astrocyte tangles. These differences may be attributable to species differences, time from injury to analyses, or other factors yet to be uncovered in human and/or mouse TBI pathogenesis.

Earlier preclinical TBI studies using tau transgenic rodent models have described changes in tau pathology similar to those we have shown after TBI. Most of these studies have been confined to mouse models with familial genetic mutations (28, 29), and only a few have exploited r-mTBI injury. For example, Tran et al (25) used triple transgenic-AD mice (at 5–7 months of age) from the B6/SJL129 background, which overexpresses β-amyloid and tau (53). Using the single controlled cortical impact injury model, which induces subdural hemorrhage and inflicts direct tissue damage to the dura mater and the ipsilateral cortex, they reported increased tau accumulations and phosphorylation at S-199 and PHF-1 epitopes in the ipsilateral fimbria, amygdale, and hippocampi after immunohistochemical and biochemical analyses (20). Some of these changes were detected at subacute time points, with phospho-tau immunoreactivity rising in a biphasic manner after 1 hour and 24 hours after injury and remaining elevated 7 days after injury (26, 27). These investigators also used the double PSAPPsw mice and the TauP301L mice to demonstrate a similar pattern of accelerated phospho-tau immunoreactivity in the same regions after single controlled cortical impact injury (26, 27). Despite the differences in time points examined and the ages of animals used, these observations...
(including ours) seem to support the contention that “tau abnormalities appear to be virtually ubiquitous sequelae” underlying the fundamental response of the brain to mTBI (26).

The precise incidence and severity of tau pathology after mTBI in animal models remain unknown. One elaborate study addressed this question in a similar (closed head repetitive injury) model involving middle-aged 12-month-old T44 mice. Animals received 16 different impacts, which involved 2 impact injuries at each side of the skull and a total of 4 series of injuries per day, repeated every week for 4 weeks (29). The authors found that repetitive TBI dramatically accelerated the formation of tau lesions at 9 months post-r-mTBI, which was accompanied by cerebral atrophy, oxidative stress, and cognitive deficits. Most transgenic mice (92%) in this study were resistant to tangle formation and the induction of potentially pathogenic phospho-tau pathology. The reduced incidence of tau pathology in these mice, despite the increased frequency of concussive injury (compared with ours), is intriguing. However, it is noteworthy that the T44 mice express the shortest isoform of human wild-type tau, rather than the complete human tau gene profile, which could explain their inability to demonstrate mTBI-dependent tau pathology as we have done.

The mechanism behind tau pathology in repetitive concussive head injury is unknown. Although tau is a useful

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*FIGURE 8.* Repetitive mild traumatic brain injury (r-mTBI) modulates microglial activation in the corpus callosum (Cc), motor/somatosensory cortex (Cortex), and hippocampus (Hippo) of aged hTau mice. (A-T) In the r-mTBI group (D, H, L, P, T), CD45 immunoreactivity and CD45-positive cell density are concomitantly upregulated in all regions versus controls (A-C, E-G, I-K, M-O, Q-S). There is a marked increase in CD45 immunoreactivity in the superficial layer of the cortex (D, D’ insert). CD45-positive microglia also showed a ramified thick bushy appearance with increased processes (D’). There was also evidence of phagocyte-like microglia with prominent cell body and shortened processes (arrows in H’) and cells resembling perivascular activated microglia (H’, L’, P’, T). There were no major alterations in CD45 immunostaining in the s-mTBI mice (C, G, K, O, S), r-sham mice (B, F, J, N, R), or single sham mice (A, E, I, M, Q). r-mTBI, repetitive (‘×5’) mild traumatic brain injury; s-mTBI, single mild traumatic brain injury; r-sham, repetitive (‘×5’) anesthesia exposure; s-sham, single anesthesia exposure. Scale bars = (A-D) 125 μm; (D’) 75 μm; (E-H, M-T) 85 μm; (H’) 35 μm; (I-L) 485 μm; (L) 25 μm; (P’) 50 μm; (T’) 75 μm.
Clinicopathologic biomarker for the diagnosis of CTE, it is not yet clear that tau is the principal mediator of pathogenesis; it could rather be the result of cumulative insidious biochemical changes initiated after repeated mechanical injury. Several pathogenic mechanisms have been suggested to contribute to this tau-specific pathologic profile. These may include 1) covalent modifications of tau (27, 54), 2) imbalance in the activity levels of tau-specific kinases (27, 54, 55), 3) proteolytic stress and dysfunction in protein clearance mechanism (56, 57), 4) transsynaptic secretion of hTau protein–like conformers (58), and 5) increased genetic susceptibility (ApoE4 allele) (59).

Effect of Single or Repetitive Anesthesia on Tau Pathology in Aged hTau Mice

Cross-sectional longitudinal studies have revealed that elderly patients who undergo general anesthetic surgery have an increased likelihood of developing tau pathology (60, 61). Previously, a serendipitous finding reported that anesthesia (by chloral hydrate, sodium pentobarbital, and isoflurane vapors) conspicuously induced tau hyperphosphorylation, 1 hour after exposure, in 4- to 8-month-old JNPL3 tau mutant transgenic mice (35). The authors attributed this phenomenon to anesthesia-induced impairments in the normal autonomic

FIGURE 9. Repetitive mild traumatic brain injury (mTBI) induces apoptosis and alters the morphology of pyramidal neurons in the motor/somatosensory cortex (Cortex) and CA3 (Hippocampus) of aged hTau mice. (A–F) Representative micrographs stained with cresyl violet show sagittal sections taken from the cortex and hippocampus of sham and mTBI animals. Single sham and mTBI animals have normal-appearing neuronal nuclei (A, C, F). There are numerous pyramidal cells with altered nuclear morphology and darkly stained perikarya in the cortex (D, circles) and CA3 (E and Ec, arrows) in r-mTBI animals and to a much lesser extent in r-sham controls (B). Some nuclei are pyknotic. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)–positive apoptotic nuclei are stained dark brown and vary in their size and shape (A–D'). Single sham and single TBI animals were almost devoid of TUNEL-positive cells (A', C). TUNEL-positive nuclear abnormalities were detected mainly in the cortex of the r-sham (B') and r-mTBI (D') animals. r-mTBI, repetitive (‘×5’) mild traumatic brain injury; s-mTBI, single mild traumatic brain injury; r-sham, repetitive (‘×5’) anesthesia exposure; s-sham, single anesthesia exposure. Scale bars = (A–D) 110 μm; (E, F) 225 μm.
thermoregulatory control (hypothermia). Restoring normothermia in these animals 30 minutes after exposure in a ventilated incubator at 37°C completely rescued tau phosphorylation to normal levels, demonstrating that tau phosphorylation is not mediated by anesthesia per se but rather by the consequent induction of hypothermia (35). In the present study, control hTau mice were given either a single exposure or 5 separate exposures of 3% isoflurane, and their temperatures were monitored and regulated carefully during anesthesia. We did not find any evidence that there are significant changes in phospho-tau immunoreactivity after repetitive exposure to anesthesia, thereby ruling out artifactual anesthesia-induced tau pathology in this study. However, in contrast to young hTau mice, it might be possible that the existing phospho-tau pathology in the aged hTau mice used in this study already superseded that which could be induced by anesthesia.

**Glial Activation After r-mTBI**

Glial activation and inflammation are common features of neurodegenerative diseases, such as CTE (8). Herein we report that, after r-mTBI, there were prominent alterations in astrocytes and microglia. Resident protoplasmic populations of astrocytes directly beneath the epicenter of repetitive injury (in the motor/somatosensory cortex) markedly upregulated GFAP by 4-fold and underwent morphologic transformation to a more activated state. Single mTBI had a minimal (albeit significant) effect on astrocytic activation in the CA1/CA3 regions. Indeed, differences were noticeable in the hippocampus after r-mTBI in which GFAP immunoreactivity was significantly reduced by 2-fold. This novel observation is intriguing and might indicate that these resident GFAP-positive cells in the hippocampus are a heterogeneous population of astroglia, which have distinct functional properties in response to TBI. Microglia beneath the impact site after r-mTBI also underwent morphologic transformation, mostly displaying prominent cell bodies with thickened processes and a bushy ramified morphology. Some few cells resembled phagocyte-like microglia, with prominent cell somata and shortened processes. They were also observed around vessels in the basal region of the brain and in the superficial layer of the cortex beneath the injury site. These morphologic and phenotypic changes were accompanied by robust upregulation of CD45 immunoreactivity, highlighting an increase in the activation status of these cells (48, 49). Our data showing astrocytic and microglial alteration are further supported by numerous other in vivo observations of inflammatory consequences at acute and chronic time points after repetitive closed head injury (34, 62–64).

Current ideas about the mechanisms behind neuroinflammatory responses after TBI have been dominated by extrapolations from in vivo studies (65–67). Although activated astrocytes and microglia can be neuroprotective, preserving tissue integrity and restricting postinjury inflammation, their roles in TBI superimposed on an aged background may be different. Glial cells in aging are considered to be in a potentially primed state, whereby they gradually lose their threshold of tolerance and become vulnerable to subsequent stimuli such as head injury (65, 68). In the event of repetitive (insidious) concussive injury, these primed cells might trigger a hyperactive response and further stimulate a self-perpetuating cycle of damaging events that involve a sustained increase in proinflammatory and cytotoxic factors. However, it is noteworthy that, in humans, such glia cell changes do not occur only in isolation but in the context of a parallel increase in cellular age-related stress responses or deficiencies in cellular coping mechanisms, collectively associated with the accumulative effects of r-mTBI (65, 69).

In conclusion, we report that repetitive injury shows potentially pathogenic phospho-tau and inflammatory-specific changes in aged hTau mice. This injury model and this mouse model may each be of particular relevance to exploration of human TBI particularly because tau is now thought to play a key, although poorly understood, role in TBI pathogenesis. Our use of aged hTau mice may be of relevance given the high incidence of falls in the elderly population and their increased risk of developing age-related neurodegenerative diseases. However, the use of younger hTau mice will be pivotal for future studies to mimic the ages at which combat and sports-related concussions are typically sustained. This will also facilitate longitudinal studies at extended time points for neurobehavioral assessment, identification of biomarkers, and possibly preclinical drug testing. More importantly, it will also enable a detailed characterization of the events specific to tau pathobiology, such as the different profile of changes specific to a variety of phospho-tau–specific epitopes (from oligomers to fibrillar conformations), including adequately addressing the question as to whether tau pathology is a permanent, progressive, or reversible component implicated in repetitive head injury (70, 71).

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