

α -Synuclein oligomers and fibrils: partners in crime in synucleinopathies

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Abstract

The misfolding and aggregation of α -synuclein is the general hallmark of a group of devastating neurodegenerative pathologies referred to as synucleinopathies, such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. In such conditions, a range of different misfolded aggregates, including oligomers, protofibrils, and fibrils, are present both in neurons and glial cells. Growing experimental evidence supports the proposition that soluble oligomeric assemblies, formed during the early phases of the aggregation process, are the major culprits of neuronal toxicity; at the same time, fibrillar conformers appear to be the most efficient at propagating among interconnected neurons, thus contributing to the spreading of α -synuclein pathology. Moreover, α -synuclein fibrils have been recently reported to release soluble and highly toxic oligomeric species, responsible for an immediate dysfunction in the recipient neurons. In this review, we discuss the current knowledge about the plethora of mechanisms of cellular dysfunction caused by α -synuclein oligomers and fibrils, both contributing to neurodegeneration in synucleinopathies. **Key Words:** amyloid aggregation; neurodegeneration; Parkinson's disease; protein aggregation; protein misfolding

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Introduction

α -Synucleinopathies are a group of devastating neurodegenerative disorders characterized by the abnormal deposition of insoluble aggregates, both in neurons and glial cells, whose major filamentous component is the α -synuclein (α S) protein. This group includes Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and other rare neuroaxonal dystrophies (McCann et al., 2014). Among these conditions, PD is the most widespread, affecting 1–2% of the global population aged 65 years, and more than 4% of the population aged 85 years or older; thus, age can be considered the most relevant risk factor for PD (GBD 2016, 2018). This pathology is characterized by the death of dopaminergic neurons in the *substantia nigra pars compacta*; their loss results in a severe depletion of the neurotransmitter dopamine, which is in turn responsible for the cardinal motor dysfunctions of the disease, represented by bradykinesia, muscular rigidity, resting tremor, postural and gait impairment (Kalia et al., 2015). DLB is the second most common neurodegenerative dementia, characterized by progressive memory loss, visual hallucinations, parkinsonism, and cognitive decline, which have

been linked to the presence of insoluble α S assemblies in the cortex (McKeith et al., 2017). Finally, MSA is a rare and fatal neurodegenerative condition defined by the presence of filamentous α S inclusions in oligodendrocytes, referred to as Papp-Lantos bodies (Roncicvic et al., 2014). It is characterized by motor disturbances such as akinesia, rigidity, and postural tremor, but also by autonomic failure and cerebellar ataxia (Wenning et al., 1994). Importantly, aggregated forms of α S were also found to be present in a high percentage (more than 50%) of Alzheimer's disease (AD) patients (Lippa et al., 1998; Koehler et al., 2013; Mattson et al., 2013; Robinson et al., 2018; Twohig et al., 2019), with pure AD representing a minority of cases (Robinson et al., 2018). Lewy body (LB) pathology in the brain of AD patients does not show the same pattern of accumulation observed in PD (De Ture et al., 2019), occurring in the olfactory bulb, amygdala, and other limbic structures (Serrano-Pozo et al., 2011). Its presence has been associated with faster cognitive decline, but also with visual hallucinations and extrapyramidal signs (Twohig et al., 2019). Similarly, α S accumulation and toxicity have also been linked to lysosomal storage disorders such as Gaucher Disease (GD), caused by biallelic mutations in the *GBA1* gene, encoding the lysosomal acid β -glucocerebrosidase (GCase), an enzyme deputed to the degradation of lysosomal glucosylceramide. GD is clinically characterized by hepatosplenomegaly, anemia, and bone crises (Daykin et al., 2021). In the last two decades, accumulated evidence indicated that people carrying *GBA1* mutations possess a high probability to develop PD (Horowitz et al., 2022), with some GD patients showing forms of parkinsonism concomitantly with the occurrence of LB pathology (Blanz et al., 2016), which is also present in other lysosomal storage disorders such as GM1/GM2 gangliosidosis, Sanfilippo syndrome, Niemann-Pick type C and α - and β -Mannosidosis (Shachar et al., 2011). α S is a 14-kDa intrinsically disordered protein first discovered in the electric organ of the cartilaginous fish *Torpedo californica* (Maroteaux et al., 1988), that became the object of intense investigation upon its association with PD. The primary structure of α S is reported in **Figure 1**, and consists of three main portions: the positively charged and amphipathic N-terminal region (residues 1–60), that mediates the association with phospholipid membranes and, after binding, forms α -helical structures (Chandra et al., 2003; Ferreon et al., 2009); the hydrophobic non- β -amyloid component (NAC) region (residues 61–95), responsible for α S aggregation (Giasson et al., 2001), and the negatively charged highly acidic C-terminal region (residues 96–140), that remains largely unstructured and is involved in many different interactions, such as those with ions, polycations, and polyamines (Brown, 2007; Lautenschläger et al., 2018). Interestingly, the NAC region was observed in senile plaques isolated from a single AD patient (Ueda et al., 1993), and its presence was lately confirmed in a larger number of AD-affected subjects (Masliah et al., 1996). α S populates a dynamic equilibrium between the soluble, monomeric, and unfolded state acquired in the cytosol of neuronal cells, and a membrane-bound one, in which it assumes a partial α -helical secondary structure (Chandra et al., 2003; Bussell et al., 2005; Burré et al., 2013, 2018). In contrast to the physiological conformations, a persistent β -sheet amyloid secondary

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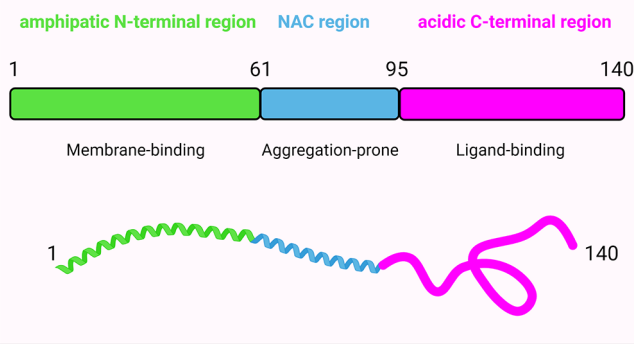


Figure 1 | Schematic representation of the primary structure of wild-type full-length monomeric α S.

NAC: Non- $A\beta$ -amyloid component. Created with BioRender.com.

structure is acquired in pathological conditions and is associated with the formation of oligomers and fibrils, ultimately deposited into Lewy bodies (Conway et al., 1998; Lashuel et al., 2002; Uversky, 2007), as represented in **Figure 2**. Such conformations, having different structures, sizes and morphologies, co-exist and convert each other depending on environmental conditions (Uversky, 2007). A growing body of evidence suggests that protein aggregation, including that of α S, is responsible for a toxic-gain-of-function mediated by misfolded aggregates, and plays a cardinal role in the pathogenesis of the associated diseases (Chiti et al., 2017; Tofaris, 2022). Over the last decades, the relationship between structure and toxicity of the different α S aggregates and the specific contribution of these conformers to neurodegeneration have been a matter of intense research. Mounting evidence suggests that prefibrillar oligomeric species, formed during the very early phases of the aggregation process, rather than mature fibrils or deposits, are by far the most neurotoxic (Bengoa-Vergniory et al., 2017; Bigi et al., 2021; Cascella et al., 2021, 2022). In particular, such assemblies were reported to have a central pathogenic role in the onset and progression of synucleinopathies, impairing the functionality of neuronal cells and ultimately leading to their death. Simultaneously, other researchers proposed that fibrillar α S species could also have an important role in neurodegeneration, by seeding the self-assembly of soluble endogenous α S into aggregated forms (Luk et al., 2009; Volpicelli-Daley et al., 2011; Cascella et al., 2022), and because of their ability to interact aberrantly with the plasma membrane, thus inducing its permeabilization, with subsequent ionic alterations and impairment of cytosolic organelles (Pemberton et al., 2011; Pieri et al., 2012). Here, we describe the main structural features of oligomeric and fibrillar α S conformers and their detrimental effects on neuronal cells.

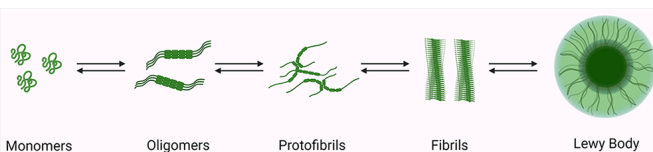


Figure 2 | Under specific conditions, the natively unstructured monomer can convert into oligomers, which can further aggregate into protofibrils and finally in highly ordered cross- β -sheet fibrils.

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Alpha-Synuclein Oligomers

An oligomer is a form of soluble aggregated α S that has not still raised the fibrillar conformation, and the term refers to a range of considerably different species varying in size, structural and biophysical properties, such as β -sheet content, and solvent-exposed hydrophobicity (Lashuel et al., 2002; Bengoa-Vergniory et al., 2017; Cremades et al., 2017). α S oligomers are usually described as on-pathway when they further aggregate forming mature fibrils, or off-pathway, if they are kinetically stable and remain in the oligomeric state without converting into fibrillar assemblies (Gosh et al., 2015). Small oligomeric conformers, intermediates within the aggregation pathway, are actually considered to be the most neurotoxic species in synucleinopathies (Bengoa-Vergniory et al., 2017), and, as a consequence, promising biomarkers for the early diagnosis and relevant putative disease-modifying therapeutic targets for such diseases (Majbour et al., 2016; Kulenkampff et al., 2021). Despite considerable effort, methods for the *in vivo* identification and isolation of α S oligomers are still lacking, given their metastable nature and very low concentration in human samples, such as the cerebrospinal fluid and plasma (Blömeke et al., 2022). For these reasons, studies aimed at identifying their specific role in neurodegeneration actually take advantage of oligomeric conformers synthesized *in vitro* from the monomeric protein. A large variety of preparation methods is described in the literature, giving rise to a plethora of species with extremely high levels of heterogeneity in terms of size, secondary structure, and solvent-exposed hydrophobic regions (Cremades et al., 2017); the formation of α S oligomers in solution can be promoted by

incubating the monomeric protein with a range of different molecules, such as lipids, metal ions, or other small molecules (Cremades et al., 2017; Alam et al., 2019). Given the metastable and transient nature of these species, numerous approaches have been used to stabilize them and to generate “kinetically-trapped” conformers remaining stable for time intervals that allow their structural, biophysical, and biological characterization (Cremades et al., 2017; Alam et al., 2019). Lyophilization is one of the most commonly used methods to stabilize oligomers, promoting at the same time the generation of conformers that are recognized by the oligomer-specific A11 antibody (Kayed et al., 2003); these species were reported not to further aggregate forming fibrils (Celej et al., 2012; Chen et al., 2015). Moreover, a range of natural compounds with the ability to bind specific oligomeric conformations was reported to block their elongation; among them, there are polyphenols such as curcumin, rosmarinic acid, baicalein and epigallocatechin gallate (Cremades et al., 2017). Oligomers have been shown to form upon α S interaction with brain-derived membranes (Ding et al., 2002) or other phospholipid bilayers and vesicles resembling biological membranes; their interactions give rise to conformational modifications that lead to oligomerization (Eliezer et al., 2001; Volles et al., 2001). Among the plethora of oligomeric conformers that could assemble during α S aggregation, single molecule techniques have suggested that the neurotoxic capability is associated with proteinase K resistance, which is, in turn, correlated with a β -sheet-rich structure (Cremades et al., 2012; Cascella et al., 2022). Consistently, two distinct oligomeric populations, referred to as Type A* and Type B* oligomers (OA* and OB*, respectively), with significantly different toxic properties, have been identified (Cremades et al., 2012); such species, presenting similar dimensions and molecular configuration, exhibit a considerable different secondary structure: while OA* are largely disordered, negative to the conformation-sensitive A11 antibody and non-toxic to neuronal cells, OB* possess a relevant β -sheet secondary structure, are positive to the A11 antibody and highly toxic to neuronal cells (Chen et al., 2015; Fusco et al., 2017; Cascella et al., 2021). These species closely match those of the previously identified unstable forms of nontoxic (type A) and toxic (type B) α S oligomers (Cremades et al., 2012). The structural determinants of their differential neurotoxicity have been identified by using the solution and solid-state nuclear magnetic resonance (ssNMR). While OA* remain largely unstructured and only binds to the membrane surface, OB* expose the highly lipophilic N-terminal region, responsible for the interaction with the membrane surface, and a structured and rigid core rich in β -sheet able to insert into the lipid bilayer, thus disrupting the neuronal membrane integrity (Fusco et al., 2017). Accordingly, the use of a specific antibody that targets the N-terminus of α S significantly reduced OB* toxicity when they were incubated with neuronal cells (Cascella et al., 2019).

Alpha-Synuclein Fibrils

Highly stable and ordered fibrils are the end product of α S aggregation, and represent the major filamentous components of the intracellular inclusions found in the brain of people affected by synucleinopathies (Arima et al., 1998; Spillantini et al., 1998; Goedert et al., 2017). However, whether the formation of such inclusions is cause or consequence of neurodegeneration is still a matter of intense debate. α S fibrils formed *in vitro* possess considerably different morphological and biological properties, depending on the various aggregation conditions (Kim et al., 2016; De Giorgi et al., 2020; Shrivastava et al., 2020). Despite such heterogeneities, these mature fibrillar conformers share cardinal amyloid properties, including an elongated and filamentous morphology, the capability to bind Thioflavin T, and a persistent β -sheet structure (Serpell et al., 2000; Sulatskaya et al., 2018; Gracia et al., 2020). The filament core approximately includes amino acid residues 30–100 of α S (Miake et al., 2002); a range of different techniques has been employed for the structural characterization of α S fibrils, including ssNMR, micro-electron diffraction, electron paramagnetic resonance, circular dichroism, hydrogen/deuterium exchange NMR, and cryo-electron microscopy (cryo-EM) (Vilar et al., 2008; Comellas et al., 2011; Gath et al., 2012; Tuttle et al., 2016; Cremades et al., 2017). In this way, the existence of different fibril polymorphs at the molecular level has been elucidated. Their heterogeneity is essentially associated with differences in the number of protofilaments, in their organization, as well as in the strength of the amino acid interactions determining the β -sandwich fold (Vilar et al., 2008; Comellas et al., 2011; Tuttle et al., 2016; Cremades et al., 2017; Li et al., 2018; Guerrero-Ferreira et al., 2019; Gracia et al., 2020), and directly related to the differential toxicity they evoke when applied to neuronal cells (Peelaerts et al., 2015). The heterogeneity of α S strains needs, however, to be validated by isolating *ex vivo* fibrils from patients' specimens. In this context, Strohäker and coworkers recently investigated the structural characteristics of α S fibrils amplified from brain extracts of both PD and MSA patients. Such filaments were reported not to possess markedly distinct structural properties, but to be substantially different from an array of fibrillar polymorphs synthesized *in vitro*, thus suggesting the existence of potentially considerable dissimilarities between *in vitro* and *in vivo* fibrils, and highlighting the urgency to use brain-derived conformers in future studies (Strohäker et al., 2019). In a subsequent study, Schweighauser and collaborators used cryo-EM to explore α S filaments, revealing that fibrils from the brain of MSA patients are structurally different from those present in individuals with DLB; moreover, they revealed that DLB-derived fibrils do not appear to twist, and are thinner than those present in the brain of MSA-affected subjects (Schweighauser et al., 2020). Again, despite some topological similarities, the structure of fibrils arising from MSA patients resulted to be different from *in vitro* assembled conformers (Schweighauser et al., 2020). In a very recent and elegant work, Yang et al. (2022) reported the cryo-EM microscopy structures of α S fibers extracted

from the brain of PD, PDD, and DLB patients. Such conformers were composed of a single protofilament (referred to as Lewy fold) remarkably different from the protofilaments observed in MSA subjects, thus probing the existence of different α S conformations in equally different neurodegenerative conditions. Even though the increasing relevance given to α S oligomers as the major culprits of neurodegeneration in synucleinopathies, important contributions of fibrillar species have also been reported. Such conformers are actually considered to be prion-like seeds able to transfer from neuron-to-neuron and propagate between interconnected vulnerable brain areas, thus driving the pathogenesis of α -synucleinopathies (Kordower et al., 2008; Li et al., 2008). Specifically, the addition of α S preformed fibrils (PFFs) to the extracellular medium of cultured neuronal cells was reported to corrupt the endogenous monomeric protein, that aggregates acquiring a pathological misfolded conformation finally culminating in the formation of inclusions morphologically and biochemically comparable to LBs (Volpicelli-Daley et al., 2011). The presence of these LB-like structures has been reported to evoke the loss of dopaminergic neurons in the *substantia nigra* (SN), as well the development of PD-like motor phenotypes in relevant animal models (Luk et al., 2012; Masuda-Suzukake et al., 2014; Osterberg et al., 2015). In a recent study, Bassil et al. (2020) revealed that the injection of α S PFFs in AD 5xFAD mice induced a significant increase in A β plaque burden by growing β -amyloid production from the amyloid precursor protein, in good agreement with previously reported evidence (Roberts et al., 2017).

Alpha-Synuclein Oligomers and Fibrils: A Complex Interplay

Since both α S oligomers and fibrils have been described as neurotoxic agents in synucleinopathies, the identification of the main culprit starting neurodegeneration is still a matter of debate. Although the presence of large intracellular α S inclusions in the brain of affected people is a cardinal hallmark of synucleinopathies, their abundance does not directly correlate with the disease phenotype observed in patients (Colosimo et al., 2003). We have recently demonstrated that fibrils can release highly toxic prefibrillar oligomeric species that promptly cause a massive dysfunction in neighboring neurons (Bigi et al., 2021, 2022; Cascella et al., 2021). Taking advantage of specific conformation-sensitive antibodies, we revealed that α S fibrils interacting with neuronal membranes progressively release A11-reactive oligomers resembling on-pathway oligomers formed during α S aggregation (Bigi et al., 2021; Cascella et al., 2021). Such conformers were internalized by neurons, where they induced ionic dyshomeostasis and oxidative stress, finally culminating in cell death (Cascella et al., 2021). Thus, we propose that both oligomeric and fibrillar aggregates of α S play a crucial role in neurodegeneration: fibrillar conformers act as a reservoir of soluble oligomeric species, which are the toxic counterpart promptly triggering neuronal dysfunction. In order to probe the nature of the aggregated species that penetrated into the cytosolic compartment, we performed immunofluorescence experiments using the A11 antibody in human induced pluripotent stem cells (iPSC)-derived dopaminergic neurons exposed to *in vitro*-synthesized fibrils (Figure 3; Bigi et al., 2021; Cascella et al., 2021). Confocal images revealed a remarkable A11-positive signal in cells treated for 24 hours with the previously described OB* and fibrils as compared to untreated ones, thus pointing out the presence of α S oligomeric species similar to OB* inside dopaminergic neurons upon treatment with extracellularly-added fibrillar assemblies (Figure 3).

The Pathological Spreading of Alpha-Synuclein

Our evidence clearly suggests the existence of a complex interplay between the different aggregated forms of α S, which can continuously convert each other under specific environmental conditions, as extensively reviewed in Bigi et al. (2022). The release of toxic oligomers from mature fibrils is one of the possible mechanisms explaining the fiber neurotoxicity, together with their widely explored capability to diffuse among neurons, thus contributing to LB pathology spreading between synaptically-connected brain areas. Importantly, α S fibrils are also uptaken by microglial cells, that preferentially phagocytose such conformers with respect to monomeric and oligomeric α S (Hoffmann et al., 2016). Fibrils promptly activate microglia, inducing the production of pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-1 beta (TNF- α and IL-1 β , respectively; Hoffmann et al., 2016). A detailed description of all the mechanisms by which neuron-to-neuron and neuron-to-glia spreading occurs was recently provided by Cascella et al. (2022) and Bigi et al. (2022) with their collaborators in two distinct reviews. The hypothesis of a prion-like spreading of α S strains through neuroanatomically connected areas in PD patients first emerged from the Braak hypothesis (Braak et al., 2002, 2003a). This theory postulates that Lewy pathology is induced by an unknown pathogen or toxin penetrated in the nasal cavity that subsequently reaches the gut and then spreads into the brain via the vagus nerve, finally joining the *substantia nigra pars compacta* (Braak et al., 2003a, b). This hypothesis was lately corroborated by Holmqvist et al. (2014) through the intestinal injection of lysates of human PD brains containing monomeric, oligomeric, and fibrillar α S in animal models, demonstrating the existence of a retrograde transport of such conformers through the vagal nerve, finally reaching the brainstem in a time-dependent manner. Consistently, further experimental evidence supported the notion that α S aggregates are present in the enteric system before they occur in the brain (Chandra et al., 2017). An alternative scenario, characterized by the bidirectional spreading of misfolded α S (from the brain to the gut and *vice versa*) has been recently speculated by

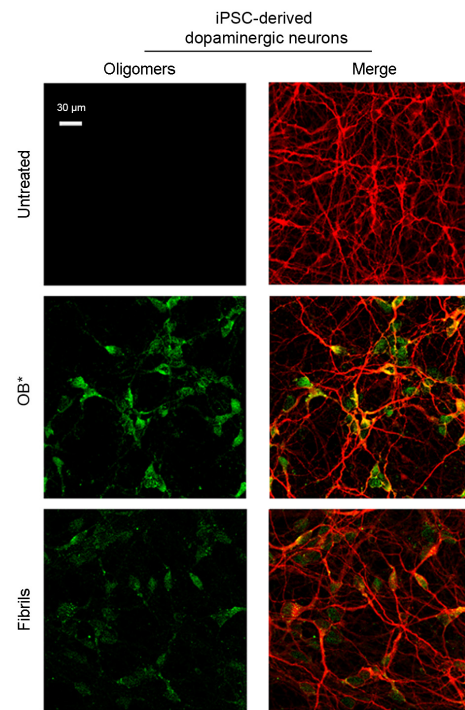


Figure 3 | Representative confocal scanning microscope images showing human induced pluripotent stem cell (iPSC)-derived dopaminergic neurons treated for 24 hours with type B* oligomers (OB*) and fibrils of α S at 0.3 μ M.

Red and green fluorescence indicates mouse anti-MAP-2 antibodies and the A11-positive prefibrillar oligomers, respectively. Images adapted and reprinted from Cascella et al. (2021), licensed under Creative Commons Attribution 4.0 International Public License (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0/>).

Arotcarena et al. (2020) upon striatal or enteric injection of PD-brain extracts in non-human primates. Even if the Braak's theory does not fit with all PD cases, this hypothesis is supported by several clinical observations, such as the presence of hyposmia (Doty, 2012), as well as a range of gastrointestinal problems (Del Tredici et al., 2016) in PD patients carrying LB pathology both in olfactory neurons and in the enteric system (Lebouvier et al., 2010). Thus, it is of remarkable importance to shed light on the gut involvement in PD, with particular attention to the crosstalk with the brain, commonly referred to as the "gut-brain axis" (Carabotti et al., 2015). Indeed, fecal samples from PD patients revealed relevant alterations of gut microbiota, that were found to be associated with specific motor phenotypes (Scheperjans et al., 2015), raising the hypothesis that microbial alterations could have a role in PD pathogenesis. More recently, abnormal concentrations of stool immune factors were revealed in the feces of early PD patients, confirming the existence of a massive inflammatory intestinal process (Houser et al., 2018; Schwiertz et al., 2018). Many *in vivo* studies on animal models of PD have shown that chronic intestinal inflammation hastens and increases neuroinflammation and dopaminergic neuropathology (Garrido-Gil et al., 2018; Kishimoto et al., 2019).

Alpha-Synuclein Pathogenic Pathways

The neurotoxicity of α S misfolded aggregates involves a range of distinct cellular pathways and organelles, such as the plasma membrane, the endoplasmic reticulum (ER) and Golgi, mitochondria, nucleus, and synaptic vesicles.

Perturbation of the Plasma Membrane Integrity

The maintenance of the plasma membrane integrity is of fundamental importance for every cell type, for normal functionality and survival. Its loss has been widely described as a cardinal pathogenic mechanism of PD and proteinopathies in general (Cenini et al., 2010; Evangelisti et al., 2016). α S aggregates, and in particular oligomers, were reported to exert their cytotoxic effects upon an abnormal interaction with lipid membranes (Iyer et al., 2019). Specifically, they can induce changes in the permeability and fluidity of the lipid bilayer, and influence the normal distribution of exposed proteins (Shrivastava et al., 2017). Their binding is generally mediated by their positively charged core, which establishes electrostatic interactions with negatively charged lipids present in the plasma membrane (Stöckl et al., 2012). This step is then followed by the permeabilization of the lipid bilayer, occurring through different mechanisms; the first one is the pore formation, or "poration" (Zakharov et al., 2007; Tosatto et al., 2012). This model assumes that annular α S oligomers with a central pore embed into lipid bilayers forming transbilayer proteins that allow the passage of small molecules and ions (Stöckl et al., 2013). Specifically, such pores were reported to possess an ion channel-like activity, resulting in an abnormal calcium influx (Figure

4; Danzer et al., 2007; Kim et al., 2009). Another possible mechanism is that in which α S oligomers cause the thinning of the lipid bilayer upon their binding, thus inducing an increase of membrane permeability and causing its leakage. According to this model, α S oligomers establish electrostatic interactions between their positively charged core and the negatively charged lipid headgroups; then, they are incorporated between the tightly packed lipids, thus evoking the thinning of the hydrophobic core of the bilayer and increasing its permeability (Stöckl et al., 2013). Finally, α S oligomers were also reported to bind to existing packing defects present in the bilayer, and extract phospholipids (Chaudhary et al., 2016); specifically, the increased exposure of lipid acyl chains at the edges of defects was reported to facilitate the interaction between oligomers and membranes, resulting in the growth of fractal domains in which there are no lipids (Chaudhary et al., 2016). Consistently, Reynolds and coworkers reported that the addition of monomeric wild-type and mutated A53T and E57K α S on the surface of supported lipid bilayers induced the protein adsorption to the membrane, whose subsequent aggregation caused both membrane thinning and lipid extraction in the near proximity of the growing aggregates (Reynolds et al., 2011). The previously described OB* and, to a minor extent, short fibrils (SF), were recently reported to alter the permeability of the membrane of synthetic synaptic-like small unilamellar vesicles and cultured neuronal cells, by inducing the release of the calcein-AM fluorescent probe (Cascella et al., 2021). A significant leakage of intracellular calcein, corresponding to the low green fluorescent signal in **Figure 5A**, was shown in rat primary cortical neurons following the addition to the culture medium of OB* and SF (Cascella et al., 2021), suggesting a permanent dysfunction of neuronal bilayers following the aggregates interaction with the lipid bilayer. Specifically, the propensity of OB* to permeabilize plasma membranes has been associated with their ability to anchor to the lipids through the N-terminus α -helical structure and to later insert their rigid β -sheet structure into the hydrophobic core of the membrane (Fusco et al., 2017). By contrast, all other α S species, such as α S monomer, OA* and long fibrils (LF), caused negligible calcein release (**Figure 5A**), indicating a low propensity to permeabilize neurons (Cascella et al., 2021).

Calcium Dyshomeostasis

Calcium (Ca^{2+}) is an essential metal ion and the most ubiquitous intracellular second messenger, involved in the modulation of almost all neuronal processes, such as gene expression, synaptogenesis, synaptic transmission, energy production, membrane excitability, neuronal plasticity, learning and memory (Berridge, 1998; Kawamoto et al., 2012). Neuronal Ca^{2+} signaling depends both on highly transient and reversible elevation of cytosolic concentrations via an influx from the extracellular space through specific plasma membrane channels, as well as from its release from intracellular stores (Brini et al., 2014). α S aggregates, and particularly oligomers, were reported to enhance the plasma membrane permeability to Ca^{2+} ions through the perforation of the lipid bilayer by forming pore-like complexes (Danzer et al., 2007). Several observations have been performed after the addition of different α S species to the external medium of cultured cells. Angelova and coworkers revealed that both monomeric and oligomeric α S can induce a massive influx of Ca^{2+} ions in neurons and astrocytes, but only oligomers evoke neuronal cell death (Angelova et al., 2016). Consistently, we recently observed that a 15 min treatment with OB* and SF and, to a lesser extent, LF, evoked an extensive influx of Ca^{2+} both in cortical neurons and SH-SY5Y cells, whereas OA* and monomer caused a negligible elevation of intracellular calcium (**Figure 5B**; Cascella et al., 2021). Simultaneously, the interaction with a range of membrane channels and receptors, with the consequent modulation of their activity, has also been revealed. A prolonged treatment of rat hippocampal slices with α S oligomers, but not with the monomeric protein or fibrillar conformers, was demonstrated to cause a massive activation of N-methyl-D-aspartate receptors (NMDARs), which triggered in turn an increased activation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), so promoting the insertion into the postsynaptic membrane of higher conductance Ca^{2+} -permeable *versus* GluR2-containing AMPARs, involved in basal synaptic transmission, ultimately leading to impaired long-term potentiation (Diógenes et al., 2012). More recently, Ferreira and coworkers revealed that the α S oligomers-induced impairment of long-term potentiation was due to the interaction with the cellular prion protein (PrP^{C}), with the consequent phosphorylation of the Fyn kinase via metabotropic glutamate receptors 5, finally culminating in the phosphorylation and activation of NMDARs, through which a massive increase of intracellular Ca^{2+} levels occurs (**Figure 4**), ultimately triggering synaptic dysfunction in hippocampal neurons and synaptic and cognitive deficits in mice (Ferreira et al., 2017). More recently, Trudler et al. (2021) demonstrated that both oligomeric and fibrillar α S species evoke a rise in astrocytic intracellular Ca^{2+} , which is responsible for a massive release of glutamate, that activates aberrantly extra-synaptic NMDARs on neighboring neurons, directly contributing to synapse loss. In the same work, α S oligomers were also reported to directly activate extra-synaptic NMDARs on neurons, thus exacerbating their damage (Trudler et al., 2021). Aggregated - but not monomeric - α S has also been shown to lead to Ca^{2+} dyshomeostasis upon intracellular accumulation; once in the cytoplasm, α S aggregates bind to the sarco/endoplasmic reticulum Ca^{2+} -ATPase, thus stimulating its activity and triggering an initial reduction of cytosolic Ca^{2+} concentration and an overload in the ER (**Figure 4**). This phase is thought to be characterized by a significant imbalance of Ca^{2+} -dependent processes, that is responsible for cellular dysfunction, and it is followed by a later increase in cytosolic Ca^{2+} levels, that precedes cell death (Betzer et al., 2018).

Lysosomal and Autophagy Impairment

Cell-to-cell propagation of neurotoxic and pathogenic α S conformers has been widely described as a cardinal mechanism in the progression of synucleinopathies. This necessarily implies that such species are released from donor cells and then taken up by neighboring neurons, where they induce the aggregation of endogenous α S (Luk et al., 2012; Volpicelli-Daley et al., 2014). Thus, it is likely that internalized aggregates circulate, or either impair, cellular degradation pathways, most importantly the autophagy-lysosome one (Bae et al., 2015; Sacino et al., 2017), which is actually considered to be the elective mechanism for the degradation of α S assemblies by neuronal cells (Cuervo et al., 2004; Mak et al., 2010). This process encompasses a range of distinct pathways, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), to name the most relevant. In particular, aggregated forms of α S were reported to colocalize with both early and late endo/lysosomal markers (Lee et al., 2008; Brahic et al., 2016; Karpowicz et al., 2017; Bayati et al., 2022). Human α S overexpression was observed to impair macroautophagy in mammalian cells and in transgenic mice, where it affects Rab1a-specific secretory pathways, hampering omegasome (autophagosome precursor) formation (Winslow et al., 2010), as well as to damage the autophagic flux in aging adult neurons through the inhibition of mitophagosome maturation and autophagosome-mediated α S clearance (**Figure 4**; Sarkar et al., 2021). CMA was also reported to be affected by aggregated α S. This specific type of autophagy implies protein recognition by the cytosolic chaperone heat shock cognate protein 70 (HSC70), with the subsequent delivery to the lysosomal-associated membrane protein 2A (LAMP2A), and the final translocation into lysosomes (Cuervo et al., 2004). While wild-type α S is efficiently degraded by CMA, the pathogenic mutants A53T and A30P are poorly degraded, despite their higher binding ability to LAMP2A with respect to the wild-type protein. In this way, such mutants also block the lysosomal uptake and degradation of other target substrates (Cuervo et al., 2004). Accordingly, reductions in LAMP2 and LAMP2A have been observed in the brain tissue of PD patients (Alvarez et al., 2010; Murphy et al., 2014). CMA impairment was also revealed in the presence of dopamine-modified α S, which also blocked the degradation of other substrates by the same pathway (Martinez-Vicente et al., 2008). As previously reported, a range of mutations of the *GBA1* gene are important genetic risk factors for PD onset (Horowitz et al., 2022). Among these mutations, those responsible for the GCcase mislocalization on the lysosomal surface, instead of in the lumen, have been associated with a significant inhibition of CMA, with subsequent intracellular accumulation of CMA protein targets, including α S (Kuo et al., 2022). This evidence is substantially in line with previous reports revealing that the reduction of the GCcase physiological activity can be responsible for the accumulation of high molecular weight α S aggregates (Kim et al., 2018; Zunke et al., 2018). Recently, the inhibition of GCcase activity has been reported to be evoked by extracellularly added PFFs, but not by the monomeric protein (Gegg et al., 2020; Henderson et al., 2020a).

Endoplasmic Reticulum and Golgi Functionality

ER plays an essential role in protein synthesis, folding and secretion, as well as in lipid synthesis and in the regulation of intracellular Ca^{2+} homeostasis (Baumann et al., 2001). The misfolding and accumulation of disease-related proteins, including α S, is responsible for the impairment of ER functionality, and evoke a spectrum of detrimental effects, finally culminating in cell death. In such conditions, the ER initiates a multi-signaling pathway called unfolded protein response (UPR), aiming at restoring its functionality (Hetz et al., 2011). The UPR is orchestrated by three major ER stress sensors, including the activating transcription factor 6, inositol-requiring kinase 1 α , and protein kinase RNA-activated-like ER kinase (PERK) (Manié et al., 2014).

During UPR, the cell attenuates protein synthesis, increases that of chaperones to facilitate the proper protein folding, and enhances the translation of proteins implicated in the ER-associated protein degradation, in order to increase the elimination of misfolded proteins (Shröder et al., 2005). An overactivation of the UPR response has been observed in the SN of PD patients as compared to control cases (Hoozemans et al., 2007), as well as in postmortem brain tissue of people affected by DLB and PDD (Baek et al., 2016), strongly suggesting that the activation of this process should be evoked by α S accumulation (**Figure 4**). Consistently, Bellucci and coworkers (2011) showed that the overexpression of human full-length and truncated α S both in cultured cells and in a transgenic mouse model of PD resulted in the accumulation of the protein within the ER and in the activation of the PERK-dependent pathway of the UPR. Accordingly, the induction of ER-stress and UPR has been demonstrated in a synucleinopathy-affected transgenic mouse model expressing a range of human α S variants (Colla et al., 2012), with α S oligomers accumulating in the ER lumen (Colla et al., 2012; Kalia et al., 2013). Moreover, the subsequent inhibition of the vesicular trafficking between the ER and the Golgi apparatus has been demonstrated *in vitro* (Cooper et al., 2006). More recently, Liu et al. (2018) showed that the overexpression of both wild-type and mutant α S (A30P and A53T) was able to induce the dysfunction of the ER-Golgi compartment in astrocytes, thus leading to a massive reduction of the glial cell line-derived neurotrophic factor levels, which is, in turn, responsible for the suppression of neurite outgrowth. The addition of oligomeric, but not monomeric and fibrillar forms of α S, to the extracellular medium of cultured human neuroblastoma cells was reported to evoke the activation of the ER stress response factor XBP1 (X-box binding protein 1), which exerts a protective effect: this evidence clearly indicates the prominent capacity of oligomeric α S to compromise the cellular homeostasis, including that of ER (Castillo-Carranza et al., 2012).

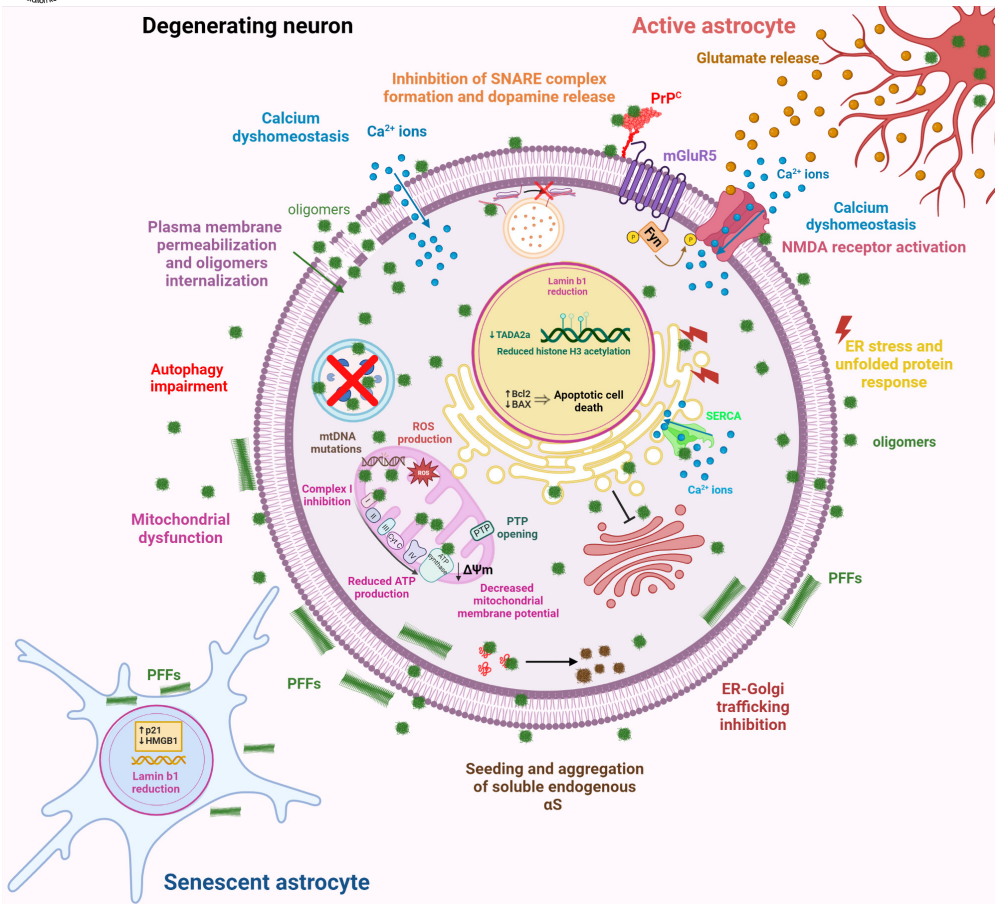


Figure 4 | An overview of the main neurotoxic cascades evoked by α S oligomers and PFFs.
BAX: Bcl-2-associated X protein; Bcl2: B-cell lymphoma 2; Ca^{2+} : calcium; $\Delta\Psi_m$: mitochondrial membrane potential; ER: endoplasmic reticulum; HMGB1: high mobility group box 1; mGluR5: metabotropic glutamate receptor 5; mtDNA: mitochondrial DNA; NMDA: N-methyl-D-aspartate receptors; PFFs: pre formed fibrils; PrPC: cellular prion protein; PTP: permeability transition pore; ROS: reactive oxygen species; SERCA: sarco/endoplasmic reticulum Ca^{2+} -ATPase; TADA2a: transcriptional adapter 2-alpha. Created with BioRender.com.

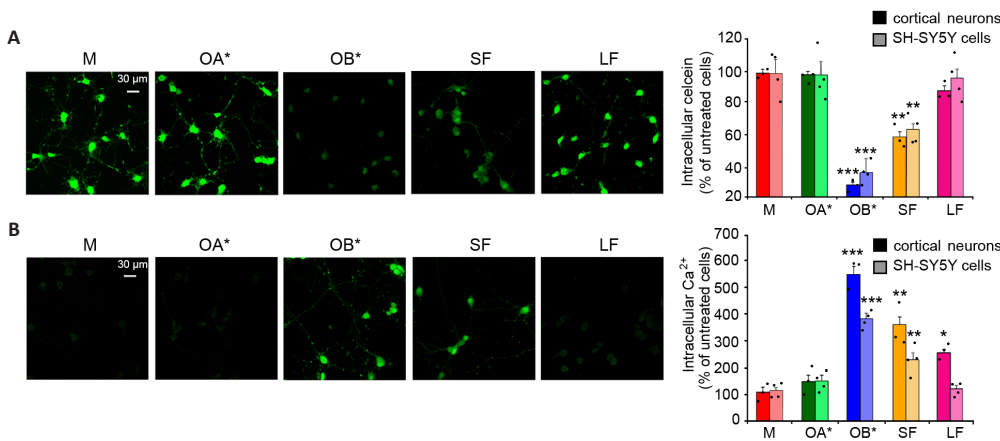


Figure 5 | Disruption of the plasma membrane integrity and Ca^{2+} dyshomeostasis evoked by α S oligomers and fibrils.
(A) Representative confocal microscope images showing primary rat cortical neurons loaded with the calcein-AM probe for 10 minutes and then treated for 1 hour with α S monomer (M), type A* oligomers (OA*), type B* oligomers (OB*), short fibrils (SF), and long fibrils (LF) at 0.3 μ M. Semi-quantitative analyses of the calcein-derived fluorescence signal in primary rat cortical neurons and SH-SY5Y cells. (B) Representative confocal microscope images showing the Ca^{2+} -derived fluorescence in primary rat cortical neurons treated for 15 minutes with the indicated 0.3 μ M α S species and then loaded with the Fluo-4 AM probe. Semi-quantitative analysis of the intracellular Ca^{2+} -derived fluorescence in primary rat cortical neurons and SH-SY5Y cells. Images reprinted from Cascella et al. (2021), licensed under Creative Commons Attribution 4.0 International Public License (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0/>).

Mitochondrial Dysfunction

Mitochondrial dysfunction is actually considered to be a prominent toxic event in PD and synucleinopathies in general. Mitochondria are abundant organelles where adenosine 5'-triphosphate (ATP) synthesis takes place; they are also involved in the regulation of Ca^{2+} storage, lipid metabolism, and neuronal survival (Nunnari et al., 2012). Neuronal cells, and in particular dopaminergic neurons, due to their high synaptic connectivity, need high amounts of ATP and, as consequence, elevated mitochondrial activity. In physiological conditions, ATP synthesis occurs through an electron transport chain composed of four different complexes (I–IV), responsible for the formation of a protonic gradient over the mitochondrial inner membrane, required for ATP production. α S is a predominantly cytosolic protein but, in

specific conditions, can interact and localize in mitochondria-associated ER membranes (Guardia-Laguarta et al., 2014); the occurrence of pathogenic point mutations in α S resulted in a reduced association with mitochondria-associated ER membranes, causing, in turn, a significant decrease of mitochondria-associated ER membranes functionality, as well as in an increase in mitochondrial fragmentation, thus pointing out a prominent role of α S in the control and modulation of mitochondrial morphology (Guardia-Laguarta et al., 2014). Concomitantly, an α -helical-mediated binding of monomeric α S with the inner mitochondrial membranes has been described by Robotta and collaborators (2014). Not surprisingly, the existence of a mitochondrial targeting sequence, comprising the first N-terminal 32 amino acids has been suggested (Devi et al., 2008). Specifically, such sequence is recognized by the translocase of the outer membrane (TOM) receptors. Upon

recognition, target proteins are translocated through the TOM complex to the inner membrane and finally into the matrix (Devi et al., 2008). When applied exogenously, monomeric α S was reported to localize to mitochondria, to interact with ATP synthase subunit α , and increase its activity, thus playing a role in the physiological regulation of mitochondrial bioenergetics (Figure 4; Ludtmann et al., 2016). In this regard, Di Maio et al. (2016) revealed that oligomeric, dopamine-modified, and S129 phosphorylated (but not monomeric and nitrated) α S was able to bind with high affinity the TOM20 receptor of the mitochondrial protein import machinery. Such interaction inhibited the binding of TOM20 with its co-receptor, TOM22, and impaired mitochondrial protein import. Consequently, deficient mitochondrial respiration, enhanced production of reactive oxygen species (ROS), and loss of mitochondrial membrane potential were observed (Di Maio et al., 2016). Consistently, the concomitant overexpression of human α S and TOM20 in the SN of Lewis rats was reported to prevent the mitochondrial damage evoked by α S (De Miranda et al., 2020).

Importantly, defects in mitochondrial protein import determine the dysfunction of complex I, which is a cardinal pathological hallmark of PD (Franco-Iborra et al., 2018); consistently, a reduction in complex I activity is responsible for ROS formation, and has been observed in the SN of patients with sporadic PD (Hattingen et al., 2009), as well as in the skeletal muscle (Bindoff et al., 1991) and platelets of PD patients (Haas et al., 1995), supporting the notion that systemic mitochondrial alterations are implicated in PD. The α S oligomers-linked complex I inhibition also leads to slow depolarisation of the mitochondrial membrane potential (Ludtmann et al., 2018), to a rapid accumulation of mitochondrial DNA (mtDNA) mutations (Helley et al., 2017), as well as to the selective oxidation of the beta subunit of ATP synthase, and to mitochondrial lipid peroxidation. Such oxidation events dramatically increase the probability of permeability transition pore opening (Figure 4), triggering mitochondrial swelling, and ultimately leading to cell death (Ludtmann et al., 2018). Oligomers formed upon α S overexpression were reported to reduce axonal mitochondrial transport in human neurons derived from induced pluripotent stem cells of a PD patient, in turn responsible for reduced axonal density and severe synaptic degeneration (Prots et al., 2018). Also, extracellularly-added oligomers were described for their ability to impair mitochondrial biosynthesis due to the parkin-dependent reduction of the protein levels of the peroxisome proliferator-activated receptor- γ coactivator (Wilkaniec et al., 2021). Other studies investigated the effect of α S PFFs on mitochondrial functionality; to this aim, Tapias et al. (2017) added PFFs to the culture medium of primary ventral midbrain neurons, revealing a significant increase of mitochondrial ROS, together with a massive impairment of mitochondrial respiration. Concomitantly, a significant depletion of optic atrophy-1 expression was observed, with consequent perturbation of the mitochondrial fission-fusion cycle (Tapias et al., 2017). The mitochondrial accumulation of phosphorylated α S in a similar neuronal model was confirmed by Wang and coworkers, that reported that PFFs possess a high binding capability to purified mitochondria with respect to the monomeric protein, revealing a preferential mitochondrial binding by aggregated α S (Wang et al., 2019).

Nuclear Function

The nuclear localization of α S has been posited since its discovery (Maroteaux et al., 1988), but its occurrence here, as well as the effect of its nuclear localization, are still a matter of investigation (Nishie et al., 2004; Huang et al., 2011; Davidi et al., 2020). Importantly, the presence of nuclear α S inclusions has been observed in MSA patients (Nishie et al., 2004). Extracellularly added α S was reported to evoke ROS production, to decrease mitochondrial membrane potential and to activate programmed cell death through the induction of the expression of the pro-apoptotic protein Bax and the reduction of the activity of the anti-apoptotic protein Bcl2 (Figure 4; Motyl et al., 2018). The largest majority of studies on the effects of α S in the nucleus has been performed by injecting PFFs in the mouse brain, or adding such conformers to the extracellular medium of cultured cells; their administration to various cell types such as dopaminergic N27 cells, primary cortical neurons, microglia, and astrocytes, as well as their injection in mouse brain tissues, resulted in a substantial reduction in the levels of Lamin B1 and high mobility group box 1, both established markers of cellular senescence, together with an increase in the levels of p21, a cell cycle-arrest and senescence marker, in both reactive astrocytes and microglia of mouse brains (Figure 4; Verma et al., 2021). In a recent study, Lee et al. (2021) revealed that the injection of PFFs in mice also resulted in a significant decrease in transcriptional adapter 2- α in the striatum and SN; the block of the activity of this component of the p300/CBP-associated factor led to a reduced histone H3 acetylation. Therefore, histone modification through α S-transcriptional adapter 2- α interaction may be associated with α S neurotoxicity in PD pathology.

Synaptic-Vesicle Trafficking and Neurotransmitter Release

Since its discovery, α S has been reported to be mainly localized at presynaptic terminals (Maroteaux et al., 1988); this observation, together with its reported capability to bind synaptic vesicles (Man et al., 2021), strongly suggests a regulatory function in synaptic activity and plasticity, neurotransmitter release, and in the management of the synaptic vesicle pool and trafficking, as reviewed in Burré et al. (2015). Neurotransmitter release is a complex mechanism firstly requiring the mobilization and clustering of the reserve pool of synaptic vesicles near synapses, and then the docking at active

zones of such vesicles, that become the readily releasable pool. This step is followed by the formation of the synaptic soluble NSF attachment protein receptor (SNARE) complex, constituted by the proteins syntaxin-1, SNAP-25, and VAMP2/synaptobrevin-2 (Söllner et al., 1993), then by the fusion of synaptic vesicles with the presynaptic plasma membrane upon Ca^{2+} signaling, and finally by the disassembly of the SNARE complex and endosomal recycling of synaptic vesicles (Sudhof, 2004). α S has been reported to chaperone the SNARE complex assembly through its binding to the VAMP2/synaptobrevin-2 protein, thus promoting synaptic vesicle fusion at the presynaptic terminal (Burré et al., 2010). Choi et al. (2013) reported that large α S oligomers bind the N-terminal domain of synaptobrevin-2, thus blocking SNARE-mediated vesicle docking (Figure 4). More recently, the same group pointed out a cooperative pathological effect in an *in vitro* liposome fusion setting, occurring when α S monomers at submicromolar concentrations were co-incubated with oligomers. In such condition, oligomeric α S exclusively interacted with synaptobrevin-2, without binding vesicle lipids. The addition of small peptide fragments from the C-terminal domain of α S blocked α S oligomers binding to synaptobrevin-2, thus restoring the formation of the SNARE complex and vesicle fusion (Yoo et al., 2021). Consistently, Volpicelli-Daley and coworkers previously revealed a significant reduction in the level of many synaptic proteins, such as the complex SNARE proteins SNAP25 and VAMP2, as well as other proteins involved in SNARE complex assembly or in the exocytotic synaptic vesicle cycle, such as synapsin II (which regulates the motility of synaptic vesicle pools) and cysteine string protein α (that chaperones the SNARE complex assembly very similarly to α S), 2 weeks after the addition of α S PFFs to primary neuronal cultures (Volpicelli-Daley et al., 2011).

Aging and Its Impact on Neurodegeneration

It is well-known that motor performance declines with age (Seidler et al., 2010; Hoogendam et al., 2014). Several age-dependent changes, including alterations in dopamine metabolism, uptake and synthesis, receptor sensitivity, mitochondrial function, calcium homeostasis, and proteostasis make dopaminergic neurons more susceptible to dysfunction and senescence with respect to other neuronal cells (Reeve et al., 2014; Zucca et al., 2017; Surmeier, 2018). The manifestation of PD is complex and heterogeneous, however, several features that differently contribute to brain aging might help the differential diagnosis with other synucleinopathies (Chen et al., 2022). Although it is well-known that age is the highest risk factor in the development of neurodegenerative diseases, including PD, the mechanisms and resulting effects have only been explored to a limited extent. In the last few years, experimental studies on animal models generated conflicting results, generally due to an increased vulnerability of aged animals (Klæstrup et al., 2022). The first animal models have been focused mainly on reflecting the disease in the CNS with different motor phenotypes. However, most studies failed to induce pathology in the CNS and/or neurodegeneration and a motor phenotype. In addition, different effects on treatment efficacy have been reported between young and old animals, indicating that the models actually used in preclinical research may be suboptimal and that the translational outcomes are limited. As reported above, it has been postulated that PD may start in the gut through a pre-motor phase (Reichmann, 2017). Indeed, gut dysbiosis has been shown in PD patients (Romano et al., 2021; Klann et al., 2022) and potential alterations in our microbiome during aging might also contribute to PD risk. Recent studies on gut-seeding properties demonstrated that old age is a crucial factor to obtain effective gut-to-brain propagation of α S pathology (Challis et al., 2020; Van Den Berge et al., 2021). Importantly, gut microbiota also influences the immune environment of the brain, contributing to the inflammation both in the brain and in the peripheral body and to immunosenescence, with functional consequences both in neurons and microglia (Ogrodnik et al., 2021). Overall, identifying age-associated features in disease presentation and progression could be useful both on the individual level, impacting positively the patient management, and for clinical research developing new therapeutic strategies.

Alpha-Synuclein-Targeting Therapeutic Approaches

Disease-modifying therapies aimed at targeting α S pathology actually include various approaches to prevent and mitigate its neurotoxicity; such strategies involve: (I) the reduction of α S expression; (II) the inhibition of its misfolding and aggregation; (III) the enhancement of cellular degradation mechanisms; (IV) the clearance of α S pathogenic aggregates from the brain; (V) the prevention of cell-to-cell transmission of α S aggregates. A potential strategy to diminish the level of α S aggregates implies the reduction of SNCA expression; several experimental evidences obtained in cultured cells, mouse models and human primates revealed that the use of small interfering RNA, as well as short hairpin RNA, significantly decreased SNCA expression and accumulation of misfolded α S (Lewis et al., 2008; Khodr et al., 2014; Zharikov et al., 2019). However, questionable results emerged from *in vivo* evidence, suggesting potential detrimental effects on dopaminergic neurons upon total abolition of SNCA expression (Menon et al., 2022). Thus, partial silencing is actually suggested, considering that moderate α S reduction is enough to prevent misfolding and pathology spreading (Menon et al., 2022). The reduction of α S mRNA transcription with beta-2 adrenoceptor antagonists such as metaproterenol, clenbuterol, and salbutamol, has been proposed as a disease-modifying strategy (Mittal et al., 2017); more recently, small molecules able to bind the iron-responsive element of SNCA mRNA, a region involved in translation regulation, were designed to reduce its translation (Zhang et al., 2020). α S aggregation can be inhibited taking advantage of

small molecules as epigallocatechin gallate, previously reported to reduce the formation of oligomeric α S and their associated cytotoxicity (Kurnik et al., 2018). Consistently, epigallocatechin gallate has been recently tested in a Phase III trial in MSA patients, revealing no clinical efficacy, and adverse effects in some patients (Levin et al., 2019). Other compounds, referred to as naturally occurring aminosterols, such as squalamine (ENT-01) and trodusquemine were recently reported to modulate α S aggregation and to inhibit the binding of toxic oligomers to the plasma membrane of neuronal cells (Perni et al., 2017, 2018; Limbocker et al., 2021). Thanks to promising results obtained in animal models, ENT-01 has been tested in the phase IIa RASMET study, which was conducted on 50 PD patients with constipation. Its administration was safe, and bowel function resulted to be rapidly improved, as well as neuropsychiatric symptomatology, confirming that targeting enteric α S might be a beneficial approach in PD therapy (Hauser et al., 2019). The IIb study KARMET, a randomized and placebo-controlled trial involving another 150 PD patients, was conducted to determine the safety and efficacy of up to 25 days of ENT-01 treatment, confirming its ability to improve both bowel motility and gut-brain axis (Camilleri et al., 2022). Another promising therapeutic approach consists of the enhancement of the degradation of α S misfolded aggregates. To this aim, rapamycin was proposed as potential disease-modifying therapy given its ability to enhance macroautophagy through the inhibition of the mechanistic target of rapamycin. Despite promising results obtained both in cellular and animal models (Moors et al., 2017), the low specificity of this drug, together with the severe adverse effects it evoked, limited its potential therapeutic use (Brundin et al., 2017). A valid alternative to enhance the autophagic process through the downregulation of the mechanistic target of rapamycin pathway is represented by tyrosine kinase inhibitors. Specifically, the tyrosine kinase c-Abl has been identified as a potent inducer of α S aggregation with subsequent neuronal cell death (Brahmachari et al., 2016); consistently, its inhibition has been reported to be neuroprotective in PD animal models (Brahmachari et al., 2017; Lee et al., 2018). Despite some promising results observed in nonblinded safety trials of the c-Abl inhibitor nilotinib in DLB and PD patients (Pagan et al., 2016, 2019), a subsequent efficacy trial conducted in PD subjects did not reveal any significant improvement with respect to the placebo group (Simuni et al., 2021). Immunoregulatory approaches are based on the ability of specific antibodies to bind and clear both intracellular and extracellular α S aggregates (Bhatt et al., 2013; Spencer et al., 2017; Messer et al., 2020), and many clinical trials employing both passive and active immunization are being developed and tested (Valera et al., 2016; Wang et al., 2019; Kwon et al., 2020). In particular, active immunization consists of vaccination through the administration of a specific antigen; this treatment requires rare and low-cost administrations, at the same time the immune response elicited does not follow the same pattern in different individuals, as the immune response pattern cannot be predicted (Schneeberger et al., 2016). Passive immunization is based on the administration of antibodies by specialized personnel; at the same time, this procedure guarantees the possibility to control systemic antibody concentrations and to interrupt treatment when side effects occur (Bergström et al., 2016). The largest majority of such antibodies are raised against small epitopes predominantly localized at the C-terminal domain of α S (Knecht et al., 2022). These approaches, targeting various α S epitopes, have shown neuroprotection in transgenic mouse models of PD, DLB, and MSA (Masliah et al., 2011; Mandler et al., 2015; Tran et al., 2014; Spencer et al., 2017; Henderson et al., 2020b; Lemos et al., 2020), thus leading to reduced accumulation of toxic oligomers and limited neurodegeneration. Nevertheless, the existence of potential detrimental effects against physiological α S, as well as the logistical challenges related to the crossing of the blood-brain barrier must be considered (Knecht et al., 2022). Very recently, the first two phase II trials of therapies aimed at targeting α S in early PD did not pursue the primary endpoints. Specifically, the humanized monoclonal immunoglobulin G1 antibody Prasinezumab, targeting an epitope in the C-terminal domain of α S and previously reported to easily penetrate the brain and to evoke the reduction of serum α S levels (Schenk et al., 2017; Jankovic et al., 2018) did not produce any significant positive effect on global or imaging measures of PD progression as compared with placebo (Pagano et al., 2022). Similarly, the human-derived monoclonal antibody Cinpanemab, previously reported to target aggregated α S (Brye et al., 2019) and to reduce α S spreading and motor dysfunction in PD mice (Weihofen et al., 2019), failed in modifying disease progression in early PD subjects as compared to the placebo group (Lang et al., 2022). Finally, given the prominent role of receptor-mediated endocytosis in the uptake of α S misfolded aggregates (Mao et al., 2016; Thom et al., 2022) the inhibition of their membrane receptors could be a valuable strategy to prevent their cell-to-cell transmission, as extensively reviewed by Henderson et al. (Henderson et al., 2019).

Concluding Remarks

α S misfolding and aggregation in specific cellular subpopulations is the cardinal hallmark of a range of devastating neurodegenerative conditions, collectively referred to as synucleinopathies. During this process, a vast range of distinct aggregates is formed, including oligomers, protofibrils, and fibrils. Such species differentially exert detrimental effects on neuronal cells: while oligomeric aggregates seem to be directly implicated in the induction of neurotoxicity, given their ability to directly affect multiple cellular pathways, fibrillar assemblies not only play a crucial role in disease spreading and progression, but they also release harmful oligomeric conformers, promptly evoking cellular dysfunction in the recipient neurons. Actual research attempts to identify possible disease-modifying therapeutic strategies aiming at reducing the neurotoxicity of α S misfolded aggregates, by inhibiting their

formation from the monomeric protein, by reducing their accumulation, or by enhancing their degradation. To this aim, a deeper understanding of the triggers of α S misfolding and aggregation, as well as of the molecular mechanisms of their spreading, and of the plethora of neurotoxic pathways they evoke will be of fundamental importance.

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