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Short Communication

Aggregation of α-Synuclein in Lewy Bodies of Sporadic Parkinson's Disease and Dementia with Lewy Bodies

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Lewy bodies (LBs) are hallmark lesions of degenerating neurons in the brains of patients with Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Recently, a point mutation in the gene encoding the presynaptic α-synuclein protein was identified in some autosomal-dominantly inherited familial PD pedigrees, and light microscopic studies demonstrated α-synuclein immunoreactivity in LBs of sporadic PD and DLB. To characterize α-synuclein in LBs, we raised monoclonal antibodies (MAbs) to LBs purified from DLB brains and obtained a MAb specific for α-synuclein that intensely labeled LBs. Light and electron microscopic immunocytochemical studies performed with this MAb as well as other antibodies to α- and β-synuclein showed that α-synuclein, but not β-synuclein, is a component of LBs in sporadic PD and DLB. Western blot analyses of highly purified LBs from DLB brains showed that full-length as well as partially truncated and insoluble aggregates of α-synuclein are deposited in LBs. Thus, these data strongly implicate α-synuclein in the formation of LBs and the selective degeneration of neurons in sporadic PD and DLB. ***(Am J Pathol 1998, 152:879–884)***

Parkinson's disease (PD) is the most common neurodegenerative disorder affecting brainstem extrapyramidal neurons (eg, dopaminergic substantia nigra neurons) of middle-age individuals.[1] Spherical filamentous inclusions known as Lewy bodies (LBs) form in perikarya of degenerating brainstem neurons, and these lesions are diagnostic of PD.[2–4] However, LBs also are found in a subset of cortical neurons in a late-life neurodegenerative disorder known as dementia with LBs (DLB), the second most frequent dementia after Alzheimer's disease.[5–7] Neurofilament subunits[8–12] and ubiquitin (Ub)[13–17] are implicated as major components of LBs in PD and DLB, but the precise building blocks of LBs still remain unclear. Recent studies showed that α-synuclein, a 140-amino-acid protein localized to presynaptic terminals,[18,19] harbors an Ala to Thr substitution at position 53 (A53T) due to a missense mutation of the α-synuclein gene in four pedigrees with autosomal-dominantly inherited familial PD.[20] Furthermore, immunoreactive α-synuclein has been demonstrated by light microscopy in LBs of sporadic PD and DLB.[21]

To demonstrate unequivocally that α-synuclein is the building block of LBs, we have used two complementary strategies. In the first, we have raised monoclonal antibodies (MAbs) to highly purified LBs and obtained a MAb specific to α-synuclein. In the second, we use light and electron microscopic immunocytochemical as well as biochemical studies to examine isolated LBs and demonstrate that full-length, partially truncated, and insoluble α-synuclein is incorporated into LBs of sporadic PD and DLB. Thus, in addition to the A53T α-synuclein mutation in familial PD, alterations in the biophysical properties of

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wild-type α-synuclein may play a mechanistic role in the formation of LBs and the degeneration of affected neurons in sporadic PD and DLB.

Materials and Methods

Cases

Brains were obtained at autopsy from seven patients with PD (three ethanol fixed and four formalin fixed) and nine patients with DLB (six ethanol fixed and three formalin fixed) diagnosed according to published criteria.[5–7] Sections from midbrains were examined in all cases, and sections from cingulate or medial temporal cortices were examined in the DLB cases. Fixation of some brains in 70% ethanol with 150 mmol/L NaCl was performed as described,[9] whereas other brains were fixed in 10% formalin. Frozen cerebral cortices from four DLB cases were used for the isolation of LBs.

Purification of LBs and Synucleins

LBs were isolated and purified by sucrose density gradient centrifugation followed by fluorescence-activated particle sorting as previously described,[17] except that detergent treatment after cell sorting was omitted. Human α- and β-synuclein proteins were purified from the soluble, heat-stable fraction of homogenates of frozen human cerebral cortex as described,[22–25] except that α- and β-synucleins were separately recovered on a DEAE-cellulose column.

Antibodies

Here, MAbs to LB immunogens were generated using ~3 × 10^6 LBs purified from DLB cortices, and hybridoma supernatants were screened by immunostaining unfixed smears of the post-sucrose fractions of isolated LBs as described,[17] except that the immunogens were disrupted by repeated ultrasonication in 0.5 ml of 50 mmol/L Tris-buffered saline (pH 7.6) without denaturation by formic acid. The specificity of the MAbs were further characterized by immunoblot analyses using a heat-stable, high-salt extractable fraction from homogenates of human brain (see below). For the production of polyclonal antibodies to α- and β-synuclein, we used synthetic synuclein peptides as immunogens, and these peptides included nonhomologous amino acid sequences in α- and β-synuclein that were well conserved in the rat and human forms of these proteins (93.8% for α-synuclein and 100% for β-synuclein). The amino termini of each of the peptides were conjugated to keyhole limpet hemocyanin via a cysteine residue for use as immunogens in rabbits to generate antisera 253 and 259 as described,[22–25] whereas antiserum 77 was raised against purified bovine PNP14 (β-synuclein) as described.[22–25]

Immunoblot Analyses

Purified human α- and β-synuclein proteins (50 ng for MAb LB509 and antisera 77 and 259 and 100 ng for antiserum 253) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 13% gel, transferred to a nitrocellulose membrane, incubated with mouse MAb LB509 (culture supernatant, 1:10) or rabbit antisera 77 (1:2000), 259 (1:1500), and 253 (1:1500), respectively, and visualized by enhanced chemiluminescence as described.[22–25] The specificity of both the MAbs and polyclonal antibodies were further assessed using a heat-stable soluble fraction (100,000 × *g*) of human cingulate gyrus homogenized in 1.5 vol of a buffer containing 0.75 mol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol, 0.1 mol/L 4-morpholineethanesulfonic acid, pH 7.0, and a cocktail of protease inhibitors as well as purified human α- and β-synucleins separately recovered on a DEAE-cellulose column. For immunoblot analysis of purified LBs, approximately 200,000 purified LBs were solubilized in 70% formic acid, desiccated, resolubilized in SDS sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with LB509.

Immunocytochemistry and Immunoelectron Microscopy

Paraffin-embedded sections were immunostained with the anti-synuclein or anti-Ub antibodies, (diluted at 1:1000 for polyclonal antibodies to synucleins, 1:1~100 for the LB509 MAb supernatant, and 1:400 for anti-Ub antibodies) by a standard avidin-biotin complex method using 3,3′-diaminobenzidine as chromogen and lightly counterstained with hematoxylin.[17] Double-label confocal microscopy with anti-tau (MAb C5, gift of Dr. Y. Ihara) and anti-α-synuclein (259) antibodies was performed as described.[26] Immunoelectron microscopic studies were performed using the immunoperoxidase method,[13] and the immunogold method was used on isolated LBs.[17]

Results

A Monoclonal Antibody (LB509) Raised against Purified LBs Specifically Recognizes Human α-Synuclein

Among the MAbs we raised to LBs purified from DLB brains, we identified one α-synuclein-specific MAb (clone LB509) that intensely stained numerous intraneuronal LBs throughout the substantia nigra (Figure 1A) and neocortex (Figure 1B) by immunohistochemistry performed on tissue sections from the postmortem brains of sporadic PD or DLB patients. LB509 stained LBs equally well in brains fixed with 70% ethanol or 10% formalin, and it also stained many LBs in unfixed smears of these isolated LBs (Figure 1C). LB509 specifically reacted with an ~18-kd polypeptide in the heat-stable soluble fraction of human cingulate gyrus

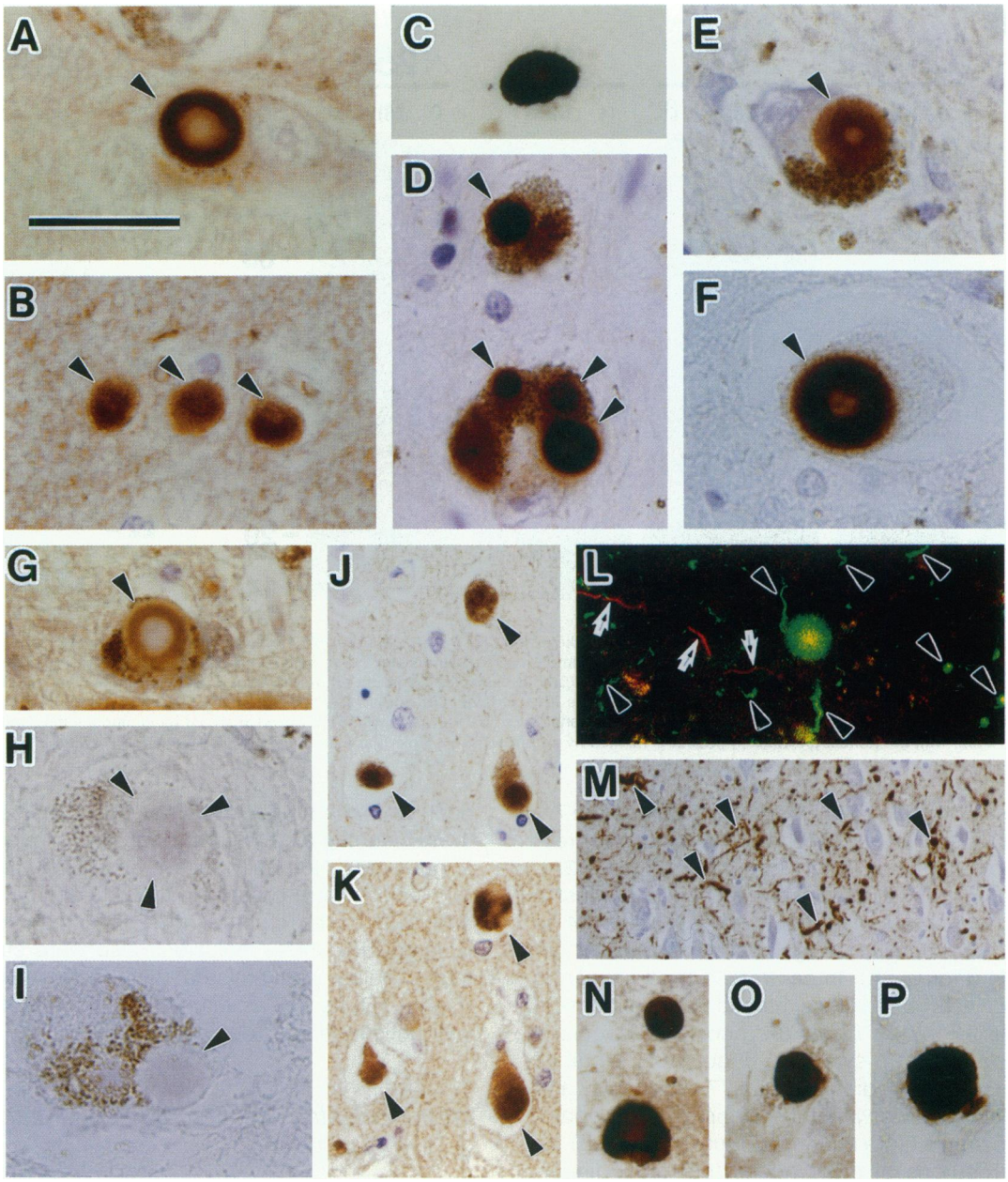

Figure 1. Light microscopic immunohistochemistry of LBs. Brainstem LBs in pigmented neurons of the substantia nigra (**A**, **D**, **E**, and **G** to **I**) as well as in neurons of the Edinger-Westphal nucleus (**F**) and LBs in neurons of the cingulate cortex (**B**, **J**, and **K**) are immunostained with MAb LB509 (**A** and **B**) and antisera 259 (**D**, **F**, and **J**) and 77 (**E**) as well as with anti-ubiquitin (Ub) antibodies (**G** and **K**). However, LBs are not stained by antiserum 259 after adsorption with its corresponding peptide immunogen (**H**) or by antiserum 253 to β-synuclein (**I**). **Arrowheads** indicate immunostained (**A**, **B**, **D** to **G**, **J**, and **K**) and unstained (**H** and **I**) LBs. Temporal DLB neocortex (**L**) double labeled with anti-tau (C5) and anti-α-synuclein antibodies (259) and the CA2/3 region of the hippocampus (**M**) of a DLB brain immunostained with antiserum 259 show an α-synuclein-positive LB (large round green fluorescent profile in **L**) as well as α-synuclein-positive abnormal neurites (green fluorescent profiles identified by arrowheads in **L**; brown profiles identified by **arrowheads** in **M**) that are distinct from tau-positive neuropil threads (red fluorescent profiles identified by **arrows** in **L**). Tissue fixation in **B**, **D**, and **F** to **L** was with ethanol and in **A**, **E**, **G**, and **M** with formalin. Unfixed, isolated cortical LBs were immunolabeled with MAb LB509 (**C**), antisera 259 (**N**) and 77 (**O**), and anti-Ub (**P**). Scale bar in **A** is equivalent to 30 μm in **A** to **I** and **N** to **P**, 60 μm in **J** to **L**, and 120 μm in **M**.

on Western blots (see Figure 2B). This protein was shown to be α-synuclein by demonstrating that LB509 specifically reacted with purified human α-synuclein, which migrated at ~18 kd, but not with β-synuclein (also known as PNP14), which ran with a slightly slower mobility (Figure 2A).[22–25]

Antibodies to α-Synuclein, but Not to β-Synuclein, Immunolabel LBs

To further confirm the presence of α-synuclein in LBs, we raised antibodies to synthetic synuclein peptides or purified synuclein proteins and examined the ability of

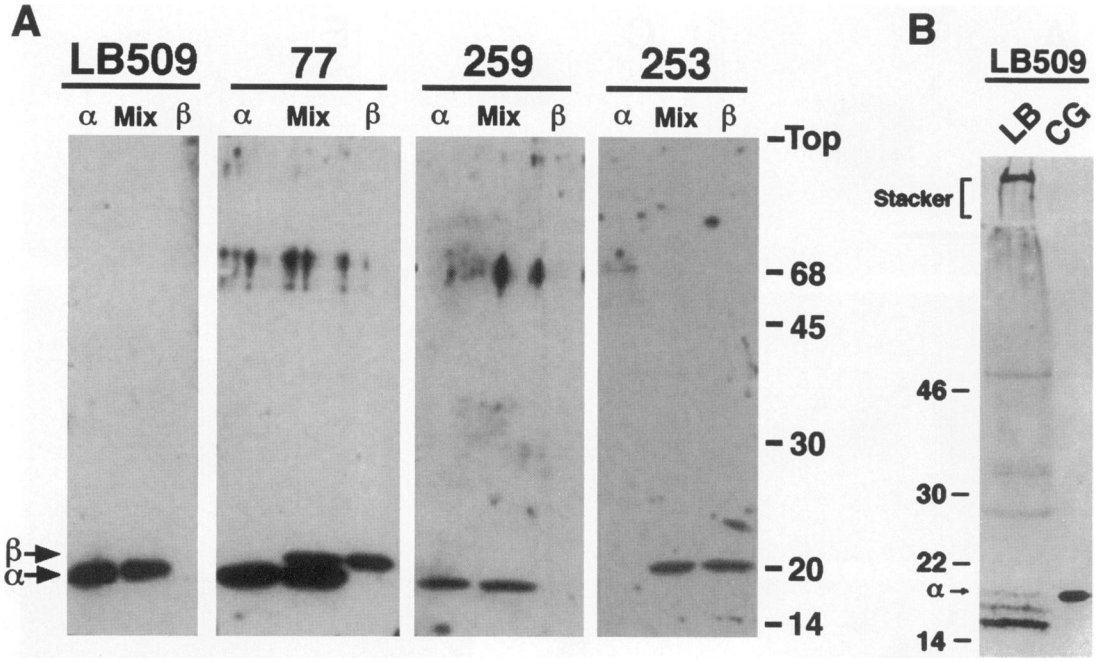

Figure 2. A: Characterization of the anti-LB and anti-synuclein antibodies. MAb LB509 and antiserum 259 specifically reacted with purified human α-synuclein migrating at ~18 kd, but never cross-reacted with β-synuclein. Antiserum 77 reacted with α-synuclein as well as with β-synuclein, the latter migrating at a slightly slower position compared with α-synuclein, and antiserum 253 specifically recognized β-synuclein. α, α-synuclein; β, β-synuclein, Mix, mixture of α-synuclein and β-synuclein. **B:** Western blot analysis of purified LBs. Purified LBs extracted with 70% formic acid (LB; **left lane**), together with the heat-stable, high-salt extractable fraction from a normal human cingulate gyrus (CG; **right lane**), were fractionated by SDS-PAGE and analyzed by immunoblotting with MAb LB509. A band co-migrating with the normal α-synuclein protein is marked by an **arrow** (α→). Molecular mass standards are shown in kilodaltons.

these antisera to stain LBs. For example, antiserum 77, which was raised to purified bovine β-synuclein, recognized both human α- and β-synucleins (Figure 2A), whereas antiserum 259, which was raised against a synthetic peptide corresponding to residues 104 to 119 of rat α-synuclein, recognized human α- but not β-synuclein (Figure 2A). In contrast, a third antiserum (253), which was raised to a synthetic peptide identical to residues 91 to 105 of rat β-synuclein, recognized only purified human β-synuclein (Figure 2A). Notably, just like LB509, antibodies 259 and 77 specifically and intensely labeled LBs within the cytoplasm of dopaminergic substantia nigra neurons (Figure 1, D and E), as well as in neurons of other nuclei (eg, the Edinger-Westphal nucleus; Figure 1F). The α-synuclein immunoreactivity in brainstem LBs was more intense than that obtained using anti-Ub antibodies (Figure 1G). Adsorption of antiserum 259 with its peptide immunogen completely abolished LB staining (Figure 1H), and the β-synuclein-specific antiserum (253) never immunolabeled LBs (Figure 1I). Antisera 259 and 77 also immunolabeled cortical LBs (Figure 1J) as strongly and frequently as anti-Ub antibodies (Figure 1K). Notably, τ-positive neurofibrillary tangles and neuropil threads in some of these sections were never labeled with the anti-synuclein antibodies in double-label confocal microscopy studies (Figure 1L). However, α-synuclein-positive neurites (which were distinct from τ-positive neuropil threads) were found in regions of DLB cortex showing severe neuronal loss and numerous LBs (Figure 1L). Although hitherto unrecognized in DLB cortex, these abnormal processes closely resembled CA2/3 neurites in the hippocampus of DLB patients,[27,28] and these neurites also were strongly α-synuclein positive (Figure 1M). Notably, these α-synuclein-labeled neuritic lesions were present in the brains of all of the PD and DLB cases studied here. In smears of unfixed isolated LBs, these inclusions also were strongly immunolabeled with antisera 259 (Figure 1N) and 77 (Figure 1O) as well as with anti-Ub (Figure 1P), but not with antiserum 253 (data not shown).

Immunoelectron Microscopic Localization of α-Synuclein in LBs

To characterize the ultrastructural localization of α-synuclein immunoreactivity in LBs, we performed immunoelectron microscopy with antiserum 259. The peripheral portions of brainstem LBs were immunolabeled with antiserum 259 (Figure 3A), which primarily decorated filaments and associated granular material in these inclusions (Figure 3B, arrowheads). Unfixed cortical LBs also were examined by an immunogold immunoelectron microscopic technique, and the gold particles localized to amorphous material that often was associated with the filaments in LBs (Figure 3C, arrowheads).

Immunoblot Analysis of α-Synuclein in Purified LBs

To biochemically characterize the α-synuclein in LBs, we performed Western blot analyses on more highly purified,

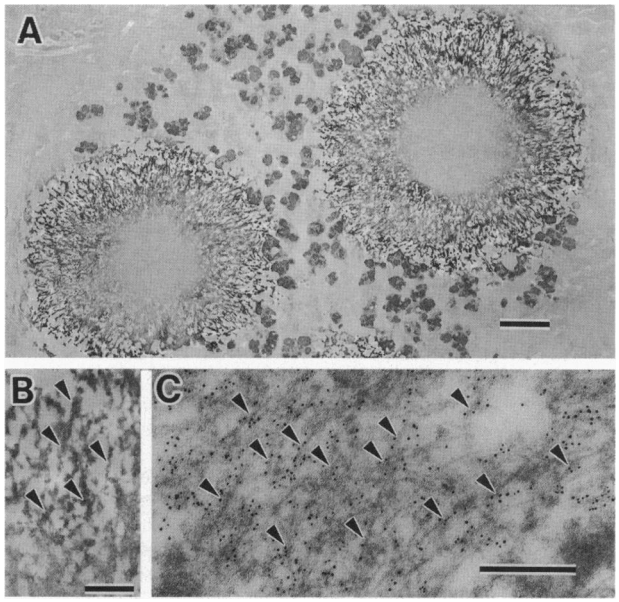

Figure 3. Immunoelectron microscopy of LBs. **A:** Two brainstem LBs immunolabeled with antiserum 259 and visualized by the immunoperoxidase method. **B:** A high-power view of the periphery of a LB in **A**. **C:** Immunogold labeling of an unfixed, isolated cortical LB with antiserum 259. **Arrows** in **B** and **C** indicate α-synuclein immunoreactivity associated with LB filaments. Scale bars, 2 μm (**A**), 500 nm (**B**), and 200 nm (**C**).

formic-acid-treated LBs isolated from DLB cortex (Figure 2B). LB509 labeled an immunoband with a molecular weight similar to normal human α-synuclein (Figure 2B, arrow), as well as 14- to 16-kd immunobands (Figure 2B), consistent with partially truncated forms of α-synuclein. Additionally, several other immunostained bands migrating at 25 to 50 kd as well as higher molecular weight α-synuclein-immunoreactive aggregates, and labeled material that did not enter the stacking gel, also were detected by LB509 (Figure 2B). Similar protein bands also were detected in Western blots of isolated LBs probed with antiserum 259 (data not shown).

Discussion

Taken together, the data presented here show that α-synuclein is an integral component of both brainstem and cortical LBs in patients with sporadic LB disorders. Our findings significantly extended previously published light microscopic observations[21] by 1) raising a MAb against purified LBs that specifically recognized α-synuclein, 2) demonstrating α-synuclein in LBs stained with α-synuclein-specific antibodies at the light and ultrastructural level, and 3) detecting full-length and altered α-synuclein in highly purified LBs by Western blots. In the brainstem LBs, the immunoreactions for α-synuclein were predominantly observed in the peripheral portion; however, it remains possible that the core portions of brainstem LBs contain some α-synuclein because the high density of LB cores often prevents the penetration of antibodies into this region.[29] Notably, our biochemical analysis showed that full-length and partially truncated forms of α-synuclein, as well as high molecular weight aggregates, are present in LBs recovered from the insoluble fraction of DLB brains, although the possibility that some of the truncation occurred postmortem during the purification of LBs cannot be completely excluded. These data suggest that the mechanisms leading to the selective incorporation of α-synuclein into LBs probably involve alterations in the metabolism of this molecule that render it insoluble and prone to aggregate. Indeed, we have recently obtained preliminary data suggesting that there is a shift of α-synuclein from a soluble to an insoluble pool in the cortex of DLB brains (P.-H. Tu, M. Baba, T. Iwatsubo, J. Q. Trojanowski and V. M.-Y. Lee, unpublished observation). Thus, wild-type α-synuclein probably plays a role in the pathogenesis of sporadic PD and DLB, whereas a mutation in the α-synuclein gene in familial PD results in an A53T substitution in α-synuclein that may augment this process.

Although the significance of this selective deposition of α-synuclein in LBs is unclear, α-synuclein is normally transported to presynaptic termini[18,19] where it is thought to play a role in learning or neuronal plasticity.[30] Furthermore, α-synuclein is thought to lack significant secondary structure, but it may interact with other proteins.[31–34] Thus, a mutation in α-synuclein in familial PD, or the aberrant interaction of altered wild-type α-synuclein with other axonal proteins (eg, neurofilament subunits) in sporadic PD or DLB may lead to the precipitation of α-synuclein with other proteins in filamentous, insoluble aggregates that become ubiquitinated and form LBs in these disorders. The long-term consequences of these aggregates could be the disruption of neuronal functions leading to the death of affected neurons. Furthermore, mutant human α-synuclein with the A53T substitution identified in familial PD is expected to acquire a higher propensity to self-aggregate, or to lose its ability to interact normally with other proteins,[31] and this could augment the pathological processes outlined above. These speculations notwithstanding, the present data strongly argue that additional investigation into the role of α-synuclein in LB formation should facilitate understanding of the pathogenesis and biological significance of LBs in PD and DLB, as well as the development of effective therapeutic strategies for these common, yet devastating neurodegenerative disorders.

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