Enhanced Neurofibrillary Tangle Formation, Cerebral Atrophy, and Cognitive Deficits Induced by Repetitive Mild Brain Injury in a Transgenic Tauopathy Mouse Model

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ABSTRACT

Traumatic brain injury (TBI) is a risk factors for Alzheimer’s disease (AD), and repetitive TBI (rTBI) may culminate in dementia pugilistica (DP), a syndrome characterized by progressive dementia, parkinsonism, and the hallmark brain lesions of AD, including neurofibrillary tangles (NFTs), formed by abnormal tau filaments and senile plaques (SPs) composed of Aβ fibrils. Previous study showed that mild rTBI (mrTBI) accelerated the deposition of Aβ in the brains of transgenic (Tg) mice (Tg2576) that over-express human Aβ precursor proteins with the familial AD Swedish mutations (APP695swe) and model of AD-like amyloidosis. Here, we report studies of the effects of mrTBI on AD-like tau pathologies in Tg mice expressing the shortest human tau isoform (T44) subjected to mrTBI, causing brain concussion without structural brain damage to simulate injuries linked to DP. Twelve-month-old Tg T44 (n = 18) and wild-type (WT; n = 24) mice were subjected to mrTBI (four times a day, 1 day per week, for 4 weeks; n = 24) or sham treatment (n = 18). Histopathological analysis of mice at 9 months after mrTBI revealed that one of the Tg T44 mice showed extensive telencephalic NFT and cerebral atrophy. Although statistical analysis of neurobehavioral tests at 6 months after mrTBI did not show any significant difference in any of groups of mice, the Tg T44 mouse with extensive NFT had an exceptionally low neurobehavioral score. The reasons for the augmentation of tau pathologies in only one T44 tau Tg mouse subjected to mrTBI remain to be elucidated.

Key words: Tau protein; transgenic mouse; repetitive head injury; neurofibrillary tangles; cerebral atrophy; cognitive deficit

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INTRODUCTION

A large body of evidence indicates that traumatic brain injury (TBI) is a risk factor for Alzheimer’s disease (AD), and repetitive TBI (rTBI) in career boxers may culminate in a disorder known as dementia pugilistica (DP) or “punch drunk” syndrome. It is characterized clinically by a progressive dementia with parkinsonism, as well as postmortem evidence of abundant senile plaques (SPs) formed by Aβ deposits and neurofibrillary tangles (NFTs) composed of abnormal tau filaments similar to AD (Geddes et al., 1996; Schmidt et al., 2001; Smith et al., 2003). Indeed, immunohistochemical and biochemical analyses showed that the NFTs and a pathological species of tau in DP brains are indistinguishable from those in AD (Schmidt et al., 2001). While the mechanisms whereby TBI increases the risk for AD or DP remain enigmatic, mild rTBI (mrTBI) accelerates Aβ deposition and cognitive impairments in transgenic (Tg) mice (Tg 2576) that over-express human Aβ precursor proteins and harbor the familial AD Swedish (APP695swe) mutations (Uryu et al., 2002). Also, it has been observed that, in non-Tg mice, cortical impacts induce α-synuclein axonal pathology in an age-dependent manner (Uryu et al., 2003). Thus, it is pertinent to further assess the potential causal relationship between TBI and AD-related pathogenesis, and to that end, we sought to elucidate the contributions of TBI to the formation of NFTs by subjecting Tg mice, over-expressing the shortest human tau isoform (T44) to mrTBI. As described earlier (Ishihara et al., 1999, 2001), these tau Tg mice developed filamentous tau inclusions in the spinal cord by 9 months of age and cortical AD-like NFTs by 18 months. These studies demonstrate that mrTBI can augment the accumulation of telencephalic NFT-like inclusions and pathological species of tau; however, this was observed in only one of the tau Tg mice and none of the wild-type (WT) mice subjected to mrTBI.

MATERIALS AND METHODS

Animals and Surgical Procedures

T44 tau Tg mice (n = 18) and WT littermate non-Tg control mice (n = 24) were used in this study and the properties of these mice have been described in previous publications (Ishihara et al., 1999, 2001). At 12 months of age, T44 (n = 12) and WT mice (n = 12) were subjected to mrTBI as described earlier with minor modifications (Uryu et al., 2002; Laurer et al., 2001; Longhi et al., 2005). Briefly, mice were anesthetized with inhalation anesthesia via a nose cone using 2% Isoflurane and placed in a modified stereotactic frame. An eye lubricant ointment (Duratears Naturale, Alcon Laboratories, Fort Worth, TX) was applied to protect the corneal membrane during surgery. A midline scalp incision was made and the skull was exposed. After disconnection from the nose cone and immediately after observing a positive response to a tail pinch, the mice were subjected to mild TBI using a controlled cortical impact (CCI) device as previously described for rodents (Dixon et al., 1991; Smith et al., 1995). In order to obtain repetitive injuries that recapitulate the features of diffuse injury without the risk or skull fracture and focal brain damage, we replaced the previously adopted rubber impactor tip with a larger diameter (9 mm) silicone impactor. The impactor used to produce these injuries was a silicone-covered impactor driven by a pneumatic piston, which was rigidly mounted at an angle of 20° from the vertical plane and driven alternatively perpendicularly onto the exposed left and right parietal bones between the bregma and lambda sutures. The zero point was obtained by lowering the impactor tip until it touched the parietal bone, midway between bregma and lambda. The impounder was driven at 4.8–5.0 m/sec to a depth of 2 mm farther than the zero point, causing a non-penetrating concussive blow to the head. A linear velocity displacement transducer (model ATC-101; Schaevitz, Pennsauken, NJ) was used to produce an analog signal for verification of the impact parameters. Closure of the scalp incision was then performed with a 4–0 silk suture. During surgery and recovery, the mice were placed on a heating pad and core body temperature was maintained at 37°C. The mice received two injuries on each side of the skull, for a total of four injuries that were sequentially performed at 20-min intervals. To simulate mrTBI here, a series of four injuries in a day were repeated once a week for 4 weeks leading to a total of 16 injuries per mouse. Sham-injured mice were subjected to the same procedures without receiving the injuries. All of these procedures were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and they were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Neurobehavioral Assessments

The Morris water maze (MWM) and the composite neuroscore (NS) studies were performed once at 6 months after mrTBI to assess cognitive function and motor function respectively. MWM and NS procedures were performed as described in detail previously (Uryu et al., 2002). Briefly, the MWM is a circular pool 1 m in diameter, painted white inside (Morris et al., 1982). The water (16–18°C) is made opaque by adding nontoxic, water-soluble white coloring. To test for mrTBI-induced
learning impairments, animals received no training in the MWM before injury and were trained to locate a stationary, submerged platform (0.5 cm below the surface) using external cues starting at 24 weeks after injury. The essential feature of the MWM is that mice can escape from the water onto the platform after being placed randomly at one of four sites in the pool. Latencies of four trials/day were recorded and averaged to obtain a measurement for the performance of each animal on a given day. Animals were tested for their ability to learn the visuospatial task in the MWM over an 8-day period that began 24 weeks after being subjected to mrTBI. Our previous studies to adapt the MWM to mice indicated that mrTBI does not cause changes in swim speed or visual acuity that influence latencies in the mouse version of the MWM (Uryu et al., 2002). NS was obtained for mice before performing MWM evaluations as described previously for TBI in rats (McIntosh et al., 1987; McIntosh et al., 1989) after modifications for use in mice (Murai et al., 1998; Nakamura et al., 1999). The NS measures the following tasks: (1) forelimb flexion response during suspension by the tail, (2) resistance to lateral pulsion, and (3) response of the hindlimb and toes (hindlimb flexion) when raised by the tail. Each animal was scored by an investigator blinded to the injury status of the animal using a scaling system ranging from 4 (preinjury control status) to 0 (afunctional).

**Histopathological Analyses**

Histopathological studies were performed on T44 tau Tg and WT non-Tg male and female mice that were sacrificed 3 \( (n = 2) \), 6 \( (n = 7) \), or 9 \( (n = 33) \) months after being subjected to mrTBI or sham injury. All mice were lethally anesthetized and perfused intracardially with PBS \( (0.1M, \text{pH } 7.4) \), and their brains were cut sagittally between each hemisphere and one hemisphere as well as the spinal cord were emersion fixed with phosphate-buffered 4% paraformaldehyde, and the other hemisphere was flash frozen and kept at \(-80^\circ C\) until biochemical analysis. Fixed brains and spinal cords were sliced into 2-mm-thick coronal slabs, embedded in paraffin, and 6-\( \mu m \)-thick coronal sections were cut from these blocks for histopathological analyses. Representative sections of brains and spinal cords were stained with Hematoxylin and Eosin (H&E), Gomori, Gallyas, Thioflavin-S, and Congo-Red histochemical methods, as described elsewhere (Ishihara et al., 1999; Uryu et al., 2002). Immunohistochemistry was performed on a similar series of sections using a polyclonal anti-tau antibody (17026), as well as a panel of epitope specific monoclonal antibodies to tau, a monoclonal antibody to glial fibrillary acidic protein (GFAP; DAKO, Glostrup, Denmark) and a monoclonal anti-ubiquitin antibody (Chemicon, Temecula, CA) as previously described (Ishihara et al., 1999; Uryu et al., 2002).

**Biochemical and Western Blot Analyses**

To examine normal and pathological species of tau proteins, brain tissues were extracted with RAB Hi-Salt buffer \( (0.1M \text{ MES, } 1mM \text{ EGTA, } 0.5mM \text{ MgSO}_4, 0.75M \text{ NaCl, } 0.02M \text{ NaF, } 1mM \text{ phenylmethylsulfonyl fluoride, and } 0.1\% \text{ protease inhibitor cocktail} \) [\( 100mg/mL \) each of pepstatin A, leupeptin, \( N\)-tosyl-L-phenylalanyl chloromethyl ketone, \( N\)-tosyl-lysine chloromethyl ketone, and soybean trypsin inhibitor], and 100 mM EDTA [\( \text{pH } 8.0 \)] and centrifuged at \( 50,000 \times g \) for 40 min at \( 4^\circ C \). The resulting pellets were extracted with RIPA buffer \( (50mM \text{ Tris}, 150mM \text{ NaCl}, 1% \text{ NP40, } 5mM \text{ EDTA, } 0.5% \text{ sodium deoxycholate, and } 0.1% \text{ SDS} \) [\( \text{pH } 8.0 \)] and centrifuged to generate RIPA-soluble samples, and the RIPA-insoluble pellets were re-extracted with 70% formic acid (FA). The RAB- and RIPA-soluble samples were boiled for 5 min, chilled on ice for 5 min, and re-centrifuged at \( 10,000 \times g \) for 20 min at \( 4^\circ C \). Protein concentration was then determined for the second supernatants with the BCA assay kit (Pierce). The samples were resolved on 7.5% SDS-PAGE gels and transferred onto nitrocellulose membranes. Western blot analyses were performed as previously described (Ishihara et al., 1999; Ishihara et al., 2001) using the 17026 anti-tau polyclonal antibody, as well as a panel of epitope specific monoclonal antibodies to tau, and immunolabeled tau bands were visualized using an alkaline phosphatase labeled secondary antibody and diaminobenzidine (DAB; Sigma St. Louis, MO).

**RESULTS**

Only one mouse showed evidence of cognitive impairments and an increased burden of tau pathology, and this was T44 tau Tg mouse L7-11-14 that was subjected to mrTBI. Immunohistochemical and biochemical analyses up to 9 months following mrTBI or sham treatment revealed that none of the WT non-Tg mice showed any evidence of tau pathology, as expected from previous studies (Ishihara et al., 1999, 2001), while the tau pathology in all the T44 tau Tg mice was indistinguishable regardless of injury status, except for the same T44 tau Tg mouse L7-11-14 that had cognitive deficits. As summarized in greater detail below, we detected a remarkably increased and more widespread burden of NFT-like tau pathology accompanied by higher levels of insoluble species of pathological tau in this 21-month-old T44 tau Tg mouse subjected to mrTBI than we have previously observed in any other T44 Tg mouse.
Histopathological Findings in the L7-11-14 T44 tau Tg Mouse

The brain of L7-11-14 was atrophic, the lateral ventricles were dilated and the thickness of the temporal cortex with was reduced (Fig. 1J, right) compared to the brains of the other T44 tau Tg and WT non-Tg mice (Fig. 1J, left). L7-11-14 showed an enormous burden of tau-positive NFT-like neuronal inclusions mainly in the hippocampus, entorhinal cortex (Fig. 1A,B), and inferolateral surface of the brain (Fig. 1J,1J). In the hippocampus, the number of NFT-like tau inclusions increased from the CA4 region to the CA1 region following the entorhinal cortex, which is similar to the distribution of NFTs in the AD brain (Fig. 1A,B). These tau inclusions were most abundant in the superficial layers of ventral cortical brain regions (Fig. 1J), and they most closely resembled the NFTs in AD, while no globose-shaped, Pick body–like tau positive inclusions were seen in the L7-11-14 mouse, as described in other lines of tau Tg mice (Lewis et al., 2000; Allen et al., 2002). Few tau pathology were found in the brains from the rest of the injured mice (Fig. 1C).

We also analyzed the histochemical properties of these tau inclusions in adjacent sections using Gallyas (Fig. 1B,E,I), Thioflavin-S (Fig. 1F), and Congo-red (Fig. 1G) staining methods. The examination using serial sections from this brain indicated that nearly 100% of the tau positive inclusions also were positive for Gallyas and Thioflavin-S. Some (~50%) Congo Red positive tau inclusions (Fig. 1G, red) exhibited birefringence (Fig. 1G, yellow) using polarizing filters. Although these histochemical properties of the tau inclusions in L7-11-14 recapitulate those seen in AD NFTs, and in the tau inclusions of other tauopathies, only about 30% of the tau inclusions in the L7-11-14 mouse were reactive with a ubiquitin antibody (data not shown). Reactive astrocytes with thick processes stained with a GFAP antibody were widely seen in the affected areas of the brain (Fig. 1H), but none of the these glial cells contained tau inclusions, and no immunoreactive Aβ deposits were observed.

Although no other focal lesions or structural abnormalities were observed in any of the mice subjected to mrTBI, the Gomori histochemical stain for iron revealed focal deposits of iron subjacent to the leptomeninges in the region of the mrTBI site and extending more ventrally in the L7-11-14 mouse brain associated with GFAP positive reactive astrocytes consistent with minor focal hemorrhage (Fig. 1L,M), while none of the other brains from mice subjected to mrTBI showed these changes.

Analysis of tau Insolubility and Phosphorylation of Soluble tau

Because the dramatically increased number of NFT-like inclusions in the L7-11-14 mouse brain suggested increased accumulations of insoluble tau, we analyzed the solubility of tau by extracting samples using three buffers with increasing extraction strengths from the other frozen half of the L7-11-14 mouse brain. The three fractions, extracted with RAB, RIPA buffer, and FA, respectively, were then immunoblotted with the polyclonal 17026 anti-tau antibody and other anti-tau monoclonal antibodies. Compared L7-11-14 with the rest of injured T44 tau Tg mouse brain samples, the FA sample from L7-11-14 showed an overt increase in the levels of insoluble tau immunoreactive bands, some of which exhibited a smeared profile (Fig. 2A) similar to those seen in AD. Interestingly, a comparison of tau immunoreactivity in RAB and RIPA fractions between L7-11-14 and other T44 tau Tg mice did not show prominent differences, but the migration of tau proteins in these fractions of the L7-11-14 mouse brain was slower, suggesting that these species of tau in the L7-11-14 mouse brain may be more highly phosphorylated. Therefore, we examined the phosphorylation state of tau in the RAB fraction from L7-11-14 using several phosphorylation-dependent antibodies and a non-phosphorylation dependent antibody. As shown in Figure 2B, all phosphorylation-specific antibodies, in particular PHF1 (pS396/pS404), PHF6 (pT231), and 12E8 (pS262), exhibited stronger signals in L7-11-14 than the rest of injured T44 Tg mouse brains.

Neurobehavioral Analysis

At 6 months following sham treatment or mrTBI, motor function was evaluated with the NS. No statistical differences in the NS scores were found between the four groups of mice, i.e. T44 with mrTBI (n = 9), T44 with sham treatment (n = 5), WT non-Tg with mrTBI (n = 9), and WT non-Tg with sham treatment (n = 10). However, the NS of L7-11-14 was 16 (average, 20.8 ± 2.7 [SEM]), which was an exceptionally low score in comparison with other mice. After the NS evaluation, all mice underwent MWM testing, and all of the mice were able to swim without visible alterations in swimming ability (swim speed and swim distance), consistent with previous reports (Smith et al., 1995). There was no significant difference between groups in learning latencies or memory scores based on two-way ANOVAs (p > 0.05). The learning latency of L7-11-14 was 52.5 sec (average, 34.2 ± 9.5 sec) for the 24 trials, and the memory score was 44.6 (average, 153 ± 55), both of which were among the lowest of all the mice tested.
FIG. 1. Histochemical analysis in a traumatized T44 mouse. Serial sections of the hippocampal region (A–F) from L7-11-14 were immunohistochemically stained with 17026 (A,D), and histochemically stained with Gallyas (B,E), Thioflavin-S (C,F), and Congo Red (G). The densities of NFTs, which are positive for tau (A) and Gallyas (B), gradually increase from the CA3 region (left upper side) to the entorhinal cortex through the CA1 region (right lower side), as seen in AD, while the rest of injured Tg mice showed few NFTs (C), which were identical to the mice from the sham group. The NFTs appeared to be morphologically similar to those in the AD brain in terms of the filamentous flame-like shape with threads. Merged images of sections stained with Congo red under fluorescence microscopy and birefringence with polarizing filters indicated that approximately half the Congo red–positive neurons exhibited birefringence (G). Reactive astrocytes, which were strongly positive for GFAP, were diffusely observed in the hippocampal area (H). In other parts of the brain, Gallyas positive NFTs distributed mainly in the orbital gyrus and inferolateral temporal cortex (I, arrowheads), and the thickness of the cortex of the traumatized T44 mouse (J, rt) was apparently thinner than that of the control mouse (J, lt). This brain, but not other T44 brains, was positive for iron staining (K,L, blue, arrows), which was found only in the region of the brain facing the skull base. Bars = 0.5 mm (in C, for A–C,H,J, and L), 20 μm (in F, for D–G), 2 mm (in I, for I and K).
DISCUSSION

Our previous studies of mrTBI in the Tg2576 mice, which carry APP696swe and model AD–like Aβ brain amyloidosis, provided the first experimental evidence to indicate that mrTBI can accelerate the deposition of Aβ and the accumulation of AD-like SPs in this animal model (Uryu et al., 2002). Thus, these findings grounded an extensive body of epidemiological data which supports the association of TBI with an increased risk for developing AD for the very first time in an experimental model system of AD-like Aβ amyloidosis. Although this mrTBI induced acceleration of SP deposition, it also was linked to increased oxidative stress, similar to that observed in AD. The Tg2576 mouse model does not completely recapitulate the spectrum of neuropathological abnormalities in DP and AD, especially since these Tg mice do not develop NFT-like tau pathology even following experimental manipulation such as mrTBI (Uryu et al., 2002).

In career boxers, DP is associated with recurrent TBI. Specifically, the neuropathology of DP includes widespread NFT formation and the histochemical and biochemical characters of DP tau pathology are indistinguishable from those seen in AD brains (Schmidt et al., 2001). Thus, the development of an animal model of DP offers a promising avenue for investigating pathophysiology of DP as well as AD-related tau pathologies. For these reasons, we undertook the present study of the T44 tau Tg mice in order to assess the effects of mrTBI on the tau pathology that these Tg mice develop with age.
Notably, while the tau pathology in these Tg mice is observed initially in the spinal cord, it also is seen later in the telencephalon (Ishihara et al., 1999, 2001). Accordingly, T44 tau Tg mice were used to assess whether TBI can accelerate formation and accumulation of NFT-like filamentous tau inclusions with aging and thereby model aspects of tau pathology in DP and AD. This Tg mouse is an appealing tauopathy model for these studies because they first develop very small numbers of telencephalic tau lesions beginning at ~18 months of age, and the frequency of NFT-like tau inclusions per 6-μm-thick section of telencephalon is three or less, even in 24-month-old T44 tau Tg mice (Ishihara et al., 1999, 2001). Thus, any TBI-induced augmentation of NFT pathology in 1–2-year-old T44 tau Tg mice would be readily evident, as was the case here for the L7-11-14 T44 mouse subjected to mrTBI. Although, mrTBI accelerated the formation of tau lesions in only one T44 tau Tg mouse, the L7-11-14 mouse, the extent of the increase of tau pathology was truly remarkable, and it far exceeded any of the tau pathology that we have observed in >100 previously examined T44, including the mice in this study. Moreover, this mouse also showed a dramatic degree of cerebral atrophy and as well as prominent cognitive deficits, all of which are observed in authentic AD and DP.

However, since only one mouse was observed with numerous TBI induced NFTs, a remarkable additional insight gleaned from these studies is that mrTBI does not routinely lead to accelerated NFT formation, and the reasons for this also need to be investigated further. Thus, additional factors must account not only for the resistance of most Tg mice to tangle formation, but also for the induction of NFTs following mrTBI. While the exact nature of these additional factors is unclear, and it is enigmatic why only one of the T44 tau Tg mice subjected to mrTBI showed this dramatic augmentation of tau neuropathology and brain atrophy, especially since the T44 mice are an inbred line of mice, we speculate that accelerated tangle formation may result from one or more stochastic consequences of TBI that have yet to be defined.

Nonetheless, it is plausible that accelerated NFT formation may be linked to the presence of iron deposits demonstrated by Gomori staining in superficial cortical layers, especially in ventral cortical regions, of the L7-11-14 mouse brain since this was not seen in any of the other T44 tau Tg mice subjected to mrTBI or sham treatment. For example, it is known that iron can be a source of damaging reactive oxygen species via the Fenton reaction, where iron (II) is stoichimetrically oxidized by H2O2 to iron (III), producing a hydroxyl radical (OH•) that plays a pivotal role in the pathogenesis of neurodegenerative disorders (Christen, 2000; Casadesus et al., 2004). Indeed, there are many reports of circumstantial evidence linking abnormal iron metabolism to mechanisms underlying AD and several other neurodegenerative disorders (Bouras et al., 1997; Yamamoto et al., 2002), while perivascular hemorrhage and meningeal breeding have been observed in about one third of ex-boxers’ brains (Adams and Bruton, 1989). Thus, it is possible that the deposition of iron in brain parenchyma following mrTBI in the L7-11-14 mouse may have contributed to the enhanced tau pathology in this mouse, but additional studies are needed to determine if other blood components or factors unrelated to focal hemorrhages are involved in this mrTBI induced augmentation of tau pathology. Given the fact that TBI is the most robust environmental risk factor for AD and that preventive measures are available to minimize TBI and the risk for subsequent development of AD, we hope that the novel observations reported here of mrTBI induced tau pathology in a tauopathy mouse model, albeit uniquely in one experimental animal, will further stimulate efforts to confirm and extend these findings in order to better elucidate the role of TBI in mechanisms of neurodegeneration.

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