**Protein Aggregation Mechanisms in Synucleinopathies: Commonalities and Differences**

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**Abstract**

Synucleinopathies are characterized by the presence of different types of α-synuclein (AS)-positive inclusion in the brain. Thus, whereas Lewy bodies are the hallmark of Parkinson disease and dementia with Lewy bodies, glial and neuronal cytoplasmic inclusions are shown by multiple system atrophy. Because the main component of all these inclusions is conformationally modified AS, aggregation of the latter is thought to be a key pathogenic event in these diseases. Although very little information has been available on AS function and aggregation mechanisms until 2 years ago, recent investigations have greatly improved our understanding of the steps involved in the pathogenesis of synucleinopathies. Additionally, important insights into the specific molecular events (e.g., differential posttranslational modifications or isoform expression profiles) underlying each of these conditions have been gained. The present review summarizes our current knowledge of the commonalities and differences shown by protein aggregation mechanisms in the various synucleinopathies.

**Key Words:** α-Synuclein, Dementia with Lewy bodies, Glial cytoplasmic inclusion, Inclusion body, Lewy body, Multiple system atrophy, Parkinson disease.

**INTRODUCTION**

Parkinson disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are considered to be synucleinopathies because they share α-synuclein (AS) aggregation into inclusion bodies as their key pathogenic event. The molecular pathways leading to AS accumulation in synucleinopathies show interesting commonalities and differences and gaining an insight into these may significantly help to develop effective diagnostic and therapeutic approaches to these conditions. After providing a brief introduction to the various synucleinopathies, in this review we describe the features of the inclusion bodies found in these disorders, discuss the mechanisms of inclusion body formation, analyze the commonalities and differences among synucleinopathies in regard to their inclusion body protein content, and propose working hypotheses and future research directions.

**THE SYNUCLEINOPATHIES**

**Lewy Body Diseases and Parkinson Disease**

First described in 1817, PD is the most common progressive movement disorder in the elderly and is characterized by tremor, rigidity, and bradykinesia. There is increasing evidence that PD is a multisystemic disorder showing both progressive degeneration of the dopaminergic nigrostriatal system and widespread extranigral pathology (1–3). In PD, Lewy body (LB) pathology first appears in lower brainstem nuclei such as the dorsal motor nucleus of the vagus and the olfactory system (stages 1–2). Afterwards, ascending progression leads to changes in the coeruleus complex, substantia nigra pars compacta, basal forebrain magnocellular nucleus, subthalamic nucleus, and amygdala (stages 3–4). Finally, involvement of the neocortex may supervene (stages 5–6) (1, 2).

In the last few years advances in the genetics of PD have revealed that mutations are responsible for a small proportion of cases, whereas most instances are of sporadic origin. So far, mutated genes have been found to be responsible for Mendelian forms of PD. The first PD-related gene identified was the AS (SNCA) gene, in which pathogenic point mutations (A30P and A53T) were initially detected (3, 4). Afterward, a third SNCA mutation (E46K) was described in a large Spanish pedigree with autosomal dominant parkinsonism, dementia, and visual hallucinations (5). SNCA gene duplication and triplication have also been observed in independent familial PD pedigrees showing a gene dose-dependent disease course (6, 7). Other familial PD cases have revealed mutations of the DJ-1 gene (8) and the phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) gene (9). On the other hand, a synphilin-1 gene I93M mutation has been linked to autosomal dominant parkinsonism, dementia, and visual hallucinations (5). SNCA gene duplication and triplication have also been observed in independent familial PD pedigrees showing a gene dose-dependent disease course (6, 7). Other familial PD cases have revealed mutations of the DJ-1 gene (8) and the phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) gene (9). On the other hand, a synphilin-1 mutation has been described in patients with sporadic PD (10). Additionally, the ubiquitin C-terminal hydrolase L1 (UCH-L1) gene I93M mutation has been linked to autosomal dominant PD (10), whereas the S18Y polymorphism of the same gene has been associated with a significantly lower risk of PD (11).

Interestingly, mutations of the leucine-rich kinase 2 (LRRK2) gene encoding dardarin, a multidomain protein of uncertain function, have been related to both familial PD...
Multiple System Atrophy (3%–10%) and sporadic PD (1%–8%) in several European-derived populations (12). Intriguingly, although the clinical features of LRRK2-related PD are similar to those of idiopathic PD, LRRK2-related PD cases show only a small number of nigral LBs and very occasional cortical LBs (13). Finally, parkin (PRKN) gene mutations are responsible for approximately 50% of all autosomal recessive parkinsonism cases of early onset and for 77% of cases with onset before 21 years of age (14). The vast majority of patients with PD associated with PRKN mutations lack LBs (15) and consequently are outside the scope of the present review.

Dementia With Lewy Bodies

DLB is the second most frequent cause of dementia in the elderly after Alzheimer disease (AD) (16) and is clinically characterized by progressive dementia, often accompanied by parkinsonism and psychiatric symptoms (17). Widespread distribution of LBs in virtually every brain area is a typical feature of DLB, although the frontal cortex, pigmented midbrain and brainstem nuclei, dorsal efferent nucleus of the vagus, basal forebrain nuclei, and limbic cortical regions are particularly involved (18). DLB cases may be grouped into types according to their LB distribution: the brainstem-predominant type, the limbic or transitional type, and the diffuse neocortical type (17).

Recently revised consensus guidelines (19) for the pathologic diagnosis of DLB propose a semiquantitative severity grading of LB-related pathology into mild, moderate, severe, and very severe cases. This system is based on semiquantitative assessment of LB density rather than on previously used counting methods (19). DLB is often associated with variably extensive AD and is then termed common Lewy body disease (18). In these cases, the likelihood that the patient’s clinical syndrome is caused by LB pathology is directly related to LB density and inversely related to the severity of concurrent AD-type pathology (19).

An AS gene mutation (E46K) has been described in a large Spanish pedigree encompassing patients with both DBL and PD (5). Interestingly, an UCH-L1 gene 193M mutation has also been detected in another large family with patients with both PD and DBL (10). Additionally, mutations (V70M and P123H) of β-synuclein, an AS homolog, have been found in independent DBL cohorts (20).

Multiple System Atrophy

The term MSA was first proposed by Graham and Oppenheimer in 1969 for a progressive neurodegenerative disorder with parkinsonism, ataxia, and autonomic failure (21). The etiology of this condition remains unknown (21). Phenotypically, MSA forms with predominantly parkinsonian symptoms are known as MSA-P and show overall atrophy of the putamen. On the other hand, MSA forms with predominantly cerebellar symptoms are designated as MSA-C and show cerebellar atrophy with Purkinje cell depletion, as well as atrophy of the middle cerebellar peduncles, basis pontis, and inferior olivary nuclei (22).

Glial cytoplasmic inclusions (GCIs) in oligodendrogial cells, a MSA neuropathologic hallmark, are accompanied by neuronal loss and astrocytosis in the striatonigral system (16). Whereas in stage I MSA there is widespread distribution of GCIs, neuronal loss is restricted to the substantia nigra and locus coeruleus, sparing the striatum (23). These findings suggest that MSA pathogenesis differs substantially from that of most other neurodegenerative disorders, in which neurons are primarily targeted (24).

When neurons finally become involved in MSA, they develop neuronal cytoplasmic inclusions that are particularly abundant in the anterior central gyrius and supplementary motor cortex (25). Neuronal cytoplasmic inclusions are round or ovoid structures that occupy the greater part of the neuronal cytoplasm and, as shown by immuno-electron microscopy, contain both granular and filamentous AS (26). Some MSA cases may also show rare ubiquitin-positive neuronal nuclear inclusions resembling those seen in motor neuron disease. A semiquantitative 4-tiered grading system has recently been proposed for both types of MSA (27).

Although no point mutations have been described in MSA as yet, cases associated with either minimal GAA1 expansion of the gene frataxin (28) or FMN1 gene premutation status have been reported (29). These findings are in accord with MSA phenotypical variability.

INCLUSION BODIES IN SYNucleinopathies

Lewy Bodies of PD and DBLs

Morphologically, LBs may be divided into brainstem and cortical types. Whereas brainstem LBs are found in the brainstem nuclei and diencephalon, cortical LBs are preferentially seen in the cerebral limbic cortex and amygdala.

Classic brainstem LBs are spherical, intraneuronal cytoplasmic inclusions that measure 8 to 30 μm in diameter and are characterized by hyaline eosinophilic cores, concentric lamellar bands, narrow pale halos, and immunoreactivity for AS and ubiquitin (30). In contrast, cortical LBs typically lack a halo (31). AS immunolabeling of PD brains has revealed that AS immunostaining can be detected in approximately 64% of nigral LBs and 31% of cortical LBs (32).

Ultrastructurally, brainstem LBs are composed of radially arranged 7- to 20-nm intermediate filaments associated with granular electron-dense material and vesicular structures. Their core shows tightly packed filaments and dense granular material, whereas radiating fibers occupy their periphery (30). As expected from their uniform density on light microscopy, cortical LBs are composed almost entirely of circular or oval fibrillary material (33).

Although a number of proteins have been identified in LBs, their precise biochemical composition has not yet been elucidated (Table 1). Granulofilamentous accumulation of AS has recently been shown in Purkinje cell axons in PD and DBL brains, albeit if cerebellar pathology has not been unequivocally demonstrated in LB diseases (34).

GCIs of Multiple System Atrophy

GCIs are faintly eosinophilic, sickle-shaped, oval or conical inclusions that displace the nucleus eccentrically. Their localization to microglia has been established by double staining techniques (21). Ultrastructurally, GCIs consist of loosely aggregated filaments with cross-sectional...
TABLE 1. Main Protein Components Found in Lewy Bodies (18, 35) and Glial Cytoplasmic Inclusions (18, 39)

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<thead>
<tr>
<th>Lewy Bodies</th>
<th>Glial Cytoplasmic Inclusions</th>
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<tr>
<td>AS</td>
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<tr>
<td>Ubiquitin</td>
<td>Ubiquitin</td>
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<tr>
<td>α-,β-Crystallin</td>
<td>α-,β-Tubulin</td>
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<tr>
<td>Complement proteins (C3d, C4d, C7, C9)</td>
<td>bcl-2</td>
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<tr>
<td>Cyclin-dependent kinase 5 (cdk-5)</td>
<td>Carbonic anhydrase isoenzyme II</td>
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<td>Cytochrome c</td>
<td>cdc-5</td>
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<tr>
<td>Dorfin</td>
<td>Dorfin</td>
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<td>Heat shock protein 90 (Hsp90)</td>
<td>Heat shock protein 90 (Hsp90)</td>
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<tr>
<td>Microtubule-associated proteins</td>
<td>MAP-5</td>
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<tr>
<td>Multicatalytic protease</td>
<td>Mitogen-activated protein kinase (MAPK)</td>
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<tr>
<td>Neural precursor cell expressed,</td>
<td>NEDD8</td>
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<td>developmentally downregulated 8 (NEDD8)</td>
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<tr>
<td>Neurofilaments</td>
<td>Phosphatidylinositol 3-kinase (PI3K)</td>
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<tr>
<td>Nuclear factor-κB</td>
<td>p25</td>
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<td>p25</td>
<td>Tau 2 protein</td>
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<td>Sphingomyelin</td>
<td>Sept4</td>
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<td>Sept4</td>
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<td>Synaptic vesicle-specific protein</td>
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<td>Synaptophysin</td>
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<td>Torsin A</td>
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<td>Tubulin</td>
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<td>Tyrosine hydroxylase</td>
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<td>Ubiquitin C-terminal hydroxylase</td>
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diameters of 20 to 40 nm, in addition to granulated material, that often entrap cytoplasmic organelles such as mitochondria and secretory vesicles (21). Further immunoelectron microscopic analysis of GCI s has revealed the presence of different types of AS filaments whose width may either be uniform or show periodic variations (35). It is suspected that other cytoskeletal proteins, such as α-tubulin and β-tubulin, may be involved in AS filament formation (36).

AS is the main component of GCI s, but the latter contain many other proteins such as ubiquitin and E3 ubiquitin ligases. The main GCI protein components detected by immunocytochemistry are listed in Table 1.

MECHANISMS OF INCLUSION BODY FORMATION IN SYNUCLEINOPIATHIES

Brainstem LBs

AS aggregation is now accepted as the key step preceding LB formation. Although the precise events responsible for AS aggregation remain unknown, accruing evidence suggests a complex mechanism encompassing phenomena as diverse as posttranslational modification and alternative splicing (37). Furthermore, mutations affecting AS intrinsic structure are also responsible for aggregation enhancement, as suggested by the occurrence of accelerated fibril formation in the presence of A30P, E46K, and A53T mutated AS (38, 39). In LB diseases there is altered solubility of aggregated AS as well as substantial accumulation of detergent-soluble and detergent-insoluble AS species of various molecular weights, particularly in the grey matter (30).

An immunoelectron microscopic study of AS in PD and DLB showed AS filaments in LBs, pale bodies, and perikaryal threads (40). In LBs, AS material was largely detected in radially arranged peripheral filaments, whereas the cores showed just a small number of grains. Pale bodies showed AS labeling to be related to loosely aggregated filaments, whereas perikaryal threads revealed a loose meshwork of small filament bundles that were immunoreactive for AS but not for ubiquitin. The ultrastructural similarities among filaments in LBs, pale bodies, and perikaryal threads prompted the hypothesis that AS perikaryal threads are an early stage of filament assembly that may then progress to pale bodies and, finally, to classic LBs (40). Other studies confirmed these data and showed that pale bodies occur after incorporation of p62 and assimilation of less aggregated forms of AS (31, 41). Afterwards, the appearance of LBs would be coincidental with increasing ubiquitin immunoreactivity and further AS incorporation (41).

Interestingly, initial perikaryal AS accumulation seems to be specifically constituted by AS112, a major AS isoform resulting from alternative splicing. AS112 lacks part of the unfolded protein C terminus but conserves intact membrane-protein and protein-protein binding domains. Therefore, AS112 seems to be an aggregation-prone isoform with enhanced membrane-binding properties (37, 42). Additionally, the fact that aggregation of AS is preceded by its interaction with membranes (43) and that the small fraction of membrane-bound AS seeds the accumulation of the far more abundant cytosolic form (44) point to the AS112 membrane-protein interaction domain as a crucial element in early aggregation steps (37).

Cortical LBs

The development of cortical LBs has been divided into stages based on morphologic and immunoelectron microscopic observations (45). In Stage 1 there is cytoplasmic granular AS accumulation in the neuronal cell body. Subsequent addition of filamentous AS leads to stage 2. In stage 3, AS accumulation gives rise to typical round LBs composed of dense granulofilamentous material. Stage 4 is characterized by involvement of dendrites. In stage 5 LBs are distorted and acquire a loose periphery. Finally, in stage 6 LBs undergo degradation, become extracellular, and show loose filamentous components, decreased AS immunoreactivity, and astroglial process involvement (45). Brains with mild LB pathology show stages 1 to 2 LBs in the neocortex, whereas brains with severe LB pathology show stages 5 to 6 LBs in the limbic cortex.

Glial Cytoplasmic Inclusions

As happens in LBs, profound solubility modifications and pathologic accumulation of AS precedes GCI formation.
further compaction (55).

Incorporation of additional proteins into GCIs and their progression is marked by ubiquitin-positive GCIs increases as the disease evolves. Ubiquitin-negative GCIs decreases and the number of AS-negative, ubiquitin-positive GCIs increases as the disease evolves. The total number of MSA GCIs does not vary with disease duration, it changes with disease duration. Thus, although full-length AS has been detected in GCIs, the predominant form of the aggregated protein seems to be C-terminally truncated (49).

Two main hypotheses have been proposed to explain the presence of aggregated AS in oligodendroglia. The first hypothesis is based on the observations that AS is transiently expressed in cultured rat oligodendrocytes (50) and that glias show low AS levels in vivo (51). Impaired ability to degrade AS or selective upregulation of AS expression in glial cells would lead to its accumulation and aggregation in these cells (52). The second hypothesis claims that oligodendrocytes actively uptake AS previously released by dying neurons (25, 53). In agreement with this, a patient with early-stage MSA presented a number of neuronal inclusions that drastically exceeded the number of GCIs (54). Because later MSA stages are characterized by the presence of numerous GCIs, translocation of aggregated AS from dying neurons to oligodendroglial cells can be invoked as a possible mechanism of GCI formation.

It has been suggested that GCI evolution is different according to brain region and that GCI composition changes with disease duration. Thus, albeit the total number of MSA GCIs does not vary with disease duration, it has been shown that the number of AS-positive, ubiquitin-positive GCIs decreases and the number of AS-negative, ubiquitin-positive GCIs increases as the disease evolves. This finding indicates that MSA progression is marked by incorporation of additional proteins into GCIs and their further compaction (55).

THE PATHOGENESIS OF SYNUCLEINOPATHIES: COMMUNALITIES

An important commonality of synucleinopathies, also shared by most neurodegenerative conditions, is the genetic and neuropathologic heterogeneity shown by each of these disorders. Whereas the ever-increasing number of gene mutations associated with synucleinopathies undermines their genetic heterogeneity, the variety of protein components found in inclusion bodies bears witness to their neuropathologic heterogeneity. Of these proteins, some are present in all inclusion bodies discussed in this review, whereas others are specific markers of certain inclusion forms.

Elevated phosphorylated AS levels have been detected in all synucleinopathies. AS is constitutively phosphorylated at serine residue 129 (56), and repeated phosphorylation and dephosphorylation within the AS C-terminal domain, mediated by casein kinases CK-1 and CK-2, has been observed in vivo (57). Extensive AS phosphorylation may be a significant pathogenic event and, probably, a necessary step in LB formation. This hypothesis is suggested by the fact that more than 90% of insoluble AS is phosphorylated in brains with synucleinopathies, whereas phosphorylation is a feature of only about 4% of normal AS (58).

Another common early event of inclusion body formation in synucleinopathies is the occurrence of AS/rab3a binding. This phenomenon, absent in the normal brain, is characterized by indirect binding of rab3a to AS with the aid of rabphilin intermediation (59). In MSA, AS/rab3a binding has been shown in both GCI-rich cerebellum and pons and GCI-free cerebral cortex, pointing to a specific role for rab3a in AS aggregation preceding GCI formation (60).

The incorporation of ubiquitin into all inclusion bodies in LB diseases and MSA is a further commonality of synucleinopathies. Nevertheless, the deposition of mono- or diubiquitinated phosphorylated AS in LB diseases and MSA indicates that AS ubiquitination occurs after AS aggregation (61). Consequently, AS ubiquitination seems to be an advanced step in LB as well as GCI formation (62).

One of the common components of LBs and GCIs is oxidative stress-related, ubiquitin-binding protein p62, whose immunostaining patterns are markedly similar to those of ubiquitin (63). It has been shown that the association of p62 with polyubiquitinated proteins enhances aggregation and plays an important role in protecting cells against the toxicity of misfolded proteins, particularly in advanced disease stages (64).

Synphilin-1 was initially identified as an AS-binding protein and, in association with AS, constitutes a major component of LBs (65). As shown by immunocytochemical and ultrastructural studies, most PD brainstem LBs show intense central core positivity for synphilin-1. On immunoelectron microscopy, the reaction product is seen to be located in filamentous and circular structures. In contrast, synphilin-1 is not present in pale bodies and only a small fraction of DLB cortical LBs contains synphilin-1. Similarly to brainstem LBs, numerous GCIs are positive for synphilin-1, suggesting that the role of abnormal synphilin-1 accumulation is not restricted to brainstem LB formation (66).

Interestingly, dorfin colocalizes with ubiquitin in LBs and GCIs. This E3 ubiquitin ligase participates in the formation of ubiquitinated AS-positive inclusions in PD, DLB, and MSA (67). Because dorfin physically binds and ubiquititates synphilin-1 through its central portion but does not ubiquitinate AS (68), it may be surmised that the synphilin-1 central domain plays an important role in inclusion body formation.

Intensive investigations on AS aggregation and inclusion body formation have recently identified additional proteins in LBs and GCIs. One of them is neural precursor cell expressed, developmentally downregulated 8 (NEDD8), an ubiquitin-like protein that controls vital biologic events through its conjugation to culling ubiquitin E3 ligases (69). Its presence in LBs and GCIs indicates that NEDD8 is involved in the formation of various ubiquitinated inclusions via the ubiquitin-proteasome system. Studies of heat shock proteins (Hsp), which facilitate refolding of denatured polypeptides, revealed that Hsp90 and AS colocalize in
LBs and GCIs. These findings suggest a role for Hsp90 in AS inclusion body formation (70).

Sept4, a member of the septin protein family, is consistently found in LBs and GCIs (71). Because tagged Sept4 and AS synergistically accelerate cell death induced by the proteasome inhibitor and this effect is further enhanced by LB-associated synphilin-1 expression, another LB-associated protein, it may be postulated that Sept4 is a potential cofactor in inclusion body formation.

Finally, investigation of other AS proaggregation proteins identified brain-specific p25α as a candidate that preferentially binds to AS in its aggregated state. In vitro stimulation of AS aggregation by purified recombinant human p25α and detection of p25α in LBs and GCIs prompted the suggestion that this protein plays a proaggregation role in neurodegeneration associated with AS aggregates (72).

THE PATHOGENESIS OF SYNUCLEINOPATHIES: DIFFERENCES

Lewy Bodies

Oxidative stress is a strong inducer of cell protein alterations. Mainly affected are mitochondria, as shown by mitochondrial complex I decreased activity and increased reactive oxygen species production in PD substantia nigra (73). An important protein target of oxidative damage is Cu,Zn-superoxide dismutase, a key antioxidant enzyme whose function is altered by oxidative modifications (74). Because AS seems to be a further target of oxidative stress in PD presymptomatic stages, AS oxidation may be a significant enhancer of AS aggregation properties (75).

Colocalization of parkin and AS in both brainstem and cortical LBs indicates that functional parkin protein may be required for LB formation (76). Parkin, in common with both doroﬁn and 7 in absentia homolog (SIAH), acts as an E3 ubiquitin ligase and is involved in protein turnover via the ubiquitin-proteasome system. Substrates ubiquitinated by parkin are thought to be destined for proteasomal degradation. Besides, it is currently known that parkin interacts with synphilin-1 and ubiquitinates it in a nonclassic, proteasomal-independent manner that involves lysine 63 (K63)-linked polyubiquitin chain formation (77). Furthermore, parkin-mediated ubiquitination of proteins in LB-like inclusions coexpressing synphilin-1, AS, and parkin occurs predominantly via K63 linkages and formation of these inclusions is enhanced by K63-linked ubiquitination (78).

LBs may contain tau as well. In fact, about 80% of PD cases show tau-immunoreactive LBs. Tau positivity is detected at the periphery of LBs, regardless of Braak stage. LBs and neuroﬁbrillary tangles (NFTs) keep an intriguing relationship inasmuch as the proportion of tau-immunoreactive LBs is greater in NFT-vulnerable neurons and lesser in NFT-resistant neurons (Table 2). These observations suggest that tau may coaggregate with AS in LBs, especially in neuronal populations that are vulnerable to both NFTs and LBs (79).

Finally, it is worth noting that AS shares physical and functional homology with 14-3-3 proteins, to which it binds. In agreement with this finding, 14-3-3 proteins have been detected in LBs (Table 2) (80), although it is not clear which 14-3-3 proteins are specifically involved in LB formation.

Brainstem LBs

Because certain proteins are present only in brainstem LBs and not in the other inclusion types discussed in this review (Table 3), it may be assumed that specific mechanisms are responsible for brainstem LB formation (Fig. 1).

Synphilin-1, an AS interacting protein, accumulates in brainstem LBs but its presence in cortical LBs is only occasional. Through its interaction with AS, synphilin-1 promotes AS aggregation and ubiquitination. Evidence of localization of Pael-R, a parkin substrate, in LB cores suggests that Pael-R is involved in the early phases of LB formation (81). PINK1, a protein with both mitochondrial targeting and serine/threonine kinase domains, is detected in a proportion of brainstem LBs in both sporadic PD and PINK1 gene mutation-associated PD. The presence of PINK1 in brainstem LBs in addition to its mitochondrial

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<th>Proteins</th>
<th>Brainstem</th>
<th>Cortical</th>
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<tbody>
<tr>
<td>Synphilin-1</td>
<td>+</td>
<td>–</td>
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<td>Parkin</td>
<td>+</td>
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<td>SOD1</td>
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<td>SOD2</td>
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<td>Pael-R</td>
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<td>Pink-1</td>
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<td>Clusterin</td>
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<td>DJ-1</td>
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<td>Elk-1</td>
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<tr>
<th>Proteins</th>
<th>Brainstem</th>
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<tr>
<td>Partially detected</td>
<td>+/+/−</td>
<td>+/+/−</td>
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<tr>
<td>Tau</td>
<td>+/+/−</td>
<td>+/+/−</td>
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<tr>
<td>Clusterin</td>
<td>–/+/−</td>
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+, positive; −, negative; ++/−−, most inclusion bodies are positive; +/−−, many inclusion bodies are positive; +/+/−−, 50% of inclusion bodies are positive; +−−−, some inclusion bodies are positive; +/−−−−, a few inclusion bodies are positive; +−−−−−, very few inclusion bodies are positive.

GCIs, glial cytoplasmic inclusions.
localization, underpins the significance of mitochondrial dysfunction in the pathogenesis of nigral cell degeneration in PD (82). Further evidence in favor of the existence of distinctive production mechanisms for each of the two LB types is the absence of clusterin in brainstem LBs but not in cortical LBs (83).

In addition to dorfin, synphilin-1 interacts with E3 ubiquitin ligases SIAH-1 and SIAH-2, which ubiquitinate synphilin-1 and promote its degradation by means of the ubiquitin-proteasome system. Inability of the proteasome to degrade the synphilin-1/SIAH complex leads to robust formation of ubiquitinated cytosolic inclusions. AS also associates with SIAH (mainly SIAH-2) and is monoubiquitinated by it. Detection of SIAH immunoreactivity in PD LBs has implicated this protein in inclusion body formation (84).

**Cortical LBs**

Several alterations have been described to be specific for cortical LBs in DLB (Table 3; Fig. 2). First, it has been seen that AS112 is overexpressed in DLB brains but not in control or AD brains. This suggests that AS112 is related to LB formation and probably constitutes a primary step in DLB pathogenesis. Additionally, UCHL-1 is selectively downregulated in the frontal cortices of DLB brains (85). Because synphilin-1 is present only in a small fraction of cortical LBs (66), it may be proposed that LB formation in DLB (or at least in a DLB subset) is promoted by...
overrepresentation of aggregation-prone AS112 in the absence of AS-interacting synphilin-1. Moreover, about 50% of cortical LBs are immunoreactive for clusterin, a molecular chaperon with a role in the refolding of misfolded proteins. In agreement with this function, clusterin colocalizes with AS, as indicated by the almost complete overlap of immunoreactivity of both proteins. Identification of some LBs showing high clusterin but low AS levels suggests that clusterin may influence LB formation (83).

**Glial Cytoplasmic Inclusions**

In contrast to cortical LBs, clusterin is detected in only approximately 10% of GClIs (Table 2) (83). On the other hand, only a subset of GClIs (or none in some cases) contain hypophosphorylated tau and 14-3-3 proteins (Table 2) (86). When present, 14-3-3 proteins associate with AS in GClIs. A negative correlation between the degree of tissue degeneration and the density of 14-3-3 proteins suggests that the latter are AS cofactors in GCI formation (87), at least in some MSA cases. The absence of Pael-R and parkin in MSA GClIs points to the existence of different formation pathways for LBs and GClIs.

DJ-1, one of the GCI-specific components detected to date (Table 3), is present as an insoluble, modified protein and seems to incorporate into GClIs after undergoing upregulation in reactive astrocytes and neurons (88). Another GCI-specific protein is Elk-1, which does not directly bind to AS but shares with AS its ability to bind to extracellular signal-related kinase (ERK)-2, a mitogen-activated protein (MAP) kinase. Interaction of AS with the MAP kinase pathway might cause dysfunction of neurons and oligodendrocytes and lead to neurodegeneration (89). Immunohistochemical detection of midkine in GClIs and the subsequent immunoelectron microscopic identification of midkine-positive, granule-coated fibrils indicated that this new neurotrophic factor could be an essential, specific constituent of GClIs (90).

A further specific feature of MSA seems to be the extensive accumulation of β-synuclein, an AS aggregation inhibitor, in Purkinje cells. The latter cells show no abnormal AS accumulation in MSA, in contrast to the increased AS levels shown in PD and DLB (54).

**WORKING HYPOTHESES**

**Brainstem and Cortical LBs**

The occurrence of significant differences between brainstem and cortical LB components suggests that their formation mechanisms are markedly disparate (Figs. 1, 2). Brainstem LBs contain the AS-interacting protein synphilin-1, whose core location would indicate that interaction between AS and synphilin-1 is an early event. Subsequent failure of protein refolding agents would result in protein sequestration within LBs. E3 ubiquitin ligases would ubiquitinate synphilin-1 and AS for their targeting for proteasome degradation, and parkin would carry out K63-mediated ubiquitination, thus enhancing protein aggregation and LB formation. Intervention of additional E3 ubiquitin ligases, such as dorfin and SIAH, would result in their sequestering within LBs. As cell stress increases, mitochon-
instrumental in neuronal differentiation, cell proliferation, and apoptosis (91). Widespread detection of midkine in GCI granule-coated fibrils suggests that this neurotrophic factor, initially associated with fetal morphogenesis, participates in GCI formation in a probable attempt to repair AS accumulation damage.

WHERE TO FROM HERE? FUTURE RESEARCH DIRECTIONS

Whereas synucleinopathies show an unexpected homology in regard to their neuropathologic findings, these disorders demonstrate an equally unexpected phenotypic heterogeneity, mainly due to genetic variations. Thus, PD, DLB, and MSA are each constituted by different subgroups that seem to be the result of idiosyncratic molecular mechanisms, as suggested by the heterogeneous brain findings of these conditions (Table 2).

Although AS aggregation is a common event preceding inclusion body formation in synucleinopathies, increasing evidence strongly suggests that different factors trigger and/or enhance this aggregation in different settings (Fig. 2). Accurate characterization of all PD, DLB, and MSA subgroups is necessary for the development of efficient preventive treatments. This would initially require comprehensive neuropathologic studies for accurate determination of protein content variations in LBs and GCIs in different settings. These studies should be carried out on brain areas that are differentially involved in the earlier and later stages of these conditions, as well as on uninvolved brain regions. In this regard, a very recent work analyzed the brain distribution of AS pathology independently of clinical phenotype. Interestingly, the authors identified cases showing a high AS pathology burden in both brainstem and cortical areas in the absence of clinical symptoms (92). The question then arises as to why some individuals become symptomatic and others do not while showing similar neuropathologic changes. The aforementioned observations provide further evidence in favor of the heterogeneity of factors conditioning the development of synucleinopathies (92). Delineating the differences in protein distribution and contents in inclusion bodies would help to elucidate the mechanisms of formation for each subgroup. If inclusion body protein distribution and contents can be correlated with the disease genotype or phenotype, the choice of therapeutic strategies in each patient could be guided by knowledge of the operating mechanism as deduced from the individual genotype or phenotype.

CONCLUSIONS

The basic feature of neurodegenerative disorders is the accumulation of misfolded proteins that subsequently undergo sequestration and aggregation as proteinaceous inclusions. It is thought that these inclusions protect the cell from the toxicity of soluble misfolded proteins, although conflicting evidence suggests that the presence of inclusion bodies may also lead to cell dysfunction and death. Therefore, it is of paramount importance to determine which proteins are misfolded and which are sequestered as they attempt to refold altered proteins.

Synucleinopathies share AS aggregation as the main pathogenic event preceding inclusion body formation, but different molecular mechanisms seem to be responsible for the development of LBs in PD and DLB and of GCIs in MSA. It thus seems mandatory to accurately delineate different subsets of these diseases and their molecular mechanisms. Appropriate diagnosis and treatment of individual patients will not be possible until the complete characterization of the genotypic and phenotypic alterations specifically associated with each synucleinopathy subgroup has been achieved. Only elimination of primary causes through either enhancement of AS solubility or inhibition of AS aggregation will bring about effective disease control. It should be emphasized, however, that the precise pathogenic factors underlying each synucleinopathy subtype must be fully unraveled before we are able to influence AS solubility or aggregation.

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