Enhanced Lysosomal Pathology Caused by β-Synuclein Mutants Linked to Dementia with Lewy Bodies*\$1

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Two missense mutations (P123H and V70M) of β-synuclein (β-syn), the homologue of α-syn, have been recently identified in dementia with Lewy bodies. However, the mechanism through which these mutations influence the pathogenesis of dementia with Lewy bodies is unclear. To investigate the role of the β-syn mutations in neurodegeneration, each mutant was stably transfected into B103 neuroblastoma cells. Cells overexpressing mutated β-syn had eosinophilic cytoplasmic inclusion bodies immunopositive for mutant β-syn, and electron microscopy revealed that these cells were abundant in various cytoplasmic membranous inclusions resembling the histopathology of lysosomal storage disease. Consistent with these findings, the inclusion bodies were immunopositive for lysosomal markers, including cathepsin B, LAMP-2, GM2 ganglioside, and ATP13A2, which has recently been linked to PARK9. Notably, formation of these lysosomal inclusions was greatly stimulated by co-expression of α-syn, which was dependent on the phosphorylation of α-syn at Ser-129, and was more efficient with the A53T familial mutant of α-syn compared with wild type. Furthermore, the inclusion formation in cells overexpressing mutant β-syn and transfected with α-syn was significantly suppressed by treatment with autophagy-lysosomal inhibitors, which were associated with impaired clearance of syn proteins and enhanced apoptosis, indicating that formation of lysosomal inclusions might be protective. Collectively, the results demonstrated unambiguously that overexpression of β-syn mutants (P123H and V70M) in neuroblastoma cells results in an enhanced lysosomal pathology. We suggest that these missense mutations of β-syn might play a causative role in stimulating neurodegeneration.

The synuclein (syn) family of peptides is a group of presynaptic proteins with three members: α-syn, β-syn, and γ-syn (1, 2). These proteins are characterized by natively unfolded structures with highly conserved N terminal and divergent C-terminal acidic regions. Importantly, α-syn is distinct from other members of the syn family in that it possesses a highly hydrophobic central region that has been identified as a non-amyloid-β component (NAC) of Alzheimer disease amyloid (3). Since the discovery of the linkage of two missense mutations (A53T and A30P) to familial Parkinson disease (PD) (4, 5), numerous histopathological studies have shown that α-syn fibrils are the major constituent in Lewy bodies and glial cell inclusions in a wide range of Lewy body disorders, including sporadic PD, dementia with Lewy bodies (DLB), and multiple system atrophy (6–9). Furthermore, another missense mutation E46K was recently identified for DLB (10). All the mutant proteins have a greater propensity for self-association and aggregation compared with wild-type (wt) α-syn (11, 12), suggesting that α-syn aggregation may play a causative role in stimulation of neurodegenerative disorders.

In contrast to α-syn, β-syn may be neuroprotective, because this molecule has a natural deletion in the middle of the NAC-associate region. Supporting this notion, neuropathological features of α-syn transgenic (tg) mice, such as formation of Lewy bodies and motor function deficits (13), are significantly ameliorated in α- and β-syn bigenic mice compared with α-syn single tg mice (14, 15). Furthermore, β-syn directly inhibited aggregation and protifibrillar formation of α-syn under cell-free conditions (14, 16, 17) and overexpression of β-syn in cell cultures up-regulates Akt signaling pathway through a chaperone-like action (18). Thus, these results suggested that β-syn is protective against α-syn-related neurodegeneration. Although γ-syn also inhibits α-syn aggregation (16), the role of this molecule in neuroprotection is less clear.

It is natural to speculate that alteration of the neuroprotective β-syn might be relevant to the pathogenesis of neurodegenerative disorders. Indeed, a limited number of investigations have suggested that both α-syn and other syn proteins are involved in the pathogenesis of neurodegenerative disease. α-syn, β-syn, and γ-syn have been reported to accumulate abnormally in

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† The abbreviations used are: syn, synuclein; NAC, non-amyloid-β component; PD, Parkinson disease; DLB, dementia with Lewy bodies; wt, wild type; tg, transgenic; J-MA, J-methyldenine; GM1, Gaq1, 3GalNAc2, 4GalNAc2, 3Gal; Neu5Ac-v2,3-Gal1,4Glc1; 1-ceramide; GM2, Ga2Nac2,4GalNAc3GalβGlc(C1)-ceramide; GM3, NeuAcx3GalβGlc(C1)-ceramide; BSA, bovine serum albumin; TBS, Tris-buffered saline; LSCM, laser-scanning confocal microscope; PBS, phosphate-buffered saline; DAPI, 6-diamino-2-phenylindole dihydrochloride; H&E, hematoxylin and eosin; TUNEL, TdT-mediated dUTP nick end labeling.

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dystrophic neurites in PD and DBL brains (19), and accumulation of β- and γ-syn has been found in the axonal spheroid bodies formed in gracile axonal dystrophy mice with a naturally truncated LCHL-I (PARKS) gene (20, 21). Moreover, both α- and β-syn were accumulated in lysosomal vacuoles formed in presenilin-1 knock-out mice (22). In this context, it is of note that two missense mutations of β-syn have been identified in unrelated DBL cases (23): a proline to histidine substitution at position 123 (P123H) identified in several members in a familial DBL case in Seattle, and a valine to methionine change at position 70 (V70M) found in one case of sporadic DBL in Japan. The P123H substitution may alter the charge state of the C-terminal domain of β-syn, whereas the V70M substitution may change the hydrophobicity of the NAC corresponding region. Because the phosphorylation state of the C-terminal domain and the hydrophobicity of the NAC region are critical for aggregation of β-syn, it is possible that these missense mutations of β-syn may confer aggregation properties on β-syn, leading to stimulation of neurodegeneration. Nonetheless, it is unclear whether these missense mutations of β-syn are causative for neurodegeneration (23).

The main objective of the current study was to determine if missense mutations (P123H and V70M) of β-syn play causative roles in neurodegeneration. For this purpose, each β-syn mutant was stably transfected into B103 neuroblastoma cells. These cells had a characteristic enhanced lysosomal pathology, suggesting that P123H and V70M might be functionally relevant in the pathogenesis of DBL.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Reagents, including 3-methyladenine (3-MA), ammonium chloride (NH₄Cl), and rapamycin (obtained from Sigma), were applied to cell cultures at the indicated concentrations.

The following antibodies were used in this study: monoclonal anti-α-syn (syn-1) and anti-β-syn antibodies (BD Biosciences, Franklin Lakes, NJ), monoclonal anti-phosphorylated-α-syn antibody (Wako Pure Chemical Industries, Ltd., Osaka, Japan), monoclonal anti-β-actin (AC-15) and anti-α-tubulin (B-5-1-2) antibodies (Sigma), monoclonal anti-ubiquitin antibody (Chemicon, Temecula, CA), monoclonal anti-19S proteasome S6a subunit antibody (Biomol, Plymouth Meeting, PA), rabbit polyclonal anti-cathepsin B (EMD Biosciences, San Diego, CA), monoclonal anti-LAMP-2 (H4B4) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and polyclonal anti-ATP13A2 antibody (ab43075, Abcam, Tokyo, Japan). Cholera toxin subunit B conjugated with Alexa Fluor 488 was purchased from Molecular Probes (Eugene, OR). We also used rabbit polyclonal antibodies, anti-C-terminal α-syn and anti-β-syn, which we reported previously (7, 27), murine monoclonal antibodies for gangliosides, GM2 and GM3, have also been described previously (28). Alexa Fluor 488-conjugated anti-goat and anti-rabbit antibodies and Alexa Fluor 555-conjugated anti-mouse antibody (Molecular Probes) were used as second antibody.

**Construction of Expression Vectors**—To create a β-syn P123H expression vector, PCR was performed using primers based on the wt human β-syn sequence (BT006627): sense primer 5′-ACATCGCCGCCGATGACGTGTTACATGAA-GGGCCTG-3′ (NotI-β-syn: N-terminal positions 1–24 of human β-syn and a NotI site) and antisense primer 5′-ACAT-CGGATCTACGCTTGCTAGATCTCTCTGTTGCAGG-CTG-3′ (C-terminal 362–405 of human P123H β-syn and a BamHI site). The PCR product was digested with NotI and BamHI and ligated into a pCEP4 expression vector (Invitrogen) previously digested with NotI and BamHI to generate pCEP4-β-syn P123H.

Next, to create β-syn V70M cDNA, a two-step PCR strategy was performed. Briefly, two sets of primer pairs, sense primer 5′-ACATCGCCGCCGATGACGTGTTACATGAA-GGGCCTG-3′ (NotI-β-syn: N-terminal 1–24 of human β-syn and a NotI site) and antisense primer 5′-CCAGAGAACACAGCTC-CTCC-3′ (reverse sequence 189–208 of human V70M β-syn), and sense primer 5′-GGAGGAGCTATGTTCTTG-3′ (sense sequence 189–208 of human V70M β-syn) and antisense primer 5′-ACATCGGATCCTACGCTTGCTAGATCTCTCTGTTGCAGG-CTG-3′ (BamHI-C-β-syn: C-terminal 383–405 of human β-syn and a BamHI site) were individually incubated with pCEP4-β-syn (29) as a template in the first PCR reaction. The PCR products were gel-purified, combined, and incubated with NotI-N-β-syn and BamHI-C-β-syn primers to synthesize full-length β-syn V70M cDNA in the second PCR reaction. The resulting product was digested with NotI and BamHI and inserted into NotI and BamHI sites of pCEP4 to generate pCEP4 β-syn V70M.

In other sets of experiments, two artificial Ser-129 mutants of α-syn, S129A and S129E, were produced by one-step PCR, and three familial PD-linked α-syn mutants, A30P, E46K and A53T, were created by two-step PCR. The α-syn mutant cDNAs were subcloned into a mammalian expression vector p-TARGET (Promega Biotech, Madison, WI). The fidelity of the sequence was confirmed for each plasmid construct.

**Cell Cultures and Transfection**—Rat B103 neuroblastoma cells have been used previously to investigate the roles of α- and β-syn in neurodegeneration (18, 27). These cells were cultured in Dulbecco’s modified Eagle’s medium (high glucose) containing 10% fetal calf serum (BioWest, Nuaillé, France) and 1% penicillin/streptomycin (Invitrogen) in a 5% CO₂/95% air atmosphere. For stable transfection, cells were transfected with pCEP4 or pCEP4 containing human wt β-syn, P123H β-syn, or V70M β-syn using Lipofectamine 2000 (Invitrogen). After incubation for 2–3 weeks in the presence of 200 μg/ml hygromycin B (EMD Biosciences), resistant colonies of cells (~20) were isolated. These stable cell lines were maintained routinely in the presence of 50 μg/ml hygromycin B.

**Immunoblot Analysis**—Unless specifically indicated, experimentally growing cells under semi-confluent conditions were harvested and dissolved in lysis buffer (1% Nonidet P-40, 50 mm HEPES, 150 mm NaCl, 10% glycerol, 1.5 mm MgCl₂, 1 mm EGTA, 100 mm sodium fluoride, and protease inhibitor mixture (Nacalai Tesque, Tokyo, Japan). Protein concentrations of cell lysates were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). In some experiments, insoluble fractions of the lysis buffer were solubilized with SDS-PAGE sample buffer. Ten μg of detergent-soluble fractions and the corresponding volume of detergent-insoluble fractions were subjected to analysis.
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**FIGURE 1.** Inclusion body formation in B103 neuroblastoma cells overexpressing mutant β-syn. A, immunoblot analysis for mutant β-syn proteins in transfected B103 cells. Cell extracts (10 μg) were analyzed by immunoblotting using anti-β-syn monoclonal antibody (top panel), anti-α-syn monoclonal antibody syn-1 (middle panel), and anti-actin antibody (bottom panel). Three wt β-syn clones (βw-3, -4, and -10, lanes 3–5), three high expresser clones for P123H β-syn (ph-3, -7, and -12, lanes 6–8), and V70M β-syn (vm-5, -8, and -13, lanes 9–11) are shown in addition to vector-transfected cells lane 1) and α-syn-overexpressing cells (aw-4) (lane 2). The aw-4 clone has previously been referred to as clone α-4 (27). Recombinant α- and β-syn proteins are used as positive controls (lanes 12 and 13). B, representative immunofluorescence/LSCM images for β-syn expression in transfected B103 cells. Cells overexpressing α-syn (aw-4) (a), wt β-syn (βw-4) (b), β-syn P123H (ph-12) (c, e, and f) or β-syn V70M (vm-8) (d) were immunostained with antibodies against α-syn (green; a), β-syn (red; b–d), and ubiquitin (Ub) (green; f), followed by observation by LSCM. In e, primary antibody was not added. Arrows indicate inclusion bodies. The bar represents 20 μm, and all figures are at the same magnification.

Immunoblot analysis was performed as described previously (30). Briefly, cell extracts were resolved by SDS-PAGE (16%) and electroblotted onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ) with 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11.0). The membranes were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl) plus 0.2% Tween 20, followed by incubation with primary antibodies in TBS containing 3% BSA. After washing, the membranes were incubated with a secondary antibody conjugated with horse-radish peroxidase (GE Healthcare) in TBS (1:10,000). Recombinant α- and β-syn proteins were used as positive controls (14).

**Immunofluorescence/Laser Scanning Confocal Microscopy**—An immunofluorescence study was performed as described previously (30). Briefly, cells were seeded on poly-L-lysine-coated glass coverslips, grown to 70% confluence, fixed with 4% paraformaldehyde in 0.1M sodium cacodylate buffer at 4 °C for 20 min, and subjected to freezing and thawing to rupture cell membranous structures, and centrifuged at 15,000 rpm for 10 min. The supernatants (10 μl) were then incubated either with benzylxycarbonyl-Arg-Arg-Glu-amidomethylcoumarin fluorescent cathepsin B substrate (40 μM) or benzylxycarbonyl-Leu-Leu-Glu-amidomethylcoumarin fluorescent proteasome substrate (40 μM, both purchased from Chemicon). The enzymatic activities were assayed by continuous recording of the fluorescence activity released from fluorogenic substrate using Berthold Mithras LB940 microplate reader (Berthold, Bad Wildbad, Germany) for 1 h at 37 °C (excitation, 380 nm; emission, 460 nm), and the reaction rates were analyzed. The activities were described as arbitrary units/min/mg of protein.

**Electron Microscopy**—Electron microscopic analysis was performed as described previously (30). Briefly, cells were harvested using trypsin-EDTA and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C for 2 h. After centrifugation, cells were washed with 0.1 M sodium cacodylate buffer three times. Cell pellets were obtained by centrifugation, post-fixed in 1% osmium tetroxide and 1% potassium ferrocyanide at room temperature for 2 h, and processed for embedding in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead nitrate and observed using a Hitachi H-7500 electron microscope.

For immunoelectron microscopy, washed cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h at 4 °C. After washing in PBS, cell pellets were processed for embedding in LR-White resin (Nissin EM, Tokyo, Japan). Ultrathin sections were treated in 10% hydrogen peroxide in methanol for 30 min for etching. After blocking with 1% BSA, the sections were incubated with the primary antibody (or antibodies for double staining) in PBS (1:200–400) overnight, followed by incubation with goat anti-mouse antibody (1:50) labeled with 10 nm gold particles (or the goat anti-rabbit antibodies labeled with 5 nm gold particles for double staining) (BB International, Cardiff, UK) for 3 h. Finally, the sections were stained with uranyl acetate and lead citrate and subjected to imaging.

**Evaluation of Lysosomal (Cathepsin B) and Proteasomal Activities**—The assays were done as described previously (30). Briefly, cells growing in sub-confluent conditions were harvested in buffer containing 50 mM HEPES (pH 7.4), 10 mM EDTA, and 10 mM NaCl, subjected to freezing and thawing to rupture cell membranous structures, and centrifuged at 15,000 rpm for 10 min. The supernatants (10 μl) were then incubated with either benzylxycarbonyl-Arg-Arg-Glu-amidomethylcoumarin fluorescent cathepsin B substrate (40 μM) or benzylxycarbonyl-Leu-Leu-Glu-amidomethylcoumarin fluorescent proteasome substrate (40 μM, both purchased from Chemicon). The enzymatic activities were assayed by continuous recording of the fluorescence activity released from fluorogenic substrate using Berthold Mithras LB940 microplate reader (Berthold, Bad Wildbad, Germany) for 1 h at 37 °C (excitation, 380 nm; emission, 460 nm), and the reaction rates were analyzed. The activities were described as arbitrary units/min/mg of protein.

**Hematoxylin and Eosin and Thioflavin S Staining**—H&E staining was performed according to the manufacturer’s instructions (Sigma). The number of cells with eosinophilic inclusions was counted in 6 different fields of 1000 cells under each experimental condition. Inclusion bodies with a maximum diameter of >2 μm were counted by an investigator who...
was blinded to the experimental condition. For thioflavin S staining, transfected cells were washed with PBS and incubated with 0.01% thioflavin S (Sigma) for 8 min.

**Co-immunoprecipitation Experiments**—The assay was performed as described previously (14) with minor modifications. Briefly, cells were solubilized in lysis buffer (0.5% Tween 20, 10% glycerol, 50 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM EGTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Lysates (200 μg) were pre-absorbed with protein G-Sepharose (GE Healthcare) for 1 h, and the precleared lysates were incubated with either syn-1 antibody or mouse IgG (1 μg each) overnight at 4°C, followed by incubation with protein G-Sepharose. The immune complexes were then washed three times with lysis buffer. The samples were then heated in the SDS sample buffer for 5 min, and then subjected to immunoblot analysis.

**TUNEL Assay**—This procedure was performed as described previously with some modifications (31). Briefly, cells were fixed in 4% paraformaldehyde, rinsed in PBS, left overnight in 70% ethanol, and then processed for TUNEL labeling as recommended by the manufacturer (Roche Applied Science kit). Staining was assessed by LSCM. All cells were stained with DAPI to quantify the percentage of neurons undergoing cell death.

**Statistical Analysis**—All values in figures are expressed as means ± S.D. To determine statistical significance, the values were compared by two-group t-tests with differences considered significant for p values <0.05.

**RESULTS**

**Inclusion Body Formation in B103 Neuroblastoma Cell Overexpressing Mutant β-Syn (P123H and V70M)**—To investigate the role of missense mutations (P123H and V70M) of β-syn in neurodegeneration, B103 neuroblastoma cells were stably transfected with P123H or V70M β-syn cDNA. Three clones (ph-3, -7, and -12) with high expression of P123H β-syn, and three (vm-5, -8, and -13) with high expression of V70M β-syn were selected based on immunoblot analysis (Fig. 1A). Similarly to cells overexpressing wt β-syn (clones βw-3, -4, and -10), little immunoreactivity of oligomeric β-syn was detected in cells overexpressing mutant β-syn. In the majority of experiments, the high expresser clones ph-12 and vm-8 were used for comparison with the wt β-syn-overexpressing clone, βw-4. Cellular growth rates among the clonal cell line showed little difference under regular growth conditions (data not shown).

To analyze the intracellular distribution of syn proteins, immunofluorescence was performed (Fig. 1B). In agreement with a previous study (27), both wt α-syn and wt β-syn were diffusely distributed in their cell bodies in staining by syn-1 (a) and monoclonal anti-β-syn antibody (b), respectively. A similar pattern of immunoreactivity was observed in cells overexpressing P123H β-syn. However, one striking difference was that these cells were occasionally burdened with large inclusion bodies in the cytoplasm (c). No immunoreactivity was detected in P123H β-syn overexpressing cells in the absence of anti-β-syn antibody (d). Similar formation of anti-β-syn immunoreactive inclusions was observed in V70M β-syn overexpressing cells (e), and the inclusion bodies were frequently ubiquitin positive (f).

**Ultrastructural Characterization of Inclusion Bodies**—Electron microscopy was performed to investigate the ultrastructure of inclusion bodies formed in cells overexpressing mutant β-syn (Fig. 2A). Compared with both vector-transfected and α-syn overexpressing cells (b and c), cells overexpressing P123H β-syn were characterized by the presence of various types of lysosomal inclusion bodies (a, d–f). Especially, two types of large inclusions were frequently observed: one type contained numerous cystic membranous inclusions (d), and the other had electron-high dense myelinosome-like inclusions composed of concentric and/or multilamellar periodic membranes (e). Some inclusion bodies had mixed characteristics of the two types (f), suggesting that the myelinosome-like inclusion bodies might be derived from the former type. Numerous small inclusions were also present in cells overexpressing P123H β-syn. Similar lysosomal inclusion bodies...
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FIGURE 3. Immunofluorescence/LSCM characterization of lysosomal inclusion bodies. A, representative double immunofluorescence/LSCM for cells overexpressing P123H β-syn (ph-12). Cells are doubly stained for β-syn (red; a, d, g, m, and p) and cathepsin B (CatB) (green; b, LAMP-2 (green; e), GM1 (green; h), GM2 (green; k), GM3 (green; n), or γ-tubulin (green; q). Nuclei are simultaneously stained with DAPI in the merged figures (c, f, i, l, o, and r). The bar represents 20 μm. Arrows and arrowheads indicate inclusion body formation. Note that CatB and LAMP-2 are specifically co-localized with P123H β-syn in inclusion bodies, whereas γ-tubulin is only partially co-localized. In addition, GM2 and to a lesser extent GM1, but not GM3, are co-localized with P123H β-syn in inclusion bodies. B, representative double immunofluorescence/LSCM for cells overexpressing wt β-syn (j–l) and P123H β-syn (ph-12). Cells are doubly stained for β-syn (red; a and d) and ATP13A2 (green; b and e). Nuclei are simultaneously stained with DAPI in the merged figures (c and f). The bar represents 20 μm. Arrows indicate co-localization of ATP13A2 and P123H β-syn in inclusion bodies. were observed in V70M β-syn-overexpressing cells (data not shown).

To investigate the localization of mutant β-syn in the lysosomal inclusion bodies, immunoelectron microscopy was performed (Fig. 2B). Gold particles with mutant β-syn were associated with fibril-like structures (g and j) and amorphous structures (h and k) in the inclusion bodies in cells overexpressing P123H β-syn. Furthermore, when these cells were transfected with wt α-syn, immunogold markers of mutant β-syn and α-syn were both associated with fibril-like structures in the inclusion bodies (i and l). Similar granular type fibril formation occurred with recombinant mutant β-syn (supplemental Fig. S1). However, further studies are required to determine if the fibril-like structures in the inclusion bodies in cells overexpressing mutant β-syn are amyloid-fibrils composed of syn proteins. Taken together, these results demonstrate that overexpression of the β-syn mutants leads to formation of enhanced lysosomal structures.

Immunofluorescence/LSCM Characterization of Lysosomal Inclusion Bodies—Unique ultrastructures, such as multiple cystic membraneous inclusions and electron dense myelinosome-like inclusions, have been well characterized in ganglioside-related lysosome storage disease, and therefore we predicted that the inclusion bodies formed in cells overexpressing mutant β-syn might be derived from lysosomal structures. To test this possibility, double immunostaining study was performed using antibodies for various lysosomal markers (Fig. 3A). The inclusions in cells overexpressing P123H β-syn were consistently immunoreactive for both cathepsin B (a–c) and LAMP-2 (d–f). These cells were also immunopositive for GM2 (j–l) and to a lesser extent with GM1 (g–i), but were negative for GM3 (m–o). In contrast, immunoreactivity with γ-tubulin (p–r), an aggresome marker (32), was only partially detected in the inclusions. Similarly, immunoreactivity of the proteasome subunit S6a was not localized in the inclusion bodies (data not shown).

It has recently been shown that missense mutations of ATP13A2, a type 5 p-type ATPase, are linked to an autosomal recessive early onset parkinsonism (PARK9), and transient transfection of ATP13A2 into COS7 cells resulted in localization of this molecule in lysosomes (33). Therefore, we performed an immunofluorescence study for the lysosomal ATP13A2 (Fig. 3B). Immunoreactivity of ATP13A2 showed considerable overlap with that of mutant β-syn in lysosomal inclusion bodies and was higher in cells overexpressing mutant β-syn than in other cell types. Collectively, these results show that various lysosomal markers and ATP13A2 co-localized with mutant β-syn in lysosomal inclusion bodies in cells overexpressing mutant β-syn.

Up-regulation of Lysosomal Activity in Cells Overexpressing Mutant β-syn—To determine if the activity of lysosome is altered in cells overexpressing mutant β-syn, the activity of cysteine protease cathepsin B, one of the major lysosomal proteases, was evaluated (Fig. 4A). The activity of cathepsin B in cells overexpressing mutant β-syn was much higher than those in other cell types. Under the same experimental conditions, the activity of proteasome was little affected in cells overexpressing mutant β-syn (Fig. 4B). These results suggest that mutant β-syn stimulates the lysosomal activity without interfering with the proteasomal activity.

On the other hand, the activity of cathepsin B in cells overexpressing α-syn was slightly but significantly increased (Fig. 4A), whereas the activity of proteasome in these cells was significantly decreased compared with those in vector-transfected cells and cells overexpressing wt β-syn (Fig. 4B). Thus, these results suggest that the increased lysosomal activity in cells overexpressing α-syn might reflect a compensatory mechanism for the compromised proteasomal activity. In support of the above notion, immunofluorescence showed that the immuno-
reactivity of the proteasome subunit S6a was well merged with that of α-syn in cells overexpressing α-syn, but not with that of β-syn in cells overexpressing mutant β-syn (data not shown).

Formation of Lysosomal Inclusion Bodies by Mutant β-Syn Is Increased by Co-expression of α-Syn—H&E staining is effective for detection of eosiophilic lysosomal structures, and this method was used to stain lysosomal inclusion bodies formed in cells overexpressing mutant β-syn (Fig. 5A). The number of inclusion bodies counted by H&E staining was well correlated with the number of P123H β-syn immunoreactive inclusion bodies formed in wt B103 cells transfected with wt α-syn and mutant β-syn were estimated to be 2.6 ± 0.2% for P123H β-syn and 1.9 ± 0.3% for V70M β-syn (lanes 7 and 8, respectively), whereas the combination of wt α-syn and wt β-syn resulted in little inclusion formation (0.4 ± 0.1%) (lane 6). Inclusion bodies in P123H β-syn- and V70M β-syn-transfected cells formed at levels of 1.2 ± 0.1% and 0.8 ± 0.2%, respectively (lanes 4 and 5), which were slightly but significantly higher than the level in vector-transfected control cells (lane 1). Given the relatively high efficiency (~40–50%) of transfection in B103 observed by immunofluorescence (data not shown). The number of cells overexpressing P123H β-syn with inclusion bodies (Fig. 5B) was estimated to be 2.1 ± 0.3% of the total number of cells (lane 4), which was slightly higher than that (1.8 ± 0.2%) for cells overexpressing V70M β-syn (lane 5). In contrast, inclusion body formation was observed at the level of <0.5% in other cell types, including vector-transfected cells (lane 1), and cells overexpressing wt α-syn (lane 2) and wt β-syn (lane 3).

Next, to investigate the effect of α-syn co-expression on the inclusion body formation by mutant β-syn, cells overexpressing β-syn mutants were transiently transfected with α-syn, followed by evaluation of inclusion body formation by H&E staining. Co-expression of α-syn with mutant β-syn resulted in a dramatic increase in the number of inclusion bodies: 13.2 ± 1.4% in P123H β-syn-overexpressing cells transfected with wt α-syn (lane 8), and 8.2 ± 0.2% in V70M β-syn-overexpressing cells transfected with wt α-syn (lane 9). In contrast, transient transfection of α-syn in either vector-transfected cells or cells overexpressing wt β-syn had little effect on inclusion formation (lanes 6 and 7). Similarly, when cells overexpressing wt α-syn were transfected with mutant β-syn, the levels of inclusion bodies were 10.5 ± 1.4% for P123H β-syn and 9.4 ± 0.5% for V70M β-syn (lanes 11 and 12, respectively), whereas no inclusions were induced by transfection of wt β-syn (lane 10).

To investigate the effect of transient co-expression of both mutant β-syn and α-syn on inclusion formation, double transfections were performed (Fig. 5C). The levels of
To define inclusion formation by mutant \( \beta \)-syn with \( \alpha \)-syn is due to an interaction of these two molecules, co-immunoprecipitation was performed. Either wt or mutant (A30P, E46K, and A53T) \( \alpha \)-syn was transiently transfected into cells overexpressing P123H \( \beta \)-syn (Fig. 7B, left panel). Cell extracts were immunoprecipitated with syn-1 or mouse IgG, followed by immunoblotting with anti-\( \beta \)-syn antibody. Co-immunoprecipitation of \( \beta \)-syn with A53T \( \alpha \)-syn occurred more efficiently compared with wt, A30P, or E46K \( \alpha \)-syn (left panel, upper). Under the same experimental conditions, similar amounts of \( \alpha \)-syn were precipitated by syn-1 antibody in each sample (left panel, lower). In contrast, transfection of A53T \( \alpha \)-syn into cells overexpressing wt \( \beta \)-syn resulted in negligible co-immunoprecipitation of wt \( \beta \)-syn with A53T \( \alpha \)-syn (right panel, upper and lower). Taken together, these results show that A53T \( \alpha \)-syn efficiently stimulates inclusion body formation by mutant \( \beta \)-syn, suggesting that the aggregation properties of \( \alpha \)-syn play an important role in the combined effects of mutant \( \beta \)-syn and \( \alpha \)-syn in inclusion formation.

**FIGURE 6. Phosphorylation of \( \alpha \)-syn is required to stimulate inclusion body formation by mutant \( \beta \)-syn**. 

A, quantification of eosinophilic inclusion bodies by \( \text{H&E} \) staining. Cells overexpressing wt \( \beta \)-syn (\( \beta \)-4 lane 1) or P123H \( \beta \)-syn (\( \beta \)-12 lane 2, 4, 5) were transfected with wt \( \alpha \)-syn (lanes 1 and 2), S129A \( \alpha \)-syn (lanes 3 and 4), or S129E \( \alpha \)-syn (lanes 5 and 6). Data are shown as means \( \pm \) S.D. (\( n = 4 \)). **, \( p < 0.01 \) versus cells with wt \( \alpha \)-syn transfection. B, immunofluorescence/LSCM for detection of syn expression in transfected cells. Cells overexpressing P123H \( \beta \)-syn (lane 1) were transfected with wt \( \alpha \)-syn. Cells were then doubly immunostained for \( \beta \)-syn (red; a, d, g, and j) and either \( \alpha \)-syn (green; b) or S129-phospho-\( \alpha \)-syn (green; e and h). Cells were also probed with \( \beta \)-syn, followed by additional staining with Thioflavin S (green; k). Nuclei are simultaneously stained with DAPI in the merged figures (c, f, i, and l). The bar represents 20 \( \mu \)m, and all figures are shown at the same magnification.

Moreover, these inclusions were positive in immunostaining with anti-S129-phospho-\( \alpha \)-syn antibody. In addition to the giant inclusion bodies (\( g \)–\( i \)), a number of small inclusion bodies were also positively stained (\( d \)–\( f \)). Some inclusion bodies also stained positively with thioflavin-S, suggesting that mutant \( \beta \)-syn and \( \alpha \)-syn formed amyloid-fibril-like structures in the inclusion bodies (\( j \)–\( l \)). Collectively, these results suggest that phosphorylation of \( \alpha \)-syn is required to stimulate inclusion body formation by mutant \( \beta \)-syn.

Mutant A53T \( \alpha \)-Syn Efficiently Stimulates Inclusion Formation by Mutant \( \beta \)-Syn—A link of mutant \( \alpha \)-syn (A30P, E46K, and A53T) to familial cases of PD and DLB has been established with the mutant proteins exhibiting enhanced aggregation compared with wt \( \alpha \)-syn (11, 12). Therefore, we asked if these \( \alpha \)-syn mutants might increase inclusion formation together with mutant \( \beta \)-syn. To test this possibility, wt and mutants (A30P, E46K, and A53T) \( \alpha \)-syn were transiently transfected into cells overexpressing P123H \( \beta \)-syn, and inclusion bodies were evaluated by \( \text{H&E} \) staining (Fig. 7A). Inclusion formation was significantly increased by expression of A53T \( \alpha \)-syn (23.3 \( \pm \) 0.8%) compared with wt (13.3 \( \pm \) 0.8%) and other mutations (A30P:16.7 \( \pm \) 0.6%, E46K:15.0 \( \pm \) 0.6%). In contrast, when cells overexpressing wt \( \beta \)-syn were transfected with the same \( \alpha \)-syn mutants, little formation of inclusion bodies was observed.

To determine if inclusion formation by mutant \( \beta \)-syn with \( \alpha \)-syn is due to an interaction of these two molecules, co-immunoprecipitation was performed. Either wt or mutant (A30P, E46K, and A53T) \( \alpha \)-syn was transiently transfected into cells overexpressing P123H \( \beta \)-syn (Fig. 7B, left panel). Cell extracts were immunoprecipitated with syn-1 or mouse IgG, followed by immunoblotting with anti-\( \beta \)-syn antibody. Co-immunoprecipitation of \( \beta \)-syn with A53T \( \alpha \)-syn occurred more efficiently compared with wt, A30P, or E46K \( \alpha \)-syn (left panel, upper). Under the same experimental conditions, similar amounts of \( \alpha \)-syn were precipitated by syn-1 antibody in each sample (left panel, lower). In contrast, transfection of A53T \( \alpha \)-syn into cells overexpressing wt \( \beta \)-syn resulted in negligible co-immunoprecipitation of wt \( \beta \)-syn with A53T \( \alpha \)-syn (right panel, upper and lower). Taken together, these results show that A53T \( \alpha \)-syn efficiently stimulates inclusion body formation by mutant \( \beta \)-syn, suggesting that the aggregation properties of \( \alpha \)-syn play an important role in the combined effects of mutant \( \beta \)-syn and \( \alpha \)-syn in inclusion formation.
FIGURE 7. Effects of familial mutations of α-syn on inclusion body formation by mutant β-syn. A, quantification of eosinophilic inclusion bodies by H&E staining. Cells overexpressing wt β-syn (lanes 1, 3, 5, and 7) or P123H β-syn (lanes 2, 4, 6, and 8) were transfected with wt α-syn (lanes 1 and 2), A53T α-syn (lanes 3 and 4), A30P α-syn (lanes 5 and 6), or E46K α-syn (lanes 7 and 8). Data are shown as means ± S.D. (n = 4). *, p < 0.05 versus cells with wt α-syn transfection. B, co-immunoprecipitation study of mutant β-syn with α-syn. In the left-hand panels, cells overexpressing P123H β-syn (ph-12) were transfected with wt α-syn (lanes 3 and 4), A53T α-syn (lanes 5 and 6), A30P α-syn (lanes 7 and 8), and E46K α-syn (lanes 9 and 10). Cell extracts (200 µg) were immunoprecipitated with syn-1 (lanes 4, 6, 8, and 10) or nonimmune IgG (lanes 3, 5, 7, and 9), followed by immunoblotting with anti-β-syn monoclonal antibody (upper panel) or syn-1 (lower panel). Extracts (2 µg) of cells overexpressing P123H β-syn (ph-12) transfected without wt α-syn (lane 1) or with wt α-syn (lane 2) were loaded as positive controls. In the right-hand panels, cells overexpressing P123H β-syn (ph-12) were transfected with either vector (lanes 3 and 4) or A53T α-syn (lanes 5 and 6). Cells overexpressing wt β-syn (8w-4) were also transfected with A53T α-syn (lanes 7 and 8). Immunoprecipitation and immunoblotting was performed exactly as in the left panel. Extracts (2 µg) of cells overexpressing P123H β-syn (ph-12) transfected with either vector (lane 1) or A53T α-syn (lane 2) were loaded as positive controls.

Suppression of Lysosomal Inclusion Body Formation Results in Accumulation of Mutant Syn Proteins and Cell Death—To determine if alteration of the autophagy-lysosomal pathway affects inclusion body formation, both wt β-syn- and P123H β-syn-overexpressing cells were transfected with either wt or A53T α-syn and then treated with reagents known to modulate the activities of the autophagy-lysosomal pathway (Fig. 8A). 3-MA, an inhibitor of early stage macroautophagy, significantly decreased the number of inclusion bodies in P123H β-syn-overexpressing cells transfected with A53T α-syn and to a lesser extent in the same cells transfected with wt α-syn. Under the same experimental conditions, immunofluorescence for β-syn showed that small immunoreactive aggregates somewhat increased in parallel with the decrease in large inclusion body formation (data not shown). Suppressive effects on inclusion body formation were also observed with NH4Cl, a general lysosomal inhibitor. In contrast, formation of inclusion bodies was not significantly affected by treatment with rapamycin, a well characterized stimulator of autophagy.

Down-regulation of autophagy-lysosomal activities by inhibitors might exacerbate accumulation of both mutant β-syn and α-syn proteins, and this possibility was evaluated by immunoblot analysis (Fig. 8B). In P123H β-syn-overexpressing cells transfected with A53T α-syn, the immunoreactivities of P123H β-syn monomer and possible oligomers in detergent-soluble fractions were increased by treatment with NH4Cl and 3-MA, and slightly decreased by rapamycin (panel a). Similarly, immunoreactivity of P123H β-syn in detergent-insoluble fractions was increased by NH4Cl and 3-MA and decreased by rapamycin (panel b). The immunoreactivities of A53T α-syn in both fractions were also increased by NH4Cl and 3-MA and decreased by rapamycin (panels c and d). The syn proteins showed a decreased tendency to accumulate in both fractions from wt β-syn-overexpressing cells transfected with wt α-syn (panels e–h).

To determine if an increased level of syn proteins may lead to increased cellular toxicity, cell viability was evaluated using a TUNEL assay (Fig. 9). For this purpose, cells overexpressing wt β-syn or P123H β-syn were transfected with either wt or A53T α-syn and treated with 3-MA. TUNEL staining (Fig. 9A) indicated cellular apoptosis in P123H β-syn-overexpressing cells transfected with A53T α-syn. Quantification of the TUNEL-positive cells indicated greater induction of apoptosis by 3-MA in cells overexpressing P123H β-syn than in those overexpressing wt β-syn (Fig. 9B). Apoptosis was significantly increased by transfection of A53T α-syn compared with wt α-syn. Similar results were observed with NH4Cl treatment (data not shown). Collectively, these results show that inhibition of the autophagy-lysosomal pathway suppresses inclusion body formation, which is associated with accumulation of syn proteins and increased cytotoxicity.

DISCUSSION

Our results show that overexpression of DLB-linked P123H and V70M β-syn mutants in B103 neuroblastoma cells leads to formation of eosinophilic cytoplasmic inclusion bodies immunopositive for mutant β-syn (Fig. 1). Ultrastructurally, cells overexpressing mutant β-syn showed abundant formation of lysosomal inclusion bodies, with numerous membranous cystic inclusions and electron dense myelinosome-like inclusions (Fig. 2), similar to the histopathology in various types of lysosomal storage diseases, especially gangliosidosis (34). Consistent with this, the inclusion bodies in cells overexpressing mutant β-syn were immunopositive for lysosomal markers, including cathepsin B, LAMP-2, and ganglioside GM2 (Fig. 3), and the lysosomal activity in these cells was dramatically increased (Fig. 4). In contrast, inclusion bodies were rarely observed in cells overexpressing wt α-syn or wt β-syn. An additional study using a cell-free system revealed that the β-syn mutants have increased aggregation properties (supplemental data 1). con-
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**FIGURE 8.** Suppression of inclusion body formation by treatment with autophagy-lysosomal inhibitors. A, quantification of eosinophilic inclusion bodies by H&E staining. Cells overexpressing wt \( \beta \)-syn-overexpressing cells (\( \beta \)-w4) or P123H \( \beta \)-syn (ph-12) were transfected with wt \( \alpha \)-syn (lanes 1–8), and cells overexpressing P123H \( \beta \)-syn (ph-12) were also transfected with A53T \( \alpha \)-syn (lanes 9–12). After 48 h of transfection, cells were incubated with vehicle, 3-MA (10 mM), NH\(_4\)Cl (20 mM), or rapamycin (200 nM) for an additional 16 h, followed by staining. Data are shown as means \( \pm \) S.D. (\( n = 4 \)). *, \( p < 0.05 \) versus cells treated with vehicle. B, immunoblot analysis of \( \beta \)-syn proteins in transfected cells. Cells overexpressing P123H \( \beta \)-syn (ph-12) were transfected with A53T \( \alpha \)-syn (panels a–d), and cells overexpressing wt \( \beta \)-syn (\( \beta \)-w4) were transfected with wt \( \alpha \)-syn (panels e–h). After 48 h of transfection, cells were incubated with vehicle (lane 1), 3-MA (10 mM) (lane 2), NH\(_4\)Cl (20 mM) (lane 3), or rapamycin (200 nM) (lane 4) for an additional 16 h. Cell extracts (10 \( \mu \)g) of both detergent-soluble (a, c, e, and g) and insoluble (b, d, f, and h) fractions were prepared and analyzed by immunoblotting using anti-\( \beta \)-syn monoclonal antibody (a, b, e, and f) or syn-1 (c, d, g, and h). Recombinant \( \alpha \)- and \( \beta \)-syn proteins were used as positive controls (lane 1).

Sustained with a recent report demonstrating that \( \beta \)-syn forms fibrils in the presence of stimuli, such as metals and glycosaminoglycans (35). Lentivirus-mediated expression of \( \beta \)-syn mutants in an \( \alpha \)-syn tg mouse model also resulted in an increase of inclusion body formation (supplemental data 2). Taken together, these results suggest that \( \beta \)-syn mutant (P123H and V70M) proteins have increased aggregation properties and that accumulation of these proteins leads to an enhanced lysosomal pathology in neuroblastoma cells. This appears consistent with the current view that the autophagy-lysosomal pathway plays a crucial role in the clearance of amyloidogenic proteins, such as \( \alpha \)-syn and Huntingtin (36–38).

Notably, formation of lysosomal inclusions by mutant \( \beta \)-syn was stimulated greatly by co-expression of \( \alpha \)-syn (Fig. 5). Furthermore, the stimulatory effects of \( \alpha \)-syn on inclusion formation by mutant \( \beta \)-syn was dependent on the phosphorylation of \( \alpha \)-syn at Ser-129, and both mutant \( \beta \)-syn and S129-phospho-\( \alpha \)-syn were co-localized in the inclusion bodies (Figs. 2 and 6). Moreover, compared with wt \( \alpha \)-syn and other familial PD and DLB mutants (A30P and E46K) of \( \alpha \)-syn, A53T \( \alpha \)-syn was more effective in stimulating inclusion formation by mutant \( \beta \)-syn, and this was correlated with the efficiency of co-immunoprecipitation (Fig. 7). Phosphorylation of \( \alpha \)-syn at Ser-129 stimulates \( \alpha \)-syn aggregation (24), and A53T \( \alpha \)-syn has greater aggregation properties than other mutant (A30P and E46K) \( \alpha \)-syn proteins (11, 12), making it reasonable to speculate that the combined effect of syn proteins on lysosomal inclusion body formation depends on their aggregation properties and possible interaction. Curiously, the combined effect of syn proteins on inclusion formation is somewhat similar to those of synphilin-1 and \( \alpha \)-syn. Engleender and coworkers (39) showed that synphilin-1 associates with \( \alpha \)-syn and promotes inclusion formation in HEK293 embryonic kidney cells. Subsequently, it was shown that synphilin-1A, an alternative splicing variant of synphilin-1, is more prone to aggregate and more efficiently forms inclusions with \( \alpha \)-syn compared with synphilin-1 (40). Finally, S129-phospho-\( \alpha \)-syn-immunopositive fibrillar structures are formed in SH-SY5Y neuronal cells co-expressing synphilin-1 and \( \alpha \)-syn, suggesting that synphilin-1 stimulates formation of \( \alpha \)-syn fibrils (41). Interestingly, inclusion bodies formed by synphilin-1 and \( \alpha \)-syn were eosinophilic similar to the inclusions formed by mutant \( \beta \)-syn and \( \alpha \)-syn in the current study. Therefore, the mechanisms of the inclusion body formation by mutant \( \beta \)-syn and \( \alpha \)-syn and by synphilin-1 and \( \alpha \)-syn may have considerable overlap.

A popular current view is that formation of lysosomal inclusion bodies may be a protective mechanism, because impaired sequestration and clearance of aggregation-prone proteins might result in increased neurotoxicity, leading to neurodegeneration (42). Supporting this notion, it has been shown that inhibition of lysosomal functions results in neuropathological features, such as protein deposition, synaptic loss, and neuronal demise in rodent models (43, 44). In a similar context, the current study shows that lysosomal inclusions might play a protec-
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In detergent-insoluble, formic acid-extractable fractions: β-syn immunoreactivity with this antibody was much stronger in sporadic DLB brains compared with control brains (supplemental data 3). Thus, re-evaluation of Pro-123 DLB brain homogenates using a sensitive anti-β-syn antibody may provide evidence of aggregated P123H β-syn in the brain. Second, although β-syn mutants are prone to aggregate and stimulate aggregation of α-syn, the aggregates of mutant β-syn might be sensitive to degradation in the brain. In support of this possibility, both mutant β-syn aggregates were more susceptible to proteinase K digestion under the cell-free conditions compared with α-syn aggregates (supplemental data 1). α-syn may be degraded not only by lysosomal proteases but also by various proteases, such as calpain (45) and neurexins (46), and aggregated mutant β-syn may be more efficiently degraded by these proteases.

The third possibility is that mutant β-syn may not be directly involved in Lewy body pathology. Instead, considering that the current study showed that overexpression of mutant β-syn in B103 neuroblastoma cells resulted in an enhanced lysosomal pathology, it is possible that lysosomal dysfunction caused by mutant β-syn might play a causative role in neurodegeneration. In support of this notion, a role of lysosomal dysfunction in the pathogenesis of PD, it was recently described that ATP13A2, a lysosomal type 5 p-type ATPase, is linked to an autosomal recessive form of early-onset parkinsonism with pyramidal degeneration and dementia: so-called Kufor-Rakeb syndrome (PARK9) (33). It was further shown that several missense mutations of ATP13A2 are associated with some types of juvenile and young onset PD (47). Interestingly, the current study shows that ATP13A2 was co-localized with mutant β-syn in lysosomal inclusions formed in cells overexpressing mutant β-syn (Fig. 3). Moreover, the expression of ATP13A2 was up-regulated compared with other types of cells (Fig. 3). Thus, although further work is clearly required, these results may suggest a pathological link between mutant β-syn and ATP13A2 through a convergent lysosomal pathology in neurodegeneration.

In conclusion, overexpression of β-syn mutants linked to DLB (P123H and V70M) results in enhanced lysosomal pathology in neuroblastoma cells. Together with the recent identification of a linkage of park 9 to lysosomal ATP13A2, we found a support to the notion that lysosomal dysfunction has a causative role in neurodegeneration. More work is required to test this scenario, either by investigation of mutant β-syn in brain tissue from patient with DLB, or through analysis of a tg mice model of mutant β-syn overexpression.

**FIGURE 9. Suppression of lysosomal inclusion bodies leads to cell death.** A, representative confocal image of TUNEL staining of syn-transfected cells. Cells overexpressing wt β-syn (βw-4) without (a and b) or with transfection of wt α-syn (c and d), and cells overexpressing P123H β-syn (ph-12) without (e and f) or with transfection of A53T α-syn (g and h) were treated with either vehicle (a, c, e, and g) or 3-MA (10 mM) (b, d, f, and h) for 16 h. The cells were then analyzed by TUNEL staining. Nuclei were simultaneously stained with DAPI B, quantification of cellular apoptosis as evaluated by TUNEL staining. Cells overexpressing wt β-syn-overexpressing cells (βw-4) without (lanes 1 and 2) or with transfection of either wt (lanes 3 and 4) or A53T α-syn (lanes 5 and 6) and cells overexpressing P123H β-syn (ph-12) without (lanes 7 and 8) or with transfection of either wt (lanes 9 and 10) or A53T α-syn (lanes 11 and 12) were treated with 3-MA (10 mM) for 16 h. The number of TUNEL-positive cells was calculated as a percentage of the total number of cells. Similar results were obtained by three independent experiments. Data are shown as means ± S.D. (n = 4). *, p < 0.05 versus non-transfected cells.

The role of missense mutations of β-syn in the pathogenesis of DLB is complicated by an original report describing little aggregation of P123H β-syn in patients’ brain (23). In this report, biochemical analysis showed no immunoreactivity of P123H β-syn in detergent-insoluble fractions. Furthermore, histopathological analysis of P123H β-syn DLB brain showed typical formation of Lewy bodies with immunoreactivity with α-syn but no immunoreactivity of P123H β-syn (23). Given these results, there are at least three possibilities that should be considered to support a pathogenic role of mutant β-syn. First, it is possible that detection of aggregation of P123H β-syn may depend on the sensitivity of the antibody probe. Indeed, immunoblot analysis of sporadic DLB and control brains using several antibodies showed that only one anti-β-syn monoclonal antibody was effective in detection of β-syn immunoreactivity.
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