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Studies of protein aggregation in A53T α-synuclein transgenic, Tg2576 transgenic, and P246L presenilin-1 knock-in cross bred mice

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ABSTRACT

Synucleinopathies are a group of neurodegenerative disorders, including Parkinson disease, associated with neuronal amyloid inclusions comprised of the presynaptic protein α-synuclein (α-syn); however the biological events that initiate and lead to the formation of these inclusions are still poorly understood. There is mounting evidence that intracellular α-syn aggregation may proceed via a seeding mechanism and could spread between neurons through a prion-like mechanism that may involve other amyloidogenic proteins. Several lines of evidence suggest that Aβ peptides and/or extracellular Aβ deposits may directly or indirectly promote intracellular α-syn aggregation. To assess the effects of Aβ peptides and extracellular Aβ deposits on α-syn aggregate formation, transgenic mice (line M83) expressing A53T human α-syn that are sensitive to developing α-syn pathological inclusions were cross bred to Tg2576 transgenic mice that generated elevated levels of Aβ peptides and develop abundant Aβ plaques. In addition these mice were bred to mice with the P264L presenilin-1 knock-in mutation that further promotes Aβ plaque formation. These mice demonstrated the expected formation of Aβ plaques; however despite the accumulation of hyperphosphorylated α-syn dystrophic neurites within or surrounding Aβ plaques, no additional α-syn pathologies were observed. These studies show that Aβ amyloid deposits can cause the local aggregation of α-syn, but these did not lead to more extensive α-syn pathology.

Introduction

Synucleinopathies are a group of neurodegenerative diseases associated with neuronal, and in some cases oligodendritic, amyloid inclusions comprised of the presynaptic protein α-synuclein (α-syn) [11,20,47,51]. Parkinson disease (PD), the most common known synucleinopathy, is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and the formation of α-syn inclusions, known as Lewy bodies (LBs) and Lewy neurites (LNs), in some of the remaining dopaminergic neurons [12,20,51]. PD is a progressive movement disorder [15,45], however it is associated with a range of nonmotor symptoms [4,40] and many other affected neuronal populations outside of the substantia nigra contribute to the progression of disease [2,7,11,12].

Aβ senile deposits or plaques, which consist primarily of aggregated Aβ peptides that vary between 39 and 43 amino acids in length, are one of the hallmark lesions of Alzheimer disease [19,43,48,53]. Aβ peptides are secreted from cells following cleavage of the trans-membrane Aβ precursor protein (βAPP) at the Aβ N-terminal (β-secretase cleavage) and C-terminal (γ-secretase cleavage) [43,53]. Aβ deposits can be observed in several other neurodegenerative diseases, including dementia with Lewy bodies (DLB) and LB variant of Alzheimer disease (LBVAD), where concomitant α-syn intraneuronal inclusions are present [11,20,38,51].

The most direct and compelling evidence for a fundamental role of α-syn in the pathogenesis of synucleinopathies is the causal relationship between genetic mutations and disease [6,8,34,51]. The missense mutation (c.G209A) in the α-syn gene (*SNCA*) resulting in the amino acid substitution A53T was first identified in a large Italian family (Contursi) and three small Greek families with autosomal dominant PD [41], and this mutation enhances the propensity of α-syn to form amyloid [5,18]. However, the biological events that initiate and lead to the formation of α-syn inclusions are still poorly understood. Several lines of evidence suggest that extracellular Aβ deposits may directly or indirectly promote intracellular α-syn aggregation. Besides the frequent co-occurrence of α-syn inclusions and Aβ deposits in the brains of patients with PD, DLB or LBVAD [11,20,38,51], α-syn inclusions are commonly observed in patients with familial Alzheimer disease where genetic defects in the *APP*, *presenilin-1* (PS1) or *presenilin-2* (PS2) genes affect biological pathways that promote the formation of Aβ aggregates

Abbreviations: βAPP, Aβ precursor protein; DLB, Dementia with Lewy bodies; LBVAD, LB variant of Alzheimer disease; LB, Lewy body; LN, Lewy neurite; α-syn, α-Synuclein.

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[23,27,35,46,55]. PS1 and PS2 are enzymatic components of the transmembrane γ-secretase complex [29,39] that cleaves βAPP. Over 100 mutations in the *PS1* and *PS2* genes have been identified in familial Alzheimer disease and these mutations result in increased production of the longer Aβ 1–42(43) species [13,43,53]. Aβ 1–42(43) peptides have been shown to have a greater propensity to form amyloidogenic fibrils compared to the shorter Aβ 1–40 peptide [25]. In addition, Aβ 1–42 is deposited early and selectively in senile plaques [24], but the nature and mechanism of Aβ toxicity are still debated [1,3,30,42,53].

To investigate the possibility that Aβ peptides or amyloid plaques may promote/initiate the aggregation of α-syn, α-syn transgenic mice (line M83) expressing A53T human α-syn that are sensitive to developing α-syn pathological inclusions [16] were cross bred to the previously characterized transgenic mice that overexpress human βAPP (695 amino acid splice form) with the "Swedish" double mutation K670M/N671L (line Tg 2576) that develop abundant age-dependent Aβ plaques [22,28]. In addition these mice were bred to mice with the P264L PSI knock-in mutation that increase Aβ 1–42 production and further promote Aβ plaque formation [10,44].

Materials and methods

Antibodies. pSer129 is a mouse monoclonal antibody specific to α-syn phosphorylated at S129 [52]. Syn505 and Syn506 are conformational anti-α-syn mouse monoclonal antibodies that preferentially detect α-syn in pathological inclusions [50]. Syn 211 is a mouse monoclonal antibody specific for human α-syn [17]. Rabbit anti-Aβ antibody was purchased from Cell Signaling Technologies (Danver, MA). The anti-Aβ mouse monoclonal antibody 6E10 was purchased from Covance (Princeton, NJ). Karen, a goat polyclonal anti-N-terminal APP antibody and NAB228, a monoclonal antibody raised against Aβ1-11 synthetic peptide [32], were generous gifts from Dr. Virginia Lee. Affinity purified mouse anti-actin (clone C4) monoclonal antibody was purchased from Millipore (Billerica, MA).

Transgenic mice. The previously described M83 A53T human α-syn [16] and Tg2576 βAPP [22] transgenic mouse lines were used in these studies. In addition, P264L PS1 knock-in mice were previously described [10,44]. For genotyping, genomic DNA samples were isolated from mouse tails with proteinase K digestion followed by purification with the Wizard® SV Genomic DNA Purification System (Promega, Madison, WI). The α-syn and βAPP transgenes were screened by Southern blot analysis with ^{32}P-labeled oligonucleotide-primed specific DNA probes respectively, as previously described [16,22]. The presence of the P264L PSI mutation that results in a larger DNA product was screened by PCR using the forward primer GCTGGAGCAATGCTGTGTTA and the reverse primer GAGATGGCTTACGGGTTGAG [10].

Western blot analysis. Brain tissues were harvested and lysed in 3% SDS/50 mM Tris, pH 6.8 by sonication and heating to 100 °C for 10 min. Total protein extracts were quantified using the BCA assay using bovine serum albumin as the standard. Equal amounts of protein extracts (5 μg) were separated by electrophoresis onto 15% polyacrylamide gels for α-syn analysis or 8% polyacrylamide gels for βAPP analysis. Immunoblotting was performed as previously described [52].

Immunohistochemical analysis. Mice were sacrificed with CO_2 euthanization as approved by the University of Pennsylvania Institutional Animal Care and Use Committee and perfused with PBS/heparin, followed by perfusion with either 70% ethanol/150 mM NaCl or PBS buffered formalin. The brain and spinal cord were then removed, fixed and processed for paraffin infiltration as previously described [9]. Embedded tissue paraffin blocks were cut into 7 μm sections. Immunostaining of the sections was performed as previously described [9]. Immunocomplexes were visualized with the chromogen 3,3′-diaminobenzidine and the sections were counterstained with hematoxylin.

Double-labeling immunofluorescence analysis of mouse brain tissue. Paraffin-embedded tissue sections were hydrated and stained with primary antibodies as previously described [52]. Sections were incubated with goat anti-mouse secondary conjugated to Alexa 594 and goat anti-rabbit secondary conjugated to Alexa 488 (Invitrogen, Eugene, OR) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The sections were coverslipped with Fluoromount-G (SouthernBiotech, Birmingham, AL) and visualized using an Olympus BX51 microscope mounted with a DP71 Olympus digital camera to capture images.

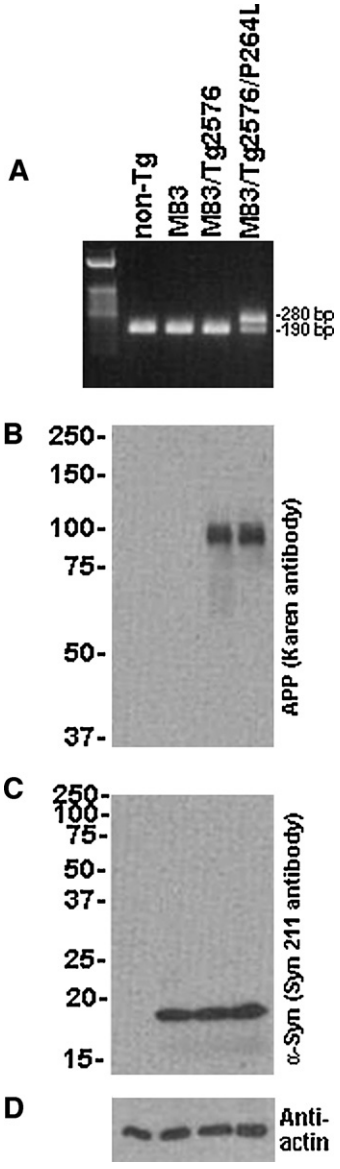

Fig. 1. PCR and Western blot analysis confirming the genotypes of the mice. (A) Ethidium bromide stained TBE/1.5% agarose gel loaded with PCR products amplified as described in "Materials and methods" from the mice genotyped indicated above each lane. (B) Western blot analysis with polyclonal antibody Karen specific for βAPP. (C) Western blot analysis with antibody Syn 211 specific for human α-syn. (D) Western blots were also probed with an actin antibody to assess equal protein loading. The mobility of protein markers in kDa are indicated on the left.

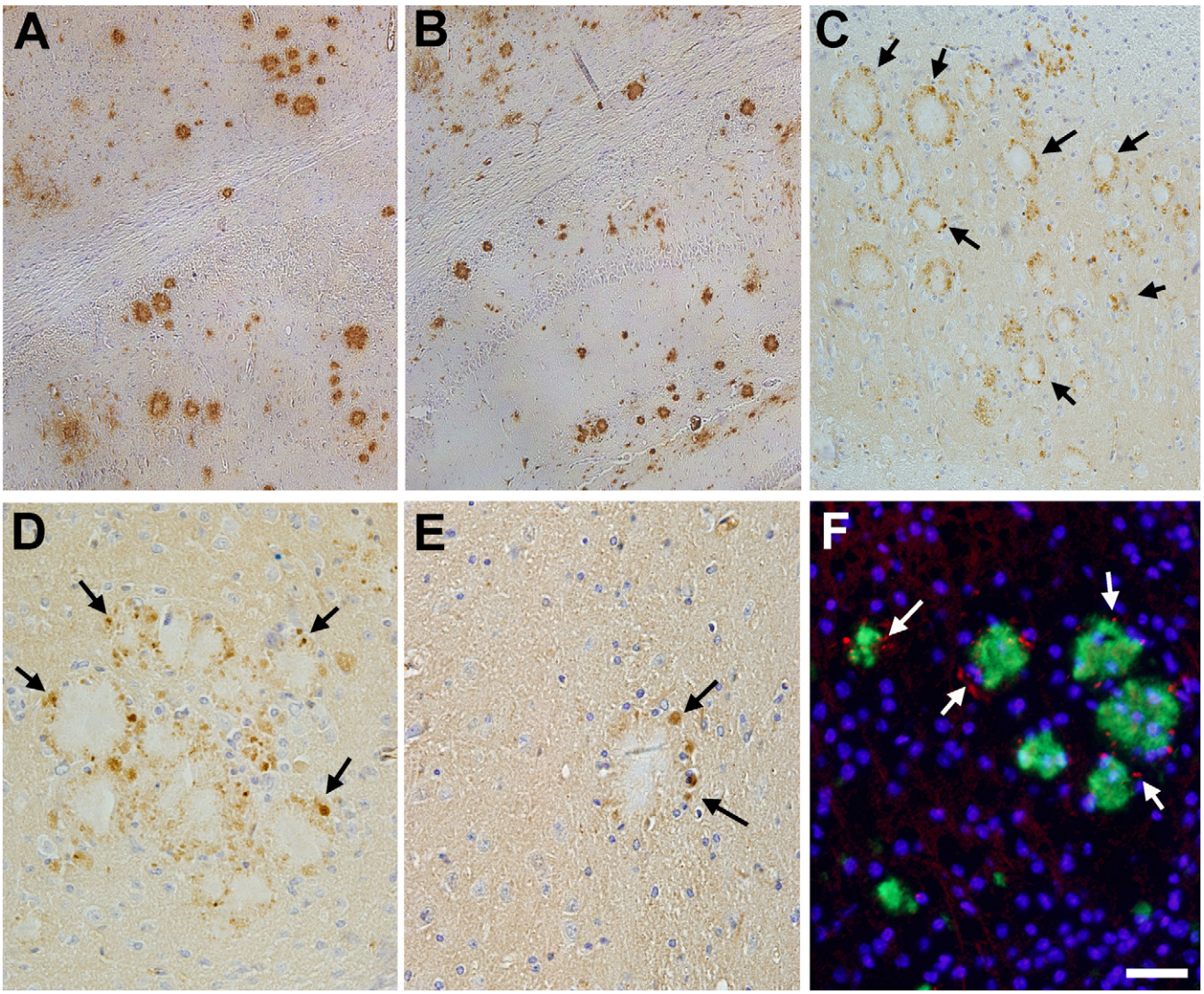

Fig. 2. Representative images of Aβ plaques and α-syn dystrophic neurites in Tg2576/M83 bigenic mice. Immunocytochemistry and immunofluorescence analyses were performed as described in "Materials and methods". Abundant Aβ plaques stained with antibodies 6E10 (A) or NAB228 (B) in the cortex and hippocampus of a 21-month-old Tg2576/M83 bigenic mouse. Accumulation of α-syn dystrophic neurites surrounding Aβ plaques detected with antibodies Syn 505 (C and D) and pSer129 (E). Double immunofluorescence of Aβ plaques with a rabbit anti-Aβ antibody (green) and antibody Syn 506 (red) showing accumulation of α-syn dystrophic neurites surrounding amyloid plaques (F). For immunocytochemistry sections were counterstained with hematoxylin and for immunofluorescence sections were counterstained with DAPI. Arrows indicate α-syn dystrophic neurites. Bar = 200 μm in A and B, 100 μm in C and F and 50 μm in D and E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Results and discussion

The A53T human α-syn transgenic mice (line M83) are prone to develop an age-dependent lethal motor phenotype associated with the formation of neuronal α-syn inclusions in the brain and spinal cord, however heterozygous mice from this transgenic line do not develop these phenotypic and cellular pathological changes until at least 22 months [16]. Consequently, this line provides a unique opportunity to study the effects of Aβ expression on the initiation and promotion of α-syn aggregation. To this end, A53T human α-syn transgenic mice (line M83) were crossbred with Tg2576 βAPP transgenic mice to generate mice that are heterozygous for each gene. In addition, these mice were further bred with knock-in mice carrying the P264L PSI mutation (Fig. 1A). The expression of human α-syn and human βAPP for the respective genotypes was further confirmed by Western blot analysis (Fig. 1B and C). The Tg 2576 transgenic mice start to develop extracellular amyloid deposits around 10–12 months and the presence of these inclusion increase rapidly thereafter [10,22,28]. The analysis of M83/Tg2576 bigenic mice from 7 to 22 months ($N = 22$) demonstrated the predicted profile of Aβ deposits with some initial detection of plaques beginning at 11–12 months and increasing abundance with age (Fig. 2). Further histological analysis revealed the presence of abundant α-syn dystrophic neurites within and surrounding many Aβ plaques (Fig. 2C–F) and many of these neurites accumulated α-syn phosphorylated at S129 (Fig. 2E), a modification that is persistent in pathological inclusions [14,52]. However, no additional α-syn pathologies were observed beyond these changes.

α-Syn/Tg2576 bigenic mice were further crossbred mice with the mice carrying the P264L PSI knock-in mutation. The P264L PSI knock-in mutation increases the amount Aβ 1-42 production, promoting Aβ plaque formation [10,44]. These mice were studied between 5 and 14 months of age ($N = 18$). Similar to previously reported Tg2576/P264L PSI mice [10,44], this triple crossed line developed amyloid plaques earlier than the α-syn/Tg2576 bigenic mice, starting around 6 months of age with an increasing

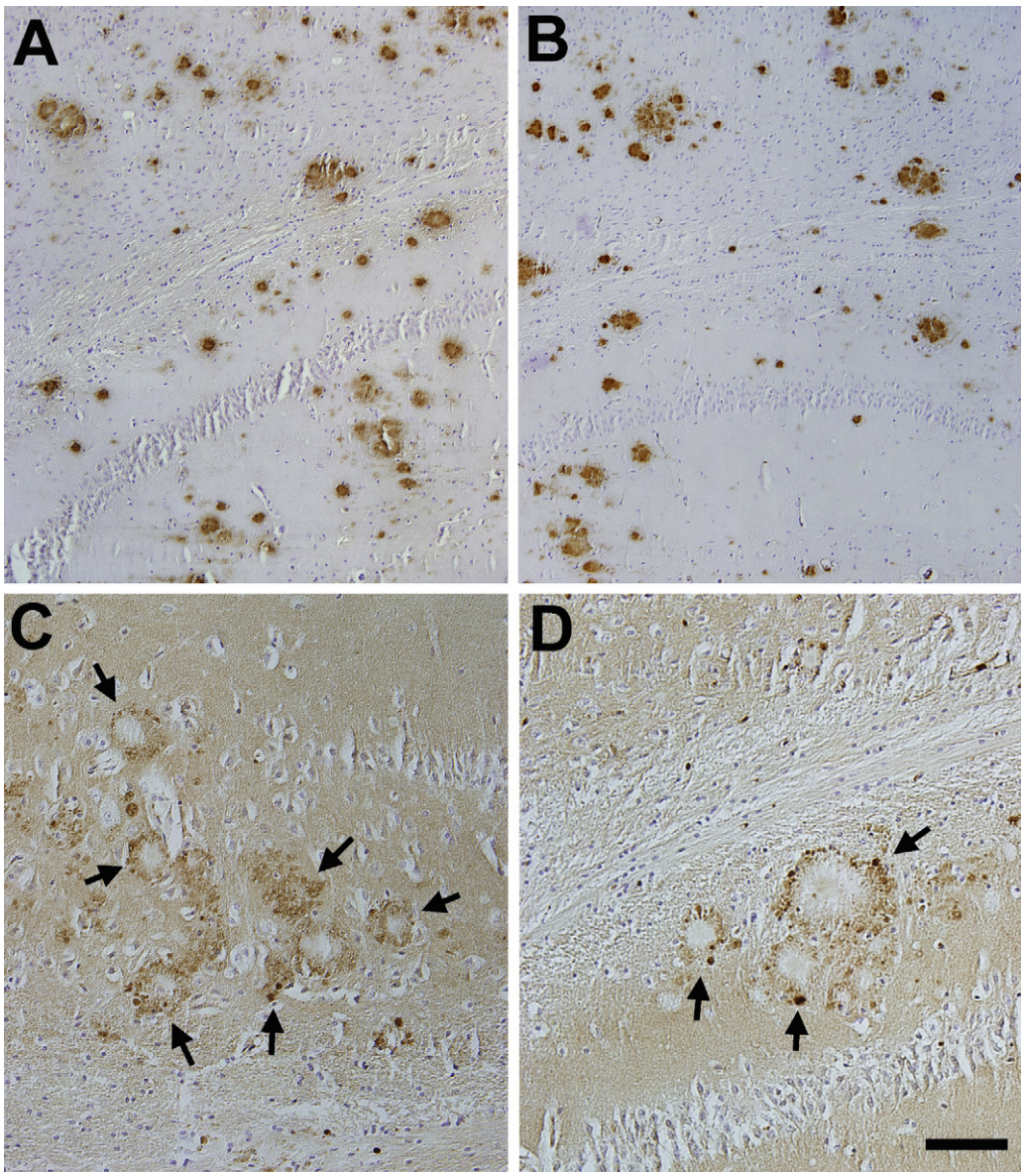

Fig. 3. Representative images of Aβ plaques and α-syn dystrophic neurites in M83/Tg2576/P264L PSI transgenic mice. Immunocytochemistry analysis was performed as described in "Materials and methods". Abundant Aβ plaques stained with antibodies 6E10 (A) or NAB228 (B) in the cortex and hippocampus of a 12-month-old Tg2576/M83/PSI P264L transgenic mouse. Accumulation of α-syn dystrophic neurites surrounding Aβ plaques stained with antibodies Syn 505 (C) and pSer129 (D). Arrows indicate α-syn dystrophic neurites. Sections were counterstained with hematoxylin. Bar = 200 μm in A and B and 100 μm in C and D.

abundance of plaques at older time points (Fig. 3). Many of these plaques displayed abundant α-syn dystrophic neurites that were hyperphosphorylated at S129 (Fig. 3C and D), but further α-syn pathology was not observed.

Several lines of evidence suggest that extracellular Aβ deposits may directly or indirectly promote intracellular α-syn aggregation. Besides the frequent co-occurrence of Aβ deposits and α-syn inclusions in the brains of patients with various synucleinopathies such as PD, DLB or LBVAD [11,20,38,51], α-syn inclusions are frequently observed in brains from patients with familial Alzheimer disease where genetic defects in the *APP*, *presenilin-1* (PS1) or *presenilin-2* (PS2) genes affect biological pathways that promote Aβ aggregation [23,27,35,46,55]. In addition, in vitro studies showed that Aβ peptides have the ability to promote polymerization of α-syn [26,36] and cell culture studies indicated that the addition of Aβ 1–42 can also promote the formation of α-syn inclusions [36]. Moreover, in studies of bigenic mice over-expressing βAPP (line J9) and wild-type α-syn (line D), it was reported that the presence of Aβ (or perhaps other APP products) significantly enhances the formation of filamentous α-syn aggregates [36]. J9 transgenic βAPP mice express human APP that is modified with both the "Swedish" (K670N/M271I) mutation and the "Indiana" (V717F) mutation resulting in an increase in total Aβ production as well as the amount of the longer forms of the Aβ 1–42/43 peptides with amyloid deposit starting at 5–7 months [37]. Similarly, cross breeding Tg2756 mice on a P264L PSI background has been shown to increase Aβ 1–42 production [10,44], which is responsible for the earlier formation of amyloid plaques.

Previous analysis of Tg2576 βAPP transgenic mice showed the accumulation of α-syn dystrophic neurites within amyloid plaques [49,54]. In addition, similar α-syn dystrophic neurites were reported with Tg2576 βAPP transgenic mice crossed to transgenic L286V mutant PSI mice [31]. The presence of the L286V PSI mutation accelerated the formation of amyloid plaques and the presence of α-syn dystrophic neurites within the plaques.

Given mounting evidence that α-syn aggregation may proceed intracellularly via a seeding mechanism, and even between neurons through a prion-like spreading disease mechanism [21,33], it was of interest to assess whether α-syn dystrophic neurites resulting from amyloid plaques, and perhaps other changes induced by Aβ expression, could lead to further α-syn pathology in M83 A53T human α-syn transgenic mice that are prone to develop α-syn inclusions. Tg2576/M83 bigenic mice on a wild-type or P264L PSI background developed abundant age-dependent α-syn dystrophic pathologies within and surrounding Aβ amyloid plaques, but it is surprising that additional α-syn pathologies did not present beyond these changes. It is possible that the aggregated forms of α-syn within these neurites are not compatible with the seeding of α-syn amyloid formation. However, it is also possible that additional cellular insults are required for the spreading of α-syn to occur in vivo. Nevertheless, the relationship between the formation of α-syn pathological inclusions and alteration in Aβ aggregation and/or processing remain enigmatic and further studies will be required to understand their possible association.

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