Reverse engineering Lewy bodies: how far have we come and how far can we go?

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Abstract | Lewy bodies (LBs) are α-synuclein (α-syn)-rich intracellular inclusions that are an important pathological hallmark of Parkinson disease and several other neurodegenerative diseases. Increasing evidence suggests that the aggregation of α-syn has a central role in LB formation and is one of the key processes that drive neurodegeneration and pathology progression in Parkinson disease. However, little is known about the mechanisms underlying the formation of LBs, their biochemical composition and ultrastructural properties, how they evolve and spread with disease progression, and their role in neurodegeneration. In this Review, we discuss current knowledge of α-syn pathology, including the biochemical, structural and morphological features of LBs observed in different brain regions. We also review the most used cellular and animal models of α-syn aggregation and pathology spreading in relation to the extent to which they reproduce key features of authentic LBs. Finally, we provide important insights into molecular and cellular determinants of LB formation and spreading, and highlight the critical need for more detailed and systematic characterization of α-syn pathology, at both the biochemical and structural levels. This would advance our understanding of Parkinson disease and other neurodegenerative diseases and allow the development of more-reliable disease models and novel effective therapeutic strategies.

Parkinson disease (PD) typically exhibits three major manifestations: clinical motor and non-motor symptoms; neurodegeneration in several brain regions, with a greater degree of dopaminergic neuronal loss in the substantia nigra pars compacta (SNc); and the formation of intracellular inclusions termed 'Lewy bodies' (LBs) in neuronal cell bodies and 'Lewy neurites' (LNs) in neuronal processes[1]. Although it has been more than 100 years since LBs and LNs were discovered as major pathological hallmarks of PD, their composition, the mechanisms that trigger their formation, propagation and clearance, and how they contribute to PD pathogenesis remain poorly understood. Two of the major reasons for this knowledge gap are the unavailability of cellular and animal models that develop LBs and LNs that exhibit biochemical and ultrastructural properties similar to corresponding inclusions in brain tissue from individuals with PD and the lack of in-depth characterization of aggregates in PD brain tissue and existing models of PD. This has hindered deciphering the sequence of molecular events leading to the formation of these pathological hallmarks and their contributions to the neuronal dysfunction and degeneration observed in PD.

Genomic mutations in or multiplications of *SNCA*, the gene that encodes α-synuclein (α-syn), have been directly linked to LB and LN formation and cause familial PD[2–4]. α-syn is one of the major proteinaceous constituents of LBs; it abnormally fibrillizes and forms filamentous structures that accumulate within LBs and LNs and underlies their ultrastructural organization[5]. Increasing evidence suggests that the process underlying the conversion of α-syn from its benign soluble form into fibrillized pathological inclusions entails a complex series of events that extend beyond a simple increase in protein levels. Multiple post-translational modifications (PTMs), including phosphorylation, ubiquitination, nitration and truncation, have been implicated in modulating α-syn misfolding and aggregation and the processes of inclusion formation and maturation[6–16]. Moreover, LBs and LNs are complex structures rich in lipids, cytoskeletal proteins, organelles and membranous fragments, and the dynamics of α-syn interactions with these structures probably has a key role in regulating the process of inclusion formation, the biochemical and organizational heterogeneity of the inclusions formed, and downstream consequences of neurodegeneration[17–20]. Indeed, α-syn pathology in

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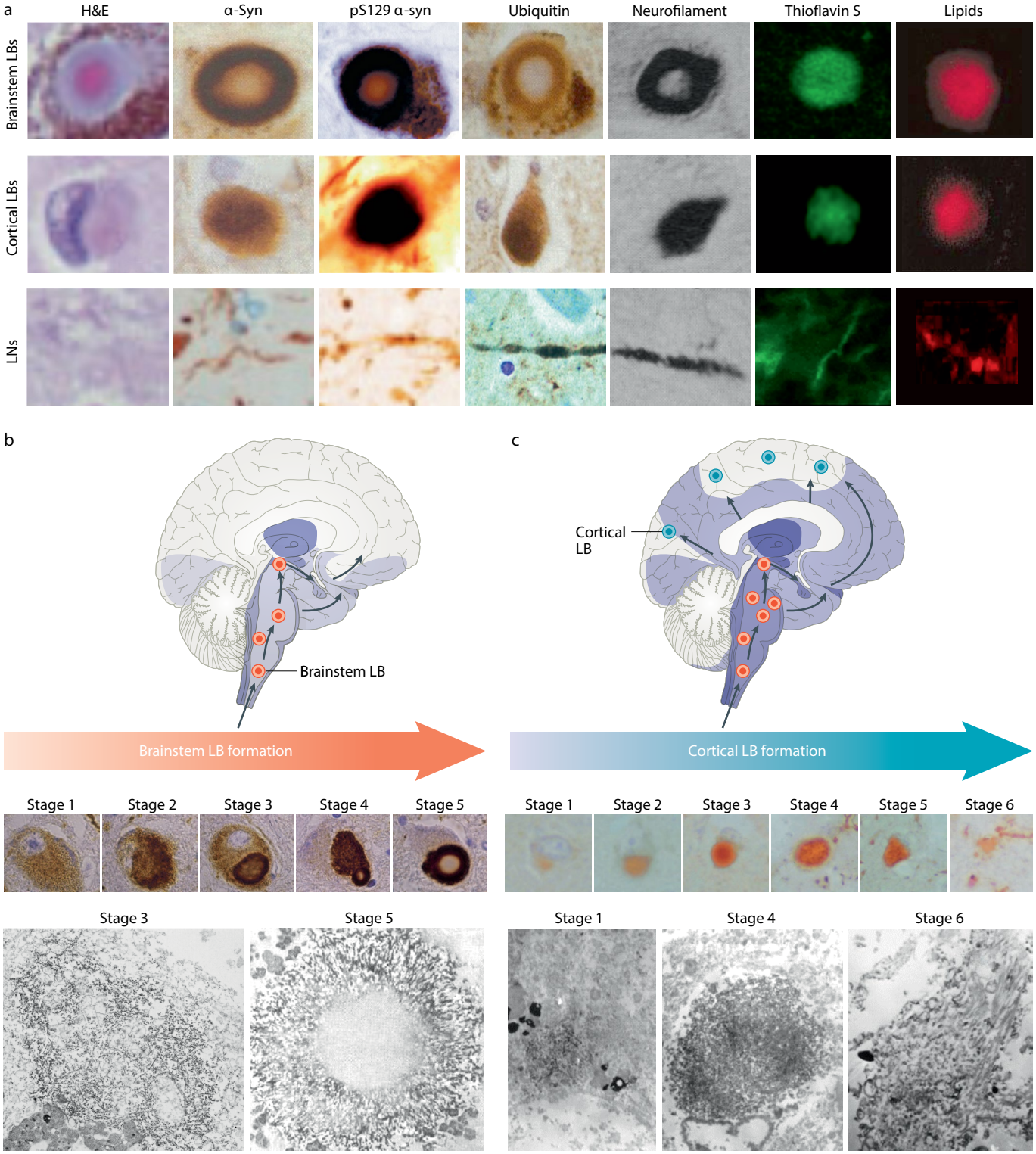


brain tissue from individuals with PD is highly polymorphic, with different α-syn conformers — such as oligomers, protofibrils, fibrils and mature LBs — being purified from such tissue[21–25]. Furthermore, LBs exhibit diverse morphological, structural and molecular compositions across different brain regions and different patient subpopulations[26–30]. Therefore, it remains unclear whether the heterogeneity of LBs and α-syn immunoreactive inclusions reflects distinct pathologies or a continuum of conformations reflecting different stages of LB formation, maturation and processing over time.

A systematic understanding of the molecular, structural and cellular determinants of LB formation is essential for delineating the mechanisms of pathology formation and spreading, and how these processes contribute to the pathogenesis of PD and other neurodegenerative diseases that are characterized by

◀ Fig. 1 | **Histological properties and proposed staging of cortical and brainstem Lewy bodies and Lewy neurites from patients with Parkinson disease. a** | Morphology of brainstem Lewy bodies (LBs), cortical LBs and Lewy neurites (LNs) from histological sections of brains from individuals with Parkinson disease stained with haematoxylin and eosin (H&E)[37], antibodies to α-synuclein (α-syn)[9,37], α-syn phosphorylated at S129 (pS129)[6,287], ubiquitin[9,288] and neurofilament[53–57], thioflavin S[45–47] and Nile red (for lipid staining)[19,81]. **b** | Brainstem LBs have been proposed to go through five stages before reaching maturity. The upper panel shows the anatomical distribution of brainstem LBs and proposed routes for their spreading, the middle panel shows α-syn-immunostained human neurons at different stages and the lower panels show transmission electron microscopy (TEM) analysis of accumulations at early and late stages. Stage 1 features abnormal cytoplasmic accumulations of α-syn, which become irregularly shaped, large structures in stage 2. Stage 3 is characterized by well-defined pale bodies that lack a halo and consist of a granular, vesicular and filamentous ultrastructure. In stage 4, pale bodies exhibit peripheral condensation into smaller LBs having a halo. Stage 5 features large mature LBs having a central core and a surrounding halo of α-syn-immunopositive fibrils. **c** | Six stages have been proposed to underlie cortical LB formation. The upper panel shows the anatomical distribution of brainstem and cortical LBs and proposed routes for LB spreading, the middle panel shows α-syn-immunostained human neurons at different stages and the lower panels show TEM analysis of accumulations at early, intermediate and late stages. In stage 1, the abnormal α-syn accumulations are loose, granular and intermingled with organelles. In stages 2 and 3, the structures are more well-defined and show intense staining for α-syn but they exhibit granular ultrastructures with few filaments. By stage 4, the structures have an irregular shape, consist of a dense granulofilamentous ultrastructure and are associated with curly neurites. In stages 5 and 6, the accumulations are ill-defined, exhibit a looser granulofilamentous structure and show weaker reactivity with anti-α-syn immunostaining (stage 6). Part **a** H&E panels adapted with permission from REF.[37], Wiley; α-syn panels for brainstem LBs and cortical LBs adapted with permission from REF.[9], American Society for Investigative Pathology; α-syn panel for LNs adapted with permission from REF.[37], Wiley; pS129 α-syn panels for brainstem LBs and cortical LBs adapted from REF.[6], Springer Nature Limited; pS129 α-syn panel for LNs adapted with permission from REF.[287], Elsevier; ubiquitin panels for brainstem LBs and cortical LBs adapted with permission from REF.[9], American Society for Investigative Pathology; ubiquitin panel for LNs adapted with permission from REF.[22], PNAS. Copyright (1998) National Academy of Sciences, USA; neurofilament panels adapted with permission from REF.[57], AAAS; thioflavin S brainstem LBs panel adapted with permission from REF.[45], CC BY 2.0 (https://creativecommons.org/licenses/by/2.0/); thioflavin S cortical LBs panel adapted from REF.[46], Springer Nature Limited; thioflavin S LNs panel adapted with permission from REF.[47], Oxford University Press; lipids panels for brainstem LBs and cortical LBs adapted with permission from REF.[81], Elsevier; lipids LNs panel adapted from REF.[19], Springer Nature Limited. Parts **b** and **c** brain schematics adapted with permission from REF.[289], Wiley. Part **b** middle row (stages 1–5) panels adapted with permission from REF.[207], Wiley; bottom row stage 3 panel adapted from REF.[43], Springer Nature Limited; bottom row stage 5 panel adapted with permission from REF.[9], American Society for Investigative Pathology. Part **c** middle (stages 1–6) and bottom (stages 1, 4 and 6) row panels adapted with permission from REF.[208], Elsevier.

the accumulation of α-syn aggregates and inclusions in the brain, hereafter referred to as 'synucleinopathies'. Furthermore, an in-depth understanding of the biochemical and structural bases underlying the pathological heterogeneity in PD, and across other synucleinopathies, is crucial for developing biochemical and imaging biomarkers for the detection and imaging of diverse forms of LBs at early stages, monitoring of disease progression and differential diagnosis of different synucleinopathies. In this Review, we first highlight the structural, morphological and molecular features of LBs observed in different brain regions that might reflect PD heterogeneity and subtypes. We then provide an α-syn-centric review of the most commonly used cellular and animal models of de novo LB formation and LB propagation, evaluate to what extent these models reproduce cardinal features of authentic LBs, and present an up-to-date mechanistic overview of molecular events underlying the de novo formation of α-syn inclusions and their subsequent propagation. Finally, we propose an integrative approach that would enable current knowledge gaps to be addressed, and pathological heterogeneity in synucleinopathies to be dissected at the molecular, biochemical and structural levels.

Inclusions
A term commonly used to describe the localized accumulation of proteins in cells or tissues as determined by their immunoreactivity to specific antibodies or detection of fluorescent puncta when the protein of interest is fused to fluorescent proteins. Not all inclusions represent the accumulation of aggregated proteins, unless verified by other biophysical or biochemical techniques.

Lewy bodies

Anatomical distribution

In 1912, Fritz Heinrich Lewy identified eosinophilic cellular inclusions in the dorsal motor nucleus of the vagus nerve, the basal nucleus of Meynert, the globus pallidus, the lateral nucleus of the thalamus and the periventricular nucleus of the thalamus in post-mortem brain tissue from individuals with PD[31]. Later, Konstantin Tretiakoff discovered similar aggregates in the SNc and named them 'Lewy bodies' (LBs) and 'Lewy neurites' (LNs) depending on the subcellular compartment in which they were observed[32]. Since then, LBs and LNs have been consistently observed within and beyond the basal ganglia, reaching anatomically higher brain regions such as the hippocampus and cortex. Indeed, it has been proposed that the appearance of LB pathology throughout different brain regions could follow a specific sequential pattern that correlates with disease progression. In 2003, Heiko Braak hypothesized, on the basis of post-mortem analyses of hundreds of brains from individuals with PD at different stages of the disease, that LB pathology could be classified into six stages, with pathology progressing via neural connections from the olfactory bulb to interconnected regions via the olfactory system, and from the dorsal motor nucleus of the vagal nerve through the brainstem to cortical regions[33]. In this proposed staging system, at early stages (stages 1 and 2), LBs and LNs are first detected in the olfactory bulb, as well as in lower brainstem regions, then appear in anatomically higher brain regions such as the SNc and amygdala at later stages (stages 3 and 4) and are finally observed in the neocortex (stages 5 and 6)[33]. On the basis of Braak's hypothesis[34], it has been proposed that PD and α-syn pathology in the brain could be originating from non-dopaminergic parts of the brainstem, from the olfactory system, or even from the peripheral nervous system (for example, the gut)[35]. This model is also in line with increasing evidence demonstrating the presence of α-syn pathology in peripheral tissues and organs that are associated with early non-motor symptoms of PD (including olfactory disturbances and gastrointestinal problems)[36].

Structure and composition

Initial identification and classification of LBs relied on conventional histological stains, such as haematoxylin and eosin, which typically revealed brainstem LBs as dense spherical structures with a hyaline eosinophilic core and a small pale halo[37–41] (FIG. 1a). By contrast, cortical LBs and LNs were difficult to detect by haematoxylin and eosin staining and lacked a central halo, suggesting different ultrastructural properties. Indeed, transmission electron microscopy (TEM) has consistently revealed that although both brainstem and cortical LBs exhibit a similar underlying ultrastructure, comprising filamentous fibrillar proteins mixed with lipids, the arrangement

Box 1 | α-Synuclein aggregation and fibril polymorphism

The current models of α-synuclein (α-syn) fibrillization suggest that the process of α-syn aggregation occurs via nucleation-dependent polymerization mechanisms, showing a characteristic sigmoidal kinetic profile with three different phases[295]. During the initial, slow nucleation phase, α-syn monomers adopt a partially folded conformation and/or form ordered oligomers composed of many α-syn units[271,290]. During the elongation phase, ordered oligomers induce the misfolding of other monomers, which are then recruited to form larger oligomers that eventually grow into fibrils[296]. The now 'mature' fibrils are typically insoluble, non-branched, β-sheet-rich, twisted structures with an average diameter of ~10 nm (REFS[297,298]). In the last, steady-state phase, an equilibrium is reached between the rates of incorporation of monomers into fibrils and their dissociation from fibrils[299]. Thus, the fibrils no longer grow, and aggregation reaches a plateau. Therefore, the formation of the initial 'seed' in this model is the slow rate-limiting step, after which monomer addition into the seeding-competent oligomers and fibrils is a fast process.

Another recently emerging notion regarding α-syn aggregation is that it can lead to the generation of different strains of fibrils. In other words, the same aggregating α-syn polypeptide can generate multiple fibrillar conformers that exhibit differences in morphology, structure, stability, seeding efficiency and even toxicity in neurons[234,300–302]. This idea suggests that the heterogeneous clinical manifestations in individuals with PD could be attributed to the formation of distinct strains of pathological α-syn fibrils[268]. The structural basis underlying fibril polymorphism was only recently elucidated, owing to advancements in cryogenic electron microscopy techniques, which enabled the determination of the atomic structure of fibrils generated in vitro of different α-syn variants[48,241,303–306] and of fibrils isolated from human brain tissues[307]. These studies revealed that α-syn fibrils are made of two intertwining protofilaments and that the differences in the packing or assembly of these two protofilaments give rise to different morphologies or conformations of fibrils, including straight fibrils[304], unbranched fibrils[241], and rod and twister forms of fibrils[48]. Notably, the structures of fibrils derived from the brains of individuals with multiple system atrophy are different from those generated in vitro; they exhibit different protofilament interfaces and extra densities, suggesting the presence of post-translation modifications or other non-proteinaceous components[307]. Indeed, mass spectrometry studies on the same sample revealed the presence of multiple post-translation modifications, including acetylation, ubiquitination and phosphorylation at multiple residues. These differences are clearly reflected in the distinct seeding activity of multiple system atrophy-derived α-syn aggregates compared with recombinant preformed fibrils or PD-derived aggregates[308,309].

Lewy bodies
(LBs). One of the key pathological hallmarks of Parkinson disease and other synucleinopathies. They comprise intracellular globular cytoplasmic inclusions that contain complex mixtures of proteins, lipids and membranous organelles, and are enriched in aggregated forms of α-synuclein, predominantly insoluble fibrillar species.

Lewy neurites
(LNs). A pathological hallmark of Parkinson disease. These are dystrophic processes containing protein accumulations that share many of the biochemical and immunohistological properties of Lewy bodies, including the presence of phosphorylated filamentous α-synuclein aggregates, although their ultrastructural features and biochemical composition remain less well characterized.

of fibrils is different between both LB subtypes (FIG. 1b,c). Brainstem LBs exhibit a dense core with a halo of irradiating filaments, whereas cortical LBs show a more diffuse distribution of disorganized filamentous fibrils[9,42–44]. The high levels of insoluble β-sheet-rich fibrillar constituents in both cortical and brainstem LBs and LNs suggested that these filaments exhibit amyloid-like properties, including the cross-β structure seen in amyloid plaques, neurofibrillary tangles and other amyloid protein-containing deposits. Subsequent studies showed that amyloid-specific dyes that recognize this unique cross-β structure, such as thioflavin S (ThS), consistently exhibit strong reactivity with both brainstem and cortical LBs[45–47] (FIG. 1a). Recent cryogenic electron microscopy (EM) studies[48] and analysis of LBs using microbeam X-ray diffraction[49] have confirmed that α-syn aggregates in LBs isolated from the brain tissue of people with multiple system atrophy are indeed rich in cross-β structures. Moreover, as observed for inclusions found in other neurodegenerative diseases, such as Alzheimer disease and Pick disease[50–52], both brainstem and cortical LBs are strongly reactive to antibodies against ubiquitin and neurofilaments[53–57] (FIG. 1a).

Between 1965 and 1997, a consensus emerged that LBs and LNs are composed of filamentous structures and that the different arrangements and packing of these structures underlie different types of Lewy pathologies. However, the nature of the protein building blocks forming these filamentous structures remained unknown. The main suspects were initially neurofilaments and other cytoskeletal proteins[26–28,58–62]. However, subsequent studies showed that neurofilaments are found mainly in the periphery of LBs and that their diameter and solubility properties are different from those of filamentous aggregates found in the core of LBs[21,63].

In 1997, a major breakthrough in the PD research field was achieved when α-syn was discovered as the main constituent of LBs and LNs[5]. This seminal finding followed a genome-wide linkage analysis conducted in an Italian pedigree, which identified *SNCA* as the first gene implicated in familial PD[40]. α-Syn, a 140 amino acid presynaptic nerve terminal protein, was originally identified in Alzheimer disease amyloid plaques as the precursor of the non-amyloid component[64]. Later, α-syn was shown to abnormally aggregate in vitro and form fibrils that are similar to those underlying the structure of LBs[5] (BOX 1). Biochemical studies have consistently shown that α-syn within LBs is mostly detergent-insoluble[9,65,66] and post-translationally modified, with phosphorylation at serine 129 (S129)[6], ubiquitination and amino-terminal (N-terminal) and carboxy-terminal (C-terminal) truncations being the predominant modifications[7,8]. The ubiquitin-binding protein p62 has also emerged as one of the most consistently detected proteins within α-syn inclusions in PD brain tissue[67]. Therefore, antibodies to total α-syn, α-syn phosphorylated at S129 (pS129 α-syn), ubiquitin and p62 were established as the most consistent probes for detecting α-syn aggregates and inclusions, including LBs and glial cytoplasmic inclusions[68] in post-mortem brain tissue from individuals with PD or multiple system atrophy, respectively[5,9,30,43,69,70], with pS129 α-syn staining being the most widely used and robust marker of α-syn pathology (FIG. 1a).

To date, more than 100 additional proteins have been detected within LBs by immunohistochemical analyses. Initial studies identified proteins that were found in pathological inclusions associated with other diseases, as well as cytoskeletal proteins (for example, neurofilaments, tubulin and tau paired helical filaments[26–28,58–62,71]). Subsequent antibody-based profiling studies revealed proteins involved in various cellular processes, including proteasomal and lysosomal degradation, phosphorylation, mitochondrial function and cell cycle regulation (Supplementary Table 1). As this approach has limited throughput for investigating biological pathways involved in or impacted by LB formation and maturation during disease pathogenesis, a few studies opted to perform mass spectrometry analyses to obtain complete, unbiased proteomic profiles of LBs from brains of people with PD. For instance, proteomic analyses of isolated cortical LBs identified a total of 296 proteins involved in various cellular processes, such as the ubiquitin–proteasome system, folding and intracellular trafficking, oxidative stress, synaptic transmission and vesicular transport, and signal transduction and apoptosis[72]. Other studies performed proteomic analysis of LB-containing brain tissue from areas such as the SNc[73–76], locus coeruleus[77] and frontal cortex[78]

and identified proteins that are potentially enriched in PD brains.

In addition to proteins, lipids are present in LBs; this finding was described in the literature as early as 1969 (REF.[79]). Membranous fragments, organelles, vesicular structures and various subtypes of lipid constituents later emerged as key components of LBs, and their presence was established by a variety of techniques, including immunohistochemistry (FIG. 1a) and Fourier transform infrared spectroscopy[27,63,79–82]. However, their role in LB formation or LB-mediated neurodegeneration has been, for the most part, overlooked. Two recent studies have put the spotlight again on lipids and membranous organelles in LBs. Through the use of correlative light and electron microscopy to unravel the ultrastructure of LBs in freshly autopsied (non-fixed) post-mortem brains from individuals with PD[19,83], Shahmoradian et al. obtained tantalizing images of LB structures that are enriched in lipids and membranous structures, including mitochondria. They also suggested that LBs contain mainly non-fibrillar forms of α-syn, claims that we have recently challenged[84]. Using a similar approach, we recently showed that formation of LB-like inclusions — similar to those observed by Shahmoradian et al. — requires the formation of α-syn fibrils, which undergo significant remodelling and PTMs, and interact with membranous structures as they convert into LB-like inclusions comprising proteins, fragmented membranes, vesicular structures and dysmorphic organelles (mitochondria, lysosomes and autophagosomes)[20].

De novo LB formation

As ageing is the most prominent risk factor for PD, it is likely that the process of LB formation requires several months, if not years, to occur. Indeed, it has been suggested that the average lifespan (survival time) of a given LB from its initial formation to neuronal death is 6.2 months[85]. Given the difficulty to model such a complex phenomenon in cell culture models and even in animals, much of our current understanding of the spatio-temporal events underlying LB formation stems either from snapshots of the morphology or ultrastructure of inclusions in histological brain sections from deceased individuals with PD, or from reductionist in vitro approaches investigating the aggregation behaviour of purified α-syn. By combining information obtained from both approaches, as well as our current understanding of factors influencing α-syn aggregation and other pathways implicated in PD from cellular and animal models, a hypothetical model on how LBs form de novo can be extrapolated. In the following sections, we describe and critically evaluate current cellular and animal models of LB formation in relation to the extent to which these models reproduce α-syn fibrillization and LB formation, and then discuss the staging of LB formation based on histological analyses of the brains of patients with PD. Last, we present a speculative, but testable, model of molecular events underlying the formation of α-syn inclusions that integrates current knowledge obtained from clinical studies, cellular and animal models, and in vitro approaches.

Models of α-syn aggregation

Although studies on post-mortem brain tissue have helped to establish a strong association between LBs and PD and have led to disease staging systems based on the analysis of Lewy pathologies, the role of LBs and their formation in the pathogenesis of PD remains a subject of intense debate and active investigation. To address this knowledge gap and pave the way for elucidating the relationship between LB formation and neurodegeneration and disease progression, it is crucial to recapitulate these processes in different cellular and animal systems. It has been consistently argued that for this approach to be successful, the models generated should develop aggregates that reproduce the main features of authentic LBs, such as decreased solubility, α-syn ubiquitination[7,8] and hyperphosphorylation at S129 (REFS[6,7]), reactivity to amyloid-binding dyes[45–47], fibrillar ultrastructural organization[9,42–44] and the presence of lipids and fragments of organelles[5,27,63,79–82]. In this respect, two major strategies have been undertaken to model de novo LB formation in various cellular and animal models: first, increasing levels of α-syn (alone or in combination with other proteins) and, second, treatment with compounds and toxins leading to proteasomal impairment or oxidative/metabolic stress. In the following sections, we evaluate current cellular and animal models generated from these strategies in relation to the extent to which authentic features of α-syn fibrillization and LB formation are reproduced at the biochemical and ultrastructural levels.

Cellular models of α-syn overexpression. The identification of α-syn as one of the major constituents of LBs, combined with strong genetic evidence linking duplications, triplications and point mutations of *SNCA* to PD[40,86–92], suggested that increasing levels of wild-type (WT) or disease-associated mutant forms of α-syn could be used to model LB formation and different aspects of PD in cells and different model organisms. Unfortunately, this strategy has shown limited success in recapitulating the formation of fibrillar LB-like structures in cellular models (Supplementary Table 2). Most studies reported diffused localization of ectopically expressed α-syn without the formation of inclusions[47,93–95]. In a few reports, some inclusions were detected, but these did not exhibit the biochemical and ultrastructural features of LBs found in PD brains. One of the major limitations of these studies is the loose definition of an aggregate or LB-like inclusion. Frequently, immunofluorescence is used as the primary method of monitoring or characterizing aggregation without biochemical and ultrastructural characterization and validation of the models. For instance, although cytoplasmic aggregates were observed in HEK293 cells[96] or SH-SY5Y cells[97,98] upon α-syn overexpression, either EM analysis was not performed to investigate their ultrastructural properties or it showed that the inclusions comprised amorphous aggregates that do not resemble cortical or brainstem LB-like structures[96–98] (Supplementary Fig. 1a).

To investigate the dynamics and mechanisms of α-syn aggregation and inclusion formation in cells, several cellular models based on the overexpression of α-syn fused to fluorescent reporter proteins were developed[94,99–102].

Propagation
A term that refers to the process underlying the spreading of pathological aggregates in the brain through the transfer of seeding-competent protein aggregates from one cell to another.

Oligomers
Soluble peptide or protein assemblies (dimers or multimers) that exhibit different sizes, secondary structures and shapes (for example, spherical, pore-like and curvilinear structures).

Fibrils
Insoluble filamentous structures (8–12 nm in diameter) that form as a result of the misfolding and self-assembly of a peptide or a protein (for example, α-synuclein) via formation of repeating β-sheet structures, also known as cross-β structure, which is responsible for their unique binding to specific dyes (for example, thioflavin T, thioflavin S, Amytracker and Congo red), and allows them to be distinguished from other β-sheet-rich proteins.

Amorphous aggregates
Aberrant protein aggregates that do not exhibit a specific ordered organization or morphology, that is, they are non-fibrillar.

For instance, α-syn fused to enhanced green fluorescent protein was reported to form inclusions when expressed in yeast[99–102] and even in primary neurons[94]. Nevertheless, the number of neurons with aggregates was very low (~5%)[94], and detailed EM studies later revealed that inclusions detected in yeast were composed of vesicular structures that contained non-fibrillar α-syn[99,101]. To circumvent the possible influence of large fusion proteins on α-syn aggregation, several groups generated cellular models by fusing α-syn to smaller peptide-based tags or fluorescent probes (for example, a 23 amino acid V5/6×His tag[94] or a 12 amino acid tetracysteine tag[103]). Overexpression of these constructs also led to α-syn accumulation in primary neurons, HeLa cells and SH-SY5Y cells[94,103]. However, in these studies, α-syn aggregation was monitored mostly by the accumulation of fluorescence signals rather than by direct biochemical or structural characterization of aggregates and inclusions. Therefore, whether the observed inclusions comprised fibrillar α-syn and the extent to which they truly recapitulated cortical or brainstem LBs remains unclear.

One alternative strategy that has been used to induce α-syn aggregation and LB formation relies on the co-expression of α-syn with one of its interacting partners, synphilin 1, which co-accumulates with α-syn in LBs[104]. Strikingly, co-expression of both proteins enhanced α-syn aggregation and produced inclusions exhibiting several LB features, including being ubiquitin positive, ThS positive and hyperphosphorylated at S129 (REFS[94,105–111]). Importantly, filamentous and granular aggregates were detected within these inclusions by TEM[109] (Supplementary Fig. 1b), some of which colocalized with lipids or endomembranes[110,112]. Interestingly, the filaments within the inclusions were not prominent and did not exhibit an ordered arrangement with a dense core as observed in mature brainstem LBs. Whether these features reflect early stages of cortical or brainstem LB formation or different types of α-syn inclusions is unknown.

In line with previous findings suggesting that murine α-syn homologues could attenuate the aggregation of human α-syn, we recently showed that human α-syn overexpression in mouse *Snca*$^{-/-}$ primary neurons enhances inclusion formation. The inclusions formed exhibit several features of authentic LBs, including reduced solubility, presence of filamentous structures, reactivity with ThS and immunoreactivity for antibodies to pS129 α-syn and ubiquitin[113] (Supplementary Fig. 1c,d). One of the limitations of this model, however, was that the ultrastructural organization of filaments within inclusions did not resemble the structural and biochemical complexity of LBs found in PD brains[9,30,58].

Together, these observations show that many of the protein and PTM markers that are commonly used to assess the LB-like nature of α-syn aggregates in cellular and animal models are not sufficient to differentiate α-syn aggregates from bona fide LBs[20,113]. Moreover, these results suggest that LB formation and maturation may require conditions that occur only in human brains or neurons. Indeed, it is interesting that despite harbouring one of the disease-associated *SNCA* mutations (A53T) and forming fibrils in vitro and in seeding models, the endogenous α-syn mouse analogue does not aggregate or form de novo LBs in cells. Understanding what makes this protein, which differs from human α-syn by only seven amino acids, resistant to aggregation could provide novel insights into mechanisms that could be exploited to prevent human α-syn aggregation and LB formation.

iPS cell-derived models of PD. Missense and multiplication mutations in *SNCA* can lead to autosomal dominant forms of PD[86–90,92,114–118]. Thus, many studies have sought to generate induced pluripotent stem cell (iPS cell) lines from individuals with PD harbouring different *SNCA* mutations and differentiating them into disease-specific vulnerable cell types[119–130]. In a few studies using iPS cell lines generated from individuals with the A53T *SNCA* mutation, neurons showed several pathological features that were not observed in mutation-corrected control neurons, including nitrosative and mitochondrial stress, the accumulation of endoplasmic reticulum-associated degradation substrates, endoplasmic reticulum stress[120], α-syn aggregation into ThS-positive and pS129 α-syn-positive aggregates, axonal neuropathy and transcriptional alterations in genes involved in synaptic signalling[123]. Likewise, studies on iPS cell lines generated from patients harbouring *SNCA* triplication reported increased α-syn expression and phosphorylation of α-syn at S129, and the formation of punctate 'oligomeric' structures in cell bodies[130], which were coupled with multiple features of neuronal dysfunction[125–129]. Nevertheless, as ultrastructural characterization of the aggregation state of α-syn in all these models was not performed, beyond the use of a proximity ligation assay (Supplementary Table 2), it remains unknown what aspects of α-syn aggregation or LB formation were recapitulated in these iPS cell models of PD.

Animal models of α-syn overexpression. A wide range of models have been used to investigate the effects of α-syn overexpression in vivo, ranging from invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* to rodents and non-human primates (Supplementary Table 3). Early attempts to generate α-syn-overexpressing transgenic *Drosophila* showed promise in reproducing key aspects of molecular PD pathology and neuronal loss, as LB-like inclusions with a core and peripherally radiating filaments were detected in these models (Supplementary Fig. 2a), and these were accompanied by loss of dopaminergic neurons and locomotor dysfunction[131–133]. These inclusions were later shown to be positive for pS129 α-syn[134,135], to exhibit proteinase K resistance[135] and to contain insoluble α-syn species[135,136]. A recent *Drosophila* model with even higher levels of α-syn expression than previous models has been described[137], but it remains unknown whether this increased expression influences the formation and maturation of aggregates, as the characterization of this model was restricted to an assessment of ubiquitin immunoreactivity[137] (Supplementary Table 3). In *C. elegans*, α-syn overexpression has been shown to provoke dopaminergic neuronal loss and motor deficits[138–140]. Importantly, although punctate staining (which reflects clusters of cells expressing

α-syn) has been described during assessment of α-syn expression in these models, the intracellular distribution of α-syn is typically diffuse, without the formation of LB-like inclusions, as established by ultrastructural evaluation (Supplementary Fig. 2b).

To test whether rodents could be used to model α-syn pathology, α-syn has been overexpressed using different transgenic and viral approaches, and by implementing different promoters. Unfortunately, transgenic α-syn expression in rodents has not been successful in generating robust LB-like pathology within SNc neurons[141,142]. Although the first transgenic mice overexpressing WT human α-syn under control of the human platelet-derived growth factor-β promoter displayed α-syn-positive intraneuronal inclusions in multiple brain regions, they did not exhibit fibrillar ultrastructures, and no inclusions or loss of neurons was detected in the SNc[70] (Supplementary Fig. 2c). Similar results were obtained when the *Thy1* cassette was used to drive WT or mutant α-syn expression[66,143–148]: some of the inclusions detected were ubiquitin positive and exhibited disorganized granular ultrastructure, but they were mostly confined to the spinal cord[145] (Supplementary Fig. 2d). Notably, expression of mutant E46K or A53T α-syn under the control of the prion promoter led to the formation of inclusions that were proteinase-K resistant[148], insoluble and fibrillar in nature (as shown by TEM)[149,150] (Supplementary Fig. 2e), and often contained mitochondria. Moreover, these inclusions did not exhibit classic LB organization with a dense core and radiating filaments, suggesting that these may reflect a different type of LB pathology.

To express exogenous α-syn at levels closer to its physiological state, several groups generated bacterial artificial chromosome (BAC) transgenic mice and rats carrying the complete human *SNCA* locus (WT or with a mutation)[151–153]. With the use of this approach, more accurate spatio-temporal protein expression should be achieved compared with the use of traditional cDNA constructs and constitutive promoters. Unfortunately, none of the BAC α-syn transgenic models developed fibrillar inclusions that are positive for common LB markers such as pS129 α-syn, ubiquitin or p62 in SNc neurons. Whereas BAC α-syn transgenic rats manifest an age-dependent accumulation of insoluble α-syn that is coupled with some nigrostriatal alterations[151,152] (Supplementary Fig. 2f), BAC transgenic mouse counterparts showed functional abnormalities and α-syn aggregates only in enteric neurons[153].

The lack of nigral pathology in the aforementioned models could be, among other factors, due to weaker expression of α-syn in the SNc than in other regions. Therefore, the tyrosine hydroxylase (TH) dopaminergic neuron-specific promoter was used to target WT or mutant α-syn expression to the SNc[154–159]. Strikingly, although some of the generated transgenic mice exhibit reduced dopamine levels and some motor deficits, no discrete LB-like inclusions were detected in any of the generated mice. Expression of truncated α-syn (comprising amino acids 1–120) under the TH promoter in the absence of endogenous mouse α-syn showed promise, as these mice developed key symptoms of PD, including SNc dopaminergic neuronal death, motor deficits and the formation of partially proteinase K-resistant α-syn inclusions[160]. Unfortunately, no detailed biochemical and structural characterization of these aggregates was reported. Altogether, these studies suggest that α-syn expression levels in the SNc may still not have been high enough, or that certain compensatory mechanisms could be occuring throughout the development of transgenic mice, thereby interfering with the fibrilization potential of exogenously expressed α-syn.

To address these challenges and limitations, an alternative approach for overexpressing α-syn was applied that uses stereotactic injection of lentivirus and adeno-associated virus (AAV) vectors encoding α-syn into the SNc. This approach allows targeted expression of high levels of α-syn in the SNc of adult animals, thereby bypassing issues of developmental compensation. Although this approach successfully provoked reproducible nigrostriatal degeneration in rats, no authentic LB-like aggregates were detected in SNc neurons following α-syn overexpression[161–166]. Indeed, whereas some inclusions exhibited hyperphosphorylation of α-syn S129 and were shown to be proteinase-K resistant[166,167], they were mostly ubiquitin-negative, exhibited a granular ultrastructure with sparse filaments and were often associated with specific organelles[166] (Supplementary Fig. 2g,h). Notably, this effect was not due to a peculiar property of the rats, as similar results were later obtained in mice and in non-human primates into which lentiviruses or AAVs encoding WT α-syn or a mutant α-syn were injected[168–174].

Cellular and animal models based on treatment with toxins. Given that individuals with sporadic PD consistently exhibit reduced levels of 20S and 26S proteasomes, concomitant with reduced proteasomal activity in the striatum and SNc compared with controls[175], several groups investigated whether proteasomal inhibition would prompt the formation of LB-like inclusions (Supplementary Table 2). Although dopaminergic PC12 cells and primary neurons treated with proteasomal inhibitors developed numerous ubiquitinated inclusions, some of which were α-syn positive and ThS positive[176–179], it remained unclear whether aggregated α-syn or LB-like structures were their main underlying component[179]. Indeed, in cellular models overexpressing α-syn and co-treated with these inhibitors, aggregates either comprised α-syn but without ubiquitination[94,97] or were ubiquitin-positive but did not contain α-syn[180]. Further complicating the utility of these models was the pronounced toxicity induced by proteasomal inhibition itself[176–179] and the lack of EM information on inclusion ultrastructure. Notably, expression of a proteasomal subunit that disrupts 26S proteasomal degradation in conditional knockout mice resulted in neurodegeneration and formation of LB-like inclusions that are positive for ubiquitin, α-syn, ThS and p62 (REF.[181]). Most importantly, ultrastructural studies revealed immunogold-labelled α-syn filaments and granular material in central regions of inclusions, and mitochondria or membranous vesicles in the periphery (Supplementary Fig. 3a). These structures could be reminiscent of pale bodies (see later), as they do not exhibit features of mature brainstem LBs such as a central core with radiating filaments.

Pale bodies
Usually referred to as precursors to Lewy bodies, pale bodies are proteinaceous accumulations enriched with aggregated α-synuclein but do not exhibit many of the morphological, organizational and biochemical properties of mature Lewy bodies.

As mitochondrial dysfunction is also a prominent pathway implicated in PD[180,182–185], and as it has been reported that LB pathology is more prevalent in older individuals (around 60 years old) with mitochondrial disease than in controls[186], several studies focused on treating neuroblastoma cell lines with pharmacological agents causing oxidative or nitrative stress, which induced the accumulation of endogenous or overexpressed α-syn into insoluble inclusions[180,182–185]. Notably, although these aggregates reproduce some LB features, such as ThS fluorescence and ubiquitin immunoreactivity, the inclusions detected in most of these models exhibit amorphous ultrastructure with some disorganized fibrils by TEM[180,183], and thereby may reflect early stages of LB formation. Moreover, investigation of toxic mechanisms proved to be challenging, given the toxicity induced by the treatment with some of the oxidative agents themselves.

Since the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in humans by Langston and Irwin more than 30 years ago[187], there have been tens of attempts to model PD in animals by treatment with metabolic neurotoxins (Supplementary Table 3). Although some of these models exhibit α-syn inclusions, authentic LB-like structures have not been consistently reproduced. This is not too surprising since the humans that were exposed to the drug themselves did not develop classic LB pathology 20 years following their initial encounter[188]. In mice, although both short-term and long-term MPTP administration solicit neuronal degeneration[189], only some models with long-term treatment develop neuronal inclusions[190,191], mostly when cotreated with probenocid to block MPTP clearance[192,193]. However, this effect has not been consistently replicated[194,195], and the inclusions formed comprise concentric membranes with disorganized α-syn fibrils[190] (Supplementary Fig. 3b), or granular and filamentous accumulations of proteins and lipofuscin granules[192,193] (Supplementary Fig. 3c). In non-human primates, long-term MPTP treatment similarly provokes progressive dopaminergic neurodegeneration, coupled with the formation of inclusions[196–198]. These brainstem aggregates, however, have not been consistently detected[199], and their ultrastructure differs markedly from that of mature brainstem LBs in terms of the lack of a central core and the presence of filaments with a much larger diameter (20–23 nm)[190,192,196,198] (Supplementary Fig. 3d).

Rotenone is another neurotoxin that has also been used to induce PD-like phenotypes in animals. The use of this pesticide, which disrupts complex-I function in mitochondria, gained increased attention with reports associating PD with agricultural work[200,201]. Notably, peripheral treatment of rats with rotenone provokes dopaminergic degeneration and prominent formation of LB-like inclusions that replicate many features of authentic LBs[202,203]. These inclusions are ubiquitin positive and granular or filamentous, and display a dense core with a peripheral fibrillar halo[202] (Supplementary Fig. 3e). Although much smaller than typical LBs, these rotenone-induced aggregates bear the greatest resemblance to mature brainstem LBs compared with inclusions observed in any other animal model. Nevertheless, the drawback of rotenone treatment is non-specific toxicity, high mortality and variability in neurodegeneration[189]. It is worth noting that many other neurotoxins — including 6-hydroxydopamine, bacterial endotoxin lipopolysaccharide, paraquat, maneb, and methamphetamine — and blockage of protein degradation, with PSI, epoxomicin and lactacystin[189,204,205], have been used in attempts to produce reliable PD models[206]. Unfortunately, none of these models manifested the formation of well-characterized fibrillar LB-like α-syn inclusions.

Morphological staging of LB formation

Given the lack of success in modeling the lengthy and complex process of LB formation in different cellular and animal models, several groups have tried to infer mechanisms of LB formation by studying the heterogeneity of structures observed in brain tissue from individuals with PD. To this end, chronological staging of LB pathology has been proposed by compiling data from histological analyses of such tissue taken from individuals who died at different stages of the disease. Given the previously discussed difference in the structure of cortical and brainstem LBs, these two LB subtypes have been staged separately. Five stages of LB maturation based on morphological and ultrastructural changes have been proposed for brainstem LBs[207] (FIG. 1b). In the first stage, neurons manifest granular α-syn accumulations in the cytoplasm that later form large, irregularly shaped structures (stage 2). These structures then concentrate to form pale bodies, which typically lack a halo and comprise granular, vesicular and filamentous ultrastructures (stage 3). Some pale bodies subsequently show signs of peripheral condensation, and then one or more small LBs (with halos) emerge out of these condensations (stage 4). Finally, these 'early' LBs mature into larger LBs exhibiting the typical ring-like staining with a central core that is strongly ubiquitin-positive and surrounded by an α-syn-immunopositive halo of irradiating fibrils (stage 5).

Cortical LB staging is quite different as these inclusions pass through six stages before reaching maturity[208] (FIG. 1c). At the first stage, similar to brainstem LBs, abnormal somatic α-syn accumulations that are loose, granular and intermingled with organelles are observed. These become more defined and intensely stained for α-syn with stage progression and form granular structures with few filaments (stages 2 and 3). Granular and curly neurites are then found associated with these structures that later become irregular in shape and consist of a dense granulofilamentous ultrastructure (stage 4). At later stages, these accumulations become ill-defined, exhibit a looser granulofilamentous structure (stage 5) and ultimately show weak reactivity with anti-α-syn (stage 6). This latter observation is attributed to the probable involvement of astroglial processes that degrade LBs into extracellular components[208].

It is important to note that in both cases of brainstem and cortical LBs, α-syn inclusions detected at each of the stages show variable morphologies, heterogenous composition and differential antibody staining patterns. For instance, whereas heterogenous staining patterns have been reported for ubiquitin in cortical LBs, ranging from uniform staining to enriched staining of the core

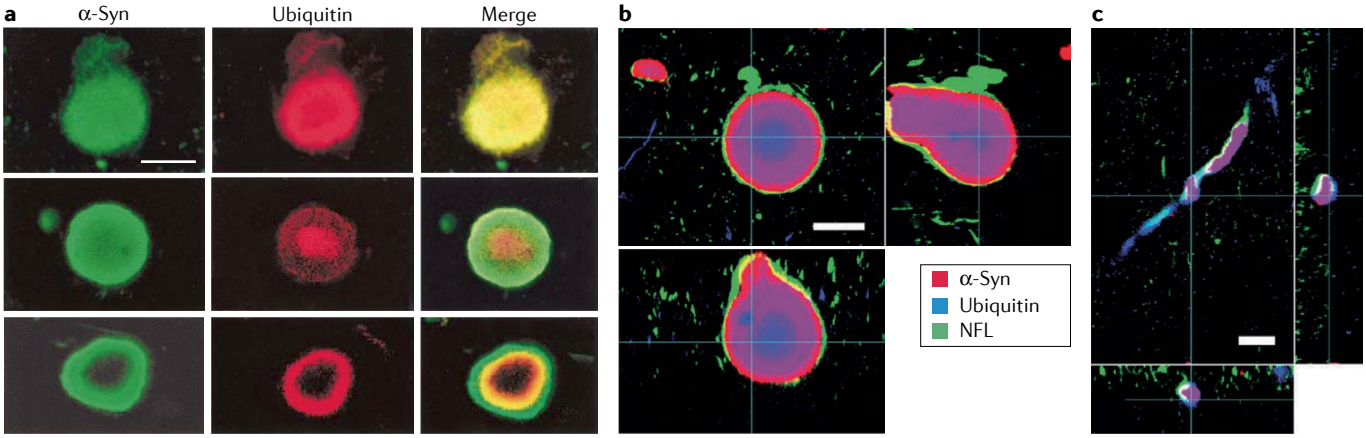


Fig. 2 | **Diversity of immunostaining patterns of cortical and brainstem Lewy bodies from patients with PD. a** | Different cortical Lewy bodies (LBs) co-stained with antibodies to α-synuclein (α-syn) and ubiquitin exhibit heterogenous staining patterns ranging from uniform staining (upper panels) to enriched staining of the core (middle panels) and preferential staining of the halo (lower panels)[81]. **b** | Orthogonal projection of a three-dimensionally reconstructed brainstem LB co-stained with α-syn (red), ubiquitin (blue) and neurofilament light chain (NFL; green) shows concentric layered architecture with ubiquitin detected in the core, surrounded by consecutive layers of α-syn and NFL[209]. **c** | Similarly, co-staining with α-syn (red), ubiquitin (blue) and NFL (green) shows that the three-layered concentric architecture is also present in Lewy neurites[209]. Scale bars denote 12 μm in part **a** and 20 μm in parts **b** and **c**. Part **a** adapted with permission from REF.[81], Elsevier. Parts **b** and **c** adapted with permission from REF.[209], Wiley.

and preferential staining of the halo[81] (FIG. 2a), a concentric layered architecture has been described for brainstem LBs and LNs, with ubiquitin detected in the core and surrounded by consecutive layers of α-syn and neurofilament light chain[209] (FIG. 2b,c). For α-syn, it appears that the detection pattern could also depend on the epitope targeted, as concentric staining patterns have been described for antibodies to different α-syn epitopes within the same brainstem LB, with antibodies targeting the N-terminus detecting the core and C-terminal antibodies staining consecutive outer layers[13]. Altogether, these findings highlight that the heterogeneity in LB morphology and staining patterns observed could reflect differences in the stages of pathology progression, the composition of α-syn pathology, PTMs or strains of seeds initiating pathology or the antibodies used to detect pathology.

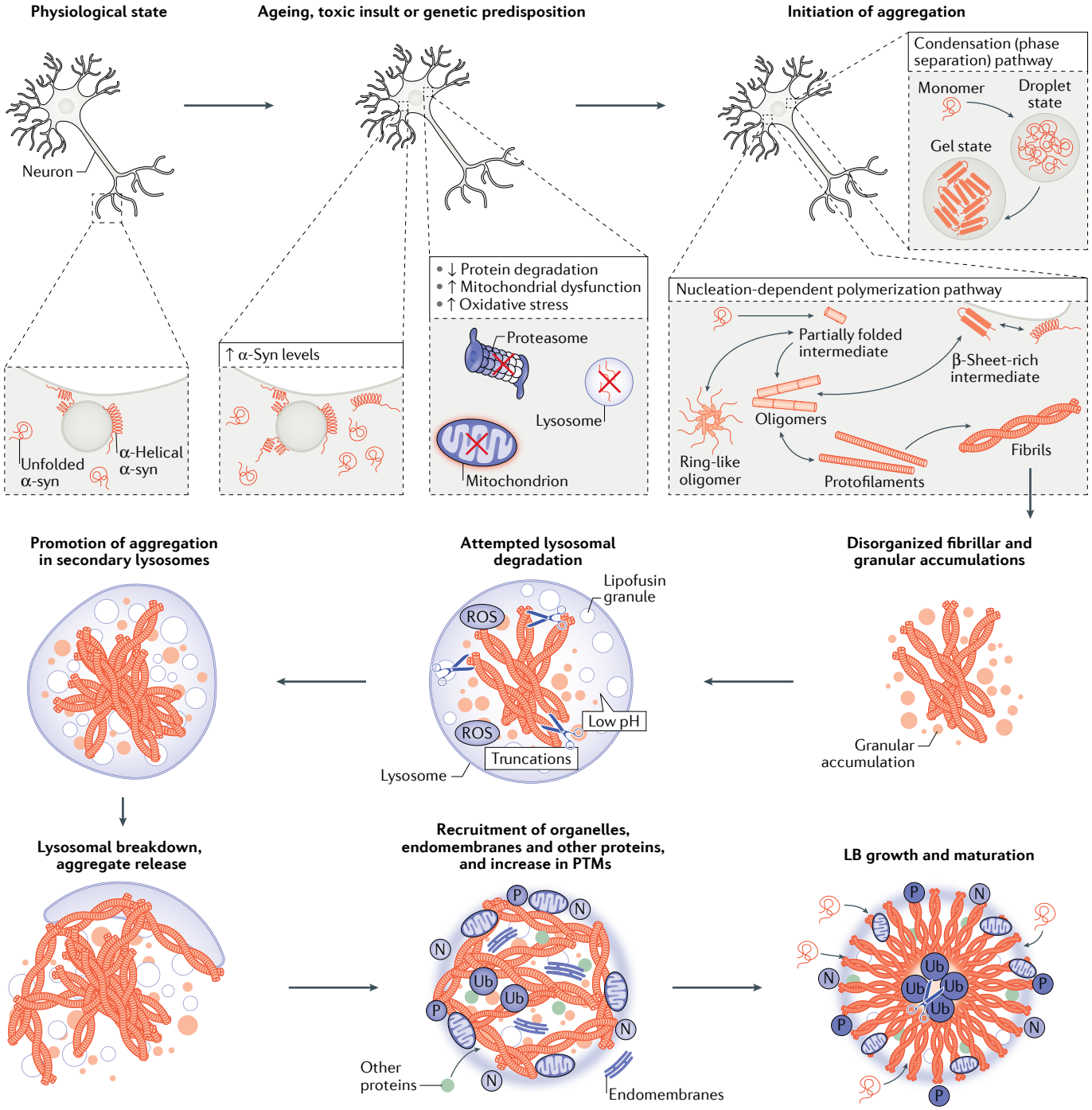
A model for de novo LB formation

The presynaptic localization of α-syn in healthy individuals[210–212] and its granular accumulation in neuronal soma during early stages of LB formation suggest that factors that increase α-syn levels, induce its misfolding or provoke its translocation from its physiological presynaptic compartment could trigger de novo LB pathology (FIG. 3). This hypothesis is supported by the observation of spontaneous 'dose-dependent' pathology in individuals with PD who have extra copies of *SNCA*[86,87], and the increased risk of sporadic PD with increased α-syn expression[213–216]. Impairment of the protein degradation machinery with ageing, following a toxic insult or by genetic predisposition could also lead to the accumulation of physiological or misfolded α-syn. Disruption of α-syn clearance systems has been observed in PD brain tissue, and familial PD-linked mutations have been identified in genes encoding proteins implicated in degradation[2]. The increase in α-syn levels coupled with induction of PTMs that enhance α-syn aggregation, such as C-terminal truncation, could then facilitate α-syn aggregation either in the cytosol or at the plasma membrane. This process could start with the formation of nucleation-competent oligomers of α-syn, which go on to form fibrillar seeds that then grow into fibrils, that then elongate by incorporation of α-syn monomers at their growing ends (FIG. 3). The nascent de novo-formed fibrils would exist at this stage in the cytosol as an ensemble of disorganized filaments accompanied by granular material of amorphous aggregates, oligomers and monomers.

It has also recently been suggested that the conversion of α-syn from the monomeric state to the amyloid state may proceed via a condensation pathway (FIG. 3). Therein, α-syn undergoes liquid–liquid phase separation to form liquid droplets, which results in a highly saturated and concentrated environment that promotes the formation of α-syn aggregates that gradually mature into amyloid-rich hydrogels[217,218]. This phenomenon has been shown to occur only in the presence of crowding agents in vitro. Although it has been suggested that this process also occurs in cell lines and *C. elegans* overexpressing α-syn[217,218], further studies are needed to characterize the biochemical and biophysical properties of the α-syn species in these models and to establish whether phase-separated α-syn could actually convert into fibrils or LB-like inclusions in neurons.

In response to the accumulation of misfolded proteins, the cell may attempt to degrade these abnormal and potentially toxic structures via different pathways, including lysosomal-mediated pathways (FIG. 3). If these degradation pathways are blocked or stalled, this could exacerbate the situation and provoke further aggregation and accumulation of α-syn. For example, the characteristic low pH within lysosomes, the presence of reactive oxygen metabolites and exposure of α-syn to lysosomal proteases are all conditions that promote the misfolding of α-syn, fragmentation of fibrils or the production of highly aggregation-prone modified forms of the protein. Moreover, since cellular membranes are normally recycled by lysosomes, these could accumulate within fibrils and form lipofuscin granules that exacerbate aggregation

Seeds
Relatively stable aggregates that can be prepared in vitro (such as fragmented fibrils) or isolated from pathological inclusions (such as Lewy bodies or glial cytoplasmic inclusions), total brain homogenates or the cerebrospinal fluid of patients with Parkinson disease or other synucleinopathies. When added to solutions or cells that have monomeric α-syn subunits, seeds accelerate their conversion to fibrils (in vitro) or to fibrils and Lewy body-like structures in neurons.



and the recruitment of misfolded α-syn monomers. Therefore, the insoluble fibrillar material increases in quantity within the now-called 'secondary lysosomes', possibly by further incorporation of monomers released from entrapped aggregates[219].

At some point, such overloaded lysosomes could break down and expel their contents into the cytoplasm (FIG. 3). The released lipofuscin–fibrillar accumulations would then act as potent nucleation centres that rapidly incorporate free α-syn molecules. As they grow, these aggregates could interact with and grow on membranes of organelles in their proximity (such as mitochondria), thereby recruiting and clustering them into inclusions, and gradually transforming these from a pale body phase with a disorganized structure into a more refined ultrastructure with a dense core and irradiating filaments (FIG. 3). These de novo-formed mature LBs, being large, insoluble and of very low free energy, would represent sinkholes that actively sequester soluble proteins and other organelles in their proximity. Finally, the now-diseased neuron would use up possible resources to tag this pathological structure for degradation by introducing PTMs, including ubiquitination and phosphorylation at α-syn S129 and other modifications that serve as signals to remodel fibrils or recruit other key players to facilitate their sequestration and eventual clearance. As suggested for amyloid

◀ Fig. 3 | **A proposed model for the de novo formation of Lewy bodies.** In the physiological state, α-synuclein (α-syn) exhibits a predominant presynaptic localization in natively unfolded and α-helical membrane-bound forms. Lewy body (LB) pathology could be triggered by a toxic environmental insult, ageing or genetic predisposition that affects the structure/protein levels of α-syn, or impairs pathways implicated in protein degradation or mitochondrial homeostasis. This abnormally increases α-syn levels in neuronal somas and terminals and facilitates the initiation of nucleation-dependent polymerization events. Therein, membrane-bound and cytosolic α-syn undergo conformational changes to form β-sheet-rich or partially folded intermediates, which then assemble into oligomers. These then grow into protofilaments, which then assemble to form mature fibrils. Different types of oligomeric species have been observed during the aggregation process, including off-pathway ring-like structures[271,290]. Moreover, it was recently proposed that the conversion of α-syn into fibrils may also proceed via a condensation (phase separation) pathway, in which α-syn first forms a droplet state, which then gradually matures and adopts an amyloid conformation via gel intermediates[217,218]. In either case, the nascent, disorganized granular or fibrillar accumulations are then engulfed within lysosomes for attempted degradation. However, α-syn aggregation could be promoted even further within these lysosomes, as α-syn could be truncated (denoted by the scissors), and the lysosome microenvironment consists of a low pH, reactive oxygen species (ROS) and lipofuscin granules, all of which are known to promote α-syn aggregation. The now 'secondary lysosomes', overwhelmed with insoluble fibrillar material, eventually break down and release the aggregates in the cytosol. The released fibrils then readily incorporate soluble α-syn, grow and interact with and/or grow on membranes of organelles in the vicinity (such as mitochondria), which induces the clustering of fibrils and gradual transformation from disorganized pale bodies to refined mature LBs having a dense core and radiating α-syn fibrils. With time, the generated fibrils continue to grow in size, and are hyperphosphorylated at serine 129 (P) and tagged with ubiquitin (Ub) by the cell, which attempts to clear these pathogenic structures. Nitration (N) of α-syn could also occur at this stage due to the prevalent presence of ROS within the neurons. PTM, post-translational modification.

plaques[220], these inclusions could also act as reservoirs for toxic oligomers, which are sequestered during inclusion formation, represent by-products of cellular processes aimed at dissociating and clearing fibrils or are formed within these deposits or inclusions.

Clearly, this model is correlative, predictive and hypothetical, as the process of α-syn de novo fibrillization into LBs has not been observed in any of the cellular and animal models. We think that having this global overview that integrates current knowledge and experimental observations could help identify key areas that need further investigation and could help develop different approaches to test or disprove different aspects of this model. Moreover, it emphasizes how unravelling and establishing the true and precise sequence of molecular events leading to the de novo formation of LBs is a major challenge that could hold huge potential for advancing the field of PD research.

LB propagation

As LB pathology is not restricted to the SNc but is also observed in many other brain regions, it has been postulated to spread in the brain following a specific sequential pattern that correlates with disease progression[33,221,222]. Specifically, it has been suggested that PD and LB pathology could be originating from non-dopaminergic regions of the brainstem (or even from peripheral tissues) and then physically spreading to higher brain regions to gradually provoke neurodegeneration and motor deficits. This model of pathology spreading in PD gained ground when two independent groups reported in 2008 that fetal dopaminergic neurons engrafted in the striatum of patients with PD developed LBs that were indistinguishable from those present in surrounding tissues ~15 years later[223,224]. This constituted the first evidence that host neurons that have developed LBs could propagate some sort of pathology to neighbouring ones that were previously completely healthy, a finding that was later confirmed in many other patients with PD[225–227]. Nevertheless, the propagation of pathology could be due to either the engrafted neurons developing de novo LBs as a consequence of the pathogenic microenvironment in which they were placed, or the physical spread of a specific LB element from the sick neurons that specifically seeds the formation of an identical LB in recipient neurons. This latter possibility fuelled the interest of research groups to investigate whether α-syn aggregates, one of the main components of LBs, could be 'the' element that is transferred among neurons and propagating LB pathology in a 'prion-like' manner. In the following sections, we evaluate current cellular and animal models of α-syn seeding and late events of LB formation generated by treatment with extracellular α-syn aggregates derived from recombinant or synthetic α-syn or from post-mortem PD brain tissue, with an emphasis on assessing the extent to which these aggregates reproduce different aspects of LB formation and maturation in PD.

Cellular models of LB propagation

To assess whether treatment with exogenous α-syn preformed fibrils (PFFs), as seeds, could provoke LB formation in cellular and neuronal models, multiple groups explored whether PFFs can recruit and initiate the aggregation of endogenous α-syn (Supplementary Table 2). This approach proved to be successful and consistently led to the induction of pathological α-syn aggregates in cell lines and primary neurons[228–237] and, later, in animal models[160,238–240]. Notably, seeding-mediated induction of α-syn aggregation could be robustly and reproducibly induced in cultures that are engineered to overexpress α-syn, as well as in naive untransfected neurons. The aggregates formed exhibited many features of brainstem and cortical LBs, including ThS reactivity, hyperphosphorylation at α-syn S129, reduced α-syn solubility, ubiquitination and accumulation of fibrillar ultrastructures. In addition to the formation of LB-like inclusions, the neuronal seeding model developed neuritic pathology, with abundant aggregates that are reminiscent of LNs[228,229]. Despite possessing a PTM profile that is similar to that of α-syn in LBs, the fibrillar aggregates described in these studies (monitored for up to 14 days) do not possess the classic organization and composition of mature LBs, being round and rich not only in aggregated α-syn but also in other proteins, lipids and membranous organelles[19,20,27,58,63,79,241], and therefore may represent an earlier stage of LB formation. Moreover, the spatio-temporal events that govern the biogenesis of these fibrillar aggregates at the ultrastructural level were not investigated and therefore remain unknown.

Recently, we reported a neuronal seeding model in which a transition from fibrillar accumulations into LB-like inclusions was observed during the period of 14–21 days after treatment with nanomolar concentrations of α-syn PFFs[20,241]. After 21 days, the inclusions found in α-syn PFF-treated neurons shared many features of bona fide LBs, including: the presence of insoluble

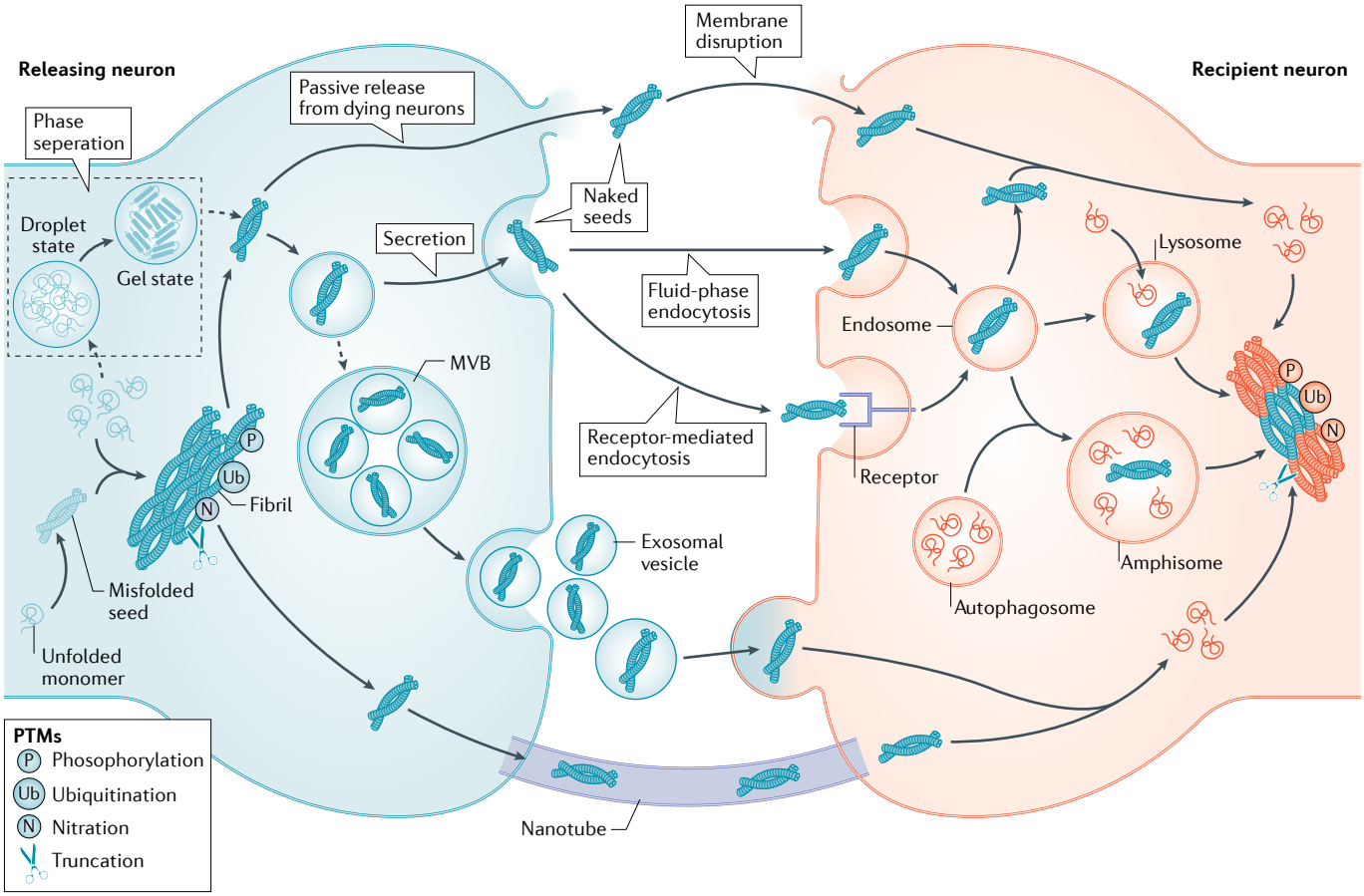


Fig. 4 | **A model for the spreading of fibrillar α-synuclein pathology.** Following nucleation-dependent polymerization or phase-separation events, de novo-formed α-synuclein (α-syn) fibrils in neurons undergo different post-translational modifications, including phosphorylation, ubiquitination, nitration and truncation, and could fragment into misfolded 'seeds'. These seeds could then be released by passive diffusion across membranous pores, active secretion via non-classical exocytic or endocytic pathways, incorporation into the intraluminal vesicles of multivesicular bodies (MVBs) and release as exosomal vesicles, or direct translocation through nanotubes. The released seeds could be internalized by other neurons via direct disruption of the plasma membrane, fluid-phase or receptor-mediated endocytosis, or fusion of plasma–exosomal membranes. Once inside the recipient neuron, if the seeds are in a 'naked', non-membrane-bound form, they could directly interact with recipient α-syn monomers and provoke their fibrillization. If the seeds are internalized via endocytosis, the seeds could either directly disrupt endosomal membranes to gain access to the cytosol or they could interact with endogenous α-syn monomers delivered by chaperone-mediated autophagy or with α-syn in autophagosomes, thereby forming amphisomes. This interaction would provoke further fibrillization events that eventually disrupt the vesicles and lead to the cytoplasmic release of the seeds. These 'free' seeds could then undergo different post-translational modifications (including phosphorylation, ubiquitination, nitration and truncation) and interact with the bulk of endogenous α-syn monomers and other cellular components to promote further fibrillization events.

fibrillar α-syn aggregates that are positive for ThS and immunoreactive for pS129 α-syn, ubiquitin and p62 (REF.[30]); the enrichment of C-terminally truncated α-syn species[9,12,241]; and the recruitment of organelles, such as mitochondria, autophagosomes and membranous vesicular structures[19,27,63] (Supplementary Fig. 1e). Importantly, this model also shows that most LB-associated PTMs and proteins are already present when fibrils are formed, suggesting that these modifications and protein interactions play important roles in the remodelling of fibrils and regulating their interactomes, and that both processes are important for LB formation and maturation. Moreover, neuronal dysfunction and neurodegeneration were observed only during the transition from fibrils to LBs, possibly explaining why the formation of α-syn fibrils or other types of aggregates was not sufficient to induce neuronal loss in many of the previous cellular and animal models of synucleinopathy.

Animal models of pathology spreading

Given the reproducible and robust induction of α-syn aggregation in cellular systems following treatment with PFFs, many groups investigated whether a seeding-based strategy would be as effective in vivo (Supplementary Table 3). In an early report, aggregated α-syn material was obtained by homogenization of brains of old symptomatic transgenic mice overexpressing A53T α-syn and then injecting this into the brains of their young, asymptomatic transgenic counterparts overexpressing the same mutant form of α-syn[242]. This treatment triggered early-onset motor deficits and exacerbated α-syn accumulation, leading to the formation of insoluble inclusions[242], an effect that was reproduced by several independent groups[239,243,244]. Similar results were also obtained when brain homogenates from individuals with multiple system atrophy[243] or recombinant α-syn PFFs[239,245,246] were intracerebrally injected into

transgenic mice, thereby directly linking the observed pathology with α-syn aggregates. Further demonstrating the pathogenicity of α-syn fibrillar species, studies reported widespread α-syn inclusion formation following intracerebral injections of α-syn recombinant PFFs[240,246–249] or brain extracts from patients with PD or dementia with LBs into WT non-transgenic mice[248,250]. Notably, and similarly to the case with models of de novo LB formation, increasing evidence suggests that the induction of pathology is most prominent when the injected PFF material is derived from α-syn protein that matches the sequence of the endogenously expressed host protein[113,251,252]. Importantly, the inclusions detected were consistently shown to reproduce many LB features, including ThS reactivity, ubiquitination, phosphorylation at α-syn S129 and the presence of fibrillar ultrastructure as revealed by TEM[249]. A dual strategy of injecting seeds of human α-syn fibrils into the rat SNc in combination with AAV-mediated overexpression of human α-syn has been also shown to replicate many cellular and behavioural features of PD, such as progressive degeneration of dopaminergic neurons in the substantia nigra, neuritic swelling, reduced striatal dopamine release and impaired motor behaviour[253]. Nevertheless, as limited biochemical and structural characterization of the detected inclusions was performed, the extent to which this model reproduces human LB-like pathology remains unknown.

Several groups have also used non-human primates to investigate the process of LB propagation in a more clinically relevant and translatable model. Inoculations of LB extracts derived from patients with PD in the striatum and SNc of rhesus monkeys led to progressive nigrostriatal neurodegeneration, with pS129-α-syn positive staining appearing in neuronal somas and presynaptic terminals, and α-syn fibrils propagating via both anterograde and retrograde transport to anatomically-interconnected brain regions[250]. In other reports, intrastriatal injections of α-syn PFFs resulted in the loss of TH-positive neurons and the spreading of α-syn pathology from the striatum to the substantia nigra. Importantly, the detected inclusions were positive for pS129 α-syn, ubiquitin, p62 and ThS[254–256]. Unfortunately, however, EM characterization of these inclusions was not performed.

A model for LB pathology spreading

Following the initial de novo formation of brainstem LBs in lower brain regions or in peripheral tissues, such as in the gut, a 'prion-like' mechanism could provoke the spread of this pathology to anatomically higher brain areas. In this model, fibrillar fragments of α-syn (or high molecular weight oligomeric α-syn species[257]) are released from neurons that have hosted nucleation-dependent fibrillization or phase separation events, and then these seeds enter neighbouring neurons and provoke the abnormal aggregation of their native α-syn monomers. Although the precise sequence of events underlying this phenomenon in human brains is largely unknown, animal and cellular models in this case have provided significant insights into the mechanism of release and uptake of pathogenic seeds.

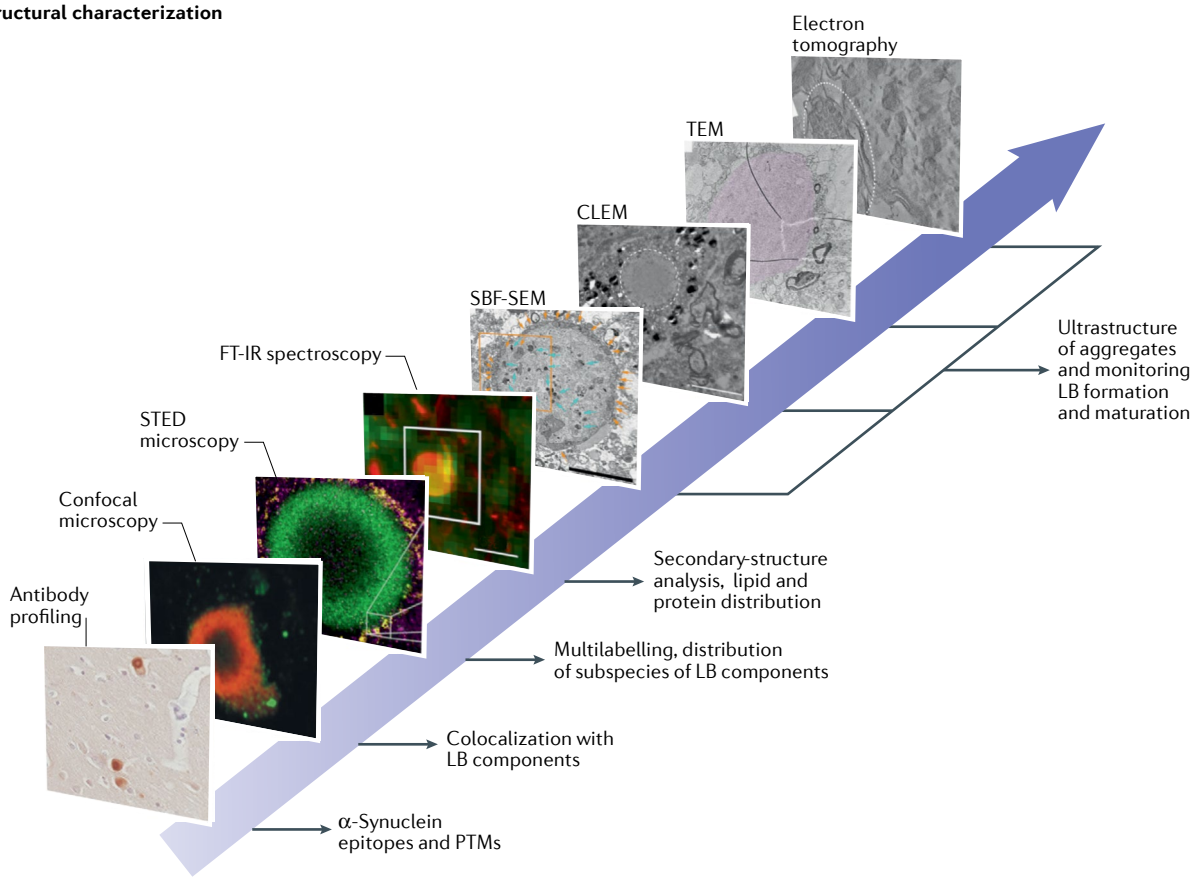
For the release of α-syn, a number of different possibilities have been proposed[258] (FIG. 4). In the simplest scenario, fibrillar or oligomeric α-syn species could escape from degenerating neurons with damaged

Table 1 | **Known properties of different α-syn species along the pathway to Lewy body formation**

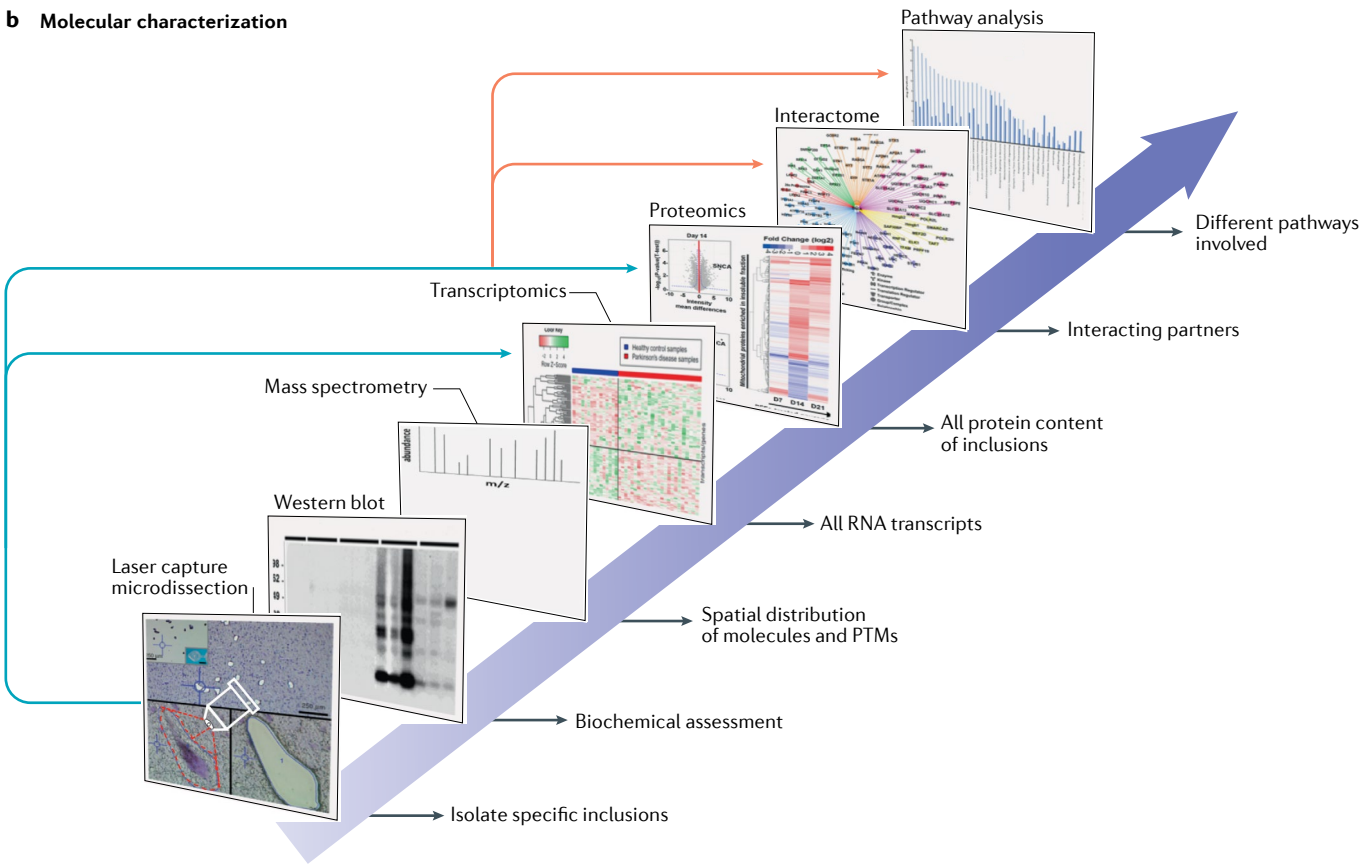
Properties	Aggregation state of α-synuclein					
	Diffuse or soluble	Oligomeric aggregate	Vesicular accumulation	Amorphous aggregates	Filamentous aggregates	Lewy body aggregates
Insoluble	No	No	No	Yes	Yes	Yes
Positive for markers of endolysosomal pathway	No	ND	Yes	ND	Yes	Yes
Thioflavin S positive	No	No	No	No	Yes	Yes
Fibrillar structure (revealed by electron microscopy)	No	No	No	No	Yes	Yes
Positive for all of the following immunomarkers of Lewy bodies (pS129 α-synuclein, p62 and ubiquitin)	No	No	No	ND	Yes	Yes
Proteinase-K resistant	No	No	No	ND	Yes	Yes
Increase in size with time	No	Yes	ND	Yes	Yes	Yes
Seeds aggregation of soluble monomers	No	ND	ND	ND	Yes	Yes
Modulated by pharmacological or genetic inhibitors of aggregation	No	Yes	No	ND	Yes	Yes
Co-accumulation with membranous fragments or organelles	No	Yes	No	Yes	ND	Yes
Detection by proximity ligation assay	No	Yes	ND	Yes	Yes	Yes

ND, not determined; pS129, phosphorylated serine 129.

a **Structural characterization**



b **Molecular characterization**



◀ Fig. 5 | **Tools and platforms for dissecting the diversity of Lewy bodies and evaluating the α-synuclein aggregation states in cellular and animal models.** Tools and platforms for characterizing Lewy body (LB)-like aggregates and inclusions at the structural (part **a**) and molecular (part **b**) levels. CLEM, correlative light and electron microscopy; FT-IR spectroscopy, Fourier transform infrared spectroscopy; PTM, post-translation modification; SBF-SEM, serial block-face scanning electron microscopy; STED microscopy, stimulated emission depletion microscopy; TEM, transmission electron microscopy. Part **a** antibody profiling panel adapted with permission from REF.[291], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/); confocal microscopy panel is adapted with permission from REF.[167], Society for Neuroscience; STED microscopy, FT-IR spectroscopy, SBF-SEM, CLEM, TEM and electron tomography panels adapted from REF.[19], Springer Nature Limited. Part **b** laser capture microdissection panel adapted with permission from REF.[292], Oxford University Press; western blot panel adapted with permission from REF.[167], Society for Neuroscience; transcriptomics panel is adapted with permission from REF.[293], CC BY 3.0 (https://creativecommons.org/licenses/by/3.0/); proteomics and pathway analysis panels adapted with permission from REF.[20], PNAS. Copyright © 2020 the Author(s). Published by PNAS distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND); interactome panel adapted with permission from REF.[294], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

plasma membranes, or they could propagate between two neurons that are directly connected via cytoplasmic bridges termed 'nanotubes'[259]. Alternatively, the seeds could be packaged into secretory vesicles and could be secreted by non-classical endoplasmic reticulum–Golgi apparatus-independent exocytosis[260–262] or could be translocated to early endosomes and released extracellularly via recycling endosomes[263,264]. In all of these cases, the fibrillar or oligomeric seeds are released in a 'naked' form; that is, they are not membrane-bound. Notably, exosomal vesicles have also been implicated in the release of α-syn[265–267], as following the incorporation of the seeds into early endosomes, the vesicles could coalesce into large multivesicular bodies that are secreted in a calcium-dependent manner[266]. The fusion of these multivesicular bodies with the plasma membrane would release exosomal vesicles containing the α-syn seeds; that is, in a membrane-bound form.

To provoke pathology, the released seeds have to access the cytosol of neighbouring cells, interact with the bulk of cytosolic, endogenous α-syn and prompt its misfolding and conversion into amyloid structures[268] (FIG. 4). For seeds that are engulfed within exosomes, the fusion of the exosomal and plasma membranes would result in their direct release into the cytosol. Aggregates that are released in a 'naked' form may undertake several different routes before interacting with cytoplasmic α-syn of recipient cells. As recently suggested by our group, the seeds could bind the plasma membrane and aggregate into larger structures by incorporating soluble extracellular α-syn monomers[269]. This process could disrupt the plasma membrane and grant cytoplasmic access to the seeds. This membrane-disruptive capability of α-syn has also been extensively reported in vitro, either by directly forming amyloid pore-like channels[270–274] or by membrane thinning[275,276], stripping[277,278] or tubule formation[279,280]. Otherwise, the naked seeds could be taken up via adsorptive[228] or receptor-mediated endocytosis[233,281,282] and end up entrapped within endosomes inside the recipient neuron. The seeds could again damage the endosomal membranes at this stage and access the cytoplasm. Alternatively, they could meet endogenous α-syn monomers if the endosomes fuse with autophagosomes, or if soluble α-syn monomers are delivered into endosomes by chaperone-mediated autophagy[283]. In either case, this would provoke α-syn fibrillization events that break down the vesicles and subsequently release the fibrillar material.

Moreover, a key event following internalization and release of seeds entails efficient C-terminal cleavage of α-syn seeds, which rapidly recruits the bulk of endogenous α-syn and forms growing fibrillar intracellular structures[228,233]. These could be then phosphorylated at S129 and ubiquitinated, leading to the recruitment of p62. Subsequent fragmentation of these fibrils would possibly occur, causing structural changes that regulate the transition from fibrils to LB-like inclusions, including lateral association of higher-order fibrils and sequestration into mature LB-like inclusions, and the recruitment of membrane-bound organelles, as we have recently shown[20]. Moreover, the fragmentation events would generate novel seeds that can be released in turn and provoke α-syn fibrillization in other neurons. Therefore, the outcome of a single spreading event following the de novo formation of LBs in the brainstem could theoretically be a vicious cycle of pathology that gradually spreads among interconnected neuronal circuits.

Having proposed this model describing the spread of α-syn pathology, it is important to stress that, for the moment, it remains controversial whether such a 'prion-like' propagation scheme truly accounts for the observed widespread occurrence of LBs in patients with PD. The essence of this model is that misfolded exogenous seeds can be transferred within, and possibly between, individuals and can prompt the templated aggregation of native endogenous α-syn into fibrils with the same characteristic properties, as, for example, is consistently shown for the scrapie prion protein[284]. However, it remains to be established whether α-syn propagates in exactly the same manner as prion proteins. Although α-syn has been shown to be transmissible between cells in culture and in animals, to date no evidence has shown that PD can be transmitted from one individual to another. Therefore, further research is needed to unravel the true implication and potential mechanisms governing the spreading of LB pathology in PD.

Conclusions and perspectives

Our comprehensive analysis of the different cellular and animal models of α-syn aggregation and pathology formation suggests that the value of some of the existing cellular and animal models could have been underestimated. Although the great majority of these models do not reproduce all biochemical and structural features of LBs observed in post-mortem PD brains, many reproduce different stages of α-syn aggregation and exhibit some features of Lewy pathologies. These observations suggest that many of these models may still serve as useful tools to investigate specific aspects of the different stages of α-syn aggregation and LB formation. Indeed, one has to recognize that it may be impossible to model all aspects of LB formation and neurodegeneration in PD in one model and over the scale of days to weeks, as these processes have been suggested to evolve over

months[85] to possibly years in patients' brains. However, it is unfortunate that despite the huge time and financial investments made in generating these models, the aggregation state of α-syn is not characterized sufficiently well to judge what aggregation stage they actually represent. In most cases, 'inclusion formation' per se is vaguely reported as an 'aggregation event', and EM analysis is rarely performed to unravel inclusion ultrastructure. In addition, the large disparity in the tools and methods used to assess α-syn aggregation in these models makes it difficult to compare neuropathological observations. In TABLE 1, we describe current knowledge on the properties of different α-syn species along the pathway to LB formation, which could facilitate determining the aggregation state of observed α-syn inclusions in cellular or animal models of synucleinopathy. Indeed, the combination of thorough immunocytochemical, biochemical and ultrastructural analyses would not only allow the validation of the aggregation state of α-syn but would also elucidate the biochemical and structural basis of the pathological and potentially clinical heterogeneity of PD and other synucleinopathies. Indeed, such an approach has recently shown its potential in deciphering the clinical heterogeneity observed in Alzheimer disease, where biophysical, biochemical and cell- and animal-based bioactivity assays were used to characterize tau in different patients with Alzheimer disease[285]. Importantly, that study not only showed that tau seeding activity correlates with clinical pathology, but also found that some tau PTMs could be associated with enhanced seeding activity and worse clinical outcomes.

With the advent of recent imaging and super-resolution technologies, including stimulated emission depletion microscopy, correlative light and electron microscopy, and electron tomography[286], as well as recent advances in genomic and proteomic approaches, the heterogeneity of LBs at the structural and biochemical levels in tissues and brain homogenates should also be assessed. This would also enable a thorough understanding of the genes, proteins, and molecular pathways that might be involved in regulating the formation and maturation of detected inclusions (FIG. 5). This level of characterization is essential for evaluating new cellular and animal models and assessing their utility and validity as tools to investigate different stages and aspects of α-syn aggregation and LB formation. Having such models reproducing specific features of authentic LBs and LNs is of critical importance to do the following: investigate the key cellular and molecular determinants that regulate different stages of LB formation and maturation for mechanistic studies; screen potential therapeutic agents to slow down or halt pathology; identify toxic species, processes and pathways; and elucidate the role of α-syn aggregation and Lewy pathology formation in the pathogenesis of PD (that is, whether LBs are protective or neurotoxic). Although seeding-based models may recapitulate the late processes of interneuronal spreading and α-syn pathology formation and maturation, they do not address the early events that could be provoking the disease. Therefore, further efforts are needed to establish models that recapitulate earlier events in PD pathology to enable the development of therapeutic strategies that prevent α-syn misfolding and self-assembly.

Finally, it must be acknowledged that the biochemical and structural bases underlying the diversity of Lewy pathology in human brains remains understudied. The great majority of studies on the ultrastructural properties of LBs focused primarily on late-stage PD and mature LBs and the filamentous structures within LBs. However, increasing evidence suggests that α-syn pathology in PD brains is polymorphic and diverse in terms of biochemical composition, the pattern of α-syn PTMs, the extent of α-syn fibrillization and the presence of membranous organelles. Therefore, efforts to model human pathology should begin with concerted efforts to revisit human PD pathology and correlate it with disease symptoms and/or disease progression. To achieve this goal and capture the full diversity of α-syn aggregates and Lewy pathologies at the ultrastructural and biochemical levels, it is also critical to use an expanded set of validated tools and apply integrative approaches that leverage the latest advances in high-resolution imaging and omics (FIG. 5).

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