Lewy bodies

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Lewy bodies (LB) in the substantia nigra are a cardinal pathological feature of Parkinson's disease, but they occur in a number of neurodegenerative diseases and can be widespread in the nervous system. The characteristics, locations, and composition of LB are reviewed, with particular attention to α-synuclein (α-SYN), which appears to be the major component of LB. The propensity for α-SYN, a presynaptic protein widely expressed in the brain, to aggregate is because of an amyloidogenic central region. The factors that favor the aggregation of α-SYN and mechanisms of toxicity are examined, and a mechanism through which aggregates of α-SYN could induce mitochondrial dysfunction and/or release of proapoptotic molecules is proposed.

Lewy bodies (LB) and loss of pigmented neurons in the substantia nigra (SN) pars compacta (SNpc) are the cardinal pathological features of Parkinson's disease (PD) (1). In 1912, Friedrich Lewy (2) first described the intraneuronal inclusions in PD brains, but he described them not in the SNpc but in the nucleus basalis of Meynert and the dorsal motor nucleus of the vagus. The pathology of PD includes not only spherical LB in the neuronal perikarya but also thread-like Lewy neurites (LN) and neuroaxonal spheroids in neuronal processes (3–5).

LB have been noted in a number of cases of PD associated with specific mutations in genes of various functions, including *α-synuclein* (6, 7) and *leucine repeat rich kinase 2* (*LRRK2*) (8, 9). LB-like inclusions have been described in animal models of PD that use inhibitors of mitochondria (10, 11) and the ubiquitin proteasome system (UPS) (12).

LB occur in a number of other neurological disorders, including LB dementia (LBD) (13, 14); Alzheimer's disease (AD) (15), including cases of familial AD with mutations in the *presenilin 1,2* and *amyloid precursor protein* genes (16); Down's syndrome (17); neurodegeneration with brain iron accumulation type 1 (also known as Hallervorden–Spatz disease) (18); progressive autonomic failure (19); rapid eye movement sleep disorder (20); Parkinsonism–dementia complex of Guam (21); Gaucher's disease (22); and Pick's disease (23).

Description and Composition of LB

Light Microscopy. The classical appearance of the LB in pigmented nigral neurons with hematoxylin/eosin staining is one or more eosinophilic spherical body with a dense core surrounded by a halo (3) (Fig. 1, which is published as supporting information on the PNAS web site). Staining with hematoxylin/eosin has largely been supplanted by immunolabeling for ubiquitin and α-synuclein (α-SYN) (24, 25), which more readily reveal LB and pale bodies, diffuse inclusions that are weakly stained with eosin. LB in the neocortex typically lack a halo (14) and may be inconspicuous with hematoxylin/eosin. However, Sakamoto *et al.* (26) immunolabeled for α-SYN in PD brains and found that ≈36% of nigral LB lacked peripheral accentuation of α-SYN, and 31% of LB in the cingulate cortex had this peripheral α-SYN accentuation. Gómez-Tortosa *et al.* (14) immunolabeled for α-SYN and ubiquitin in the SN and cortical regions from patients with LBD and noted a spectrum of intracytoplasmic inclusions from diffuse or "cloud-like" α-SYN staining, which were typically ubiquitin-negative and not apparent with hematoxylin/eosin staining, to pale bodies, which had variable immunolabeling for ubiquitin, to classical LB with a halo, usually with α-SYN labeling in the halo and ubiquitin labeling in the core. The spectrum of inclusions could be seen in the SN of a single case, and pale bodies and classical LB could be seen in the same neuron. Gómez-Tortosa *et al.* (14) noted a spectrum of neuritic inclusions in the SN. They hypothesized that the spectrum of α-SYN inclusions represent different stages in the evolution of LB, the cloud-like α-SYN structures represent an early stage; these evolve to more compact structures that may become tagged with ubiquitin and could be interpreted as pale bodies, which become more condensed with a halo to form the classic LB. The data suggest that pale bodies can progress to become classical LB (14, 26).

Electron Microscopy. Roy and Wolman (27) performed ultrastructural studies of the SN in PD brains and noted LB to be intracytoplasmic, sometimes with multiple LB in a neuron, with an occasional extracellular LB. They noted two types of LB; the more common type was composed of granular and fibrillar components, with the granular material being more prominent in the core. Fibrils at the periphery appeared to radiate; this more common type seemed to be the classical LB with a central core when observed with light microscopy. The second type of LB was composed almost entirely of circular or oval fibrillar material and appeared to be the LB of uniform density with light microscopy. Other groups have also reported that LB have a matted network of filaments in the core surrounded by a looser array of radiating filaments in the halo (28–30).

The study of the ultrastructure of α-SYN in LB was carried out by Arima *et al.* (31). They noted α-SYN filaments in LB, pale bodies, and perikaryal threads. In LB, the α-SYN material was largely noted in the radially arranged LB filaments in the periphery, with a few α-SYN silver grains in the central core (tight packing may have impeded labeling in the core). In pale bodies, the loosely aggregated filaments were labeled for α-SYN. They noted perikaryal threads, which were a loose mesh-work of small bundles of filaments immunoreactive for α-SYN but not for ubiquitin (dystrophic neurites were identified by antisera to both α-SYN and ubiquitin). The ultrastructural features of the filaments in LB, pale bodies, and perikaryal threads were similar, and Arima *et al.* (31) hypothesized that the α-SYN perikaryal threads represent an early stage of filament assembly that may develop into LB or pale bodies. Although α-SYN and ubiquitin are major components of LB, a large number of other constituents, such as neurofilaments,

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Abbreviations: α-SYN, α-synuclein; AD, Alzheimer's disease; DA, dopaminergic; KO, knockout; LB, Lewy body; LBD, LB dementia; LN, Lewy neurite; PD, Parkinson's disease; SN, substantia nigra; UPS, ubiquitin proteasome system; NAC, non-Aβ component of AD amyloid.

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have been reported in LB (see *Supporting List*, which is published as supporting information on the PNAS web site).

Location of LB

In 1942, Lewy (32) commented that "pathological changes were widespread over the whole central nervous system." Ohama and Ikuta (33) reported that the distribution of LB followed that of the monoaminergic systems in the brain, but more recent work has confirmed Lewy's observation. Braak *et al.* (1) immunolabeled for α-SYN to examine brains from PD cases, cases with no clinical evidence of PD but with LN or LB in sites involved in PD, and cases with no history of PD and no LN or LB in the dorsal motor nucleus of the vagus (but no cases of clinical LBD or AD). The examination led to division of the cases into six stages. In stage 1, the pathology was confined to the medulla oblongata with LN and LB within the projection neurons of the dorsal motor nucleus of the vagus, which contain large amounts of lipofuscin granules, and in the projection cells of the intermediate reticular zone. Typically, LN could be observed without LB, suggesting that they occurred earlier. LN and LB were not found in neurons containing neuromelanin, a product of oxidation of catecholamines. Stage 2 was characterized by the addition of LN and LB in the caudal raphe nuclei, the gigantocellular reticular nucleus, and the locus ceruleus–subceruleus complex (the ceruleus being the first neuromelanin complex to be involved). In stages 3 and 4, the melanin-containing [dopaminergic (DA)] neurons in the dorsal nucleus of the vagus and the intermediate zone began to develop LB. The most striking feature of stage 3 was that the DA neurons of the SN and the lipofuscin-laden neurons in the magnocellular nuclei of the basal forebrain contained LN and LB (LN appeared before LB), but there was no obvious loss of neuromelanin-containing nigral neurons. The cortical and subcortical regions joined to anterior olfactory nucleus showed mild involvement. Stage 4 was characterized by marked loss of the neuromelanin-containing neurons in the SN, and other mesencephalic nuclei containing neuromelanin show destruction. Stage 4 was characterized by severe involvement of the magnocellular nuclei of the basal forebrain and the hypothalamic tuberomamillary nucleus. Cortical involvement was confined to the temporal mesocortex and the CA2 plexus. In stage 5, the Lewy inclusions were found in the high-order sensory areas of the neocortex and the prefrontal neocortex. The distinguishing feature of stage 6 was the involvement of nearly the entire cortex. The level of proteinase K-resistant α-SYN in brain regions correlated with the severity of α-SYN pathology (34). A common feature of neurons that are affected early appears to be the presence of markers of oxidative stress, i.e., lipofuscin (35) and neuromelanin (5), but cells containing neuromelanin do not necessarily have LB early.

Jellinger (36) evaluated the extent of LB and LN in brains from elderly persons with PD, AD, LBD, and essential tremor- and age-matched control subjects. All of the PD cases had α-SYN inclusions in the dorsal motor nucleus of the vagus and SN. Fifty-two percent of the AD cases had α-SYN inclusions, but in 73% of these cases, α-SYN inclusions were not found in medullary nuclei. Jellinger (36) concluded that in PD cases, the Braak stages were valid, but in some AD cases, deviation from Braak's proposed stereotypic pattern of expansion occurred, and that incidental LB and LN could affect only the LC and SN. The AD pathology in the AD cases could have contributed to the development of LN and LB (see below).

Parkkinen *et al.* (37) examined 106 autopsy cases and found α-SYN inclusions in the dorsal motor nucleus of the vagus, SN, or the basal forebrain nuclei and reported, at variance with the scheme of Braak *et al*. (1), a number of cases in which LN and LB were noted in the SN, the basal forebrain, and cortical regions but not in the dorsal motor nucleus of the vagus.

The data reviewed above indicate that the pattern of progression proposed by Braak *et al.* (1) is often, but not invariably, found in series of cases with α-SYN inclusions; for example, α-SYN inclusions may occur in the basal forebrain nuclei and cortical areas but not in the medulla, particularly in cases with a coexistent pathology of AD. It is plausible that formation of α-SYN inclusions can be driven by a number of processes (see below), and that these underlying processes will determine the pattern of progression.

α-SYN, a Major Component of LB

A large number molecules have been identified in LB (see *Supporting List*), but the most prominent is α-SYN (38). α-SYN was first cloned from the *Torpedo californium* (39) and later identified in humans as the non-Aβ component of AD amyloid (NAC) precursor (NACP) (40). Attention focused on α-SYN when Polymeropoulos *et al.* (6) reported that in a kindred with familial PD, a mutation, A53T, was associated with disease. A number of groups quickly reported that α-SYN is a major component of LB (41–45). Two additional missense mutations, A30P (46) and E46K (47), are also associated with familial PD. A triplication of the region on chromosome 4 containing the *α-SYN* gene and an estimated 17 genes was found to be associated with familial PD (7). The polymorphic NACP-Rep1 element in the promoter region has been reported to affect the risk of PD (48, 49), and this region affects gene expression (50). Increased expression of α-SYN has been reported with injury to the SN (51).

Structure. α-SYN is a member of a highly conserved family of proteins consisting of α-, β-, and γ-SYN. It consists of 140 amino acids, and the *α-SYN* gene contains seven exons, five of which are protein coding (52–54). The N-terminal 89 amino acids are composed almost entirely of six variants of a degenerate 11-amino acid consensus motif with slight variations of the sequence KTKEGV. The 11-mer repeats comprise an apolipoprotein-like-class-A_2 helix, which mediates binding to vesicles of phospholipids. In neutral solution, α-SYN is unfolded, but binding to lipid shifts the protein secondary structure from ≈3% to >70% α-helix. The central region contains two of the 11-mer repeats and the hydrophobic NAC portion between residues 61 and 95. The NAC region of α-SYN appears to be crucial to fibrillation, because α-SYN lacking the domain and β-SYN, which lacks 11 amino acids in the NAC region, have reduced susceptibility to form fibrils. The C terminus is less conserved than the N terminus and contains a preponderance of acidic residues.

Distribution. Solano *et al.* (55) examined levels of α-SYN mRNA in autopsy cases without a history of neurological illness and noted expression in regions in which LB are commonly found in PD and LBD. However, they noted substantial expression in regions in which LB are not routinely described, indicating that the level of expression of the *α-SYN* gene is not the sole determinant of the formation of LN and LB.

α-SYN protein has been estimated to comprise 0.5–1.0% of brain cytosolic protein (56), and α- and β-SYN proteins are widely distributed in the neuropil in the brain (57, 58). Mori *et al.* (58) reported that in the adult rat, α-SYN protein is also present in the neuronal cytoplasm in the olfactory bulb, cortex (especially layer IV), SN, and pontine nuclei.

α-SYN, first described in presynaptic terminals (56), is enriched in synaptosomal preparations but is not isolated in purified preparations of synaptic vesicles

(59, 60). Electron microscopic studies demonstrated α-SYN in an amorphous matrix, beneath the regions of abundant synaptic vesicles (61), positioning it to participate in the release of neurotransmitters from the reserve or resting pools (see below).

Function. The function(s) of α-SYN is not fully understood, but studies of α-SYN knockout (KO) mice have been informative. Abeliovich *et al.* (62) reported that α-SYN KO mice were viable and fertile and had normal brain architecture, particularly of the nigrostriatal DA system, indicating that α-SYN is not necessary for normal development of this system. The dopamine content in the striatum, but not the midbrain, was reduced. Open field activity was normal, but amphetamine-induced locomotion was reduced. Simple electrical stimulation of the nigrostriatal DA system produced a normal pattern of dopamine discharge and reuptake. Paired-pulse stimulation had accelerated recovery of release of dopamine after an initial stimulus, suggesting an inhibitory role for α-SYN in the activity-dependent modulation of nigrostriatal DA transmission. Chandra *et al.* (63) used single and double α- and β-SYN KO mice and reported that the SYNs are not crucial to neurotransmitter release but may be involved in long-term regulation and/or maintenance of presynaptic function. Cabin *et al.* (64) carried out similar studies in α-SYN KO mice and noted, in the hippocampus, a reduction in the pool of undocked vesicles, and that prolonged stimulation in slices, which would rely on the reserve vesicle pool, was impaired. In cultured hippocampal cells (65), long-lasting potentiation of synaptic transmission between cells was accompanied by an increase in the number of α-SYN clusters, and in cultures derived from α-SYN KO mice, this potentiation was not observed. Cabin *et al.* (64) concluded that α-SYN is required for the genesis, localization, and/or maintenance of at least some of the vesicles that make up the reserve or resting pools of presynaptic vesicles, and the data appear to be consistent with this conclusion. The mechanism by which α-SYN modulates neurotransmitter release is uncertain, but it may be through inhibition of phospholipase D2, with effects on levels of diacyl glycerol and phosphatidic acid and their effects on the size and recovery rate of the readily releaseable pool of synaptic vesicles and membrane trafficking (62).

The α-SYN KO mice have shed light on certain pathogenic mechanisms important in PD. Four lines of α-SYN KO mice have been demonstrated to be resistant to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which is a mitochondrial inhibitor relatively selective for the nigrostriatal DA system (11, 66, 67), and the protective effect appeared to be upstream of the mitochondria (66).

Degradation. The UPS removes misfolded and damaged proteins from the cytosol, nucleus, and endoplasmic reticulum. Attachment of multiple molecules of ubiquitin serves as the signal for degradation of the protein by the proteasome (68–70). Ubiquitin target protein ligation is accomplished through a series of three steps: activation of ubiquitin in an ATP-dependent reaction by ubiquitin-activating enzyme (E1), transfer of ubiquitin to one of several ubiquitin-conjugating enzymes (E2s), and ligation of ubiquitin to a lysine on the target protein by a member of the E3 ligase family. Additional activated ubiquitin molecules can be attached to lysines within the ubiquitin molecule to form polyubiquitin chains, which is the signal for recognition by the proteasome. The polyubiquinated molecule is then processed by proteasomes in the cytoplasm, perinuclear regions, and nucleus. The 26S proteasome consists of a cylindrical catalytic core, the 20S proteasome, with 19S complexes capping either end (68). The inner surface of the 20S core contains three catalytic sites: chymotrypsin-like, trypsin-like, and peptidylglutamyl–peptide hydrolytic sites. The 19S complex selectively opens the channel through the 20S proteasome, unfolds the polyubiquinated protein, and cleaves from the targeted protein the polyubiquitin, which is broken down into monoubiquitins by ubiquitin C-terminal hydrolase. Attention was drawn to the UPS by the discoveries that a common form of autosomal recessive PD is due to mutations in the *Parkin* gene, which encodes for an E3 ligase (71, 72). Another member of the UPS, ubiquitin C-terminal hydrolase L1, was reported to have a missense polymorphism in two siblings with PD (73).

The role of the UPS and Parkin in the degradation of α-SYN is not fully resolved. Bennett *et al.* (74) reported that α-SYN was degraded by the UPS, but other groups have reported that inhibition of proteasomes did not increase levels of α-SYN (75, 76), suggesting alternate pathways for degradation. *In vitro* α-SYN can be degraded by the 20S proteasome through a ubiquitin-independent mechanism (77).

Infusion of the proteasome inhibitor lactacystin into the SN (78) and lactacystin and epoxomycin (a naturally occurring inhibitor) into the striatum (79) created models of PD in the rat. McNaught *et al.* (12) extended this research by systemically administering epoxomicin to rats and found that the animals developed behavior and pathology with α-SYN inclusions reminiscent of PD. Mutant forms of α-SYN (80–82) and protein aggregates (83) can reduce proteasomal activity.

The finding that *Parkin* KO mice have a normal level of α-SYN (84) raised the possibilities that it is not a normal substrate for Parkin or that other degradation systems are involved. Inhibition of both the proteasome and autophagy increased the levels of α-SYN in PC12 cells with α-SYN in autophagic vacuoles and lysosomes (85). In cultured cortical neurons, proteasome inhibition led to activation of macroautophagy and the lysosomal pathway (86). In cultured neuronal cells, α-SYN oligomers, but not mature fibrillar inclusions, were cleared by the lysosomal pathway (87). Cuervo *et al.* (88) reported that WT α-SYN was internalized and degraded in lysosomes by chaperone-mediated autophagy, and A30P and A53T forms of α-SYN blocked lysosomal uptake and degradation of α-SYN by chaperone-mediated autophagy. Of note, four groups have reported an increased prevalence of heterozygous carriers of mutant forms of glucocerebrosidase, the lysosomal enzyme involved in Gaucher's disease, in Parkinsonian patients, (89–93), and one series described cortical and/or hippocampal LB (91). Subclinical lysosomal dysfunction in cases with mutant glucocerebrosidase has been hypothesized to lead to impaired clearance of α-SYN (89).

The data indicate that α-SYN can be degraded by both the proteasome (through ubiquitin-dependent and -independent processes) and autophagy.

Mechanisms of α-SYN Aggregation

α-SYN has been reported to aggregate under a number of conditions: in simple solutions, incubation with lipids, cell culture, and animal models of disease.

Concentration. A number of groups have demonstrated that α-SYN can aggregate to form protofibrils (94) and amyloid-like fibrillar structures (94–96). In solution, the mutations A53T and A30P increased the rate of α-SYN oligomerization (97) and formation of β-sheets (98). The rate of formation of mature fibrils was increased by A53T and reduced by A30P (99). Overexpression of α-SYN in a number of cell lines resulted in the formation of aggregates (80, 100, 101). Masliah *et al.* (102) overexpressed WT α-SYN in mice and found amorphous and granular (not fibrillar) aggregates of α-SYN in various brain regions

in which LB are found. The mice exhibited injury to the nigrostriatal DA system and motor deficits. Adenoviral vector-selective overexpression of WT and A53T α-SYN in the SN in nonhuman primates caused α-SYN inclusions and degeneration of DA neurons (103). Overexpression of WT and mutant α-SYN in *Drosophila* resulted in intraneuronal α-SYN inclusions, loss of DA neurons, and motor deficits (104). Overexpression of mutant and WT α-SYN in *Caenorhabditis elegans* resulted in DA cell loss (105). The concentration dependence of aggregation may be relevant to PD, because triplication of the α-SYN gene is associated with familial PD (7).

Oxidation. Evidence of excessive oxidation is found in the brains of PD patients (106–108). Hashimoto *et al.* (109) demonstrated that treatment of α-SYN with H_2O_2 and ferrous iron in solution led to the formation of insoluble aggregates of α-SYN. Treatment of neuroblastoma cells engineered to overexpress A53T, A30P, and WT α-SYN with iron resulted in the formation of α-SYN aggregates, and the mutants were more susceptible (110). Increased amounts of iron occur in the SN in PD brains (111). Considerable evidence has accumulated that mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of PD (112). Chronic treatment of rats with rotenone, a mitochondrial inhibitor, resulted in injury to the nigrostriatal DA system and formation of α-SYN aggregates (10), and continuous exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice resulted in a PD phenotype and formation of LB-like inclusions (11). α-SYN inclusions in brains of PD patients contain oxidized α-SYN (113).

Phosphorylation. Fujiwara *et al.* (114) isolated α-SYN phosphorylated at Ser-129 from LBD brains. Immunohistochemical studies demonstrated phosphoserine α-SYN in brains from patients with PD, multiple system atrophy, and neurodegeneration with brain iron accumulation type 1. Immunolabeling for phosphoserine αSYN revealed intense labeling of LB and LN but not in the neuropil. Under normal physiological conditions, ≈4% of α-SYN is phosphorylated at Ser-129 in the rat, but in LB, 89% of α-SYN was phosphorylated at this residue (114). Hasegawa *et al.* (115) demonstrated that the phosphoserine α-SYN was mono- and diubiquinated. The effects of phosphorylation of Ser-129 on α-SYN′s conformation are not known, but phosphorylation at Ser-129 caused a 4-fold increase in insoluble α-SYN (114) and inhibited interaction of α-SYN with phospholipids and phospholipase D2 (116). G protein-coupled receptor kinases phosphorylate α-SYN at this single residue (116, 117), and casein kinase 1 and 2 have been suggested as a responsible kinase (118). Chen and Feany (119) created a series of transgenic *Drosophila* to explore the role of phosphoserine 129 α-SYN in neurotoxicity to DA neurons. Mutation of Ser-129 to alanine (to prevent phosphorylation) completely suppressed DA neuronal loss produced by expression of human α-SYN, and substituting aspartate for Ser-129 (to mimic phosphorylation) significantly enhanced α-SYN toxicity. Overexpression of G protein-coupled receptor kinase 2 enhanced α-SYN toxicity. Blocking phosphorylation at Ser-129 substantially increased aggregation of α-SYN, which may seem counter to the accumulation of LB in human diseases, but Chen and Feany (119) hypothesized that phosphorylation at Ser-129 favors maintenance in a soluble (perhaps toxic) form. Smith *et al.* (120) reported that expression of both WT α-SYN and synphilin-1 in human neuroblastoma SH-SY5Y cells yielded cytoplasmic inclusions with some features resembling LB, whereas interference of phosphorylation of Ser-129 by coexpression of S129A α-SYN and synphlin-1 or treatment with a casein kinase 2 inhibitor reduced formation of inclusions.

Glycosylation. Shimura *et al.* (121) isolated a novel *O*-glycosylated α-SYN in autopsy brain, which interacted with normal but not mutant Parkin.

Crosslinking. A transglutaminase-crosslinked intramolecular bond in α-SYN has been reported present in the SN of PD patients, and it increased with disease progression (122). Exposure of α-SYN to nitrating agents resulted in α-SYN aggregation (123) and formation of oligomers due to crosslinking through oxidation of tyrosine to form *o,o′* dinitrotyrosine (124).

Tau. The observation that up to half of AD cases also contain LB suggested there might be a commonality in the processes that form LB and the senile plaques and neurofibrillary tangles (NFT), the pathological hallmarks of AD (15, 125, 126). Cases of familial AD can have LB (16), and patients with the A53T mutation in the gene for α-SYN have tau, as well as α-SYN, inclusions (127). Giasson *et al.* (128) reported that α-SYN could initiate polymerization of tau *in vitro*, and that tau significantly increased the assembly of α-SYN into fibrils. However, both tau and α-SYN preferentially form homopolymers rather than heteropolymers, suggesting that each may participate in an initiation phase (seeding) of formation of fibrils, but that enlargement into larger fibrils is specific for each protein (125), and that these and other amyloidgenic proteins can induce both α-SYN and tau pathology (126). In AD, neurons tend show a predilection to form NFT or LB, and tau and α-SYN tend to segregate into separate filaments (126). This commonality might also be due to processes that underlie the formation of tau and α-SYN inclusions.

Possible Toxic Mechanisms of Aggregated Protein (α-SYN). The data reviewed above are consistent with roles for LB and α-SYN in the pathogenesis of disorders with LB, and gene expression in LB-positive and -negative nigral neurons in PD brains suggests that neurons with LB are more compromised (129). Other data suggest that LB may not be injurious (130). Kholodilov *et al.* (51) reported increased expression of α-SYN in the surviving nigral neurons in a model of SN injury. Transgenic mice overexpressing WT α-SYN had reduced injury to nigral neurons after treatment with paraquat (131).

Protofibrils. Lansbury and coworkers (132–134) have extensively evaluated the possibility that α-SYN fibrils may not be toxic; rather, intermediates, which they have termed protofibrils, may be the toxic compounds. The protofibrils, which, like α-SYN fibrils, contain β-sheet structures, occur early in the fibrillation process. Atomic force microscopic images at steps in the fibrillation process indicated that α-SYN first formed spherical protofibrils, which progressed to chain and annular protofibrils, and some or all of these were converted to fibrils (133). In cultured cells, overexpression α-SYN or treatment with a mitochondrial inhibitor resulted in two distinct forms of aggregated α-SYN; small spheres of α-SYN, which formed first; and larger inclusions of α-SYN (135, 136). Membrane-bound α-SYN can create forms of α-SYN that serve to aggregate cytosolic α-SYN (136). α-SYN was shown to form adducts with dopamine, which appeared to slow the conversion of protofibrils to fibrils, and these may explain the propensity of nigral DA neurons to injury (137). Although most of the studies are consistent with a process through which protofibrils become fibrillar inclusions, Cole *et al.* (138) presented data to suggest that covalent association of α-SYN into protofibrils and filament formation can form through separate processes. Although

protofibrils have yet to be described in PD, in a study of glial cytoplasmic inclusions (GCI), which are the cardinal feature of multiple system atrophy, another Parkinsonian disorder, and are composed primarily of α-SYN, atomic force microscopy of particles extracted from detergent-treated GCI revealed annular particles having "some similarity in morphology" to the annular oligomers described by Lansbury's group, with a central cavity of 2–3 nm (139). Protofibrils have been reported to permeabilize phospholipid vesicles, perhaps through a pore-like mechanism, and α-SYN may injure cells through this mechanism (140) (see below a discussion of how protofibrils might contribute mitochondrial dysfunction and release of proapoptotic molecules). Although there is evidence that protofibrils form and can be injurious to cells, Volles and Lansbury (141) pointed out that a contribution of them to the pathogenesis of PD needs further study in animal and/or cellular models (141).

Interference with Normal Cellular Physiology. It is plausible that excessive amounts of α-SYN oligomers might overwhelm the mechanisms responsible for clearance of aberrant proteins, thereby interfere with the normal physiology of the cell, and eventually lead to cell injury and death. Recent attention has turned to the role of the proteasomal system in the pathogenesis of PD (see above). Impairment of proteasome function can obviously be the primary event (as occurs in patients who have mutations in the *Parkin* gene but lack LB), but investigators have also shown that mutant forms of α-SYN (80–82) and protein aggregates (83) can reduce proteasomal activity. The inability to normally degrade misfolded and damaged proteins would lead to increasing dysfunction and death of cells. As discussed above, there is evidence of proteasomal dysfunction in PD, and animal models in which proteasome inhibitors have been reported to create the behavioral and pathological features of PD. Interestingly, Parkin KO mice exhibited mitochondrial dysfunction and oxidative damage (142). Mitochondrial dysfunction has been established to occur in PD (112), and overexpression of α-SYN has been reported impair mitochondrial function (100).

Disruption of Normal Cellular Topography. An extension of the idea above is that the presence of large inclusions such as LB and LN so disrupts normal cellular architecture that cell function is compromised, eventually leading to cell death.

Conclusions, Questions, and Hypotheses

LB are found in a number of neurological disorders that occur increasingly with aging, e.g., PD, AD, and LBD, and in a number of hereditary disorders in which the specific primary etiological trigger is known, e.g., mutations in the *α-synuclein* and *LRRK2* genes. LB-like inclusions also occur in animal models of PD that recreate pathogenic mechanisms occurring in the disorder by using mitochondrial and proteasomal inhibitors. These data indicate that a number of primary pathogenic events can lead to the development of LB.

LB occur in various neuronal cell types, indicating that the processes that lead to their formation are common to many types of neurons. LB have been considered to be toxic, protective, or innocuous. If they are protective, they are not very effective, because there is not an increasing number of LB-laden neurons in the SN with advancing PD; rather, the affected neurons die.

The major component of LB (as revealed by current techniques) appears to be α-SYN. The propensity for α-SYN to form aggregates appears to be due to two factors. First, it is widely expressed in relatively high amounts. Second, the NAC region, with its tendency to form β sheets, appears to be crucial. The factors that lead to aggregation are not fully understood, but a number of factors that may contribute to aggregation have been identified, including phosphorylation at serine 129, oxidation, exposure to tau, and proteasomal dysfunction. Clearly, more research needs to be done to elucidate these factors. For factors that have already been identified as likely to play a role in aggregation of α-SYN, e.g., phosphorylation at serine 129, we need to develop and test in animal models interventions in these processes.

Specific mechanisms through which α-SYN aggregates injure neurons have not been identified. Our current understanding of the pathogenic mechanisms in PD involves three pathways, protein aggregation (143, 144); impaired protein degradation, i.e., dysfunction of the proteasome and lysosome systems (38, 68, 69, 88); and mitochondrial dysfunction/oxidative stress (10, 112, 143). Other processes, such as inflammation, may also contribute to the death of cells (145, 146). These three processes likely overlap and interact (38, 143, 147). For example, overexpression and aggregation of α-SYN can lead to mitochondrial dysfunction (100), and inhibition of mitochondrial function with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and rotenone leads to aggregation of α-SYN (10, 11). Impairment of the proteasomal system in Parkin KO mice and in PC12 cells leads to mitochondrial dysfunction (142, 148), and mitochondrial dysfunction results in impairment of proteasomal function (149). Inhibition of the proteasomal system results in formation of α-SYN aggregates (12, 78, 79), and aggregates of α-SYN can impair proteasomal function (83).

Malfunction of mitochondria or the proteasome and lysosome systems would result in the accumulation of proteins, many abnormal, and obviously interfere with function of the cell. However, specific mechanisms through which these aggregates of proteins are injurious to cells have been surprisingly unexplored. The processes involved in the formation of LB reviewed above suggest a mechanism through which the processes may interact, lead to α-SYN aggregation, and result in mitochondrial dysfunction and potentially release of proapoptotic molecules. It may not be coincidence that mitochondria are a conspicuous component of LB (27, 150, 151). Aggregation of α-SYN can be triggered by a number of factors reviewed above. Mitochondria, which both produce ATP for the cell and are central to apoptosis, are composed of an outer membrane, an intermembrane space, and an inner membrane and matrix (112, 152). The electron transport chain (ETC), which is composed of five complexes, is located on the inner mitochondrial membrane, and energy released by passage of electrons down the ETC is used by complexes I, III, and IV to transport protons from the matrix to the intermembrane space. The transport of protons creates a proton and electrochemical gradient across the inner membrane, and the energy stored in the electrochemical proton gradient is used to drive complex V to form ATP. In addition to its role in the bioenergetics of the cell, or perhaps because of its central position in the energetics of the cell, the mitochondrion has evolved to play a central role in apoptosis through the release of proapoptotic molecules from the mitochondrial intermembrane space (153–155).

The mitochondrial outer membrane is freely permeable to molecules smaller than 2 kDa but not to larger molecules (156). Aggregated α-SYN can form annular pore-like structures, and the inner diameters of pores range from 1 to 3 nm (132, 157). Insertion of such pores into the outer mitochondrial membrane could allow leakage of molecules from the intermembrane space. Leakage of proteins involved in mitochondrial maintenance, e.g., Tim9,10 and/or bioenergetics [mitochondria contain ≈1,500

different proteins (158)] would obviously lead to impaired mitochondrial function. Of note, the activity of complex I, which is the largest complex in the electron transport chain with at least 43 subunits and would likely be affected by impaired mitochondrial maintenance, is reduced in PD patients (159–161).

It is also conceivable that pores of this size could lead to release of proapoptotic molecules. For example, annular protofibrils might release cytochrome *c* from the intermembrane space, as Bax has been reported to in liposomes and mitochondria. Saito *et al.* (162) reported that the Stokes diameter of cytochrome *c* is 1.7 nm (12 kDa), and that insertion of Bax into liposome vesicles formed pores with 2.2-nm inner diameter (a pore size similar to that for annular protofibrils) and released cytochrome *c* from the vesicles. In proteoliposomes prepared from the mitochondrial outer membrane, a channel appeared with apoptosis, which had a pore diameter of 4 nm (larger than that in simple liposome vesicles) and appeared to be Bax (163). Volles and Lansbury (140) did not find that protofibrils allowed passage of cytochrome *c* from artificial vesicles of phospholipids (140) but these vesicles obviously differ from the mitochondrial outer membrane, which has evolved to allow release of proapoptotic molecules. Release of proapoptotic molecules may not be as simple as passage through a channel or pore and may involve interaction with components of the outer membrane, such as voltage-dependent anion channels, to form large pores (164) or fission of the mitochondria (155).

It is plausible that annular protofibrils of α-SYN may insert into the outer mitochondrial membrane, as well as other membranes, and form pores that could allow leakage of molecules from the intermembrane space necessary for mitochondrial function and integrity and/or proapoptotic molecules. The possibility could be addressed with purified annular protofibrils and mitochondria and examination of mitochondrial morphology, mitochondrial membrane potential, the activities of the electron transport chain, and release of molecules in the intermembrane space, such as cytochrome *c*. Further studies using an antibody that recognizes the oligomeric state of α-SYN (165) to evaluate the location of such oligomers, possibly with mitochondria, in models of PD and human tissue should also be performed.

Lewy's observation of intraneuronal inclusions in PD alerted investigators that intracellular aggregates might play a role in the pathogenesis of the disorder, and recent research has focused attention on α-SYN aggregates. Further research is needed to better understand the mechanisms that favor α-SYN aggregation, the specific mechanisms of toxicity of the aggregates, and interventions in these processes.

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