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**Journal Title:** Proceedings of the National Academy of Sciences

**Volume:** Volume 114, Number 40

**Publisher:** National Academy of Sciences | 2017-10-03, Pages 10773-10778

**Type of Work:** Article | Final Publisher PDF

**Publisher DOI:** 10.1073/pnas.1713969114

**Permanent URL:** https://pid.emory.edu/ark:/25593/s92pc

Final published version: [http://dx.doi.org/10.1073/pnas.1713969114](http://dx.doi.org/10.1073/pnas.1713969114)

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Accessed May 12, 2019 1:42 PM EDT
TrkB neurotrophic activities are blocked by α-synuclein, triggering dopaminergic cell death in Parkinson’s disease

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Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved August 24, 2017 (received for review August 7, 2017)

BDNF/TrkB neurotrophic signaling is essential for dopaminergic neuronal survival, and the activities are reduced in the substantia nigra (SN) of Parkinson’s disease (PD). However, whether α-Syn (alpha-synuclein) aggregation, a hallmark in the remaining SN neurons in PD, accounts for the neurotrophic inhibition remains elusive. Here we show that α-Syn selectively interacts with TrkB receptors and inhibits BDNF/TrkB signaling, leading to dopaminergic neuronal death. α-Syn binds to the kinase domain on TrkB, which is negatively regulated by BDNF or Fyn tyrosine kinase. Interestingly, α-Syn represses TrkB lipid raft distribution, decreases its internalization, and reduces its axonal trafficking. Moreover, α-Syn also reduces TrkB protein levels via up-regulation of TrkB ubiquitination. Remarkably, dopamine’s metabolite 3,4-Dihydroxyphenylacetaldehyde (DOPAL) stimulates the interaction between α-Syn and TrkB. Accordingly, MAO-B inhibitor rasagiline disrupts α-Syn/TrkB complex and rescues TrkB neurotrophic signaling, preventing α-Syn–induced dopaminergic neuronal death and restoring motor functions. Hence, our findings demonstrate a noble pathological role of α-Syn in antagonizing neurotrophic signaling, providing a molecular mechanism that accounts for its neurotoxicity in PD.

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, with typical movement abnormalities that include resting tremor, rigidity, bradykinesia, and postural instability as major clinical manifestation. PD is characterized by selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and a corresponding decrease in dopaminergic innervation of the striatum, along with the accumulation of intraneuronal protein aggregates [Lewy bodies (LBs) and Lewy neurites (LNs)] primarily composed of the protein alpha-synuclein (α-Syn) (1, 2). The physiological functions of α-Syn remain unclear, but several studies have demonstrated that it might be implicated in dopamine (DA) biosynthesis, synaptic plasticity, and vesicular dynamics (3, 4). A pathogenic role for α-Syn in PD is supported by various genetic data. For instance, multiplications of the gene encoding α-Syn (SNCA) and various point mutations in this gene (e.g., A53T, A30P, and E46K) result in dominant familial parkinsonism (1, 5, 6). Moreover, certain polymorphisms in SNCA are major risk factors for sporadic PD (7). The abnormal accumulation of α-Syn, resulting from an unbalanced production and/or degradation of the protein, is thought to trigger DAergic neuronal death in both familial and sporadic cases of PD (8). So far, several cellular mechanisms, such as aberrant protein folding, oxidative stress, and mitochondrial dysfunction have been linked to the development and progression of PD, however the exact molecular mechanisms of how α-Syn exerts neurotoxicity remain elusive.

Neurotrophins (NTs) are growth factors that regulate the development and maintenance of the peripheral and the central nervous systems. Brain-derived neurotrophic factor (BDNF) is a member of the NT family, which includes nerve growth factor (NGF), NT-3, and NT-4/5. BDNF, like the other NTs, exerts its biological functions on neurons through two transmembrane receptors: the p75 neurotrophin receptor (p75NTR) and the TrkB receptor tyrosine kinase (NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 preferentially binds to TrkC) (9). TrkB is one of the most widely distributed neurotrophic receptors (NTRs) in the brain and is highly enriched in the neocortex, hippocampus, striatum, and brainstem. BDNF binding to TrkB triggers its dimerization through conformational changes and autophosphorylation of tyrosine residues in its intracellular domain (ICD), resulting in activation of the three major signaling pathways involving mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C-g1 (PLC-γ1), mediating neural differentiation, survival, and neurogenesis. BDNF colocalizes with DAergic neurons in the SN (10), and promotes DAergic neuronal survival in vivo (11). PD patients show markedly decreased levels of BDNF (12–14), indicating that reduced levels of this neurotrophic factor may be involved in the etiology and pathogenesis of PD. Transplantation of modified fibroblasts that express BDNF into either the striatum or the midbrain attenuates 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal

Significance

Alpha-synuclein plays an important role in the pathophysiology of Parkinson’s disease (PD), however the molecular mechanisms related to α-synuclein in neurodegeneration of PD remain unknown. We show that α-synuclein specifically inhibits BDNF/TrkB signaling, leading to dopaminergic neuronal death. The disruption of this interaction rescues TrkB signaling, preventing α-Syn–induced dopaminergic neuronal death and restoring motor functions. This study reveals the mechanism related to α-synuclein–induced neurotoxicity of PD via regulation of TrkB neurotrophic signaling.

Author contributions: S.S.K., Z.Z., J.X., and K.Y. designed research; S.S.K., Z.Z., and X.L. performed research; F.P.M., M.J.B., X.C., and Y.E.S. contributed new reagents/analytic tools; S.S.K., J.X., and K.Y. analyzed data; and J.X. and K.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1713969114/-/DCSupplemental.

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α-Synuclein selectively interacts with TrkB receptors. (A) α-Syn specifically interacts with TrkB receptors. GST pull-down assay was conducted from HEK293 cells cotransfected with mammalian GST-α-Syn and HA-Trks. (B) α-Syn N terminus is implicated in binding TrkB. Different mGST-tagged α-Syn truncated were cotransfected with HA-TrkB into HEK293 cells. A GST pull-down assay was performed, and coprecipitated proteins were analyzed by immunoblotting with anti-HA (Top). Schematic diagram of α-Syn truncations (Bottom). (C) Mapping assay for TrkB ICD required for binding to α-Syn. (Bottom) Schematic diagram of TrkB domains. (D) BDNF inhibits α-Syn/TrkB association. Cortical neurons were pretreated with K252a (100 nM) for 15 min, followed by BDNF treatment (50 ng/mL) for 30 min. Immunoprecipitation was performed with anti-α-Syn, and the coprecipitated proteins were analyzed by immunoblotting with anti-TrkB. (F) TrkB colocalizes with α-Syn in the LBS of PD patients. Immunofluorescent costainings with anti-TrkB or p-TrkB (Green) and α-Syn (Red) were conducted with human PD brain sections. The nuclei were stained with DAPI. (Scale bar, 20 μm.)

Fig. 2. Overexpression of α-Syn blocks BDNF/TrkB signaling. (A) α-Syn inhibits BDNF/TrkB signaling. TrkB stably transfected SH-SYSY (BR6) cells were transfected with α-Syn, followed by treatment with BDNF for 10 min. p-TrkB and its downstream effectors, p-Akt and p-MAPK, were analyzed in the cell lysates. (B) BDNF/TrkB signaling is blocked in SNCA overexpressing transgenic neurons. Wild-type, SNCA KO, and SNCA transgenic neurons were treated with BDNF for 15 min, 1 h, or 2 h, and the pTrkB signaling cascade was probed with various antibodies. (C) α-Syn overexpression decreases the neuroprotective effects of BDNF against MPP+-induced neuronal cell death. MPP+ sensitized α-Syn-induced neuronal cell death. Shown are SNCA transgenic, SNCA KO, or wild-type dopaminergic neurons in the presence or absence of BDNF, treated with MPP+ (200 μM) or not for 24 h. LDH assay was conducted with cell medium. (E and F) Overexpression of α-Syn decreases TrkB levels and elevates neuronal cell death. Primary cortical cultures were infected with AAV virus expressing α-Syn or GFP control, followed by treatment with anti-BDNF or anti-IgG, then treated with MPP+ (200 μM) for 24 h. Immunoblotting analysis of cell lysates with various antibodies (E) and LDH assay of the treated cells (F). Data are shown as mean ± SEM. n = 3 each group. *p < 0.05, **p < 0.01. The relative intensities of the band that were quantified with Image J were indicated in the immunoblots.
the interaction between TrkB and α-Syn, we pretreated rat primary cortical neurons with the Trk receptor inhibitor K252a, followed by BDNF treatment. Remarkably, BDNF completely disrupted the formation of the α-Syn/TrkB complex, and inhibition of TrkB barely affected the interactions (Fig. 1D, Top). As expected, BDNF-stimulated phosphorylation of TrkB (p-TrkB), and its downstream p-Akt and p-MAPK signals, was strongly blunted by K252a. Notably, BDNF strongly elicited α-Syn Y125 phosphorylation regardless of the treatment with K252a (Fig. 1D, Bottom). Interestingly, we found that α-Syn also bound to TrkB in human cortex samples from brains of patients with Lewy body dementia (LBD) but not in control brain tissue samples (Fig. 1E). Immunofluorescent staining also verified that TrkB colocalized with α-Syn in LBs in PD patients with p-TrkB signals much dimmer than total TrkB levels (Fig. 1F). Hence, α-Syn interacts with TrkB, which can be inhibited by BDNF treatment.

α-Synuclein Inhibits BDNF-Mediated TrkB Signaling. To examine the biological effect of α-Syn on BDNF/TrkB signaling, we transfected BR6 cells (SH-SY5Y cells stably transfected with the TrkB receptor) with α-Syn, followed by BDNF stimulation. BDNF-induced phosphorylation of TrkB and its downstream effectors was blocked by α-Syn overexpression (Fig. 2A). We also extended the experiment into primary cortical neurons from SNCA transgenic overexpressing mice or SNCA KO mice. In wild-type neurons, BDNF elicited prominent p-TrkB/p-Akt/p-MAPK signaling, which was significantly suppressed in SNCA transgenic neurons. In SNCA KO neurons, the BDNF-triggered signaling cascade remained intact, though the temporal onset of TrkB activation was delayed (Fig. 2B). One of the key physiological functions of the BDNF/TrkB pathway is to promote neuronal survival. Consequently, we performed a lactate dehydrogenase (LDH) release cytotoxicity assay to quantify spontaneous cell death. The LDH assay demonstrated that SNCA transgenic neurons exhibited much higher spontaneous cell death compared with wild-type or SNCA KO neurons (Fig. 2C). Treatment of primary neurons with BDNF significantly repressed neuronal cell death regardless of genotype (Fig. 2D). To investigate how α-Syn affects BDNF-mediated neuroprotection in the face of PD-associated pathology, we next treated primary neurons with the neurotoxicant 1-methyl-4-phenylpyridinium (MPP+) and analyzed LDH release. MPP+-induced LDH release was strongly reduced in both wild-type and SNCA KO neurons by BDNF treatment. However, the neuroprotective effect of BDNF was much weaker in SNCA transgenic neurons (Fig. 2D). To further investigate the biological role of α-Syn in BDNF-mediated neuronal survival, we transduced primary cortical neurons with AAV-expressing human α-Syn. And neurons underwent BDNF deprivation (by treatment with an anti-BDNF antibody) either in the presence or absence of MPP+. Within neurons transduced with the AAV-GFP control vector, depletion of BDNF diminished p-TrkB, MPP+ also reduced p-TrkB, and the combination of both BDNF depletion and MPP+ treatment resulted in an additive effect to further decrease p-TrkB (Fig. 2E, second panel, left four lanes). Strikingly, AAV-mediated overexpression of α-Syn strongly repressed BDNF-induced phosphorylation of TrkB. The combination of α-Syn overexpression with MPP+ treatment completely abolished p-TrkB levels, and importantly this effect was independent of the presence or absence of BDNF. Moreover, total TrkB levels were reduced following α-Syn overexpression (Fig. 2E, top two panels). The downstream phosphorylation of Akt followed the p-TrkB pattern. P-MAPK displayed similar effects, except that p-MAPK 42 was not further diminished in the AAV–α-Syn-infected neurons treated with anti-BDNF (Fig. 2E, panels 3–6). Immunoblotting of α-Syn showed increased α-Syn, which was prominently displayed as oligomers in the presence of MPP+ and anti-BDNF. Overexpression of α-Syn induced notable oligomerization (Fig. 2E, Bottom). We next analyzed cytotoxicity within these treatment groups. The LDH assay revealed an inverse correlation between levels of p-TrkB/p-Akt and LDH release. Neurons transduced with AAV–α-Syn exhibited more cell death
α-Synuclein Blocks TrkB Signaling by Inhibiting Its Internalization and Lipid Raft Distribution. TrkB receptors reside within lipid rafts, and this localization is mediated by Fyn tyrosine kinase. Disrupting lipid rafts prevents the full activation of TrkB (21). To test whether α-Syn mediates TrkB cellular localization, we conducted a subcellular fractionation experiment. We prepared primary cortical neuronal cultures and transduced the cells with AAV overexpressing α-Syn or a short-hairpin RNA (shRNA) targeting endogenous α-Syn, followed by BDNF treatment for 0, 15, or 60 min. In control neurons, BDNF treatment resulted in a TrkB enrichment in fraction #2, where Fyn was also eluted (i.e., this fraction also contained lipid rafts, as Fyn is also used as a marker for these; Fig. 3A). In addition, TrkB and α-Syn coeluted in fraction #7 and #8. When α-Syn was overexpressed, it distributed into fraction #2 as well. Notably, TrkB levels in lipid raft fractions were clearly reduced following α-Syn overexpression, whereas Fyn levels remained intact. Following α-Syn knockdown, TrkB levels in fraction #2 were increased upon BDNF stimulation, similar to control cells (Fig. 3A). The NT–TrkB complex internalization is essential for the signal transduction that initiates the cellular response to target-derived NTs, and this process is regulated through clathrin-mediated endocytosis, leading to the formation of signaling endosomes (22, 23). Next, we examined whether α-Syn also regulates TrkB internalization in BR6 cells. BDNF strongly stimulated TrkB internalization in control cells and α-Syn shRNA-treated cells, associated with robust p-TrkB, p-Akt, and p-MAPK signals. Nonetheless, these effects were substantially antagonized by α-Syn overexpression (Fig. 3B). To further assess the effect of α-Syn on TrkB endocytosis, we performed immunofluorescent staining to monitor the subcellular localization of TrkB as well as the colocalization of TrkB with EEA1, a marker for early endosomes. BDNF induced the internalization of both TrkB and p-TrkB as indicated by colocalization with EEA1. These effects were reduced in SNCA transgenic neurons (Fig. S1). The trafficking of NT/Trk complexes within the endosome plays a critical role in neurotrophic signaling cascade. Accordingly, we performed a live-cell imaging assay using wild-type, SNCA transgenic, and SNCA KO dopaminergic neurons. We found that the mobility of TrkB fluorescent particles within the axon of SNCA4 overexpressing transgenic neurons was significantly impaired compared with that observed in wild-type or SNCA4 KO cells (Fig. 3C), fitting with previous reports that formation of α-Syn LN-like aggregates in axons impedes the transport of distinct endosomes (24).

α-Synuclein Blocks the Prosurvival Effects of BDNF/TrkB in Dopaminergic Neurons. To explore the biologic effect of α-Syn-mediated blocking of BDNF/TrkB neurotrophic activities in DAergic neuronal survival, we stereotaxically delivered AAV-GFP into the left SNpc and AAV-BDNF into the right SNpc of WT SNCA KO or SNCA transgenic overexpressing mice. Mice then received a daily i.p. injection of MPTP (30 mg/kg) treatment for 5 d. Immunofluorescent staining demonstrated that GFP and BDNF were appropriately expressed in the respective SNpc regions (Fig. S2A). BDNF– expression induced the p-TrkB/p-Akt/p-MAPK signaling cascade in WT and SNCA KO mice, however this effect was suppressed in SNCA transgenic mice (Fig. S2B). As expected, MPTP administration induced substantial DAergic neurodegeneration in all of the genotypes, but tyrosine hydroxylase (TH)-positive cell loss was significantly greater in SNCA transgenic mice compared with WT or SNCA KO mice (Fig. 4 A–C). Immunoblotting analysis demonstrated that TH immunoreactivity was reduced in SNCA transgenic mice compared with wild-type or SNCA4 KO mice, and MPTP treatment almost completely eliminated TH immunoreactivity in SNCA transgenic mice (Fig. 4D). Hence, α-Syn inhibits TrkB signaling, rendering DAergic neurons more vulnerable to the neurotoxin MPTP.

Disruption of α-Syn/TrkB Association Promotes Dopaminergic Neuronal Survival. The DA metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) induces α-Syn binding to TrkB, leading to suppression of TrkB neurotrophic signaling and escalation of DAergic neurodegeneration (Fig. S3). Next, we tested whether the inhibition of DOPAL by MAO-B inhibitor (rasagiline) could block α-Syn/TrkB association, promoting DAergic neuron survival in vivo as well as in BR6 cells (Fig. S4). We injected mice with AAV-GFP or AAV–α-Syn into the left and right substantia nigra (SN) of the same mice, respectively, followed by rasagiline administration. As expected, overexpression of α-Syn elicited DAergic neurodegeneration in the SN and nigrostriatal denervation of the striatum compared with the expressing hemisphere. This effect was alleviated by rasagiline treatment (Fig. 5A–C). Consequently, DA concentrations were significantly elevated in both SN and striatum (Fig. 5D and E). Since MAO-B was potently inhibited by rasagiline, its oxidation product DOPAC was substantially reduced (Fig. 5 F and G). Motor behavioral assays indicated that α-Syn–induced motor dysfunctions were rescued by rasagiline (Fig. 5 H–J). Moreover, immunoprecipitation assays showed that rasagiline strongly inhibited the association between α-Syn and TrkB, restoring BDNF/TrkB neurotrophic signaling (Fig. 5K).
Conceivably, rasagiline disrupts α-Syn/TrkB complex via inhibiting MAO-B–produced DOPAL.

Discussion

In the current study, we provide compelling evidence that α-Syn directly interacts with the TrkB receptors. This interaction is in turn negatively regulated by BDNF and Fyn tyrosine kinase activity, resulting in the phosphorylation of α-Syn on Y125, causing it to dissociate from TrkB receptors (Fig. S5). Strikingly, α-Syn binds TrkB and completely suppresses its neurotrophic activities, increasing the vulnerability of DA neurons to degeneration. Moreover, α-Syn potently reduces TrkB protein levels by stimulating TrkB ubiquitination (Fig. S6). The data presented herein suggest that α-Syn blocks TrkB signaling by diminishing TrkB endocytosis, internalization, and axonal transport. Though α-Syn overexpression in neurons from SNCA overexpressing transgenic mice greatly inhibits BDNF/TrkB neurotrophic signaling, the absence of α-Syn in neurons of SNCA KO mice has little effects on these events. Here, we observed that α-Syn KO exhibit a slight delay in the kinetics of BDNF-induced phosphorylation of TrkB and its downstream effectors. This delay did not affect the neurotrophic activity of BDNF, as the effects of BDNF treatment were comparable in both wild-type and SNCA KO DA neurons in the face of MPP+-induced neurotoxicity (Fig. 2 C–F). These observations indicate that SNCA is not required for BDNF/TrkB neurotrophic activities, despite the finding that increased levels of α-Syn can inhibit BDNF neurotrophic signaling. Previous studies indicate that BDNF stimulates the association between endogenous TrkB and Fyn. In neurons derived from Fyn knockout mice, the translocation of TrkB to lipid rafts in response to BDNF is compromised, while inhibiting TrkB translocation to lipid rafts prevents the full activation of TrkB and downstream signals (21). In the current study, we replicated the finding that BDNF treatment translocates TrkB to lipid rafts and extended these findings to show that α-Syn overexpression diminishes TrkB lipid raft residency, whereas α-Syn depletion does not affect TrkB subcellular distribution. Using TrkB stably transfected dopaminergic SH-SY5Y cells, we showed that BDNF-triggered TrkB internalization is abolished by α-Syn overexpression. These findings are consistent with previous reports that BDNF-induced TrkB accumulation at lipid rafts is prevented by blocking the internalization of TrkB (21). Conceivably, BDNF treatment activates Fyn, which subsequently phosphorylates α-Syn on Y125, resulting in a dissociation from TrkB receptors and restores p-TrkB signaling. Communoprecipitation assay with anti-α-Syn from SN tissues treated with or without rasagiline and coprecipitated proteins were analyzed by immunoblotting. SN lysates were probed by various indicated antibodies.

Fig. 5. Rasagiline disrupts α-Syn/TrkB complex and rescues dopaminergic neurons from α-Syn–induced cell death. (A) Rasagiline reduces TH loss induced by α-Syn. C57BL/6 mice were injected with AAV-GFP or AAV–α-Syn into the left and right SN, respectively, followed by rasagiline (3 mg/kg) treatment for 10 d. TH expression in SN and striatum was analyzed by immunofluorescent staining. (Scale bar, 200 μm.) (B and C) Quantification of TH-positive fluorescent signaling in SN (B) and striatum (Str) (C). Data are shown as mean ± SEM. n = 6 sections each group. *P < 0.05. (D and E) DA concentrations in SN and striatum were increased by rasagiline in α-Syn–overexpressed mice. (F and G) DA metabolite DOPAC concentrations in SN and striatum were decreased by MAO-B inhibitor, rasagiline, in α-Syn–overexpressed mice. Data are shown as mean ± SEM. n = 3 each group. *P < 0.05. (H–J) Motor behavioral assays. Overexpression of α-Syn induced motor impairment, and rasagiline significantly improved the motor functions. Data are shown as mean ± SEM. n = 6 each group. *P < 0.05, **P < 0.01. (K) Rasagiline disrupts α-Syn/TrkB complex and restores p-TrkB signaling. Communoprecipitation assay with anti–α-Syn from SN tissues treated with or without rasagiline and coprecipitated proteins were analyzed by immunoblotting. SN lysates were probed by various indicated antibodies.
to TrkB receptors, completely blocking TrkB neurotrophic signal- naling, a phenomenon which was partially attenuated by BDNF. Accordingly, α-Syn overexpression-induced neuronal cell death was further escalated by DOPAL. Moreover, blocking DA bio-
synthesis by α-methyl-p-tyrosine (AMPT), which inhibits TH activity, significantly attenuates α-Syn–elicited neuronal cell death (Fig. S3). Elevation of DOPAL levels may contribute to the specific vulnerability of DAergic neurons to complex I inhibition (29). DOPAL covalently modifies α-Syn and stimulates its ag-
gregation and is neurotoxic in vivo (30). Fitting with in vitro results (Fig. S4), we found that rasagiline strongly dissociates the α-Syn/ TrkB complex in the SNpc of mice injected with AAV–α-Syn or MPTP, resulting in a subsequent up-regulation of p-TrkB/p-Akt/ p-MAPK signaling. Consequently, rasagiline significantly attenuated α-Syn–induced D4ergic neuronal death in the SN with the concomitant preservation of DA terminals within the striatum and improvements in motor functions. These findings are consistent with previous reports that rasagiline promotes regeneration of SN dopaminergic neurons in post–MPTP-induced parkinsonism via activation of tyrosine kinase receptor signaling pathway (31, 32).

Here we show that α-Syn binds to, and inhibits, TrkB neuro-
 trophic activities. The current study provides a pathologic func-
tion for α-Syn in the binding of the TrkB receptor and resultant inhibition of BDNF/TrkB neurotrophic signaling. This interaction is strongly up-regulated by DA’s metabolite DOPAL, resulting in increased dopaminergic neuronal death. This discovery provides a model for the underlying molecular etiology of α-Syn–mediated neurotoxicity and D4ergic neuronal loss in PD.

Methods

Animals. All mice were obtained from the Jackson Laboratory. Eight- to 12-wk-
old C57BL/6 were used as controls. SNCA null mice (B6;129 x 1-SncaFtm1Rosl stock no. 003692) or human SNCA overexpressing mice [B6.Cg-Tg(SNCA)Ovx37Rwm Sncatm1Rosl/J, stock no. 023837] on pure genetic background were backcrossed. Identification was performed by PCR using genomic DNA isolated from the tail tip. PCR was performed using mutant primers F (5′-TCA GCC ACC ATA AAA CTG AGG3′), R (5′-GCC TGA AGA AGC TGA TCA G3′) and transgene primers F (5′-CCT CTT GGT AGC TGG GCT TT3′), R (5′-ACC CCC TTC TGG GTT GTT TT3′). Animal care and handling were performed according to the Declaration of Helsinki and Emory Medical School guidelines. Investigators were blinded to the group allocation during the animal experiments. The protocol was reviewed and approved by the Emory Institutional Animal Care and Use Committee.

Human Tissue Samples. Postmortem brain frozen samples of LBD patients (n = 3) and paraffin-embedded sections of PD patients (n = 3) were provided from the Emory Alzheimer’s Disease Research Center. The study was approved by the biospecimen committee at Emory University. LBD and PD cases were clinically diagnosed and neuropathologically confirmed. Informed consent was obtained from all subjects.

Plasmid Clones and Viral Genomes. AAV viral genome contained either the human α-syn or the GFP coding sequence controlled by the hybrid chicken beta-actin/cytomegalovirus promoter. AAV2/SV and LV pseudotyped with VSV-G was produced as described previously (33).

Statistical Analysis. Statistical analysis was performed using either Student’s t test (two-group comparison) or one-way ANOVA followed by LSD post hoc test (more than two groups), and differences with P values less than 0.05 were considered significant.

ACKNOWLEDGMENTS. We thank Alzheimer’s Disease Research Center at Emory University for human PD and LBD patients and healthy control sam-
ple. This work was supported by M. J. FOX Foundation Grant 11137 and NIH Grant RF1 AG051538 (to K.Y.); National Key Basic Research Program of China Grant 2010CB945202 (to Y.E.S.) and NSFC Grants 81461138037, 31471029, and 31671055 (to J.X.).