**β-III Tubulin Fragments Inhibit α-Synuclein Accumulation in Models of Multiple System Atrophy**

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**Background:** Neuronal and oligodendrocytic aggregation of insoluble α-synuclein contributes to neuropathology in multiple system atrophy.

**Results:** α-Synuclein binds directly to β-III tubulin. A fragment carrying the α-synuclein-binding site inhibited α-synuclein-β-III tubulin complex formation and intracellular α-synuclein accumulation.

**Conclusion:** Inhibition of α-synuclein binding to β-III tubulin may be an effective strategy for preventing α-synuclein accumulation.

**Significance:** Small β-III tubulin fragments may inhibit neurodegeneration in multiple system atrophy.

Multiple system atrophy (MSA) is a neurodegenerative disease caused by α-synuclein aggregation in oligodendrocytes and neurons. Using a transgenic mouse model overexpressing human α-synuclein in oligodendrocytes, we previously demonstrated that oligodendrocytic α-synuclein inclusions induce neuronal α-synuclein accumulation and progressive neuronal degeneration. α-Synuclein binds to β-III tubulin, leading to the neuronal accumulation of insoluble α-synuclein in an MSA mouse model. The present study demonstrates that α-synuclein co-localizes with β-III tubulin in the brain tissue from patients with MSA and MSA model transgenic mice as well as neurons cultured from these mice. Accumulation of insoluble α-synuclein in MSA mouse neurons was blocked by the peptide fragment β-III tubulin (residues 235–282). We have determined the α-synuclein-binding domain of β-III tubulin and demonstrated that a short fragment containing this domain can suppress α-synuclein accumulation in the primary cultured cells. Administration of a short α-synuclein-binding fragment of β-III tubulin may be a novel therapeutic strategy for MSA.

Multiple system atrophy (MSA) is a neurodegenerative disease clinically characterized by parkinsonism, cerebellar dysfunction, and autonomic nervous system failure (1, 2). MSA is caused by progressive aggregation of insoluble α-synuclein (α-syn) in oligodendrocytes and neurons. Two pathological hallmarks of MSA are glial cytoplasmic inclusions (GCIs) and neuronal inclusions, both of which are composed of α-synuclein (α-syn). The GCIs are confined to oligodendrocytes and are the first neuropathological manifestations of MSA (3–7). Neuronal inclusions ultimately lead to treatment-resistant neurodegeneration and neurological deficits. It remains controversial whether the source of the α-syn that accumulates in oligodendrocytes is of endogenous or exogenous origin, although it was recently shown that SNCA mRNA is present in MSA and control oligodendrocytes (8). We hypothesize that oligodendrocytic degeneration is a consequence of α-syn inclusion and that GCIs contribute to neuronal degeneration in MSA.

Mouse models of MSA have revealed that early formation of GCIs, the first histopathological sign of MSA, is necessary for later neuronal aggregation and resultant neuronal degeneration in the central nervous system (CNS) (9–11). Our transgenic (Tg) mouse model of MSA-expressing human α-syn in oligodendrocytes under the control of the 2′,3′-cyclic nucleotide 3′-phosphodiesterase promoter exhibited eventual formation of neuronal α-syn aggregation and neuronal degeneration associated with motor impairments, brain atrophy, decreased neuronal numbers, and reactive gliosis (10). In Tg mice, we clearly distinguished oligodendrocytic (exogenous) α-syn from neuronal (endogenous) α-syn. The exogenous α-syn in oligodendrocytes was of human origin, and the endogenous α-syn in neurons was of mouse origin in the mouse CNS. These Tg mice not only recapitulated many features of MSA neuropathology but also provided evidence that formation of GCIs triggers neuronal accumulation of insoluble α-syn, leading to neuronal dysfunction and degeneration in the mouse CNS (10, 12, 13). This is consistent with the neuronal pathology in the human pathology of MSA (14). We subsequently identified cystatin C as an intercellular signal released from oligodendrocytes containing GCIs using oligodendrocyte primary cultures derived from Tg mice (15). Treatment of wild-type neuronal cultures with cystatin C up-regulated α-syn expression and led to the accumulation of insoluble α-syn, strongly suggesting that cystatin C is an oligodendrocytic signal triggering neurodegeneration in MSA.

In primary cultured cells derived from Tg mice, we identified the neuron-specific microtubule component β-III tubulin as a major α-syn-interacting protein. Binding to β-III tubulin...
resulted in the formation of an insoluble protein complex that progressively accumulated in the neurites of Tg neurons, leading to neuronal dysfunction (12). Furthermore, we demonstrated that accumulation of insoluble α-syn was suppressed by a microtubule-depolymerizing agent, indicating that free β-III tubulin-α-syn molecules are less prone to inclusion formation (12, 16).

Although the binding of neuronal α-syn to β-III tubulin is a key pathogenic process in the MSA mouse model, it is still unclear whether and how α-syn binds to β-III tubulin in patients with MSA. In the present study, we demonstrate α-syn/β-III tubulin co-localization in the brains of mice and human patients with MSA and identify the β-III tubulin domain responsible for α-syn binding. A fragment containing this domain inhibited α-syn binding and inclusion formation in neurons. This study suggests an alternative treatment strategy for MSA, inhibiting α-syn/β-III tubulin complex formation to suppress insoluble α-syn accumulation in neurons.

**EXPERIMENTAL PROCEDURES**

**Anti-α-synuclein Antibodies—**syn4469 is a polyclonal antibody raised against mouse α-syn that selectively recognizes a mouse α-syn epitope between amino acid (aa) residues 115 and 125 (12). To generate an antibody specific to human α-syn, a synthetic peptide corresponding to the human wild-type α-syn aa 100–110 (LGKNEEGAPQE) was conjugated with keyhole limpet hemocyanin and used to immunize rabbits. The antisemum was purified using affinity chromatography. The isolated polyclonal antibody, syn4470, and anti-phosphorylated α-syn antibody (pS129) were used for immunohistochemistry, immunocytochemistry, and probing of immunoblots.

**Immunohistochemical Studies—**Brain tissues from wild-type and Tg mice were examined for the presence of co-localized α-syn and β-III tubulin in inclusions. In addition, we examined co-localization in patients with MSA pathologically confirmed by the presence of abundant GCIs in post-mortem brain tissue. Wild-type and Tg mice were sacrificed using a lethal dose of anesthesia and perfused transcardially with phosphate-buffered saline (PBS). Brains were fixed in 10% neutral buffered formalin or 70% ethanol, 150 mM NaCl, and slices were prepared for immunofluorescence double-labeling to evaluate the subcellular localization of α-syn and β-III tubulin in neurons. After incubation with the primary antibodies syn4469 and anti-β-III tubulin (Tuj1, Covance), immunolabeling was visualized using Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen). For thioflavin staining, sections were incubated with 0.05% thioflavin T and incubated with primary antibodies against α-syn (syn4470) and β-III tubulin. Immunolabeling was visualized as above. Alternatively, tissues from patients and age-matched controls were immunolabeled with syn4470 or pS129, and the immunoreaction was visualized by a biotinylated secondary antibody and developed with diaminobenzidine using the Vectastain ABC kit (Vector Laboratories) as described previously (17). Experiments involving human subjects were undertaken with the understanding and written informed consent of each individual and family. The National Center for Geriatrics and Gerontology Institutional Review Board approved the experiments involving human subjects.

**Transgenic Mice and Primary Cell Culture—**Transgenic mice were generated expressing human wild-type α-syn selectively in oligodendrocytes under the control of the murine 2′,3′-cyclic nucleotide 3′-phosphodiesterase promoter (10). Primary cultures of neurons and glial cells were prepared as described previously (12, 15). Briefly, cerebral cortices were dissected from P0 to P1 Tg mice and dissociated. The cells were plated on 15-mm glass coverslips coated with polyethyleneimine (Sigma-Aldrich) for immunocytochemistry or on polyethyleneimine-coated 25-cm² flasks for biochemical experiments. For biochemical studies, cultured cells were lysed, and proteins were sequentially extracted in three fractions: high salt (HS), radioimmune precipitation assay (RIPA), and formic acid (FA) fractions (15). Namely, the cells were homogenized in HS buffer (50 mM Tris-HCl, pH 7.4, 750 mM NaCl, 20 mM NaF, and 10 mM EGTA). The resulting pellet was dissolved in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EGTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS), and the RIPA buffer-insoluble proteins were extracted from the pellet by sonication in 70% (v/v) FA. Equal amounts of protein per gel lane were separated using SDS-PAGE and analyzed by immunoblotting. For immunocytochemistry, primary cultured cells on glass coverslips were fixed and immunostained with syn4469, syn4470, Tuj1, pS129, and anti-ubiquitin antibody (15). After incubation with primary antibody, cells were incubated for 1 h with fluorophore-conjugated secondary antibodies (Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG). For thioflavin staining, cells were incubated with 0.05% thioflavin T and washed with PBS before immunostaining. Nuclei were visualized by counterstaining with DAPI. All experiments on mice were approved by the National Center for Geriatrics and Gerontology Animal Care and Use Committee.

**Mutagenesis—**The complete cDNA sequence of β-III tubulin (Tubb3) was cloned into pT7Blue (12). An EcoRI site was induced at the 5′ end of Tubb3 by polymerase chain reaction (PCR), and the new sequence was transferred to the vector pGEX-6P (GE Healthcare) expressing GST. A HindIII site was added at the 5′ end of GST, and the GST-Tubb3 fusion gene was excised by digestion with HindIII and XhoI and subcloned into pcDNA3.1 (Invitrogen). N-terminal and C-terminal deletion mutants of β-III tubulin were created by inserting an EcoRI site at the chosen 5′ end and a stop codon at the chosen 3′ end using PCR. Internal deletion mutants of β-III tubulin were created by inverse PCR using Tubb3-pGEX6 as a template (18). The primers used for these mutations are listed in Table 1. Tubb3 mutants were excised by EcoRI and XhoI digestion and ligated to EcoRI-XhoI-digested GST-Tubb3-pcDNA3.1.

**GST Pull-down Assay and Immunoblotting—**The mouse α-syn gene Snca with a FLAG tag was cloned into the pcDNA3.1 vector (12). COS-7 cells were co-transfected with...
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| Primer sequences used in this study | Usage | Primer name | Primer sequence (5’ → 3’) |
|-------------------------------------|-------|-------------|--------------------------|
| β-III tubulin deletion mutant       | Δ1–116-Forward | GGGAATTCTAGCTAGATGTGCTGGAGAAGGTT | |
|                                     | Δ1–116-Reverse | GAACTCTTTCACCTGTGGGTCCTGCTGCTGCTGCA | |
|                                     | Δ117–234-Forward | GACGTGACCACCATCCCGCGCGCGCG | |
|                                     | Δ117–234-Reverse | GCTACCTGACAGACACCAAGCAG | |
|                                     | Δ225–331-Forward | GCCCGGACGGCAGGTGGCG | |
|                                     | Δ225–331-Reverse | CTACATGTCGACCTGCAACAG | |
|                                     | Δ332–451-Forward | GCCATGGTGGCAGACACAG | |
|                                     | Δ332–451-Reverse | GCTACCTGACAGACACCAAGCAG | |
|                                     | Δ235–282-Forward | GCCCTGACGGTGCTGCTGCTGCTGCTG | |
|                                     | Δ235–282-Reverse | CTCTAGAGTCGACCTGCAACAG | |
|                                     | Δ283–331-Forward | GCAATCTGACGCGCG | |
|                                     | Δ283–331-Reverse | ACTCATGGTGCGACGACACAG | |
| β-III tubulin fragment              | 235–282-Forward | GAACTGACTGCTGGCTGCGC | |
|                                     | 235–282-Reverse | GCTACCTGACAGACACCAAGCAG | |
|                                     | 283–331-Forward | GAACTGACTGCTGGCTGCGC | |
|                                     | 283–331-Reverse | ACTCMAGACGCCAAGCAGAC | |

The Snca vector together with a GST-tagged wild-type or mutant β-III tubulin vector for 48 h using polyethylenimine (Sigma-Aldrich) (19). Co-transfected cells or controls transfected with Snca and GST-pcDNA3.1 were harvested from two 100-mm dishes and homogenized in PBS containing protease inhibitors. Glutathione-Sepharose beads (GE Healthcare) were added to the respective cell lysates, and the mixture was rotated for 2 h at 4 °C. To assess direct binding of α-syn with wild-type or mutant β-III tubulin, proteins were produced using the pET system and purified as described (20). The purified α-syn (100 μg) was incubated for 2 h at 4 °C with GST-β-III tubulin (100 μg) prebound to glutathione-Sepharose beads. The beads were washed three times with 1% Nonidet P-40 in PBS, and bound proteins were eluted by boiling in SDS-PAGE sample buffer. Eluted proteins were electrophoretically separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Membranes were incubated in 5% nonfat milk powder in TBS and incubated with syn4469 and anti-GST antibodies (Santa Cruz Biotechnology, Inc.), followed by incubation with an HRP-conjugated secondary antibody. Immunoreactive proteins were visualized using an ECL prime system (GE Healthcare).

**Neuro2a Cell Culture**—The mouse neuroblastoma cell line Neuro2a was maintained in DMEM supplemented with 10% FBS. FuGENE 6 (Roche Applied Science) was used for the introduction of exogenous DNA into Neuro2a cells (15). After 24 h of transfection, the medium was replaced with DMEM supplemented with 2% FBS and 20 μM retinoic acid (Sigma-Aldrich) to induce neuronal differentiation. After 72 h of transfection, protein expression was examined by immunocytochemistry as described for Tg-derived neurons.

**Treatment of Tg-derived Neurons with β-III Tubulin Fragments**—A peptide containing the putative α-syn-binding sequence (amino acids 235–282) as defined by GST pull-down assays and a control peptide (amino acids 283–331) were synthesized in a bacterial expression system. Partial cDNA fragments encoding these sequences were amplified, and a BamHI site was added at the 5’ end and a stop codon to the 3’ end by PCR (Table 1). The cDNA fragments were cloned into pT7Blue, excised using BamHI and EcoRI enzymes, and ligated into pRSETB (Invitrogen), which introduces a His6 tag. The resulting plasmids were transformed into *Escherichia coli* BL21(DE3)pLyS5 (Merck Millipore). Expression of the recombinant peptides were induced as described previously (20). Bacteria were then suspended in PBS and disrupted by ultrasonication. The cell lysates were centrifuged at 20,000 × g for 10 min, and the pellet was resuspended in 8 M urea in PBS (pH 7.4). The recombinant peptides were purified using Ni-Sepharose 6 Fast Flow (GE Healthcare), dialyzed with PBS, pellets by centrifugation at 20,000 × g for 10 min, and resuspended in DMSO. To examine the effects of the peptide on α-syn binding to β-III tubulin, COS-7 cells were transfected with α-syn, and the lysate was exposed to recombinant β-III tubulin (decoy) peptide for 1 h at 4 °C. Peptide-treated α-syn was mixed with the GST-β-III tubulin fusion protein isolated from other transfected COS-7 cells, and the mixture was subjected to a GST pull-down assay. Primary cultured cells derived from Tg mice were transfected with decoy and control peptides (0.5 μg/cm² each) using Xfect protein transfection reagent (Clontech) at DIV8 and DIV15. At DIV23, cells were harvested for immunoblotting or immunostained with anti-α-syn and anti-GST.

**Real-time PCR Analysis**—Harvested cells were immediately soaked in RNAlater stabilization reagent (Qiagen). Total RNA was isolated from cells by using NucleoSpin RNA (Takara Bio). Purified total RNA (1 mg) was converted to cDNA with the use of the High Capacity cDNA reverse transcription kit (Invitrogen). Gene expression levels were quantified with Power SYBR Green PCR Master Mix (Invitrogen). The primer sequences were described previously (15).

**RESULTS**

α-Synuclein Co-localizes with β-III Tubulin in Vivo—α-Syn binds to β-III tubulin in primary cultured neurons derived from Tg mice (12). To determine whether α-syn binds to β-III tubulin in vivo, leading to insoluble α-syn aggregation, we examined the spatial correlation between α-syn and β-III tubulin expression in the Tg mouse CNS and brain tissues from patients with MSA. First, we found that oligodendrocytic accumulation of α-syn shows aggregation in the primary cultured cells derived from Tg mice. pS129 stained oligodendrocytic inclusions, and the inclusions were also immunoreactive to anti-ubiquitin antibody (Fig. 1). Thioflavin T stained oligodendrocytic inclusions but did not detect neuronal aggregation of α-syn in the neurons. Second, immunoreactivity to the mouse α-syn anti-
body syn4469 was observed in the cytoplasm and axons co-localized with β-III tubulin immunoreactivity (Fig. 2, A and B). Neuronal cytoplasmic inclusions were detected by thioflavin T in Tg mouse brain tissues (Fig. 2C). Neuronal cytoplasmic inclusions and dystrophic neurites in the brain tissues of patients with MSA were examined by immunohistochemistry using the human α-syn antibody syn4470; ~0.5% of neurons in

![Image](https://example.com/image1)

**FIGURE 1.** Aggregation of α-synuclein in primary neural cells derived from MSA model mice. A–C, primary cultured neural cells derived from Tg mice were double-stained with anti-human α-syn (syn4470; red) and either anti-phosphorylated α-syn antibody (pS129) (A), thioflavin T dye (ThT) (B), or anti-ubiquitin antibody (Ubi; green) (C). D, primary cultured neural cells derived from Tg mouse were double-stained with anti-mouse α-syn (syn4469; red) and anti-ubiquitin antibody. A double-labeling immunofluorescence study showed that oligodendrocytic inclusions were stained by pS129, ThT, and anti-ubiquitin antibody. Additionally, the anti-ubiquitin antibody stained neuronal aggregation of α-syn in Tg cultured cells. Nuclei were counter-stained with DAPI (blue). Scale bar, 10 μm.

![Image](https://example.com/image2)

**FIGURE 2.** Co-localization of β-III tubulin with α-synuclein in the brains of MSA model mice and patients with MSA. A–D, brain stem tissues from a 12-month-old wild-type mouse (A), a 12-month-old and a 24-month-old Tg mouse (B), and a patient with MSA (D) were double-stained with anti-α-syn (syn4469 or 4470; green) and anti-β-III tubulin (Tuj1; red). Brain tissues from a 24-month-old Tg mouse were double-stained with thioflavin T dye (green) and syn4469 (red) (C). α-Synuclein co-localized with β-III tubulin in axons and cytoplasm of Tg mouse neurons and in the MSA brain. The punctate α-syn staining pattern is consistent with aggregation of insoluble inclusions. Thioflavin T stained neuronal aggregation of α-syn in Tg mouse brain tissues; 36.3 ± 7.0% of neurons were positive. E, immunohistochemical staining of brain stem tissues in a patient with MSA and an age-matched control using the syn4470 or pS129 and diamino benzidine- HRP staining (brown). syn4470 labeled the GCIs (arrows) and punctate aggregation of α-syn in axons (arrowheads), whereas no labeling was observed in brainstem tissue from an age-matched control. The quantitative change of α-syn in the affected areas of control and MSA brain tissues was assessed on immunoblots. Scale bar, 10 μm.
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FIGURE 3. Direct interaction between α-synuclein and β-III tubulin. The interaction of β-III tubulin to α-syn was examined by a GST pull-down assay. A, COS-7 cells were co-transfected with α-syn and GST-β-III tubulin or α-syn and GST alone. Lysates were then treated with glutathione-Sepharose beads, and eluted proteins were detected by immunoblotting. α-Syn was precipitated by GST-β-III tubulin bound to glutathione-Sepharose beads (right-hand gel) but not by GST alone on glutathione-Sepharose beads (left-hand gel). B, recombinant mouse α-syn and GST-β-III tubulin were expressed in E. coli. Purified α-syn and GST-β-III tubulin were mixed with glutathione-Sepharose beads. Immunoblots stained by anti-mouse α-syn (syn4469) and anti-GST antibodies showed that α-syn bound to β-III tubulin in the absence of mammalian proteins.

The affected areas of temporal cerebral cortex contained syn4470-positive cytoplasmic inclusions, and ~0.5% of the neurons associated with syn4470-positive dystrophic neurites in those areas. We then examined α-syn-β-III tubulin co-localization in brain tissues of patients with MSA by co-immunostaining with anti-β-III tubulin antibody and syn4470. The antibodies co-labeled dystrophic neurites and axons in the medulla of patients with MSA (Fig. 2D). syn4470 selectively labeled dystrophic neurites and axons in the lesions of patients with MSA but not in the brain tissue from controls (Fig. 2E). Immunoblots showed that there were no differences in total protein amounts of α-syn and β-III tubulin between the two brain tissues. These results suggest that the interaction between α-syn and β-III tubulin participates in the neuronal aggregation of α-syn in MSA.

α-Synuclein Directly Binds to β-III Tubulin—We previously demonstrated that α-syn binds to wild-type β-III tubulin but not to other β-tubulin isoforms in primary neuronal cells from Tg mice (12). In the current study, we directly examined β-III tubulin-wt-syn complex formation by GST pull-down assays using whole-cell extracts prepared from COS-7 cells co-expressing GST-β-III tubulin and α-syn or GST alone and α-syn. The GST–β-III tubulin fusion protein was used as bait. Immunoblot analysis of protein extracts following pull-down assays showed that α-syn co-precipitated with GST–β-III tubulin but not with GST alone (Fig. 3A). To exclude the possibility that the interaction between α-syn and β-III tubulin was indirectly mediated by other mammalian proteins, assays were also performed on α-syn and GST–β-III tubulin purified from expressing E. coli. Similar to lysates from COS-7 cells, purified GST–β-III tubulin precipitated α-syn (Fig. 3B), indicating that α-syn and β-III tubulin directly interact in mammalian cells.

Mapping the α-Synuclein-binding Region of β-III Tubulin—To determine the domain of β-III tubulin required for α-syn binding, we conducted GST pull-down assays using lysates from COS-7 cells co-expressing α-syn with GST alone (control), full-length β-III tubulin, or one of the following β-III tubulin deletion mutants: Δ1–116, Δ117–234, Δ235–331, or Δ332–451 (Fig. 4A). Full-length β-III tubulin and the deletion mutants Δ1–116, Δ117–234, and Δ332–451 precipitated α-syn, whereas Δ235–331 and GST alone did not (Fig. 4B), indicating that the binding region lies between residues 235 and 331. To precisely define the α-syn binding region, we generated two more restricted deletion mutants of β-III tubulin, Δ235–282 and Δ283–331 (Fig. 4C). The deletion mutant Δ235–282

FIGURE 4. Mapping the α-synuclein-binding domain on β-III tubulin. A, GST–β-III tubulin and deletion constructs of β-III tubulin are illustrated. Deleted regions are designated by Δ, and the numbers following Δ are the deleted amino acids of β-III tubulin in mutant constructs. The putative α-syn-binding region of β-III tubulin (aa 235–281) was generated as a decoy peptide to block the binding of α-syn to β-III tubulin. B, the α-syn-binding site on β-III tubulin was examined by GST pull-down assay. α-Syn was co-expressed with GST-tagged full-length β-III tubulin or deletion mutants. Glutathione-Sepharose beads were used for pull-down assays, and proteins eluted from the beads were detected by immunoblotting using syn4469 and anti-GST antibodies. Immunoblots show that α-syn immunoreactivity is lost when the beads were bound to GST–β-III tubulin Δ235–331, although full-length β-III tubulin and the other deletion mutants did not precipitate α-syn. C, the α-syn-binding region within aa 235–331 was further refined using 235–282 and 283–331 deletion mutants and a GST pull-down assay. Immunoblots revealed that β-III tubulin Δ283–331 but not Δ235–282 precipitated α-syn. Thus, α-syn binds within aa 235–282 of β-III tubulin. D, to verify this α-syn-binding domain, the fragment β-III tubulin 235–282 was co-expressed with α-syn in COS-7 cells. Immunoblots showed that this fragment but not GST alone bound α-syn.
did not pull down α-syn, whereas Δ283–331 did (Fig. 4C), indicating that aa 235–282 of β-III tubulin are required for the interaction with α-syn.

To confirm this binding site in neurons, α-syn was co-expressed with full-length GST-β-III tubulin or the deletion mutants in Neuro2a cells. Immunofluorescence studies showed that α-syn co-localized with full-length β-III tubulin or Δ283–331, appearing as intense fluorescent granules in the cytoplasm and neurites (Fig. 5) and resembling the immunostaining pattern of α-syn inclusions in the Tg mouse brain. In contrast, α-syn did not co-localize with β-III tubulin in cells expressing Δ235–282. Thus, a region within aa 235–282 of β-III tubulin is responsible for binding and concomitant formation of α-syn inclusions in neurons. In addition, a GST fusion protein with the β-III tubulin sequence 235–282 was prepared and subjected to the GST pull-down assay. Immunoblotting showed that α-syn bound to the short aa 235–282 fragment of β-III tubulin (Fig. 4D). Thus, the β-III tubulin region 235–282 is necessary and sufficient for α-syn binding.

The β-III Tubulin Fragment Containing aa 235–282 Blocks α-Synuclein Inclusions in Tg-derived Neurons—The interaction between α-syn and β-III tubulin plays a crucial role in the neuronal accumulation and aggregation of insoluble α-syn and degeneration of neurons in Tg mice; therefore, we examined whether a β-III tubulin fragment containing the α-syn-binding region (aa 235–282) can disrupt α-syn inclusion in COS-7 cells and neurons derived from Tg mice. The β-III tubulin 235–282 fragment was expressed in E. coli as a His tag fusion protein and purified (Fig. 4A). The purified fragment dissolved in DMSO was first used as a decoy peptide in GST pull-down assays. Lysate from COS-7 cells overexpressing α-syn was preincubated in the presence of the purified decoy peptide or vehicle and subjected to the pull-down assay using GST-β-III tubulin as bait. Treatment with the β-III tubulin 235–282 fragment reduced the binding of α-syn to bait GST-β-III tubulin, indicating that it acted as an inhibitor of β-III tubulin-α-syn binding (Fig. 6A). To examine this inhibitory effect on neuronal accumulation of insoluble α-syn, primary neural cultures derived from Tg mice were examined for α-syn inclusions (punctate immunoreactivity) following treatment with the β-III tubulin 235–282 fragment (decoy), 283–331 fragment (control), or vehicle. Quantitative analyses of the immunoblots confirmed that the total amount of α-syn in Tg mouse neural cells treated with decoy peptide is not different from that treated with control peptide or vehicle (Fig. 6B). Moreover, to study the biochemical properties of α-syn in Tg mouse cells, α-syn in cultured cells was sequentially extracted into three fractions, HS, RIPA, and FA (Fig. 6C). Treatment of Tg neurons with the β-III tubulin 235–282 decoy peptide decreased the immunoreactivity of insoluble α-syn in FA fraction, whereas it did not decrease that of α-syn in HS fraction (Fig. 6C). We also studied the changes in α-syn accumulation resulting from decoy peptide treatment. Immunocytochemical staining with an α-synuclein antibody demonstrated that the punctate accumulation of α-syn in somata and axons characteristic of Tg neurons was reduced by decoy peptide treatment (Fig. 6D). Furthermore, the decoy peptide treatment decreased the α-syn accumulation in a dose-dependent manner (Fig. 6D). The decoy peptide did not decrease the total amount of endogenous α-syn mRNA (vehicle, 100 ± 4; decoy peptide, 89 ± 14; control peptide, 107 ± 40; mean ± S.D. percentage of vehicle; n = 3), indicating that the reduction of α-syn accumulation is not a down-regulation of natural α-syn expression but results from the suppression of patho-

![FIGURE 5. Co-localization of α-synuclein with β-III tubulin in Neuro2a cells depends on the aa 235–282 region.](image-url)
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A, α-Syn was transiently expressed in COS-7 cells, and the whole cell lysate was preincubated with the decoy peptide prior to GST pull-down assay. Proteins eluted from the beads were analyzed by immunoblotting using syn4469 (top) and anti-GST (bottom). Syn4469 staining revealed no α-syn in immunoblots of COS-7 cells treated with the decoy peptide, suggesting removal by binding to the decoy. B, primary cultured neural cells derived from Tg mice were treated with decoy peptide, control peptide, and vehicle from DIV8 to DIV23 and compared with untreated cultures. Total amount of α-syn was quantified by immunoblotting analysis (n = 3). A bar graph shows that decoy peptide did not decrease the total amount of α-syn protein. α-Syn signals on the immunoblots were normalized to the signals for β-actin. C, primary cultured neural cells derived from Tg were treated with the decoy peptide or vehicle from DIV8 to DIV23, and proteins were sequentially extracted in three fractions: HS buffer, RIPA buffer, and 70% FA. Immunoblots demonstrated that insoluble α-syn was reduced in Tg mouse primary neurons by treatment with the decoy peptide. A bar graph quantitatively shows the α-syn amount in each fraction on the immunoblots of cultured neural cells derived from Tg mice with and without decoy peptide treatment (n = 3). Neurofilament (NF160) was used as a marker for insoluble proteins. D, primary cultured neural cells derived from Tg mice were treated with decoy peptide, control peptide, and vehicle from DIV8 to DIV23. Cells were double-stained with syn4469 and Tuj1. Immunocytochemistry revealed that treatment with the decoy peptide decreased co-localization of α-syn with β-III tubulin. Scale bar, 10 μm. The bar graph illustrates the ratio of neurons containing α-syn inclusions to the total number of β-III tubulin-positive neurons in primary cultures with or without decoy peptide treatment (mean percentage of α-syn-positive cells from 10 different microscopic fields). A dose-response curve of neuronal α-syn inclusions in response to decoy peptide is plotted. Data are expressed as mean ± S.D. (error bars), *, p < 0.05. Student’s t test. E, primary cultured neural cells derived from Tg mice were treated with decoy peptide, control peptide, and vehicle from DIV8 to DIV23. Cells were double-stained with syn4469 and anti-ubiquitin antibody (red). Treatment of decoy peptide decreased the immunoreactivity that co-localized α-syn and ubiquitin in the neurites of Tg mouse primary cultured cells. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm.

DISCUSSION

We previously demonstrated that α-syn binds to β-III tubulin, leading to neuronal accumulation of insoluble α-syn and neurodegeneration in an MSA mouse model (10, 12, 13). The present study showed that α-syn co-localizes with β-III tubulin in the brain tissues of patients with MSA. In addition, we iden-
tified the α-syn-binding domain of β-III tubulin. A short peptide fragment that carries this domain interfered with native β-III tubulin-α-syn complex formation and suppressed the accumulation of α-syn in primary cultured neurons from MSA model mice.

Previous studies reported α-syn co-localization with tubulin in neurons (12, 21–23) and α-syn binding to free tubulin (20), although the physiological role of this interaction is still not fully understood. Our previous studies on the MSA mouse model demonstrated α-syn binding specifically to the neuron-specific β-III tubulin, resulting in neuronal accumulation of insoluble α-syn (12). In the present study, α-syn accumulation in Neuro2a cells required co-expression with β-III tubulin, suggesting that an aberrant increase in free β-III tubulin contributes to α-syn accumulation in neurons. Tubulin was also shown to initiate and promote α-syn fibril formation under physiological conditions in vitro (15, 22). It was also reported that α-syn binds to monomeric tubulin and stimulates microtubule polymerization (24). Indeed, blocking tubulin polymerization using nocodazole resulted in reduced α-syn accumulation (12, 16). Furthermore, ingestion of nocodazole attenuated impaired synaptic function in MSA mice (13). Although the antibiotic rifampicin, which inhibits α-syn self-aggregation, decreased recombinant α-syn aggregation in vitro, it had little effect on the aggregation of α-syn in primary cultured neurons derived from MSA mice (16). These results strongly suggest that free β-III tubulin binds to α-syn and then polymerizes to form microtubules. This in turn reduces α-syn solubility, resulting in formation of inclusions. Moreover, α-syn aggregation may lead to the disruption of the microtubule network and microtubule-dependent trafficking (23), and a reduction in acetylated tubulins, indicating a loss of microtubule stability (25). Loss of microtubule stability may disrupt the neuroplastic changes critical for neural repair and adaptation (learning). Furthermore, α-syn binding may depolymerize microtubules and increase free tubulin concentration. These free tubulin monomers may in turn bind even more α-syn, thereby generating a positive feedback cycle resulting in reduced α-syn solubility, inclusion formation, and cytoskeletal disruption.

In general, the therapeutic use of peptides is limited due to poor bioavailability and stability. Recently, however, new strategies for increasing protein half-life and delivery systems capable of transporting peripherally administered peptides across the blood-brain barrier have renewed interest in peptides as therapeutic agents (26, 27). Several strategies for non-invasive drug delivery to the CNS has been reported, including molecular Trojan horse approaches and nanocarrier systems, such as liposomes or nanoparticles (28–30). α-Synuclein forms filamentous inclusions in synucleinopathies, including MSA and Parkinson disease. Because α-syn self-associates to form oligomers and fibrils, the first treatment strategies for synucleinopathies such as Parkinson disease attempted to disrupt self-association (31, 32) (e.g. by using peptide inhibitors derived from the α-syn sequence) (33, 34). However, the formation of neuronal α-syn inclusions in an MSA mouse model is also facilitated by α-syn-β-III tubulin binding (12), necessitating an alternative strategy.

Microtubules are critical for maintaining the morphological stability and adaptive responses of neurons and thus for the stability and neuroplasticity of the nervous system. The cytoskeleton is a dynamic structure because of the constant exchange of tubulins between soluble and filamentous forms. Thus, the restoration of appropriate microtubule dynamics can provide therapeutic benefits for MSA and other synucleinopathies. Indeed, the octapeptide NAP derived from an activity-dependent neuroprotective protein bound to tubulin and conferred neuroprotection (35). NAP-treated Parkinson disease model mice exhibited improved motor performance and a reduction in proteinase K-resistant α-syn inclusions in the substantia nigra (36). In MSA, we speculate that the inhibition of β-III tubulin-α-syn binding may be a more effective strategy for preventing neuronal α-syn accumulation and preserving cytoskeletal dynamics. This study demonstrates that a peptide derived from β-III tubulin containing the α-syn binding domain blocks the interaction between α-syn and β-III tubulin and reduces the accumulation of insoluble α-syn in neurons. We propose a drug design strategy for the selective mitigation of neuronal α-syn accumulation caused by α-syn-β-III tubulin complex formation. This could be the first step toward the development of an effective treatment for MSA.

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