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β-synuclein modulates α-synuclein neurotoxicity by reducing α-synuclein protein expression

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by fibrillar aggregates of α-synuclein in characteristic inclusions known as 'Lewy bodies'. As mutations altering α-synuclein structure or increasing α-synuclein expression level can cause familial forms of PD or related Lewy body disorders, α-synuclein is believed to play a central role in the process of neuron toxicity, degeneration and death in 'synucleinopathies'. β-synuclein is closely related to α-synuclein and has been shown to inhibit α-synuclein aggregation and ameliorate α-synuclein neurotoxicity. We generated β-synuclein transgenic mice and observed a marked reduction in α-synuclein protein expression in the cortex of mice over-expressing β-synuclein. This reduction in α-synuclein protein expression was not accompanied by decreases in α-synuclein mRNA expression. Using the prion protein promoter α-synuclein A53T mouse model of PD, we demonstrated that over-expression of β-synuclein could retard the progression of impaired motor performance, reduce α-synuclein aggregation and extend survival in doubly transgenic mice. We attributed the amelioration of α-synuclein neurotoxicity in such bigenic mice to the ability of β-synuclein to reduce α-synuclein protein expression based upon I^{125} autoradiography quantification. Our findings indicate that increased expression of β-synuclein protein results in a reduction of α-synuclein protein expression. As increased expression of α-synuclein may cause or contribute to PD pathogenesis in sporadic and familial forms of disease, this observation has important implications for the development of therapies for PD.

INTRODUCTION

A key turning point in our understanding of Parkinson's disease (PD) and related disorders characterized by Lewy body inclusions came in 1997 when mutations in the α-synuclein gene were found to co-segregate with an autosomal dominant form of familial PD (1). Soon after this discovery, analysis of Lewy body inclusions revealed that α-synuclein is a major component of these proteinaceous deposits, forming the central core of the Lewy body (2). Interestingly, the α-synuclein protein had already been implicated in the process of protein aggregation in Alzheimer's disease, as amino acid residues 61–95 in the mid-portion of the α-synuclein protein had been identified as the so-called 'non-amyloid component' (NAC) of amyloid plaques (3). The presence of α-synuclein within Lewy body inclusions thus implicated this protein in the cellular pathology of PD and a host of related disorders (now known as 'synucleinopathies') characterized by accumulation of α-synuclein fibrillar deposits (4,5).

The α-synuclein protein is a small polypeptide, consisting of just 140 amino acids, that is highly expressed in presynaptic nerve terminals (6). α-synuclein is a member of a family of at least three proteins that include α-synuclein, β-synuclein and γ-synuclein (7). Although the members of the synuclein family share considerable sequence homology, α-synuclein is unique in its possession of a highly amyloidogenic amino acid domain within its NAC region. Indeed, α-synuclein is

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the only member of the synuclein family to display a strong propensity to form fibrillar structures *in vitro* (8). Of the other synuclein family members, β-synuclein is the most closely related and also shows a highly overlapping subcellular pattern of expression within the central nervous system, localizing to presynaptic nerve terminals (6,7). Although β-synuclein does not co-localize with α-synuclein in Lewy body inclusions (9), a role for β-synuclein in modulating α-synuclein pathology has been suggested by a variety of reports. Two independent studies of α-synuclein fibrillization have shown that β-synuclein can inhibit the process of α-synuclein aggregation and fibril formation *in vitro* (10,11), whereas analysis of β-synuclein transgenic mice crossed with α-synuclein transgenic mice has suggested that β-synuclein over-expression can reduce α-synuclein aggregation and toxicity *in vivo* (12). This potential anti-amyloidogenic effect of β-synuclein upon α-synuclein led us to screen familial and sporadic dementia with Lewy bodies (DLB) patients for mutations in β-synuclein, yielding two unique, non-conservative amino acid alterations in the β-synuclein gene in unrelated DLB probands (13). These findings suggest a role for β-synuclein in synucleinopathy disorders involving α-synuclein misfolding and aggregation.

To better understand the role of β-synuclein in neurodegeneration and in the genesis of α-synuclein neuropathology, we have produced lines of transgenic mice that over-express wild-type β-synuclein. We report that β-synuclein transgenic mice are phenotypically normal. However, upon careful analysis of endogenous murine α-synuclein expression, we found that over-expression of β-synuclein reduces expression of α-synuclein at the protein level without affecting its RNA levels. When we crossed our β-synuclein transgenic mice with the α-synuclein-A53T prion protein (PrP) promoter mouse model of PD (14), we observed a marked improvement in behavioral phenotype, neuropathology and survival and correlated these findings with a reduction in α-synuclein protein expression. As increased α-synuclein expression levels can produce PD and DLB phenotypes in humans, modulation of α-synuclein protein metabolism by β-synuclein may represent a viable option for therapeutic intervention in synucleinopathy disorders.

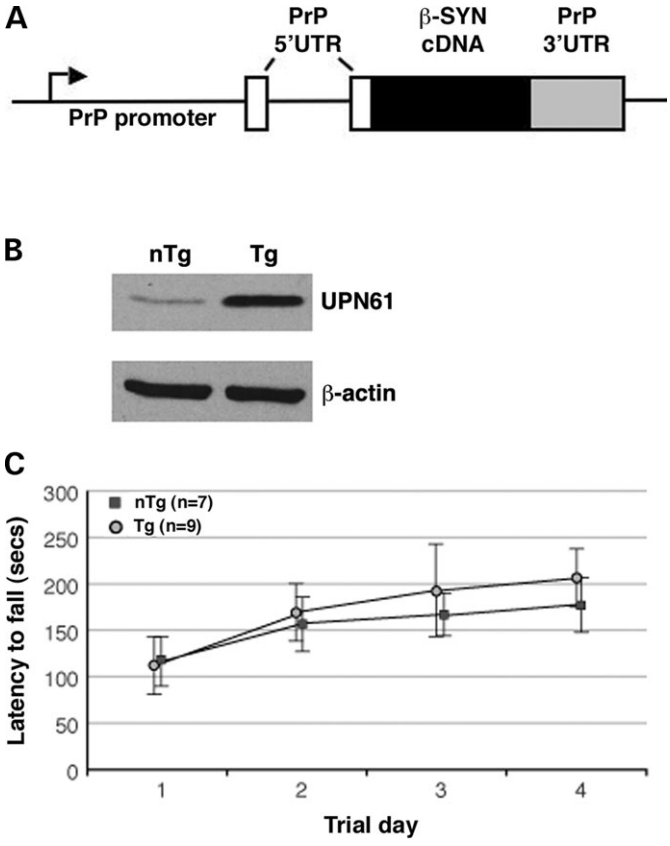

Figure 1. Generation and characterization of β-synuclein transgenic mice. (**A**) Diagram of the transgene vector. Wild-type human β-SYN cDNA was cloned into the *Xho*I site of the murine PrP promoter (MoPrP) expression vector (31). The resulting construct contains the murine PrP promoter, the 5′-UTR of the PrP gene with an intron, the human β-SYN cDNA and the 3′-UTR of the PrP gene. (**B**) Representative western blot probed with the anti-β-synuclein (UPN61) or anti-β-actin antibody. Here, we see the results for cortex samples obtained from age-matched (3-month-old) non-transgenic control (nTg) and PrP-β-SYN 7500 (Tg) mice. (**C**) Rotarod test to assess coordination function. Cohorts of 14-month-old PrP-β-SYN 7500 (Tg; $n = 9$) and non-transgenic (nTg) littermate control mice ($n = 7$) were placed on an accelerating rotarod apparatus. Latency-to-fall times were not different between the two groups ($P = 0.46$ by ANOVA).

RESULTS

Production and characterization of β-synuclein transgenic mice

To evaluate the role of β-synuclein in neurodegeneration, we generated transgenic mice that over-express wild-type human β-synuclein (β-SYN) by inserting the cDNA for human β-synuclein into the murine PrP expression vector (Fig. 1A). We thereby derived two PrP-β-SYN transgenic lines that express β-SYN protein at levels greater than endogenous murine β-synuclein (β-syn) (Fig. 1B). These two lines, β-SYN 7500 and β-SYN 8272, respectively, over-express β-SYN at ~8× and ~2.5× endogenous β-syn on the basis of western blot and RT–PCR analysis. We therefore expanded the β-SYN 7500 and β-SYN 8272 transgenic lines and characterized them for behavioral or histopathological abnormalities. We studied multiple cohorts of transgenic and non-transgenic littermate controls at 14 months of age and noted no visible phenotype, clasping upon tail suspension, or movement disorder. There were no significant differences in size, weight or activity level (data not shown). We performed rotarod analysis upon cohorts of β-SYN 7500 and β-SYN 8272 transgenic mice and observed no significant difference in rotarod performance (Fig. 1C). We also examined cortical sections from β-SYN 7500 and β-SYN 8272 transgenic mice at 16 months of age for β-SYN protein aggregation in light of a previous report of such aggregates in DLB patients (15), but did not detect any evidence of β-SYN aggregation in transgene-positive individuals (data not shown). We have continued to observe β-SYN 7500 and β-SYN 8272 transgenic mice and report that they have normal life-spans, do not display any signs of neurological abnormality and retain normal weight until death.

Reduction of α-synuclein protein expression levels in PrP-β-SYN mice

In a previous study of β-SYN transgenic mice, a role for β-synuclein in the regulation of α-synuclein conformation was proposed on the basis of the observation of decreased α-synuclein aggregation, α-synuclein–β-synuclein co-immunoprecipitation and improved behavioral phenotype in α-synuclein–β-synuclein doubly transgenic mice (12). In that report, β-SYN transgene expression from the mThy-1 vector did not affect platelet-derived growth factor B (PDGF-B) promoter-driven wild-type human α-synuclein (α-SYN) transgene expression at the RNA or protein level; expression of endogenous murine α-synuclein (α-syn), however, was not measured (12). When we analyzed the expression of endogenous α-syn in the cerebral cortex of our PrP-β-SYN transgenic mice, we documented no effect upon α-syn mRNA expression, but observed a marked reduction of α-syn expression at the protein level in the higher expressing PrP-β-SYN 7500 transgenic line (Fig. 2A–E). Using I^{125} autoradiography to accurately quantify this reduction, we performed a temporal profile of α-syn protein expression in the PrP-β-SYN 7500 transgenic line. We documented significant reductions in α-syn protein expression from 5–12 months of age in PrP-β-SYN transgenic mice (Fig. 3). I^{125} autoradiography analysis of endogenous α-syn protein expression in PrP-β-SYN 8272 transgenic mice, which over-express less β-synuclein, also revealed reductions in α-syn protein, but these reductions were modest in comparison to PrP-β-SYN 7500 mice (Fig. 2A–D).

Over-expression of β-SYN ameliorates the disease phenotype in PrP-α-SYN-A53T mice

To test the hypothesis that β-synuclein might be modulating α-synuclein protein metabolism to improve Parkinsonian-like phenotypes and synuclein histopathology, we obtained the PrP-α-SYN-A53T transgenic (line M83) mouse model of PD (14). In this model, homozygous PrP-α-SYN-A53T (but not wild-type) transgenic mice develop a severe and complex motor phenotype at 8–12 months of age and succumb to their disease usually by 16 months of age (14). We crossed heterozygous PrP-β-SYN 7500 transgenic mice (β-SYN+/−) with homozygous PrP-α-SYN-A53T transgenic mice (α-SYN-A53T+/+) to derive double heterozygous progeny (α-SYN-A53T+/−; β-SYN+/−). α-SYN-A53T+/−; β-SYN+/− mice were then backcrossed with α-SYN-A53T+/+ to yield cohorts of α-SYN-A53T+/+; β-SYN+/− and α-SYN-A53T+/+; β-SYN−/− transgenic mice. Prior to 10 months of age, only a few mice from any cohort displayed an obvious phenotype. However, by 11 months of age, considerable numbers of mice became hypoactive and tremulous. When we compared rotarod performance at 11 months of age, we found that α-SYN-A53T+/+; β-SYN+/− transgenic mice did not display a deficit, whereas α-SYN-A53T+/+; β-SYN−/− transgenic mice exhibited a significant impairment (Fig. 4A). We charted survival, and although all α-SYN-A53T+/+; β-SYN−/− mice died between 10–15 months of age (as expected on the basis of their initial characterization) (14), more than half of the α-SYN-A53T+/+; β-SYN+/− cohort were still alive at 16 months of age (Fig. 4B). We also examined α-SYN aggregation in subcortical regions of the brain at 12 months of age and observed a significant reduction in α-SYN aggregate burden in the α-SYN-A53T+/+; β-SYN+/− transgenic mice (Fig. 5A–D). Thus, as previously reported (12), we found that over-expression of human β-synuclein could ameliorate α-synuclein-induced behavioral abnormalities and neuropathology in a mouse model of α-synuclein neurotoxicity.

Over-expression of β-SYN lowers α-SYN protein expression in doubly transgenic mice

The mechanistic basis of β-synuclein amelioration of α-synuclein neurodegeneration *in vivo* remains unknown. To address this question in the context of our studies of α-SYN-A53T+/+; β-SYN+/− transgenic mice, we performed I^{125} quantitative western blot analysis of α-SYN protein expression on cortex samples obtained from 12-month-old homozygous α-SYN-A53T singly transgenic mice or doubly transgenic mice co-expressing β-SYN. Sequential fractionation of α-SYN yielded soluble protein and lipid-associated protein, and for both soluble α-SYN and lipid-associated α-SYN, we observed significant reductions in α-SYN protein levels in cortex samples from α-SYN-A53T+/+; β-SYN+/− transgenic mice (Fig. 6A–C). We also obtained RNA samples from the cortex of α-SYN-A53T+/+; β-SYN+/− transgenic mice and measured α-SYN RNA expression, but found no differences in α-SYN RNA expression between α-SYN-A53T+/+; β-SYN+/− and α-SYN-A53T+/+; β-SYN−/− mice (data not shown). To determine whether α-SYN might correspondingly modulate β-synuclein protein expression, we quantified endogenous β-synuclein protein in α-SYN+/+ transgenic mice, but did not observe any alterations in β-synuclein protein expression (Supplementary Material, Fig. S1). Comparison of β-synuclein protein expression between β-synuclein transgenic mice and α-SYN-A53T+/+; β-SYN+/− bigenic mice similarly showed no differences (data not shown).

DISCUSSION

Although the precise molecular basis of PD remains ill-defined, numerous independent lines of investigation strongly suggest a central role for α-synuclein in the pathogenesis of sporadic and inherited forms of classic PD: (i) three different amino acid alterations (A53T, A30P and E46K) of α-synuclein are known to co-segregate with PD in pedigrees displaying autosomal dominant inheritance (1,16,17); (ii) α-synuclein shows a propensity to form protofibrils and to aggregate in an oxidative cellular milieu that is believed to be characteristic of substantia nigra neurons destined to degenerate in PD (18,19); (iii) triplication of the α-synuclein gene in humans resulting in increased expression of α-SYN protein is sufficient to produce PD or DLB (20,21); (iv) representative animal models of PD-like phenotypes and neuropathology can be generated in fruitflies

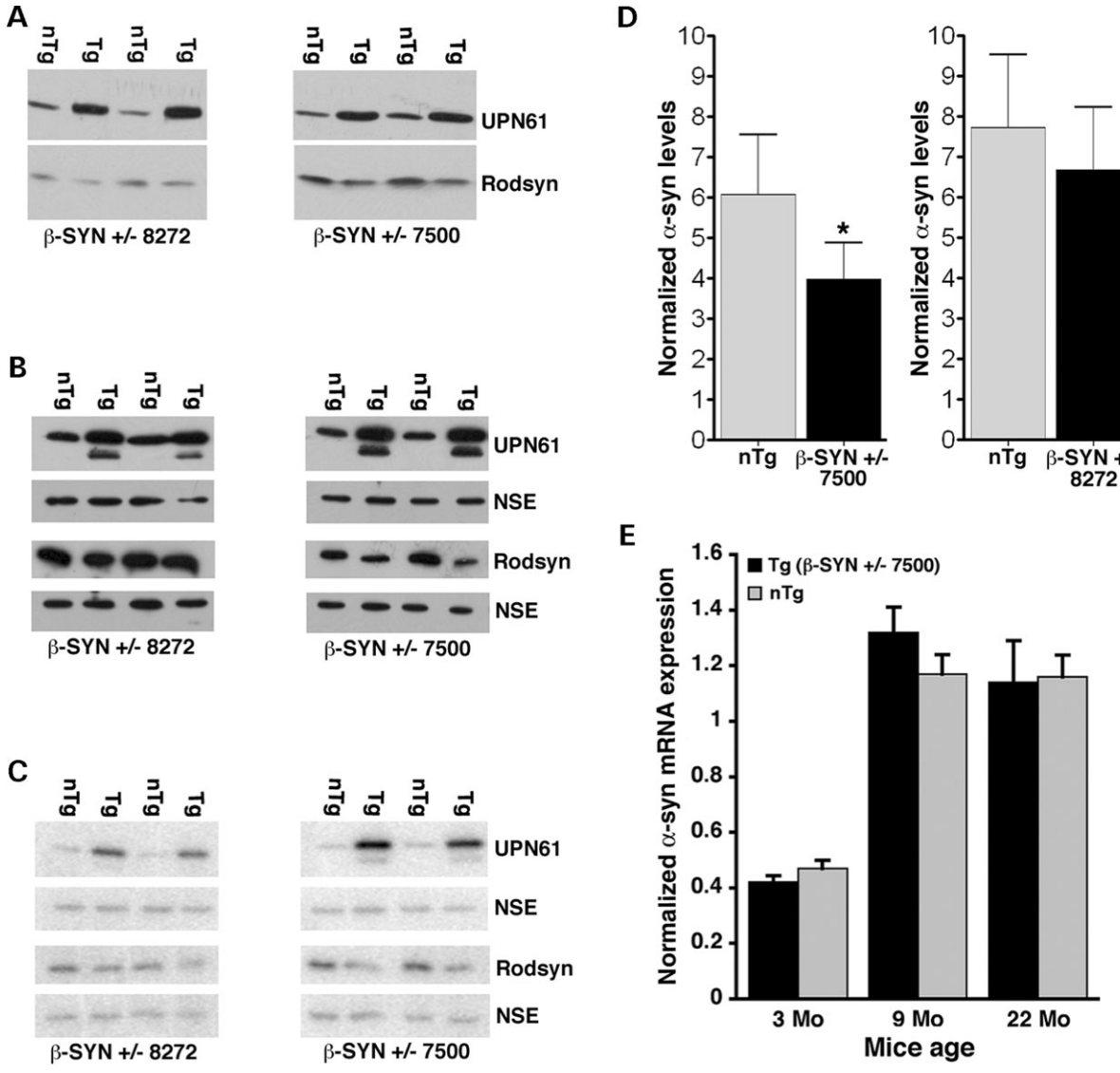

Figure 2. β-synuclein transgenic mice show reduced α-synuclein protein expression but display no change in α-synuclein RNA expression. (**A**) Soluble cortex protein lysates were analyzed by immunoblotting with an anti-β-synuclein antibody (UPN61) or an anti-α-synuclein antibody (Rodsyn). For transgenic (Tg) samples, β-synuclein protein expression is increased and α-synuclein protein is decreased when compared with age- and sex-matched littermate controls (nTg). Equivalent protein loading was confirmed by probing with anti-neuron-specific enolase (NSE) antibody (data not shown). (**B**) Unfractionated total brain protein lysates were analyzed by immunoblotting with anti-β-synuclein (UPN61), anti-α-synuclein (Rodsyn) or anti-NSE antibodies. Although reduction of α-synuclein protein is apparent for the PrP-β-SYN 7500 line, the decrease in α-synuclein protein expression is modest for the PrP-β-SYN 8272 line. (**C**) I^{125} immunoblotting of the samples in (B). (**D**) PhosphoImager analysis of the I^{125} immunoblotting normalized to NSE. PrP-β-SYN 7500 transgenic mice display a significant reduction in α-synuclein protein expression when compared with non-transgenic (nTg) littermate controls ($n = 7$/group; $P < 0.05$ by two-way ANOVA). Only a trend toward reduction of α-synuclein protein expression is apparent in the lower expressing PrP-β-SYN 8272 line ($n = 6$/group; $P = 0.37$ by two-way ANOVA). (**E**) Real-time RT–PCR analysis of α-synuclein gene expression in the cortex of β-synuclein transgenic mice. RNA samples were isolated from sets ($n = 4$/group) of PrP-β-SYN 7500 (Tg) and age- and sex-matched littermate controls (nTg) at the indicated ages and subjected to quantitative real-time RT–PCR. At all time points, α-synuclein RNA expression was comparable between Tg and nTg mice ($P > 0.05$ by Student's *t*-test). All reactions were performed in duplicate and normalized to β-actin RNA expression.

and mice by simply over-expressing normal or mutant forms of α-synuclein (22).

Given a likely central role for α-synuclein in the pathogenesis of disorders characterized by Lewy body pathology, we have focussed our attention upon the effect of β-synuclein on the α-synuclein toxicity pathway. There is indeed much evidence suggesting that β-synuclein may interact with or regulate α-synuclein. β-synuclein is highly homologous to α-synuclein, with >50% amino acid sequence identity in the N-terminal half of the two proteins (7). β-synuclein shows a nearly identical subcellular localization, as it is also enriched in presynaptic nerve terminals and displays extensive co-localization in both mouse and human brain (6). Although such commonalities are intriguing, most compelling is the observation that β-synuclein can prevent α-synuclein from forming protofibrils and fibrils (10,11). Furthermore,

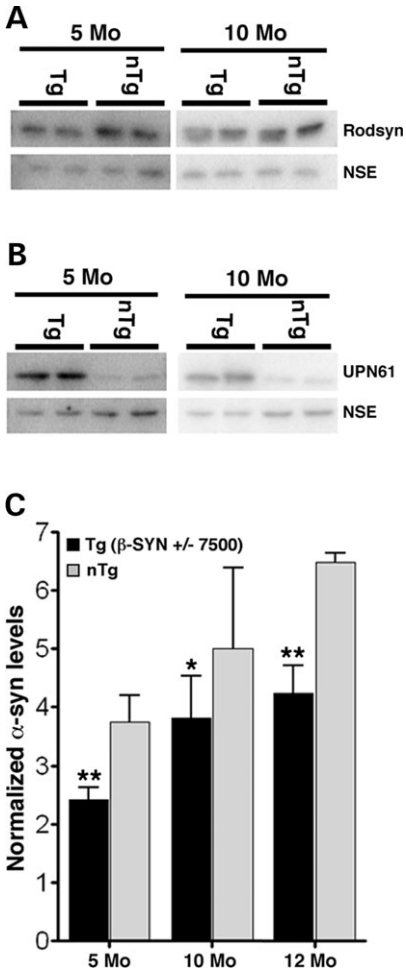

Figure 3. Quantification of α-synuclein protein expression in PrP-β-SYN 7500 mice reveals significant reductions throughout adulthood. (**A**) Representative I^{125} immunoblots of total cortex lysates probed with antibodies for α-synuclein (Rodsyn) or NSE. (**B**) Representative I^{125} immunoblots of total cortex lysates probed with antibodies for β-synuclein (UPN61) or NSE. (**C**) Comparison of α-synuclein protein expression for sets of PrP-β-SYN 7500 transgenic mice and age- and sex-matched non-transgenic (nTg) littermate controls ($n = 4–5$/group). α-synuclein protein expression was measured by I^{125} immunoblotting and normalized to NSE. Significant reductions in α-synuclein protein levels ranging from ~20 to 40% were noted in 5, 10 and 12-month-old PrP-β-SYN 7500 transgenic mice ($P < 0.001$, $P < 0.05$ and $P < 0.01$ by two-way ANOVA).

it appears that β-synuclein is capable of preventing aggregated α-synuclein from inhibiting the proteasome (23). Finally, independent evidence supporting a suppressor-like effect of β-synuclein upon α-synuclein neurotoxicity has come from studies of transgenic mice in which co-expression of β-synuclein with α-synuclein inhibited α-synuclein aggregation and ameliorated α-synuclein neuropathology (12).

To better understand the mechanistic basis of β-synuclein modulation of α-synuclein toxicity pathways, we generated independent lines of transgenic mice over-expressing wild-type β-synuclein. These mice displayed no phenotype and appear entirely normal throughout their lifespans, suggesting that increased dosage of β-synuclein is not pathogenic. We then examined whether over-expression of β-synuclein modulated α-synuclein expression and observed a dose-dependent reduction in α-synuclein protein expression, as more marked reductions in α-synuclein were consistently documented in our higher expressing line. Interestingly, reductions in endogenous α-synuclein protein expression were not accompanied by decreased levels of α-synuclein RNA based upon real-time RT–PCR quantification of α-synuclein gene expression. Our discovery of β-synuclein-dependent reductions in α-synuclein protein expression led us to test the hypothesis that this process might be responsible for the 'anti-Parkinsonian' effect of β-synuclein upon α-synuclein neurotoxicity reported in an earlier transgenic cross (12). Although we confirmed that transgenic over-expression of β-synuclein does indeed ameliorate α-synuclein neurotoxicity in mice, using in this case the PrP-α-SYN-A53T homozygous mouse model of PD for the cross (14), our analysis of α-synuclein protein expression suggested that the beneficial effect of β-synuclein over-expression is due to its ability to reduce α-synuclein protein expression. To insure the accuracy of our measurements, we employed I^{125} autoradiography for quantification of α-synuclein protein expression on cortex samples obtained from littermates who differed only in their possession of the β-synuclein transgene. We observed an ∼25% reduction in soluble α-synuclein protein and an ∼50% reduction in lipid-associated α-synuclein protein after sequential fractionation of the samples. As α-synuclein is a lipid-binding protein that is highly enriched in synaptic vesicles and membranes (6), this likely accounts for the greater reduction in the lipid-associated α-synuclein fraction. In a previous study of bigenic β-synuclein–α-synuclein transgenic mice, β-synuclein modulation of α-synuclein protein expression levels was not reported (12). Although the reasons for this are not entirely clear, the method for protein quantification in this earlier study involved soluble cytosolic fractions from brain homogenates. As the reduction in soluble α-synuclein protein expression, which we observed, is not as marked as for lipid-associated α-synuclein, and the earlier study employed a semi-quantitative approach, their methodology may not have been robust enough to detect any decreases. Furthermore, wild-type α-synuclein transgene expression driven by the PDGF-B promoter in that study was lower than in the PrP-α-SYN-A53T homozygous transgenic mice, making the detection of a significant alteration in α-synuclein protein expression even more difficult.

Our discovery of β-synuclein modulation of α-synuclein protein expression has important implications for the development of therapies for PD. If one accepts the model that α-synuclein over-expression is one pathway to neurotoxicity in PD, as suggested by the discovery of α-synuclein gene triplications in human PD families (20,21), then the prospect of down-regulating α-synuclein protein expression by increasing β-synuclein expression is an appealing treatment option. As β-synuclein does not form fibrils and does not produce any apparent abnormalities when over-expressed in transgenic mice, the likelihood for untoward side effects from such an intervention seems low. Advances in gene delivery strategies based upon improvements in viral

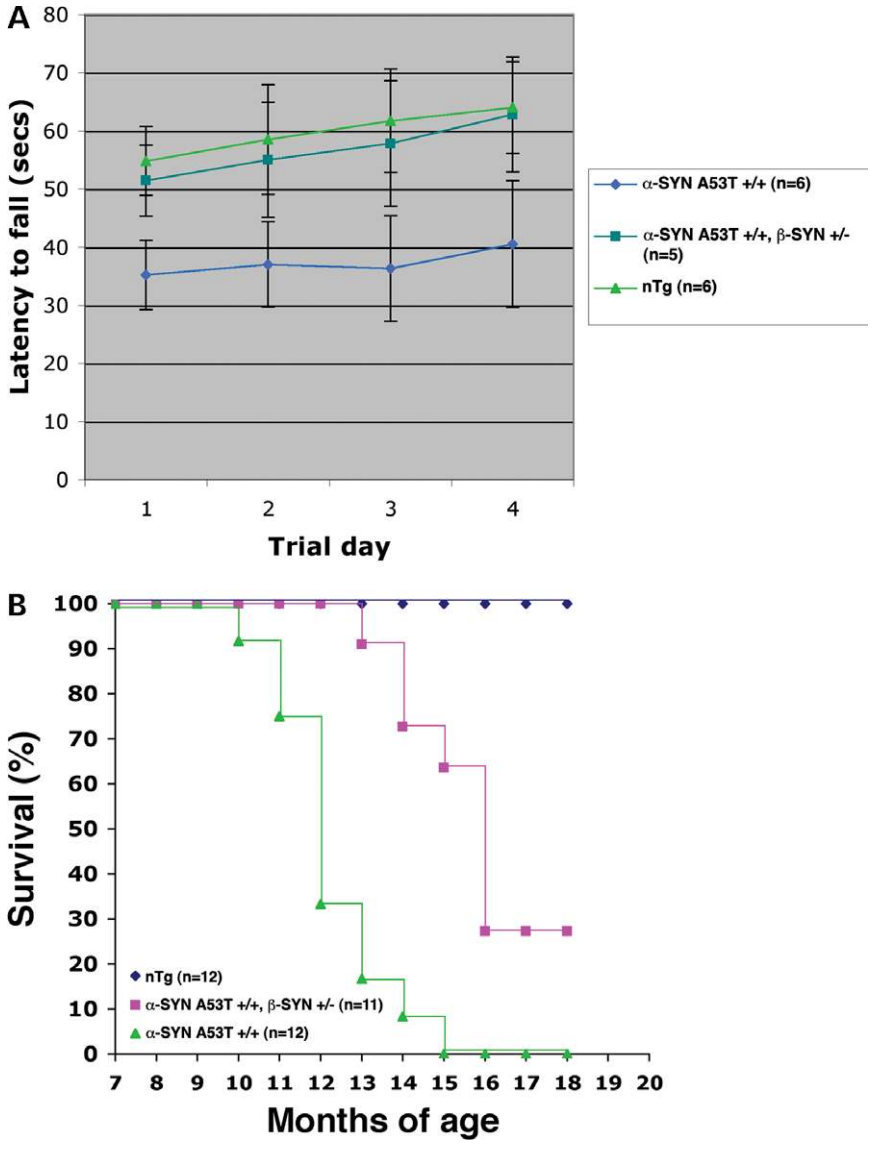

Figure 4. β-SYN rescues α-SYN neurotoxicity and extends survival in transgenic mice. (**A**) β-SYN retards the progression of impaired rotarod performance in α-SYN transgenic mice. Cohorts of 11-month-old α-SYN-A53T+/+; β-SYN+/− transgenic mice, α-SYN-A53T+/+(;β-SYN−/−) and non-transgenic (nTg) littermate mice were assessed for their ability to remain on the rotarod. α-SYN-A53T+/+; β-SYN+/− mice performed comparably to nTg controls and displayed significantly longer mean latency-to-fall times compared with α-SYN-A53T+/+(;β-SYN−/−) transgenic mice ($P < 0.01$ by two-way ANOVA). (**B**) β-SYN prolongs survival in α-SYN transgenic mice. Here, we see a Kaplan–Meier plot for survival for cohorts of α-SYN-A53T+/+; β-SYN+/− transgenic mice, α-SYN-A53T+/+(;β-SYN−/−) and non-transgenic (nTg) littermate mice. Beginning at 12 months of age, a marked difference in survival is apparent ($P < 0.005$ by Fisher's exact test), as the entire cohort of α-SYN-A53T+/+; β-SYN+/− transgenic mice were still alive, whereas half of the α-SYN-A53T+/+(;β-SYN−/−) transgenic mice had already expired by this time.

vectors (24), together with the small size of the β-synuclein gene, suggest that such gene therapy-based approaches could be feasible. Alternatively, screening drugs for their ability to up-regulate β-synuclein gene expression, as was done for the glutamate transporter GLT-1 in a recent study that yielded ceftriazone as a potential therapy for amyotrophic lateral sclerosis (25), would be another option. Of course, more direct modes of therapeutic intervention could be envisioned once the mechanistic basis of β-synuclein modulation of α-synuclein protein expression has been worked out. As α-synuclein and β-synuclein share considerable sequence homology, it is possible that the two proteins undergo identical post-translational modifications that regulate protein stability. For example, both α-synuclein and β-synuclein are phosphorylated on shared conserved residues and may be subject to proteolytic processing (26–28). If such events regulate protein stability, then increasing β-synuclein concentration in the presynaptic region may reduce access of α-synuclein to such modifying enzymes, thereby promoting its degradation, a process that may involve chaperone-mediated autophagy (29). Although such a model is speculative, at least one study has documented a balance between α-synuclein and β-synuclein protein expression levels in one synucleinopathy and proposed that

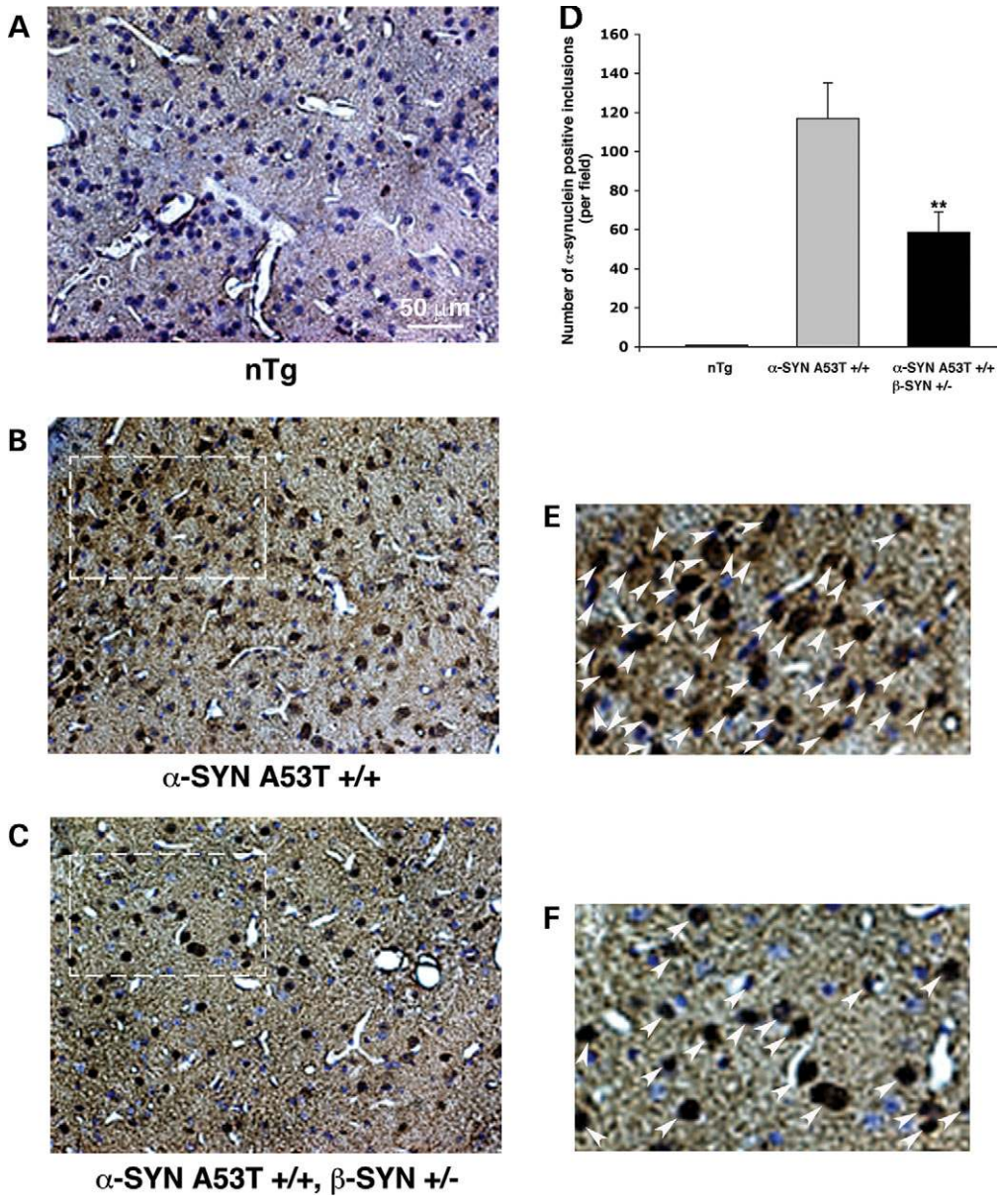

Figure 5. β-SYN reduces PD-like pathology in α-SYN transgenic mice. (**A**–**C**) Immunostaining of subcortical brain sections from 12-month-old transgenic mice. Subcortical sections were obtained from α-SYN-A53T+/+; β-SYN+/− transgenic mice, α-SYN-A53T+/+(; β-SYN−/−) and non-transgenic (nTg) littermate mice ($n = 3$/group) and immunostained with anti-human α-synuclein antibody Syn 303 (brown) and counterstained with hematoxylin (blue). Here, we see representative sections from the dorsal raphe nucleus for non-transgenic (A), α-SYN-A53T+/+(;β-SYN−/−) (B) and α-SYN-A53T+/+; β-SYN+/− (C) mice, revealing no α-synuclein-positive inclusions in nTg sections (A), but extremely numerous α-synuclein positive inclusions in α-SYN-A53T transgenic mice that do not co-express β-SYN (B). An obvious reduction in the number of α-synuclein positive inclusions in α-SYN-A53T–β-SYN bigenic mice is apparent (C). (**D**) Here, we see a summary of the numbers of α-synuclein positive inclusions per field counted from the immunostaining analysis shown in (A–C). For each individual, three fields per section were chosen, and three sections per individual were examined. Singly transgenic α-SYN-A53T transgenic mice display about twice as many α-synuclein positive inclusions per field as α-SYN-A53T transgenic mice co-expressing β-SYN ($P < 0.01$ by Student's t-test). The mean number of α-synuclein positive inclusions per field are shown with SEM. (**E**) Inset of (B) at high power to more clearly reveal α-synuclein positive inclusions (marked by arrowheads). (**F**) Inset of (C) at high power to more clearly reveal decreased number of α-synuclein positive inclusions (marked by arrowheads) in a doubly transgenic α-SYN-A53T–β-SYN mouse.

the reciprocal accumulation of α-synuclein and β-synuclein underlies aggregate formation (30). Future studies aimed at understanding how β-synuclein modulates α-synuclein protein stability or turnover thus seem warranted and may provide insights into how to develop targeted therapies for PD and related disorders.

MATERIALS AND METHODS

Transgenic construct

Human β-synuclein cDNA was obtained from pHBsyn (pcDNA3.1; Invitogen) by PCR amplification using primers h-β-syn-5′F (5′-gac ttt ccg cca cca tgg acg tgt tca tga agg

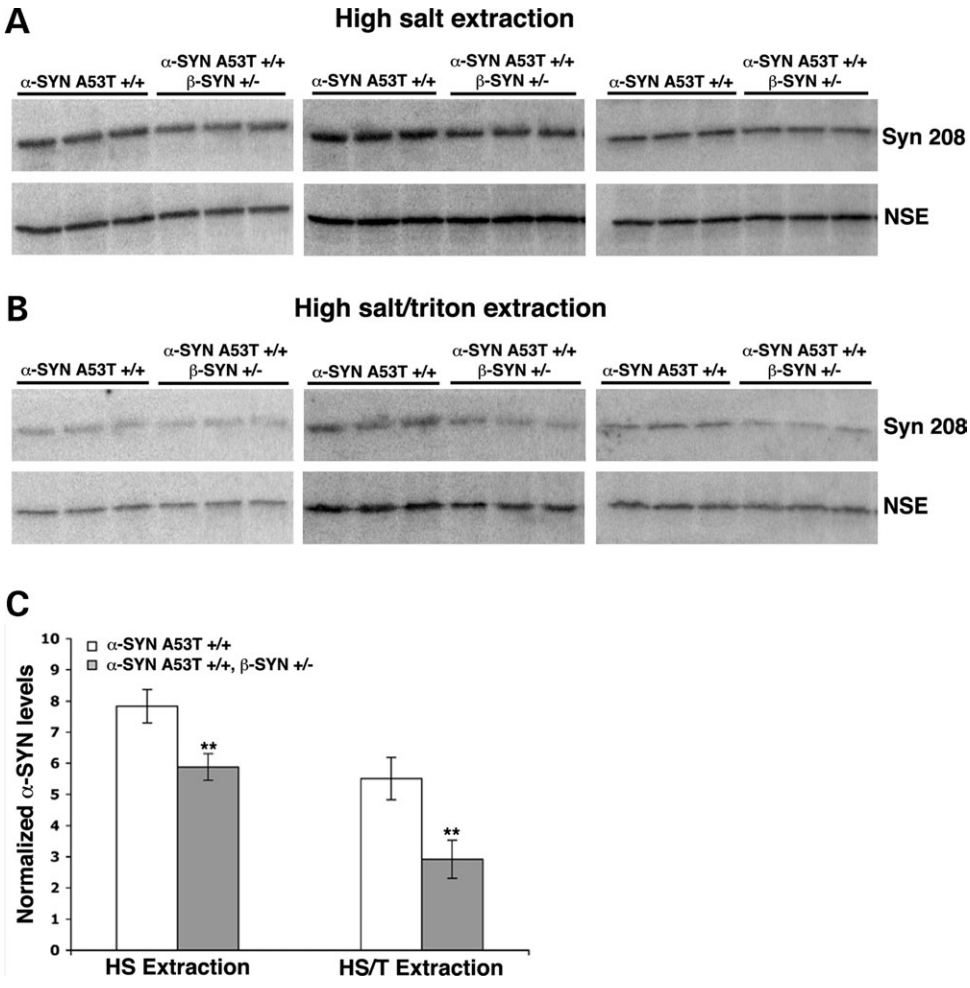

Figure 6. β-SYN down-regulates mutant α-SYN-A53T protein level *in vivo*. (**A** and **B**) Quantitative western blot analysis of mutant α-SYN-A53T protein using sequential biochemical fractionation and I^{125} quantification. Cortex protein lysates were prepared through HS extraction (A) and HS/Triton extraction (B) from sets ($n = 3$) of 12-month-old singly transgenic (α-SYN-A53T+/+), doubly transgenic (α-SYN-A53T+/+; β-SYN+/−) and non-transgenic (nTg) littermate control mice, immunoblotted with a human α-synuclein-specific antibody (Syn 208) and reprobed for NSE as a loading control. Each sample was loaded in triplicate, and blots were developed using a PhosphoImager. (**C**) Densitometric measurement of α-SYN-A53T and NSE bands. Comparison of α-SYN-A53T protein between the various cohorts revealed a significant decrease in α-SYN-A53T protein in the α-SYN-A53T+/+; β-SYN+/− group when compared with the α-SYN-A53T+/+(;β-SYN + /−) group ($P < 0.01$ by Student's *t*-test).

gcc tg-3′) and h-β-syn-3′R (5′-gac ttt ccg aat tcc cct acg cct ctg gct cat act c-3′). The PCR was performed with *Pfu Taq* polymerase (Stratagene), and the PCR product was ligated into the *Xho*I cloing site of the MoPrP expression vector (31). The reading frame was verified by PCR sequencing.

Generation of transgenic mice

Translational competence of the transgenic construct was verified by transient transfection into HEK293 cells using Lipofectamine 2000 (GIBCO-BRL). The vector backbone was removed by *Not*I digestion and then microinjected into oocyte pronuclei from C57BL/6J×B6C3 F1 hybrids. Founder mice were identified by PCR analysis of tail DNAs, using primers that flank both murine PrP (Prp-5′F: 5′-cct gag tat att tca gaa ctg aac-3′ and PrP-3′R: 5′-cgt aag ctg act cat tat cag agc tac-3′) and MoPrP vector sequences (Prp-5′F: 5′-cct gag tat att tca gaa ctg aac-3′ and PrP-3′R: 5′-cct caa ttg aaa gag cta cag gtg-3′). PCR amplification conditions are available upon request. Transgene-positive founders yield both 550 bp and 1 kb fragments, whereas transgene-negative founders only show the 1 kb fragment from the endogenous mouse PrP gene.

Behavioral analysis

Visual inspection and rotarod testing for locomotor function were conducted on groups of mice ($n = 5–9$) in a blinded manner. For the rotarod task, an Economex treadmill (Columbus Instruments, Columbus, OH, USA) was used. Mice were given four trials a day for four consecutive days, the speed was set at 5 r.p.m. and accelerated at a rate of 0.2 r.p.m./s until reaching a speed of 20 r.p.m. at which it was maintained. When the speed reached 20 r.p.m., this was designated as time $t = 0$, and the latency to fall from the apparatus after that point was measured in seconds in the best four of five trials over a

course of four trial days. Performance on the rotarod was statistically compared using two-way ANOVA.

Antibody production

Synuclein polyclonal antibodies UPN61 (β-synuclein) and Rodsyn (α-synuclein) were obtained by immunization of rabbits (Covance, Richmond, CA, USA) with synthetic peptides corresponding to amino acids 86–131 of human β-synuclein and 118–130 of mouse α-synuclein, respectively (HM Keck Institute, Yale University), which were crosslinked to BSA and KLH, respectively (Pierce, Rockford, IL, USA). The polyclonal antibodies were affinity purified against their respective antigen coupled to Affigel 15 (Pierce). Neuron-specific enolase (rabbit anti-rat NSE; Polysciences Inc., PA, USA) and monoclonal β-actin (Sigma) antibodies are commercially available. The production, characterization and use of Syn 208 and Syn 303 have been previously described (32,33).

Western blot analysis

Protein lysates were obtained by homogenizing brain tissues in 1× Laemmli buffer or sample buffer (62.5 mM Tris–HCl pH 6.8, 4% SDS, 200 mM dithiothreitol, 10% glycerol, 0.001% bromophenol blue) at a ratio of 1:10 (w/v) and then boiling for 10 min. Protein samples were immunoblotted as previously described (32,33), with antibodies to α-synuclein Rodsyn and Syn 208 (1:500) or rat NSE (1:1000) and β-synuclein UPN61 (1:500) or monoclonal β-actin (1:10 000). For I^{125} quantification, 10 μg samples of extract protein were loaded in duplicate or triplicate. Primary antibodies were used as mentioned earlier, and Protein A I^{125} (NEN Life Sciences) was added for 4–6 h at 1 μCi/ml in 4% BSA in PBS pH 7.4, instead of the secondary antibody, at RT. The blot was washed thrice (first wash for 30 min and the last two washes for 1 h each) in TBS-Tween at pH 7.4, dried and exposed to a PhosphoImager plate for 2 days. The plate was developed using a PhosphoImager (Storm 840, Molecular Dynamics) and bands quantified using ImageQuant (Molecular Dynamics), normalized to NSE. Quantities between groups were compared using two-way ANOVA.

Immunohistochemistry

Mice were euthanized by an intraperitoneal overdose of xylazine and ketamine, perfused with PBS and brains were surgically removed and fixed in 70% EtOH/150 mM NaCl for histochemical analysis or frozen for biochemical analysis as described (14). Sections of 6 μm were deparaffinized in xylene followed by graded alcohol in descending ethanol concentrations. Endogenous peroxidase activity was reduced by incubation in methanol/H_2O_2 (150 and 30 ml of 30% H_2O_2). The sections were then immunostained using α-synuclein antibody Syn 303 (1:3000). Immunoreactivity was visualized with the avidin–biotin complex detection system (Vector Laboratories) using 3,3-diaminobenzidine as the chromogen and counterstained with hematoxylin. The number of α-synuclein positive inclusions was quantified with the Image J particle analysis program (Version 1.36b; NIH) to ensure an unbiased determination of inclusion number. We created an algorithm (available upon request) to count the number of α-synuclein positive inclusions. Immunoreactive foci needed to meet two criteria: (i) a particle size of 50–200 μm^2 and (ii) a circularity of 0.3–1.0. (Note that a circularity of 1.0 corresponds to a perfect circle, whereas values approaching 0.0 correspond to increasingly elongated polygons.) The number of α-synuclein positive inclusions was quantified in this way for three individual mice/group. Quantifications were performed in a blinded manner, and three fields per animal were counted for a total of nine separate quantifications/cohort. The difference between transgenic groups was compared using Student's *t*-test.

Sequential biochemical fractionation

Half mouse cortices were homogenized in 10 ml/g of high salt (HS) buffer at pH 7.4 (50 mM Tris, 750 mM NaCl, 10 mM NaF, 5 mM EDTA plus protease inhibitors) and centrifuged at 40 000 r.p.m. at 4°C for 20 min. Pellets were re-extracted once with HS buffer and the supernatants pooled. These supernatants contain soluble proteins. All pellets were then twice extracted in 10 ml/g HS containing 1% Triton X-100 (Triton X buffer), pooling the supernatants. This was followed by twice extracting the pellets at room temperature in 10 ml/g Triton X buffer containing 2% SDS (SDS buffer), with brief sonication on the second extraction. The SDS fractions were pooled. Lastly, the resulting pellets were resuspended in 2 ml/g SDS buffer containing 8 M urea, with brief sonication.

Quantitative RT–PCR

Total RNA was isolated from mouse cortex by homogenizing in Trizol (Invitrogen), and the integrity of the RNA was verified on an Agilent 2100 Bioanalyzer. Quantitative real-time PCR was performed on a Light Cycler in a one-tube RT–PCR reaction using RNA master SYBR Green I protocol (Roche Diagnostics) with α-synuclein primers (Alp1-F: 5′-gca gag gca gct gga aag aca a-3′ and Alp1-R: 5′-cct ctg aag gca ttt cat aag cct cac t-3′). All reactions were performed in triplicate with β-actin as a control and results were compared using Student's *t*-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement.The authors declare that there are no competing financial interests or other conflicts of interest.

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