



● REVIEW

Subcellular localization of alpha-synuclein aggregates and their interaction with membranes

Fabiana Miraglia^{1,2,#}, Alessio Ricci^{1,#}, Lucia Rota^{1,#}, Emanuela Colla^{1,*}

1 Bio@SNS Laboratory, Scuola Normale Superiore, Pisa, Italy

2 Department of Pharmacy, University of Pisa, Pisa, Italy

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Abstract

For more than a decade numerous evidence has been reported on the mechanisms of toxicity of α -synuclein (α S) oligomers and aggregates in α -synucleinopathies. These species were thought to form freely in the cytoplasm but recent reports of α S multimer conformations when bound to synaptic vesicles in physiological conditions, have raised the question about where α S aggregation initiates. In this review we focus on recent literature regarding the impact on membrane binding and subcellular localization of α S toxic species to understand how regular cellular function of α S contributes to pathology. Notably α S has been reported to mainly associate with specific membranes in neurons such as those of synaptic vesicles, ER/Golgi and the mitochondria, while toxic species of α S have been shown to inhibit, among others, neurotransmission, protein trafficking and mitochondrial function. Strategies interfering with α S membrane binding have shown to improve α S-driven toxicity in worms and in mice. Thus, a selective membrane binding that would result in a specific subcellular localization could be the key to understand how aggregation and pathology evolves, pointing out to α S functions that are primarily affected before onset of irreversible damage.

Key Words: *alpha-synuclein; oligomers; aggregates; subcellular localization; membranes binding; Parkinson's disease; neurodegeneration; alpha-synucleinopathies*

Introduction

Parkinson's disease (PD) is the most frequent neurodegenerative disease among α -synucleinopathies, a family of illnesses that share as a common feature the accumulation of intracellular proteinaceous inclusions made mainly of α -synuclein (α S) [for a review (Goedert et al., 2012)]. In PD, α S inclusions are predominantly present in the soma, named Lewy bodies (LB), or in neurites, named Lewy neurites (LN), of neurons of the central nervous system. Although PD has been previously considered a motor disease, the involvement of peripheral neurons, both sympathetic and parasympathetic bearing LBs/LNs has been shown in recent years (Braak et al., 2003) and has been furthermore suggested to correlate with the presence of numerous non-motor dysfunctions, which represents an important aspect of PD symptomatology and negatively impacts the quality of life of patients.

Pathological accumulation of α S inclusions has been shown to correlate with the degree of neurodegeneration and dysfunction in a variety of animal models [as example flies, worms, mice (Feany and Bender, 2000; Masliah, 2000; Lakso et al., 2003)] and it is thought to be a cardinal step in the pathogenesis of the disease. Formation of α S inclusions is a complex nucleation reaction where α S, a small soluble protein, becomes trapped in an insoluble β -sheet conformation and tightly packed in long filamentous protofibrils and fibrils (Lashuel et al., 2002; Cremades et al., 2012; Tuttle et al., 2016). Chemical and structural variables such as pH, ionic salts and point mutations can influence fibril formation (Buell et al., 2014) and intermediate multiple high molecular weight (HMW) species with different structures, defined

collectively as oligomers, can form during the aggregation process, raising the issue about their relative toxicity in α S driven pathology. While for long time α S toxic species have been thought to have a cytoplasmic localization, α S ability to bind membranes and associate with cellular organelles and synaptic vesicles has prompted the question about how cellular localization impacts on pathology and whether membrane interaction influences aggregation.

In this review we attempt to piece together recent findings regarding the subcellular localization of α S toxic HMW species and their relationship with biological membranes. Initially we will discuss α S conformation in native and physiological conditions as well as during aggregation and then we will focus our attention on the impact of membrane binding on α S structure and cellular localization *in vitro* and *in vivo*. Finally we will evaluate the impact of subcellular localization of α S and its membrane binding preference on α S pathology in PD.

α S

α S, together with β -synuclein and γ -synuclein, belongs to a family of proteins called synucleins which were discovered in 1988 (Maroteaux et al., 1988). Initially observed to localize in the nucleus and in the presynaptic terminals of neurons, α S was linked to the autosomal dominant form of PD, when a missense mutation of α S, a threonine substitution to an alanine at position 53 (A53T) was found in a family pedigree with early onset PD (Polymeropoulos, 1997). At the same year, α S was found to be the main constituent of LBs/LNs, providing strong evidence that the α S gene, mutated and wild-type isoforms, is associated to familial and sporadic PD

*Correspondence to:

Emanuela Colla, Ph.D.,
emanuela.colla@sns.it

#These authors contributed equally to the study.

orcid:

0000-0002-4755-7450
(Emanuela Colla)

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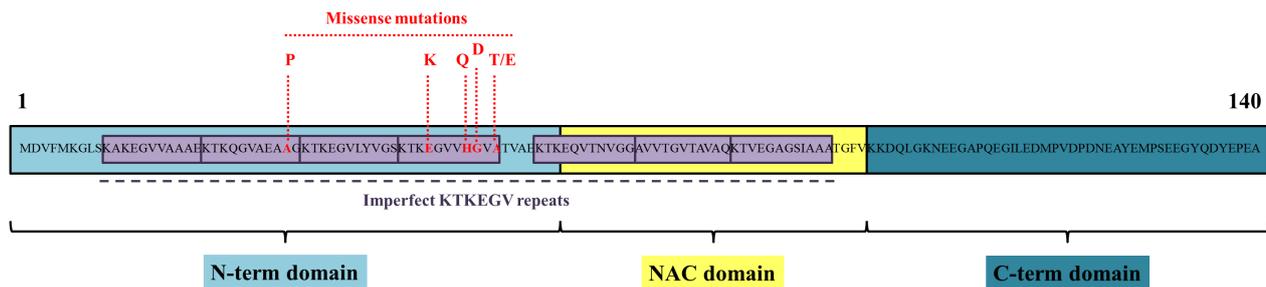


Figure 1 Human α -synuclein (α S) protein sequence.

α S is a small protein of 140 amino acids where point mutations (in red) have been associated with familial forms of Parkinson's disease (PD) (Polymeropoulos, 1997; Krüger et al., 1998; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Lesage et al., 2013). The protein can be divided in three domains: an N-terminal domain (light blue), important for membrane binding; the non amyloid β -component (NAC) domain (yellow), important for fibril formation (El-Agnaf et al., 1998) and a C-terminal domain (blue) important for protein interaction. Seven 11-amino acids imperfect repeats (purple), a unique motif predicted to form α -helix and highly conserved, are also shown (George et al., 1995). This motif is located within the N-terminal domain and the NAC domain. Notably, missense point mutations that have been found thus far are all located within the N-terminal domain, suggesting that membrane binding may influence α S aggregation.

and other α -synucleinopathies (Spillantini et al., 1997).

The SNCA gene, which encodes for α S protein, in humans is located in the long arm of chromosome 4 at position 22.1. Besides the A53T mutations, which is so far the most frequent and thus better characterized (Polymeropoulos, 1997), several missense mutations linked to a genetic form of PD and dementia with LBs have been mapped in SNCA gene more recently such as A30P (Krüger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013; Proukakis et al., 2013), G51D (Lesage et al., 2013) and A53E (Pasanen et al., 2014). Furthermore, duplication or triplication of the SNCA gene have also been found and linked to familial PD, suggesting that increasing the amount of the wild-type protein is also pathogenic (Singleton et al., 2003; Ibáñez et al., 2004). All missense mutations and amplifications of the SNCA gene were associated with a dominant inheritance and an early onset of the disease compared to the sporadic forms. Since the overexpression of wild-type or mutated α S causes neurodegeneration in different animal models [as examples (Feany and Bender, 2000; Masliah, 2000; Lakso et al., 2003)] while its ablation has little or no effect in mice (Abeliovich et al., 2000), α S toxicity has been explained through a gain-of-function mechanism in which a modified version of the protein is responsible for causing neuronal demise.

Because of its presynaptic localization (Maroteaux et al., 1988) and its ability to bind biological membrane, it was proposed that α S physiological function was implicated in neurotransmission. More recent findings (Burré et al., 2010; Nemani et al., 2010; Diao et al., 2013; Wang et al., 2014), have strengthened this view and now it is largely accepted that α S can act as a molecular chaperon and promote synaptic transmission by facilitating clustering, recycling and docking of synaptic vesicles to the cell membrane. In addition α S has been involved in intracellular protein trafficking such as vesicles transport from the endoplasmic reticulum (ER) to Golgi (Cooper et al., 2006; Gitler et al., 2008; Thayanidhi et al., 2010; Oaks et al., 2013) and from the Golgi to endosomes/lysosomes (Chung et al., 2013; Volpicelli-Daley et al., 2014; Breda et al., 2015; Mazzulli et al., 2016). An

active role in axonal transport has also been reported for α S in which the protein acts as a molecular dynamase, binding directly to microtubule and promoting their assembly and stability (Cartelli et al., 2016).

Native α S Protein Structure

The α S protein, an acidic protein of 140 amino acids with a predicted molecular weight of approximately 14 kDa, is expressed mainly in neurons and possibly oligodendrocytes of the CNS (Asi et al., 2014), but also, under physiological conditions, in the PNS, in circulating blood cells and in hematopoietic cells of the bone marrow (Nakai et al., 2007; Gardai et al., 2013).

Biochemically and functionally the α S protein can be divided into three distinct regions (Figure 1):

- i) the amphipathic N-terminal domain (residues 1–60), which interacts with phospholipid membranes and micelles;
- ii) the hydrophobic non amyloid β -component (NAC) of Alzheimer's disease (AD) (residues 61–95), which plays a strong role in α S self-aggregation (El-Agnaf et al., 1998);
- iii) the acidic C-terminal domain (96–140), a major site for post translational modifications, protein truncation (Li et al., 2005) and interaction with modulators of α S aggregation such as metal cations (Binolfi et al., 2006).

The overall protein contains seven imperfect 11-residues repeats with a conserved KTKEGV sequence: four included in the N-terminal region and three in the NAC core (George et al., 1995).

Soluble cytosolic α S has been described as an intrinsically disordered protein due to an unfolded native conformation (Weinreb et al., 1996). In fact, although α S, purified from mouse brain by gel-filtration, elutes as a single peak with an apparent molecular mass of 63 kDa, close to a folded tetramer, mass spectrometry and circular dichroism analysis reveal a monomer conformation with a single mass of 17 kDa (larger than the expected size, probably due to an *in vivo* N-terminal acetylation) (Burré et al., 2013). In agreement with these latest data, NMR studies show how acetylated α S, which is the predominant form in physiological conditions, is a disordered monomer but adopts a more compact con-

formation in solution that shields the NAC domain from other interaction in the cytosol (Theillet et al., 2016). Thus the higher molecular mass obtained previously in native conditions after gel filtration was associated with α S's tendency to adopt an extended conformation, thereby yielding a larger mass, rather than a tetramer structure. The unfolded and disordered monomer conformation was confirmed in rat, human brain and erythrocytes isolated under denaturing and non-denaturing conditions as well as in bacteria expressed α S, while no oligomer species were found (Fauvet et al., 2012) under physiological conditions.

In contrast with previous evidence, however, Bartels and collaborators have shown how α S extracted in non-denaturing conditions and upon crosslinking in living cells (*i.e.*, human erythrocytes, cell lines and brain tissue), is mainly a metastable homo-tetramer of 58 kDa. This α S tetramer is in a dynamic equilibrium with the unfolded monomer, which on the contrary is more susceptible to aggregation (Bartels et al., 2011). Under conventional extraction protocols, the 58 kDa tetramer disappears resulting in an increase in monomer concentration. Interestingly in the same study 80–100 kDa α S homo-oligomers (*i.e.*, hexamers and octamers) were also detected together with the tetramer in native conditions. A homo-tetramer structure in physiological conditions was also suggested by an independent study (Wang et al., 2011), which found how subunits in the α S tetramer are held together by hydrophobic interactions and each subunit is characterized by two transient α -helices structure in the first 100 N-terminal residues, followed by a disordered C-terminal region. Thus it was postulated that α S tetramer and monomer would co-exist in native conditions and any perturbation of this dynamic equilibrium with an increased accumulation of the monomer would be associated with aggregation and pathology. In agreement with this, it was found that certain missense mutations could decrease the tetramer:monomer ratio and initiate neurotoxicity (Dettmer et al., 2015a, b).

Although the tetramer model was and is still widely debated, more recent work by Burré et al. (2014) showed how α S binds synaptic vesicles *in vivo* not as a monomer but in a folded α -helical multimer conformation, larger than an octamer. This conformation has a defined structural orientation and occurs only upon binding with vesicles that are docked at the cell membrane. In accordance with this finding, Bartels et al. (2011) described how the α S tetramer isolated upon crosslinking from human erythrocytes had a greater lipid-binding ability than the single monomer although the NMR structure obtained by Wang and coworkers did not show any phospholipid molecule (Wang et al., 2011). Thus while tetramer and membrane-bound multimer might be in reality part of the same complex, more evidence is necessary to fully understand the physiological structure of α S.

α S Aggregation

Besides the controversy about α S native physiological state, it is known that transition to an aggregated β sheet conformation is the necessary step for the formation of insoluble inclusions or LBs. In its amyloid form, α S monomers form

antiparallel in-register β -sandwich fold, which in turn stack into a parallel arrangement forming the fibril protofilament (Vilar et al., 2008; Tuttle et al., 2016). Protofilaments further assemble into fully mature fibrils.

The aggregation process (summarized in **Figure 2**) is a nucleation-type reaction thought to occur in a sequential series of steps, even though “ramifications” of this path are likely to occur. The *in vitro* characterization of the fibrillation process revealed a precise time course, with an initial lag phase, in which the monomers convert into an oligomer-type of conformation (nucleation), a growth phase and a steady state that terminates with the accumulation of α -sheet rich amyloid fibrils (Cremades et al., 2012). Oligomers are defined in general as low-molecular weight aggregates, soluble or insoluble, that have not acquired a fibrillary organization. Once the seeds are formed, α S fibrils are believed to grow through the addition of monomers rather than oligomers (Buell et al., 2014). At least two different aggregate polymorphs, fibrils and ribbons, that present different biochemical and seeding properties, have been described *in vitro*, depending on the aggregation protocol used (Bousset et al., 2013; Guo et al., 2013).

Extensive literature has focused on the role of α S oligomers and aggregates in PD pathology. Despite the presence of fibrillar α S in LBs strongly suggests an involvement of the aggregation process in α -synucleinopathies, it has been proposed that fibrils formation could constitute an innocuous by-product or even a neuroprotective response. For instance, LBs deposition is not always associated with neurological symptoms (Braak et al., 2003), whereas in some forms of familial PD there are no signs of α S aggregation (Schneider and Alcalay, 2017). However, Peelaerts et al. (2015) showed that all the *in vitro*-generated α S aggregates (fibrils and ribbons) are potentially toxic and can elicit distinct histopathological phenotypes, posing a structural base for heterogeneity among α -synucleinopathies.

Just as with α S fibrils, multiple forms of oligomers have been described *in vitro*, differing in size and morphology, including spherical, annular and tubular structures (Lashuel et al., 2002). Some of them are described as on-fibrillation pathway, while others generate amorphous, nonfibrillar assemblies. Since the fibrillation process can be influenced by numerous factors, including protein concentration, specific physicochemical conditions, the presence of certain ligands (including dopamine) and cross-linking (Buell et al., 2014), it is still unclear whether this heterogeneity in the oligomers population is due to the aggregation protocol used or has physiological relevance. More recent data obtained by directly following the oligomerization reaction using single molecule fluorescence technique showed how the oligomers population is mainly composed of two different species, named type A and type B, that form during early stages of aggregation (Cremades et al., 2012; Chen et al., 2015). These two oligomer populations seem to differ for chemical, structural and toxic properties. Type B is more resistant to protease K digestion than type A and requires a longer lag time for formation, suggesting that these species could derive

from the conversion and rearrangement of type A oligomer. In addition, type B has a higher content in β -sheet structures that is instead negligible in type A (Fusco et al., 2017). *In vivo*, type B oligomers were shown to induce cell death in neuronal cells, *via* disruption of cellular ion homeostasis and production of reactive oxygen species with concomitant mitochondrial dysfunction (Danzer et al., 2007; Fusco et al., 2017), while type A were able to enter cells and induce intracellular aggregation, leading indirectly to cell death. Recently the structural basis of these different mechanisms of pathogenicity has been investigated by Fusco et al., who showed that while both oligomers bind biological membranes, only type B form a rigid β -sheet core that is able to insert into the lipid bilayer and induce directly membrane disruption and cellular toxicity (Fusco et al., 2017). Thus based on this model, both α S oligomers and aggregates are toxic. On-pathway type A oligomers are converted in compact protofibrils and fibrils and are responsible for seeding formation of new aggregates and propagate the α S pathology, while off-pathway type B oligomers are still largely detrimental, acting directly on biological membranes but do not self-propagate. In agreement with this, our group found that microsomes-associated α S toxic species behaved differently, in terms of seeding abilities of intracellular aggregates, when isolated from diseased α S transgenic (Tg) mice as opposed to aged presymptomatic littermates, suggesting the presence *in vivo* of at least two types of α S HMW species depending on the stage of α S pathology (Colla et al., 2018). For instance when isolated from presymptomatic mice, microsomes-associated α S oligomers induce cell death of primary neurons without seeding the formation of intracellular aggregates as opposed to microsomes-associated α S species (probably a mixture of oligomers and aggregates) isolated from diseased mice that had both properties. Thus the heterogeneity of α S toxic species, that is coming to light with recent findings shows a complicated picture of α S aggregation in which both aggregates and oligomers are toxic for cellular functions and the biochemical and functional diversity of α S toxic species is pathologically translated in at least two different and interconnected mechanisms of disease. It becomes evident that targeting one single HMW species of α S is not sufficient to stop successfully aggregation and α S-driven neurodegeneration.

α S Binding to Biological Membranes and Subcellular Localization

α S is known to bind lipids and biological membranes *in vitro* and *in vivo*. But does it bind them with the same efficiency? The answer is probably no. Physical properties and chemical composition of biological membranes or presence of cationic compounds able to bind lipids (Perni et al., 2017) dictate the strength of α S binding. *In vitro* α S binding preference is toward membranes composed of negatively charged phospholipids [such as phosphatidylethanolamine (PE), phosphatidic acid (PA) and phosphatidylinositol (PI) over phosphatidylserine (PS), or phosphatidylcholine (PC) (Middleton and Rhoades, 2010; Galvagnion et al., 2016)] or containing lipid packing defects, such as cone-head phospholip-

ids (Ouberai et al., 2013). In addition, α S senses membrane curvature, preferring to bind to small, highly curved vesicles such as synaptic vesicles, rather than large multilamellar bodies (Middleton and Rhoades, 2010).

Membrane interaction is mediated by the α S N-terminal that, upon binding, undergoes a conformational transition from random coil to α -helix, adopting a long extended one-single α -helix (Ferreon et al., 2009) in the presence of big vesicles (diameter larger than 100 nm) or two anti-parallel curved α -helices linked with a short residues chain (Chandra et al., 2003) in the case of small vesicles. In both conformations the amphipathic helices insert between the lipids polar groups at a depth corresponding to that of the glycerol group (Fusco et al., 2016). While both structures seem interchangeable and physiologically relevant (Georgieva et al., 2010), it is not clear if other conditions *in vivo*, besides vesicles size, would dictate a conformational change toward one structure over the other. Also, of note, is that part of the N-terminal α S region binding lipids contains the NAC domain, which is responsible for α S fibril formation (El-Agnaf et al., 1998). Interaction with membranes is known to modify not only α S conformation, but also the membranes' physical properties, *e.g.*, inducing changes in melting temperature (Galvagnion et al., 2016) and membrane remodelling (Jiang et al., 2013) such as lateral expansion of membrane lipids and lipid packing modifications (Ouberai et al., 2013). In addition α S binding to membrane promotes clustering of synaptic vesicles (Diao et al., 2013).

How does α S preference for membranes translate in a cell context, *in vivo*? Physiologically, α S is believed to shift between a free, cytosolic and a membrane-bound state in a dynamic equilibrium with the membrane-bound state accounting for 10–15% of the total protein amount. In line with this, membranes of specific organelle such as the mitochondria and the ER, and synaptic vesicles have been shown to be associated at different extent with α S (Figure 3).

α S can bind synaptic vesicles at the synapses and this binding is believed to mediate its synaptic function in neurotransmission. For instance, α S acts as a molecular chaperon to promote SNARE-complex assembly (Burré et al., 2010), which is necessary to regulate docking of synaptic vesicles to the cell membrane. Vesicle binding is mediated not only by the interaction with acidic phospholipids such as PE, PC, PS, or cholesterol, of which the synaptic vesicles are rich, but also by specific proteins such as SNARE-protein synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP2) (Diao et al., 2013). α S interaction with synaptic vesicles that occurs through a multimer conformation, promotes vesicular clustering and thus reducing synaptic vesicles trafficking and recycling (Wang et al., 2014).

Moreover, α S has been found to be associated with the ER and Golgi in mice and human cells cultures (Colla et al., 2012a). Protease K sensitivity assays have shown how microsomes-associated α S is partially protected from digestion, therefore associating with the luminal side of the microsomes (a membrane fraction including ER/Golgi and synaptic vesicles) in mice and human cell lines (Lee, 2005;

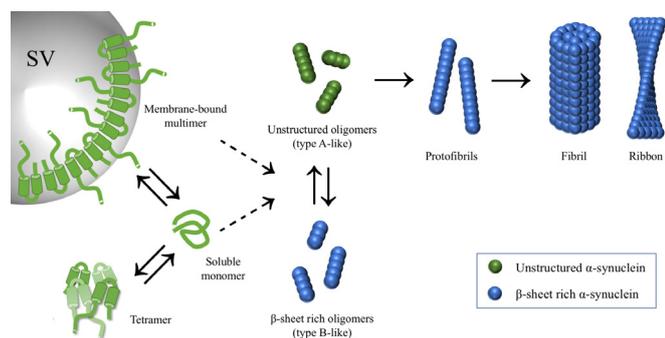


Figure 2 α-Synuclein (αS) fibrils formation.

In physiological conditions, αS has been reported to be a highly disordered monomer in a dynamic equilibrium with a multimer conformation when bound to synaptic vesicles (Weinreb et al., 1996; Burré et al., 2010, 2014; Fauvet et al., 2012; Theillet et al., 2016). Others have suggested that αS native structure is a homo-tetramer and dissolution of this latest conformation gives rise to an increase of the monomer form that drives pathology (Bartels et al., 2011; Wang et al., 2011; Dettmer et al., 2015a, b). Apart from its physiological state, it is still unclear which conformation is more susceptible to aggregation. Formation of αS fibrils is a nucleation reaction in which soluble αS monomer is converted to an insoluble β-sheet rich structure, tightly stacked in a parallel configuration, that give rise to protofibrils and fibrils (Lashuel et al., 2002; Vilar et al., 2008; Tuttle et al., 2016). During this process, a heterogeneous population of intermediate configurations, collectively called oligomers, has been described *in vitro* (Cremades et al., 2012; Chen et al., 2015). αS fibrils obtained with *in vitro* fibrillation can have a ribbon or a fibril structure (Bousset et al., 2013; Guo et al., 2013). Both oligomers and fibrils can be toxic though with different mechanisms of pathology (Danzer et al., 2007; Peelaerts et al., 2015; Fusco et al., 2017).

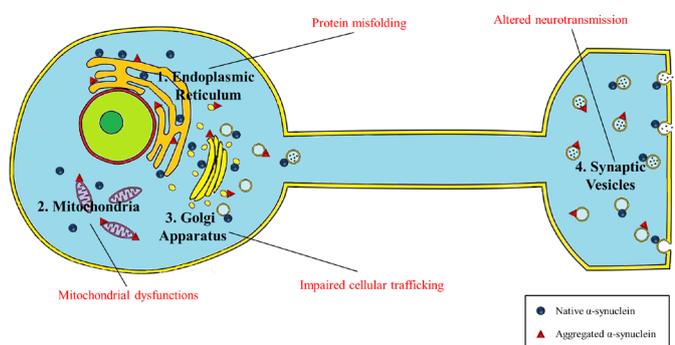


Figure 3 Influence of subcellular localization on α-synuclein (αS) oligomers/aggregates toxicity.

αS has been found to selectively bind to synaptic vesicles, endoplasmic reticulum (ER)/Golgi and the mitochondria and membrane binding seems to be part of its physiological function (Devi et al., 2008; Nakamura et al., 2008; Burré et al., 2010; Colla et al., 2012a). Although it is still unclear if association with membranes interferes with or accelerates αS aggregation, accumulation of αS toxic species selectively along these subcellular locations can primarily impact specific cellular functions such as neurotransmission, protein trafficking and mitochondrial respiration directly linked to the above-mentioned organelles.

Colla et al., 2012a). While no lipid binding involvement has been described yet, αS was found to bind, in αS Tg mice and human cell lines overexpressing αS, to gpr78/binding immunoglobulin protein (BIP), an ER chaperone bound to the luminal side of the ER, transiently associated with the ER translocon import pore, and directly implicated as a sensor of protein misfolding and initiator of the unfolded protein response (Bellucci et al., 2011; Colla et al., 2012a). Moreover overexpression of αS was shown to impair vesicular trafficking at the ER-Golgi level in yeast and other organisms (Cooper et al., 2006) causing accumulation of ER proteins with induction of ER stress, Golgi fragmentation and depletion of lysosomal enzymes (Oaks et al., 2013; Mazzulli et al., 2016). Interestingly, this transport defect was rescued by overexpression of proteins implicated in vesicles transit from the ER to the cell membrane such as Rab1 (ER-Golgi), Rab8 (Golgi) and Rab3A (post-Golgi) (Gitler et al., 2008) but also from endolysosomal pathway such as Rab-11A (recycling endosomal) (Breda et al., 2015), implicating a major role for αS in vesicle trafficking and recycling, outside the synapses.

In addition, αS has been found to bind mitochondrial outer and inner membrane [(Devi et al., 2008; Nakamura et al., 2008) and our lab (unpublished results)]. Since most of the data were obtained from *in vivo* observations, it is not clear whether this binding was based on lipids, according to αS preference to cardiolipin, which is rich in the mitochondria membranes, or was also mediated by specific proteins. Interestingly a translocase of the mitochondria outer membrane has been described as responsible for the import of αS into the mitochondria and one of its subunit, TOM20, has been

shown to bind αS *in vivo* (Di Maio et al., 2016). Moreover overexpression of αS was found to promote mitochondria dysfunction in αS Tg mice (Martin et al., 2006, 2014) and mitochondria fragmentation *in vitro* and in primary neurons. This last effect was dependent on the direct interaction of αS with mitochondria since disruption of αS N-terminal membrane-binding domain, rescued mitochondria morphology (Nakamura et al., 2011).

Impact of Membrane Binding and Subcellular αS Localization *In Vivo* on αS Pathology

While αS cytosolic and membrane bound-state are both physiologically relevant, it is still unclear how their localization affects αS pathology and where aggregation initiates. Accumulating evidence has shown that membrane binding and lipid interaction can stimulate but also attenuate αS fibrillation (Narayanan and Scarlata, 2001; Lee et al., 2002; Jo et al., 2004; Burré et al., 2015; Galvagnion et al., 2016).

In line with this controversial aspect is the observation that point mutations in αS associated with familial PD are located in the N-terminal lipid-binding domain, suggesting that lipid binding can be implicated in αS acquired pathogenicity. This is somewhat true for some point mutations such as A30P, where membrane binding is reduced while aggregation increased, but not others. In fact pathological amino acid substitutions in αS such as A53T, E46K and H50Q, lead to an increase in fibril formation without affecting lipid binding (Bussell and Eliezer, 2004; Fredenburg et al., 2007; Khalaf et al., 2014) while other mutations such as G51D,

attenuate both membrane binding and aggregation (Fares et al., 2014). Thus while membrane binding and aggregation may not always be directly correlated, other factors, besides point mutations, such as intramolecular interaction between the N and C termini or protein binding to the C-terminal can influence the propensity of α S to aggregate and compensate for amino acid substitutions (Ulrih et al., 2008; Burré et al., 2010). In addition the presence of oxidative stress-induced posttranslational modifications of α S [such as nitrosylation, metal ion-catalyzed oxidation and presence of dopamine (or its oxidative metabolites) adducts] has been shown to increase oligomerization and, possibly, influence α S ability to bind vesicle membranes as a monomer or in an oligomer conformation (Binolfi et al., 2006; Xiang et al., 2013; Follmer et al., 2015; Plotegher et al., 2017).

More recently, two independent papers proposed how two different small compounds, one of synthesis, NPT100-18A and the other naturally produced, squalamine, could reduce α S oligomers/aggregates content and subsequently rescue behavioural deficits in mice and worms, by interfering with α S binding to membranes (Wrasidlo et al., 2016; Perni et al., 2017). Notably, while NPT100-18A and squalamine have a different origin, they both bind only to membrane-associated α S, inducing a rearrangement of the protein structure that would lead to a displacement from the lipids, therefore reducing the amount of aggregation-prone α S. Thus while these results greatly suggest that aggregation might initiate from a pathological conversion of the membrane-bound α S fraction, because α S membrane-binding is required for α S function, development of therapeutic strategy that would block aggregation by interfering with α S membrane binding has to be taken with caution.

In vivo subcellular localization of α S and association with specific membranes can determine pathobiology processes connected to aggregation and neuronal degeneration. Our group and others (Colla et al., 2012a, b; Fagerqvist et al., 2013) have shown that α S aggregates can be selectively associated with the secretory pathway including the ER and Golgi, in pathogenic conditions in α S Tg mice but not with other organelles such as the mitochondria. Notably in absence of pathology, aged Tg mice already accumulated specifically oligomer species associated with the ER, Golgi and synaptic vesicles before neuronal degeneration appearance. When compared to other α S species that co-precipitate at lower g, microsomes-associated α S oligomers (*i.e.*, HMW species associated with membranes from the secretory pathway) obtained from healthy aged Tg mice with no overt accumulation of α S insoluble aggregates, were increasingly harmful and able to induce apoptosis, after exogenous administration to murine neuronal cultures (Colla et al., 2018). Additionally, α S oligomers have been found to decrease axonal transport and influence microtubule stability, a condition that could exacerbate synaptic vesicles traffic dysfunction (Prots et al., 2013).

At the synapse, exogenous administration of large oligomers of recombinant α S was shown to bind synaptic vesicles through synaptobrevin-2 causing vesicle docking inhibition

to the membrane (Choi et al., 2013) and to lower synapsin-I/II abundance (Larson et al., 2017). Although direct measurement of ER-Golgi traffic was not assessed in these conditions, it is plausible that accumulation of toxic species of α S might affect the whole protein transport system from the ER to the membrane. Moreover, electrophysiology studies showed that α S oligomers impair long-term potentiation (Diogenes et al., 2012; Martin et al., 2012) and reduces neuronal excitability (Kaufmann et al., 2016).

α S oligomers have been shown to be particularly toxic to mitochondria. Mitochondrial damage such as inhibition of complex I activity with subsequently increase of reactive oxygen radical production and oxidative stress (Devi et al., 2008; Cremades et al., 2012), alteration of membrane potential and Ca^{2+} homeostasis, induction of mitochondrial fragmentation (Nakamura et al., 2011), mitochondrial protein import impairment (Di Maio et al., 2016), and, more recently externalization of cardiolipin to the outer mitochondrial membrane, a process that stimulates mitophagy in response to cellular stress (Ryan et al., 2018), has been associated with accumulation of toxic α S. High-ordered α S structure such as a small oligomer but not the monomer was found to be able to associate and cluster artificial mitochondria and induced fragmentation, a process similar to that described in the case of synaptic vesicles (Diao et al., 2013). In addition cardiolipin has been shown to associate and promote refolding of α S fibrils *in vitro*, a process negatively affected and reduced by the presence of PD-related α S point mutations (Ryan et al., 2018).

Because of most of those evidence were obtained *in vitro*, it is not clear whether mitochondria could be a primary site of aggregation or if mitochondrial dysfunction, due to toxic α S, might be the result of a secondary generalized spreading of α S aggregates in the neuron. Thus while more evidence will be necessary to fully understand the influence of membrane binding on α S pathology, it is plausible to hypothesize that the initial pathologic transition of α S toward a toxic conformation might occur in proximity of the membranes in the above mentioned specific locations and then spread to other sites in neurons.

Conclusions

α S has been found to shift between a free native conformation and a membrane-bound state in a dynamic equilibrium. What dictates this transition is not clear but α S has been found to associate with specific membranes in neurons such as that of synaptic vesicles and some cellular organelles, like the ER/Golgi and the mitochondria. Although it is still not clear whether membrane-bound α S is more susceptible to aggregation or binding to membrane prevents the pathologic conversion to toxic species, initial phases of α S aggregation might begin selectively along those specific membranes and compromise, as has been reported, processes linked to these sites such as synaptic transmission, protein folding and trafficking, energy production. Initial damage from these sites would spread to other cellular functions, exacerbating α S aggregation and lead ultimately to neuronal demise. In line

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with this, compounds that would modify α S interaction with membranes might inhibit the initiation of α S aggregation and clarify whether membranes are necessary for the initiation of α S aggregation. At the same time though, a widespread inhibition of α S binding to membranes might result in a decrease in cellular functions mediated by α S, therefore the use of such strategies in mammals might be difficult and not directly result in a pathology improvement. A way to bypass this problem could be to implement strategies that would act on the initial phases of cellular dysfunction described above. New data will be necessary in the near future to clarify the impact that membrane binding and subcellular localization have on α S toxicity and to understand how to intervene in the early phases of the α S aggregation process before a generalized damage occurs.

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Open peer review reports:

Reviewer 1: Hailong Song, University of Missouri Columbia, USA.

Comments to authors: The authors provided a comprehensive review with nice illustrations and detailed explanation of some current research. The scientific significance, quality, and novelty of this review are high. In this manuscript, the authors reviewed α S's association with membrane binding and its subcellular localization to further understand the regulation of α S contributing to PD pathophysiology. Specifically, this review included α S conformation, the impact of membrane binding on alpha-synuclein structure and cellular localization, and the subsequent alpha-synuclein pathology in PD. Overall, the authors provided a comprehensive review with nice illustrations and detailed explanation of some current research. The scientific significance, quality, and novelty of this review are high.

Reviewer 2: Darrin Jason Lee, University of Toronto, Canada.

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