Neurotherapeutics (2023) 20:67–82
https://doi.org/10.1007/s13311-023-01365-5

REVIEW

α-Synuclein Structural Diversity and the Cellular Environment in α-Synuclein Transmission Models and Humans

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Accepted: 4 March 2023 / Published online: 13 April 2023
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Abstract

Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are termed synucleinopathies, disorders that are characterized by the intracellular aggregation of the protein α-synuclein. The cellular tropism of synuclein pathology in these syndromes is notably distinct since in the Lewy disorders, PD and DLB, αSyn forms aggregates in neurons whereas in MSA αSyn forms aggregates in oligodendrocytes. Studies examining αSyn pathology in experimental models and in human brain have now identified fibrillar αSyn with unique but distinct molecular signatures, suggesting that the structure of these αSyn fibrils might be closely tied to their cellular ontogeny. In contrast to the native structural heterogeneity of αSyn in vitro, the conformational landscape of fibrillar αSyn in human brain and in vivo transmission models appears to be remarkably uniform. Here, we review the studies by which we propose a hypothesis that the cellular host environment might be in part responsible for how αSyn filaments assemble into phenotype-specific strains. We postulate that the maturation of αSyn strains develops as a function of their in vivo transmission routes and cell-specific risk factors. The impact of the cellular environment on the structural diversity of αSyn might have important implications for the design of preclinical studies and their use for the development of αSyn-based biomarkers and therapeutic strategies. By combining phenotype-specific fibrils and relevant synucleinopathy transmission models, preclinical models might more closely reflect unique disease phenotypes.

Keywords Synucleinopathies · Parkinson's disease · Multiple system atrophy · Alpha-synuclein · Animal models · Strain · Transmission

Introduction

The synucleinopathies are a group of degenerative brain disorders that are characterized by intracellular deposits of the protein α-synuclein (αSyn). Inclusions of aggregated αSyn are the pathognomonic feature of synucleinopathies as they are invariably found in affected areas of the peripheral and central nervous system. Synucleinopathies include Parkinson's disease (PD), dementia with Lewy bodies, multiple system atrophy (MSA), and other related but less common disorders, such as pure autonomic failure. Genetic variants as well gene duplications and triplication of αSyn give rise to early onset Parkinson's disease directly linking αSyn to familial and sporadic cases of PD [1, 2]. PD and Lewy body dementia are characterized by cell-specific deposits of αSyn in neurons [3], termed Lewy body inclusions, whereas in MSA αSyn aggregates are observed in oligodendrocytes named glial cytoplasmic inclusions [4]. Because of the neuronal tropism of synucleinopathy in PD and DLB, these disorders are collectively referred to as Lewy diseases.

The main constituent of Lewy bodies or glial cytoplasmic inclusions is fibrillar αSyn. These filamentous forms of αSyn have been extracted from postmortem brain but can also be isolated from cerebrospinal fluid of patients with Lewy diseases or MSA [5–8]. Purified αSyn filaments from the brain of people with the same clinical diagnosis show a remarkable overlap in their conformation [5–7]. Contrastingly, when comparing Lewy disorders and MSA, the conformation of the αSyn fibrils is noticeably distinct. Fibrils of αSyn thus have disease-specific conformations, reflecting a molecular fingerprint that is intimately linked to clinical diagnosis [5–7]. Diagnosis of PD, DLB, or MSA is currently based on clinical consensus criteria but because of the conformational uniformity of αSyn filaments in Lewy diseases and MSA, αSyn filaments are being investigated as a proximal diagnostic marker via seeded amplification assays [9, 10].

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Many studies have shown that fibrillar αSyn is intrinsically pathogenic [11–13], and that αSyn aggregates can cause cellular dysfunction resulting in white and gray matter degeneration in experimental models. Fibrillar aggregates of αSyn can be transmitted between cells in vitro and in vivo [14, 15]. Stable filamentous seeds of αSyn can replicate intracellularly with endogenous αSyn and form new αSyn fibrils in a manner that resembles infectious proteins. Because of the compelling evidence for the pathobiology of fibrillar αSyn in the neurodegenerative process, fibrillar assemblies of αSyn are now widely used for animal modeling or preclinical studies that inform clinical trials [11].

However, there are still several challenges in translating αSyn-related pathology from preclinical models to humans. First, we need to better understand the molecular basis of the clinical diversity in synucleinopathies, as it has been difficult to reconcile the observation of αSyn as a key pathogenic substrate with different pathologies across a spectrum of related human syndromes. Adding to this complexity is the species barrier between rodent and human, considering that the structural features of human αSyn have mostly been studied after intracellular replication in rodent models. More accurately capturing the structural diversity of human αSyn assemblies within this experimental setting would benefit the development of future preclinical trials and allow to make better predictions for αSyn-targeting therapeutic strategies in humans.

Another not well understood key factor in αSyn transmission is the intracellular or in vivo environment. With recent methodological developments, the role of the host environment in αSyn pathobiology becomes increasingly appreciated as the cellular environment appears to be closely tied to the structural identity of its populating conformers [16, 17]. Next to characterizing the structural properties of αSyn aggregates, understanding how aggregation is initiated and proceeds within the cellular host environment might be crucial to understand the similarities and the differences in the development of synucleinopathies. Preclinical animal models would thus need to mimic and establish the conditions wherein the folding landscape of pathogenic αSyn resembles that of the human brain—on the cellular and the structural level. Here, we will discuss emerging insights into the native function and structural diversity of αSyn and the transmission of pathology in the context of the host cellular environment in preclinical αSyn transmission models. We further highlight the advantages and shortcomings of transmission models and propose how to address outstanding research gaps.

Native and Aggregated Assemblies—The Conformational Diversity of α-Synuclein

The relationship between the conformation and the function of αSyn has been intensively studied for more than two decades, but its native role remains elusive partly because of structural heterogeneity of the protein. There are significant methodological challenges to study αSyn as it is a small intrinsically disordered protein, with an apparent lack of conformation. It is expressed widespread in various tissues of the body, in- and outside the brain, underscoring its functional diversity. It is highly abundant in the human central and the peripheral nervous system [18, 19] and in rodent brain it is one of the most abundant synaptic proteins [20]. The expression of αSyn in the peripheral nervous system is also evident at barrier sites, such as the skin or visceral organs. Often, deposits of αSyn are observed within these barrier sites but in the absence of clear signs of cellular pathology or neurological illness [21, 22]. These peripheral deposits could also be tied to the presence of αSyn in the blood, as αSyn is also present in leukocytes and erythrocytes, where it is highly enriched [23].

αSyn is a small protein of 140 amino acids which lacks a defined structure in solution. When incubating the protein with negatively charged membranes, the N-terminus of αSyn adopts an α-helical structure similar to apolipoproteins via inserting its positively charged and hydrophobic KTKEGV repeats (Fig. 1) into the phospholipid bilayer [13]. This is followed by binding of the central core of the protein via its more hydrophobic non-amyloid component (NAC) region with the membrane [24, 25]. The carboxy terminus of protein remains unstructured as it is highly negatively charged and does not bind with the membrane [26, 27].

In neurons of the peripheral and central nervous system, αSyn is predominantly located in the neuronal synapse where it preferentially binds with the outer membrane of synaptic vesicles to mediate synaptic vesicle endo- and exocytosis [28]. αSyn is also present in the cytoplasm as it interacts with membranes of high curvature such as mitochondria, granules, or other smaller organelles [29, 30]. More recently, it was shown that αSyn binds with cytosolic mRNA proteins on membraneless organelles via its N-terminus [31]. By doing so, it stabilizes translationally repressed mRNA transcripts in granular processing bodies, slowing down their degradation [31].

As an intrinsically disordered protein with no discernable conformation, αSyn can undergo conformational changes when it inserts itself into the outer lipid bilayer of curvature-rich membranes. Upon binding with the membrane, the protein transitions to a membrane bound helical state via which it stabilizes lipid packaging by relieving the elastic membrane stress on the outer membrane of the membrane vesicle [32]. On these small vesicles, membrane-bound αSyn can transiently interact with other αSyn monomers, to form helical multimers on the membrane [33]. These structural changes of αSyn occur fast, and thus strongly rely on the conformational flexibility of the protein [34, 35].

Although this has been frequently overlooked, next to its ubiquitous expression in the nervous system, αSyn is

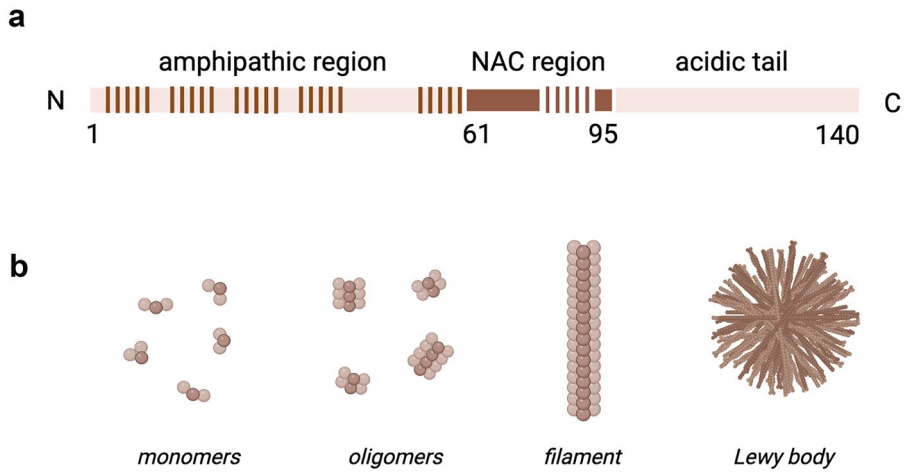

Fig. 1 Schematic of αSyn native and pathological conformations. **a** αSyn is a small protein of 140 amino acids with an N-terminal amphipathic region, a central hydrophobic region, and a highly charged acidic tail. Interactions with phospholipid membranes are mediated via consensus KTKEGV repeats in the N-terminus and the NAC region. **b** Monomeric αSyn is unstructured and natively unfolded in solution. Under pathological conditions, αSyn monomers will form unwanted intermolecular interactions via β-sheets through its polypeptide backbone. Upon assembly into stable oligomeric species, larger αSyn filaments can form, which are deposited into Lewy bodies and which are characteristic of synucleinopathies

highly abundant in the blood [23], the epithelial vasculature [36, 37], and in cells of the innate immune system such as granulocytes and other white blood cells [38, 39]. The transcriptional expression levels of αSyn in some human leucocytes, such as dendritic cells, are comparable to the levels of expression in other cell types of the brain [39, 40]. αSyn expression is low in cells of the adaptive immune system, such as in lymphocytes [39, 40]. In different types of innate leukocytes, αSyn is enriched on vesicular and granular membranes. Because its membrane binding role and granule stabilizing capacities, it likely plays a role in granule vesicle dynamics in leukocytes [41]. However, it is still unclear what its role is of αSyn in leucocytes and especially erythrocytes, where αSyn is highly abundant.

Recent studies have shown that αSyn is important for the host–pathogen immune response [42] as it plays a critical role in innate immune defense mechanisms [43–45]. Next to its putative role in the peripheral immune system, αSyn function is also critical to mediate the central immune response. Expression of αSyn in the brain during infection has been shown vital for protection against acute viral encephalitis as mice without αSyn lack a host pathogen response and succumb to infection [44–46]. Neurotropic infection triggers neuronal αSyn expression in the brain. This causes the protein to transiently accumulate in the neuronal cytoplasm and redistribute intracellularly as it relocates into the cell nucleus to supports the expression of interferon-related genes [44, 47].

αSyn is thus expressed in a variety of cells in and outside the brain. The structural diversity or structural flexibility of αSyn is imperative for its function as the protein has a broad impact on a wide variety of cellular functions. With its cellular distribution in the cytosol and membranes, αSyn needs to efficiently transition between different subcellular compartments to fulfill its native function. The conformational flexibility is therefore a functional advantage since it allows αSyn to rapidly perform complex dynamic interactions. However, unwanted interactions between αSyn monomers can as quickly turn this advantage into a disadvantage. Folding intermediates of αSyn on the membrane or in the cytosol can interact because of exposed hydrophobic residues in the NAC region, which are normally shielded by charged residues of the protein itself. However, exposure of these residues allows multiple molecules to bind and form high molecular weight assemblies. These aggregates of αSyn can set off a pathogenic cascade of protein aggregation with potentially far-reaching complications.

Although the structural flexibility of αSyn serves an important functional role, it can thus lead to unwanted interactions between multiple αSyn molecules. Because of the dynamic interactions of αSyn monomers with its environment, the protein can expose hydrophobic sequences via an intermediate assembly state that now allows direct interactions with other αSyn monomers [48, 49]. The polypeptide backbone of two or more αSyn molecules can transiently bind and form β-sheets leading to their aggregation into small but unstable oligomers [50, 51]. These amorphic oligomers need to undergo structural reorganization before they become stable and assemble into an aggregate core that forms the basis of a protofilament [48–53]. The filament can now serve as a seeding nucleus, with which new αSyn monomers can nucleate at the fibrillar surface catalyzing

the formation of new aggregates or with which monomers can bind at the ends of the filament resulting in fibrillar elongation [54].

Oligomers and stable filaments of αSyn can exist in a range of sizes of shapes. There have been extensive efforts to characterize the functional or pathological effects of different types of αSyn conformers. The smaller variants of αSyn oligomers are particularly challenging to study due to their kinetically unstable nature, their vast conformational heterogeneity, or subgroups of oligomeric assembly states. These oligomers can have distinct structures with various extents of β-sheet content or hydrophobicity.

Nevertheless, many studies have found that several of the smaller oligomeric assemblies can have detrimental effects on a wide range of cellular biological processes in vitro [50, 55]. Structural conversion of newly formed oligomers into more stable assemblies often leads to the formation of assemblies with more lasting and therefore more damaging effects on cellular health [53, 56, 57]. Even upon transient rearrangement into intermediate folding states in vitro, there can be high kinetic barriers during structural conversion [53]. More structurally defined oligomers can insert into the membrane bilayer via binding of amphipathic helices to the outer membrane and inserting their β-sheet into the deeper layers, thereby disrupting membrane integrity [58].

The existence of distinct subgroups of oligomers is likely due to the multiplicity of pathways in the misfolding process although the exact determinants required for the structural assembly into unique small oligomers are not yet fully understood. However, assembly conditions during in vitro aggregation can significantly influence how oligomeric or filamentous aggregates assemble, with slight variations in buffer composition or shaking conditions yielding different structural outcomes in the assembly state of the aggregate [48, 59]. Indeed it was shown that different in vitro assembly conditions via varying the composition of salts in the aggregation buffer led to the assembly of two types of fibrils with distinct structures as defined by the fibrillar morphological, biophysical, and biochemical properties [60]. The resulting assemblies were termed fibrils and ribbons, in accordance with their morphological appearance. In cell culture, the two types of fibrils amplified with endogenous αSyn but they exhibited distinct cellular toxicities [60]. There is thus a structural functional relationship between αSyn assemblies and it cellular phenotypes. Because of this direct relationship between aggregate structure and function, it is imperative that studies investigating the biological effects of αSyn assemblies are well controlled, by clearly describing the purification and assembly conditions and by making efforts to work with structurally related or homogeneous assemblies instead of heterogeneous mixtures, to infer reliable and consistent results within and between studies.

Fibrillar Assemblies—The Conformational Uniformity of α-Synuclein Strains

Contrasting with the heterogeneity of structural assemblies formed in vitro is the structural homogeneity of αSyn filaments formed in vivo. Using cryo-electron microscopy, recent studies have shown that fibrils postmortem extracted from the brain of six unrelated people with Lewy disease contained only a single filament with strikingly matching conformations between isolated filaments [5, 7]. These filaments had a typical fold, seen in the outer and inner layers of the fibril core, and it very closely matched the structure of the fibrils isolated from the brain of the people with either PD, DLB, or PD with dementia [5, 7]. Even though ultrastructural differences were still noticeable in the fibrillar rotation between the filaments analyzed from people with a clinical diagnosis of PD and DLB, the structure or the fold within the fibrillar core of the αSyn fibrils was almost indistinguishable. To indicate the structural resemblance of this unique fold from fibrils isolated from patients with Lewy disorders, this conformation was termed a "Lewy fold" [5, 7].

In MSA, different assembly conditions likely exist, as the cellular tropism of synucleinopathy is mostly directed towards oligodendrocytes instead of neurons and in which the fibrillar assembly of αSyn thus might be differentially influenced. Postmortem structural analysis of fibrils derived from the brain of three people with MSA has shown that αSyn fibrils are composed of two filaments that interact via their outer surface [5]. The outer filament layers pack and stabilize the conformation of the resulting fibril via a shared interface in which an unidentified substrate screens charged residues in the fibril cavity that allow the two filaments to closely interact [5]. The two filaments slightly differ in their conformation so that the fibril is asymmetrically composed by two non-identical filaments that twist around a central axis [5].

Although the fibrils from PD and DLB (or PD with dementia) show slight differences in their overall structure, the core of the two types of PD and DLB fibrils is surprisingly similar. They both have a typical Lewy fold but with a slightly different pitch, in which the fibrils turn around their own axis [5, 7]. Although these results remain to be replicated in larger studies, the similarities between fibrils of Lewy disorders tentatively suggest that these fibrils have shared ontologies and that Lewy disorders could be part of an overlapping or continuous disease spectrum where cellular conditions are present under which structurally similar proteopathic seeds can assemble in neurons. This largely contrasts with the disease conditions in MSA, where oligodendrogliopathy or oligodendroglial cellular risk factors might influence the formation of a different type of fibril [16, 17].

Because of the unique folds in the conformation of the fibrils from Lewy diseases and MSA brain, the pathogenic blueprint of distal pathology might be imprinted within the conformation of these fibrillar strains. The cellular interactome and the conditions in which fibrils assemble can have a strong impact on the amplification and the structure of the resulting fibril [6]. Nevertheless, establishing the cellular conditions in which αSyn strains form and why they develop their typical structural features remains largely unexplored. To understand the etiopathogenesis of synucleinopathies, it will be important to further address these questions.

Fibrillar Transmission in Animal Models of Synucleinopathy

An important aspect of fibrillar in vivo toxicity is its sustained or infectious pathogenicity via filaments that seed with endogenous monomers to form mature inclusions, thereby disrupting central cellular processes [61]. Initial evidence that recombinant αSyn fibrils could cause pathology in vivo came after injecting fibrillar αSyn in transgenic mice (M83) bearing the familial A53T mutation of αSyn [62]. Inclusions of αSyn phosphorylated on serine at position 129 (pSer129-αSyn) in the striatum, substantia nigra, and connected areas progressively worsened over time. This accumulation of pSer129-αSyn was accompanied by moderate dopaminergic degeneration and behavioral impairment [62]. Following this, a study by the same group showed that striatal injection of fibrillar αSyn could also trigger de novo formation of pSer129-αSyn pathology in wild type mice (C57BL6). The authors again observed a spatiotemporal pattern of pSer129-αSyn pathology from the site of injection with neurodegeneration [63]. These findings were important in the light of the transmission hypothesis and provided the first evidence that αSyn fibrils can seed pathology in vivo.

In support of this view, subsequent studies independently confirmed that injection of in vitro assembled αSyn fibrils in wild type mice and rats can seed in vivo αSyn aggregation [64–67]. Direct live imaging of αSyn inclusion formation in mouse cortex showed that cortical neurons selectively degenerate during inclusion formation [68]. This is in accordance with other studies that established a close relationship between αSyn inclusion formation and cellular toxicity during seeding [61]. Injection of fibrillar αSyn and not oligomeric αSyn seems to be crucial for inclusion formation [69]. Although oligomeric αSyn can cause local neuronal damage, only stable fibrillar seeds can trigger the conversions of endogenous αSyn into new aggregates akin to Lewy pathology [69, 70]. Small fibrillar aggregates (in the range of 50–100 nm) are potent seeders and more efficiently cause inclusions and pathology than their larger fibrillar counterparts [71].

However, cellular degeneration also appears to be cell-specific and selectively affect vulnerable populations of cells over time [72, 73]. Some neurons might therefore remain resilient and show no degenerative changes, even after fibrillar uptake. This cell-type specific vulnerability and resilience is an intricate mechanism of αSyn cell-to-cell transmission and will be further discussed in one of the following sections. Altogether, αSyn transmission models using in vitro generated fibrils have now become invaluable for designing and testing new strategies that intervene with αSyn-related mechanisms of neurodegeneration.

Strain-Specific Transmission in Animal Models of Synucleinopathy

Given the cellular tropism of pathology in synucleinopathies and the neuropathological changes seen in experimental models during αSyn transmission, research has focused on whether phenotype-specific effects could be transferred by the fibril conformation. As it was shown by in vitro intracellular seeding, different strains of αSyn, ribbons and fibrils, amplified into distinct types of inclusions that conferred phenotype-specific effects [60]. These two type of strains were subsequently injected into the substantia nigra of rats [70]. Both fibrils amplified in vivo and caused phenotype-specific effects while retaining their conformation-specific properties [70]. Because of the relationship between structure of ribbons and fibrils and the cellular phenotypes that were inseparable from the two fibrillar types, the two in vitro generated fibrils thus behaved as protein strains.

The relationship between the structural diversity of αSyn strains and developing in vivo phenotypes was further shown via other approaches. This involved the generation of αSyn fibrils via altering the pH or other salts in the buffer composition, via repetitive seeding or via adding cell-specific or environmental cofactors relevant to PD or MSA that might influence the folding of αSyn [70, 74–78]. As such, repetitively in vitro seeded αSyn fibrils caused tau pathology in vivo whereas first generation or de novo assembled fibrils did not [58]. Incubation of αSyn with the bacterial endotoxin lipopolysaccharide, or the oligodendroglial protein p25, resulted in the assembly of unique fibrils that amplified in vivo with strain-specific toxicities. The conformational variation of distinct fibrils also largely determined which cellular populations were targeted and the brain regions that developed pathology [76, 77]. These phenotypes were furthermore retained after serial passaging between different animals emphasizing a mechanism that involves conserved templating-directed amplification of the injected strain in vivo [77]. Collectively these studies thus show that recombinant fibrils of αSyn with a defined conformation can act as strains, providing strong evidence for a

structural-pathological relationship between the conformation of the αSyn fibril and the developing phenotype [58].

Next to using recombinant assembled fibrils, strain-related pathologies have also been studied by using brain extracts or by purifying and amplifying αSyn fibrils from patient brain for in vivo transmission (Fig. 2). In the case of brain extracts, pathological brain is homogenized and directly injected intracerebrally in wild type or transgenic animals [64, 79–86]. Several studies have compared intracerebral injection of brain homogenates from people with PD, DLB, or MSA with similar findings: MSA brain homogenates are generally more toxic and cause more aggressive neurodegeneration with neuroinflammation than brain homogenates derived from people with Lewy disorders [64, 81–85].

In line with these observations, experiments with fibrils purified and amplified from human brain have shown similar outcomes [81, 87]. Here, after isolating αSyn fibrils from patient brain, fibrils are amplified via seeded amplification and the residual brain material after extraction is diluted to levels at which it becomes undetectable (Fig. 2). By controlling the in vitro assembly conditions of αSyn, seeded amplification will generate αSyn fibrils that are directly derived from human fibrillar seeds. After serial sonication and amplification by shaking and incubation with recombinant monomers, the structural information from brain-derived seeds is at least partially templated onto the newly assembled fibrils [6, 88] (Fig. 2). After injection of fibrils derived from PD, DLB, and MSA brain in rodent brain, animals develop distinct phenotypes, depending on the type of fibril [81]. Reminiscent of experiments with brain homogenates, fibrils derived from MSA brain induced the most progressive phenotype, with more pronounced PSer129-αSyn inclusions around and away from the injected site [81]. In human induced pluripotent stem cells (iPSC) derived dopaminergic neurons, the aggregate burden of MSA or PD fibrils was determined by αSyn expression levels and the type of strain administered to the cells [89]. Fibrils derived from the brain of people with distinct clinical syndromes thus uniquely affect cellular health and transmit pathology to distinct cell types or brain areas, again illustrating that αSyn strains and the cellular environment strongly interact.

Both materials, brain homogenates or seeded and templated aggregates, have advantages as well as disadvantages for preclinical animal studies as injection of brain homogenates will contain unmodified αSyn aggregates with their post-translational modifications whereas amplified fibrils do not have the same modifications. Fibrils isolated from Lewy bodies have several post-translational modifications, including phosphorylation, acetylation, ubiquitinylation, C-terminal truncations, among many others [90–93]. Notable differences are also found in the distribution of these modifications in αSyn isolated from brains with either Lewy or MSA pathology [93]. Modified soluble αSyn, such as PSer129-αSyn, can influence the seeding, transmission, and

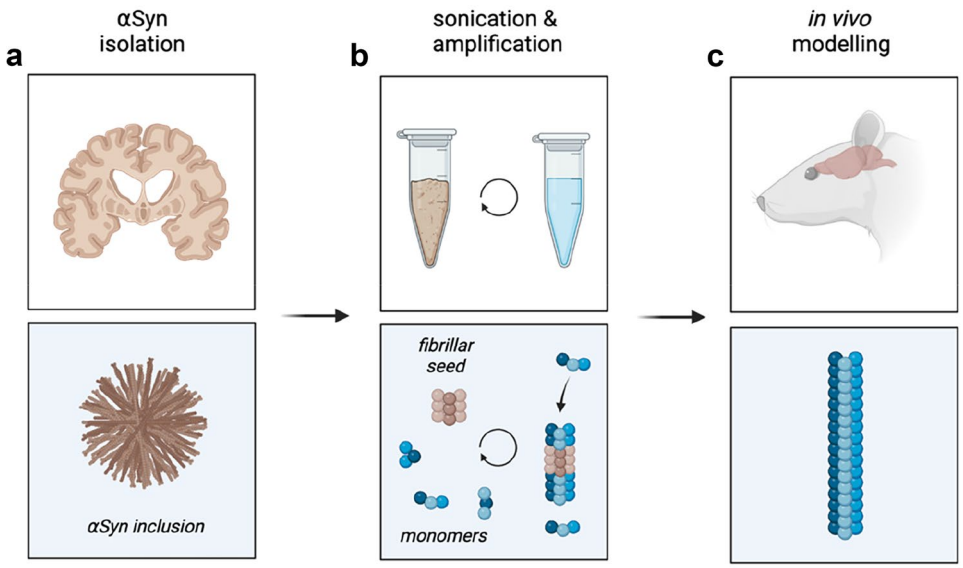

Fig. 2 Seeded amplification of αSyn from human brain. **a** For the amplification of αSyn from human brain, brain tissue is isolated from selected brain regions and homogenized. Samples are sonicated to fractionate αSyn fibrils into smaller fibrillar seeds. **b** Monomeric αSyn is added with amplification buffer and in vitro templated amplification takes place under shaking conditions using the fractionated seeds from the brain homogenate. Fibrils will elongate after incorporation of new monomers at the fibril ends via templating-directed amplification and building on the original input conformation. After each reaction, amplified samples are diluted and sonicated to generate new fibrillar fragments. **c** When this process is repeated several times, highly homogenous fibrillar assemblies, free from brain material, will be available for subsequent experimental work in animal models

pathogenicity of αSyn fibrils in vitro and in vivo [93–95]. In contrast, the protein misfolding cyclic amplification method amplifies fibrils with recombinant αSyn that is largely free of post-translational modifications; however, using this method, it more strictly avoids inoculating any potential host-specific pathological triggers, present in brain homogenate, such as inflammatory mediators, that could set off unwanted αSyn aggregation. Altogether, studies using either brain homogenates or brain-derived amplified seeds provide additional support that αSyn strains can be amplified from human brain and that unique structural information can be transferred via seeded amplification. This structural information can also transmit disease-specific pathology in vivo.

The Cellular Milieu Impacts Fibrillar Transmission—Lewy Disorders

Significant evidence thus supports a role for αSyn strains in the development of synucleinopathies and αSyn fibrils from antemortem and postmortem samples have a unique disease-specific fold that is intimately tied to a clinical diagnosis of Lewy disease or MSA [5–8]. But why is the fold of fibrils between different syndromes so different—or, how do αSyn fibrils obtain their unique conformation?

The structural and cellular heterogeneity of native αSyn is astounding, and the potential conformations that αSyn can adapt are almost limitless. Within these lines, multiple studies have shown that by varying aggregation conditions, numerous structural variations in the fibrillar conformation can be obtained [48, 59, 60, 70, 74–77]. Nevertheless, there seems to be a remarkable uniformity between the conformation of αSyn fibrils that are isolated from unrelated patients that share a similar clinical diagnosis. The solved structures of fibrils from patients with Lewy diseases—albeit a limited group—are almost indistinguishable, and although the fibrils from MSA patients do show subtle differences, depending on the region from where the fibrils were isolated, their quaternary organization and filament outer and inner core layers are closely similar [5]. Together with the experimental evidence that patient-derived fibrils of αSyn can cause disease-specific phenotypes in experimental models, it can be concluded that the conformational landscape of aggregated αSyn in patient brain is relatively narrow, which is counterintuitive from the many possible conformations that αSyn fibrils could potentially and experimentally adapt. This now leaves the question of how these disease-specific assemblies arise or why only a restricted population of fibrillar strains exists within a patient's brain.

Although addressing this question can be experimentally challenging, cellular and in vivo models have provided important clues as to whether cell autonomous factors might limit or promote the transmission of pathology. Concurrently, these cellular factors could be equally responsible for the maturation of αSyn into phenotype-specific fibrils. On the level of cellular connectome, there are several determinants that could influence the transmission of αSyn fibrils. First is the susceptibility or resilience of the incipient or recipient cell in which fibrils assemble or transmit (the cell type). Secondly, transmission is strongly dependent on αSyn levels with which fibrils can amplify (αSyn expression). A last factor is the strength of projections between the cells in which fibrils amplify and to which the seeds are transmitted (the connectome).

In normal human brain, as well as in the brain of people with PD and MSA, the highest levels of *SNCA* gene expression is in neurons, followed by microglia and mature oligodendrocytes, where expression is still significant but lower [19, 40]. Although directly comparing αSyn expression between human and mouse brain has not yet been possible, a similar distribution is seen in mouse brain [18]. Several studies have examined the pattern of transmission in mouse brain, by injecting fibrillar αSyn in defined brain areas. Based on the observation of pSer129-αSyn inclusions, the transmission of pathology was shown to be dependent on the levels of endogenous αSyn but also on the cell type in which pathology develops [96–98]. As such, these experimental models show that seeded aggregation as well as transmission of αSyn is a function of brain connectivity and αSyn protein levels.

Notably, pSer129-αSyn-specific pathology can be transient, as regions that develop pSer129-αSyn inclusions have been shown to eventually clear pSer129-αSyn pathology [72], indicating that in addition to the neuronal connectome and cell-autonomous factors, there can be neuronal resilience that counteracts in vivo transmission. Contrarily, genetic or environmental risk factors can facilitate αSyn fibrillar permissivity. For instance, GBA or LRRK2 genetic variants can unlock a disease-associated cellular environment in which the barriers for fibrillar transmission are lowered [99–102], although the exact mechanisms behind this are not yet clear [103]. The propagation of αSyn appears thus restricted in certain subsets of cells but facilitated by others. These features thereby define the cellular subtype as a last determinant of fibrillar transmission, in which fibrils can either become degraded or amplify in conjunction with PD-associated host risk factors.

In light of the strain hypothesis, the serial transmission through a permissive neuronal route could thus be a conditio sine qua non for the assembly of conformation-specific filaments as it would almost invariably lead to a defined path down a folding landscape in which only a restricted set of fibrillar assemblies with high thermodynamic stability can exist. As the host cellular environment both restricts as well as facilitates the formation of fibrillar strains, a predominant filament can further amplify within its disease-associated cellular environment on the background of the aforementioned neuronal determinants.

What further illustrates this idea is that although strains propagate phenotype-specific effects in vivo, in vivo amplification within a defined cellular environment can lead to measurable conformational changes in the folding landscape (Box 1). By directly measuring the conformational spectrum using brain-penetrant conformation-sensitive fluorescent probes, it was shown that the conformation of pSer129-positive αSyn aggregates in Lewy bodies is strongly restricted, with a low degree of conformational variability [104]. Similarly, the folding landscape of pSer129-positive αSyn aggregates in oligodendrocytes is narrowly distributed, but shows a conformational spectrum that does not overlap with that of Lewy bodies [17, 104]. The use of these oligothiophene probes does not require any extraction or fibril amplification and they measure the structural state of aggregates in their respective environments in situ. These results therefore corroborate that αSyn fibrils in neurons and oligodendrocytes adopt distinct conformations within their respective cellular environment.

Other studies that examined the composition of Lewy bodies in situ have also found remarkable findings. Lewy bodies are complex intracellular deposits composed of complex lipid structures and multiple αSyn assemblies with distinct PTMs, depending on its distribution within the Lewy body [91, 92]. The initial steps of αSyn aggregation might take place on the membrane, and more in particular on membranes that have a particular affinity for αSyn, thereby acting as an aggregation hot spot, as is for instance seen for mitochondria, the nuclear membrane, or the Golgi membrane [61, 105]. There is thus a strong enrichment in Lewy bodies of proteins and undigested membranes, from dystrophic mitochondria, lysosomes, or other lipid-rich damaged organelles in between which αSyn fibrils are sequestered [90–92, 106, 107].

The cellular proteome and lipidome could thereby act as a crucial cofactor for templated seeding of unique αSyn assemblies into fibrillar inclusions or αSyn strains. Future studies will have to identify the cellular identity of the transmissible connectome and the cellular cofactors that conduct the amplification of heterogeneous assemblies into phenotype-specific strains. Reconstructing the protein and lipid interactome as well as the connectome of sequentially transmitted αSyn fibrils in humanized models could provide important new clues as to which cofactors and cellular risk factors might govern these processes. Identifying the molecular and cellular identity of a strain-permissive environment could be very valuable as this could inform the development of novel in vitro or in cellulo seeding assays for assembling phenotype-specific recombinant PD, DLB, or MSA αSyn strains, as it has been successfully done for other amyloid proteins.

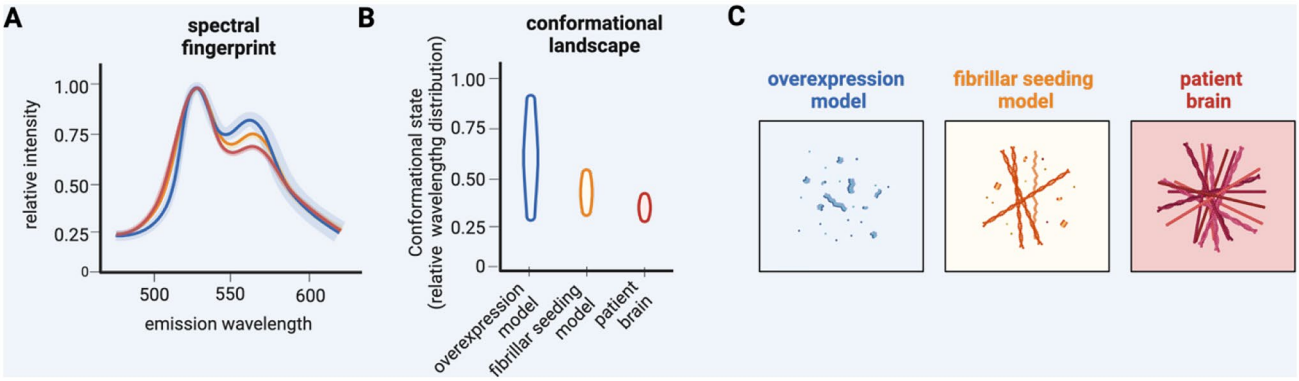

Figure box 1 Measuring the structural state of αSyn in situ. By using conformation-sensitive oligothiophenes, the relative conformation and the structural heterogeneity of aggregated αSyn can be directly measured in the brain (adapted from [17]). **A** Oligothiophenes indirectly provide the relative conformational state of aggregated αSyn via their emitted fluorescent spectrum adjusted to its relative intensity. Assemblies with a comparable structure will have unique spectral emission fingerprint, shown by an overlapping spectral profile, whereas differences in the structural state are seen by changes in the emission spectra. **B** The overall heterogeneity of the structural state is reflected by variations in the distribution of superimposed spectra from multiple inclusions. This allows to visualize the effects of templated-directed seeding as seen by a reduction in the variability or an overall narrowing of the conformational landscape for fibrillar seeding models (yellow), whereas aggregates in overexpression models lack a predominant structure (blue). **C** An illustration of the aggregated states of αSyn in an overexpression model, a seeding model, and human brain. In the αSyn overexpression model, the folding landscape of aggregated αSyn is relatively heterogeneous whereas after seeding with fibrillar αSyn more defined inclusions will form. By seeding in vivo with αSyn strains, the conformational variation between the populating assemblies becomes more uniform and more closely matches the spectral profile of the human brain

Box 1 A tool for studying the conformational landscape of αSyn in brain tissue

To study the conformational distribution of aggregated assemblies in rodent or human brain tissue, conformation-sensitive oligothiophenes (luminescent conjugated oligothiophenes or LCOs) can be directly applied in situ [108]. These luminescent probes have a flexible backbone, and they bind with β-sheet rich amyloid protein, such as αSyn. Depending on the orientation of the probe, it will emit a fluorescent spectrum upon stimulation that is representative of the structure of the aggregate it binds with. Via direct labeling of brain tissue, the conformation of aggregated αSyn in individual cells can be measured and the tissue and composition of the aggregate remain intact. By using LCOs, the conformational profile of various types of aggregates can be measured and directly compared with the conformational profile of aggregates in PD or MSA brain. This method is relatively accessible and easy to implement as it only requires standard immunohistochemical techniques and a confocal microscope to determine the fluorescent emission spectrum of cellular inclusions in vitro or in vivo.

The Cellular Milieu Impacts Fibrillar Transmission—MSA

In MSA, pathological changes are accompanied by a high burden of oligodendroglial αSyn in areas with dominant oligodendropathy and neuronal degeneration [109, 110]. Although it is not yet known how MSA arises, several experimental findings point to a strong link between αSyn aggregation, oligodendropathy, and the degenerative process. Experiments with various animal models of MSA that selectively overexpress human αSyn in oligodendrocytes have shown that αSyn overexpression is sufficient to cause oligodendropathy, resulting in structural and functional in vivo changes that in part resemble those of MSA (reviewed in [11]). Intracerebral injection of MSA brain homogenates or amplified fibrils from MSA brain causes neurodegeneration in wild type and transgenic animal models [79, 81, 82, 84, 85]. When compared to αSyn assemblies derived from the brain of people with Lewy disorders, MSA-derived fibrils are invariably more pathogenic.

The more aggressive nature of MSA fibrils might explain why MSA is a more progressive disease than Lewy disorders, but some crucial aspects as to how the typical pathology of MSA is caused remain puzzling. For example, direct intracerebral injection of MSA fibrils in animal models causes primarily neuronal pathology but has mostly failed to trigger oligodendroglial dysfunction. There is thus still a discrepancy between the strain-related phenotypic effects and the development of oligodendropathy in experimental models of MSA.

As such, there might be a unique but elusive role of the oligodendroglial cellular environment in the development of MSA. Although the insoluble proteome in human PD and MSA brain shows a significant overlap in mitochondrial and neuronal synaptic proteins [111], the potential interactome of αSyn in oligodendrocytes could be very different from that in neurons as both the proteome and especially the lipidome in oligodendrocytes are unique [112, 113]. The role of αSyn in oligodendrocytes remains largely unexplored and it has been questioned if oligodendrocytes can express sufficient endogenous αSyn to form intracellular aggregates [114, 115]. Nevertheless, despite some contradictory reports, expression of αSyn is detected in oligodendrocytes of the human and rodent brain [18, 19, 116, 117], albeit at considerably lower levels compared to neurons. Some disease-associated subtypes of oligodendrocytes in human brain have also been shown to express higher levels of αSyn [118]. Experimental findings point towards an active role of oligodendrocytes that involves template-directed amplification, and which would require the expression of αSyn in oligodendrocytes [16, 17]. It has been shown that by passaging fibrillar αSyn in the oligodendrocytes, a unique type of fibril develops as a consequence of repeated passaging within the oligodendroglial milieu [16, 17]. The conformation of MSA filaments is unique as they have a distinct fold with two filaments positioned around a polar cavity between the two central layers, which are screened by an unidentified residue [5]. This residue allows the two protofilaments to come closely together and interact. Why MSA fibrils have this unique feature is not yet known, but it likely happens via yet unidentified oligodendroglial factors that facilitate the templated seeding of MSA-specific fibrils.

The oligodendroglial cellular environment can thus introduce significant structural variations in the conformational state of the fibril and as a result an oligodendroglial-specific strain will develop [16]. Oligodendroglial strains amplify faithfully in oligodendrocytes, and interestingly, the same conformation can be transmitted to neurons, which apparently cannot convert the new conformation back to a neuronal strain [16]. Although this remains to be experimentally tested, it raises the possibility that MSA strains can be transmitted between oligodendrocytes and neurons, and that the predominant strain, formed in oligodendrocytes, could be passaged between neurons and oligodendrocytes while retaining most of its oligodendroglial structural features and its aggressive cellular pathogenicity. This would eventually lead to a conformational landscape with a restricted number of fibrillar variants in MSA brain [5, 17, 104, 119], as its conformation would be largely determined by host-specific cellular factors.

Furthermore, the interactions during templated amplification in oligodendrocytes seem to be bidirectional, as interactions also exist between the fibrillar strain and the intracellular milieu. Indeed, intrastriatal injection of two different types of αSyn strains, ribbons and fibrils, in MSA transgenic mice overexpressing oligodendroglial αSyn revealed that the αSyn conformational landscape not only depends on cell autonomous factors, but also on the transmitted strain [17]. For instance, compared to fibrils, injections of ribbons more closely represented the conformational profile of glial cytoplasmic inclusions of the human brain [17]. This was accompanied by a narrowing of the conformational landscape, indicating that a predominant form arose after amplification in vivo (Box 1 and [17]). Thus, combining the selective overexpression of oligodendroglial αSyn with intracerebral injection of αSyn fibrils resulted in a phenotype that more closely mimics MSA [16, 17]. Together, it can be postulated that for Lewy disorders as well as for MSA αSyn aggregation can occur actively in either neurons or oligodendrocytes via templated seeding with endogenous αSyn.

From Cellular Triggers to Strain Maturation

Although αSyn has been historically viewed as a protein important for synaptic or neuronal function, it has multiple functional roles, since, as we described earlier, the protein is expressed in multiple cell types in tissue outside the central nervous system. αSyn is abundant in peripheral tissue of barrier organs and an increasing number of studies have shown that exposure to pathogens within these visceral sites can lead to accumulation of αSyn via infiltrating immune cells or alternatively, via triggering its expression in peripheral neurons as part of an innate immune response [21, 22, 38, 39, 42–46, 120]. Next to this cellular diversity, native αSyn interacts with multiple subcellular structures and across cellular compartments and depending on its precise role it can be located within the synapse, the soma, or the nucleus of the cell [121].

To fulfill these distinct roles, the conformational flexibility of αSyn provides the protein with an advantage. However, because of its pleomorphic nature, the protein may also form unwanted interactions when its expression levels are triggered to levels that exceed its own solubility [59]. Inflammation and infections may trigger expression of αSyn, especially at visceral barrier sites, where pathogens can more freely interact with αSyn-expressing cells [42, 122]. Pesticides or infectious pathogens can directly stimulate the expression of αSyn in enteric or peripheral innervations [123, 124]. Viral infections have been associated with aggregated αSyn within peripheral sites [120, 125], but also in the brain during encephalitis with neurotropic agents [126, 127].

Multiple triggers could thus potentially lead to the aggregation of αSyn with a sustained proteopathic burden. Inflammation or infection triggered aggregation appears to be a general effect that could take place in any individual exposed to an environmental trigger. Aggregated αSyn has indeed been found in visceral sites, for instance in the gut or the appendix [128], of apparently normal individuals, without any signs of neurological illness. A popular hypothesis is that aggregated αSyn can escape these visceral sites and spread to the central nervous system via parasympathetic or sympathetic connections [22, 129]. Indeed, injection of fibrils in the gut of wild type mice and transgenic rats was shown to induce transmission to the brainstem via the vagal nerve after which it further propagates to distant areas [130, 131]. Parkinsonian risk factors or aging could further influence these effects, leading to a more permissive transfer of pathogenic αSyn from the periphery to the brain [132, 133]. Similarly, injection of fibrillar αSyn in the urinary tract was recently demonstrated to lead to its transmission to the brainstem via sympathetic and spinal projections [134].

Based on these observations, a hypothetical model can be constructed that leads to fibrillar maturation of phenotype-specific αSyn strains during in vivo transmission (Fig. 3). In this model, fibrillar assemblies will passage between multiple cells via their connectome and will be repeatedly subjected to distinct intracellular interactomes. The transmission route will depend on cell-autonomous factors such as the expression levels of αSyn, cellular or disease-specific risk factors, and the selective vulnerability of the recipient cell. For each cellular passage, the transmitted seed will be subjected to new cellular environmental conditions and amplify with heterogeneous αSyn folding intermediates. When αSyn filaments amplify with endogenous αSyn, cell-specific folding intermediates can incorporate their structural changes, inherent to the environment as their templating and the intracellular amplification of αSyn will directly depend on their cellular cofactors. Similarly, the quaternary arrangement of two filaments can also be affected since some cells might express certain cofactors required for the interaction of these filaments. By passaging and amplification within different environments, the conformation of the transmitted seed can be significantly impacted. Repeated passages between different cells, such as neurons or oligodendrocytes, could thus lead to the formation of distinct and mature αSyn strains that will not undergo anymore structural changes and have cellular or disease-specific post-translational modifications as opposed to the greater heterogeneity of transient assemblies en route to a more stable conformational state that can be maintained by the host. The conformational state of these mature assemblies will thereby reflect syndrome-specific transmission pathways between a trigger site and central structures with mature pathology.

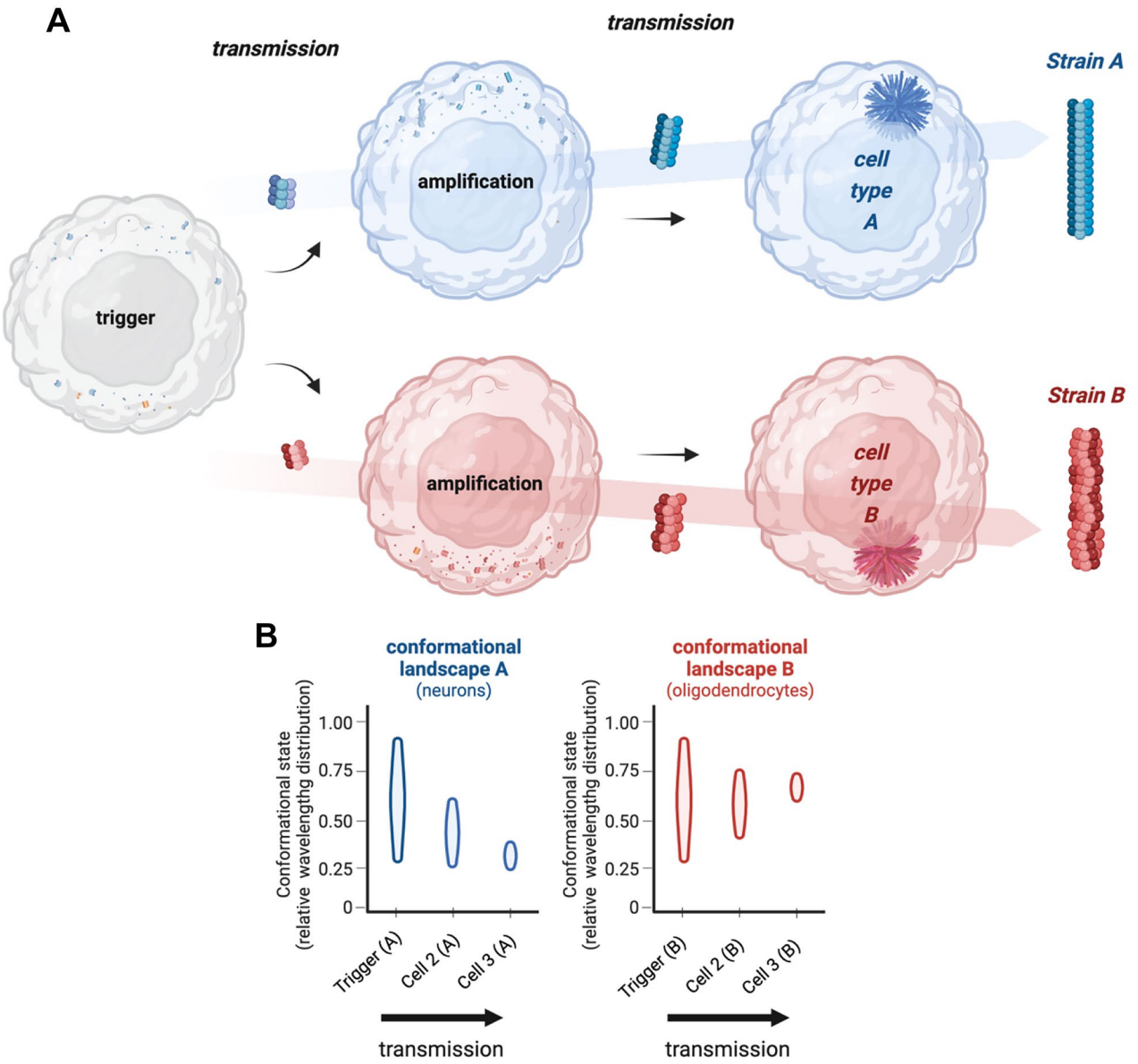

Fig. 3 A model of αSyn strain maturation during sequential in vivo transmission. **A** A trigger causes transient upregulation of αSyn, leading to its intracellular aggregation. Stable αSyn aggregates can transmit to neighboring cells via connected transcellular pathways. In the presence of sufficient endogenous αSyn in the recipient cell, amplification can occur with the transmitted seed. The transfer of αSyn fibrillar seeds to resilient cells or cells that lack sufficient endogenous αSyn will lead to unsuccessful amplification and halt serial transmission. Different cell types (A or B) can uniquely influence templated amplification as αSyn aggregates will interact with unique cellular risk factors. During in vivo amplification, cell-specific cofactors will differentially impact the conformational state of αSyn folding intermediates or αSyn filaments and introduce structural variations during templated amplification. The final conformation of the mature fibril is the function of the in vivo connectome, cellular αSyn expression, and cellular risk factors. **B** The conformational landscape of αSyn aggregates after sequential transmission via alternative transmission pathways. The maturation of αSyn fibrils from heterogeneous assemblies into phenotype-specific strains is shown over serial passages. An αSyn trigger elicits expression of αSyn, of which the assemblies lack a dominant conformation and this is reflected by the heterogeneous folding landscape of the populating assemblies. After transmission of αSyn seeds to the recipient cell, mature seeds will amplify intracellularly, whereas non-amplifying seeds will be degraded, leading to a narrowing of the conformational spectrum. Only a limited number of cell types will sustain intracellular amplification (positive selection) whereas other cell types will inhibit amplification (negative selection) resulting in a further structural selection. Templated amplification during sequential transmission will lead to the in vivo formation of mature αSyn fibrils and formation of a predominant strain in vivo

Conclusion

The conformational features of αSyn during health and disease have been a long-studied topic in synucleinopathies. The native assembly of αSyn into functional or folding intermediates in a rapidly changing environment needs to be strictly controlled to avoid the protein to form unwanted interactions. Inflammatory interactions at visceral barrier sites may trigger αSyn expression during high metabolic burden and lead to its assembly into potential pathogenic aggregates. In vivo transmission models have demonstrated how these aggregates can escape peripheral sites and transmit transcellularly to the central nervous system [12, 14]. It appears that αSyn transmission routes follow a spreading pattern determined by their cellular connectome and this spread is facilitated by cellular risk factors inherent to Lewy disorders or MSA.

By experimentally varying in vitro assembly conditions, αSyn aggregation into recombinant fibrils can yield a great variety of structural states. In contrast, the number of possible structural conformations of αSyn aggregates in human brain and in vivo seeding models appears to be much more restricted [5–8, 17, 104, 119]. There is also a significant structural overlap between αSyn filaments isolated from patient brain with a similar clinical diagnosis [3–5]. This illustrates that there is a remarkable and maybe unexpected biological uniformity between the conformations of fibrillar αSyn.

Even if it is not yet clear why such a disease-specific structural fingerprint exists, the cellular conditions in which αSyn forms stable fibrils might be instrumental for these assemblies to form. This phenotype-specific conformational uniformity could point to a molecular signature of disease and as such, αSyn strains could potentially serve as a biological substrate of synucleinopathy subtypes. This has important but promising implications for αSyn biomarker-based diagnostics that are currently under development.

It remains to be investigated how structural information is translated onto elongating fibrils during seeded amplification in vivo. The cellular environment appears to have a significant impact on the in vivo assembly of fibrillar αSyn during sequential transmission. By comparing the structure of amplified and non-amplified fibrils from patient brain, it was shown that aggregation conditions can impact seeded amplification and that information might be partially lost during in vitro templating [6]. Although some phenotypic effects are translated and propagated robustly during fibrillar transmission in vivo, this discrepancy also reflects an important shortcoming of current preclinical models.

To improve the translational value of these models as well as to improve cross-laboratory findings, the effects of isolating and amplifying recombinant strains from native disease-specific assemblies for transmission studies need to be better understood. Within the same lines, it will be important to provide the correct αSyn substrate in vitro and in vivo, to allow faithful amplification of disease-specific conformations. This might require working with assemblies with disease-specific post-translational modifications or providing protein or membrane substrates for reliable templating during seeded amplification. Providing a physiological environment that is both permissive but also resilient for serial transmission and allowing sufficient time for assemblies to seed and transmit so that Lewy- or MSA-like pathology can develop in a progressive manner could promote the formation of mature, compact inclusions that more closely mimic those of the human brain. By standardizing analyses methods that leave aggregates and brain tissue intact, and that are fast to implement in the lab, for instance via in situ labeling, additional quality controls could ensure further standardization and improve the translational value of these preclinical transmission models. Hence, it will be important to determine what the exact contribution of the cellular environment is, and whether in vitro conditions can be further optimized so that experimental Lewy and MSA phenotypes can be more robustly reproduced.

Future studies will need to confirm that this structural uniformity of αSyn between diseases indeed exists, and if so, the field will need to devise new methods and guidelines for the standardization and development of recombinant disease-specific fibrils and their use for in vitro, in cellulo, and in vivo studies as slight variations between assembly conditions could significantly impact the experimental outcome. Doing so will further improve the validity of preclinical studies, potentially via humanizing animal models, removing the species barrier, and assuring full sequence homology for reliable templated seeding with human αSyn strains in vivo. Collectively, these new insights will facilitate the development of novel biomarkers and facilitate αSyn-targeting drug discovery efforts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13311-023-01365-5.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author Contribution WP conceptualized and wrote the manuscript; VB corrected and finalized the manuscript.

Funding We acknowledge financial support from the FWO-Vlaanderen (postdoctoral fellowship to WP and project G081121N) and the KU Leuven (project C14/18/102).

Declarations

Conflict of Interest The authors declare no competing interests.

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