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Review

Defining a Lewy Body: Running Up the Hill of Shifting Definitions and Evolving Concepts

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Abstract. Lewy bodies (LBs) are pathological hallmarks of Parkinson's disease and dementia with Lewy bodies, characterized by the accumulation of α-synuclein (αSyn) protein in the brain. While LBs were first described a century ago, their formation and morphogenesis mechanisms remain incompletely understood. Here, we present a historical overview of LB definitions and highlight the importance of semantic clarity and precise definitions when describing brain inclusions. Recent breakthroughs in imaging revealed shared features within LB subsets and the enrichment of membrane-bound organelles in these structures, challenging the conventional LB formation model. We discuss the involvement of emerging concepts of liquid-liquid phase separation, where biomolecules demix from a solution to form dense condensates, as a potential LB formation mechanism. Finally, we emphasize the need for the operational definitions of LBs based on morphological characteristics and detection protocols, particularly in studies investigating LB formation mechanisms. A better understanding of LB organization and ultrastructure can contribute to the development of targeted therapeutic strategies for synucleinopathies.

Keywords: α-synuclein, Lewy body, Parkinson's disease, aggregation, liquid-liquid phase separation

INTRODUCTION

The accumulation of Lewy bodies (LBs) in the brain is a pathological hallmark of Parkinson's disease (PD) and dementia with LBs (DLB). The presence of α-synuclein (αSyn), a synaptic protein implicated in the regulation of synaptic vesicle cycle, is a defining feature of LBs. The synuclein family is a group of short, disordered proteins, which are highly abundant in the brain tissue, accounting for 1% of total cytosolic protein [1]. In fact, αSyn is a *bona-fide* presynaptic protein that is able to interact with many other synaptic proteins—stabilizing SNARE complexes [2], VAMP2 [3], synapsin 1 [4], as well as lipid membranes [5].

While LB inclusions were first described more than a century ago, the mechanisms involved in their formation and morphogenesis are still incompletely

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understood. However, in recent decades, the availability of new research tools has provided various new insights. Particularly, the development of antibodies against αSyn and other markers, advanced imaging methods, and model systems contributed to the appreciation of widespread inclusion pathology in PD and DLB and expanded the spectrum of heterogeneous inclusion morphologies.

The present paper addresses historically applied and current criteria for a 'LB', guided by a selection of seminal findings. For a more detailed and comprehensive review on LBs, see [6]. Here, we emphasize the importance of semantics and definitions when describing inclusions in the human brain and in model systems to prevent potential ambiguity, particularly when operating at the interface of different research fields/perspectives (e.g., neuropathological diagnostics versus disease modeling). Based on recent findings in PD/DLB and related neurodegenerative diseases, we finally address the possibility of the involvement of emerging concepts such as liquid-liquid phase separation in the formation of LBs, and a potential role for αSyn in orchestrating their morphogenesis.

A BRIEF HISTORY OF EVOLVING LEWY BODY DEFINITIONS

Lewy bodies as eosinophilic hyaline inclusions

Exactly one century ago, Friedrich Lewy published his seminal book '*Die Lehre vom Tonus und der Bewegung*', which was the culmination of Lewy's work on *Paralysis Aagitans* (Pa; which was later termed PD), including detailed descriptions of the neuronal inclusions he discovered in postmortem brain specimens of his former patients [7, 8]. In particular, Lewy applied a series of histochemical staining techniques, such as Mann's methyl blue-eosin stain, and observed concentric 'hyaline' intraneuronal masses that were positively stained by eosin, which is an acidic (basophilic) dye that binds to positively charged basic components in tissue, including cytoplasmic proteins [9]. This work confirmed his previous findings (1912) in Meynert's nucleus in the substantia innominata and the dorsal motor nucleus of the vagus nerve—the first ever description of cellular inclusions in PD—in additional cases. The presence of eosinophilic inclusion bodies in Pa brains was soon confirmed by others, and called after their discoverer by both Lafora and Tretiakoff, who demonstrated their abundance in an additional subcortical structure, the substantia nigra pars compacta (SNpc) [10].

According to Lewy's descriptions, morphologies of eosinophilic inclusion bodies were highly heterogeneous, ranging from serpent-like to globoid, and did not differentiate between inclusions with perikaryal or neuritic localizations (the term 'Lewy neurite' was only described many years later; see below). Although most described structures were observed in subcortical regions, Lewy routinely examined neocortical brain regions. While many of the cases he studied were demented—some of them without apparent tangles or plaques—Lewy made mention of only one case that showed acidophilic granules in the neocortex [8], which was here identified by staining with fuchsin, which is a basophilic dye similar to eosin. Only in 1961, Okazaki et al. described eosinophilic inclusions in the cortex of two donors with dementia as well as extrapyramidal rigidity during life, and named this phenomenon diffuse LB disease [11]. In the years after, some more characterization was done on cortical eosinophilic LBs. As these appear ambiguous in hematoxilin/eosin stainings (H&E), it was noted that the detection of such inclusions in the cortex is challenging, while their eosinophilicity and morphology were variable, with descriptions of their shapes ranging from globular to angular or reniform [12, 13]. Based on their different appearances, in 1978, Kosaka introduced the terms 'midbrain-type' LBs and 'cerebral-type' (now commonly referred to as 'cortical-type') LBs [14].

While both midbrain-type and cortical-type LBs have historically been defined by their reactivity to eosin, a subset of H&E-negative inclusions in the midbrain of PD donors were additionally described, and named 'pale bodies' (PB) [15, 16]. These structures are best detected in the pigmented cells in locus coeruleus and SNpc, as they are accentuated by the displacement of neuromelanin. Although the initial definition of PBs was based on the lack of eosinophilicity, later on, certain PBs have been described as 'weakly' eosinophilic [17, 18] (although this has also been described as LB-like matter [16]). While cortical PBs have been mentioned by, e.g., Gibb et al. [15], these structures are not commonly referred to in the field, possibly due to lacking definitions and protocols for their unambiguous detection. Moreover, in later reports, the term PBs became more restricted to the SNpc and locus coeruleus [16].

In addition to their essential contribution to detection and differentiation of PD-related inclusions such as LBs and PBs, conventional histochemi-

cal techniques provided important indications about the chemical composition of LBs, and even within LB. While most consistently stained by basophilic dyes, extensive characterizations of LB reactivity in other histochemical staining methods showed they are also reactive to other dyes, including various phospholipid-, sphingomyelin- [19, 20], and also histone-binding [21] agents. These findings provided the first suggestions that proteinaceous LBs additionally contain accumulations of lipid-rich material. Moreover, intriguingly, for many of these techniques, differential reactivity patterns have been reported between the LB core and peripheral area of the inclusions, suggesting they possess different biochemical properties. Importantly, similar to the above-discussed findings, these analyses were largely focused on LBs in subcortical structures such as the SNpc and locus coeruleus, as biochemical characterization of cortical inclusions by histochemical techniques is limited, probably due to their more challenging detection without available antibody-based stainings ([12]; more later).

Contribution of early ultrastructural studies to 'LB' definition

Following Lewy's initial description of eosinophilic neuronal inclusions, subsequent work established their visualization using a variety of histochemical techniques, including toluidine blue staining, as well as phase contrast microscopy. Using the latter technique, Duffy and Tennyson identified inclusions in human brain samples and analyzed their ultrastructure using transmission electron microscopy [22]. They reported a moderately dense inner core, surrounded by radially oriented filaments, consistent with the 'laminar' appearance of LBs described using histochemical techniques. The presence of filamentous structures in PD inclusions has been reported frequently afterwards, and such filaments have been characterized in great detail, as was reviewed by Forno (1996) and more recently by Lashuel (2020) [23, 24]. In words of Forno (pre-dating the discovery of αSyn in LBs), in a polymorphic landscape of inclusions, "*the most important element which all LBs have in common is, however, the filamentous cytoskeletal component, now considered to consist of neurofilaments*" [23].

When relating this to literature, this description would include a specific subset of filamentous midbrain and cortical inclusion morphologies, while it technically does not discriminate between LBs and PBs, as both of them were reported to contain filamentous material in variable amounts [25]. However, it should be noted here that only limited work has been done on the ultrastructure of pale bodies (and also cortical LBs), partially due to their more challenging unambiguous detection, particularly in correlative histochemical/phase contrast microscopy and electron microscopy (EM) studies. Still, a 'filamentous' ultrastructure has been commonly referred to as a cardinal hallmark of LBs.

Immunolabeling by antibodies against neurofilament, ubiquitin, and p62

The landscape of PD neuropathology changed dramatically after finding that pathologic inclusions in PD are, similar to neurofibrillary tangles and plaques [26–29], immunoreactive to antibodies against ubiquitin and cytoskeletal components (neurofilament; tubulin). Goldman and colleagues provided the initial description (1983) that immunolabeling using antibodies against neurofilament enabled the detection of Lewy bodies in the midbrain and cortex, particularly showing staining of the periphery of core-shell inclusion morphologies in the midbrain [18]. A few years later, LBs were shown to be immunoreactive to antibodies against ubiquitin [30]. Moreover, ubiquitin antibodies allowed unambiguous staining of inclusions (somatic and neuritic) in the cortex that were previously difficult to identify based on eosinophilicity [31]. Although ubiquitin antibodies are not specific for PD-related inclusions, their enhanced sensitivity greatly facilitated the detection of a condition formerly referred to as "diffuse LB disease", which was far more common than originally anticipated [32]. In addition to LBs, strong uniform immunolabeling was observed for many other intraneuronal inclusion morphologies. Most notably, different ubiquitin-positive morphologies with neuritic localization were observed, for instance, in the amygdala and hippocampus [33, 34]. The appearance of such neuritic inclusions varied dramatically, ranging from delicate and thread-like to swollen and dystrophic-appearing, including structures with a core-shell appearance. The original description of the term 'Lewy neurite' (LN), which was only introduced around this time (the mid-90 s) based on ubiquitin-immunopositive neuritic structures, does not further categorize between such morphologies.

In addition to neurofilament and ubiquitin, around the same time p62 antibodies were also demonstrated to consistently label LBs [18, 35]. These antibod-

ies showed overlapping patterns with ubiquitin, with strong staining of core-shell structures in the midbrain and also cortical LBs and PB morphologies. Similar to neurofilament (NF) and ubiquitin, p62 is not specific for PD-related inclusions as antibodies against this protein also labeled other protein deposits [35, 36].

Lewy bodies and α-synuclein: a new era

Today, it is hard to think of LBs without linking them to αSyn. After the initial discovery that PD inclusions are immunopositive for αSyn by Spillantini and colleagues [37, 38], antibodies against αSyn, and particularly its Serine 129 phosphorylation (Ser129-p) [39], were established as the most sensitive marker for inclusion pathology in PD. Moreover, in contrast to previously applied markers, αSyn-antibodies are far more specific for neuronal inclusions in PD, with limited cross-reactivity for other neuropathological hallmark structures such as tangles and plaques.

αSyn antibodies strongly label neuronal somatic inclusions of different morphologies, including core-shell structures; uniformly labeled midbrain structures with different appearances (including smaller aggregates); cortical-type inclusions. Combined immunohistochemistry approaches showed that αSyn stains a pallet of eosinophilic and non-eosinophilic structures, including pale bodies [18, 40]. In addition to neuronal perikaryal inclusions, αSyn antibodies highlight many other features, including diffuse, punctate or granular perikaryal staining; synaptic-like and neuritic profiles, punctalike inclusions, while certain anti-αSyn antibodies also highlight a variety of glial inclusions in oligodendrocytes [41, 42] and astrocytes [43, 44]. Thereby, αSyn provided a previously unestablished pathological link between different diseases that display αSyn-positive inclusions (e.g., neuronal or glial), which are now collectively known as synucleinopathies. Moreover, the finding that a large proportion of αSyn in LBs is post-translationally modified—most notably Ser129-p [39, 45]—led to the development of antibodies against this epitope that are more sensitive for detection of pathology-associated structures compared to physiological αSyn.

By increased sensitivity, (Ser129-p) αSyn antibodies dramatically expanded the known spectrum of inclusions' morphological appearances in PD and related diseases, further underlining widespread pathologies over brain regions localizing to different subcellular compartments. Using these new research tools, in 2003, Braak and colleagues published their landmark paper proposing a neuropathological staging of PD pathology based on a systematic caudo-rostral distribution of αSyn-immunoreactive inclusions over brain regions during the disease course [46]. Braak staging is now considered the standard for neuropathological verification of a clinical diagnosis for PD.

Moreover, the discovery that αSyn accumulation is a key aspect of LB-related pathology in PD and αSyn mutations/triplications cause (familial forms of) PD greatly stimulated the development of αSyn-based cellular and animal models of PD [47–51], which are discussed in more detail later. Several of these models have described the formation of αSyn-containing inclusions, which were commonly detected by (Ser129p) αSyn immunostaining, and represent an important foundation for these models. However, while these inclusions have been sometimes termed LBs or LB-like, the extent to which these inclusions represent Lewy's original descriptions or other inclusion types in the human brain is not always clear. Therefore, recent studies have advocated for the need for in-depth characterization of inclusions in model systems, including their reactivity towards different LB-detecting antibodies [6].

In summary, substantial progress in recent decades have dramatically improved the detection of PD-related pathologies, thereby increasing the appreciation of the extent and distribution of PD pathology throughout the brain, its morphological heterogeneity, and different affected cell types. Moreover, implication of αSyn in PD provided an important foundation for the development of αSyn-based model systems, which are pivotal for a better mechanistic understanding of PD-related inclusion pathology and development of new treatment strategies.

What is a Lewy body—and is this important?

Since the original description of PD-relevant neuronal inclusions by Lewy, different additional aspects have been incorporated in the definition of LBs over the years. While initially defined based solely on eosin staining, currently applied definitions for LBs involve other markers and/or different additional factors, depending on the examined brain region.

In the midbrain, in addition to eosin staining, immunoreactivity for markers such as αSyn, ubiquitin and neurofilament have all been used as

defining criteria of LBs. Moreover, the combination of immunoreactivity with morphological criteria such as core-shell appearance is required to enable discrimination of LBs from, e.g., PBs, which are strongly stained for αSyn and ubiquitin (Fig. 1A). The somatic (perinuclear) subcellular localization of the inclusion is an additional prerequisite to allow discrimination between LBs and LNs. Of note, Lewy's original definition of these inclusions did not include the subcellular localization as a criterion, and some of the described serpent-like eosinophilic structures would likely be categorized as (dystrophic) LNs by our current definitions.

Thereby, one could argue that over the years, the definition of LBs in the midbrain was refined.

In sharp contrast, cortical inclusions, which are challenging to discern based on eosinophilic staining—and hence not discussed in detail by Lewy and his contemporaries—are now referred to as cortical-type LBs based on reactivity to ubiquitin or αSyn antibodies, regardless of their positivity for eosin and morphological appearance (Fig. 1B).

This discrepancy in nomenclature (e.g., between brain regions) complicates the conception of what a LB is and can introduce ambiguity. For instance, according to these definitions, the same structure could be termed LB when detected in the cortex or a PB when detected in the midbrain [52]. While commonly referred to as "Braak LB stage", this staging system does not differentiate between different αSyn-positive morphologies, as neuritic pathology (for instance, in the hippocampus) and somatic inclusions with either diffuse or core-shell appearances, are all considered 'positive' in this assessment. While the field has since adopted the term 'Lewy pathology' in this context to collectively refer to neuritic and somatic inclusions, this term still does not clarify that the staging procedure is based on αSyn antibody labeling.

As the formation of neuronal inclusions has been described for different model systems, to avoid confusion, it can be argued that the field would benefit from more operational definitions, based on morphological characteristics and applied detection protocol (i.e., ring-shaped Ser129p αSyn-positive inclusion; Braak αSyn staging). This is particularly important for studies focused on the mechanisms underlying the formation and morphogenesis of LB-like inclusions, which have recently received renewed attention [51, 53–56]. In particular, several studies were published on the detailed organization and ultrastructure of αSyn-immunopositive inclusions in the PD postmortem brain, which was re-appraised using state-of-the-art imaging techniques such as correlative light and electron microscopy and super-resolution microscopy in combination with label-free and proteomic techniques [53–55]. In addition, around the same time, different groups have made significant progress in modeling neuronal inclusions in cellular and animal model systems that display features of αSyn inclusions in the human brain by different approaches [47–51].

A better mechanistic understanding of how neurons process and organize different inclusion morphologies is needed, especially as the formation of these inclusions might have major consequences on cellular function. Understanding these mechanisms can contribute to developing possible cell-empowering therapeutic strategies in PD. To streamline this big collective effort of different fields, careful wording and use of definitions will be of utmost importance.

THE DETAILED ANATOMY OF LEWY BODIES

Recent ultrastructural insights renewed emphasis on non-proteinaceous Lewy body components

Historically, LBs are considered as predominantly proteinaceous structures, based on 1) the co-localization of different protein markers (i.e., reviewed in [9]; 2) the detection of hundreds of individual proteins by unbiased proteomic approaches, for instance following isolation of cortical LBs by laser capturing dissection [57]; 3) the observation of electron-dense elements and radiating filamentous structures of LBs by EM. Moreover, the findings that midbrain LBs and filaments extracted from insoluble fractions of cingulate cortex tissue of PD patients both demonstrated immunogold labeling for αSyn, suggested a crucial role for the aggregation of this protein in the pathogenesis of PD [38, 58]. As αSyn was shown to form fibrils *in vitro* under certain conditions [59–61], it was hypothesized that accumulation of αSyn fibrils could represent a starting point for LB formation, analogously to Aβ in Alzheimer's disease [62, 63]. For example, recent studies have provided evidence that exogenous addition of fibrils formed *in vitro* from recombinant αSyn to cells or animal models leads to propagation of inclusion pathology and toxicity [47–51].

In addition to a proteinaceous component, various studies provided indications for an enrichment of

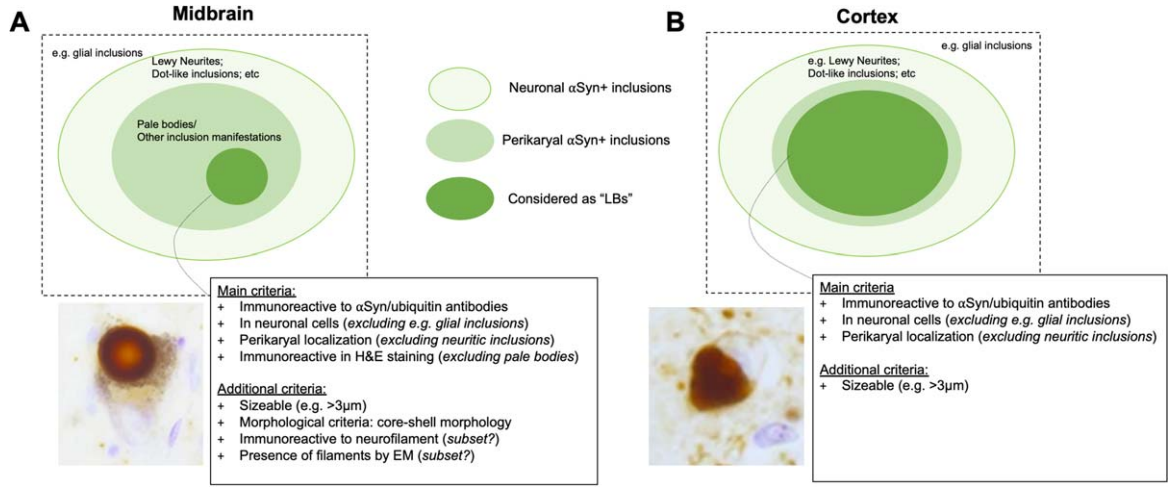

Fig. 1. Conceptualization of 'LBs' in the midbrain versus the cortex based on most commonly used markers. In the midbrain (A), a LB is often referred to as a subset of neuronal perikaryal inclusions that are immunopositive for αSyn and ubiquitin, and further fulfill several other criteria (e.g., morphological) criteria, and positive H&E staining. Together, this highlights the conceptual difference between a subset of well-defined inclusions among a larger group of different-appearing heterogeneous inclusion types (e.g., pale bodies). In contrast, in the cortex (B), any perikaryal inclusion that is immunopositive for ubiquitin or αSyn is termed a 'LB', consequently encompassing a large spectrum of ill-defined structures, ranging from, e.g., compact and globular to expanding uniform cytoplasmic fillings (discussed further in text). Micrographs showing Ser129-immunoreactive (antibody: 11A5) inclusions in SNpc and cortex are modified from Moors et al., 2021 [54], where the images were published under a CC-BY 4.0 license.

organellar and (sphingo)lipid components in LB-like inclusions as well, for instance, using EM [64–66] histochemical staining methods [20, 67] or label-free imaging techniques [52, 54, 68]. This aspect was further highlighted by a recent study which showed that LBs in the SNpc and hippocampus of PD patients, selected and analyzed using immunoreactivity for Ser129p αSyn by correlative light and electron microscopy, were abundant in (vesicle-derived) membranes and organelles, including fragmented mitochondria and autophagosomal/lysosomal structures [32]. These findings were corroborated by the detection of lysosomal and mitochondrial markers in Ser129p αSyn-positive LBs by STED microscopy. Moreover, the enrichment of lipids in LBs was also detected by coherent anti-Stokes Raman scattering, Fourier-transform infrared imaging, and lipidomics [53]. While all studied inclusions were abundant in organelles and membranous structures, a subset of inclusions additionally contained proteinaceous filaments. Although it was suggested that some of the more delicate filaments may have been missed in this study [24], these findings nevertheless highlighted more heterogeneity in the ultrastructural composition of LBs than anticipated and has raised the question whether fibrils of αSyn are formed and incorporated in inclusions during their maturation process [69]. The finding of abundant membranes in LBs has particular significance given the clear physiological association of αSyn with (synaptic vesicle) membranes and contributed to a shift from PD as a protein-centered disease to also a lipidopathy [70].

On the heterogeneity versus consistency in Lewy body research

With the availability of novel more sensitive tools for detection of PD-relevant inclusions, the appreciation of the heterogenous manifestation of αSyn-positive pathologies in the brain with PD has grown substantially. Increased insights into the variety of PD-related features, involving accumulations in various affected cellular sites or even cell types, and morphologies with different properties, will be of great help in identifying processes underlying cellular dysfunction in PD.

However, within this multitude of morphologies, studies using selective antibodies against αSyn domains and post-translational modifications (PTMs) further underlined that a subset of inclusions in the SNpc displays remarkably consistent lamellar-appearing organizations [54, 67, 71, 72]. By combining multiple labeling approaches with STED microscopy, a recent study demonstrated recurring distributions of antibody labeling for αSyn epitopes in core-shell inclusion morphologies both within

and between PD patients [54]. In particular, the distribution of antibodies against Ser129p αSyn was localized at the periphery of such inclusions while truncated αSyn species were enriched particularly in their core, in line with a previous study [71]. In addition, novel antibodies specifically directed against the C-terminal and N-terminal regions of αSyn showed a differential distribution at the periphery and core of this inclusion subtype, respectively. This separation of epitopes with specific biochemical properties in LBs may suggests gradual local differences in their composition (i.e., solubility; hydrophobicity; other biochemical/electrostatic factors).

Moreover, such layered morphologies were generally paired with immunoreactivity for antibodies against neurofilament and beta-tubulin, revealing a structured cage-like organization of cytoskeletal components, and the peripheral arrangement of mitochondria [53, 54]. While the abundance of neurofilaments particularly at LB periphery has been previously reported in studies using conventional confocal microscopy [71, 72], the superior resolution of STED imaging further revealed that neurofilaments are not only incorporated in LBs but intricately arranged in a structured-appearing framework [54].

Interestingly, similar cage-like structures by the redistribution of cytoskeletal elements (e.g., vimentin) have been described in aggresomes, which are hierarchically organized proteinaceous inclusion bodies that form *in vitro* upon proteasomal inhibition [73]. Moreover, similar to LBs, aggresomes recruit markers for proteolysis (i.e., ubiquitin, p62, and HDAC6). Additionally, the peripheral alignment of mitochondria observed in LBs has also been described as a feature of aggresomes [73]. Together, these similarities led to the hypothesis that LBs represent failed aggresomes [74]. As it is doubtful whether LBs are degraded in the brain, the encapsulation of accumulated material by cytoskeletal elements may still represent an active process to stabilize inclusions, and to shield the cellular cytoplasm from their toxic microenvironments. Furthermore, the transition towards morphologies was speculated to favor compaction of expanding inclusions [18]. Although such suggestions of 'compaction-like' maturation of LBs has been repeatedly suggested in the studies based on observations in the human brain [18, 75], this still needs to be recapitulated in model systems of inclusion formation.

Further, while the resemblance of consistent midbrain-type 'classical' LBs to aggresomes is thus compelling, these features are less evident or absent for other morphologies such as pale bodies, respectively, raising questions about the relevance of common pathways between LBs and aggresomes in LB biogenesis. Together, these observations suggest there may be a convergence of various heterogeneous inclusions into a hierarchical, 'end-point' state, highlighting the LB maturation and morphogenesis are highly regulated processes beyond the aggregation of αSyn.

Explaining morphological heterogeneity of LBs demands the integration of conceptual models

The sections above highlight that PD-relevant inclusions such as LBs form a wide spectrum of morphologies, containing complex and heterogenous mixtures of structural components, including many different proteins, lipids, and membrane-bound organelles. This range of morphological manifestations generated discussion in the field [24], particularly as the classical view suggests that aggregated αSyn fibrils are a starting point for LB formation (extensively reviewed in, e.g., [76]). Moreover, propagation and cytoplasmic seeding of exogenous αSyn fibrils led to development of valuable cellular and animal models [47–51].

However, while a subset of inclusions displays prominent radiating filaments readily recognized in EM, this feature is less pronounced in some lipid-rich inclusion types that were detected by αSyn immunolabeling in CLEM experiments [53]. The less prominent appearance of fibrils in αSyn-immunopositive inclusions might be in part explained by the challenging detection of short αSyn filaments [24]. Yet, the biogenesis of inclusions may not be fully captured by paradigms centered around cytoplasmic αSyn aggregation. Therefore, the development of additional conceptual frameworks and their validation in experimental models is needed to explain the observed morphological and ultrastructural heterogeneity of LB-like inclusions seen in the brains of PD patients, including morphologies without apparent or prominent filamentous structures.

Liquid-liquid phase separation: a potential bridge between αSyn physiology at synapses and Lewy bodies

One possible scenario that we will discuss here, is that initial stages of LB formation may origi-

nate following demixing of various components from the crowded perinuclear cytoplasm by liquid-liquid phase separation (LLPS), forming biomolecular condensates. In recent years, LLPS has emerged as a major mechanism for the formation of functional intracellular compartments and pathological inclusions in various neurodegenerative diseases [77, 78]. Moreover, some recent findings have attributed a role for LLPS in the biology of αSyn, the main specific constituent of LBs, in physiological and pathological contexts [79–81].

In the context of biology, LLPS is a process in which macromolecules demix from a surrounding environment into a dense phase, non-limited by a membrane or a scaffold. To undergo LLPS, macromolecules need to be able to engage in multivalent, low-affinity interactions at the concentration above the so-called critical concentration that will allow for their demixing into a dense, condensed phase [82]. Initially, proteins containing intrinsically disordered regions, regions that do not fold into any stable secondary or tertiary structure, were perceived as the key drivers of LLPS [83]. However, it is clear by now that many well-folded motives can also undergo LLPS [84]. Moreover, RNAs and even the entire membrane-bound organelles, such as synaptic vesicles (SVs), are now known to form biomolecular condensates [85, 86]. Similar to membrane-bound organelles, many of the biomolecular condensates play an important role in cellular physiology. For instance, in neurobiology, LLPS is implicated in the organization of both presynaptic boutons and postsynaptic sites [87, 88]. There are several functional consequences from distinct synaptic territories organizing into condensates, as this allows for the local coalescence of organelles and proteins into functional units generating sufficient force for membrane remodeling [89, 90] or clustering of signaling proteins and receptors [91, 92], to name a few.

The total αSyn pool is in a dynamic equilibrium between free (soluble) and membrane-bound forms [93], which might explain its diverse functions such as vesicular transport, facilitating exocytosis, help the turnover of SVs at the synapse, or controlling vesicle distribution among distinct functional pools (reviewed in detail in [1]). The N-terminal region of αSyn, which contains 11-amino acid repeats, is highly conserved and adopts an alpha-helical secondary structure when bound to phospholipids [94]. Early studies also showed that αSyn can bridge several small lipid vesicles into microclusters [95]. Moreover, αSyn itself was demonstrated to have the ability to undergo LLPS albeit at very high, non-physiological concentrations and crowding conditions [79]. While such conditions are impertinent to αSyn concentrations in synaptic boutons [96], the recent data indicate that synapsin-driven SV condensates can sequester αSyn, locally increasing its concentration substantially [80] (Fig. 2A). While enriched in these synapsin-condensates, αSyn maintains its high mobility (Fig. 2B). In fact, αSyn actively modulates the kinetics and the extent of SV condensation, emphasizing the crucial relevance of molar ratios (i.e., not only which proteins but also how much of these are biochemically accessible) [80]. This functional coupling between the local concentration of αSyn and synapsins is essential for proper neurotransmission and the synaptic vesicle cycle [4].

One of the central aspects of biomolecular condensates is their reversibility in response to physiological stimuli that could trigger their formation or dissolution. These stimuli often include posttranslational modifications (e.g., depolarization-driven activity of CaMKII at the presynapse [97] or spatial localization of specific proteins that can amend the condensate [98]. Importantly, failure to regulate the formation of condensates leads to their transition into insoluble aggregates [77]. This process was shown for numerous neuronal proteins implicated in the formation of inclusion bodies relevant to the pathology of amyotrophic lateral sclerosis and frontotemporal dementia [99], in which TDP-43, FUS, hnRNPA1 form complex, heterogeneous RNA-containing granules. Interestingly, within synaptic boutons (i.e., within SV condensates), αSyn can also form amyloid-like structures in pathology [81]. In fact, *in vitro* analysis suggested that αSyn undergoes LLPS on path to the formation of pre-formed fibrils [100]. However, this does not necessarily extrapolate into the formation of inclusions, which may not all contain αSyn fibrils. In this regard, antibodies against αSyn's intact C-terminus and pS129 immunoreactivities were shown to accumulate mainly at the interface of midbrain-type LBs (Fig. 2C) [54]. Together, less prominent abundance of filamentous components in αSyn-containing inclusions and stereotypic distributions of Ser129-p at the interface of midbrain-type LBs, are conceptually important observations indicating that the chemical properties of αSyn prompt a new outlook at LB morphogenesis.

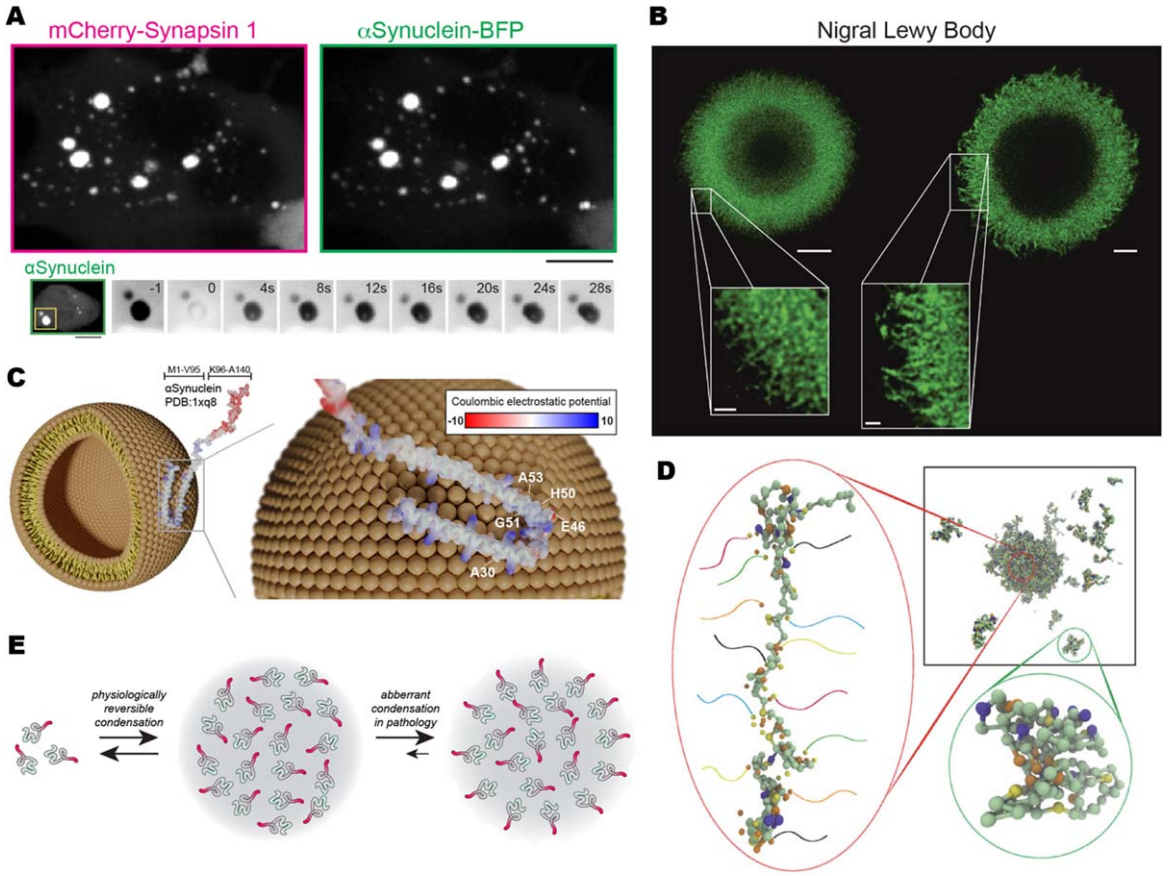

Fig. 2. αSyn as a potential surfactant of complex inclusions in synaptic physiology and Lewy body pathology. A) Synaptic condensates readily recruit α-synuclein. Top: αSyn interacts with synapsin 1 droplets in HEK cells. Bottom: αSyn readily recovers fluorescence after photobleaching indicating its high mobility in condensates. B) 3D reconstruction of a nigral Lewy body indicating the accumulation of phosphorylated αSyn at the interface of pathological inclusions. C) Scheme of the interaction between a lipid vesicle and α-synuclein. The size of folded domains of α-synuclein (PDB: 1XQ8) and lipid vesicle are to scale. αSyn residues mutated in some patients with familial Parkinson's Disease are highlighted in magenta. D) Computational examples of a protein-like polymer indicating the different chemical potential and interaction landscape in a condensate (i.e., dense phase; red circle) in comparison to a dilute phase (green circle). E) A model suggesting that soluble proteins such as αSyn reversibly form condensates in heathy cells. In pathology, however, the proteins within a condensate might undergo internal rearrangements with an ensemble of molecules accumulating with a stereotypic orientation at the interface resulting in the formation of an outer layer and reducing the overall reversibility of condensates. The images in A, B and D are modified from Hoffmann et al., 2021 [80]; Moors et al., 2021 [54]; Farag et al., 2022 [114], where the images were published under a CC-BY 4.0 license.

LEWY BODY MORPHOGENESIS: α-SYNUCLEIN AS A POTENTIAL SURFACTANT OF LEWY BODIES?

Several chemical features and physiological functions of αSyn suggest it could act as a potential surfactant of LBs. Surfactants are molecules that modulate the interface (i.e., the surface tension) between two phases (liquid/liquid or liquid/solid) [101]. In the context of LBs, it would refer to a protein that modulates the interface between the pathological inclusion and the surrounding cytosol.

αSyn is an intriguing predominantly synaptic protein comprised of an amphipathic, membrane-binding region and a highly charged, acidic tail (Fig. 2C) [102]. With only 140 amino acids, αSyn is intrinsically disordered but simultaneously can form an alpha-helix upon binding to negatively charged membranes [103]. The full-length αSyn protein can be divided into three domains: the N-terminal domain, the Non-Amyloid-Component (NAC) region, and the C-terminal domain. The NAC region, which is centrally hydrophobic, is linked to the protein's propensity to adopt a beta-sheet

configuration and form amyloid fibrils [104, 105]. The C-terminal domain is highly acidic and proline-rich and fully lacks a stable tertiary structure. αSyn interacts stably with negatively charged synthetic phospholipid membranes [5, 106] as well as biological membranes such as neuronal membranes [107, 108], native synaptic vesicles [109] (Fig. 2C), with particular affinity for highly curved vesicles (i.e., high surface-to-volume ratio) [110]. The association of αSyn with negatively charged membranes promotes the formation of alpha-helix form even if it is structurally unfolded in solution [103, 111].

Proteins undergoing LLPS might exhibit distinct chemical properties depending on whether they are in a dilute phase, such as cytosol, or within a condensate or inclusion [112, 113]. Thus, αSyn in a dense, condensed phase could engage in a range of heterotypic interactions both with proteins and with membranes distinct from the interaction in a surrounding, dilute phase. For instance, theoretical and computational analyses of a protein-like polymer indicate the different interaction landscape of a same protein when it is in a condensate (i.e., dense phase) in comparison to a dilute phase (Fig. 2D) [114]. With respect to membranes, physiologically, αSyn is an abundant protein at the SV clusters [115]. Here, αSyn was shown to form oligomers on the surface of SVs, and additionally interacts with beta- and gamma-synucleins enhancing SV binding and raising the overall local concentration of synucleins [116].

The acute perturbations of αSyn suggests synucleins crosslink small vesicles and influence the structure of SV clusters [117]. This is further substantiated by the observations that, in animals, the genetic deletion of all three synucleins resulted in the exact opposite phenotype to the deletion of synapsins [118, 119]. *In situ* investigations of nerve terminals in animals lacking all three synucleins revealed a highly organized three-dimensional packing of SVs, in contrast to the wild-type situation. The presence of SV clusters is not surprising given that synuclein knockout animals still expressed synapsin, which is both necessary and sufficient for SV cluster formation [120]. Synuclein triple-KO animals have smaller synaptic terminals [121], which could in part explain the tight packing; nonetheless, the highly-ordered architecture of these clusters reveals a crucial change in their material properties and suggest αSyn acting as a 'molecular lubricant' of SV condensates [80]. Herein, SV membranes may provide a direct platform for synapsin and αSyn interaction, particularly via their intrinsically disordered regions. The overexpression of αSyn in wild-type murine synapses reduces SV release and recycling [4]. However, when αSyn is overexpressed in neurons generated from synapsin TKO mice, this phenotype is missing. This further strengthens the notion that SV mobility and cluster density are dependent on a delicate equilibrium of synapsin and αSyn concentrations.

In PD pathology, LBs and their precursors are structures with a heterogeneous composition including a range of different proteins and membrane organelles trapped within these inclusions [53]. Hence, an obvious question arises: can a heterogeneity in LB composition be partially explained by (non)specific trapping of client proteins into αSyn-containing condensates? It is tempting to speculate that αSyn, as a key component of LBs, sequesters many proteins and organelles into these inclusions. Indeed, immunohistochemical studies have revealed that LBs include more than 90 molecules, many of which are interactors of αSyn, including PD-linked gene products (e.g., DJ-1, LRRK2, Synphilin 1, parkin, and PINK-1), mitochondria-related proteins, and molecules implicated in the ubiquitin–proteasome system, autophagy, and aggresome formation [17, 122]. A few studies used mass spectrometry to obtain complete, unbiased proteomic profiles of LBs from people with PD brains. Proteomic analyses of isolated cortical LBs, for example, revealed nearly 300 proteins involved in a variety of cellular processes, including the ubiquitin-proteasome system, folding and intracellular trafficking, oxidative stress, synaptic transmission, and vesicular transport, signal transduction, and apoptosis [57, 123]. In addition, membranous fragments, organelles, vesicular structures, and other subtypes of lipid contents have been increasingly appreciated as major components of LBs [53].

The interfaces of biomolecular condensates have recently been shown to enable the accumulation of electric potential [124], which allows these surfaces to act as the chemical reaction centers both in physiology [125] and in pathology [126]. While at the synapse, together with SVs and other synaptic proteins, αSyn forms functional condensates that are readily reversible upon depolarizations, it is tempting to speculate that in the LB pathology, αSyn may undergo internal rearrangements within the inclusion over time leading to the formation of irreversible condensates (Fig. 2E). In this scenario, an ensemble of αSyn molecules would accumulate in a stereotypic orientation, leading to the local inhomogeneity and

an apparent formation of the outer layer of proteins. The evidence for a such a non-uniform orientation is supported by unique antibody staining patterns often observed in LBs, where antibodies against the C-terminal of αSyn tend to decorate the outer layer and the N-terminal antibodies stain the core of LBs [54].

While membranous structures in the center of a Lewy body were described as being coated with a high amount of possibly non-fibrillar αSyn molecules, obvious radiating filamentous structures were only observed in a subset of all LBs analyzed by CLEM [53]. An increasingly dense chemical environment during LB maturation might generate a permissive chemical environment that would propagate the formation of αSyn fibrils over time [127]. Hence, LBs and αSyn fibrils might be two distinct cellular manifestations of the disease that formed by distinct mechanistic pathways but can help propagate each other, which is supported by fibril-based experimental model systems.

OUTLOOK: CHALLENGES AND OPPORTUNITIES WITH THE CURRENT MODEL SYSTEMS FOR αSYN INCLUSIONS

In the past decades, significant efforts have been made to generate model systems that recapitulate LB-like inclusions. Various strategies have yielded some success in generating inclusions in cellular and animal models of PD, although their resemblance to LBs in the human brain is not always clear partially because of insufficient characterization [6]. However, in recent years, substantial progress has been made to model inclusions following the hypothesis that in PD αSyn may aggregate and form oligomeric and fibrillar conformers that are able to seed and propagate inclusion pathology. Indeed, when fibrils formed *in vitro* from recombinant αSyn (pre-formed fibrils; PFFs) are administered to cellular systems and animal models, this caused propagation of αSyn pathologies paired with neurodegeneration [47–51]. Moreover, the administration of αSyn PFFs to primary hippocampal mouse neurons resulted in the formation and maturation of perinuclear inclusions that resembled various aspects of LBs after 21 days, including core-shell Ser129p αSyn-containing morphologies, trapped vesicular/membranous structures, and immunoreactivity to LB-associated markers such as ubiquitin and p62 [51]. In addition, a variety of additional inclusion morphologies was observed, including filamentous neuritic inclusions and ribbon-like somatic inclusions.

These PFF-based studies have provided important proof of concept evidence that fibrils of αSyn can induce formation of inclusion bodies. However, a clear limitation of this model system is the requirement for exogenously synthesized fibrils from recombinant αSyn, which further have different cryo-EM structures compared to fibrils isolated from brains of PD patients [128]. Thereby, PFF-based models do not address spontaneous inclusion formation mechanisms or (early-stage) inclusion subtypes that do not contain fibrils. Still, these models provide important information about the mechanisms involved in processing of αSyn fibrils and their incorporation in inclusions over time, which may be particularly relevant during advanced disease stages.

Additional models thus may be required to recapitulate spontaneous formation and early stages of αSyn-positive inclusions that do not involve obvious fibrillar organizations. Numerous attempts have been made to generate inclusions in cellular systems based on αSyn overexpression, in analogy with duplications and triplications of αSyn causing PD (reviewed in detail in [6]). While the formation of (mainly diffuse-appearing) somatic inclusions has been reported in such models, these inclusions did generally not develop into filamentous or more hierarchically organized (i.e., core-shell) morphologies [6]. Notably, in order to stimulate αSyn clustering and accumulation in cell models, a recent study applied αSyn fused to cryptochrome protein 2, generating a light-inducible protein aggregation (LIPA) model of αSyn. Inclusions of insoluble αSyn were rich in fibrillar and crowded lipid membranous material, vesicular and organellar structures and showed immunoreactivity for commonly used LB markers (including ubiquitin, Ser129p αSyn) [56].

Other studies have attempted to trigger αSyn aggregation and inclusion formation by inducing proteasomal impairment using toxins or selective inhibitors (e.g., MG132), leading to the formation of inclusions, particularly when applied in combination with αSyn overexpression [129–134]. Similar to LBs, these inclusions resembled certain aspects of aggresomes, including cytoskeletal cage-like structures [133]. Interestingly, co-expression of αSyn with synphilin also stimulated formation of aggresome-resembling inclusions [135–137].

In addition to models that focus on the accumulation of αSyn and other proteins, the attention on their co-accumulation with lipids in inclusions is grow-

ing. The stimulating effect of membrane abundance on αSyn aggregation *in vitro* is well-studied [138]. For instance, overexpression of αSyn in yeast caused accumulation of lipid (droplet)-rich inclusions [139, 140]. Moreover, the presence of membranous components in inclusions of αSyn-overexpressing model systems has been repeatedly reported. Recent studies have applied targeted mutagenesis strategies to enhance αSyn:lipid interaction, in order to initiate spontaneous inclusion formation. For instance, expression of 3K αSyn - a biochemical amplification of the familial PD-causing E46K mutation by additional E→K mutations in neighboring repeat motifs (E35K;E46K;E61K)- in cellular and mouse models led to the formation of cytoplasmic inclusions rich in vesicular and membranous structures [141–143]. Although this mutation does not occur in people, such approaches allow to study the role of αSyn:lipid accumulation in spontaneous inclusion formation.

Taken together, in the past decades various model systems have been developed to study the process of αSyn-related inclusion formation. These models lead to different morphological features, possibly mimicking different aspects and (possibly) stages of αSyn-containing inclusions. An important note here is to consider confounding effects of protein tags (such as GFP), which can impact aggregation of αSyn and stimulate LLPS, altering the dynamics/morphologies of inclusion formation [144]. While none of the current models are able to recapitulate all aspects (e.g., spontaneous occurrence, maturation, morphogenesis) of LBs, the information resulting from each of these models helps in putting forward hypotheses and experimental strategies to bridge the current gaps in our knowledge of LB pathogenesis. For instance, mechanistic models can offer important insights in the early dynamics and processes underlying inclusion formation, mechanisms and factors involved in inclusion maturation, and their effects on cellular survival. To further aid this, detailed characterization and precise descriptions of modeled inclusions are pivotal for the effective translation of findings in model systems to the human brain and vice versa.

Moreover, concepts of LLPS provide a new opportunity for examining αSyn-containing heterogenous inclusions as a consequence of the faulty condensate-membrane interactions [145]. Biochemical and live-cell imaging approaches, coupled to developments in patient-derived iPSCs, hold promise to provide a platform for investigating αSyn-containing inclusions and their biophysical properties and dynamic remodeling by post-translational modifications, and their interactome in different cell/neuron types. Such a heterogenous composition of mature LB-like inclusions composed of proteins, fragmented membranes, vesicular structures, and dysmorphic organelles (mitochondria, lysosomes, and autophagosomes) could provide an environment for fibril formation and may be an attractive target for pharmacological interventions.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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