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β-Synuclein Inhibits α-Synuclein Aggregation: A Possible Role as an Anti-Parkinsonian Factor

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Summary

We characterized β-synuclein, the non-amyloidogenic homolog of α-synuclein, as an inhibitor of aggregation of α-synuclein, a molecule implicated in Parkinson's disease. For this, doubly transgenic mice expressing human (h) α- and β-synuclein were generated. In doubly transgenic mice, β-synuclein ameliorated motor deficits, neurodegenerative alterations, and neuronal α-synuclein accumulation seen in hα-synuclein transgenic mice. Similarly, cell lines transfected with β-synuclein were resistant to α-synuclein accumulation. hα-synuclein was coimmunoprecipitated with hβ-synuclein in the brains of doubly transgenic mice and in the double-transfected cell lines. Our results raise the possibility that β-synuclein might be a natural negative regulator of α-synuclein aggregation and that a similar class of endogenous factors might regulate the aggregation state of other molecules involved in neurodegeneration. Such an anti-amyloidogenic property of β-synuclein might also provide a novel strategy for the treatment of neurodegenerative disorders.

Introduction

Abnormal folding and aggregation of proteins in the central nervous system (CNS) has been extensively explored as one of the central mechanisms leading to neurodegeneration in disorders such as Alzheimer's disease (AD), Lewy body disease (LBD), Huntington's disease (HD), and Creutzfeldt-Jakob disease (CJD) (Ferrigno and Silver, 2000; Koo et al., 1999; Ramassamy et al., 1999). In LBD, a common cause of dementia and parkinsonism in the elderly, neuronal accumulation of misfolded α-synuclein has been proposed to be centrally involved in disease pathogenesis (Hashimoto and Masliah, 1999; Trojanowski et al., 1998). Supporting this possibility, recent studies have shown that: (1) this synaptic-associated molecule is the most abundant component of Lewy bodies (LBs) (Spillantini et al., 1997; Takeda et al., 1998; Wakabayashi et al., 1997), (2) mutations in the α-synuclein gene are associated with rare familial forms of parkinsonism (Kruger et al., 1998; Polymeropoulos et al., 1997), and (3) α-synuclein expression in transgenic (tg) mice (Masliah et al., 2000) and *Drososphila* (Feany and Bender, 2000) mimics several aspects of LBD.

α-synuclein, a 140 amino acid (aa) synaptic molecule, was originally identified in human brain as the precursor protein of the non-amyloid β-protein (Aβ) component of AD amyloid (NAC) (Iwai, 2000; Masliah et al., 1996; Ueda et al., 1993). NAC is a highly hydrophobic 35 aa domain within the α-synuclein molecule, which self-aggregates to form amyloid and is an efficient seed for formation of Aβ fibrils (Han et al., 1995; Iwai et al., 1995). α-synuclein was subsequently found to belong to a larger family of molecules, including β-synuclein (or phosphoneuroprotein 14) (Jakes et al., 1994; Nakajo et al., 1993), γ-synuclein (or breast carcinoma-specific factor) (Jia et al., 1999), and synoretin (Surguchov et al., 1999). The α-synuclein molecule is a natively unfolded protein (Weinreb et al., 1996) that is capable of self-aggregating to form both oligomers and fibrillar polymers with amyloid-like characteristics (Hashimoto et al., 1998). Polymerization could occur in several stages including formation of protofibrils, nucleation (Wood et al., 1999), and fibril formation (Hashimoto et al., 1998; Serpell et al., 2000). Conditions promoting this aggregation include: (1) mutations associated with familial parkinsonism (Conway et al., 1998; Narhi et al., 1999), (2) oxidative stress mediated by iron, cytochrome c, or copper(II) (Hashimoto et al., 1998, 1999a, 1999b; Hsu et al., 2000; Paik et al., 1999; Souza et al., 2000), (3) binding to lipid membrane vesicles (Jo et al., 2000; Perrin et al., 2000), and (4) interactions with amyloidogenic molecules such as NAC and Aβ (Jensen et al., 1997; Masliah et al., 2001; Paik et al., 1998; Yoshimoto et al., 1995).

In view of evidence suggesting that α-synuclein fibrils may initiate neurodegeneration in vivo, inhibition of α-synuclein aggregation may represent a feasible therapeutic strategy in LBD and related disorders. While some factors promote α-synuclein aggregation, others might block amyloidogenesis and aggregation (Hashimoto and Masliah, 1999). Interactions among the synucleins could play a role in this process, because β- and γ-synuclein do not seed α-synuclein aggregation (Biere et al., 2000) and in LBD the ratio of β- to α-synuclein is altered (Rockenstein et al., 2001), suggesting that a critical balance between pro- and anti-aggregation factors might be at play in disorders such as LBD and AD (Masliah, 2001; Rochet et al., 2000). In this context, we reasoned that β-synuclein, the non-amyloidogenic homolog of α-synuclein that naturally lacks the NAC domain, might inhibit the abnormal accumulation of α-synuclein and neurodegeneration. For this purpose, doubly transgenic (doubly tg) mice expressing human (h) α- and β-synuclein were generated and analyzed in the rotarod and by laser scanning confocal microscopy (LSCM). Additional studies directed at investigating the interactions between α- and β-synuclein were performed in double transfected cells lines and in an in vitro cell-free system. Our results raise the intriguing possibility that β-synuclein may be a natural negative regulator of α-synuclein aggregation and that a similar class of endogenous factors might regulate the aggregation state of other molecules involved in neurodegeneration.

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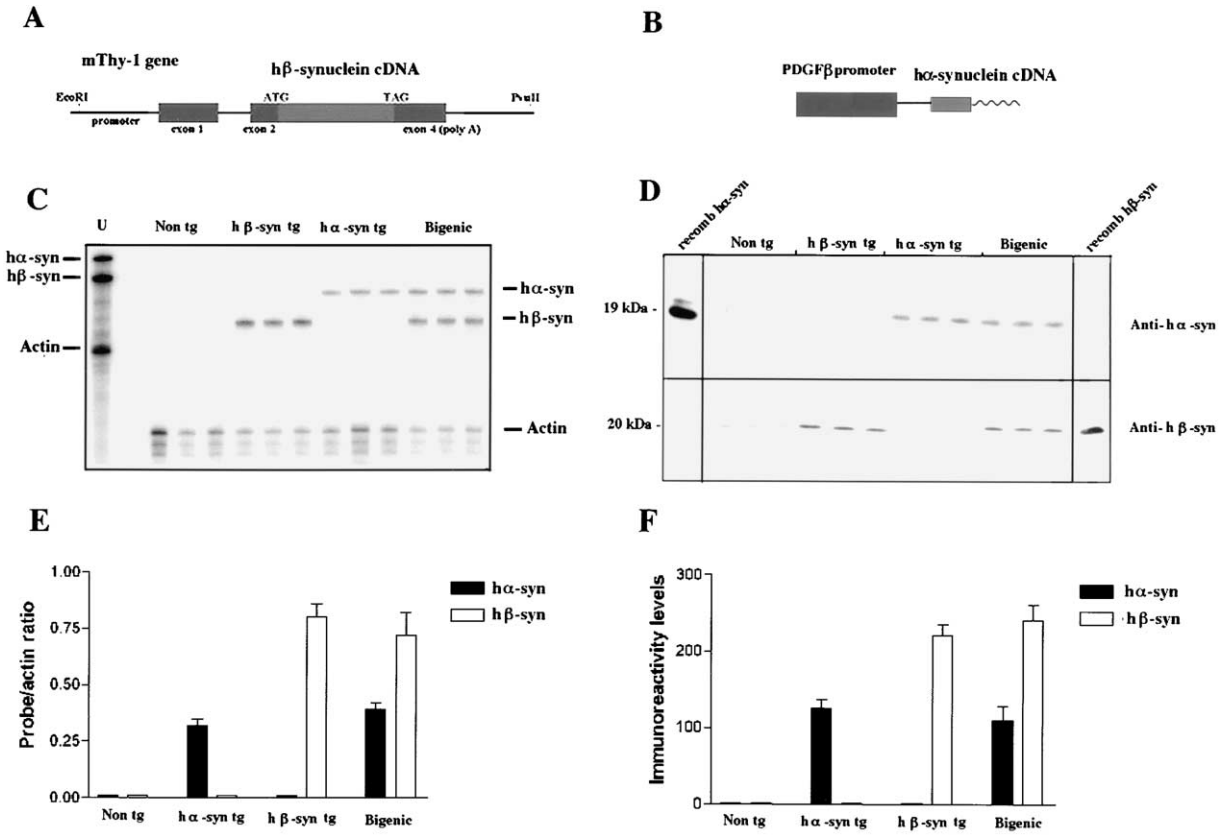

Figure 1. Generation of hα- and β-Synuclein tg and Bigenic Mice

(A) Diagrammatic representation of the Thy1-hβ-synuclein construct.

(B) Diagrammatic representation of the PDGF-hα-synuclein construct.

(C) Representative autoradiograph showing levels of α- and β-synuclein mRNA in brain tissues from nontransgenic (non-tg), hα-synuclein tg (hα-syn), hβ-synuclein tg (hβ-syn), and hα-synuclein/hβ-synuclein doubly tg mice. The leftmost lane shows signals of undigested (U) radiolabeled riboprobes (identified on left); the other lanes contain the same riboprobes plus brain RNA samples digested with RNases. Protected mRNAs are indicated on the right.

(D) Western blot analysis with antibodies against hα- and β-synuclein showed that double-tg mice expressed levels of α- and β-synuclein comparable to singly tg mice. Recombinant α- and β-synuclein were used as positive controls.

(E) Quantitative analysis of hα- and hβ-synuclein mRNA in brain extracts from non-tg and tg mice. Signals were quantified by PhosphorImager analysis and expressed as probe over signal. Singly and doubly tg mice (n = 3/genotype) did not significantly differ in the ratios of hα- or hβ-synuclein/actin (mean ± SD). Overall, levels of hβ-synuclein were about double that of hα-synuclein.

(F) Semiquantitative analysis of levels of hβ- and hβ-synuclein immunoreactivity in the cytosolic fractions of brain homogenates from non-tg and tg mice. Levels of immunoreactivity were obtained by PhosphorImager analysis and expressed as integrated pixel intensity. Bars = mean ± SD.

Results

Characterization of hα and β-Synuclein Expression in tg Mice

To test the hypothesis that in vivo interactions of β-synuclein with α-synuclein might ameliorate the alterations associated with α-synuclein accumulation in the brain, tg mice expressing hβ-synuclein under the regulatory control of the murine (m) Thy-1 promoter (Figure 1A) were generated and crossed with our highest expresser hα-synuclein tg mice (Figure 1B). Ribonuclease protection assay (RPA) showed high levels of hα- and hβ-synuclein expression in the brains of the singly and doubly tg mice (Figures 1C and 1D). However, since hβ-synuclein was expressed from the mThy-1 cassette, mRNA levels of this molecule through out the brain were 2- to 3-fold higher compared to hα-synuclein, which was expressed under the regulatory control of the platelet-derived growth factor B chain (PDGFB) promoter (Figures 1C and 1D). When compared to singly tg mice, doubly tg (bigenic) mice expressed similar levels of hα- and hβ-synuclein mRNA (Figures 1C and 1D) and protein immunoreactivity (Figures 1E and 1F).

Coexpression of hβ-Synuclein in hα-Synuclein tg Mice Ameliorates Motor Deficits and Reduces hα-Synuclein Accumulation in the Brain

In order to determine if hβ-synuclein improved the motor performance and reduced neuronal accumulation of hα-synuclein, rotarod and immunochemical analyses were performed. Rotarod analysis showed that, compared to non-tg littermates, singly tg hα-synuclein mice displayed the characteristic motor deficits, which were ameliorated in doubly tg mice coexpressing hα- and hβ-synuclein (Figure 2A). Statistical analysis showed that the deficits in rotarod performance of hα-synuclein

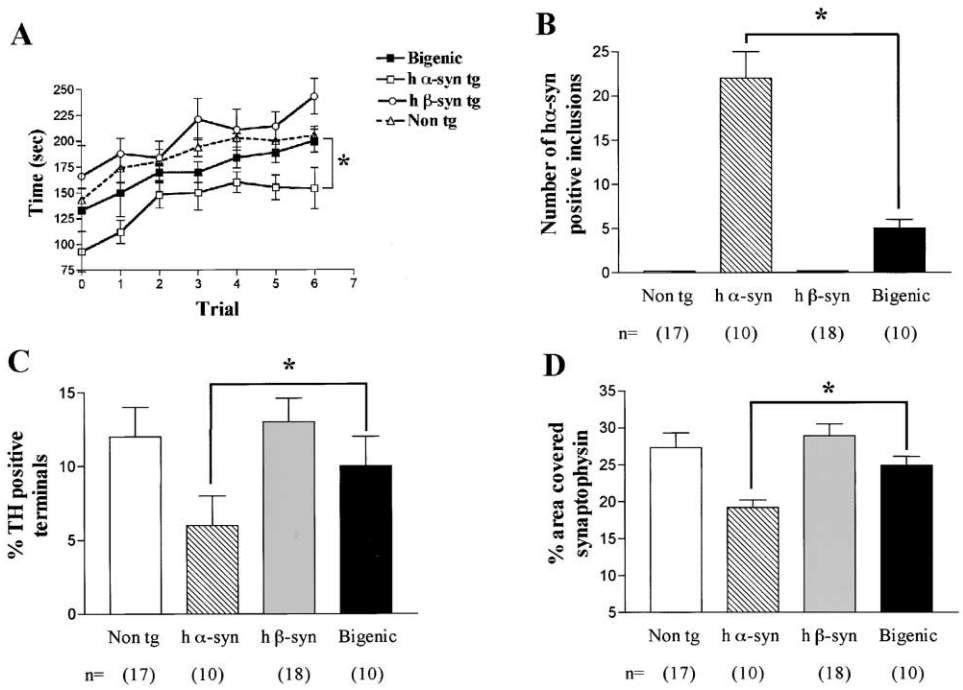

Figure 2. Characterization of Motor and Neurodegenerative Alterations in hα-Synuclein tg Mice Crossed with hβ-Synuclein tg Mice

(A) Motor assessment in the rotarod showed that 12-month-old singly tg hα-synuclein mice were significantly different from bigenic mice (*$p < 0.05$, one-way ANOVA with post-hoc Tukey-Krammer). In contrast, doubly tg (bigenic) mice performed at levels comparable to non-tg and singly tg hβ-synuclein mice. No significant differences were observed among these latter three groups (one-way ANOVA with post-hoc Dunnett's) ($n = 10$ mice per group).

(B) The numbers of the anti-hα-synuclein-immunoreactive neuronal inclusions in the temporal cortex were determined in 12-month-old mice. Compared to singly tg hα-synuclein mice, bigenic mice showed a significant reduction in the number of inclusion bodies (*$p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer).

(C) Compared to doubly tg mice, singly tg hα-synuclein mice showed a 35% decrease in the area covered by TH-positive terminals in the striatum (*$p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer). Levels of TH immunoreactivity were comparable among the bigenic, hβ-synuclein tg, and non-tg mice.

(D) Singly tg hα-synuclein mice showed a significant decrease in the percent area of the neuropil covered by anti-synaptophysin-immunoreactive terminals in the temporal cortex, compared to non-tg controls ($p < 0.05$, one-way ANOVA with post-hoc Dunnett's) and bigenic mice (*$p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer). In contrast, bigenic mice were comparable to non-tg and singly tg hβ-synuclein mice (12-month-old mice; hα-synuclein tg [$n = 10$], hβ-synuclein tg [$n = 18$], bigenic [$n = 10$], non-tg [$n = 17$]).

tg mice were significantly different when compared to bigenic and hβ-synuclein tg mice ($p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer), as well as non-tg littermates ($p < 0.05$, one-way ANOVA with post-hoc Dunnett's) (Figure 2A). No significant differences were observed between the latter three groups.

Consistent with previous studies (Masliah et al., 2000), hα-synuclein tg mice had abundant intraneuronal hα-synuclein immunoreactive inclusions in the neocortex (Figures 2B, 3A, and 3B). There was a statistically significant reduction in the numbers of inclusion bodies in the temporal cortex of bigenic mice when compared to hα-synuclein tg mice (Figures 2B, 3C, and 3D) ($p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer). No inclusions were found in the brains of hβ-synuclein tg and non-tg mice.

Furthermore, confocal analysis of double-immunolabeled sections showed that in hα-synuclein tg mice there was intense hα-synuclein accumulation in neuronal cell bodies and synapses in the neocortex (Figure 3E) and limbic system and, to a lesser extent, in the substantia nigra (data not shown). Only mild β-synuclein immunoreactivity was detected in the terminals of hα-synuclein tg mice (Figure 3E). In contrast, hβ-synuclein tg mice showed intense β-synuclein immunoreactivity in the terminals, while the levels in neuronal cell bodies were low (Figure 3F).

Bigenic mice had both hα- and β-synuclein immunoreactive presynaptic terminals (Figures 3G and 3H); however, only occasional hα-synuclein immunoreactive inclusions were observed in the neocortex (Figures 3C and 3D), limbic system, and substantia nigra (data not shown). Interestingly, in the bigenic mice, one of the inclusions there was colocalization of hα- and β-synuclein in (Figure 3G), supporting the possibility that interactions between α- and β-synuclein can occur in vivo. In order to verify this possibility, cytosolic fractions from mouse brain homogenates were immunoprecipitated with either rabbit anti-β-synuclein primary antibody or preimmune serum, followed by immunoblot analysis with anti-hα-synuclein monoclonal antibody (LB509) (Figure 4). This study showed that hα-synuclein was coimmunoprecipitated with β-synuclein only in the brains of bigenic mice (Figure 4), supporting the contention that β-synuclein might regulate the aggregation state of α-synuclein by directly binding to it.

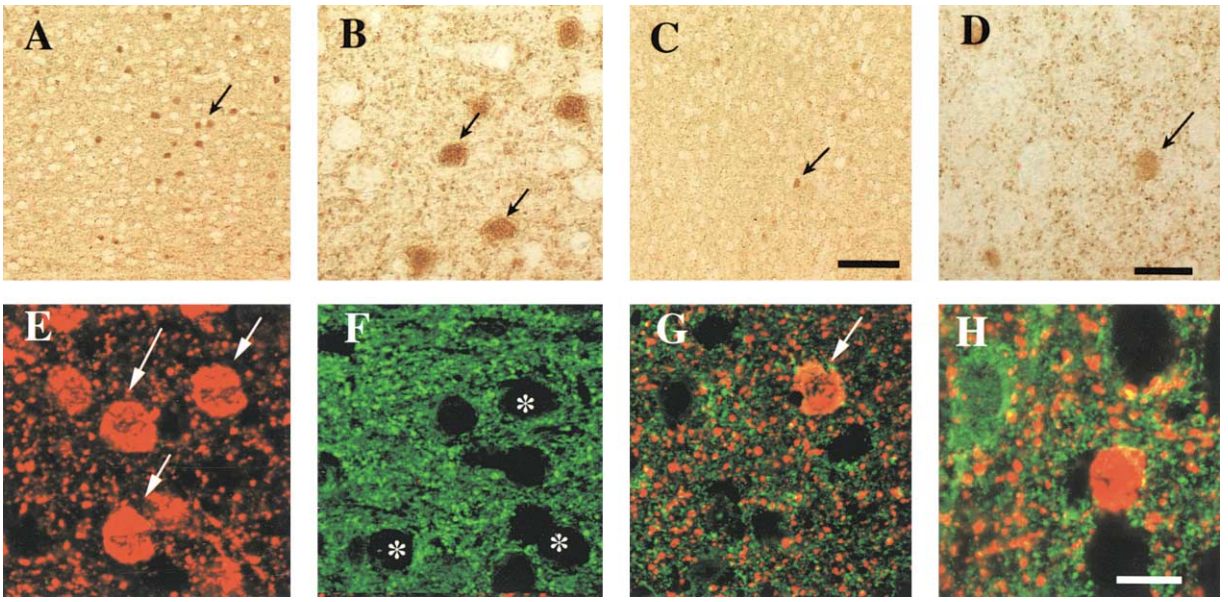

Figure 3. Neuropathological Analysis of hα-Synuclein Inclusion Formation in tg Mice

All images were obtained from the temporal cortex of 12-month-old mice.

(A–D) Sections were singly labeled with the antibody against hα-synuclein and imaged by light microscopy.

(E–H) Sections were double-labeled with antibodies against hα-synuclein (red) and β-synuclein (green) and imaged with the LSCM.

(A) Abundant anti-hα-synuclein immunoreactive inclusions were observed in hα-synuclein tg mice (low-power view).

(B) Higher magnification of the image in (A).

(C) Bigenic mice showed a reduction in the number of anti-hα-synuclein immunoreactive inclusions.

(D) Higher magnification of the image in (C).

(E) Singly tg hα-synuclein mice showed abundant anti-hα-synuclein immunoreactive inclusions (arrows). The nerve terminals in the neuropil showed low levels of β-synuclein immunoreactivity (green).

(F) Singly tg hβ-synuclein mice showed abundant anti-β-synuclein immunoreactive terminals (green). Note that neuronal cell bodies did not show β-synuclein accumulation (*) and no hα-synuclein immunoreactivity was observed.

(G and H) Bigenic mice showed both anti-hα- (red) and β-synuclein (green) immunoreactive presynaptic terminals. Occasional hα-synuclein immunoreactive inclusions were observed (arrows). Colocalization of α- and β-synuclein in the inclusions is seen in orange-yellow.

hβ-Synuclein Reduces the Neurodegenerative Alterations in hα-Synuclein tg Mice

To determine if the improved performance in the rotarod and reduced neuronal accumulation of hα-synuclein seen in bigenic mice was associated with reduced neurodegeneration, levels of tyrosine hydroxylase (TH) and synaptophysin immunoreactivity in the caudoputamen region and neocortex were analyzed. As expected, compared to non-tg mice, singly tg hα-synuclein mice showed a significant 35% decrease in dopaminergic input to the basal ganglia (Figure 2C) ($p < 0.05$, one-way ANOVA with post-hoc Dunnett's). Levels of TH immunoreactivity in the caudoputamen region of the bigenic mice were significantly different compared to singly tg hα-synuclein ($p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer). Levels of TH immunoreactivity in bigenic mice were comparable to non-tg and hβ-synuclein tg mice (Figure 2C). Furthermore, singly tg hα-synuclein mice showed a significant decrease in the percent area of the neuropil covered by anti-synaptophysin-immunoreactive terminals, when compared to bigenic synuclein ($p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer), hβ-synuclein tg and non-tg mice synuclein ($p < 0.05$, one-way ANOVA with post-hoc Dunnett's) (Figure 2D). Taken together, these results suggest that the anti-aggregation effects of hβ-synuclein also ameliorate the neurodegenerative effects promoted by hα-synuclein accumulation.

β-Synuclein Coimmunoprecipitates with α-Synuclein and Inhibits α-Synuclein Aggregation In Vitro

To further investigate the interactions between hα- and β-synuclein, coimmunoprecipitation studies in transfected HEK293 cells, utilizing the polyclonal anti-β-synuclein and monoclonal anti-α-synuclein antibodies were performed. The HEK293 cells were first transfected with muriste-rone-inducible expression vector carrying the cDNA for wild-type hα-synuclein followed by a further stable transfection with hβ-synuclein. This system was chosen because it has been previously shown that overexpression of wild-type hα-synuclein in HEK293 cells results in cytoplasmic accumulation of α-synuclein (Tabrizi et al., 2000). This study showed that hα-synuclein was coimmunoprecipitated with hβ-synuclein, specifically in the muristerone A-treated cells, by the anti-β-synuclein serum but not preimmune serum (Figure 5). hα-synuclein was also precipitated by anti-β-synuclein serum in the muristerone A-treated double transformants, including clones α3β2, α3β9, and α3β12, but not in control α3v2 cells (Figure 5). Consistent with these findings, immunocytochemical analysis showed that in the muristerone A-treated cells (not cotransfected with hβ-synuclein),

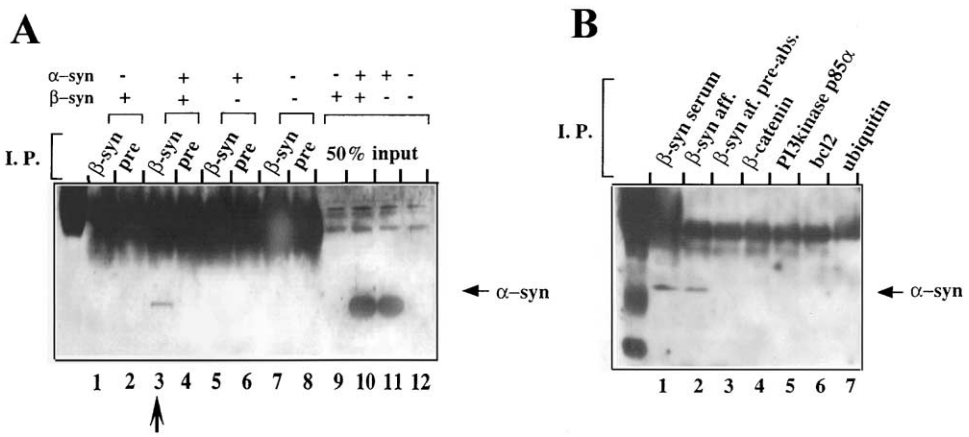

Figure 4. hα- and β-Synuclein Coimmunoprecipitates in the Brains of tg Mice

(A) Immunoprecipitation with either rabbit anti-β-synuclein (lanes 1, 3, 5, and 7) or pre-immune serum (lanes 2, 4, 6, and 8), followed by immunoblot analysis with anti-hα-synuclein (LB509) showed that β-synuclein was coimmunoprecipitated with α-synuclein only in fractions obtained from the brains of bigenic mice (lane 3, arrow). 50% input controls are simultaneously shown (lanes 9–12).

(B) To further verify the specificity of interactions between hα-and β-synuclein in tg mice, cytosolic fractions of brain homogenates from bigenic mice were subjected to immunoprecipitation with various antibodies against cytoplasmic molecules, followed by immunoblot analysis with the LB509 monoclonal antibody against hα-synuclein. The antibodies used were as follows: anti-β-synuclein serum (lane 1), anti-β-synuclein affinity purified antibody (lane 2), anti-β-synuclein affinity purified antibody preadsorbed with immunized peptides (lane 3), anti-β-catenin, C-18 (lane 4), anti-PI3 kinase p85α, Z-8 (lane 5), anti-Bcl2, N-19 (lane 6), and anti-ubiquitin (lane 7). Note that hα-synuclein was recognized specifically by either anti-β-synuclein serum (lane 1) or anti-β-synuclein affinity purified antibody (lane 2).

there was a prominent accumulation of hα-synuclein in the cytoplasm (161.5 ± 14.8 per sq mm) (Figures 6A–6F), while coexpression of hβ-synuclein in muristerone A-treated hα-synuclein-overexpressing cells resulted in reduced hα-synuclein accumulation (42.0 ± 5.5 per sq mm) (Figures 6G–6L) ($p < 0.05$, one-way ANOVA with post-hoc Tukey-Kramer). No inclusions were observed in the cells transfected with hβ-synuclein or vector alone. Taken together, these results support the contention that α- and β-synuclein interact in vivo and may form a complex in HEK293 cells.

To further confirm that hβ-synuclein can specifically block hα-synuclein aggregation, analysis was performed with recombinant proteins. Immunoblot analysis with an α-synuclein-specific antibody (101–124) showed that coincubation of hα-synuclein with recombinant hβ-synuclein (but not IgG) inhibited aggregation in a dose-dependent manner (Figure 7). More than equimolar concentrations of hβ-synuclein were effective at suppressing hα-synuclein aggregation, suggesting that heterodimerization of β-synuclein with α-synuclein inhibited homodimerization of α-synuclein, leading to inhibition of further

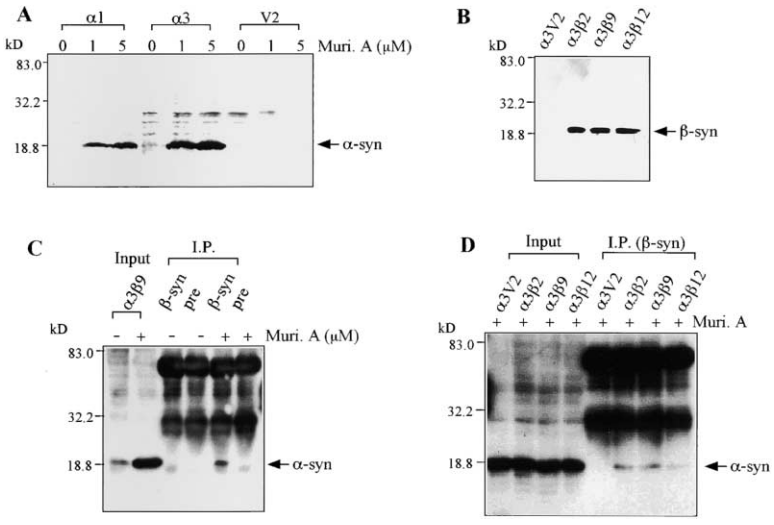

Figure 5. hα- and β-Synuclein Coimmunoprecipitate in Transfected Cell Lines

(A) α-synuclein coimmunoprecipitates with β-synuclein in cell cultures. pIND α-synuclein (α1 and α3) and pIND (v2) transfected ECR-293 cells were treated with muristerone A (0, 1, 5 μM) for 48 hr, and the cell extracts were analyzed for the inducible expression of α-synuclein by immunoblotting using LB509.

(B) The α3 cells were further transfected with pCEP4 with or without β-synuclein cDNA. Highly constitutive expressers of β-synuclein (α3β2, α3β9, and α3β12) and their control α3v2 cells were analyzed by immunoblotting using β-synuclein antibody.

(C) The α3β9 cells treated with or without muristerone A (2 μM) for 48 hr were analyzed for the interaction of α- with β-synuclein by coimmunoprecipitation experiments. The cell extracts were subjected to immunoprecipitation using either anti-β-synuclein serum or preimmune serum, followed by immmunoblotting with LB509.

(D) Each stable transformant (α3β2, α3β9, α3β12, and α3v2) was treated with muristerone A, and the cell extracts were subjected to coimmunoprecipitation studies as described in (C). In (C) and (D), 20% input controls are simultaneously shown. Results showed that hα-synuclein was coimmunoprecipitated with hβ-synuclein, specifically in the muristerone A-treated cells, by anti-β-synuclein serum but not preimmune serum. hα-synuclein was also precipitated by anti-β-synuclein in the muristerone A-treated double-transformants, including clones α3β2, α3β9, and α3β12, but not in control α3v2 cells.

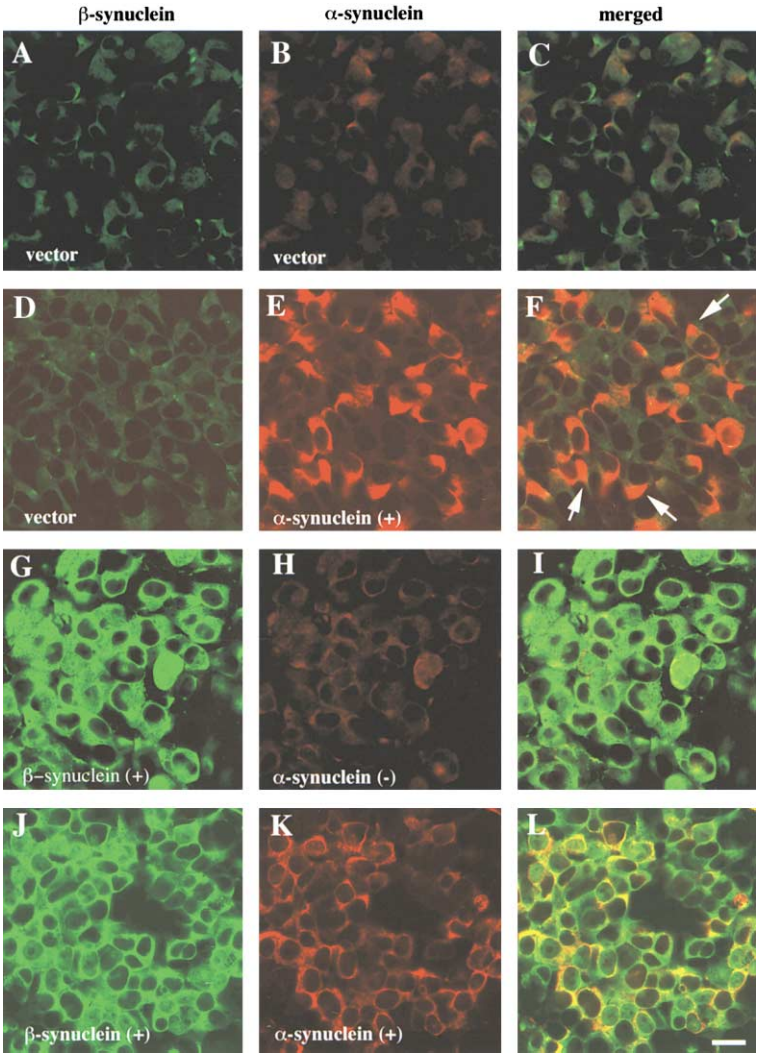

Figure 6. Analysis of hα- and β-Synuclein Expression in Stably Transfected Cell Lines

Both α3β9 (α-synuclein: inducible, β-synuclein: constitutive) and α3v2 (α-synuclein: inducible, β-synuclein: no expression) cells were grown on coverslips and incubated either in the presence or absence of muristerone A (2 μM) for 36 hr, followed by immunolabeling with antibodies against either hα- or β-synuclein and imaged with the LSCM. The panels in the left correspond to β-synuclein (green), in the center to α-synuclein (red), and in the right to merged images.

(A–C) The α3v2 cells without muristerone A treatment showed very mild levels of hα- and β-synuclein immunoreactivity corresponding to endogenous expression.

(D–F). The muristerone A-treated α3v2 cells showed increased hα-synuclein immunoreactivity, as well as accumulation of hα-synuclein as small aggregates (arrows), but not β-synuclein immunoreactivity.

(G–I) In the α3β2 cells without muristerone A treatment, only β-synuclein, but not hα-synuclein, immunoreactivity was observed.

(J–L) Muristerone A-treated α3β2 cells showed both hα- and β-synuclein immunoreactivity. However, hα-synuclein accumulation and immunoreactivity were reduced, compared to α3v2 cells (E).

Bar = 10 μm.

α-synuclein oligomerization. Moreover, histochemical and ultrastructural studies showed that α-synuclein, β-synuclein δ1 and δ2, but not β-synuclein, formed filamentous structures reminiscent of amyloid fibrils indicating that the aggregates formed by the β-synuclein mutants had amyloid-like properties (data not shown). These results indicate that interactions between synucleins might occur in the conserved regions of the molecule.

Discussion

In view of recent studies suggesting a role for abnormal interactions among CNS proteins in the pathogenesis of neurodegenerative disorders such as AD and PD (Koo et al., 1999; Masliah, 1998; Trojanowski et al., 1998), our results raise the intriguing possibility that interactions among closely homologous molecules in the same family of peptides might regulate the state of protein aggregation under pathological conditions. This is important, since in the brains of patients with parkinsonian syndromes α-synuclein abnormally accumulates in neuronal cell bodies and neurites, resulting in formation of LBs (Spillantini et al., 1997; Takeda et al., 1998; Wakabayashi et al., 1997). The present study indicates that β-synuclein might play a role in regulating the aggregation state of α-synuclein, which is consistent with previous studies showing that synucleins form both heterodimers and homodimers in vitro (Jensen et al., 1995). In addition, α-synuclein has a tendency to aggregate under stress conditions (Hashimoto et al., 1999a) while β- and γ-synuclein are less prone to aggregate (Biere et al., 2000), indicating that these molecules have opposing effects. Although the mechanisms by which β-synuclein might inhibit α-synuclein aggregation are unclear, several possibilities should be considered. For example, β-synuclein might directly bind to α-synuclein and prevent further aggregation, β-synuclein might also facilitate interactions of α-synuclein with fatty acids in the membrane, favoring the α helix rather than the β pleated conformation of α-synuclein (Davidson et al., 1998). Furthermore, it is possible that β-synuclein might have antioxidant or neuroprotective properties and since oxidative stress promotes α-synuclein aggregation (Hashimoto et al., 1999a; Osterova et al., 1999; Osterova-Golts et al., 2000; Souza et al., 2000), then decreasing α-synuclein oxidation might prevent aggregation.

Supporting the possibility that direct interaction be-

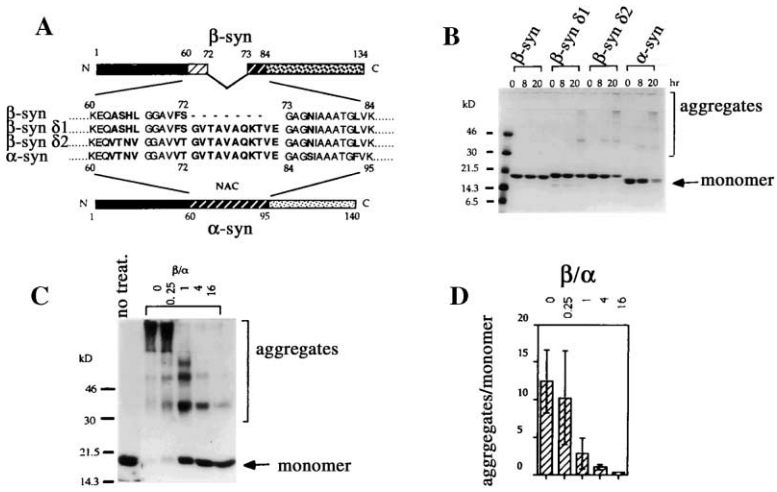

Figure 7. β-Synuclein Blocks α-Synuclein Aggregation in an In Vitro Cell-free System

(A) Schematic representation of hβ-synuclein mutants (δ1 and δ2) created by PCR-based site-directed mutagenesis.

(B) SDS-PAGE analysis of Coomasie blue stained gels showed that wild-type β-synuclein (β-syn) did not aggregate, while β-synuclein δ1 and δ2 (β-syn δ1 and δ2) and α-synuclein (α-syn) were aggregated.

(C and D) Immunoblot analysis with anti-hα-synuclein antibody showed that coincubation of recombinant hα-synuclein with hβ-synuclein for 20 hr inhibited aggregation in a dose-dependent manner.

tween α- and β- synuclein might block aggregation, a recent study showed that α- and β-synuclein were coimmunoprecipitated in a transient cotransfection system in murine Neuro2A cell line (Iwata et al., 2001). Although the authors suggested an interesting possibility that another molecule, such as elk-1, might mediate the interaction of α- with β-synuclein, it is possible that this interaction results from a nonspecific high tendency of α-synuclein to bind to other proteins. Although our preliminary studies do not support this possibility, additional coimmunoprecipitation and yeast hybrid experiments are necessary to scorroborate the observation that interactions between α- and β-synuclein are specific.

Our results in the bigenic mice and in in vitro systems support the possibility that both of these molecules might interact in vivo, which is also consistent with the observation that α- and β-synuclein concentrate in the same compartment in the synapses. This may suggest that under stress conditions such as the ones observed in neurodegenerative disorders (Jenner, 1996), β-synuclein might display anti-aggregation properties that otherwise might not be apparent under physiological conditions. If this is indeed the case, it is possible that an imbalance in the ratio of α- to β-synuclein might render α-synuclein more prone to aggregate under oxidative stress conditions. In support of this possibility, in LBD ratios among the synucleins are altered in favor of an increase in α- and a decrease in β-synuclein (Rockenstein et al., 2001). Furthermore, levels of β-synuclein mRNA in the brain are higher than α- and γ-synucleins supporting the possibility that alterations in β-synuclein expression might play a role in LBD pathogenesis.

The mechanisms by which α-synuclein might promote neuronal dysfunction leading to neurodegeneration are under intense scrutiny. Among the possibilities proposed, recent studies suggest that intracellular accumulation of α-synuclein (El-Agnaf and Irvine, 2000) leads to mitochondrial dysfunction (Hsu et al., 2000), oxidative stress (Osterova-Golts et al., 2000; Souza et al., 2000), and caspase degradation (Alves Da Costa et al., 2000) accentuated by mutations associated with familial parkinsonism (Forloni et al., 2000; Kanda et al., 2000; Zhou et al., 2000). These observations imply that the neuroprotective effects of β-synuclein might be associated with its capacity to operate as a negative regulator of α-synuclein aggregation and that a similar class of endogenous factors could regulate the aggregation of other molecules involved in neurodegeneration. This concept is supported by previous studies in sickle cell anemia showing that hemoglobin F decreases hemoglobin S fibrillation (Noguchi et al., 1989). Intracellular polymerization of hemoglobin S leads to a loss of erythrocyte deformability and eventual morphological sickling. High levels of hemoglobin F were associated with reduced disease severity, suggesting that small decreases in polymer formation at intermediate levels of hemoglobin F may give rise to a small decrease in anemia, but that greater reductions in polymer formation may be necessary to effect a significant improvement in disease severity. Similarly, previous studies relevant to amyloid formation in AD have shown that Aβ40 (Hasegawa et al., 1999) might inhibit the nucleation of the more fibrillogenic Aβ42 species. Finally, a recent study has shown that fibrillization of mα-synuclein could be inhibited by hα-synuclein (Rochet et al., 2000). mα-synuclein was more fibrillogenic than the wild-type or A53T mutant hα-synuclein, suggesting that at least one of the six mismatched residues (five located in the C-terminal region) between the two proteins plays a role in fibrillization and inhibition of fibril formation.

Taken together, these studies support the possibility that naturally occurring molecules with close homology to molecules exhibiting amyloidogenic properties might have the ability to regulate the state of aggregation of neuronal proteins. Such an anti-amyloidogenic property of β-synuclein might also provide a novel strategy for the treatment of neurodegenerative disorders.

Experimental Procedures

Generation of hα-Synuclein, β-Synuclein, and Bigenic Mice

For the present study, we used the highest expresser (line D) hα-synuclein tg mice expressing wild-type hα-synuclein in neurons from the PDGFB, generated as previously described (Masliah et al., 2000) (Figure 1B). This line was selected because early on these mice develop intraneuronal inclusions, dopaminergic deficits, and motor impairments (Masliah et al., 2000). The mThy-1 expression cassette (provided by Dr. H. van der Putten, Ciba-Geigy, Bazel) was used to generate hβ-synuclein tg mice. The hβ-synuclein cDNA fragment (405 nt, Genbank # S69965) was generated by RT/PCR from human brain mRNA, ligated into pCRII (Invitrogen, La Jolla,

CA), and sequenced for accuracy. The hβ-synuclein cDNA fragment was released from the pCRII vector and inserted into the mThy-1 expression cassette between exon 2 and 4 (Figure 1A), purified, and microinjected into one-cell embryos (C57BL/6xDBA/2 F1) according to standard procedures. For all experiments, heterozygous hα-synuclein and hβ-synuclein mice were crossed to generate bigenic mice. A total of 73 mice were generated: 11 singly tg hα-synuclein, 20 singly tg hβ-synuclein, 22 bigenic, and 20 non-tg. Genomic DNA was extracted from tail biopsies and analyzed with PCR amplification (Masliah et al., 2000; Rockenstein et al., 1995).

Assessment of Locomotor Activity

Briefly, mice were analyzed for 2 days in the rotarod (San Diego Instruments, San Diego, CA), as previously described (Masliah et al., 2000). On the first day, mice were trained for five trials: the first one at 10 rpm, the second at 20 rpm, and the third to the fifth at 40 rpm. On the second day, mice were tested for seven trials at 40 rpm each. Mice were placed individually on the cylinder and the speed of rotation increased from 0 to 40 rpm over a period of 240 s. The length of time mice remained on the rod (fall latency) was recorded and used as a measure of motor function.

Tissue Processing

For analysis of the various lines generated, mice were euthanized by transcardiac saline perfusion under anesthesia with chloral hydrate, and brains were removed and divided sagitally. For RNA or protein analysis (Masliah et al., 2000), the right hemibrain was snap-frozen in isopentane cooled in a Histobath (Shandon Lipshaw, Pittsburgh, PA) and stored at −70°C. For neuropathological analysis, the left hemibrain was immersion-fixed in 4% paraformaldehyde at 4°C for 48 hr and serially sectioned sagitally at 40 μm with the Vibratome 2000 (Leica, Deerfield, IL), as described previously (Masliah et al., 2000).

RNA Analysis

Total RNA was isolated from snap-frozen tissues using the TRI Reagent (Molecular Research Center, Cincinnati, OH). The following ^{32}P-labeled antisense riboprobes were used to identify specific mRNAs (protected nucleotides [GenBank accession number]): hα-synuclein (nt 210–475 [# L08850]); hβ-synuclein (nt 235–459 [# S69965]); and murine actin (nt 480–559 [# M18194] of mouse actin mRNA). Levels of specific RNAs were determined by RPA, as previously described (Rockenstein et al., 1995). Briefly, 10 μg of RNA hybridized to ^{32}P-labeled antisense riboprobes was digested with 40 U/ml RNase T1 (GIBCO-BRL, Grand Island, NY) and 20 μg/ml RNase A (Sigma Chemical Co., St. Louis, MO) in 100 μl volume digestion buffer. RNase was then inactivated with Proteinase K/N-Laurylsarkosine and precipitated with 4 M-guanidine thiocyanate/0.5% N-laurylsarkosine and isopropanol. Samples were separated on 5% acrylamide/8M urea TBE gels. Dried gels were exposed to Biomax film (Kodak, Rochester, NY), and signals were quantitated with a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software and expressed as integrated pixel intensities over defined volumes. Final values were expressed as ratios of (specific signal-background)/(actin signal-background) to correct for differences in RNA content/loading across samples.

Western Blot Analysis

Levels of hα- or β-synuclein immunoreactivity were analyzed in brain homogenates separated into cytosolic and particulate fractions (Masliah et al., 2000). Twelve μg of cytosolic fraction per mouse were loaded onto 10% SDS-PAGE gels, followed by transfer onto Immobilon membranes and incubation with the anti-hα- or β-synuclein antibody (1:1000). These antibodies were generated by Research Genetics, Inc. (Huntsville, AL), as previously described (Masliah et al., 2000) and affinity purified using the AminoLink Kit (Pierce, Rockford, IL) followed by followed by ^{125}I protein A. Blots were exposed to PhosphorImager (Molecular Dynamics) screens and analyzed with ImageQuant software. Experiments were done blind-coded, repeated at least once to assess reproducibility.

Immunocytochemical and Neuropathological Analysis of Transgenic Mice

Immunocytochemical analysis for α- and β-synuclein was performed in serially sectioned, free-floating, blind-coded vibratome sections from singly tg, bigenic, and non-tg mice (Masliah et al., 2000). Sections were incubated overnight at 4°C with anti-hα- or β-synuclein specific antibody (1:500), followed by biotinylated goat anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA), Avidin D-Horseradish peroxidase (1:200, ABC Elite, Vector), and reacted with diaminobenzidine tetrahydrochloride (DAB) containing 0.001% H_2O_2. Sections were analyzed with the Quantimet 570C (Leica) in order to determine the numbers of hα-synuclein immunoreactive inclusions in the neocortex. For each case, three sections were analyzed, and the results were averaged and expressed as numbers per sq mm. Double-immunocytochemical analysis was performed, as previously described (Mucke et al., 2000), to determine the relationship between hα-synuclein immunolabeled inclusions and hβ-synuclein-immunoreactive neurons. 40 μm thick vibratome sections were immunolabeled with the polyclonal antibodies against hα-synuclein (1:1000) detected with the Tyramide Signal Amplification-Direct (Red) system (1:100, NEN Life Sciences, Boston, MA) and β-synuclein (1:100) detected with the FITC-conjugated anti-rabbit secondary antibody (1:75, Vector). To determine if coexpression of hβ-synuclein ameliorated the neurodegenerative alterations associated with expression of hα-synuclein, sections were also double-immunostained with monoclonal anti-synaptophysin-Tyramide Red (1:2000, Roche Biochemicals, Indianapolis, IN) and monoclonal anti-TH-FITC (1:100, Roche) (Masliah et al., 2000). For each case, sections were immunolabeled in duplicate and analyzed with the LSCM and NIH Image 1.43 software to calculate the percent area of the neuropil covered by synaptophysin-immunoreactive terminals in the neocortex and TH-immunoreactive terminals in the caudoputamen and substantia nigra (Masliah et al., 2000). For each case, three sections were analyzed, and for each section four serial optical sections (2 μm thick) were obtained. In order to confirm the specificity of the primary antibodies, control experiments were performed where sections were incubated overnight in the absence of primary antibody (deleted), with the primary antibody preadsorbed for 48 hr with 20-fold excess of the corresponding peptide or with preimmune serum (PI).

All sections were processed simultaneously under the same conditions and experiments were performed twice in order to assess the reproducibility of results. Sections were imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss, Germany) with an attached MRC1024 LSCM system (Bio-Rad, Wattford, UK) (Masliah et al., 2000).

Cell Culture, Isolation of Stable Clones, and Induction of hα- and β-Synuclein Expression

Human α-synuclein cDNA, which is derived from the NheI and SalI fragments of pCEP4 α-synuclein (Takenouchi et al., 2001), was ligated into the NheI and XbaI site of the pIND inducible expression vector (Invitrogen, Carlsbad, CA). The pIND constructs containing either α-synuclein or no insert were transfected into EcR-293 cells, a permanent cell line derived from HEK293 that were previously transfected with the regulatory vector pVgRxR (Invitrogen). Cells were grown as a monolayer in T75 flasks in MEM with Hank's salts, supplemented with L-glutamine and 5% fetal calf serum. Transfection was performed using Lipofectamine according to the manufacturer's instructions (GIBCO-BRL, Grand Island, NY). After isolation of stable clones in the presence of 400 μg/ml G418, stable cell lines were generated from individual clones. The ability of cells to express hα-synuclein in response to induction with muristerone was assessed by Western blot analysis following a 36–48 hr induction with 5 μM muristerone A (Invitrogen, Carlsbad, CA). Among the high inducible expressers of α-synuclein, one cell line (α3) was further transfected with mammalian expression vector pCEP4 with either hβ-synuclein cDNA (Takenouchi et al., 2001) or no insert. Stable clones were selected in the presence of 200 μg/ml Hygromycin B, followed by screening for the constitutive expression of β-synuclein by immunoblotting.

Double Immunolabeling and Confocal Analysis of hα- and hβ-Synuclein Overexpressing Cells

Cells were seeded at subconfluence onto poly-L-lysine-coated glass coverslips and grown either in the presence or absence of 2 μM muristerone A for 36 hr. Cells were then washed in phosphate-buffered saline (PBS) and fixed for 20 min with 4% paraformalde-

hyde. The coverslips were first incubated overnight at 4°C with the mouse monoclonal antibody against hα-synuclein (LB509; Zymed, South San Francisco, CA) and detected with the Tyramide Red Signal Amplification-Direct (Red) system (NEN). Cells were then further incubated overnight with the rabbit polyclonal anti-β-synuclein antibody (1:1000) (Takenouchi et al., 2001) and detected by the FITC-conjugated anti-rabbit secondary antibody (Vector). The coverslips were air dried overnight, mounted on slides with anti-fading media (Vectashield, Vector), and imaged with the LSCM. Quantification of the numbers of hα-synuclein-positive inclusions in the cultured cells was performed with the Image 1.43 analysis program. Briefly, for each condition a total of ten digitized images (each image average of 0.1 sq mm) obtained from the LSCM were transfered to a personal Mac G3 computer and the number of cells containing hα-synuclein-positive inclusions was estimated. For each condition, an average of 1000 cells were analyzed.

Immunoprecipitation and SDS-PAGE Immunoblot Analysis of Transfected Cells

Cells were washed twice with PBS and incubated on ice for 20 min with a lysis buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES [pH 7.4], 140 mM NaCl, 1 mM EDTA,1 mM Na_3VO_4, 1 mM phenylmethyl-sulfonylfluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol). The cell lysates were then centrifuged (20,000 × g, 30 min), and protein concentrations in the supernatants were determined using BCA reagents (Pierce, Rockford, IL) and adjusted with 2XIP buffer. For each supernatant, 200 μg were preabsorbed with a protein G-sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 hr. The precleared lysates were then incubated at 4°C overnight with the β-synuclein antibody, and the immunocomplexes were precipitated by incubation with protein G-Sepharose at 4°C for 2 hr. Finally, the samples were washed three times with lysis buffer, washed once with PBS, boiled, and the proteins in the immunoprecipitates were separated using SDS-PAGE and analyzed by immunoblotting with the monoclonal anti-α-synuclein antibody (LB509). To further verify the specificity of the interactions between hα- and hβ-synuclein, control experiments were performed in cytosolic fractions from bigenic mice subjected to immunoprecipitation with antibodies against cytosolic proteins such as β-catenin (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), PI3 kinase p85α (Z-8, Santa Cruz), Bcl2 (N-19, Santa Cruz), and ubiquitin (DAKO, Carpinteria, CA), followed by immunoblot analysis with anti-hα-synuclein (LB509).

Preparation of Recombinant Proteins, In Vitro Aggregation Assay, and Immunoblot Analysis

Human recombinant α-synuclein, β-synuclein, and its mutants δ1 and δ2, and βsyn del2-15, were first produced using the PROEX-1 6xHis bacterial expression system (GIBCO-BRL, Grand Island, NY), as previously described (Hashimoto et al., 1998, 1999a). As to the β-synuclein mutants created by site-directed mutagenesis, δ1 contained the NAC region corresponding to aa 72–83 of α-synuclein, and δ2 was further substituted with the N-terminal part of NAC corresponding to aa 63–72 of α-synuclein. For each plasmid, fidelity of sequencing was confirmed with a detataq cycle sequencing kit (Amersham, Cleveland, OH). Aggregation studies of α-synuclein, β-synuclein, and its mutants δ1 and δ2, and βsyn del2-15 was performed as previously described (Hashimoto et al., 1998). Briefly, proteins (10–20 μM) were incubated in a total volume of 20 μl, containing 100 μM sodium acetate (pH 6.9), at 65°C for the times indicated (0, 8, and 20 hr). Immunoblot analysis was performed essentially as previously described (Hashimoto et al., 1999a). Briefly, each sample was resolved by SDS-PAGE (15%) electrophoresis and blotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked with Tris-buffered saline (TBS, pH 7.5) containing 3% BSA, followed by incubation with the affinity purified rabbit polyclonal antibody against α-synuclein (aa 101–124, 1:1000). Membranes were then incubated with ^{125}I-ProteinA (ICN, Costa Mesa, CA), followed by autoradiography and quantification of levels of reactivity with a PhosphorImager (Molecular Dynamics) (Hashimoto et al., 1999a).

Statistical Analysis

For all the different studies described, mice were coded to ensure objective assessment, and codes were not broken until the analysis was complete. Statistical analyses were carried out with the StatView 5.0 program (SAS Institute Inc., Cary, NC). Differences among means were assessed by one-way analysis of variance (ANOVA) followed, as indicated, by post-hoc Dunnett's (for comparisons between control and experimental groups) or post-hoc Tukey-Kramer (for comparisons between experimental groups) tests. Correlation studies were carried out by simple regression analysis. The null hypothesis was rejected at the 0.05 level.

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References

Alves Da Costa, C., Ancolio, K., and Checler, F. (2000). Wild-type but not Parkinson's disease-related ala-53→Thr mutant alpha synuclein protects neuronal cells from apoptotic stimuli. J. Biol. Chem. *275*, 24065–24069.

Biere, A.L., Wood, S.J., Wypych, J., Steavenson, S., Jiang, Y., Anafi, D., Jacobsen, F.W., Jarosinski, M.A., Wu, G.M., Louis, J.C., et al. (2000). Parkinson's disease associated alpha-synuclein is more fibrillogenic than beta- and gamma synuclein and cannot cross-seed its homologues. J. Biol. Chem. *275*, 34574–34579.

Conway, K.A., Harper, J.D., and Lansbury, P.T. (1998). Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nat. Med. *4*, 1318–1320.

Davidson, W.S., Jonas, A., Clayton, D.F., and George, J.M. (1998). Stabilization of alpha synuclein secondary structure upon binding to synthetic membranes. J. Biol. Chem. *273*, 9443–9449.

El-Agnaf, O., and Irvine, G.B. (2000). Review: Formation and properties of amyloid-like fibrils derived from alpha-synuclein and related proteins. J. Struct. Biol. *130*, 300–309.

Feany, M.B., and Bender, W.W. (2000). A *Drosophila* model of Parkinson's disease. Nature *404*, 394–398.

Ferrigno, P., and Silver, P.A. (2000). Polyglutamine expansions: proteolysis, chaperones, and the dangers of promiscuity. Neuron *26*, 9–12.

Forloni, G., Bertani, I., Calella, A.M., Thaler, F., and Invemizzi, R. (2000). Alpha-synuclein and Parkinson's disease: selective neurodegenerative effect of alpha-synuclein fragment on dopaminergic neurons in vitro and in vivo. Ann. Neurol. *47*, 632–640.

Han, H., Weinreb, P.H., and Lansbury, P.T., Jr. (1995). The core Alzheimer's peptide NAC forms amyloid fibrils, which seed and are seeded by β-amyloid: is NAC a common trigger or target in neurodegenerative disease? Chem. Biol. *2*, 163–169.

Hasegawa, K., Yamaguchi, I., Omata, S., Gejyo, F., and Naiki, H. (1999). Interaction between Abeta(1–42) and Abeta(1–40) in Alzheimer's beta-amyloid fibril formation in vitro. Biochemistry *38*, 15514–15521.

Hashimoto, M., and Masliah, E. (1999). Alpha-synuclein in Lewy body disease and Alzheimer's disease. Brain Pathol. *9*, 707–720.

Hashimoto, M., Hsu, L.J., Sisk, A., Xia, Y., Takeda, A., Sundsmo, M., and Masliah, E. (1998). Human recombinant NACP/α-synuclein is aggregated and fibrillated *in vitro*: Relevance for Lewy body disease. Brain Res. *799*, 301–306.

Hashimoto, M., Hsu, L.J., Xia, Y., Takeda, A., Sundsmo, M., and Masliah, E. (1999a). Oxidative stress induces amyloid-like aggregate formation of NACP/α-synuclein *in vitro*. NeuroReport *10*, 717–721.

Hashimoto, M., Takeda, A., Hsu, L.J., Takenouchi, T., and Masliah, E. (1999b). Role of cytochrome c as a stimulator of α-synuclein aggregation in Lewy body disease. J. Biol. Chem. *274*, 28849–28852.

Hsu, L.J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., Wong, J., Takenouchi, T., Hashimoto, M., and Masliah, E. (2000). α-synuclein promotes mitochondrial deficiencies and oxidative stress. Am. J. Pathol. *157*, 401–410.

Iwai, A. (2000). Properties of NACP/alpha-synuclein and its role in Alzheimer's disease. Biochim. Biophys. Acta *1502*, 95–109.

Iwai, A., Yoshimoto, M., Masliah, E., and Saitoh, T. (1995). Non-Aβ component of Alzheimer's disease amyloid (NAC) is amyloidogenic. Biochemistry *34*, 10139–10145.

Iwata, A., Miura, S., Kanazawa, I., Sawada, M., and Nukina, N. (2001). α-synuclein forms a complex with transcription factor Elk-1. J. Neurochem. *77*, 239–252.

Jakes, R., Spillantini, M.G., and Goedert, M. (1994). Identification of two distinct synucleins from human brain. FEBS Lett. *345*, 27–32.

Jenner, P. (1996). Oxidative stress in Parkinson's disease and other neurodegenerative disorders. Pathol. Biol. *44*, 57–64.

Jensen, P.H., Sorensen, E.S., Petersen, T.E., Gliemann, J., and Rasmussen, L.K. (1995). Residues in the synuclein consensus motif of the alpha-synuclein fragment, NAC, participate in transglutaminase-catalyzed cross-linking to Alzheimer-disease amyloid beta A4 peptide. Biochem. J. *310*, 91–94.

Jensen, P.H., Hojrup, P., Hager, H., Nielsen, M.S., Jacobsen, L., Olesen, O.F., Gliemann, J., and Jakes, R. (1997). Binding of Aβ to α- and β-synucleins: identification of segments in α-synuclein/NAC precursor that bind Aβ and NAC. Biochem. J. *323*, 539–546.

Jia, T., Liu, Y.E., Liu, J., and Shi, Y.E. (1999). Stimulation of breast cancer invasion and metastasis by synuclein gamma. Cancer Res. *59*, 742–747.

Jo, E., McLaurin, J., Yip, C.M., St.George-Hyslop, P., and Graser, P.E. (2000). Alpha-synuclein membrane interactions and lipid specificity. J. Biol. Chem. *275*, 34328–34334.

Kanda, S., Bishop, J.F., Eglitis, M.A., Yang, Y., and Mouradian, M.M. (2000). Enhanced vulnerability to oxidative stress by alpha-synuclein mutations and C-terminal truncation. Neuroscience *97*, 279–284.

Koo, E.H., Lansbury, P.T., Jr., and Kelly, J.W. (1999). Amyloid diseases: Abnormal protein aggregation in neurodegeneration. Proc. Natl. Acad. Sci. USA *96*, 9989–9990.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Reiss, O. (1998). Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. Nat. Gen. *18*, 106–108.

Masliah, E. (1998). The role of synaptic proteins in neurodegenerative disorders. Neurosci. News *1*, 14–20.

Masliah, E. (2001). Recent advances in the understanding of the role of synaptic proteins in Alzheimer's disease and other neurodegenerative disorders. J. Alz. Dis. *3*, 1–9.

Masliah, E., Iwai, A., Mallory, M., Ueda, K., and Saitoh, T. (1996). Altered presynaptic protein NACP is associated with plaque formation and neurodegeneration in Alzheimer's disease. Am. J. Pathol. *148*, 201–210.

Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: Implications for neurodegenerative disorders. Science *287*, 1265–1269.

Masliah, E., Rockenstein, E., Veinbergs, I., Sagara, Y., Mallory, M., Hashimoto, M., and Mucke, L. (2001). βAmyloid peptides enhance αSynuclein accumulation and neuronal deficits in a transgenic mouse model linking Alzheimer's and Parkinson's disease. Proc. Natl. Acad. Sci. USA *98*, 12245–12250.

Mucke, L., Masliah, E., Yu, G.-Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., and McConlogue, L. (2000). High-level neuronal expression of Abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: Synaptotoxicity without plaque formation. J. Neurosci. *20*, 4050–4058.

Nakajo, S., Tsukada, K., Omata, K., Nakamura, Y., and Nakaya, K. (1993). A new brain-specific 14-kDa protein is a phosphoprotein. Its complete amino acid sequence and evidence for phosphorylation. Eur. J. Biochem. *217*, 1057–1063.

Narhi, L., Wood, S.J., Steavenson, S., Jiang, Y., Wu, G.M., Anafi, D., Kaufman, S.A., Martin, F., Sitney, K., Denis, P., et al. (1999). Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. J. Biol. Chem. *274*, 9843–9846.

Noguchi, C.T., Rodgers, G.P., and Schechter, A.N. (1989). Intracellular polymerization. Disease severity and therapeutic predictions. Ann. NY Acad. Sci. *565*, 75–82.

Osterova, N., Petrucelli, L., Farrer, M., Mehta, M., Choi, P., and Hardy, J. (1999). alpha- Synuclein shares physical and functional homology with 14–3-3 proteins. J. Neurosci. *19*, 5782–5791.

Osterova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farer, M., and Wolozin, B. (2000). The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. J. Neurosci. *20*, 6048–6054.

Paik, S.R., Lee, J.H., Kim, D.H., Chang, C.S., and Kim, Y.S. (1998). Self-oligomerization of NACP, the precursor protein of the non-amyloid beta/A4 protein (A beta) component of Alzheimer's disease amyloid, observed in the presence of C-terminal A beta fragment (residues 25–35). FEBS Lett. *421*, 73–76.

Paik, S.R., Shin, H.J., Lee, J.H., Chang, C.S., and Kim, J. (1999). Copper(II)-induced self-oligomerization of alpha-synuclein. Biochem. J. *340*, 821–828.

Perrin, R.J., Woods, W.S., Clayton, D.F., and George, J.M. (2000). Interaction of human alpha-synuclein and Parkinson's disease variants with phospholipids: structural analysis using site-directed mutagenesis. J. Biol. Chem. *275*, 34393–34398.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., et al. (1997). Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045–2047.

Ramassamy, C., Aveiill, D., Beffert, U., Bastianetto, S., Tberoux, L., Lussier-Cacan, S., Cohn, J.S., Christen, Y., Davignon, J., Quirion, R., and Poirier, J. (1999). Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. Free Rad. Biol. Med. *27*, 544–553.

Rochet, J.C., Conway, K.A., and Lansbury, P.T., Jr. (2000). Inhibition of fibrillization and accumulation of prefibrillar oligomers in mixtures of human and mouse alpha-synuclein. Biochemistry *39*, 10619–10626.

Rockenstein, E.M., McConlogue, L., Tan, H., Power, M., Masliah, E., and Mucke, L. (1995). Levels and alternative splicing of amyloid β protein precursor (APP) transcripts in brains of APP transgenic mice and humans with Alzheimer's disease. J. Biol. Chem. *270*, 28257–28267.

Rockenstein, E., Hansen, L., Mallory, M., Trojanowski, J.Q., Galasko, D., and Masliah, E. (2001). Altered expression of the synuclein family in Lewy body and Alzheimer's disease. Brain Res., *914*, 48–56.

Serpell, L.C., Berriman, J., Jakes, R., Goedert, M., and Crowther, R.A. (2000). Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-β conformation. Proc. Natl. Acad. Sci. *97*, 4897–4902.

Souza, J.M., Giasson, B.I., Chen, Q., and Lee, V.-Y.I.H. (2000). Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. J. Biol. Chem. *275*, 18344–18349.

Spillantini, M.G., Schmidt, M.L., Lee, V.-Y., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). α-synuclein in Lewy bodies. Nature *388*, 839–840.

Surguchov, A., Surgucheva, I., Solessio, E., and Baehr, W. (1999). Synoretin—A new protein belonging to the synuclein family. Mol. Cell. Neurosci. *13*, 95–103.

Tabrizi, S.J., Orth, M., Wilkinson, J.M., Taanman, J.W., Warner, T.T., Cooper, J.M., and Schapira, A.H. (2000). Expression of mutant alpha-synuclein causes increased susceptibility to dopamine toxicity. Hum. Mol. Genet. *9*, 2683–2689.

Takeda, A., Mallory, M., Sundsmo, M., Honer, W., Hansen, L., and Masliah, E. (1998). Abnormal accumulation of NACP/α-synuclein in neurodegenerative disorders. Am. J. Pathol. *152*, 367–372.

Takenouchi, T., Hashimoto, M., Hsu, L.J., Mackowski, B., Rockenstein, E., Mallory, M., and Masliah, E. (2001). Reduced neuritic outgrowth and cell adhesion in neuronal cells transfected with human α-synuclein. Mol. Cell. Neurosci. *17*, 141–150.

Trojanowski, J.Q., Goedert, M., Iwatsubo, T., and Lee, V.-M. (1998). Fatal attractions: abnormal protein aggregation and neuron death in Parkinson's disease and lewy body dementia. Cell Death Differ. *5*, 832–837.

Ueda, K., Fukushima, H., Masliah, E., Yia, Y., Iwai, A., Otero, D., Kondo, J., Ihara, Y., and Saitoh, T. (1993). Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer's disease. Proc. Natl. Acad. Sci. USA *90*, 11282–11286.

Wakabayashi, K., Matsumoto, K., Takayama, K., Yoshimoto, M., and Takahashi, H. (1997). NACP, a presynaptic protein, immunoreactivity in Lewy bodies in Parkinson's disease. Neurosci. Lett. *239*, 45–48.

Weinreb, P.H., Zhen, W., Poon, A.W., Conway, K.A., and Lansbury, P.T., Jr. (1996). NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. Biochemistry *35*, 13709–13715.

Wood, S.J., Wypych, J., Steavenson, S., Louis, J.C., Citron, M., and Biere, A.L. (1999). alpha-synuclein fibrillogenesis is nucleation dependent. Implications for the pathogenesis of Parkinson's disease. J. Biol. Chem. *274*, 19509–19512.

Yoshimoto, M., Iwai, A., Kang, D., Otero, D., Xia, Y., and Saitoh, T. (1995). NACP, the precursor protein of non-amyloid β/A4 protein (Aβ) component of Alzheimer disease amyloid, binds Aβ and stimulates Aβ aggregation. Proc. Natl. Acad. Sci. USA *92*, 9141–9145.

Zhou, W., Hurlbert, M.S., Schaack, J., Prasad, K.N., and Freed, C.R. (2000). Overexpression of human alpha-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells. Brain Res. *866*, 33–43.