Inhibition of JNK by a Peptide Inhibitor Reduces Traumatic Brain Injury–Induced Tauopathy in Transgenic Mice

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Abstract
Traumatic brain injury (TBI) is a major environmental risk factor for subsequent development of Alzheimer disease (AD). Pathological features that are common to AD and many tauopathies are neurofibrillary tangles (NFTs) and neuropil threads composed of hyperphosphorylated tau. Axonal accumulations of total and phospho-tau have been observed within hours to weeks, and intracytoplasmic NFTs have been documented years after severe TBI in humans. We previously reported that controlled cortical impact TBI accelerated tau pathology in young 3xTg-AD mice. Here, we used this TBI mouse model to investigate mechanisms responsible for increased tau phosphorylation and accumulation after brain trauma. We found that TBI resulted in abnormal axonal accumulation of several kinases that phosphorylate tau. Notably, c-Jun N-terminal kinase (JNK) was markedly activated in injured axons and colocalized with phospho-tau. We found that moderate reduction of JNK activity (40%) by a peptide inhibitor, D-JNKi1, was sufficient to reduce total and phospho-tau accumulations in axons of these mice with TBI. Longer-term studies will be required to determine whether reducing acute tau pathology proves beneficial in brain trauma.

Key Words: c-Jun N-terminal kinase, Controlled cortical impact, D-JNKi1, Kinase, Phosphorylation, Tau, Traumatic brain injury.

INTRODUCTION
Progressive accumulation of hyperphosphorylated microtubule-associated protein tau into neurofibrillary tangles (NFTs) and neuropil threads is a common feature of many neurodegenerative tauopathies, including Alzheimer disease (AD), Pick disease, progressive supranuclear palsy, and frontotemporal dementias (1). Tau pathology has also been documented in individuals who suffered from a single severe traumatic brain injury (TBI) or multiple mild concussive injuries. In particular, acute axonal accumulations of total and phospho-tau have been documented within hours to weeks (2, 3), whereas NFTs have been detected years after a single severe TBI in humans (4). Furthermore, NFT pathology is widespread in patients with lifetime histories of multiple concussive injuries (5–11). Tau pathologies in AD and TBI share similar immunohistochemical and biochemical features (10, 11). In both conditions, somatodendritic tau immunoreactivity is prominent; however, tau-immunoreactive neurites observed in TBI have been suggested to have an axonal origin, which may be distinct from the threadlike forms in AD suggested to be dendritic in origin (2, 3, 8, 11–13). Furthermore, the anatomical distribution of NFTs may be different after TBI than is typically seen in AD (8). Thus, the mechanisms leading to tau hyperphosphorylation in TBI may differ from those in AD.

The physiological function of tau is to stabilize microtubules (MTs) (14). Tau binding to MTs is regulated by serine/threonine phosphorylation. Abnormally phosphorylated tau has reduced MT binding, which results in MT destabilization. This, in turn, may compromise normal cytoskeletal function, ultimately leading to axonal and neuronal degeneration (15–17). This is the basis for the hypothesis that tau hyperphosphorylation leads to neurodegeneration in tauopathies. Identification of many mutations in the tau gene, which cause frontotemporal dementia with parkinsonism linked to chromosome-17 and result in tau hyperphosphorylation, supports this hypothesis (18). Findings from experimental models in which human mutant tau is expressed provide further support for this hypothesis. In these models, hyperphosphorylation of tau often precedes axonopathy and degeneration (1). Consequently, targeting tau either by reducing its phosphorylation state or aggregation has been a focus of preclinical therapeutic development for AD and related dementias (19, 20).

Two major mechanisms proposed to underlie tau hyperphosphorylation are aberrant activation of kinases and down-regulation of protein phosphatases. Cyclin-dependent kinase 5 (CDK-5) and its coactivator p25 (21–23), glycogen synthase kinase 3β (GSK-3β) (24, 25), and protein phosphatase 2A (PP2A) (26–28) have been implicated in hyperphosphorylation of tau in vivo. Others such as protein kinase A (PKA) (29, 30), extracellular signal–regulated kinase 1/2 (ERK1/2) (31, 32), and c-Jun N-terminal kinase (JNK) (33–36) have only been shown to regulate tau phosphorylation in vitro. It is not known whether these kinases and phosphatase contribute to TBI-induced tau pathology.
We previously reported that controlled cortical impact TBI accelerated tau pathology in young 3xTg-AD mice (37). Importantly, the posttraumatic tau pathology appeared to be independent of β-amyloid (Aβ). Furthermore, TBI-induced tauopathy in these mice resembled tau pathology observed in humans in that tau immunoreactivity was evident in both axonal and somatodendritic compartments. In this study, we used this experimental TBI mouse model to investigate mechanisms responsible for increased tau phosphorylation after moderately severe brain trauma. We found JNK to be critically involved in this process.

MATERIALS AND METHODS

Animals
Five- to 7-month-old homozygous 3xTg-AD mice were used. The 3xTg-AD mice express 3 mutant human genes: PS1M146V knockin, APPsw and Taup301L mutations (38). The 3xTg-AD mice were derived from the founders received from the Laferla lab (Irvine, CA) since 2007. There was no evidence of genetic drift. Mice were housed in standard cages in a 12-hour light/12-hour dark cycle and given food and water ad libitum. Mice of both sexes were randomly assigned to experimental groups. All experiments were approved by the animal studies committee at Washington University in St. Louis, MO.

Controlled Cortical Impact TBI
The experimental TBI methods were performed as previously described (39). Briefly, a 5-mm craniotomy was performed on the left hemisphere by a motorized trephine. Experimental TBI was induced by impacting a 3.0-mm-diameter metal tip onto the cortex. Impact was centered at 3.0 mm anterior to the lambda and 2.7 mm to the left of the midline. A 2.0-mm impact below the dura was chosen, as this injury severity not only results in moderate damage to the cortex and underlying hippocampus ipsilateral to the injury, but also causes robust total and phosphorylated tau accumulations in injured axons (37). Sham-injured mice went through identical procedures but were not injured. Duration of anesthesia exposure for sham group was approximately 15 minutes ± 1 minute versus 18 minutes ± 1 minute for the TBI group. Mice were kept at 37°C via a rectal temperature probe throughout the surgery and allowed to recover on a warming pad to prevent hypothermia-induced hyperphosphorylation of tau (40).

Antibodies
All antibodies used are listed in Table. Monoclonal 3D6 antibody was biotinylated using NHS-LC-biotin from Pierce (Rockford, IL).

Western Blotting
Mice were killed by deep isoflurane anesthesia, followed by rapid decapitation at 24 hours after sham or TBI procedure. Hippocampi and surrounding white matter, including the fimbria/fornix ipsilateral to the injury site, were dissected, immediately frozen, and stored at −80°C. Tissues were homogenized in modified radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor tablets (Roche), as described (41). Homogenates were centrifuged at 13,000 rpm for 20 minutes at 4°C, and protein concentrations were determined using the bicinchoninic acid method (Pierce). Equal amounts of each sample (6 μg) were electrophoresed on 10% BisTris NUPAGE gels using 3-(N-morpholino)propanesulfonic acid buffer (Invitrogen). Gels were transferred to 0.2-μm nitrocellulose membranes, which were then blocked with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% nonfat dry milk for 1 hour at room temperature. Membranes were incubated overnight in TBS-T buffer containing 5% bovine serum albumin and the appropriate primary antibodies. Corresponding anti–rabbit horseradish peroxidase (GE Healthcare, 1:10,000) or anti–goat horseradish peroxidase (Invitrogen, 1:10,000) and ECL Advance Western blotting kit (GE Healthcare) were used for detection. Blots were washed 4 times for 5 minutes each with TBS-T between blocking and applications of antibodies. Blots were scanned, and densitometry was performed via Image J (NIH, Bethesda, MD).

Protein Phosphatase Activity Assays
Serine/threonine phosphatase activity assay kits (V2460) were purchased from Promega Corp. (Fitchburg, WI). Assays were performed on a 96-well plate format, per manufacturer’s instructions. Briefly, to remove phosphatase inhibitors and endogenous phosphates from hippocampal radioimmunoprecipitation assay lysates, samples were desalted using the Zeba microspin desalting columns (Pierce, 89877). Each sample was run in duplicate reactions; each contained 2 μL of lysates, 10 μL of appropriate 5× phosphatase reaction buffer, 5 μL of 1 mmol/L phosphopeptide, and 33 μL of deionized H2O. Protein phosphatase 2A (PP2A) reaction buffer contained 250 mMol/L imidazole, 1 mMol/L EGTA, 0.1% β-mercaptoethanol, and 0.5 mg/mL acetylated bovine serum albumin (Promega, R3961). In addition to the reagents listed for PP2A reaction buffer, PP2B (calcineurin) reaction buffer also included 50 mMol/L MgCl2, 5 mMol/L NiCl2, 250 μg/mL calmodulin (Calbiochem, 208690, EMD Millipore, Billerica, MA). Plates were incubated at 30°C for 30 minutes for phosphatase reactions to take place. Reactions were stopped by the addition of 50 μL of molybdic acid dye/additive mixture to each well. Plates were subsequently incubated at room temperature for 30 minutes to allow the molybdic acid dye to bind to free phosphates released from the reaction. Plates were read using a plate reader with a 630-nm filter. Optical densities of the samples were determined based on the optical densities of free phosphate standards. Specific activities for PP2A and PP2B were expressed as picomole phosphates per minute per microgram of total protein.

Immunohistochemistry and Double Immunofluorescence
Immunohistochemistry was done as previously reported (37). Mice were killed at 24 hours post-TBI; their brains were fixed for 24 hours in 4% paraformaldehyde and cryoprotected in 30% sucrose for 2 days before sectioning to 50-μm-thick slices via a sliding microtome. To reduce background staining on injured tissues when staining with monoclonal PHF1
antibody, an additional blocking step for 1 hour with unconjugated anti-mouse IgG monovalent Fab fragments (315-007-003, 130 μg/mL; Jackson ImmunoResearch, West Grove, PA) was performed after blocking with serum.

For double labeling of phospho-tau and activated JNK, sequential applications of primary antibodies were used. First, sections were incubated with rabbit anti-pS199, followed by goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (A11008, 2 μg/mL; Invitrogen). Sections were blocked again for 30 minutes with 3% normal rabbit serum to saturate open binding sites on the first secondary antibody with IgG. Sections were then incubated for 1 hour in excess of unconjugated goat anti-rabbit IgG monovalent Fab fragments (111-007-003, 130 μg/mL; Jackson ImmunoResearch). This was done to cover the rabbit IgG so that the second secondary antibody would not bind to it. Rabbit anti-p-JNK was subsequently applied, followed by goat anti-rabbit conjugated to Alexa Fluor 594 (A11012, 2 μg/mL; Invitrogen). Sections were washed with TBS 3 times for 5 minutes each between steps. Images were obtained using LSM 5 Pascal software (Zeiss Physiology Software) coupled to an LSM Pascal Vario 2RGB confocal system (Zeiss).

Quantitative Analyses of Histological Data

All histological analyses were done by an investigator who was blinded to treatment conditions of all mice.
brain atlas was used to identify the ipsilateral fimbria/fornix, thalamus, amygdala, and hippocampal CA1 (42).

Densitometric analysis of different kinase staining was performed on the ipsilateral fimbria/fornix of 4 sections per mouse, with each section separated by 400 μm. Phospho-c-jun staining was performed on the ipsilateral thalamus using 5 sections per mouse. These sections spanned approximately bregma -0.8 mm to -2.6 mm. Slides were scanned using a Nanozoomer HT system (Hamamatsu Photonics, Bridgewater, NJ) to obtain digitized images. Scanned images were exported with the NDP viewer software (Hamamatsu Photonics) and analyzed using the ImageJ software, as described previously (43). Briefly, images were converted to 8-bit gray scale. The “polygon” selection tool was then used to delineate either the fimbria/fornix or the thalamus. Images were thresholded to highlight stained objects using the automatic MaxEntropy thresholding function in ImageJ. The Analyze Particles function was subsequently used to quantify the percentage area occupied by each kinase in the ipsilateral fimbria/fornix and by p-c-jun in the ipsilateral thalamus.

Stereological quantifications were performed via the StereoInvestigator software (version 8.2, MBF Bioscience, Williston, VT). The optical fractionator method was used to quantify total numbers of amyloid precursor protein (APP)-, 3D6-, total tau-, pS199-, PHF1-, and pT231-positive axonal profiles per cubic millimeter of the fimbria/fornix. Axonal bulbs and swellings with spheroidal or beads-on-a-string morphologies that were 5 μm or more in diameter were counted. Axons with multiple anatomically continuous beads-on-a-string varicosities were only counted once. As we have previously noted (44), this method may result in overcounting if 2 apparently discontinuous varicosities represent 2 parts of a single disconnected axon, or undercounting if injured axons do not stain with APP or are less than 5 μm in diameter. Thus, the quantitative estimates of axonal injury should be regarded as approximate. This optical fractionator method was also used to quantify total numbers of total tau-positive somata in the ipsilateral amygdala. The spherical probe (also known as “space balls”) was used to estimate total tau-positive process length per cubic millimeter of the contralateral CA1. All parameters used for these stereological methods were as previously reported (37).

**JNK Inhibitor Treatment**

The D-JNKi1 peptide (BML-E1355) and D-TAT control peptide (BML-E1384) were purchased from Enzo Life Sciences International, Inc. (Farmingdale, NY). The D-JNKi1 peptide is a specific inhibitor of JNK, which blocks the interaction between JNK (JNK-1, -2, -3) and its substrates (45–48). The D-JNKi1 is cell permeable and has a longer half-life than its stereoisomer. The D-JNKi1 contains a 20-amino acid sequence of the JNK binding domain of the JNK interaction protein JIP1 covalently linked to the 10-amino acid HIV-TAT sequence. The D-TAT control peptide contains only the 10-amino acid HIV-TAT sequence. Before craniotomy and TBI induction, a 1-mm burr hole was drilled on the right hemisphere at -0.5 mm posterior to the bregma and 1.0 mm lateral to the midline. Mice were randomly assigned to receive either D-JNKi1 or D-TAT (5 μg, dissolved in PBS + 0.1% dimethyl sulfoxide) immediately after injury. A 33-gauge needle attached to a Hamilton syringe and KDS310 nano pump system (KD Scientific, Inc.) was lowered -2.2 mm below the dura through the burr hole to deliver peptide solutions (5 μL in total volume) at 0.3 μL/min rate into the right lateral ventricle. The duration of anesthesia exposure for the combined injury and intracerebroventricular injection procedure was similar for D-TAT– and D-JNKi1–treated groups: 50 ± 2 minutes. Mice recovered well after this combined surgical procedure. They lost approximately 10% of their original body weight, which was similar to mice that underwent only the TBI procedure.

**STATISTICAL METHODS**

All data were analyzed using Prism 5.0 (GraphPad, San Diego, CA). For pairwise comparisons of levels of tau kinases via Western blot, immunohistochemistry, and phosphatase activity between TBI and sham mice, 2-tailed Student t-test were used; values of p < 0.05 were considered significant. For comparisons of staining areas covered by activated kinases in the fimbria/fornix, a 1-way analysis of variance with Newman-Keuls posttest was used. For pairwise comparisons of quantitative histological data of D-JNKi1 experiments (e.g. D-JNKi vs D-TAT), the 1-sided Student t-test was used because unidirectional hypotheses were prespecified.

There was a trend toward reduced tau pathology when we first analyzed results from 5 D-JNKi1- and 4 D-TAT–treated mice. Therefore, 4 additional mice were added to each group, and data were reanalyzed. As such, statistical significance for these analyses was set to p < 0.025 because of the optional stopping design of the experiment. Values presented are mean ± SEM.

**RESULTS**

The Activities of Tau Kinases and Phosphatases
Were Not Different in Hippocampal
Homogenates of TBI Versus Sham 3xTg-AD Mice
at 24 Hours

Aberrant activation of tau kinase(s) (21–25, 49) or inhibition of protein phosphatases (26–28, 50) is the major proposed mechanism underlying tau hyperphosphorylation in many tauopathies. We therefore tested whether these mechanisms could account for the observed trauma-induced tau phosphorylation in our experimental TBI model. We studied overall tissue levels of the PKA, ERK1/2, GSK-3β, and JNK (31–35, 51–54). Phosphorylation of the catalytic subunit of PKA is essential for its activation by CAM (55, 56); ERK1/2 and JNK are directly activated via phosphorylation (57–59). Thus, blots were probed with phospho-specific antibodies to assess the levels of active PKA, ERK1/2, and JNK (Fig. 1A). The GSK-3β activity, on the other hand, is controlled via inhibitory phosphorylation of GSK-3β at Ser-9 by Akt/protein kinase B pathways (60). Thus, blots were probed with an antibody against phosphorylated Ser-9 of GSK-3β (Fig. 1A).

Another well-characterized tau kinase is the CDK-5. Physiological activity of CDK-5 is regulated by its association to the regulatory subunit p35, whereas association of CDK-5 to p25...
results in abnormal kinase activation and contributes to neurodegeneration (21, 22, 49). Therefore, we also measured CDK-5, p35, and p25 levels via Western blot to probe for CDK-5 activity after TBI (Fig. 1C). Western blot analyses showed no difference in the total and activated levels of all examined kinases from the homogenates of TBI compared with sham mice ($p > 0.05$, Fig. 1B, D).

Protein phosphatase 2A and PP2B are major tau phosphatases (26, 61, 62); thus, we measured the activities of these phosphatases from the same hippocampal homogenates of

FIGURE 1. Traumatic brain injury (TBI) did not affect levels of tau kinases and activities of tau phosphatases in hippocampal lysates at 24 hours. (A) Representative Western blots of ipsilateral hippocampal lysates of a sham and an injured (i.e. TBI) mouse detected with antibodies against phosphorylated protein kinase A (p-PKA), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), p-glycogen synthase kinase 3β (GSK-3β) at Ser9, and phosphorylated c-jun N-terminal kinase (p-JNK). Blots were stripped and reprobed with antibodies against total PKA, ERK1/2, GSK-3β, and JNK. The same blots were finally stripped and reprobed with α-tubulin for loading control. (B) Densitometric quantification of ratios of phosphorylated protein over total protein for each examined kinase from hippocampal lysates of sham and TBI mice expressed as percentage of sham. None were significantly different from 100%. (C) Representative Western blots of hippocampal lysates of a sham and a TBI mouse probed with antibodies against total cyclin-dependent kinase 5 (CDK-5) and its regulatory subunits, p35/p25. The CDK-5 blots were reprobed with α-tubulin for loading control. (D) Densitometric quantification of bands immunoreactive for CD5, p35, and p25 from hippocampal lysates of sham and TBI mice, expressed as percentage of sham. No significant difference was observed between TBI ($n = 4$) and sham ($n = 4$) samples, Student $t$-test. (E) Activity of protein phosphatase 2A (PP2A) and 2B (PP2B, calcineurin) from hippocampal lysates of sham and TBI mice, expressed as percentage of sham. No significant difference was observed between TBI ($n = 6$) and sham ($n = 6$) samples, Student $t$-test. Bars $=\text{mean} \pm \text{SEM}$. 

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TBI and sham mice using a phosphatase activity assay kit. Traumatic brain injury did not significantly affect activities of PP2A and PP2B when compared with sham mice (p > 0.05, Fig. 1E). In summary, changes in tau kinases and phosphatases could not be detected at the whole-tissue homogenate level 24 hours after injury in 3xTg-AD mice.

**Altered Localizations of Tau Kinases and Tau post-TBI**

Traumatic axonal injury (TAI) is a prominent feature of TBI in many contexts, including pericontusional axonal injury in our mouse model (37, 63–66). Traumatic axonal injury is thought to disrupt axonal transport thereby altering

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**FIGURE 2.** c-Jun N-terminal kinase (JNK) was markedly activated in the ipsilateral fimbria/fornix of injured 3xTg-AD mice and colocalized with phospho-tau. (A–J) Representative brain sections from a sham and a traumatic brain injury (TBI) mouse immunostained for phosphorylated protein kinase A (p-PKA) (A, B), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) (C, D), cyclin-dependent kinase 5 (CDK-5) (E, F), phosphorylated glycogen synthase kinase 3β (p-GSK-3β) at Y279 and Y216 (G, H), and p-JNK (I, J). Scale bar (A) = 50 μm. (K) Percentage of fimbria area occupied by p-PKA, p-ERK1/2, CDK-5, p-GSK-3, and p-JNK staining. Areas occupied by p-GSK-3 and p-JNK were significantly different from sham. *p < 0.05, **p < 0.001, Student t-test. The p-JNK-positive area was significantly more than the rest of the examined kinases. #p < 0.0001, 1-way analysis of variance with Newman-Keuls posttest, n = 4 mice per group. Bars = mean ± SEM. (L, M) Colocalization of tau phosphorylated at S199 (pS199) and phosphorylated JNK (p-JNK) in a TBI (M) but not sham (L) mouse.
the localizations of many proteins (67). As such, it is probable that TAI causes mislocalizations of tau and tau kinases, resulting in the observed TBI-induced tauopathy in our model. We tested this hypothesis by subjecting separate 3xTg-AD mice to TBI or sham injuries and examining their brains immunohistochemically. The brains were stained for activated forms of PKA, ERK1/2, and JNK, and for total CDK-5 using the same antibodies used for Western blotting. In a pilot experiment, we did not observe any immunoreactivity in our tissues using antibody directed against phospho-S9 of GSK-3β (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A308). Therefore, we used an antibody against phosphorylated tyrosine residues of GSK-3β.

Traumatic brain injury resulted in immunohistochemically detectible activation of most of the kinases examined, primarily in injured axons of the ipsilateral fimbria/fornix (Fig. 2). The JNK (Fig. 2I–J) appeared markedly activated compared with the rest of the examined kinases (Fig. 2A–H). Activation of JNK was also observed in the ipsilateral cortex and thalamus of injured mice (Fig. 3A–D), and increased immunoreactivity for activated PKA and GSK-3 was observed in the ipsilateral CA1 (Fig. 3E–H). Densitometric analyses showed 7.6% ± 0.8% area covered with phosphorylated (p-)JNK-positive staining and 2.5% ± 0.5% area covered with p-GSK-3 staining in the fimbria/fornix of TBI mice versus less than 0.01% p-JNK-positive area and 0.38% ± 0.1% (p-)GSK-3–positive area in sham mice. Areas covered by p-JNK (p < 0.0001) and p-GSK-3 (p < 0.05) were significantly greater in TBI versus sham mice (Fig. 2K). In comparisons with other examined kinases, p-JNK staining in the fimbria/fornix was the most prominent (p < 0.0001, Newman-Keuls posttest after 1-way analysis of variance). Furthermore, double immunofluorescence and confocal microscopy revealed that p-JNK colocalized with tau phosphorylated at Ser-199 (pS199) in the fimbria/fornix of injured but not sham mice (Fig. 2L, M).

Taken together, these data suggest that axonal coaccumulation and mislocalization of tau and tau kinases, particularly JNK, after TBI could be responsible for posttraumatic axonal tau pathology in 3xTg-AD mice.
FIGURE 5. Treatment with D-JNKi1 blocked c-jun activation in the thalamus but did not affect axonal injury as assessed by amyloid precursor protein (APP) immunohistochemistry or β-amyloid (Aß) accumulation in the fimbria of injured 3xTg-AD mice at 24 hours. (A, B) Nuclear staining of phosphorylated c-jun at S63 (p-c-jun S63) in the thalamus of injured 3xTg-AD mice that received intracerebroventricular injection of D-JNKi1 or D-TAT control peptide. Scale bar (A) = 500 μm. Insets in (A, B) are higher magnification of p-c-jun S63 staining. Scale bar = 50 μm. (C) Percentage of area occupied by p-c-jun S63 staining in D-JNKI1- and D-TAT–treated mice. Inhibition of JNK by D-JNKi1 significantly reduced the extent of c-jun phosphorylation in the thalamus of injured 3xTg-AD mice: 40% reduction, ***p < 0.001. (D, E) APP staining in the ipsilateral fimbria of injured 3xTg-AD mice treated with D-JNKi1 or D-TAT. Scale bar (D) = 50 μm. (F) Stereological quantification showed similar numbers of APP-stained varicosities in mice treated with D-JNKI1 compared with D-TAT. (G, H) Aß staining using N-terminal antibody 3D6 in the ipsilateral fimbria of injured 3xTg-AD mice treated with D-JNKi1 or D-TAT. (I) Stereological quantification showed a nonsignificant 20% reduction in the numbers of Aß-stained axonal varicosities between groups. n.s. = not significant. Student t-test, n = 8 to 9 mice per treatment group. Bars = mean ± SEM.
JNK Inhibition by D-JNKi1 Peptide Reduces TBI-Induced Tauopathy

To test the hypothesis that JNK is involved in increasing axonal tau phosphorylation and accumulation after TBI in 3xTg-AD mice, we treated mice with a specific peptide inhibitor of JNK, D-JNKi1, or control peptide, D-TAT, via intracerebroventricular injection immediately after TBI. D-JNKi1 was chosen over the ATP-competitive inhibitor of JNK, SP600125, because of its high specificity to JNK and its long half-life (45, 46). Mice were killed at 24 hours after injury, and their brains were examined by immunohistochemistry. Because c-jun is a known major target of JNK (71), we stained for c-jun phosphorylated at Ser-63 (p-c-jun) to determine the extent to which JNK activity was inhibited by

FIGURE 6. Inhibition of c-jun N-terminal kinase (JNK) by D-JNKi1 peptide reduced axonal tau accumulation but did not affect somatodendritic tau accumulation. (A-I) Total tau staining using polyclonal antibody (pAb) in the ipsilateral fimbria (A, B), ipsilateral amygdala (D, E), and contralateral (contra) CA1 (G, H) of mice that received D-JNKi1 or D-TAT by intracerebroventricular injection. Scale bar (A) = 50 μm. Stereological quantification showed D-JNKi1 treatment reduced axonal tau accumulation in the fimbria (C), but had no effect on numbers of tau-positive somata in the ipsilateral amygdala (F) or tau-positive process length in the contralateral (contra) CA1 region (I). *p < 0.025, n.s: not significant. Student t-test, n = 8 to 9 mice per group. Bars = mean ± SEM.
D-JNKi1 treatment. Traumatic brain injury resulted in c-jun activation in many pericontusional regions, most consistently the ipsilateral thalamus (Fig. 4A–D). We therefore quantified p-c-jun nuclear staining in this region and found that D-JNKi1 treatment reduced p-c-jun immunoreactivity approximately 40% when compared with D-TAT–treated mice (p < 0.0001, Fig. 5A–C).

The APP is a robust marker of axonal injury (72–75); thus, we stained these brains for APP to assess the effects of JNK inhibition on the extent of axonal injury. We also stained

![JNK inhibitor D-JNKi1 Control peptide D-TAT](image)

**FIGURE 7.** Treatment with D-JNKi1 reduces tau pathology in injured axons of 3xTg-AD mice. (A–I) Phospho-tau staining using polyclonal antibodies against tau phosphorylated at S199 (pS199, A, B), at S396 and S404 (PHF1, D, E), and at T231 (pT231, G, H) in the ipsilateral fimbria/fornix of injured 3xTg-AD mice treated with D-JNKi1 or D-TAT peptide. Scale bar (A) = 50 μm. Stereological quantification of pS199-, PHF1-, and pT213-tau puncta in the ipsilateral fimbria of injured mice treated with D-JNKi1 or D-TAT. Treatment with D-JNKi1 significantly reduced numbers of pS199- (C) and PHF1-tau puncta (F) but had no effect of pT231-tau puncta in the ipsilateral fimbria of injured mice (I). *p < 0.025, **p < 0.001, ns: not significant, 1-sided t-test, n = 8 to 9 mice per treatment group. Bars = mean ± SEM.
for the APP proteolytic product Aβ by using the 3D6 antibody, which does not recognize APP (76). Treatment with D-JNK1 did not significantly affect the degree of axonal injury as determined by the numbers of APP-positive axonal vari- cosities in the fimbria/fornix (p = 0.31, Fig. 5D–F). Treatment with D-JNK1 appeared to reduce the numbers of 3D6-positive varicosities in the fimbria, but the reduction did not reach statistical significance when compared to D-TAT treated mice (p = 0.07, Fig. 5G–I). This finding is not surprising because D-JNK1 has been shown to reduce Aβ production in vitro (77). We conclude that D-JNK1 did not affect the severity of axonal injury in this setting.

Although the D-JNK1 treatment did not fully block c-jun phosphorylation, we nevertheless asked if partial JNK inhibition was sufficient to affect posttraumatic tau pathology in this model. We assessed total tau pathology by staining with a polyclonal antibody that recognizes tau independent of its phosphorylation state (Fig. 6A, B, D, E, G, H). Stereological quantification showed a moderate but significant reduction (35%) of total tau-positive puncta in the ipsilateral fimbria/fornix (p = 0.025, Fig. 6C). As controls, we also quantified total tau-positive somata in the ipsilateral amygdala and tau-positive neurites in the contralateral CA1. These two regions exhibited increased total tau immunoreactivity (37) but lacked p-JNK staining after TBI (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A309). As expected, stereological quantification showed similar numbers of tau-positive somata and neurites in the amygdala and CA1 of D-JNK1 and D-TAT treated mice (p > 0.5, Fig. 6F, I).

We next studied effects of JNK inhibition on tau phosphorylation using phospho-specific antibodies against tau phosphorylated at Ser-199 (pS199), Ser-396, and/or Ser-404 (PHF1) (78, 79), and Thr-231 (pT231) (Fig. 7A, B, D, E, G, H). There were significant reductions of numbers of pS199-positive (55%, p < 0.0001, Fig. 7C) and PHF1-positive (34%, p = 0.014, Fig. 7F) puncta in the ipsilateral fimbria/fornix of D-JNK1– compared with D-TAT–treated mice. Numbers of pT231-positive puncta were not statistically different between treatment groups (p = 0.12, Fig. 7I). This is consistent with in vitro findings that JNK preferentially phosphorylates tau at several sites including Ser-396, but not at Thr-231 (80). In summary, we found that moderate reduction of JNK activity could ameliorate the axonal accumulations of total, pS199, and PHF1-tau in injured axons of 3xTg-AD mice.

**DISCUSSION**

In this study, we show that moderately severe TBI resulted in different regional patterns of activation of a number of tau kinases. The primary site of kinase activation and accumulation was within injured axons, particularly the ipsilateral fimbria/fornix. c-Jun N-terminal kinase was markedly activated in this region compared with the other examined kinases. Notably, JNK appeared to play a critical role in TBI-induced tau hyperphosphorylation, as activated JNK colocalized with phospho-tau and inhibition of JNK activity reduced tau phosphorylation in injured axons.

Traumatic axonal injury is thought to cause axonal transport deficits, resulting in accumulations of various organelles and proteins, including neurofilaments and APP (67). Our data suggest that axonal transport deficits induced by TAI may be responsible for the accumulation and activation of the examined tau kinases and tau. The observations that sciatic nerve ligation resulted in accumulation of total and phosphorylated ERK1/2 and JNK (81–83) lend support to this hypothesis. Nonetheless, this hypothesis can be further tested by treatment of TBI mice with drugs that rescue or reduce transport deficits, such as the microtubule stabilizer epothilone D. Epothilone D has been shown to reduce fast axonal transport defects in CNS axons and lessen axonal degeneration in tau transgenic mice (84).

The distinct spatial distributions of activated kinases, particularly JNK, GSK-3 and PKA, indicate the heterogeneous responses of different brain structures and cellular compartments to TBI. Such selective responses may be best documented using immunohistochemical techniques, which may account for the mismatch between our immunohistochemical and Western blotting data. Nevertheless, it is possible that our semiquantitative densitometric approach used to assess the levels of total and activated protein kinases in hippocampal homogenates may not be sensitive enough to detect modest but functionally important changes. It is also likely that these kinases exhibit transient patterns of activation, which our analysis at 24 hours after TBI did not capture. Indeed, a study using fluid percussion TBI in rats has reported that activated ERK1/2 and JNK in hippocampal lysates were evident within minutes but no longer detectable within hours after injury (85). As such, a more thorough analysis in which mice are killed at different time points after injury will be necessary to resolve the temporal profiles of kinase activations.

Importantly, JNK activation has been documented in contusional TBI in humans (86). This supports the validity of our TBI model. In addition, JNK was reported to be activated in a number of studies using the fluid percussion TBI model in rats (85–89). Together, these data suggest that JNK activation is a general response to brain trauma, which is consistent with the role of JNK in signaling stress signals (90). Furthermore, our findings and those from Raghupathi et al (89) suggest that JNK signaling is complex and may have distinct functions in somata versus axons. In support of this notion, many studies provide evidence for the unequivocal roles of JNK and c-jun activation in programmed cell death in neurons (91). Although JNK function in axons has received less attention, recent investigations implicate JNK in signaling axonal injury (92) and in mediating axonal degeneration (93). Because hyperphosphorylated tau is associated with axon degeneration, our findings of the role of JNK in tau phosphorylation is in line with previous reports.

Nonetheless, our study has a number of limitations. First, we have not tested the therapeutic window during which D-JNK1 can affect posttraumatic tau pathology. Borsello et al (46) showed that D-JNK1 treatment can have beneficial effects if given up to 6 hours after ischemic injury. Meanwhile, Miller et al (93) found that JNK inhibition within 3 hours after axotomy of dorsal roots ganglion axons can effectively block JNK-mediated axon degeneration. The latter time window of JNK inhibition is perhaps more applicable to our model because axonal injury is a major pathology
observed after TBI. Second, we have not systematically tested other doses and methods of delivery of this peptide inhibitor. Third, we have yet to determine which JNK isofrom is responsible for induction tau phosphorylation after injury. The JNK1<sup>−/−</sup>, JNK2<sup>−/−</sup> and JNK3<sup>−/−</sup> knockout mice subjected to similar injury paradigm will be useful for this purpose. Fourth, although our study supports JNK activation as a probable mechanism underlying TBI-induced tau pathology, we cannot rule out other mechanisms that may result in tau hyperphosphorylation, such as changes in tau conformation (94) and other posttranslational modifications of tau (95, 96). Future studies will be required to assess these alternative mechanisms. In addition, roles of GSK-3 and PKA in tau phosphorylation will require further investigation, as activated forms of these kinases were found to localize in both axons and ipsilateral CA1 regions of injured mice. Interestingly, inhibition of GSK-3 was recently shown to protect dorsal root ganglion axons from degeneration after axotomy (97). Thus, it is possible that a combined therapy involving JNK, GSK-3, and possibly PKA inhibition may be required to effect functional benefits of blocking tau hyperphosphorylation and axon degeneration. Other kinases and phosphatases not assessed here could also be involved. Lastly, it will also be important to determine if the effects of contusional TBI are similar to or different from the effects of multiple concussive injuries on pathological hyperphosphorylation and accumulation of tau.

In summary, we identified JNK as a likely kinase that phosphorylates tau in vivo in the setting of moderately severe TBI. Future studies will be required to determine whether acute axonal tau accumulation leads to NFT formation, and whether reducing acute tau pathology proves beneficial in contusional TBI.

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