Loss of Tau Expression Attenuates Neurodegeneration Associated With α-Synucleinopathy

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Abstract

Background: Neuronal dysfunction and degeneration linked to α-synuclein (αS) pathology is thought to be responsible for the progressive nature of Parkinson's Disease and related Dementia with Lewy Bodies. Studies indicate to bidirectional pathological relationships between αS pathology and tau abnormalities. We recently showed that A53T mutant human αS (HuαS) can cause post-synaptic and cognitive deficits that require microtubule-associated protein tau expression. However, the role of tau in development of αS pathology and subsequent neuronal dysfunction has been controversial. Herein, we set to determine the role of tau in the onset and progression of αS pathology (α-synucleinopathy) using a transgenic mouse model of α-synucleinopathy lacking mouse tau expression.

Methods: Transgenic mice expressing A53T mutant HuαS (TgA53T) were crossed with mTau$^{-/-}$ mice to generate TgA53T/mTau$^{-/-}$. To achieve uniform induction of α-synucleinopathy in mice, we used intramuscular injections of αS preformed fibrils (PFF) to non-transgenic (nTg), TgA53T, TgA53T/mTau$^{-/-}$, and mTau$^{-/-}$ mice. Motor behavior was analyzed at 70 days post inoculation (dpi) of PFF and tissues for biochemical and neuropathological analysis were collected at 40 dpi, 70 dpi, and end stage.

Results: Loss of tau expression significantly delayed onset of motor deficits in the TgA53T model and delayed α-synucleinopathy disease progression, as evidenced by a significant reduction in histopathological and behavioral markers of neurodegeneration and disease, and a significant improvement in survival. *In vitro* application of PFF to primary mouse hippocampal neurons demonstrated no changes in PFF uptake and processing or pS129 αS aggregation as a function of tau expression. However, PFF-induced neurotoxicity, including morphological deficits in nTg neurons, were prevented with tau removal.

Conclusions: Collectively, our data suggest that tau is likely acting downstream of αS pathology to affect neuronal homeostasis and survival. This work further supports the investigation of tau in α-synucleinopathies to identify novel disease-modifying therapeutic strategies.

Background

Parkinson's Disease (PD) and related Dementia with Lewy Bodies are progressive neurodegenerative diseases, collectively termed α-synucleinopathies, characterized by the degeneration of multiple neuronal populations, particularly the dopaminergic neurons of the substantia nigra pars compacta (SNpc). Another disease hallmark is the presence of intracellular proteinaceous inclusions called Lewy bodies (LBs) and Lewy neurites (LNs). While SNpc dopaminergic neurodegeneration is responsible for the parkinsonian motor symptoms, neuropathology and clinical abnormalities in PD extend beyond the SNpc dopaminergic neurons [1,2] leading to non-motor symptoms, including cognitive dysfunction. The presynaptic protein α-synuclein (αS) is established as a pathogenic-capable protein and genetic mutations in the αS gene (*SNCA)* are causal for autosomal dominant familial PD. Additionally, mutant αS

causes neurodegeneration in cellular and *in vivo* models, and, lastly, αS is the major structural component of LBs and LNs [3], collectively pointing to αS as a central player in disease pathophysiology.

Previously, we showed that mutant αS can cause synaptic and memory deficits that requires the expression of microtubule-associated protein tau [4,5]. While our studies showed that some of the mutant αS dependent motor deficits were not tau-dependent, other studies show that αS pathology and resulting neuronal dysfunction may be mediated by tau. Specifically, while the role of tau in the αS pathology cascade has shown mixed results [6–9], a recent study correlates αS pathology with increased tau oligomers, and that neutralization of tau oligomers via systemic injection of a tau oligomer specific antibody (TOMA) reduces αS aggregation and behavioral deficits in an A53T mutant human αS transgenic mouse model (TgA53T, line M83 [10]). However, a series of more recent studies demonstrates that tau does not impact onset and transmission of αS pathology *in vivo* [6,11]. To address these discrepancies and provide an unambiguous test of a pathological link between αS and tau, we tested whether the loss of tau expression impacts the onset and progression of overexpressed αS in our TgA53T mouse model of α-synucleinopathy. To determine the effects of tau on α-synucleinopathy, we induced pathology by intramuscular injections of αS preformed fibrils (PFF; [12]) to TgA53T mice (TgA53T; human A53T αS overexpressing mice; [3]) that are either on wild type mouse tau or mTau$^{-/-}$ (endogenous mouse tau knockout) background.

Our results show that the lack of tau expression significantly delays the onset of disease and ultimately end stage hindlimb paralysis. Analysis of neuropathology, including αS pathology, revealed that tau expression did not impact the neuroinflammation, neurodegeneration, or αS aggregation in end stage mice. However, analysis of presymptomatic mice shows that the absence of tau expression was associated with reduced αS pathology and neurodegeneration. Significantly, we failed to observe any tau abnormalities in the TgA53T mice. Our results suggest that either early initiation/progression of αS pathology or late-stage neurodegeneration is affected by tau expression.

Analysis of αS PFF uptake and processing in neurons indicates that tau does not impact initial uptake or seeding of αS aggregates in neurons. However, neurons lacking tau expression were significantly protected from dendritic neurite shortening caused by αS PFF treatment. Collectively, these results show that tau does not directly impact αS pathology but instead promotes αS-dependent neurodegeneration by acting downstream of αS pathology. This work further highlights that tau, independent of overt tau pathology and aggregation, may play a critical role in neurodegenerative diseases outside the classically recognized tauopathies. Finally, a more comprehensive understanding of the roles αS and tau play in age-related neurological diseases, particularly the α-synucleinopathies and tauopathies, is likely to improve treatment strategies in these conditions.

Methods

Animals

TgA53T animals contain a transgene expressing the human mutant A53T α-synuclein (αS, hαS^{A53T}: line G2-3) and maintained in the C57BL/6J background strain (stock 006823, Jackson Labs; Bar Harbor, ME) [3]. Non-transgenic (nTg) controls came from within these litters. To generate transgenic animals expressing hαS^{A53T} (TgA53T) and lacking endogenous mouse tau (mTau$^{-/-}$), TgA53T males were bred to Mapt$^{tm1(EGFP)Klt}$/J females (stock 004779, Jackson Labs) [13] as previously described [4]. All animal studies were performed in accordance with the NIH guidelines for the use of animals in research and approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Antibodies

Primary antibodies used for immunohistochemistry and immunoblots are listed in Additional file 1: Table S1.

Mouse perfusion and sample collection

Mice were anaesthetized using isoflurane and transcardially perfused with potassium-free phosphate-buffered saline (PBS). The brain was extracted and one hemisphere was placed in 4% paraformaldehyde (PFA) fixation solution for histological processing, while the other hemisphere was sub-dissected for biochemical analysis. [4,14–16].

Immunohistochemical analysis

After perfusion and dissection, brains and spinal cords were post-fixed in 4% PFA and embedded in paraffin. Sections were cut at 7 μm and immunostained using an immunoperoxidase method with diaminobenzidine as previously described [15–17].

Preformed fibril preparation

Human wildtype (HuWT) αS recombinant protein was isolated and purified following the protocol established by Volpicelli-Daley et al., 2014 [18]. Purified monomeric αS protein was brought to a concentration of 5 mg/mL in PBS and placed at 37°C with continuous shaking for 5-7 days using a ThermoMixer (Eppendorf; Hamburg, Germany). Assembly of amyloid αS fibrils were assessed twice-daily using Thioflavin T fluorometry. Upon Thioflavin T saturation levels, αS PFF were aliquoted and stored at -80C for future single use. Subsequently, a sedimentation assay was performed to confirm a pelletable fraction at 100,000 xg (30 PSI) using a Beckman Airfuge CLS air-driven ultracentrifuge (Beckman Coulter; Indianapolis, IN). The resulting pellet was resuspended in PBS and run alongside the supernatant using SDS-PAGE (BioRad) and Coomassie Brilliant Blue (Thermo Fisher Scientific) staining. HuWT αS preformed fibrils (PFF) were prepared for use from 5 mg/mL frozen stock diluted with PBS to a concentration of 0.25 mg/mL. The fibrils were subsequently sonicated utilizing a Fisher Scientific Branson micro probe tip sonicator (Fischer Scientific; Hampton, NH) 60 pulses, 1 second (s) ON, 1 s OFF for 120 s, at 20% amplitude immediately prior to use.

Intramuscular injections

Intramuscular injections of PFF were performed as previously described [12]. Each mouse was first anesthetized using an isoflurane chamber until unresponsive to stimuli. Immediately following, 5 µg of PFF were injected into the *bicep femoralis* muscle bilaterally. The mouse was then allowed to recover on a heat pad and placed back into its respective home cage.

Motor Behavior Tests

Rotarod and open field tests were performed as previously described [4]. For the pole test [19], mice were placed on a horizontal pole facing the top. The pole was then placed upright with the mouse facing upward. The time to turn and the time to reach the bottom of the pole were recorded with a maximum duration of 120 seconds. All animals were tested for 3 trials with 20 minutes of rest in between trials.

Western blot (immunoblot) analysis

Brain regions and spinal cord were dissected out and stored at -80°C until homogenized as described [4,14,20–23]. SDS-PAGE separation of protein in brain lysates and transfer of resolved proteins to nitrocellulose membranes were performed as previously described [4,14,20]. Briefly, frozen tissues were homogenized in complete TNE buffer consisting of TNE solution: Tris-HCl, 50 mM; NaCl, 150 mM; and EDTA, 5 mM supplemented with 0.5% NP40, 0.5% DOC, 1% SDS, and HALT protease and phosphatase inhibitors (Thermo-Fisher; Waltham, MA). The homogenates were sonicated and heated at 100°C for 10 minutes and centrifuged for 10 minutes at 16,000 xg. The supernatant was collected and BCA assay (Pierce, Thermo; Rockford, IL) was performed. Samples were prepared to equal protein concentration in reducing, SDS-sample, Laemmli buffer (Boston BioProducts; Ashland, MA). For Western blot analysis, protein lysates were run on Criterion^{TM} TGX^{TM} gels (BioRad; Hercules, CA) and transferred onto nitrocellulose membranes. Proteins on membranes were detected using appropriate primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen; Carlsbad, CA). Membranes were then developed using chemiluminescent substrates (BioRad and Thermo) and the ImageQuant LAS 4000 detection system (GE Life Sciences). Densitometry on Western blot images were subsequently analyzed using ImageQuant TL 8.1 software (GE Life Sciences).

Dot blot analysis

Dot blot analysis for proteins and oligomeric structures were performed as previously described [14,24]. Briefly, samples were prepared first by adding 10 µL of 1x TNE buffer (consisting of Tris-HCL, 50 mM; NaCl, 150 mM; and EDTA, 5mM) with protease and phosphatase inhibitors per 1 mg of tissue and mechanically homogenized. Samples were then centrifuged at 100,000 xg for 10 minutes. The supernatant was then collected and immunodepleted using Protein A and Protein G Mag Sepharose Xtra beads (MilliporeSigma; St. Louis, MO; USA). The pellet was reconstituted in 1x TNE + 1% TritonX-100 (TX-100) with protease and phosphatase inhibitors, sonicated (10 s ON, 2 s OFF, 20% amplitude) for 2 cycles, then centrifuged for 20 minutes at 16,000 xg at 4°C and supernatant separated. The TX-100 insoluble fraction was resuspended in 1x TNE with protease and phosphatase inhibitors. The samples were then diluted to 1 µg/µl (0.5 µg/µl for insoluble) and 2.5 µl droplets were deposited onto a nitrocellulose

membrane and allowed to dry for 30 minutes. The membrane was activated in transfer buffer with 10% methanol, microwaved in 50 mL PBS for 25 s, let sit for 3 minutes, followed by another 15 s in the microwave. The membrane was allowed to cool prior to blocking with tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) for 30 minutes immediately prior to overnight incubation with the primary antibody at 4°C. The membranes were then washed and incubated for 1 hour using respective IRDye secondaries and imaged using a Li-Cor Odyssey CLx (LiCor Biosciences; Lincoln, NE, USA)

Primary neuron dissection and culture

Mouse pups between postnatal day 0-2 (P0-2) were used for the isolation of primary hippocampal neurons. The hippocampus was removed and placed in cold Hibernate-A medium (BrainBits LLC; Springfield, Illinois, USA). Hippocampi were then transferred to a digestion medium containing Hibernate-A -$CaCl_2$ (BrainBits LCC), papain, L-Cysteine, and EDTA for 15 minutes at 37°C with occasional gentle shaking. The hippocampi were gently washed in a digestion inhibitor solution and subsequently triturated. The cells were plated in a plating medium at either a density of 650,000 or 250,000 cells per well of a 12-well culture dish (for protein or immunocytochemical analysis respectively) and replaced with NbActiv4 (BrainBits LCC) +FdU mitotic inhibitor the following day.

Immunocytochemistry

Cells were briefly washed with cold PBS followed by the addition of cold 4% PFA and incubated at room temperature for 30 minutes. The cells were then rinsed with PBS five times for five minutes per wash and then blocked using 50% Sniper (Biocare Medical; Pacheco, California, USA) for 15 minutes at room temperature (RT). The cells were then rinsed with PBS twice for five minutes followed by incubation with the primary antibody in 5% Sniper, 0.1% TritonX-100 in PBS overnight at 4°C. Cells were then rinsed three times for five minutes followed by incubation with the secondary antibody in 5% Sniper, 0.1% TX-100 in PBS for 2 hours at RT. Cells were incubated with DAPI prior to cover slipping in Antifade Vectashield (Vector Laboratories; Burlingame, California, USA).

Statistical analysis

All statistical analyses were performed in Prism 9.0.2 (GraphPad Software; San Diego, California, USA). Results are expressed as mean ± SEM (standard error of the mean). Student t-test was used in the comparison of only two groups. When comparing one variable across more than two groups, one-way ANOVA was performed. Two-way ANOVA was performed when comparing two variables on multiple groups. Tukey's posthoc analysis was used in multiple comparisons. For nonparametric data, Kruskal-Wallis one-way ANOVA was used when analyzing more than two groups. Statistical significance was set for $\alpha = 0.05$. Data representations are described in figure legends.

Results

Loss of tau delays onset of motor symptoms after PFF inoculation

We showed that tau is required for synaptic and memory deficits caused by A53T mutant human α-synuclein (HuαS^{A53T}) expression in primary neurons and in the TgA53T model [4,5]. While tau expression did not impact αS-dependent motor abnormalities in presymptomatic mice, studies have proposed that a pathological relationship exists between tau and αS. Interestingly, recent studies show that while αS promotes pathological spreading of tau in brain [6], tau did not promote spreading of αS pathology [6,11]. Therefore, we aimed to further evaluate the role of tau in onset and progression of α-synucleinopathy derived from HuαS.

In order to induce αS pathology in a temporally regulated manner, we used intramuscular (i.m.) injections of wild-type (WT) HuαS PFF into our TgA53T mouse model [12], as well as TgA53T mice crossed to a background lacking endogenous mTau$^{-/-}$ expression (TgA53T/mTau$^{-/-}$) and genotypic controls (mTau$^{-/-}$ and nTg; Fig. 1a). Following PFF inoculations, tissue samples for histology and biochemical analyses were collected at 40- and 70-days post inoculation (dpi), as well as when the mice reached disease end stage (classified as ataxic deterioration to the point of complete hindlimb paralysis preventing ambulation; Fig. 1a). In addition, prior to 70 dpi, mice underwent a battery of behavioral tests: pole, rotarod, and open field testing.

All PFF-inoculated TgA53T animals in wildtype background developed motor symptoms and reached end stage by ~100 dpi. Significantly, the average time to reach ataxic onset and end stage was significantly delayed in TgA53T/mTau$^{-/-}$ compared to TgA53T (p=0.0122 and p=0.0022 respectively; Fig. 1b, c). Moreover, the progression from initial onset of motor symptoms to end stage was also significantly prolonged in TgA53T/mTau$^{-/-}$ compared to TgA53T (p=0.0247; Fig. 1d), indicating that the loss of tau delayed the progression of α-synucleinopathy-associated neurodegeneration.

Consistent with the delay in disease progression associated with the loss of endogenous mTau expression, behavioral analysis at 70 dpi also showed loss of tau significantly attenuates HuαS^{A53T}-mediated behavioral abnormalities in TgA53T mice. The rotarod test of motor coordination showed that while TgA53T mice had a significantly decreased latency to fall compared to nTg and mTau$^{-/-}$ controls, TgA53T/mTau$^{-/-}$ mice had a similar latency to fall as control mice (Fig. 1e). With pole test performance, an additional indicator of motor coordination, TgA53T mice required significantly more time to perform the test than the control mice, however TgA53T/mTau$^{-/-}$ mice were not significantly different from the controls (Fig. 1f, g). As previously documented [4], both TgA53T and TgA53T/mTau$^{-/-}$ groups exhibit similar hyperactivity in open field test (Additional file 1: Fig. S1a). Interestingly, while the TgA53T group presented increased anxiety-associated behavior as they spent less time in the center, such behavior was not observed in TgA53T/mTau$^{-/-}$ mice (Additional file 1: Fig. S1b, c). Collectively, these results show that the loss of tau expression delays onset and progression of overt disease as well as intermediate behavioral deficits in the TgA53T model of α-synucleinopathy.

Loss of tau expression does not impact pS129 αS pathology in end stage TgA53T mice but leads to modest reduction in pS129 αS accumulation in presymptomatic TgA53T mice

The delay in disease/ataxic onset, as well as behavioral deficits, in the TgA53T/mTau$^{-/-}$ mice compared with TgA53T mice seems to contradict prior studies using mouse αS PFF inoculation model [6,11]. Thus, we asked whether lack of tau expression impacts subcortical α-synucleinopathy in TgA53T model.

To survey if the expression of tau was correlated with bulk changes in αS pathology, we performed biochemical analysis of lysates from spinal cord and brain stem regions that are prone to develop αS pathology in the TgA53T model [3,15]. Immunoblot analysis of spinal cord from end stage mice show similar levels of full length αS compared to age-matched controls (Fig. 2a) and a prominent increase in the phospho-serine129 αS (pS129 αS), a marker of pathological αS, in both TgA53T and TgA53T/mTau$^{-/-}$ mice (Fig. 2b**)**. Further, consistent with a prior study [6], no difference in the levels of pS129 αS/total αS was observed between TgA53T and TgA53T/mTau$^{-/-}$ mice (Fig. 2c, d). To determine if there was a difference in the aggregation of αS that is not fully reflected by total pS129 αS levels, we examined Triton X-100 detergent-soluble and insoluble fractions from the spinal cord. Our results show that while PFF inoculation increases the amount of insoluble αS, expression of tau does not significantly impact the levels of insoluble αS but there was slightly increased αS in the soluble fraction (Additional file 1: Fig. S2). Moreover, tau expression did not impact the levels of SDS-stable oligomers that resolve at ~25 kDa, ~37 KDa, and > 200 kDa (Additional file 1: Fig. S2). Analysis of brainstem region showed that PFF-inoculation leads to similar increases in pS129 αS levels in both TgA53T and TgA53T/mTau$^{-/-}$ mice, similar to the patterns observed in the spinal cord (Additional file 1: Fig. S3).

We performed histological analysis for αS pathology (pS129 αS) to determine if the pattern of αS pathology was influenced or mediated by tau expression. The immunostained spinal cord sections were used to determine the percent (%) area covered by immunoreactivity. Consistent with the bulk biochemical analysis, our results show that both TgA53T and TgA53T/mTau$^{-/-}$ mice develop similar levels of pS129 αS pathology at end stage while the control animals (nTg and mTau$^{-/-}$ alone) do not exhibit pS129 αS staining (Fig. 3a-e). In addition, we also examined the microglia and astrocyte activation via Iba1 and GFAP immunostaining, respectively. In end stage animals, quantitative analysis of microglial activation (Iba1) showed significant activation with αS pathology but did not reveal any differences as a function of tau expression (Fig. 3f-j). Similarly, while the increase in GFAP staining with αS pathology was also significant compared to nTg and mTau$^{-/-}$ controls, there were no differences as a function of tau expression (Fig. 3k-o**;** see Additional file 1: Fig. S4 for representative low magnification images). In addition, pS129 αS, Iba1, and GFAP were elevated in end stage brainstem, cerebellum, and motor cortex regions, but not hippocampus, yet were not qualitatively different between TgA53T and TgA53T/mTau$^{-/-}$ mice. (Additional file 1: Fig. S5).

Because end stage of disease is reached at a later dpi in TgA53T/mTau$^{-/-}$ mice, it is possible that αS pathology was normalized between groups at terminal stages. Thus, we evaluated tissues at the same

intermediate stage following PFF inoculation (70 dpi and 40 dpi). Similar to end stage analysis, immunoblots of spinal cord and brain stem lysates failed to show any differences in pS129 αS levels (Fig. 4 and Additional file 1: Fig. S3) or insoluble αS as a function of tau expression (Additional file 1: Fig. S6). Moreover, tau expression did not impact the levels of SDS-stable αS oligomers (Additional file 1: Fig. S6). Biochemical analysis of 40 dpi mice show lack of pS129 αS accumulation (Additional file 1: Fig. S7a, b).

We also performed histological analysis of pS129 αS and neuroinflammation as we have done for the end stage subjects above. Significantly, despite the lack of differences in bulk immunoblot analysis of total pS129 αS levels (Fig. 4), quantitative analysis of the pS129 αS staining in the spinal cord reveals a modest decrease in pS129 αS pathology in TgA53T/mTau$^{-/-}$ compared to TgA53T mice (Fig. 5a-e). This suggests that while the overall abundance of pS129 αS along the spinal cord is unchanged, there is a specific delay in the accumulation of pS129 αS in the grey matter of the cord. However, immunohistochemistry may not be sensitive enough to identify smaller changes in aggregates and levels of pS129 αS in white matter and in non-neuronal cells [25]. As such, it is possible that the loss of tau expression leads to smaller pS129 αS structures that are not readily visible by light microscopy or slow accumulation of pS129 αS in larger neurons.

The analysis of microglial (Fig. 5f-j) and astrocytic (Fig. 5k-o) activation in presymptomatic animals at 70 dpi show a significant increase in microglial activation in mice with αS pathology. Consistent with the reduced αS pathology in TgA53T/mTau$^{-/-}$ animals, the activation status of microglia and astrocytes is reduced in these mice compared to TgA53T mice (Fig. 5j and o; see Additional file 1: Fig. S8 for representative low magnification images). In the brainstem, pS129 αS, Iba1, and GFAP were modestly increased at 70 dpi in TgA53T and TgA53T/mTau$^{-/-}$ mice. The overall abundance was qualitatively similar to the quantitative results seen with spinal cord sections (Additional file 1: Fig. S9). In addition, histological analysis of 40 dpi mice shows initial onset of pS129 αS was not altered by tau loss, nor was there profound inflammatory activation (Additional file 1: Fig. S7c-i).

Loss of tau expression leads to reduced neurodegeneration in presymptomatic TgA53T mice but not in end stage mice

Thus far, our results indicate that while tau is associated with modest increases in early αS pathology, tau does not seem to impact the extent of αS pathology at end stage. However, it is possible that tau might be acting downstream of αS abnormalities [4,5]. Because loss of motor neurons is a robust neurodegenerative phenotype in the TgA53T model [16], we examined if tau expression affects the loss of motor neurons in the spinal cord of TgA53T mice.

Our analysis of end stage mice following PFF inoculation show that, as with the normal aging model, presence of αS pathology and limb paralysis in the TgA53T mice was accompanied by severe loss of motor neurons in the ventral horn (Fig. 6a-e). Further, the mean motor neurons per lumbar spinal cord section in TgA53T/mTau$^{-/-}$ mice were not different from TgA53T mice. This was further demonstrated

with loss of total NeuN+ content of the ventral horn (Fig. 6f), while dorsal horn neurons were left intact (Fig. 6g). Significantly, analysis of presymptomatic animals at 70 dpi show that the early presence of αS pathology in TgA53T mice was already associated with a significant loss of ventral horn motor neurons in the lumbar spinal cord, albeit less severe than in the end stage animals (Fig. 6h-l). More important, the loss of motor neurons was significantly attenuated in the TgA53T/mTau$^{-/-}$ mice, despite the significant presence of αS pathology, compared to the TgA53T mice (Fig. 6l; see Additional file 1: Fig. S10 for representative low magnification images). In addition, this protection was also observed after quantification of ventral horn neurons via NeuN+ staining (Fig. 6m), while dorsal horn neurons were not affected (Fig. 6n). This suggests that while there may be a mild delay of αS pathological progression as a function of tau expression, a robust delay in neuronal toxicity may be a more likely explanation for the observed delay in disease onset and behavioral deficits observed in the TgA53T/mTau$^{-/-}$ mice.

Loss of tau does not impact soluble α-synuclein oligomer formation or GSK3b activity

Using a different HuαS^{A53T}-expressing transgenic mouse line (M83), it has been previously demonstrated that TgA53T (M83) mice accumulated soluble tau oligomers with aging, and the mice treated with tau-oligomer specific antibody (TOMA) can subsequently reduce αS oligomers and aggregates [10]. Therefore, while the loss of tau expression does not alter overt αS aggregation or SDS-stable oligomers (Fig. 2, Additional file 1: Fig. S2 & S6), we tested if the loss of tau expression reduces the levels of soluble αS oligomers in our TgA53T model using the Syn33 antibody [4,10,24] with non-denaturing dot blot analysis.

Dot blot analysis of buffer soluble fractions from spinal cords of both end stage and 70 dpi spinal cord lysates from TgA53T animals reveals higher levels of αS oligomers as a function of HuαS^{A53T} expression compared to nTg and mTau$^{-/-}$ controls. Further, the accumulation of Syn33+ oligomeric species were comparable between TgA53T and TgA53T/mTau$^{-/-}$ groups (Fig. 7a, b). Consistent with specificity of Syn33 to soluble oligomers, Syn33 did not react to the detergent insoluble fractions (Fig. 7c, d). These results show that tau expression does not impact the levels of soluble oligomers recognized by Syn33 in our TgA53T model. Similar results were seen in our analysis of cortical and hippocampal tissues [4].

We also examined whether the αS pathology in the TgA53T mouse model is associated with obvious increases in pathological tau. However, our analysis for AT8-positive tau shows that, even at end stage, accumulation of hyperphosphorylated tau was not detected (Additional file 1: Fig. S11g). Increased activation of glycogen synthase kinase 3b (GSK3b) has been proposed as a mediator of αS-induced neuronal dysfunction [5,7,26,27]. Thus, we also examined spinal cord lysates for the levels of active GSK3b, as measured by phosphorylated Tyr-216 (pY216) [28]. Our results show that neither total GSK3b levels nor pY216-GSK3b activation are increased as a function of αS pathology, nor impacted by tau expression (Additional file 1: Fig. S11a-f).

Endoplasmic reticular stress and autophagy pathway protein clearance

We previously showed that α-synucleinopathy in TgA53T model was associated with chronic endoplasmic reticulum stress (ERS) [14] and dysfunction in autophagy-lysosomal pathways (ALP) [15]. Because both ERS and ALP deficits follow the onset of αS pathology, we examined whether ERS and ALP in TgA53T model are affected in a tau-dependent manner. We performed biochemical (Western blot) analysis in 70 dpi and end stage spinal cord lysates for markers of ERS and ALP (Additional file 1: Fig. S12**)**. While there was no obvious indication of ALP abnormalities at 70 dpi (Additional file 1: Fig. S12a), analysis of spinal cord lysates from end stage TgA53T mice show expected ALP abnormalities (Additional file 1: Fig. S12b). ALP markers (LC3 II/I ratio, P62, and pAMPK/AMPK ratio) in TgA53T or TgA53T/mTau$^{-/-}$ were not different from each other.

Analysis of ERS markers, Grp78 and p-eIF2α/eIF2α ratio, showed expected signs of chronic ERS in end stage TgA53T and TgA53T/mTau$^{-/-}$ mice (Additional file 1: Fig. S12b). While the levels of Grp78 was similarly increased in both TgA53T and TgA53T/mTau$^{-/-}$ mice, TgA53T/mTau$^{-/-}$ had modest but significantly reduced p-eIF2α/eIF2α ratio compared to TgA53T. No signs of ERS were seen in lysates from 70 dpi subjects (Additional file 1: Fig. S12a). Similar results for ALP and ERS markers were observed in brainstem lysates from 70 dpi and end stage animals (Additional file 1: Fig. S13). There were no signs of changes in ERS or ALP in the brainstem of 70 dpi animals (Additional file 1: Fig. S13a). However, in the brainstem, there was elevated ALP markers at end stage, but no sign of ERS in end stage subjects (Additional file 1: Fig. S13b).

Collectively, our results show that both ERS and ALP deficits associated with α-synucleinopathy in the TgA53T mouse model occurs after the αS pathology is well developed. Further, unlike the loss of motor neurons (Fig. 6) both ERS and ALP deficits seem to be independent of tau expression.

Tau expression does not affect αS PFF uptake or processing in neurons but prevents PFF induced neurotoxicity

Our *in vivo* studies using TgA53T model show that the loss of tau expression leads to a delay in αS aggregation, inflammation, and neurodegeneration. However, it is unknown if the loss of tau expression affects early processes related to the development of αS pathology, or if it makes neurons more resistant to αS pathology-induced toxic effects allowing for more efficient processing and clearance of abnormal αS. As such, we next wanted to determine whether the cellular effects of tau on the initiation of αS pathology and αS-dependent neurodegeneration is cell autonomous. To address this, we exposed mouse primary neuronal cultures to pathological αS PFF in presence and absence of tau expression.

To determine whether tau mediates the initiation and progression of intraneuronal αS pathology following PFF exposure, we examined the neuronal uptake of WT αS PFF in primary hippocampal neurons cultured from nTg and mTau$^{-/-}$ mice. Cultured neurons were exposed to αS PFF for 2 hours. After this 2-hour incubation, PFF-containing media was removed, neurons were washed with PFF-free media to remove any extracellular PFF, and then fresh PFF-free media was added. Neuronal lysates were then collected at 0, 3, 6, 16, 24 and 48 hours following the 2-hour incubation with αS PFFs. As expected,

neurons rapidly internalize αS PFF and internalized αS accumulates as a truncated protein [29] (Additional file 1: Fig. S14a, b). Quantitative analysis of uptake, truncation, and clearance of αS PFF shows that both nTg and mTau$^{-/-}$ neurons metabolize exogenous PFF almost identically (Additional file 1: Fig. S14a, b).

We next investigated whether tau expression affects αS aggregation and neuronal survival following αS PFF exposure. Primary nTg mouse hippocampal neurons were exposed to αS PFF and evaluated for the presence of pathological pS129 αS and neurodegeneration 14 days post-PFF exposure *in vitro*. αS PFF applied to primary hippocampal neurons *in vitro* led to a dose-dependent increase in pS129 αS accumulation at 14 days post-PFF in the absence of significant NeuN+ neuronal loss (Additional file 1: Fig. S14c-e). To determine if tau expression modulates PFF-induced αS aggregation in neurons, we examined the levels of pS129 αS in cultured nTg and mTau$^{-/-}$ cultures exposed to PFF treatment. To account for possible differences in the density of neurons and/or neurites between the cultures, the area of pS129 αS staining was normalized to the MAP2 stained area. These results show there was no difference in pS129 αS accumulation between nTg and mTau$^{-/-}$ neurons at 14 days post-PFF (Fig. 8a-c). The addition of αS PFF did not induce neurodegeneration in culture but did induce progressive loss of dendritic arborization (Fig. 8d, f; Additional file 1: Fig. S15). Specifically, αS PFF-induced αS aggregation in nTg neurons leads to simplification of dendritic arborization, as indicated by reduced MAP2 stained area per NeuN+ cells at 14 days post-PFF (Fig. 8d, f; Additional file 1: Fig. S15). Significantly, in mTau$^{-/-}$ neurons, while αS PFF induced comparable levels of pS129 αS staining at 14 days post-PFF (Fig. 8a-c), the loss of MAP2 was prevented (Fig. 8e, f). These results show that the loss of mTau expression attenuates PFF-induced neuronal toxicity without affecting the neuronal accumulation of pathological αS.

Discussion

Through *in vitro* and *in vivo* studies examining the expression of endogenous mouse tau on αS pathology, and α-synucleinopathy pathophysiology, we unequivocally demonstrate that tau has a central role in α-synucleinopathy disease progression. Previously, we showed that mutant αS causes synaptic and memory deficits in a tau-dependent manner [4,5]. Here, we extend our previous studies by showing that onset of motor dysfunction and reduced life span of TgA53T mice is attenuated by the loss of endogenous tau expression. While the removal of tau significantly delayed the onset of disease and disease progression to end stage, we did not observe any differences in αS aggregation and neuropathology in early stage (40 dpi) and end stage mice. However, analysis of TgA53T mice at an intermediate time point (70 dpi) revealed that while tau expression did not impact the amount of αS aggregates, loss of tau is associated with reduced neuropathology, including reduced motor neuron degeneration. This difference in αS-associated neuropathology as a function of tau expression is consistent with the fact that the lack of tau expression reverses several motor deficits associated with our TgA53T model. Considering the delay in disease onset in TgA53T/mTau$^{-/-}$ mice, it is likely that overall neuropathology was normalized upon reaching end stage between TgA53T and TgA53T/mTau$^{-/-}$ mice.

Thus, analysis of all groups at the same time point following initial αS PFF intramuscular inoculation revealed the temporal difference in the onset and progression of neuropathology and neurodegeneration.

While correlative studies with the TgA53T model establishes that tau expression is a significant pathological contributor to α-synucleinopathy *in vivo*, the overall onset and progression of α-synucleinopathy likely involves complex interactions between different cell types as well as the homeostatic condition of the neuron. Thus, while we cannot conclusively determine if tau expression directly impacts αS pathology or if tau acts downstream of αS pathology *in vivo*, our studies using αS PFF-treated primary hippocampal neurons show that tau expression does not impact PFF-mediated accumulation of pathological αS species including pS129 αS. However, the lack of tau expression was able to completely reverse the neurotoxic effects of PFF-induced pS129 αS in neurons *in vitro*. Thus, we conclude that tau is important for mediating the neurotoxic effects associated with αS pathology without directly affecting the generation of αS pathology.

Currently, there is some controversy regarding the role of tau in α-synucleinopathy. Our conclusion that tau does not impact onset and progression of αS pathology is supported by recent studies using αS PFF inoculation mouse models [6,11]. In these studies, no differences in αS pathology were seen following hippocampal [6] or intrastriatal [11] injections of mouse αS PFF to nTg and mTau$^{-/-}$ mice. However, these studies did not determine if the behavioral or neurodegeneration was attenuated in mTau$^{-/-}$ mice [6] or only examined loss of nigral neurons at end stage [11]. In our study, the lack of tau-dependent pathological differences at 40 dpi as well as at end stage, but significant delay in neurodegeneration and pathological markers at 70 dpi suggests that tau may act downstream of overt αS pathology affecting neuronal survival. In contrast, another recent study presented evidence that removal of toxic tau oligomers *in vivo* could reduce αS pathology and insoluble αS oligomers in the M83 TgA53T model, indicating to reciprocal pathological interactions between αS pathology and tau [10]. Specifically, the hypothesis is that αS pathology induces tau oligomers, which in turn, promotes αS pathology and neurological abnormalities. However, our analysis show that absence of tau expression did not reduce the abundance of soluble αS oligomers detected by Syn33 (off-pathway) or SDS-stable high molecular weight αS oligomers. Based on our results, we favor the notion that the endogenous tau expression does not directly impact αS oligomer levels or pathology. Discrepancy in the tau dependency of αS oligomers seen here and the prior study [10] could be the possibility that in M83 TgA53T mice, in contrast to G2-3 TgA53T mice used in these studies, abnormal tau accumulates as a result of mixed background strain linked to this line or differences in housing conditions.

Our lab has previously demonstrated that HuαS^{A53T} overexpression leads to tau-dependent post-synaptic deficits through GSK3β-dependent phosphorylation of tau leading to mislocalization of tau and increased AMPA receptor internalization [5]. Here, we demonstrated that exogenous αS PFF leads to dendritic simplification in nTg neurons, as well as motor neuron degeneration in TgA53T mice, that is protected and delayed respectively, with tau loss. However, given that removing tau expression does not impact levels of total or active GSK3β, the tau-dependent neuroprotection seen here does not require alterations in the state of GSK3β activation.

Conclusions

The current study represents a significant and important development in the relationship between αS and tau-dependent disease progression. We provide evidence that PFF-induced disease progression can be delayed with the removal of tau. In addition, while tau removal led to modest reductions in αS-mediated pathological changes *in vivo*, it did lead to significant improvements in spinal motor neuron survival as well as morphological markers *in vitro*.

Collectively, our data suggests that tau may act downstream of αS pathology to affect neuronal homeostasis and survival in α-synucleinopathies. These results support the use of therapeutic strategies in the treatment of α-synucleinopathies that aim to reduce overall tau expression levels, including the current Phase I clinical trial by Biogen/Ionis utilizing antisense oligonucleotides to directly impact tau expression levels. Taken together, this work provides important insight into the understanding of tau-dependent mechanisms in neurodegenerative diseases, critical to the advancement of much needed disease-modifying therapies.

Abbreviations

αS: α-synuclein; HuαS: human αS; TgA53T: transgenic A53T mutant synuclein; PFF: preformed fibril; nTg: non-transgenic; dpi: days post inoculation; PD: Parkinson's disease; SNpc: substantia nigra pars compacta; LB: Lewy body; LN: Lewy neurite; TOMA: tau oligomer specific antibody; $mTau^{-/-}$: mouse tau knockout; PBS: phosphate buffered saline; PFA: paraformaldehyde; TX-100: triton-100; TBS: tris-buffered saline; BSA: bovine serum albumin; RT: room temperature; HuαSA53T: human α-synuclein A53T; WT: wild type; pS129 αS: phosphorylated serine 129 α-synuclein; GSK3b: glycogen synthase kinase 3b; pY216: phosphorylated tyrosine 216; ERS: endoplasmic reticulum stress; ALP: autophagy lysosome pathway; IHC: immunohistochemistry; BC: biochemistry; s: seconds; A.M.: age-matched; A.U.: arbitrary units; ponc: ponceau S; ES: end stage; V. Horn: ventral horn; D. Horn: dorsal horn; SEM: standard error of the mean; HMW: high molecular weight; i.m.: intramuscular; FL: full length; trunc.: truncated

Declarations

Ethics approval

All experimental protocols involving mice were in strict adherence to the NIH Animal Care and Guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota. All applicable ethical standards required by University of Minnesota IACUC was followed. All authors declare no conflict of interest.

Consent for publication

All authors discussed and approved the results presented in the manuscript.

Availability of data and materials

Any additional data and materials are available from corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

SCV and MKL conceived the project and designed the experiments. BS and HMM performed animal husbandry, intramuscular injections, and disease staging for end stage mice. HMM and SCV performed intramuscular injections and behavioral analyses for 70 dpi mice. RK performed western blot and analyzed autophagy and ER stress pathways while SCV and AC performed all other western blot analyses. JM performed all immunohistochemical staining and JM and SCV performed histological analyses. AC performed dot blot analyses. SCV and AC cultured primary neurons and performed biochemical analysis, as well as immunocytochemical staining and analysis. MKL supervised the experiments. SCV and MKL generated the figures and wrote the manuscript. All authors discussed and approved the results presented in the manuscript.

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Figures

Figure 1

Loss of tau in TgA53T mice delays disease onset and progression following αS PFF inoculation. a Schematic of study paradigm, including genotypic groups, collection time points, behavior, and disease staging analysis. **b, c** Kaplan-Meier plots of all groups showing disease onset (onset of motor abnormalities) (**b**; $p = 0.0122$; Log-rank (Mantel Cox) test) and time to reach end stage (hind limb paralysis) (**c**; $p = 0.0022$; Log-rank (Mantel Cox) test). Both onset and time to end stage was delayed in TgA53T mice lacking tau (TgA53T/mTau$^{-/-}$). **d** Average duration from disease onset to end stage was also significantly extended with loss of tau ($p = 0.0247$; *t*-test). **e** Performance on rotarod at 70 dpi show that TgA53T mice exhibit decreased latency to fall compared to TgA53T/mTau$^{-/-}$ subjects. One-way ANOVA with Tukey's posthoc analysis; Day 3: $F_{(3,18)} = 4.925$, $p = 0.0114$; Day 4: $F_{(3,18)} = 3.150$, $p = 0.0505$. **f, g** TgA53T mice required more time to descend the pole (**f**) and perform/complete the pole test (**g**) compared to nTg and mTau$^{-/-}$ controls respectively. The performance of the TgA53T/mTau$^{-/-}$ mice were not significantly different from nTg and mTau$^{-/-}$ controls, indicating that loss of tau expression attenuates motor dysfunction. Abbreviations: preformed fibril, PFF; days post inoculation, dpi; immunohistochemistry, IHC; biochemistry, BC; seconds, s; One-way ANOVA with Tukey's posthoc analysis; time to descend: $F_{(3,20)} = 4.186$, $p = 0.0188$; time to perform: $F_{(3,20)} = 4.220$; $p = 0.0182$. *p<0.05 and **p<0.01; error bars represent mean ± SEM.

Figure 2

Biochemical abundance of pS129 αS in spinal cord not affected by tau expression in end stage TgA53T mice. a Representative immunoblots of total αS (**a**) and pS129 αS (**b**) in spinal cord of end stage mice. **c, d** Quantitation of immunoblots shown in **a** and **b**. The levels of pS129 αS was normalized to total αS. The levels of total αS and pS129 αS/total αS was not different between TgA53T and TgA53T/mTau$^{-/-}$ mice in end stage spinal cord lysates (*t*-test: $p = 0.0518$ and $p = 0.4267$, respectively). Abbreviations: age-matched, A.M.; arbitrary units, A.U.; ponceau S, ponc; N = 5 animals/genotype; error bars represent mean ± SEM

Figure 3

Loss of tau does not affect end stage neuropathology in TgA53T mice. Spinal cord sections from end stage nTg, mTau$^{-/-}$, TgA53T and TgA53T/mTau$^{-/-}$ mice were stained for pS129 αS (**a-e**), Iba1 (microglia) (**f-j**), and GFAP (astrocytes) (**k-o**). Shown are representative images and corresponding quantitative analysis of % area covered by immunoreactivity. In both TgA53T and TgA53T/mTau$^{-/-}$ mice the indices of

neuropathology were significantly increased to a similar extent. One-way ANOVA with Tukey's posthoc analysis. pS129 αS: $F_{(3,40)} = 30.64$; $p < 0.0001$. Iba1: $F_{(3,40)} = 54.81$; $p < 0.0001$. GFAP: $F_{(3,20)} = 88.16$; $p < 0.0001$. N = 2-3, 3-5 (sections, animals/genotype); scale bars = 100 mm; error bars represent mean ± SEM

Figure 4

Abundance of pS129 αS in spinal cord is not affected by tau expression in TgA53T mice at 70 dpi. a Representative immunoblots of total αS (**a**) and pS129 αS (**b**) in spinal cord of 70 dpi mice. **c, d** Quantitation of immunoblots shown in **a** and **b**. Total αS (**c**), and pS129 αS normalized to total αS (**d**) was not different between TgA53T and TgA53T/mTau$^{-/-}$ mice in 70 dpi spinal cord lysates (*t*-test, $p = 0.1575$ and $p = 0.7993$, respectively). Abbreviations: ponceau S, ponc; arbitrary units, A.U.; N = 4 animals/genotype; error bars represent mean ± SEM

Figure 5

Loss of tau attenuates intermediate neuropathology in TgA53T mice. Spinal cord sections from 70 dpi nTg, mTau$^{-/-}$, TgA53T, and TgA53T/mTau$^{-/-}$ mice were stained for pS129 αS (**a-e**), Iba1 (**f-j**), and GFAP (**k-o**). Shown are representative images and corresponding quantitative analysis of % area covered by immunoreactivity. The indices of neuropathology were all significantly reduced in TgA53T/mTau$^{-/-}$ compared to TgA53T mice at 70 dpi. One-way ANOVA with Tukey's posthoc analysis. pS129 αS: $F_{(3,23)} = 82.46$; $p < 0.0001$. Iba1: $F_{(3,26)} = 25.79$; $p < 0.0001$. GFAP: $F_{(3,18)} = 69.86$; $p < 0.0001$. N = 2-3, 3 (sections, animals/genotype); scale bars = 100 mm; error bars represent mean ± SEM

Figure 6

Loss of tau delays α-synucleinopathy-associated spinal motor neuron degeneration in TgA53T mice. Spinal cord sections from end stage (**a-g**) and 70 dpi (**h-n**) TgA53T mice and controls (nTg, mTau$^{-/-}$) were stained for NeuN (neuronal marker). Shown are representative images of lumbar ventral horn region. In end stage mice (**a-g**), TgA53T and TgA53T/mTau$^{-/-}$ mice show significant loss of ventral horn (V. Horn) motor neurons (**a-e**; $F_{(3,26)} = 82.45$, $p < 0.0001$). NeuN quantification further demonstrates significant neurodegeneration within the ventral horn (**f**; $F_{(3,20)} = 21.75$, $p < 0.0001$), but not dorsal horn (D. Horn) (**g**;

$F_{(3,20)}$ = 1.257; p = 0.3160). At 70 dpi (**h-n**), ventral horn motor neuron degeneration (**l**; $F_{(3,20)}$ = 14.15, p < 0.0001) as well as ventral NeuN+ neuronal loss (**m** - Ventral: $F_{(3,20)}$ = 6.490, p = 0.0030; **n** - Dorsal: $F_{(3,20)}$ = 0.4447, p = 0.7237) is delayed with tau loss. Abbreviation: days post inoculation, dpi; One-way ANOVA with Tukey's posthoc analysis. N = 2, 3-5 (sections, animals/genotype); scale bars = 100 mm; error bars represent mean ± SEM

Figure 7

Soluble αS oligomers detected by Syn33 are not affected by tau expression. a Dot blot detection of human αS (LB509), αS off-pathway oligomers (Syn33), and total αS (4D6) in buffer-soluble spinal cord fractions of 70 dpi and end stage mice. **b** No differences in LB509 or Syn33 were observed as a result of tau expression. **c & d** Syn33+ αS oligomers were not detected in the detergent-insoluble spinal cord fractions of both 70 dpi and end stage mice. Abbreviations: days post inoculation, dpi; end stage, ES; arbitrary units, A.U.; error bars represent mean ± SEM

Figure 8

Neurons lacking tau expression are protected from αS PFF-induced loss of dendrites without impacting the development of pS129 αS+ aggregates. a, b Primary mouse hippocampal neurons from nTg (**a**) and $mTau^{-/-}$ (**b**) were induced to develop αS pathology by αS PFF treatment. Same levels of pS129 αS+ aggregates are seen in nTg (**c**; p = 0.0001, PBS vs PFF) and $mTau^{-/-}$ (**c**; p = 0.0001, PBS vs PFF) neurons 14 days after PFF treatment. Analysis of MAP2+ neurites/dendrites at 14 days post-PBS or αS PFF addition to nTg (**d**) and $mTau^{-/-}$ (**e**) neurons. αS PFF treatment led to significant loss of MAP2 area in nTg neurons (**d, f**; p = 0.0003) but not $mTau^{-/-}$ primary hippocampal neurons (**e, f**; p = 0.4472), compared to PBS controls. Abbreviations: phosphate buffered saline, PBS; preformed fibril, PFF; Two-way ANOVA with Sidak's multiple comparisons test; ***p < 0.001. Scale bars = 100 mm; error bars represent mean ± SEM

Supplementary Files

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