



Review

Do Lewy bodies contain alpha-synuclein fibrils? and Does it matter? A brief history and critical analysis of recent reports

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A B S T R A C T

Several lines of evidence from neuropathological studies, human genetics, *in vitro* aggregation studies and cellular and animal models support the hypothesis that aSyn plays a central role in the formation of Lewy pathologies. These are cytoplasmic proteinaceous and lipid-rich inclusions that represent key pathological hallmarks of Parkinson's disease (PD) and other neurodegenerative diseases, collectively referred to as synucleinopathies. For decades, light microscopy and electron microscopy studies of these inclusions have consistently shown that they are rich in filamentous structures that exhibit distinct distribution and organizational patterns depending on where they occur in the brain (e.g., classical brain-stem Lewy bodies (LBs) and cortical LBs) and the type of synucleinopathies. Although the identity of the protein that form these filaments was a subject of debate for decades, the discovery of PD-linked aSyn mutations, the demonstration that LBs are enriched in insoluble forms of aSyn, and the ability of aSyn to form fibrils of similar dimensions have led to convergence on the hypothesis that aSyn fibrils are key components of LBs. In a recent study, Shahmoradian et al used a combination of advanced electron microscopy and immunofluorescence based imaging techniques to investigate the structure, composition, and architecture of LBs from postmortem brain tissues of individuals with PD or other synucleinopathies (Shahmoradian et al., 2019). The paper's main conclusions suggest that "lipid membrane fragments and distorted organelles together with a non-fibrillar form of aSyn are the main structural building blocks for the formation of Lewy pathology". Their proposal that LBs are devoid of aSyn fibrils or that LB formation occurs independently of aSyn fibril formation casts doubts on a substantial body of work that forms the foundation of many of the current basic and translational research programs in academia and industry. In this article, I present a critical analysis of their data and claims in the context of the existing literature. In addition, I examine the extent to which their findings and proposed models of the mechanisms of LB formation are consistent with existing data and are supported by other experimental evidence. The results from this analysis caution against overinterpretation of observations from a single report, especially given the limitations of the techniques and experimental approaches used by Shahmoradian et al and for more collaborative and systematic efforts to revisit and characterize LBs and other aSyn pathologies in the brain pathologies at the biochemical, morphological and structural level.

1. Introduction

It has been more than 100 years since Lewy bodies (LBs) were first discovered (Lewy, 1912; Tretiakoff, 1919) and ~23 years since the first report demonstrating the presence of alpha-synuclein (aSyn) within LBs (Spillantini et al., 1997). However, it seems that we are just beginning to scratch the surface and understand what LBs are made of, how they are formed and whether they are the cause or a consequence of Parkinson's disease (PD). Given that LBs are an invariant feature of PD, there has been a consensus since their discovery that understanding the

molecules and molecular events involved in their formation and their relation to disease progression could pave the way for elucidating their role in PD and possibly lead to novel therapies.

"Those of us interested in the peculiar inclusions of Lewy have reason to hope that unraveling how these bodies are formed may hold a clue to the nerve cell death and to its prevention" (Lysia Forno, 1996).

Therefore, early efforts were directed towards elucidating the biochemical composition and structural properties of LBs from different

Abbreviations: aSyn, alpha-synuclein; AD, Alzheimer's disease; PD, Parkinson's disease; MSA, multiple system atrophy; PFFs, preformed fibrils; EM, electron microscopy; CLEM, correlative light electron microscopy; STED, stimulated emission depletion microscopy

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affected brain regions of patients with PD and dementia with Lewy bodies (DLB). Between 1960 and 1997, i.e., prior to the discovery of PD-linked aSyn mutations, the development of protocols for isolating LBs, this was achieved primarily via a combination of histochemical and immunocytochemical approaches using a limited set of antibodies and chemical dyes that recognize specific proteins (ubiquitin and neurofilaments), lipids or filamentous structures (e.g., neurofilaments and paired helical filaments). Many of these molecules were selected as candidates because they were commonly found in pathological aggregates associated with other neurodegenerative diseases, including Alzheimer's disease (AD). Ultrastructural characterization of LBs was primarily carried out using a combination of light microscopy and electron microscopy (EM), which was later combined with immunogold labeling techniques. Between 1998 and 2018, the only major change that influenced the approaches by which LBs and Lewy pathologies are studied was the development of an expanded toolkit of antibodies against aSyn and other proteins found in LBs. These antibodies have enabled 1) more expanded profiling of the composition of LBs; 2) greater appreciation of the diversity of Lewy pathologies; and 3) more precise mapping of the distribution of different proteins within LBs using advanced imaging techniques (Shahmoradian et al., 2019; Goldman et al., 1983; Manetto et al., 1988; Uryu et al., 2006; Lennox et al., 1989; Kuzuhara et al., 1988; Kuusisto et al., 2003; Gai et al., 2000; Moors et al., 2018; Shahmoradian et al., 2017; Moors et al., 2019).

More recently, using a combination of advanced EM (i.e., correlative light electron microscopy (CLEM)) and immunofluorescence-based microscopy techniques (stimulated emission depletion microscopy (STED)), Shahmoradian et al sought to determine the structure, composition, and architecture of LBs from postmortem brain tissues obtained within 1 h after the death of individuals with PD or other synucleinopathies. The paper's main conclusions challenge the current thinking and evidence supporting a central role of aSyn fibrillation in LB formation and suggest that "lipid membrane fragments and distorted organelles together with a non-fibrillar form of aSyn are the main structural building blocks for the formation of Lewy pathology". Essentially, the paper makes three claims, namely, that LBs 1) are rich in lipids; 2) are composed primarily of membrane fragments and membranous organelles; and 3) do not contain fibrillar aSyn aggregates.

When discoveries that challenge this body of evidence emerge, it is crucial that we pause and review the evidence with an open mind. It is also important to carefully assess the work and claims in the context of the existing literature and to have constructive discussions and debates aimed at resolving any controversies. It is hoped that this will also help to form a consensus for future experiments to test or disprove current hypotheses of the role of aSyn in LB formation. In this spirit, I present a critical analysis of the work and main conclusions by Shahmoradian et al (Shahmoradian et al., 2019).

2. Background

LBs are cytoplasmic inclusions that were first discovered in the brain by Fridrich Heinrich Lewy in 1912 (Lewy, 1912) but were only linked to the pathogenesis of PD a few years later by Tretiakoff, who then named them after Lewy (Tretiakoff, 1919). Duffy and Tennyson were the first to report on the ultrastructural characterization of LBs from the substantia nigra and locus coeruleus using EM (Duffy and Tennyson, 1965). LB-like inclusions were first identified by phase contrast microscopy and were then sectioned and imaged by EM, similar to the approach currently used in CLEM (Shahmoradian et al., 2019; Mahul-Mellier et al., 2018; Mahul-Mellier et al., 2019). Based on their investigation of the morphology of 102 LBs via phase-contrast microscopy, Duffy and Tennyson described and defined a LB as follows: "The Lewy body in its simplest form consisted of an inner core, which appeared moderately dense, and an outer zone composed of radially oriented filaments" (Fig. 1). They also reported other more complex forms of LBs

that they referred to as multilaminated LBs that "had a dense core, surrounded by multiple concentric laminae of alternating densities". Their EM data provided compelling evidence that the different types of LBs are rich in filamentous structures that are organized in a highly specific manner. In subsequent studies, several groups reported similar findings and architectures for LB. The variation in the appearance and laminar architecture of the LBs was attributed to variations in the number, packing and organization packing of filaments, see below. This type of LB was consistently observed in the vast majority of subsequent studies on LBs; it is commonly found in the brain stem and referred to as "a classical LB". However, the exact composition of the dense core remained unclear. Several studies have suggested that the core is an area where the filaments intersect and are densely packed (Spillantini, 1998). Others have suggested that dense vesicular structures and other molecules (e.g., lipids) also accumulate in the core (Smith and Perry, 1995). These experimental observations revealed that LBs are not made of a single protein but contain a complex mixture of proteins, lipids, filamentous structures, metal ions, and organelles.

The presence of filaments in LBs has been one of the most consistent observations since their discovery. However, the nature of the protein that forms these filaments has been debated for several decades. Given the filamentous nature of the radiating structures in LBs and the implication of neurofilaments in neurofibrillary tangle formation in AD pathology at that time, it was hypothesized that LBs are made of neurofilaments or, alternatively, that neurofilaments contribute to their formation. Indeed, prior to the discovery of aSyn disease-associated mutations in 1997 and the identification of aSyn as a key component of LBs, ubiquitin, and neurofilament components were the two main proteins associated with LBs (Goldman et al., 1983; Lennox et al., 1989; Kuzuhara et al., 1988; Schmidt et al., 1991; Pollanen et al., 1992; Iwatsubo et al., 1996). In 1983, Goldman and colleagues performed immunohistochemistry on LBs using antibodies against different filamentous proteins of the central nervous system (CNS) and showed that LBs are rich in neurofilaments (Goldman et al., 1983). These findings were confirmed by Galloway et al and others, who showed that multiple antibodies against neurofilaments labeled LBs (Hill et al., 1991; Galloway et al., 1988). Until 1997, neurofilaments were still thought to be a major constituent of LBs (Trojanowski and Lee, 1998). However, the role of neurofilaments in LB formation and maturation remained unclear. Some filaments observed within LBs exhibited dimensions similar to those of neurofilaments, while others showed variable diameters ranging from 7 to 13 nm (Spillantini, 1998; Forno, 1986; Arima et al., 1998). In addition, several groups reported that different antibodies against nonphosphorylated and phosphorylated neurofilaments stained the periphery of LBs (Watanabe et al., 1977). However, several subsequent studies showed a lack of staining or faint staining of LBs by antibodies against neurofilaments, suggesting that, while neurofilaments may be present, they are not the predominant filamentous structures in LBs (Iwatsubo et al., 1996). The observation that neurofilaments in LBs were soluble in sodium dodecyl sulfate (SDS) whereas LBs were SDS-resistant, combined with the observation that LBs did not show positive staining with silver stains, which bind and stain neurofilaments, led many researchers to exclude neurofilaments as the primary constituent of LBs or the filamentous structures in the periphery of LBs. This led Iwatsubo et al to suggest that the filaments in LBs are made of one or more other proteins (Iwatsubo et al., 1996). In 1977, Watanabe et al reported that neurofilaments are localized in the LB periphery and surround LBs. Several subsequent studies confirmed these findings (Watanabe et al., 1977), suggesting that they could play a role in the LB formation, stabilization and/or maturation.

2.1. Alpha-Synuclein as a component of LBs

The discovery of PD-associated mutations in the aSyn gene (Kruger et al., 1998; Polymeropoulos et al., 1997; Polymeropoulos et al., 1996), the prompt demonstration that aSyn is a major component of LBs

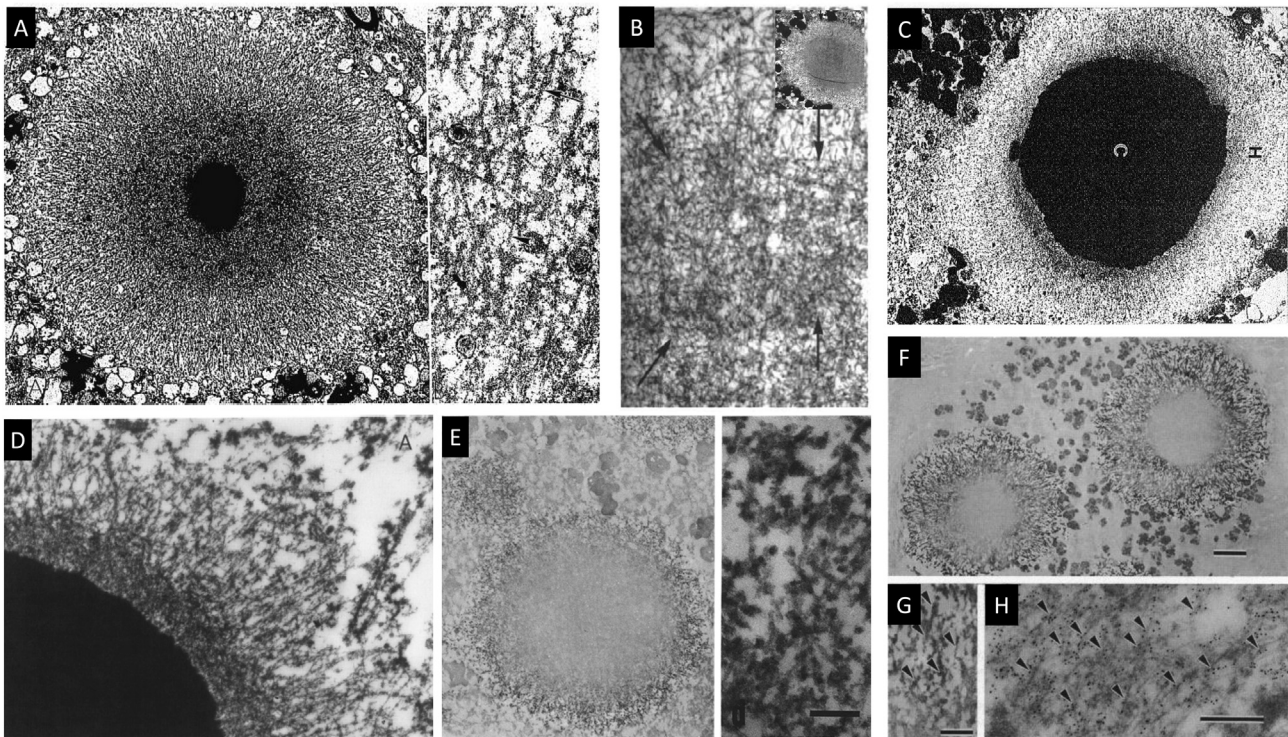


Fig. 1. Ultrastructure of LBs: A collection of published images of classical LBs from different studies revealing their common features of a dense core and radiating filaments. Independent of the nature of the protein that makes up the filaments, the images show that classical LB structures are rich in filamentous structures. Panel F presents one example showing that these filaments can be labeled with immunogold particles in LBs. A) EM image of a LB in the substantia nigra. In addition to the dense core and radiating filaments, vesicles and mitochondria in the peripheral region are clearly visible, as noted in (Forno, 1996). The right panel shows an enlarged image of the filaments and highlights the presence of filaments of different diameters (adapted from (Forno, 1996)). B) EM images of dense filaments from a LB in the substantia nigra analyzed in (Duffy and Tennyson, 1965). C) EM image of a LB from a patient with PD (adapted from (Smith and Perry, 1995)). D) EM image of a LB in intact PD brain tissue (Galloway et al., 1992). E) EM of LBs in the substantia nigra of a patient with DLBD. On the right is an enlarged image of the radiating filaments (adapted from (Kuzuhara et al., 1988)). F) EM image of two brain stem LBs. G) Enlarged image of the periphery of one of the LBs in panel F. H) Immunogold labeling of purified cortical LBs using mouse monoclonal antibodies (mAbs) specific for LBs purified from DLBD brains. The image shows specific immunoreactivity with aSyn associated with these filaments. (The images in panels F, G and H were adapted from (Baba et al., 1998).)

(Spillantini et al., 1997; Spillantini et al., 1998; Crowther et al., 2000; Irizarry et al., 1998; Mezey et al., 1998; Bayer et al., 1999) and can form filaments with dimensions similar to those of filaments found in LBs *in vitro* (Arima et al., 1998) shifted the interest of the scientific community from understanding the complexity of LBs to understanding the role of aSyn in LB formation. Since that time, studies of LBs have been centered mainly around aSyn and how other components of LBs interact with aSyn or influence its aggregation and/or contribute to LB formation.

In 1997, the same year that the mutations in the aSyn gene were linked to the autosomal dominant form of PD, Spillantini and colleagues demonstrated that antibodies raised against peptides spanning residues 116-131 and 11-34 of human aSyn stained substantia nigra from idiopathic PD and cingulate cortex from DLBD brains (Spillantini et al., 1997). Interestingly, these antibodies revealed cortical LBs with similar morphology to those detected with ubiquitin and neurofilament antibodies. The fact that antibodies against both the extreme N- and C-terminus of aSyn immunostained LBs suggested that LBs are made of the full-length protein (Spillantini et al., 1997), thus establishing aSyn as a key constituent of LBs. They proposed that the disease-associated mutation A53T could promote the aggregation of aSyn into filaments. Although no direct evidence supporting this hypothesis was presented at the time, subsequent studies by multiple laboratories have consistently shown that A53T and other disease-associated mutations (A30P, E46K), with the exception of G51D (Rutherford et al., 2014; Rutherford and Giasson, 2015; Fares et al., 2014; Lázaro et al., 2016) and A53E, enhance aSyn oligomerization and/or fibrillization *in vitro* (Rutherford et al., 2014; Conway et al., 2000; Narhi et al., 1999;

Lashuel et al., 2002; Lemkau et al., 2013; Greenbaum et al., 2005; Fredenburg et al., 2007; Khalaf et al., 2014; Ghosh et al., 2013; Mohite et al., 2018; Conway et al., 1998).

One major advance that paved the way for a better understanding of LBs and enabled the development of new tools (antibodies) to define their protein composition, ultrastructural properties and organization was the development of methods to isolate LBs from brain tissues (Iwatsubo et al., 1996). In 1992, Pollanen et al reported on the isolation of intact cortical LBs from the brains of patients with diffuse Lewy body disease (DLBD) and analysis of their composition by western blotting using antibodies against cytoskeletal proteins (Pollanen et al., 1992). In 1996, Iwatsubo T et al reported a method for the purification of LBs from the brains of DLBD patients (Iwatsubo et al., 1996; Baba et al., 1998). This method was later used by the same group to develop monoclonal antibodies that were specific for aSyn and stained LBs. These antibodies were later used to provide the first direct biochemical and immunocytochemical evidence that not only established the presence of aSyn in the brain stem and cortical LBs from patients with sporadic PD but also demonstrated that aSyn is in an aggregated and insoluble state within LBs (Baba et al., 1998). Using light microscopy and EM techniques, these researchers established the presence of filamentous aggregates that could be labeled with immunogold using aSyn-specific antiserum produced using a synthetic peptide spanning residues 104-119 of human aSyn. The EM images revealed an architecture similar to that previously described, with radiating filaments in the periphery (rim) of the LBs. These anti-aSyn antibodies primarily labeled the periphery of LBs, which were rich in filamentous structures. In addition, the gold particles labeled amorphous structures that were

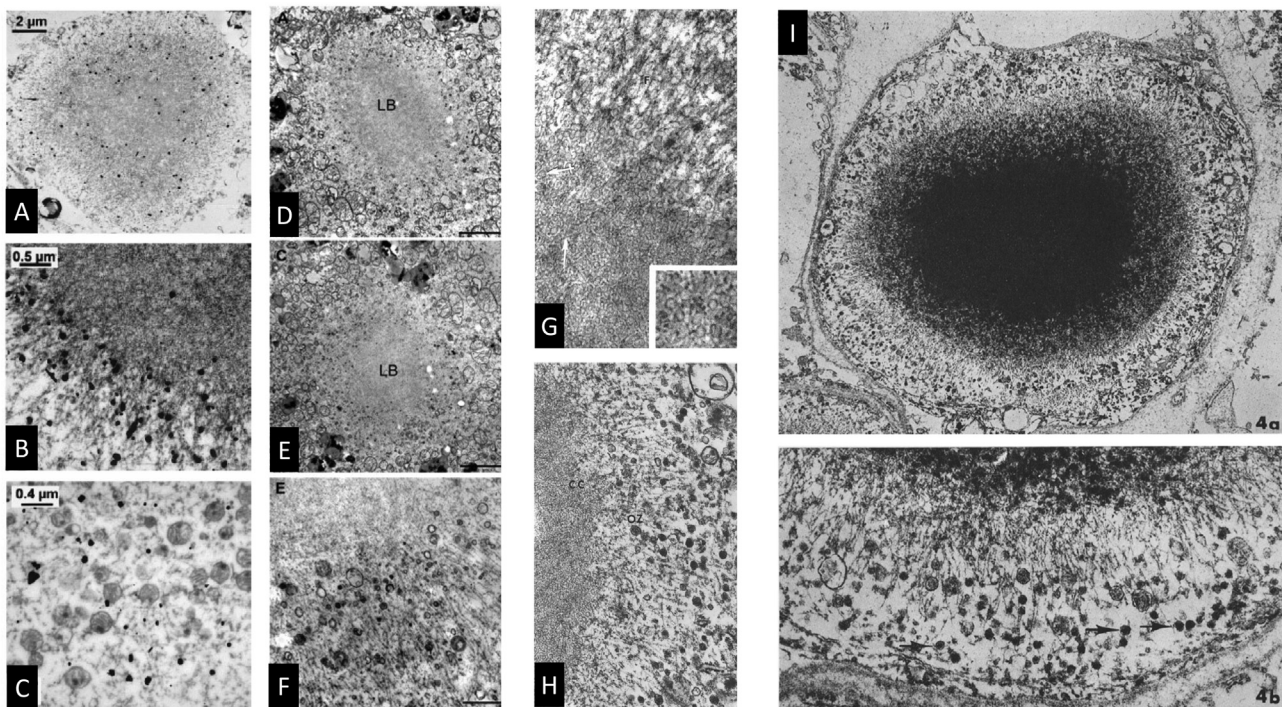


Fig. 2. LB are rich in membranous organelles. A collection of images from previous studies demonstrating the presence of vesicles and other membranous organelles in the periphery of LBs. A-C) EM images of DLB cortical LBs labeled with an anti-ubiquitin antibody showing abundant accumulation of mitochondria and membranous structures in the periphery (adapted from (Gai et al., 2000)). D-F) Electron micrographs of LBs from the substantia nigra of a patient with PD showing abundant and dense accumulation of vesicular structures and membranous organelles (adapted from (Soper et al., 2008)). G) EM image of a LB showing the accumulation of vesicular structures at the interface of the dense core and peripheral filaments (adapted from (Duffy and Tennyson, 1965)). H) EM image of LB in the locus coeruleus by Watanabe et al (Watanabe et al., 1977) confirming the results of previous studies by Forno et al (Forno, 1969) and demonstrating the accumulation of dense-core vesicles around the LB in incidental PD. I) EM image of a granular LB in the stellate ganglion with a concentric structure showing dense accumulation of vesicles in the periphery (adapted from (Forno and Norville, 1976)).

often closely associated with filaments. The quality of the antibodies and imaging procedures at the time did not allow accurate assessment of whether the particles were directly localized on filaments or filament-associated structures. The presence of substantial amounts of high-molecular-weight aggregates in LBs obtained from the insoluble fraction of DLB brain tissues further confirmed that a large percentage of aSyn within LBs exists in a highly stable and SDS-resistant aggregated form. Subsequent studies by Spillantini and colleagues also showed that LBs and Lewy neurites (LNs) from patients with PD or DLB could be stained with both N- and C-terminal-directed anti-aSyn antibodies (Spillantini et al., 1998). They were also able to extract single filaments from LBs and demonstrate by immunogold labeling EM that these filaments become extensively decorated upon incubation with anti-aSyn antibodies (Fig. 3). The quality of the preparations and lack of protein contaminants in the vicinity of the fibrils, combined with the nearby localization of the gold particles, suggested specific labeling of the fibrils. These two studies provided convincing evidence that LBs are enriched with insoluble, filamentous, and aggregated form of aSyn, thus establishing that aSyn is the most likely candidate protein responsible for the formation of the filaments found in LBs. Subsequent studies revealed that neurofilaments surround regions with strong aSyn immunoreactivity in LBs, with stronger localization in the outer layers of LBs.

The detection of aSyn within LBs (Spillantini et al., 1997), the isolation of aSyn fibrils from LBs (Spillantini et al., 1998; Baba et al., 1998), and the discovery that mutations in the gene encoding aSyn cause early-onset forms of PD provided compelling evidence that aSyn fibril formation plays central roles in LB formation and maturation. These observations are also supported by the consistent finding that aSyn forms fibrils *in vitro* that morphologically resemble fibrils isolated from brain-derived LBs (Spillantini et al., 1998; Conway et al., 1998;

Baba et al., 1998; Crowther et al., 1998; El-Agnaf et al., 1998), although recent evidence from limited PD and multiple system atrophy (MSA) cases have shown that brain-derived fibrils exhibit structural features distinct from those generated in cell-free systems (Schweighauser et al., 2020; Strohäker et al., 2019). In addition, aSyn seeding-competent aggregates, oligomers and fibrils are found in the biological fluids (cerebrospinal fluid (CSF) and plasma) of patients with PD and other synucleinopathies, and their seeding activity and structural properties show promising diagnostic potential, including enabling differentiation between PD and other types of synucleinopathies (Fenyi et al., 2019; Groveman et al., 2018; Shahnawaz et al., 2017; Kang et al., 2019; Shahnawaz et al., 2020).

Interestingly, all of the disease-associated variants were shown to enhance aSyn misfolding, oligomerization and/or fibril formation *in vitro*, and their overexpression in some cellular and animal models resulted in the accumulation and aggregation of aSyn (Delenclos et al., 2019; Visanji et al., 2016). Furthermore, the addition of aSyn pre-formed fibrils (PFFs) to mammalian cells (Luk et al., 2009) or primary neuronal cultures (Volpicelli-Daley et al., 2011) or the injection of aSyn PFFs into the brains of rodents (Luk et al., 2012) and nonhuman primates (Shimozawa et al., 2017; Dehay and Bezard, 2019) was shown to be sufficient to induce the fibrillization of endogenous aSyn. Moreover, a recent study from our group showed that the addition of aSyn PFFs (70 nM) to the media of primary neuronal cultures leads to the formation of aSyn fibrils and, eventually, inclusions that bore striking resemblance at the molecular, biochemical and ultrastructural levels to LBs found in the postmortem human brains (Mahul-Mellier et al., 2018; Mahul-Mellier et al., 2020).

Collectively, these convergent observations suggest that aSyn aggregates, fibrils, and LBs are genetically and pathologically validated targets for the development of biomarkers, diagnostic tools and

Tissue Localization	Membrane fragments and organelles ^a	Undefined filaments ^b	Undefined proteinaceous inclusions ^c	Undefined proteinaceous core ^d	Data shown in	Donor
Hippocampal (CA2)	+++	*	0	0	Fig. 1a, 2a, 2c	A
Hippocampal (CA2)	+++	0	0	0	Fig. S6a, 2b, 2d	A
Substantia nigra	+++	*	0	0	Fig. 1b	B
Substantia nigra	+++	*	++	0	Fig. S6b	B
Substantia nigra	+++	*	0	0	Fig. S6c	B
Substantia nigra	++	**	0	n/a	Fig. 3, S7	B
Substantia nigra	++	***	+	X	Fig. 1d, S8	C
Substantia nigra	+++	0	+	0	Fig. 1c	D
Substantia nigra	++	**	0	0	Fig. S6e	D
Substantia nigra	+	***⊙	***⊙	0	Fig. S6d	D
Substantia nigra	+++	**	0	0	Fig. S9	D
Substantia nigra	+++	**	0	0	Fig. S10	E
Substantia nigra	+++	**⊙	**⊙	0	Fig. S11	D
Substantia nigra	+++	**	0	0	Fig. S12	E
Substantia nigra ^N	+++	0	0	n/a	Fig. S6f	D
Substantia nigra ^N	+++	**	0	n/a	Fig. 4	E
Substantia nigra ^N	++	**	+++	n/a	Fig. 3a,b	B

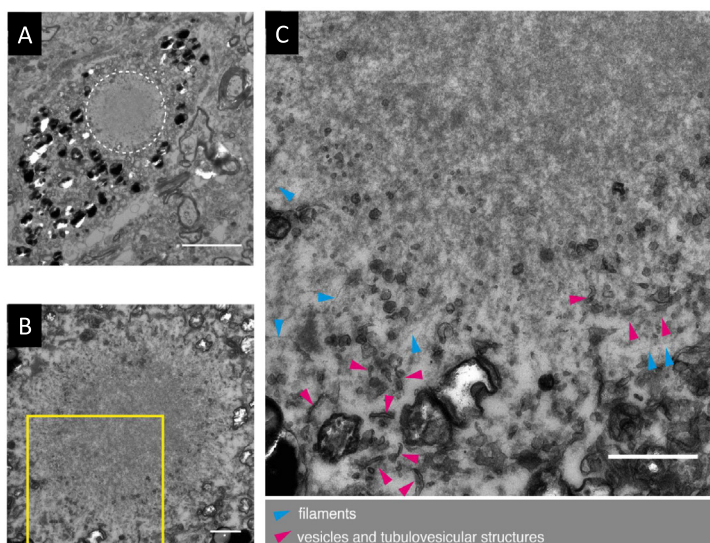


Fig. 3. The vast majority of LBs examined by Shahmoradian et al (Shahmoradian et al., 2019) contained filaments. A table from (Shahmoradian et al., 2019) summarizing the properties of the 17 LBs that they investigated. As shown underneath (“undefined filaments”), 14 of the 17 LBs examined contained filaments. However, whether the variation in the filament abundance reflects the true abundance or indicates that the sections of the LBs examined did not contain a substantial number of filaments is unclear. A-C) electron micrographs of an aSyn immunopositive inclusion aggregates at low (A) and higher magnifications (B and C) showing predominantly filamentous aggregates and the presence of vesicles and mitochondria at the periphery. Scale bars a = 5 μm; b, c = 1 μm. All panels were reproduced with permission from (Shahmoradian et al., 2019).

therapeutic strategies for PD. Therefore, unsurprisingly, the vast majority of aSyn research and development programs in both academia and industry are aimed at developing strategies to block aSyn aggregation, toxicity, pathology formation and spreading in the brain and measuring the levels of different pathology-related aSyn species in biological fluids (e.g., CSF, plasma, saliva). Whether LB formation is pathogenic or protective remains a subject of active research and intense debate. One of the main challenges in addressing this question is the lack of animal and cellular models that faithfully and simultaneously reproduce key cardinal features of PD, the formation of LBs and neurodegeneration.

3. Do LBs contain aSyn fibrils?

Shahmoradian et al (Shahmoradian et al., 2019) used an integrative approach that combines, CLEM and STED microscopy, coherent anti-Stokes Raman scattering (CARS) and confocal laser scanning microscopy (CLSM) to investigate the structural properties and composition of 17 Lewy bodies that were identified using antibodies against phosphorylated aSyn at Serine 129. They concluded that lipids and membranous organelles, rather than aSyn fibrils, are the major constituent of LBs. Several studies dating back as early as 1969, have documented the presence of lipids and membranous organelles in LB structures (Figs. 2 and 5, (Gai et al., 2000; Duffy and Tennyson, 1965; Forno et al., 1996; Forno and Norville, 1976; Jager, 1969; Hayashida et al., 1993a; Takahashi et al., 1994; Wakabayashi et al., 1998)). However, the claim that LBs contain primarily non-fibrillar forms aSyn and their proposed models of aSyn fibrillization-independent mechanisms of LB formation requires more careful analysis and discussion.

The paper starts by citing the initial immunolabeling EM studies that demonstrated that the filaments found in the periphery of LBs were immunoreactive for aSyn (Spillantini, 1998; Arima et al., 1998). This introduction is followed by a citation of a review article by Lysia Forno (Forno, 1996), which Shahmoradian et al imply argue against the presence of aSyn filaments in LBs. “By contrast, evidence from ultrastructural studies based on neuroanatomical localization points to an electron-dense core and granular features”. However, a careful review of the Lysia Forno article reveals that nowhere in the article she argued

against the presence of filaments in LBs. Indeed, it is in this review article that she presents the most commonly displayed images of LBs with a dense core and radiating filaments (Fig. 1A) and emphasizes that the presence of filaments, which at the time were thought to consist of neurofilaments, is one of the most common features of LBs (Fig. 1A) (Shahmoradian et al., 2019; Forno, 1996; Uryu et al., 2006; Kuusisto et al., 2003; Gai et al., 2000; Duffy and Tennyson, 1965; Forno, 1986; Baba et al., 1998; Forno et al., 1996; Forno and Norville, 1976; den Jager, 1969; Hayashida et al., 1993a; Takahashi et al., 1994; Wakabayashi et al., 1998; Crews et al., 2010; Dickson et al., 1991; Forno, 1969; Forno, 1987; Forno et al., 1978; Kanazawa et al., 2008; Lampert, 1967; Langston et al., 1998; Olanow et al., 2004; Soper et al., 2008; Wakabayashi et al., 1988). In fact, the granular features that Forno references were found to be embedded in the mass of filaments within LBs and were thought to reflect the presence of mitochondria, dense-core vesicles and other organelles in LBs. This article contains no reference to aSyn, because it predates the discovery of aSyn mutations and the development of aSyn-specific antibodies. I quote, “The most important element which all Lewy bodies have in common is, however, the filamentous cytoskeletal component, now considered to consist of neurofilaments”. Notably, subsequent studies established that the diameter of the filaments found in LBs did not match that of neurofilaments (Forno, 1986), and argued against neurofilaments being the primary component of the filamentous structures in LBs, see below.

The main evidence presented by Shahmoradian et al in support of the conclusion that LBs are devoid of aSyn fibrils is that they failed to detect aSyn filaments/fibrils in LBs which were identified using antibodies against aSyn and were heavily stained by antibodies against the phosphorylated form of the protein (pS129-aSyn) (Shahmoradian et al., 2019). Therefore, it is only appropriate to start by assessing the data to determine whether the LBs that they examined contained aSyn filaments.

A careful review of the text and the electron microscopy images of LBs and the properties of all the LBs as summarized in table 1 of supporting information shows that at least 14 of the 17 LBs contained filamentous structures. One of these LBs exhibited ultrastructural properties that resembled those of classical LBs, i.e., a dense core and the radiating filaments (Fig. 3). Box 1 lists excerpts from the paper that

describe the presence of filaments in several LB structures, with references to the related figures and supporting data in the paper. Careful analysis of these statements and the related figures clearly showed that the data presented provide clear evidence that the majority of the LBs examined in the paper contained filaments (see Fig. 3). However, whether these filaments were aSyn fibrils, neurofilaments or formed by other proteins was not clear. Indeed, the paper clearly stated that “clarifying the nature of the observed filaments in aSyn-immunopositive inclusions (that is, distinguishing abundant cytoskeletal filaments from aSyn filaments) was not possible in this study” (Shahmoradian et al., 2019). It would appear, therefore, by the authors own admission, that the study does not rule out the possibility that some of these filaments may represent aSyn fibrils.

“Since all Lewy pathology studied here were identified by their high content of α Syn, but primarily contained membranous material, α Syn must be present in these LB and LN in an alternate form besides filamentous” (Shahmoradian et al., 2019).

Although neurofilaments have consistently been observed in LBs, they are usually localized in the periphery of LBs (Watanabe et al., 1977; Kanazawa et al., 2008). This finding was recently confirmed using STED microscopy analysis of LBs from the brains of patients with PD (Moors et al., 2019). More specifically, Moors et al., reported that neurofilaments formed the outer layer of LBs and are usually found surrounding a layer of phosphorylated aSyn. These findings are consistent with those of previous studies showing the same distribution of aSyn and neurofilaments in LBs and demonstrating that neurofilaments are formed within or incorporated into LBs at later stages of LB formation and/or maturation (Kanazawa et al., 2008). Furthermore, while the neurofilaments may have an appearance similar to that of aSyn fibrils, they exhibit a much narrow diameter distribution (~10 nm) than the filaments found in LBs and *in vitro* (5–15/20 nm, respectively) (Spillantini, 1998; Forno, 1986; Arima et al., 1998). Interestingly, the 10 nm filaments found in LNs were identified as neurofilaments, whereas the term filaments was used to describe all filamentous structures found in LB structures examined by Shahmoradian et al.

The abundance of filamentous structures within the LBs (Fig. 3 and Box 1) suggests that it would be possible to determine whether they are composed of neurofilaments or aSyn fibrils by immunogold labeling using a panel of anti-aSyn and anti-neurofilament antibodies. Instead, the authors argued otherwise, suggesting that determining whether the gold particles are localized on the filaments or other proteinaceous material in the vicinity of the fibrils would be “challenging”. However, previous studies by several groups have shown that it is indeed possible to specifically label aSyn fibrils *in situ* in LBs (Arima et al., 1998) or after their isolation from LBs and unambiguously determine whether the filaments are made of aSyn or other proteins (Spillantini, 1998; Spillantini et al., 1998; Crowther et al., 2000; Fujiwara et al., 2002) (Fig. 4). Antibodies against the most flexible regions of aSyn (the N- and C-terminal domains) showed extensive immunogold labeling of LB-derived filaments from PD, MSA and DLB samples (Spillantini et al., 1998; Fujiwara et al., 2002). In one comparative study, aSyn filaments were found in cingulate cortex extracts from all DLB cases but not in those from age-matched controls (Spillantini, 1998) (Fig. 4). These observations argue against the claim that “immunolabeling for aSyn tends to lack specificity for filaments”.

An alternative approach to determine the chemical identity of the proteins in these filaments is to isolate the filaments and assess and characterize them using antibodies against aSyn or via mass spectrometry methods (Fig. 4). However, no efforts were made to isolate the filaments or to assess their biochemical composition. Instead, the paper argues that the existing filament isolation procedures, which were optimized for paired helical filaments, are not optimal for the isolation of aSyn fibrils from LBs. The authors referenced two studies to support their argument that Tau and Abeta (Ab) filaments are impossible to differentiate from aSyn filaments. A careful review of the cited papers

showed the following: The first study, conducted in 1996 by Iwatsubo K et al, reported a protocol for isolating LBs from DLBD cortices. This study was carried out prior to the development of aSyn-specific antibodies or the identification of aSyn as a component of LBs and, therefore, did not address the presence or absence of aSyn in LBs or report the labeling of filaments with aSyn antibodies (Iwatsubo et al., 1996). In fact, Iwatsubo K et al reported that “Anti-tau antibodies stained only Neurofibrillary tangle (NFTs) and neuropil threads (NTs) but no LBs”. The second cited paper was a comparative review of DLB and Parkinson's disease dementia (PDD) by Jellinger KA et al and did not address any topics related to the direct characterization or comparison of Tau, Ab and aSyn filaments in DLB (Jellinger and Krczyn, 2018). Recent studies have shown that it is possible not only to isolate native β (Schmidt et al., 2019; Ghosh et al., 2020), Tau and aSyn filaments from the postmortem brains of patients affected by AD, other tauopathies (Zhang et al., 2020; Arakhamia et al., 2020; Zhang et al., 2019; Falcon et al., 2018; Fitzpatrick et al., 2017) and MSA but also to determine their sequence identity and high-resolution structures, thanks to recent advances in cryo-EM (Schweighauser et al., 2020) and mass spectrometry (Zhang et al., 2020; Arakhamia et al., 2020) techniques.

It is thus puzzling why, despite acknowledging that they could not determine whether the filaments they detect in the LBs were aSyn filaments or neurofilaments and not performing any experiments to address this issue, Shahmoradian et al still concluded that these filaments are unlikely to be aSyn filaments. The models proposed in the discussion section of the paper and the conclusion statements (*see below*) restrict the description of aSyn in LBs to non-fibrillar forms of the protein and present models of LB formation that excludes the possibility of an important role for aSyn fibril formation in this process.

“Our discovery of a crowded environment of membrane fragments, mitochondria, vesicular structures, including some that resemble lysosomes and autophagosomes, intermingled with non-fibrillar α Syn in multiple pathological inclusions, provokes new theories about the mechanisms contributing to the formation of Lewy pathology in PD.” (Shahmoradian et al., 2019)

“We present here a new theoretical model in which lipid membrane fragments and distorted organelles together with a non-fibrillar form of α Syn are the main structural building blocks for the formation of Lewy pathology.” (Shahmoradian et al., 2019)

4. If LBs do not contain aSyn fibrils, then what is the aggregation state of aSyn in LBs?

The term non-fibrillar is very broad and can be used to describe a wide range of aSyn species, from monomers to oligomers to non-fibrillar aggregates. The manuscript did not report on any efforts to directly or indirectly assess the oligomerization or conformational state of aSyn in LBs or to isolate aSyn from LBs. Instead, to support their claim, the authors cited previous studies that were not directly related to investigating the composition of LBs, distribution of aSyn species in LBs or mechanisms of LB formation. For example, the paper by Grassi et al, was cited to support the paper's claim that “non-fibrillar aSyn species produced from a partial autophagosome digest appear to disrupt mitochondria in both postmortem brain tissue and cell cultures, corroborating our CLEM data” (Grassi et al., 2018). However, a careful review of the work by Grassi et al revealed that the referenced toxic aSyn species are formed as a result of autophagic degradation of fibrillar phosphorylated aSyn that interacts with and induces disruption of the mitochondrial membrane (Grassi et al., 2018).

Several studies have shown that it is possible to seed and induce aSyn fibril formation *in vitro*, in cells, and *in vivo* using brain homogenates from PD and MSA brains (Peng et al., 2018; Watts et al., 2013; Han et al., 2020; Dhillon et al., 2019; Manne et al., 2019). The very low abundance of aSyn species in these samples has historically precluded

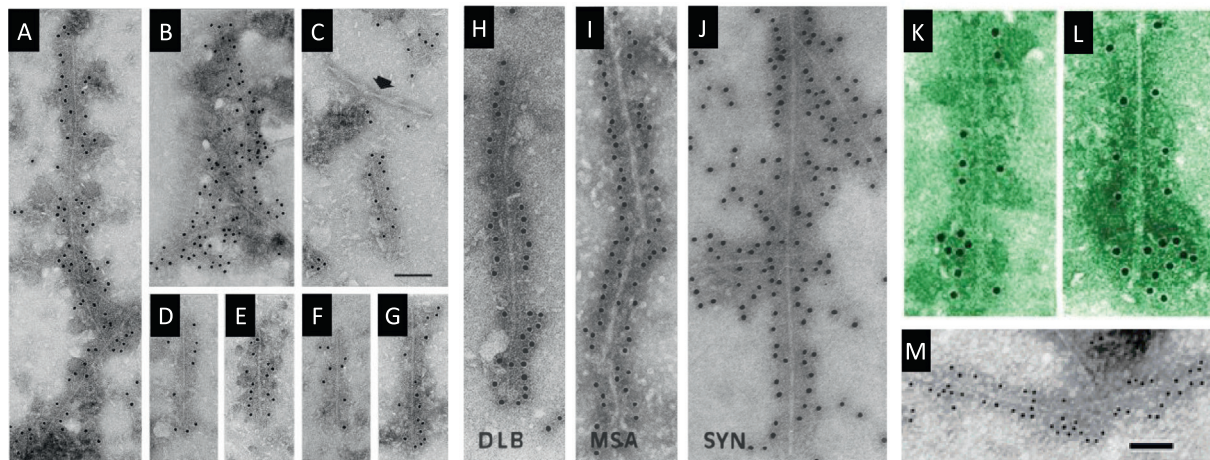


Fig. 4. A collection of EM images of aSyn fibrils isolated from human brains (A-F and I) and/or prepared from recombinant aSyn proteins (G and H) after immunogold labeling using anti-aSyn antibodies. (A-G) EM images of the cingulate cortex of patients with DLBD labeled with an anti-aSyn antibody targeting the C-terminal domain of the protein (adapted from (Spillantini et al., 1998)). EM image showing immunogold labeling of aSyn fibrils from the brains of patients with DLB and MSA and of fibrils generated in vitro from recombinant aSyn using aSyn-specific antibodies (adapted from Synucleinopathies and Tauopathies (Goedert and Spillantini, 2012)). H and I) Immunogold labeling of aSyn fibrils isolated from DLB and MSA brain. J) Immunogold labeling of fibrils produced from recombinant aSyn K-L) Immunogold labeling of aSyn fibrils isolated from the substantia nigra of a patient with PD (adapted from (Goedert et al., 2017)). M) Immunogold labeling of aSyn fibrils isolated from DLB brain using an antibody against aSyn phosphorylated at S129 (adapted from (Fujiwara et al., 2002)) (Goedert and Spillantini, 2012).

direct assessment of the aggregation state of aSyn via imaging techniques such as transmission electron microscopy (TEM) or atomic force microscopy (AFM). However, this seeding activity is not observed in brain homogenates from healthy brains, suggesting that it is linked to the presence of fibrils or other seeding-competent aggregated forms of aSyn. Given the significantly reduced fibrillization seeding activity of aSyn oligomers (Pieri et al., 2016; Fagerqvist et al., 2013), it is likely that a fibrillar form of aSyn is the primary seeding entity. These observations, however, do not exclude the possibility that an alternative form of aSyn as membrane/exosome-bound oligomers or other type aggregates could be the seeding competent species and responsible for initiating fibril formation in host cells.

5. Which form of aSyn is involved in LB formation?

If aSyn fibrillization is not required for LB formation, then how does it get incorporated into LBs? Does it play an important role in driving LB formation, or is it simply a victim of the process that gets swept into LBs? Shahmoradian et al proposed a model in which LB formation is a mechanism used by the cell to clear damaged organelles. They suggest that aSyn, under physiological conditions, acts as a membrane tether and that its interactions with membranous organelles can lead to excessive adhesion between membranes and eventually to membrane disruption and fragmentation. The process of LB formation is then activated to remove, sequester and potentially degrade these damaged structures.

The localization of aSyn and mitochondria in LBs does not support this model. If aSyn functions as a membrane tether, one would expect to see greater colocalization of aSyn and mitochondria. Instead, one observes few mitochondria dispersed mostly in the periphery of LBs, with only a low level of localization overlap with the peripheral layer of pS129-aSyn. Additionally, when mitochondria are observed in the center of LBs, the level of aSyn-mitochondrial marker colocalization is still very minimal, i.e., the vast majority of aSyn in LBs does not colocalize with mitochondria. These observations suggest that it is unlikely that the main aSyn species are associated with the mitochondrial membrane, that the vast majority of aSyn in LBs is membrane-bound, or that aSyn-mediated disruption of the mitochondrial membrane is the main driver of LB formation. The paper cited another study by Jensen PH et al to support their claim that aSyn “interacts with tubulovesicular/

vesicular structures, reminiscent of those visualized by their CLEM”. However, a careful review of this paper revealed the absence of any EM or other imaging data showing the interaction of aSyn with membranous or vesicular structures (Jensen et al., 1998). Instead, the binding of aSyn to vesicles in this study was assessed using a flotation assay based on the method of Brown and Rose and not by EM (Jensen et al., 1998).

Interestingly, although the paper suggests the absence of aSyn filaments in LBs, it still proposed an additional possible mechanism whereby the formation of disrupted and fragmented organelles and mitochondria could be driven primarily by the crowding of aSyn on membranes and membrane-catalyzed formation of aSyn fibrils.

This scenario suggests that aSyn fibril formation plays a central role in LB formation and that an abundance of membrane-bound aSyn fibrils should be easily detected in the final LB structure. However, as indicated by Shahmoradian et al, such membrane-bound aggregates would not be detectable under their experimental conditions and setups, see the discussion below. Despite this limitation, if the membrane-bound fibrils are seeding-competent, then one would expect that they would recruit monomeric or oligomeric aSyn present in or within the vicinity of LBs, leading to enhanced fibril formation. Under all these scenarios, however, one would still expect to see fibrils within LBs.

6. Study limitations

To understand why this study failed to provide decisive conclusions regarding the state of aSyn in LBs or its role in LB formation, the limitations of the techniques, tools and experimental approaches employed must be discussed. Importantly, the images presented in this study provide a single snapshot at a one-time point in the life cycle of each LB examined. Thus, attempting to reconstruct the sequence of events that led to the formation and maturation of these LBs is not possible.

6.1. EM images were acquired for only a small section of LBs

Importantly, all of the CLEM studies were performed on 100-200 nm thick cross-sections, which represent only a fraction of the LBs or LNs. Therefore, the variations in the length and abundance of the filaments in each LB may reflect a failure, in some cases, to image the sections of LBs/LNs containing the filaments. This could explain the large variability in the abundance of fibrils and membranous material across the

different LB structures examined. For example, the paper reported that seven LBs contained predominantly fragmented membranes, organelles and vesicles, and three of the 17 LBs predominantly contained filaments rather than membranes and organelles. The remaining LBs contained filaments that were dispersed among membranous structures. This finding, combined with the fact that the methods used do not allow visualization of filaments or aSyn aggregates within organelles or bound to organelle-derived membranous structures, suggest that the CLEM experiments likely underreport the number and abundance of fibrils present in the LBs/LNs, as clearly stated by Shahmoradian et al. Finally, it is possible that the LB structures examined represent precursors of LBs such as Pale bodies (PBs) (Kuusisto et al., 2003; Takahashi et al., 1994; Dale et al., 1992; Hayashida et al., 1993b; Gomez-Tortosa et al., 2000), which are known to contain S129 phosphorylated aSyn (Kon et al., 2020) and sparse and disorganized short aSyn filaments (Gibb et al., 1991). Interestingly, several reports have shown that neurofilaments are rarely detected in PBs and are localized primarily in the periphery of LBs (Takahashi et al., 1994; Gibb et al., 1991).

“Since our TEM tomograms capture the content of tissue sections spanning 100–200 nm in thickness, we can only report about the contents of these sections, which represent a fraction of each LB/LN” (Shahmoradian et al., 2019).

“The high-resolution electron microscopy imaging approach employed here may under-report the number of filaments that could be present in the LB/LN” (Shahmoradian et al., 2019).

6.2. Discrepancies between the CLEM and STED microscopy results

To assess the distribution of aSyn species and organelles in LBs, correlative multilabeling and STED microscopy approach (Fig. 5E) was used. While the paper’s interpretation of the data suggests that non-fibrillar aSyn is randomly distributed in LBs, the STED microscopy images show a different picture, a uniform and highly organized peripheral distribution of pS129-aSyn in the outer layer of the LBs (Fig. 5E). A subsequent study by the same group (Moors et al., 2019) confirmed these findings and those of previous studies (Prasad et al., 2012) demonstrating that the central core of the LBs was composed of C-terminally truncated aSyn species, consistent with previous studies by Spillantini et al showing that aSyn within the core of LBs can be detected with antibodies against the N-, but not the C-terminus of the protein (Spillantini, 1998). Both pS129-aSyn and C-terminally truncated aSyn species are usually found to accumulate in LBs and other forms of aSyn pathologies and are usually found mostly in the insoluble fractions of homogenates and lysates from PD brains and neuronal models of aSyn aggregation or inclusion formation, respectively (Baba et al., 1998; Anderson et al., 2006; Kahle et al., 2001). Furthermore, antibodies against pS129 are commonly used as the primary tool for assessing aSyn pathology and monitoring aSyn seeding and pathology spreading (Volpicelli-Daley et al., 2011; Luk et al., 2012). Therefore, the observation that these forms of aSyn are enriched in LBs is consistent with previous reports demonstrating their abundance in LBs and indicates the presence of aggregated, rather than soluble, forms of aSyn in the LBs examined by Shahmoradian et al. It is noteworthy that STED microscopy does not allow differentiation between fibrillar and non-fibrillar forms of aSyn.

6.2.1. LB composition

Shahmoradian et al. emphasize that their imaging data suggest that LBs in PD are composed mainly of lipids and membranous organelles and that if filaments (aSyn filaments or other cytoskeletal structures) are present in LBs, they are likely to be a minor component. However, it is not clear how they can arrive at this conclusion by examining only small cross-sections of LBs and without performing any biochemical studies to 1) assess the relative distribution of membranous material vs.

filaments; 2) qualitatively or quantitatively assess the biochemical composition of LBs; or 3) validate their claims using other approaches.

7. Conclusions

The idea that LBs are devoid of aSyn fibrils or that LB formation occurs independently of aSyn fibril formation casts doubts on a substantial body of work that forms the foundation of many of the current basic and translational research programs in academia and industry (Lashuel et al., 2013). This span ongoing efforts ranging from the development of animal models and reliable biomarkers, diagnostic tools, and therapeutic programs in Big Pharma, the great majority of which are focused primarily on targeting aSyn aggregation and Lewy pathology formation and spreading in PD and related synucleinopathies. There is also a risk that such claims, when published in “prestigious journals” such as Nature Review Neuroscience, could influence publication and funding decisions for PD and synucleinopathy research. Indeed, the publication of this paper has led to articles and news media reports with titles such as “forget the fibrils” (<https://www.alzforum.org>, 2019), “Shock discovery for Parkinson’s research” (<https://analyticalscience.wiley.com/>, 2019), “Parkinsonian Lewy Bodies Are Not What We Thought” (<https://www.biocompare.com/>, 2019), “Is It Time to Rethink Parkinson’s Pathology?” (<https://www.the-scientist.com/>, 2019) and to several statements that question decades of research pointing to aSyn fibrils as key components of LBs (<https://www.unibas.ch/>, 2019). Questioning conventional wisdom is healthy, and differences in opinion and thoughts should be welcomed, encouraged, and debated. However, it is also important to have healthy discussions and debates about the evidence supporting new findings and to consider the implications of these findings for the field and the research community.

Some immediate questions that come to mind after reading the paper by Shahmoradian et al are as follows: 1) What should be the fate of the current Parkinson’s therapeutic pipelines based on drugs and antibodies that target aSyn aggregates and pathology, many of which are already in clinical trials today? 2) If LBs are devoid of aSyn fibrils, should we stop ongoing programs aimed at the development of positron emission tomography (PET) tracers targeting aSyn Lewy pathology? 3) What is the relevance of investigating the mechanisms of aSyn fibrillization or continuing to solve cryogenic electron microscopy (cryo-EM) structures of brain-derived and recombinant aSyn fibrils if these structures are not relevant to Lewy pathology in PD? Before we start posing more questions or answering these questions, let us first examine the data and proposed models presented by Shahmoradian et al.

Taken together, Shahmoradian et al verify previous studies showing the presence of lipids and membranous organelles in LBs, but do not establish the absence of aSyn fibrils. Furthermore, no attempts were made to experimentally assess the aggregation state of aSyn in LBs or validate their conclusion that the aSyn in LBs exists in a non-fibrillar state. Finally, several of the models proposed to explain the possible function of aSyn in LBs were not supported by experimental data or evidence from the literature. While several *in vitro* studies support a possible role of membrane-catalyzed aSyn fibrillization in the disruption and fragmentation of organelle membranes, the literature provides no clear evidence that the formation of damaged organelles in the absence of aSyn fibrillization is sufficient to drive LB formation.

At least, three possible scenarios can be considered regarding the role of aSyn in LB formation.

7.1. aSyn is not required for LB formation

Shahmoradian et al. suggested that their findings point to a potential role of damaged organelles in the formation of LBs and LNs. Although this is an interesting hypothesis, no existing and validated data support the hypothesis that LBs can form independently of aSyn aggregation and fibril formation. Indeed, a careful review of the

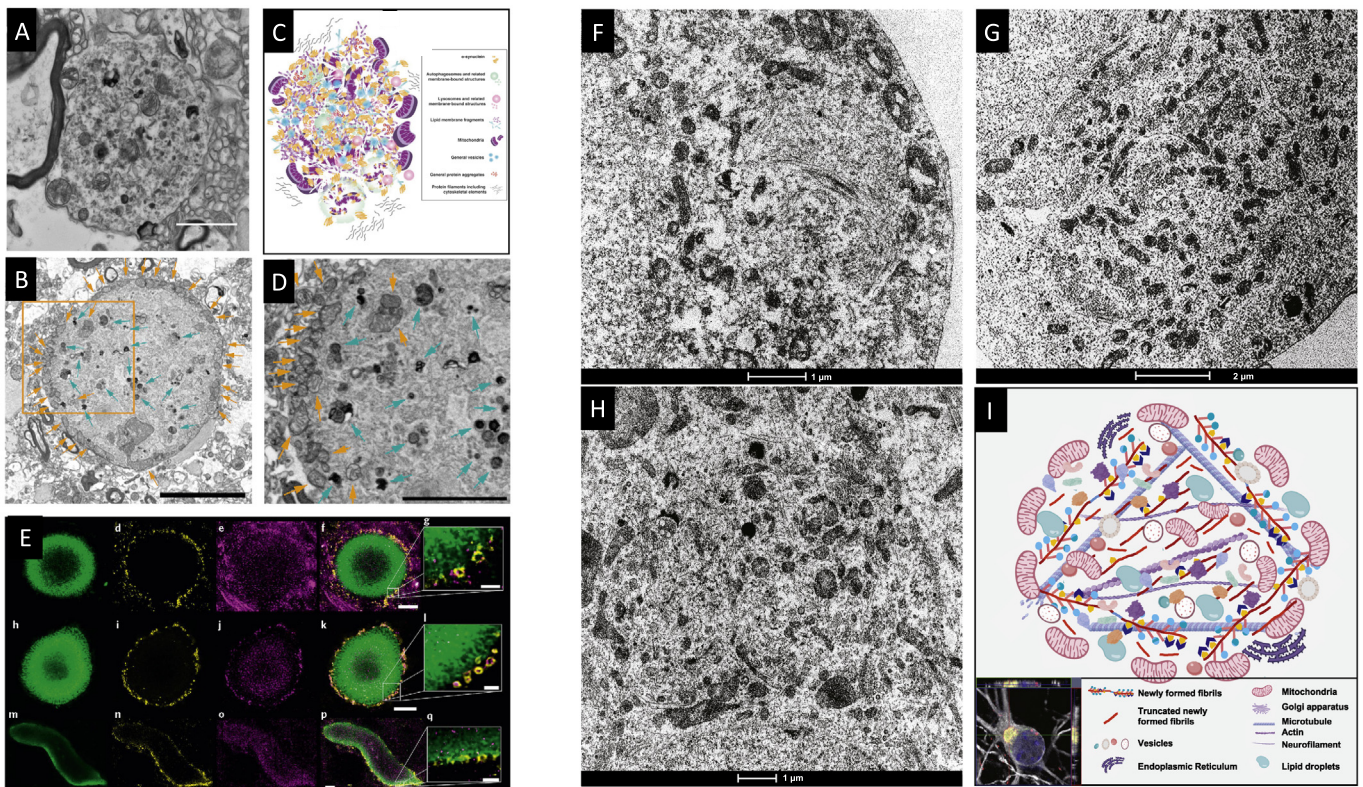


Fig. 5. CLEM image of LBs. A-D) CLEM images of LBs from PD brains showing the abundance of membranous structures and organelles within LBs. E) STED microscopy images of LBs showing dense accumulation of phosphorylated aSyn in the periphery of the LBs. F-H) CLEM images of LB-like inclusions showing the recruitment and accumulation of both aSyn and membranous structures and organelles within these round LB-like structures that formed at 21 days post-induction of aSyn aggregation using aSyn PFFs or extracts from such inclusions. Panels C and I show two models for the composition of LBs proposed by the two studies. While both models emphasize cytoskeletal proteins and membranous material and organelles, our proposed model derived from the neuronal model shows an abundance of post translationally modified and fragmented aSyn fibrillar structures (Mahul-Mellier et al., 2018; Mahul-Mellier et al., 2020), whereas the model proposed by Shahmoradian et al (Shahmoradian et al., 2019) suggests that LBs are devoid of aSyn fibrils and contain mainly non-fibrillar forms of the protein. Panels A-E were reproduced with permission from (Shahmoradian et al., 2019); panels F-I, from (Mahul-Mellier et al., 2020).

literature identified only one report suggesting the absence of aSyn in LBs. In this report, Van Duinen and colleagues described the detection of numerous inclusions in the brain of a patient who exhibited no clinical symptoms of parkinsonism or dementia that showed no immunoreactivity with anti-aSyn antibodies, despite exhibiting many of the key immunohistochemical (positive for ubiquitin and neurofilaments and negative for tau) and ultrastructural features of classical LB (van Duinen et al., 1999). Four different antibodies against full-length aSyn and the C- and N-terminal regions of aSyn were used in this study. All LBs detected in this patient were negative for all four antibodies. Although the paper suggested that these antibodies label LBs in patients with PD and DLB, those data were not presented in the paper. In addition, to the best of my knowledge, these observations have not been reproduced by other groups.

7.2. aSyn but not fibrillization of aSyn is required for LB formation

In this scenario, which was also suggested by Shahmoradian et al, aSyn plays an active role in the biogenesis of LBs but does so via mechanisms independent of its fibrillization properties. This phenomenon could explain the abundance of aSyn in LBs and further implies that the abundant filamentous structures found in LBs are not derived from aSyn—i.e., LBs are devoid of aSyn fibrils. In a recent study, Araki et al used microbeam X-ray diffraction (XRD) to analyze thin sections of LBs from the brains of PD patients (Araki et al., 2019) and established that LBs contain aggregated forms of aSyn rich in cross- β -sheet structures. Although this technique does not allow visualization of fibrils, it is well established that this specific structural signature is acquired during the

formation of aSyn fibrils and is a structural feature shared by all amyloid fibrils. The same study reported that some aSyn-positive LBs were devoid of this cross- β -sheet structure. This observation has several possible explanations: 1) this second population of LBs are devoid of fibrils; 2) the sections examined represent only a small fraction of LBs, and the researchers may not have captured the appropriate sections of the LBs; 3) the aSyn fibrils within these LB sections have undergone significant remodeling and chemical modifications that led to a decrease in their cross- β -sheet structure; or 4) that the structures examined may represent early precursors to LBs. Notably, the studies by Shahmoradian et al and Araki et al relied on the use of anti-aSyn antibodies that target the C-terminus of the protein or pS129-aSyn, thus biasing their selection for LBs that contain predominantly full-length aSyn. However, several studies have shown that LBs are highly enriched with truncated aSyn species (Prasad et al., 2012; Anderson et al., 2006; Li et al., 2005; Dufty et al., 2007; Oueslati et al., 2010; Kellie et al., 2014). Indeed, the first biochemical studies on isolated LBs (Baba et al., 1998) and glial cytoplasmic inclusions (GCIs) (Gai et al., 2000) showed that truncated species account for the vast majority (~90%) of aSyn in these inclusions. The level of truncation likely varies during the evolution and maturation of LBs, as removal of the highly negatively charged C-terminal domain facilitates the lateral association and packing of fibrils (Mahul-Mellier et al., 2018).

7.3. LBs are rich in aggregated and filamentous forms of aSyn

Several lines of evidence support the hypothesis that the accumulation of aSyn and its propensity to aggregate and form fibrils is a key

requirement for the formation of LBs. 1) since their discovery, ultrastructural characterizations of LBs and other types of aSyn pathologies have consistently shown that they are highly enriched with fibrillar aggregates that exhibit variable organization and packing depending on where the form in the brain and the stage of LB formation; 2) LBs are rich in aSyn and are extensively labeled by several antibodies targeting different epitopes and post-translational modifications in aSyn; 3) recent studies using Fourier transform infrared spectroscopy (FTIR) and XRD have shown that LBs not only are highly enriched with β -sheet structures but also contain cross- β -sheet structures, both of which are key features of all amyloid fibrils and recombinant aSyn fibrils produced *in vitro* (Araki et al., 2019; Araki et al., 2015); 4) several groups have shown that aSyn fibrils can be isolated from LBs and that antibodies against both the C- and N-terminal domains of aSyn enable efficient gold labeling of fibrils extracted from LBs isolated from different brain regions and different types of synucleinopathies (PD, MSA and DLB); 5) most aSyn species within LBs, including those associated with aggregated and highly aggregation-prone forms of aSyn (phosphorylated (pS129) and truncated species), are found in the insoluble fractions, consistent with the expected reduced solubility of aSyn fibrils; 6) aSyn species isolated from brain regions known to contain substantial levels of Lewy pathology seed aSyn fibrillization *in vitro* and induce aSyn seeding, fibrillization and pathology formation in neurons and in the brains of rodents and nonhuman primates; 7) SNCA duplication or triplication is sufficient to cause Parkinson's disease and DLB (Singleton et al., 2003; Muentner et al., 1998; Chartier-Harlin et al., 2004; Ibanez et al., 2004; Konno et al., 2016); and 8) The majority of disease-associated mutations (A30P, E46K, A53T and A53V) enhance aSyn oligomerization and/or fibrillization *in vitro* (Rutherford et al., 2014; Conway et al., 2000; Narhi et al., 1999; Lashuel et al., 2002; Lemkau et al., 2013; Greenbaum et al., 2005; Fredenburg et al., 2007; Khalaf et al., 2014; Ghosh et al., 2013; Mohite et al., 2018; Conway et al., 1998).

7.4. aSyn fibrillization is required for the recruitment of membranous organelles and the formation of LB-like inclusions

Although the images by Shahmoradian et al., provide a snapshot of different LB structures and their composition, these images do not allow for reconstructing the process of LB formation and maturation. This could be possible by collecting more images from brains of patients that died at different stages of PD. However, as Shahmoradian et al., stated, the development of cellular models that reproduce the process of LB could provide a window to gaining insight into the sequence of events and molecular and cellular determinants of this process. Indeed, while the paper by Shahmoradian et al., was in review we reported (Mahul-Mellier et al., 2018; Mahul-Mellier et al., 2019) that we can reconstruct many key aspects of the process of LB formation in primary hippocampal neuronal model (Fig. 5). In this model, it is possible to induce the formation of LB-like inclusions that bear striking resemblance at the structural and organizational levels to the LB structure described by Shahmoradian et al., and the biochemical properties of LBs. However, these LB-like inclusions are rich in aSyn fibrils in addition to lipids and membranous organelles (mitochondria, lysosomes, and autophagosomes) (Mahul-Mellier et al., 2019). In this model, the aggregation and formation of LB-like inclusions by endogenous aSyn were induced by seeding (addition of PFFs), and a significant delay was observed between the formation of fibrils and their transition from fibrils to LB-like inclusions. This process was accompanied by significant changes in the biochemical and morphological properties of the fibrils, including changes in the patterns of aSyn PTMs (extensive truncation and phosphorylation), remodeling of fibril morphology and fibril fragmentation. These phenomena could potentially explain the failure to detect long fibrils in some of the LB structures. The formation of fibrils prior to the formation of LB-like inclusions and the accumulation of fibrillar aSyn in these neuronal LB-like inclusions suggest that aSyn fibrillization is

required for, if not the main driver of LB formation.

The occurrence of these processes in PD brains could partially explain the heterogeneity of Lewy pathology seen with different antibodies against aSyn and other LB markers. Given the continued processing and fragmentation of aSyn fibrils in this model, aSyn fibrils may be responsible for initiating the process of LB formation through the recruitment of other proteins, membranous organelles, and cytoskeletal structures; however, once inclusions are formed, they continue to be subjected to fragmentation, remodeling and possibly degradation by disaggregases, lysosomal enzymes and other proteases. This possibility could explain why some LBs may not contain long filaments while others may not be rich filament or in amyloid-like aggregates (Araki et al., 2019). However, it is important to emphasize that the vast majority of currently characterized LBs exhibit an abundance of filaments (Fig. 1). Furthermore, it is important to emphasize that we know very little about the mechanisms by which LBs and other types of Lewy pathologies are degraded or cleared in neurons or after neuronal death.

7.5. The role of aSyn-lipid/membrane interactions in LB formation

The consistent observation of lipids and membranous organelles in LB structure suggest that aSyn interactions with lipids or membranes of organelles mediate different aspects of aSyn aggregation and LB formation and maturation. The recent tantalizing images from CLEM studies of LBs and LB-like inclusions in neurons further underscore the critical importance or reassessing whether these interactions represent early or late events during aSyn aggregation and LB formation. In the seeding neuronal models, sequestration of membranous organelles and mitochondrial dysfunctions seem to occur after aSyn fibrils are formed and during the transition from fibrils to LB-like inclusions (Mahul-Mellier et al., 2020). Recent studies using similar seeding-based models showed tight association between aggregated and phosphorylated aSyn and mitochondria (Grassi et al., 2018; Wang et al., 2019).

Further studies are needed to more precisely define the biochemical properties and oligomerization or aggregation state of aSyn bound to membranous organelles within LBs and in cellular/neuronal models of LB formation and aSyn toxicity. This combined with a more detailed investigation of the sequence determinants of such interactions could pave the way for the design of specific mutants and experiments that would allow for more precise investigation of the role of these interactions in regulating aSyn aggregation, LB formation and neurodegeneration in PD and other synucleinopathies. (Fanning et al., 2020)

7.6. The multiple faces of LBs and Lewy pathology

Several studies have shown that aSyn aggregates and LBs exhibit significant morphological diversity and are heterogeneous in their shape, biochemical composition, morphology, and organization and in the packing of aSyn fibrils and distribution of aSyn species and lipids (Kuusisto et al., 2003; Gai et al., 2000; Moors et al., 2019; Forno and Norville, 1976; Forno, 1969; Kanazawa et al., 2008; Gibb et al., 1991; Langston and Forno, 1978; Sakamoto et al., 2002). Since we cannot follow the evolution of a single LB, whether this morphological and biochemical heterogeneity reflects different stages of LB formation or distinct types of LBs and Lewy pathology is unclear. As suggested previously by Lysia Forno, perhaps we should not use a single term—i.e., Lewy bodies—to describe all the different forms of aSyn pathologies in the brain. The aggregation state and biochemical properties of aSyn (e.g., PTM patterns) of aSyn may differ during the evolution of LBs or among different types of Lewy pathology. Therefore, we must be careful to avoid generalization based on assessments of small sections of small numbers and few types of LB structures with limited tools that do not capture the diversity of aSyn species and types of Lewy pathology.

Although investigating the ultrastructural properties of LBs and different types of Lewy pathology using postmortem brain tissues is very informative, this approach is likely to be insufficient to enable

reconstruction of the mechanisms underlying LB formation and their role in the pathogenesis of synucleinopathies. However, this limitation by no means suggests that we should abandon such studies. Indeed, the types of the integrative approach taken by Shahmoradian et al., is what is needed, but it should be complemented by biochemical and mechanistic validation using biochemical approaches and the appropriate neuronal and animal models. Investigating the properties of LBs in brain tissues is indeed a good starting point, but we need more studies that cover a large fraction of LBs and evaluate a larger number of samples of LBs, LNs and other types of aSyn pathologies obtained from different patients and at different stages of disease progression. To translate the findings from such studies to hypotheses and working mechanistic models that could be tested and disproven in preclinical models, integrative approaches that allow morphological, biochemical and ultrastructural characterization of aSyn species and Lewy pathology are crucial. Given the large abundance of filamentous structures and cytoskeletal proteins in LBs from patients with different synucleinopathies (Figs. 1, 2, 3, 4, 5), we must revisit and systematically characterize these filaments using a larger toolkit of antibodies, different sample preparation methods and more precise techniques than previously used. Achieving these goals and comprehensively mapping and profiling the pathological diversity of Lewy pathology is possible only through collaborative efforts; the sharing of human tissue samples, tools and resources; and the integration of expertise and the latest technological advances from different disciplines.

Until further studies are carried out to systematically evaluate the models presented above or show that aSyn fibrillization is not required for LB formation, we should be cautious in how we interpret or over-interpret observations from a single report and recognize the limitations of the techniques used.

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