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Tau Protein Is Cross-Linked by Transglutaminase in P301L Tau Transgenic Mice

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The microtubule-associated protein tau is highly soluble under physiological conditions. However, in tauopathies, tau protein aggregates into insoluble filaments and neurofibrillary tangles (NFTs). The mechanisms underlying the formation of tau filaments and NFTs in tauopathies remain unclear. Several lines of evidence suggest that transglutaminase may cross-link tau into stable, insoluble aggregates, leading to the formation of NFTs in Alzheimer's disease and progressive supranuclear palsy. To further determine the contribution of transglutaminase in the formation of NFTs, we compared the levels of cross-linked tau protein from P301L tau transgenic mice that develop NFTs to four-repeat wild-type (4RWT) tau transgenic and nontransgenic mice that do not develop NFT pathology. Immunoprecipitation and immunoblotting experiments show that transglutaminase cross-links phosphorylated tau in the hindbrain of P301L tau transgenic mice but not in mice overexpressing 4RWT tau and nontransgenic mice. Cross-linked, phosphorylated tau from P301L tau transgenic mice runs as high-molecular mass aggregates on Western blots, similar to cross-linked tau from paired helical filaments of Alzheimer's disease. We also used double-label immunofluorescence to demonstrate colocalization of PHF-1-immunoreactive tau and the transglutaminase-catalyzed cross-link in the hindbrain, spinal cord, and cortex of P301L tau transgenic mice. In the spinal cord, 87% of PHF-1-labeled cells colocalize with the transglutaminase-catalyzed cross-link. Additionally, transglutaminase enzymatic activity is significantly elevated in the spinal cord of P301L tau transgenic mice. These studies further implicate transglutaminase in the formation and/or stabilization of NFT and paired helical filaments and provide a model system to investigate the therapeutic potential of transglutaminase inhibitors in tauopathies.

Key words: transglutaminase; tau; P301L tau mutation; transgenic mice; neurodegeneration; tauopathies

Introduction

Tauopathies include a clinically diverse group of sporadic and familial neurodegenerative disorders in which filamentous, intraneuronal tau aggregates constitute a hallmark pathological lesion. Tau proteins are microtubule-associated proteins that promote microtubule assembly and confer stability to the microtubule network (Weingarten et al., 1975; Cleveland et al., 1977). In tauopathies, abnormally hyperphosphorylated tau polymerizes into straight filaments and paired helical filaments (PHFs), which aggregate to form neurofibrillary tangles (NFTs). The progressive accumulation of tau protein in tauopathies such as Alzheimer's disease (AD) and progressive supranuclear palsy (PSP) implicates tau as a factor in neurodegeneration. Although several potential contributing factors have been identified, the mechanisms of PHF and NFT formation are not fully understood.

The insoluble nature of PHFs led to the hypothesis that trans-

glutaminase could contribute to PHF formation in AD (Selkoe et al., 1982a). Transglutaminases are a family of calcium-dependant enzymes that catalyze the formation of a covalent ϵ -(γ -glutamyl) lysine bond between the γ -carboxamide group of a glutamine residue and the ϵ -amino group of a lysine residue (Folk and Finlayson, 1977). Proteins containing the transglutaminase bond (i.e., cross-link) are highly stable and resistant to proteolysis and denaturation, similar to the insoluble tau polymers found in NFTs (Selkoe et al., 1982b). Mounting evidence supports the involvement of transglutaminase in the pathology of tauopathies. *In vitro* studies demonstrate that tau is a substrate of transglutaminase (Miller and Anderton, 1986; Dudek and Johnson, 1993; Miller and Johnson, 1995; Appelt and Balin, 1997; Norlund et al., 1999). Specifically, two glutamine and 10 lysine residues in tau are modified by transglutaminase *in vitro* (Murthy et al., 1998). Transglutaminase activity (Johnson et al., 1997; Zemaitaitis et al., 2003) and transglutaminase-catalyzed bonds in PHFs and NFTs (Balin et al., 1999; Norlund et al., 1999; Zemaitaitis et al., 2000; Singer et al., 2002) are significantly elevated in brain regions with abundant neurofibrillary pathology in both AD and PSP. Furthermore, transglutaminase-catalyzed bonds are present in PHF tau before NFTs are microscopically detectable, implicating transglutaminase cross-linking of tau protein as an early event in NFT formation (Singer et al., 2002). However, the role of transglutaminase in the formation of PHFs and NFTs remains unclear.

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The transgenic mice expressing mutant (P301L) tau protein developed by Lewis et al. (2000) provide an excellent system to study tau dysfunction. P301L tau mice develop NFTs in the diencephalon, brainstem, cerebellar nuclei, and spinal cord, with mainly somatodendritic tau immunoreactivity (“pretangles”) evident in the cortex and hippocampus. Mice overexpressing four-repeat wild-type (4RWT) tau or nontransgenic animals do not display NFT pathology. Similar to tauopathies, the accumulation of NFTs in P301L mice is age dependent (Lewis et al., 2000), with an increase in tau phosphorylation and insoluble tau (Sahara et al., 2002). The presence of insoluble tau in the P301L tau transgenic mice suggests that transglutaminase could be a factor in NFT formation in these animals. In this study, we hypothesized that tau protein from brain and spinal cord tissue of P301L tau transgenic mice will contain more transglutaminase-catalyzed cross-links compared with 4RWT tau and nontransgenic mice, animals that do not exhibit NFT pathology.

Materials and Methods

Antibodies. PHF-1 (kindly provided by Dr. Peter Davies, Albert Einstein School of Medicine, Bronx, NY) was used at 1:500 for immunoblots and 1:50 for immunohistochemistry. PHF-1 monoclonal antibody (mAb) recognizes phosphorylated serines 396 and 404 located within the microtubule-binding repeat on the C terminal of PHF tau protein (Otvos et al., 1994). AT8 mAb (Pierce, Rockford, IL) recognizes PHF-tau that is phosphorylated at serine 202 and was used at 1:500 on immunoblots. Tau-5 mAb (donated by Dr. Lester Binder, Feinberg School of Medicine, Northwestern University, Chicago, IL) detects an epitope from amino acids 210–241 present in all isoforms of tau protein (LoPresti et al., 1995) and was used at 1:500 for immunoblots. Anti-actin mAb (clone, C4) was purchased from ICN Biomedicals (Aurora, OH) and used at 1:10,000 on immunoblots.

Transglutaminase-catalyzed cross-links were detected with mAb 81D4 (Covalab, Lyon, France) in immunohistochemistry (1:100) and immunoprecipitation experiments. The specificity of this antibody has been demonstrated in numerous publications from our laboratory and others. Our laboratory has demonstrated previously that 81D4 specifically labels ϵ -(γ -glutamyl) lysine bonds in tissue sections by performing preadsorption control experiments with glutamyl-lysine di-peptide containing an ϵ -(γ -glutamyl) lysine bond (Singer et al., 2002; Zainelli et al., 2003). Additionally, the 81D4 antibody has been used to quantitate ϵ -(γ -glutamyl) lysine bonds in AD tissue using a competitive ELISA (Nemes et al., 2004). This antibody has also been used extensively in immunoprecipitation experiments that demonstrate the presence of ϵ -(γ -glutamyl) lysine cross-links in AD, PSP, and Parkinson’s disease tissue but not in control tissue (Norlund et al., 1999; Zemaitaitis et al., 2000; Andringa et al., 2004). The most convincing support for the specificity of the 81D4 antibody for ϵ -(γ -glutamyl) lysine cross-links comes from *in vitro* and cell culture experiments. When tau is incubated with transglutaminase *in vitro* and subsequently immunoprecipitated with 81D4, it is recognized on immunoblot by 81D1c2 [another anti- ϵ -(γ -glutamyl) lysine bond antibody] and tau antibody. Tau incubated without transglutaminase does not react with these anti-cross-link antibodies (Norlund et al., 1999). Furthermore, huntingtin is immunoprecipitated with 81D4 in cells cotransfected with truncated, mutant huntingtin protein and transglutaminase but not in cells transfected only with huntingtin (Zainelli et al., 2003). This result was confirmed by immunoprecipitating huntingtin and probing immunoblots with 81D1c2.

Animals. P301L tau transgenic mice were from the JNPL3 line previously described by Lewis et al. (2000). JNPL3 mice were assessed for presence of motor dysfunction using tests for righting reflex, escape extension, and rope hang as reported previously (Lewis et al., 2000). For righting reflex, animals were placed supine and judged for their ability to right. Unimpaired animals typically right within 1 s. To monitor escape extension, mice were elevated by their tail, and rear leg extension was assessed. Unimpaired animals generally place their legs in a wide “V” away from their bodies. For rope hang, animals were elevated by their

tail, allowed to grasp a horizontal string with both front feet, and then gently released. Unimpaired animals complete the task by stabilizing their position with placement of at least one back foot and their tail onto the string within the 2 min trial. JNPL3 mice used in this study show dramatic motor dysfunction, including slowed righting reflex and dystonia or scissoring of legs in extension tests. These JNPL3 animals were also unable to complete the rope-hang task and were unable to initially grasp the rope for >5 s without falling. JNPL3 mice used for these experiments were also characterized by reduced ability to freely ambulate, poor grooming (urine scalding/oily fur), eye irritations, and reduced weight, which have been shown previously in JNPL3 animals that have motor impairment (Lewis et al., 2000).

4RWT tau and nontransgenic mice did not exhibit any motor dysfunction. The age of the animals used in these experiments ranged from 5.5 to 15.5 months, with 4RWT tau and nontransgenic mice age matched to P301L tau transgenic mice. For immunoblots (except Figs. 1B, 4B), the same 14 mice were used for experiments and loaded on gels in the same order.

Immunoprecipitation. Hindbrain tissue from P301L tau, 4RWT tau, and nontransgenic mice was homogenized in three volumes of TBS (10 mM Tris-HCl, pH 7.5, 0.14 M NaCl), 1 mM EDTA, and 1:1000 protease inhibitor mixture (Sigma, St. Louis, MO). Sarkosyl-insoluble protein fractions rich in PHF tau protein were prepared from AD temporal cortex (provided by the Loyola University Chicago Brain Bank, Maywood, IL) following a protocol previously used in our laboratory and others (Liu et al., 1991; Norlund et al., 1999). Protein concentration was determined using the BCA Protein Assay kit (Pierce). Immunoprecipitation of proteins containing ϵ -(γ -glutamyl) lysine bonds was performed using 81D4 mAb prebound to Sepharose beads (Covalab) according to the manufacturer protocol and published methods of our laboratory (Norlund et al., 1999; Zemaitaitis et al., 2000). Immunopurified cross-linked proteins were eluted in 25 μ l of sample buffer containing 2% SDS at 90°C and stored at -80°C until Western blot analysis, in which 15 or 25 μ l was loaded in each lane.

Immunoblots. Proteins were separated on 10% SDS-polyacrylamide gels followed by electrophoretic transfer to nitrocellulose membranes. The proteins examined here are all in the SDS-solubilized fraction. Membranes were then blocked with 0.2% I-Block (Tropix, Bedford, MA) and 0.1% Tween 20 for 1 h at room temperature. Blots were incubated overnight at 4°C with primary antibodies (described above) diluted in blocking solution. The next day, blots were washed with PBS/0.1% Tween 20 and then incubated with anti-mouse secondary antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA) diluted in blocking solution (1:40,000). Blots were washed and signal was detected using ECL enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Immunoblots were quantified by calculating the integrated optical density (IOD) from the area and optical density of each protein band on film using Scion Image for Windows (Scion, Frederick, MD). Film background was measured and subtracted from each band. Measurements were done in triplicate, and the mean was calculated. The mean IOD for each band was normalized to actin (except Fig. 3B).

Double-label fluorescence immunohistochemistry. Fresh-frozen hindbrain, spinal cord, and cortical tissue was embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA). Sixteen-micrometer-thick sections were mounted on glass slides and fixed with 70% ethanol/0.9% NaCl for 10 min. Sections were washed with PBS and permeabilized with PBS/0.2% Triton X-100 for 90 s. After washes with PBS, sections were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS (blocking solution) for 1 h. Sections were then washed with PBS and incubated overnight in primary antibody (PHF-1; 1:50 in blocking solution). The next day, sections were washed with PBS and incubated with goat-anti-mouse secondary antibody (IgG γ -chain specific; 1:100 in blocking solution) conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch) for 1 h. After washes with PBS, sections were blocked again, washed with PBS, and incubated overnight with the second primary antibody (81D4; 1:100 in blocking solution). After washing sections with PBS, sections were incubated with goat-anti-mouse secondary antibody (IgM μ -chain specific; 1:100 in blocking so-

lution) conjugated to Rhodamine Red-X (Jackson ImmunoResearch) for 1 h. Slides were mounted in Vectashield with 4',6'-diamidino-2-phenylindole (Vector Laboratories) to allow visualization of cell nuclei. To control for nonspecific labeling of the tissue by secondary antibody, all experiments included tissue sections in which the primary antibody was omitted. All steps in this procedure were performed at room temperature.

The percentage of PHF-1 and 81D4 double labeling was determined in spinal cord sections from P301L tau, 4RWT, and nontransgenic mice ($n = 4$ for each group) in two independent experiments. Examination of the slides blinded to group revealed no specific PHF-1 labeling in 4RWT tau and nontransgenic animals; therefore, counts were only performed in P301L tau mice. In each experiment, PHF-1-positive cells and PHF-1 and 81D4 double-labeled cells were counted in three to five random fields with PHF-1 labeling from three nonserial sections. The percentages of PHF-1 and 81D4 double-labeled cells were calculated for each animal, and the means from the two experiments were averaged among the four P301L tau transgenic mice. Results are expressed as a percentage of PHF-1-positive cells that colocalize with 81D4 labeling \pm SEM.

Fluorescence microscopy. Hindbrain and spinal cord sections were viewed with the 40 \times objective on an Olympus (Tokyo, Japan) fluorescent inverted research microscope configured with Image-Pro Plus 4.5 software (VayTek, Fairfield, IA). Images of optical sections were captured using a Retiga EX 1350 camera and VolumeScan 3.1 software (VayTek) with eight-bit/pixel resolution. Three optical sections were deconvolved using Deconvolution Tools for Windows 9X/NT/2000 software (VayTek), and a single deconvolved optical section is shown in each figure. All deconvolved images were obtained using identical parameters. Monochrome images were pseudocolored and merged using Image-Pro Plus 4.5 software. Cortex images were acquired using standard fluorescence microscopy (40 \times objective; camera zoom factor, 2.0) with the merged image generated by overlaying the red and green images in Image-Pro Plus.

Transglutaminase activity assay. Transglutaminase enzymatic activity was determined in the spinal cord of P301L tau, 4RWT tau, and nontransgenic mice by measuring the incorporation of a radioactive polyamine donor (^3H -putrescine) into an exogenous protein acceptor (casein). Our laboratory has previously published the detailed procedure used for this assay (Zemaitaitis et al., 2003). Transglutaminase activity was measured for each animal in two separate assays, and, within an assay, each sample was measured in triplicate. The assay was repeated five times to measure samples from five animals in each group. Each sample contained 100 μg of protein. Negative controls for each sample were run in the absence of calcium and casein. Each assay also included a standard curve of varying amounts of guinea pig transglutaminase (Sigma). For each assay, transglutaminase activity for P301L and 4RWT animals was converted to a percentage control based on the mean value for the nontransgenic mice. Duplicate measurements for each animal were averaged, and data presented are the group mean \pm SEM.

Statistics. All statistical analyses were performed using GB-STAT School Pak (Dynamic Microsystems, Silver Spring, MD). Data are expressed as means \pm SEM. For Western blots, a test of homogeneity of variance revealed unequal variances for some data; thus, a nonparametric Mann-Whitney U test was used to determine significance ($p < 0.05$). Otherwise, data were analyzed using a Student's t test for separate variances. Statistical significance was determined in the transglutaminase activity assay with a one-way ANOVA and Newman-Keuls *post hoc* test.

Results

Transglutaminase-catalyzed cross-links in phosphorylated tau protein

To determine whether transglutaminase-catalyzed cross-links are present in tau protein from P301L tau, 4RWT tau, and nontransgenic mice, we immunoprecipitated all cross-linked proteins in the hindbrain and detected tau in the pool of cross-linked proteins by Western blot. The hindbrain was chosen for these experiments to enrich for brain regions with NFT, including the pons, medulla, cerebellar nuclei, and brainstem (Lewis et al., 2000). The age of the animals ranged from 5.5 to 15.5 months,

with all of the P301L tau transgenic animals exhibiting dramatic motor dysfunction.

Using PHF-1 antibody, which is directed against abnormally phosphorylated PHF-tau, we detect cross-linked PHF-1-immunoreactive tau in P301L tau transgenic mice but not in 4RWT tau or nontransgenic mice (Fig. 1A). In some replicates, high-molecular mass tau bands at ~ 120 kDa are present in P301L tau lanes (data not shown), suggesting the presence of tau homodimers or cross-linking of tau to other species of similar molecular mass. Three of the five P301L tau mice showed a smear of cross-linked, phosphorylated tau protein between 75 and 175 kDa (Fig. 1A). This protein smear is characteristic of aggregated tau protein in human tauopathies (Norlund et al., 1999; Sergeant et al., 1999; Zemaitaitis et al., 2000; Zhukareva et al., 2002) and is demonstrated in Figure 1B, in which we loaded an increased amount of cross-linked proteins from P301L animals on gels in conjunction with cross-linked PHF from the Sarkosyl-insoluble fraction of AD temporal cortex. Both P301L tau transgenic mice and AD tissue exhibit a smear of high-molecular mass cross-linked tau protein, with some cross-linked tau remaining in the stacking gel (Fig. 1B). The cross-linked proteins were solubilized in SDS sample buffer before loading on a gel, and therefore the aggregates we detect are attributable to covalent bonds, such as the transglutaminase-catalyzed cross-link. The band just above 75 kDa present in all lanes (Fig. 1A) is attributable to nonspecific binding of secondary antibody, as determined by experiments in which we omitted the primary antibody (data not shown).

We also used AT8 antibody on Western blots to detect hyperphosphorylated tau protein in the immunoprecipitated cross-linked proteins. Again, hyperphosphorylated cross-linked protein is present in P301L tau mice but is absent in 4RWT tau and nontransgenic mice (Fig. 1C). The majority of the cross-linked tau detected in P301L tau mice with AT8 is monomeric tau running at ~ 65 kDa. However, this monomeric tau could have been part of a tau aggregate, because SDS will solubilize some tau aggregates. A faint smear of cross-linked protein between 75 and 175 kDa is evident in some of the P301L tau mice even after solubilization with SDS. The band just above 75 kDa present in all lanes (Fig. 1C) is attributable to nonspecific binding of secondary antibody, as determined by experiments in which we omitted the primary antibody (data not shown).

Phosphorylated tau levels

We performed Western blots on SDS-solubilized hindbrain homogenates to compare the levels of phosphorylated tau among P301L tau, 4RWT tau, and nontransgenic mice (Fig. 2). Both P301L tau and 4RWT tau transgenic mice have PHF-1-immunoreactive tau protein in the hindbrain (Fig. 2A). Two bands are present in lanes with P301L tau mice hindbrain homogenates, whereas PHF-1 detects one band in lanes with 4RWT tau mice hindbrain homogenates. PHF-1 also recognizes two bands on Western blots prepared with lysates of Chinese hamster ovary cells transfected with the same tau isoform used in these transgenic mice (Otvos et al., 1994). Both bands in the P301L tau lanes were quantified, and the integrated optical densities were combined for statistical analysis. Phosphorylated tau levels are not significantly ($p > 0.05$; Mann-Whitney U test) increased in P301L tau mice in comparison with 4RWT tau mice (Fig. 2B), even though PHF-1-immunoreactive tau levels in the P301L tau mice (4.04 ± 2.20 IOD) are increased twofold over 4RWT mice (1.73 ± 0.50 IOD). Actin levels show equal protein loading among lanes (Fig. 2A, bottom panel).

Phosphorylated tau protein was also detected in SDS-

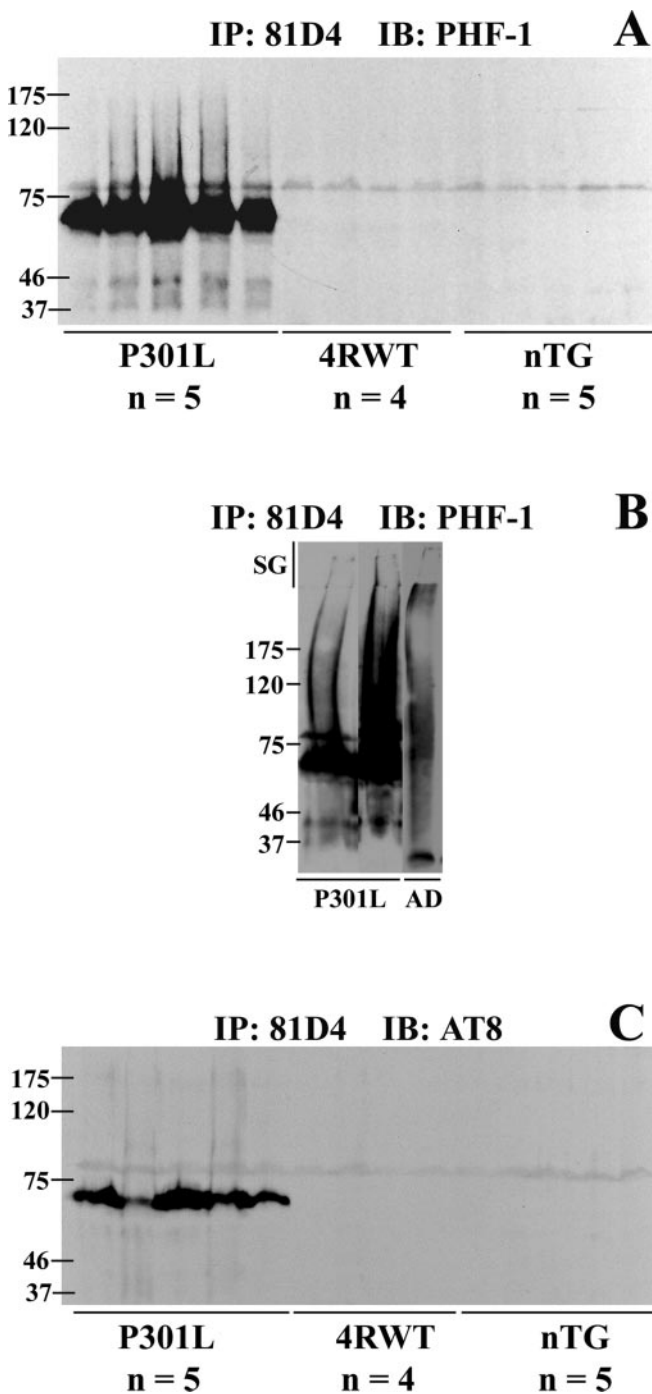


Figure 1. Phosphorylated tau is cross-linked in P301L tau transgenic mice. Cross-linked proteins were immunoprecipitated (IP) with anti-cross-link antibody (81D4) coupled to Sepharose. Phosphorylated tau protein was detected on immunoblot (IB) with PHF-1 (**A**) and AT8 (**C**) antibodies. Transglutaminase-catalyzed cross-links are present in phosphorylated tau (**A**, PHF-1; **C**, AT8) from the hindbrain of P301L tau transgenic mice but not 4RWT tau and nontransgenic (nTG) mice. Cross-linked, phosphorylated (PHF-1) tau in P301L mice is similar to cross-linked tau in PHF from the Sarkosyl-insoluble fraction of AD temporal cortex (**B**). Note the phosphorylated tau in the stacking gel (SG) of the lanes with P301L tau mice and AD (**B**). Molecular mass in kilodaltons is indicated on the left. Comparable results were obtained in three independent experiments.

solubilized hindbrain homogenates from P301L tau and 4RWT tau transgenic mice with AT8 antibody (Fig. 2C). There was no significant difference ($p > 0.05$; Student's *t* test) in the level of AT8-immunoreactive tau between P301L tau (1.02 ± 0.55 IOD)

and 4RWT tau (1.13 ± 0.33 IOD) transgenic mice (Fig. 2D). Although there is no striking difference in the levels of PHF-1 and AT8-positive tau between P301L tau and 4RWT tau transgenic mice, there is a dramatic qualitative difference in tau species detected with these antibodies. P301L tau transgenic mice clearly have high-molecular mass tau, whereas the 4RWT tau transgenic mice do not (Fig. 1).

Transglutaminase-catalyzed cross-links in tau protein

We also used the antibody Tau-5 (that detects all tau species) on Western blots of the immunoprecipitated cross-linked proteins to evaluate the amount of tau protein that was cross-linked in the SDS-solubilized protein fraction (Fig. 3A). The P301L tau transgenic animals and 4RWT tau transgenic animals both have cross-linked tau protein (Fig. 3A). Darker exposures did not reveal high-molecular mass cross-linked tau protein (data not shown). Quantification of these data shows that P301L tau transgenic mice (1.54 ± 0.58 IOD) have 4.5 times more cross-links in tau compared with 4RWT tau transgenic animals (0.34 ± 0.12 IOD), and the difference is significant ($p < 0.03$; Mann-Whitney *U* test) (Fig. 3B). Again, we did not detect cross-linked tau protein in the nontransgenic animals.

Tau protein levels

To determine whether the differences in cross-linked tau between P301L tau animals and 4RWT animals was a result of unequal transgene expression, we performed Western blots on SDS-solubilized hindbrain homogenates and probed for tau with Tau-5 (Fig. 3C). Quantification and subsequent statistical analysis demonstrated no significant difference between tau protein levels of P301L (0.63 ± 0.14 IOD) and 4RWT (0.55 ± 0.09 IOD) tau transgenic mice ($p > 0.05$; Student's *t* test) (Fig. 4E), suggesting that differences in cross-linked tau are not caused by unequal transgene expression. Tau-5 did not detect the upper band in P301L tau mice observed with the PHF-1 antibody, although this might simply reflect the phospho-specific selectivity of the PHF-1 antibody. We reprobbed immunoblots with actin (Fig. 3C, bottom panel) to demonstrate equal protein loading among lanes. Longer exposures of the blot shown in Figure 3C allow for detection of endogenous mouse tau with Tau-5 but puts P301L tau and 4RWT tau bands out of a quantifiable range. To show that Tau-5 does recognize endogenous mouse tau in nontransgenic mice, we ran a separate gel in which we loaded the same amount of protein from P301L tau and nontransgenic mice as in Figure 3C ($10 \mu\text{g}$) (Fig. 3D, lanes 1 and 2, respectively), included an additional lane with $30 \mu\text{g}$ of protein from nontransgenic mice (Fig. 3D, lane 3), and increased exposure time (Fig. 3D). Thus, the absence of cross-linked tau in the nontransgenic mice is not a result of an inability of Tau-5 to detect endogenous mouse tau.

Colocalization of transglutaminase-catalyzed cross-links and phosphorylated tau

We performed double-label immunofluorescence to determine whether phosphorylated tau and transglutaminase-catalyzed cross-links colocalize in the spinal cord, hindbrain, and cortex of P301L tau, 4RWT tau, and nontransgenic mice. PHF-1 and 81D4 double-labeled cells are present in the spinal cord, hindbrain, and cortex of P301L tau transgenic mice (Fig. 4). Sections from 4RWT tau and nontransgenic animals did not have any specific PHF-1 labeling, and therefore no double labeling is present (data not shown). Cross-link labeling in the 4RWT and nontransgenic mice is diffuse and without specific cell body labeling. In the P301L tau transgenic mice, PHF-1 labeling in the cortex is sparse;

however, PHF-1 and 81D4 double labeling predominates over PHF-1 single labeling. Examination of 1908 PHF-1-positive neurons in the spinal cord of four P301L tau transgenic mice reveals that $86.95 \pm 0.33\%$ of PHF-1-labeled neurons also label with the anti-cross-link antibody (Table 1).

Transglutaminase activity

Aberrant elevations in transglutaminase activity have been demonstrated in brain regions with neurofibrillary pathology in two human tauopathies (Johnson et al., 1997; Zemaitaitis et al., 2003). Because the spinal cord of P301L tau transgenic mice has been shown previously to develop abundant NFT (Lewis et al., 2000), we measured the transglutaminase activity in the spinal cord of P301L tau, 4RWT tau, and nontransgenic mice. The activity of guinea pig liver transglutaminase was measured in each assay in range from 0.01 to 0.1 μg and resulted in linear correlation values from 0.999 to 0.96. The activity of transglutaminase in the spinal cord samples fell within this linear range for guinea pig liver transglutaminase. Our data reveal a significant ($F_{(2,12)} = 11.5$; $p = 0.0016$) increase in the transglutaminase activity in the spinal cord of P301L tau mice ($384 \pm 80\%$ control) compared with 4RWT tau ($91.8 \pm 27.4\%$ control) and nontransgenic mice ($100 \pm 10.1\%$ control) (Fig. 5). There was no significant difference in transglutaminase activity in 4RWT tau mice compared with nontransgenic mice.

Discussion

Transglutaminase is an enzyme that cross-links proteins, rendering them stable and insoluble. The insoluble tau protein in P301L tau transgenic mice (Lewis et al., 2000; Sahara et al., 2002) prompted us to investigate the potential involvement of transglutaminase in the neurofibrillary pathology of these mice. The results presented here demonstrate that transglutaminase cross-links tau protein in P301L tau mice, an animal model previously shown to develop NFT (Lewis et al., 2000).

Immunoprecipitation experiments revealed that transglutaminase cross-links are present in phosphorylated tau (detected with PHF-1 and AT8) from P301L tau transgenic mice but not 4RWT and nontransgenic mice (Fig. 1). We also detected high-molecular mass aggregates of cross-linked, phosphorylated tau protein between 75 and 175 kDa in P301L tau mice (Fig. 1A, C). When we loaded an increased amount of cross-linked proteins on Western blot, cross-linked, phosphorylated tau protein was detected in P301L tau mice above 175 kDa and also in the stacking gel (Fig. 1B). This high-molecular mass tau is consistent with intermolecular cross-links producing tau aggregates. Similarly, cross-linked PHF-tau protein appeared as a smear on Western blots in both AD (Fig. 1B) (Norlund et al., 1999) and PSP (Zemaitaitis et al., 2000). It is important to note that these intermolecular cross-links could arise via several different scenarios. Transglutaminase may form cross-links among several tau proteins or cross-link tau to one or more other proteins found in NFT. Al-

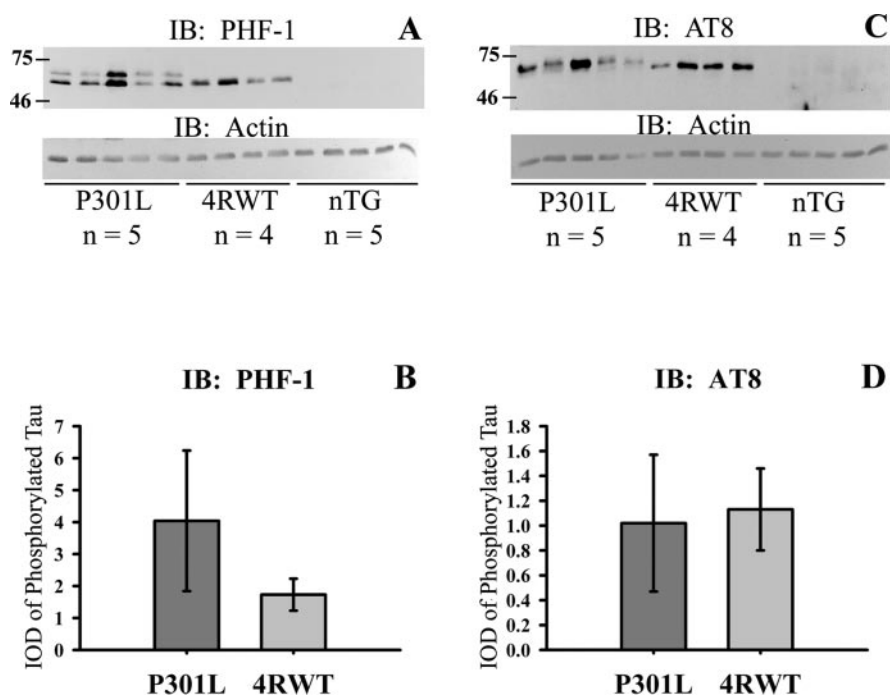


Figure 2. Phosphorylated tau levels in P301L tau, 4RWT tau, and nontransgenic (nTG) mice. Phosphorylated tau in hindbrain homogenates was detected with PHF-1 (A) and AT8 (C) antibodies. Molecular mass in kilodaltons is indicated on the left. The immunoblots (IB) were reprobed with an antibody against actin (A, C, bottom panels) to demonstrate equal protein loading among lanes. The levels of phosphorylated tau in the P301L tau animals were not significantly different from 4RWT tau mice for both PHF-1 (B; $p > 0.05$; Mann–Whitney *U* test) and AT8 (D; $p > 0.05$; Student's *t* test) antibodies. The IODs of both bands in the P301L tau mice lanes were included in the PHF-1 data analysis. All data were normalized to actin. Data shown are mean IOD \pm SEM and are representative of three experiments.

ternatively, transglutaminase may cross-link ubiquitinated tau via the ubiquitin moiety. Tau is ubiquitinated in AD (Morishima-Kawashima et al., 1993), and tau filaments in P301L tau mice are ubiquitin positive (Lin et al., 2003). Recent evidence suggests that transglutaminase cross-links ubiquitin and heat shock protein 27 in tau-positive polymers isolated from AD brain (Nemes et al., 2004).

Most of the cross-linked tau in P301L tau transgenic animals ran at ~ 65 kDa on Western blots, suggesting intramolecular cross-links in monomeric tau or incorporation of a small polyamine (Figs. 1A, C, 3A). Polyamidated tau displays increased stability (Tucholski et al., 1999); however, this does not necessarily translate into tau aggregation. 4RWT tau transgenic mice have monomeric tau with intramolecular cross-links or incorporation of a small polyamine (Fig. 3A) but do not develop tau aggregates (Figs. 1, 3A) or NFT (Lewis et al., 2000). 4RWT animals have significantly lower levels of cross-linked, monomeric tau protein detected with Tau-5 (Fig. 3A, B), and these levels may not produce enough stable tau to result in aggregate formation.

Cross-linked, phosphorylated tau was not present in 4RWT or nontransgenic animals (Fig. 1A, C), animals that do not develop NFT. However, 4RWT tau transgenic mice do have tau protein phosphorylated at the PHF-1 and AT8 sites (Fig. 2A, C). Either transglutaminase does not cross-link phosphorylated tau in the 4RWT mice or the levels are below the limits of detection. Overexpression of 4RWT tau in mice is sufficient to cause cross-linking of tau (Fig. 3A) but not cross-linking of phosphorylated tau (Fig. 1A, C). The unique aspect of the P301L tau mice was the presence of cross-linked PHF-1-labeled tau that ran at molecular masses consistent with tau aggregation. These data are consistent

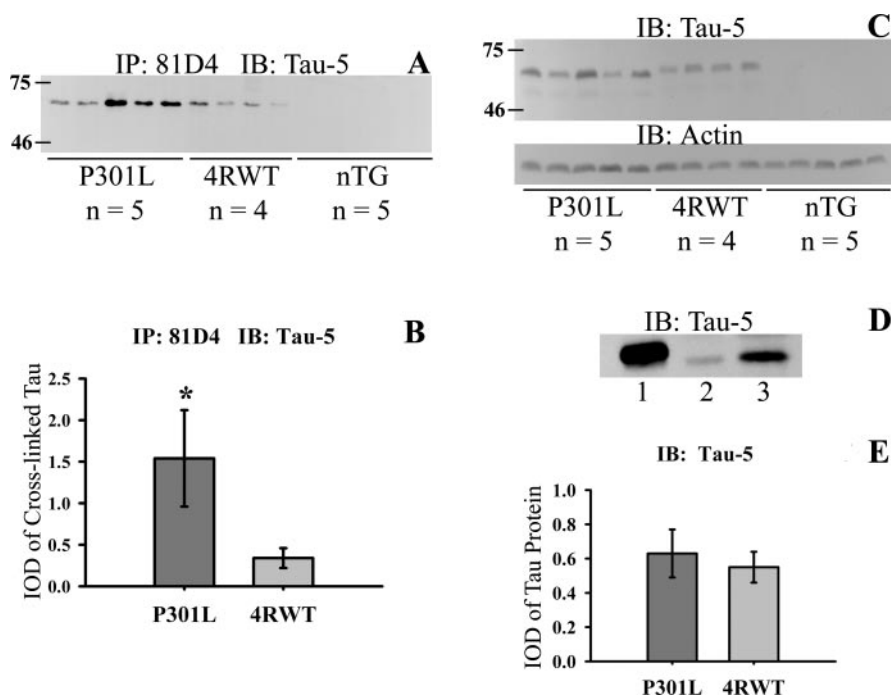


Figure 3. Tau protein is cross-linked in P301L and 4RWT tau transgenic mice. Cross-linked proteins in the hindbrain of P301L tau, 4RWT tau, and nontransgenic (nTG) mice were immunoprecipitated (IP) with anti-cross-link antibody (81D4) coupled to Sepharose, and tau protein was detected on immunoblot (IB) with Tau-5 antibody (**A**). The levels of cross-linked tau in the P301L tau animals were significantly higher than 4RWT tau mice (**B**; $p < 0.03$; Mann-Whitney *U* test). Tau protein levels in the hindbrain of P301L tau, 4RWT tau, and nontransgenic mice were detected with Tau-5 antibody (**C**). Actin levels (**C**, bottom panel) confirmed equal protein loading. To show that Tau-5 antibody recognizes endogenous mouse tau, the same amount of protein from hindbrain homogenates of P301L tau and nontransgenic mice as in **C** (10 μ g of protein; lanes 1 and 2, respectively) was loaded along with 30 μ g of protein from nontransgenic mice (lane 3) (**D**). The darker exposure shown here (compared with **C**) allows for detection of endogenous mouse tau with Tau-5. The levels of tau (normalized to actin) in the P301L tau animals were not significantly higher than those in 4RWT tau mice (**E**; $p > 0.05$; Student's *t* test). Molecular mass in kilodaltons is indicated on the left. Data shown are mean IODs \pm SEM and are representative of three experiments.

with the hypothesis that cross-linking of phosphorylated tau is dependent on initial tau aggregation.

In P301L tau mice, phosphorylated tau and the transglutaminase-catalyzed bond colocalized in the hindbrain and spinal cord, regions previously shown to develop NFT, and cortex, a region with pretangle pathology (Fig. 4) (Lewis et al., 2000). Both neurons and oligodendrocytes of P301L tau mice contain tau filaments (Lin et al., 2003), and therefore the PHF-1 and cross-link colocalization detected here could arise from either cell type. Quantification of double labeling in the spinal cord of P301L mice indicated that 87% of PHF-1-positive cells also labeled with an anti-cross-link antibody (Table 1). Approximately 13% of PHF-1-positive cells in the spinal cord of P301L tau mice were not double labeled with the cross-link antibody. PHF assembly appears to occur in stages (Friedhoff et al., 1998), and this noncross-linked, phosphorylated tau protein may represent a stage of PHF formation in which tau aggregates are unstable. When anionic inducing agents stimulate PHF formation *in vitro*, the stability of the tau aggregates and resistance to denaturation is low (Li et al., 2002). However, combining transglutaminase with tau *in vitro* results in stable, insoluble tau filaments (Dudek and Johnson, 1993; Appelt and Balin, 1997) that are biochemically similar to PHF from AD (Selkoe et al., 1982a). Together, these data suggest that the formation of PHF and the stabilization of PHF by transglutaminase in tauopathies can occur independently. The transglutaminase cross-linking of tau protein present in transgenic mice and human disease with neu-

rofibrillary pathology may be necessary for the stabilization of PHF *in vivo*.

Lysine residues involved in transglutaminase-catalyzed cross-links are located adjacent to the microtubule-binding repeats in tau protein (Murthy et al., 1998), which form the core of PHF in AD (Crowther et al., 1989). Conceivably, transglutaminase would not have access to these sites while tau is bound to microtubules. An elevation in unbound tau could contribute to the cross-linking of tau observed in the P301L tau transgenic mice (Fig. 1). *In vitro*, recombinant P301L tau protein displays a reduced ability to bind to microtubules (Hong et al., 1998; DeTure et al., 2000). Tau mutations seem to have an inconsistent effect on tau-microtubule interactions in cell culture (Dayanandan et al., 1999; DeTure et al., 2000; Perez et al., 2000; Lu and Kosik, 2001; Nagiec et al., 2001), but the impact of mutant tau on microtubule interactions may not be immediate and thus not apparent in transient expression systems. Additionally, tau in these systems has not been reported to form aggregates. However, in cells that stably express mutant tau at levels that saturate microtubules and elevate free cytoplasmic tau, mutant tau, but not wild-type tau, forms filaments (DeTure et al., 2002). Presumably abnormal, unbound tau in P301L mice eventually achieves high-enough levels to result in initial tau aggregation. Work by Zhang et al. (2004) supports this theory by showing

that mutant tau (R406W) expressed in transgenic mice displays a reduced ability to bind microtubules compared with wild-type tau expressed in transgenic mice.

Calcium positively regulates transglutaminase activity (Folk and Finlayson, 1977). Dysregulation of calcium homeostasis is hypothesized to exert a pathological role in AD (LaFerla, 2002) and could underlie the increase in transglutaminase activity measured in AD (Johnson et al., 1997). We found a significant increase in transglutaminase activity in P301L tau mice compared with 4RWT tau and nontransgenic mice (Fig. 5). Furukawa et al. (2000, 2003) demonstrated in cell culture that mutant tau (V337M) causes elevations in intracellular calcium. An increase in intracellular calcium precipitated by mutant tau could elicit the elevation in transglutaminase activity in P301L tau mice, resulting in pathological cross-linking of tau protein.

Aberrant transglutaminase activity has been implicated in PHF/NFT formation in AD and PSP (Kim et al., 2002). The basis for this hypothesis and supporting data is the result of primarily human postmortem and *in vitro* studies. Although these types of studies provide extremely valuable insight into disease processes, they cannot always lend insight into the progressive nature of disease mechanisms. P301L tau transgenic mice develop NFTs and have cross-linked tau protein; thus, they can be used to examine cross-linking of tau protein during disease progression and determine whether transglutaminase plays an early role in PHF/NFT formation. P301L tau transgenic mice can further be used to appreciate the impact of transglutaminase inhibition on

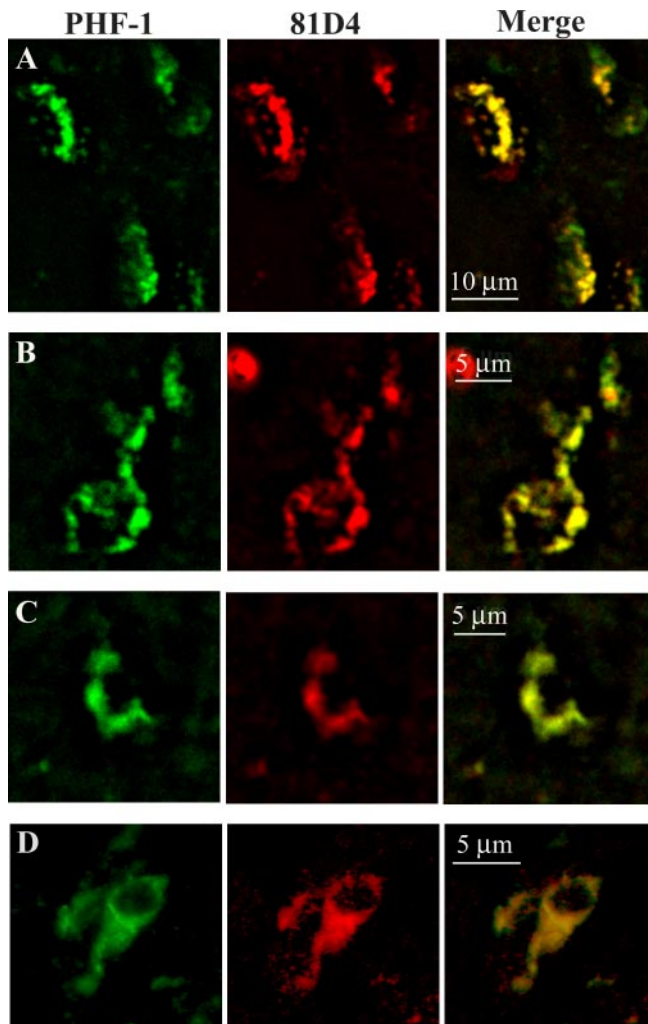


Figure 4. Phosphorylated tau and transglutaminase-catalyzed cross-links colocalize in P301L tau transgenic mice. Spinal cord (**A**, **B**), hindbrain (**C**), and cortical (**D**) tissue sections from P301L tau mice were double labeled with an antibody against phosphorylated tau (PHF-1 plus FITC; green) and anti-cross-link antibody (81D4 plus Rhodamine Red-X; red). Images in **A–C** were generated by deconvolution fluorescence microscopy. Standard fluorescent microscopy was used for images in **D**. Calibration bars are included in the merged images.

Table 1. Phosphorylated tau and cross-link colocalization in P301L tau mice spinal cord

Animal	Trial	Percentage double label	Animal average	Mean \pm SEM
1	1	84.8 ($n = 277$)	85.1%	
	2	85.3 ($n = 381$)		
2	1	88.7 ($n = 142$)	87.7%	
	2	86.6 ($n = 262$)		
3	1	85.8 ($n = 267$)	86.1%	
	2	86.4 ($n = 279$)		
4	1	86.7 ($n = 151$)	87.0%	
	2	87.2 ($n = 158$)		

PHF/NFT formation and phenotypic manifestations of the animal model. Similar experiments have been executed to illuminate the importance of transglutaminase in the pathology of Huntington's disease, in which intranuclear huntingtin aggregates have been shown to contain transglutaminase-catalyzed cross-links (Zainelli et al., 2003). Two separate studies treated a transgenic mouse model of Huntington's disease with cystamine, an inhib-

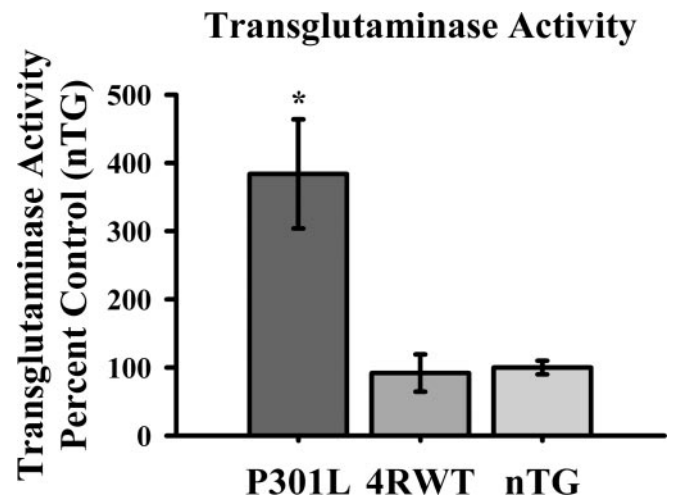


Figure 5. Transglutaminase activity is increased in the spinal cord of P301L tau transgenic mice. Transglutaminase enzymatic activity was measured by incorporation of ^3H -putrescine into casein. The transglutaminase activity in P301L tau transgenic mice is significantly different from both 4RWT tau and nontransgenic (nTG) mice ($*p < 0.01$). Data for each group ($n = 5$) are the mean of two trials and expressed as percentage control. Error bars indicate SEM.

itor of transglutaminase. Results indicate that cystamine inhibited transglutaminase activity, prolonged survival, improved motor performance (Dedeoglu et al., 2002; Karpuj et al., 2002) and, if administered before the onset of motor disturbances, decreased the amount of huntingtin-positive inclusions (Dedeoglu et al., 2002). It should be noted that cystamine may have other beneficial effects in addition to inhibiting transglutaminase. However, strategies aimed at specifically inhibiting transglutaminase may offer therapeutic potential in neurodegenerative diseases with protein aggregation as a pathological hallmark.

The data presented here demonstrate that phosphorylated, high-molecular mass tau protein is cross-linked and transglutaminase activity is elevated in transgenic mice that develop NFT. The electrophoretic pattern of cross-linked, phosphorylated tau strongly resembles that of PHF from AD and PSP. Furthermore, phosphorylated tau and the transglutaminase-catalyzed cross-link are highly colocalized in P301L tau transgenic mice. Mice that do not develop NFT do not have high-molecular mass aggregated tau protein or colocalization of phosphorylated tau and the transglutaminase bond. These results support the hypothesis that transglutaminase-catalyzed cross-linking of phosphorylated tau protein is involved in NFT formation and/or stabilization in human tauopathies, such as AD and PSP. P301L tau transgenic mice provide an excellent model to further study the role of transglutaminase in tau aggregation and neurofibrillary pathology.

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