

α -Synuclein in Parkinson's Disease: 12 Years Later

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α -Synuclein (AS) is a small presynaptic protein that is genetically, biochemically, and neuropathologically linked to Parkinson's Disease (PD) and related synucleinopathies. We present here a review of the topic of this relationship, focusing on more recent knowledge. In particular, we review the genetic evidence linking AS to familial and sporadic PD, including a number of recently identified point mutations in the *SNCA* gene. We briefly go over the relevant neuropathological findings, stressing the evidence indicating a correlation between aberrant AS deposition and nervous system dysfunction. We analyze the structural characteristics of the protein, in relation to both its physiologic and pathological conformations, with particular emphasis on posttranslational modifications, aggregation properties, and secreted forms. We review the interrelationship of AS with various cellular compartments and functions, with particular focus on the synapse and protein degradation systems. Last, go over the recent exciting data indicating that AS can provide the basis for novel robust biomarkers in the field of synucleinopathies, while at the same time results from the first clinical trials specifically targeting AS are being reported.

Since the initial discovery in 1997 of the genetic link between Parkinson's Disease (PD) and *SNCA*, the gene encoding for the presynaptic neuronal protein α -synuclein (AS), the evidence supporting the importance of AS in PD pathogenesis and evolution has continued to mount. The combination of further genetic discoveries and the understanding of AS biology and its impact on cellular processes, through the development of relevant cellular and animal models, has been instrumental in this regard. This has cul-

minated recently in the emergence of the first wet biomarker for PD, based on the aggregation properties of AS, and in the execution of the first clinical trials targeting AS. In this paper, which follows a similar article published by Stefanis (2012), we attempt to summarize the main aspects of AS biology, focusing on its normal function, its aggregation properties and cellular pathogenic effects, its genetic and neuropathological link to PD, its potential as a biomarker and finally its targeting in clinical trials.

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THE ORIGINS OF THE LINK: SNCA AS AN IMPORTANT GENETIC CONTRIBUTOR TO PARKINSON'S DISEASE

In PD, unlike Alzheimer's Disease (AD) where the neuropathological discovery of β -amyloid deposition preceded the genetic discovery of mutations in the amyloid precursor protein (APP) leading to autosomal-dominant AD, genetics came first. In 1997, Polymeropoulos et al. (1997) reported for the first time a specific genetic defect leading to familial PD. This involved a large family of Italian origin, the Contursi kindred, with an autosomal-dominant pattern of inheritance. The genetic defect identified was a missense p.A53T mutation in the SNCA gene. This was conceptually very important, as it ran against the perceived notion of PD as a sporadic disease initiated by environmental factors. Importantly, in the same publication (Polymeropoulos et al. 1997), Greek PD patients with an autosomal-dominant inheritance pattern, from seemingly unrelated families, were identified with the same mutation, solidifying the etiological link and demonstrating a founder effect, likely of thousands of years old. Since then, further research on carriers of this particular mutation has refined the clinical and other manifestations of the disease. Despite noticeable heterogeneity, ranging from incomplete penetrance to extremely aggressive manifestations, the general pattern is that of a disease that presents early, with a mean age of onset of 45, and is more severe compared to idiopathic PD (iPD)

(Papadimitriou et al. 2016). Over this period of 27 years, a number of other point mutations in the SNCA gene have been identified, all leading to autosomal-dominant PD (Fig. 1; Krüger et al. 1998; Zarraz et al. 2004; Appel-Cresswell et al. 2013; Kiely et al. 2013; Lesage et al. 2013; Proukakis et al. 2013; Pasanen et al. 2014; Kapasi et al. 2020; Fevga et al. 2021; Liu et al. 2021; Daida et al. 2022; Diaw et al. 2023). Such cases are rarer, compared to those harboring the p.A53T mutation, so their clinical picture is not as well-defined, but seems to vary by the specific mutation. An insertion of seven amino acids leading to an elongated peptide conferring novel aggregation properties has also been reported in a case of juvenile onset (Yang et al. 2023). Although a toxic gain of function is a common denominator, not all mutations lead to enhanced aggregation propensity; for example, the clinically aggressive G51D mutant form decreases the rate of fibrillization (Rutherford et al. 2014), but G51D fibrils, once formed, have altered properties that may lead to enhanced seeding and neurotoxicity (Hayakawa et al. 2020; Sun et al. 2021). It is fair to say that no single mechanism has been identified through which SNCA point mutations lead to PD; this could either indicate that such a common mechanism has yet to be identified, or that such point mutations lead to the disease through different mechanisms.

Yet another conceptual leap was the discovery in 2003 that excess copies of the SNCA gene could lead to autosomal-dominant disease. In particular, the Iowa kindred was the first to be

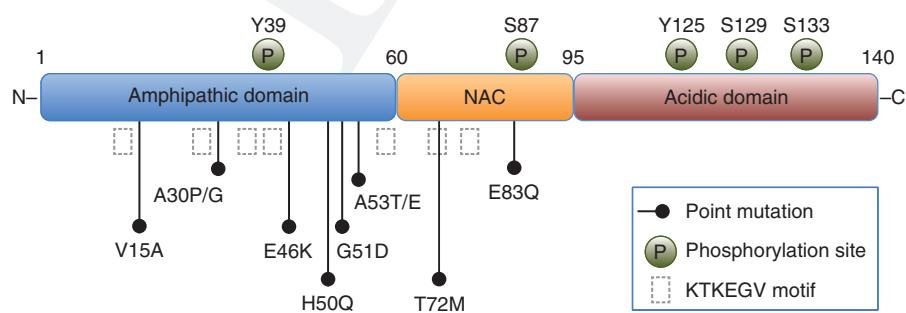


Figure 1. Simplified schematic illustration of α -synuclein primary structure. Within the three basic protein domains (amphipathic, hydrophobic nonamyloid component [NAC], and acidic), the KTKEGV motifs, the known point mutations and the phosphorylation sites are depicted.

discovered to harbor a triplication of the SNCA locus (Singleton et al. 2003). Subsequently, duplications were also identified (Chartier-Harlin et al. 2004). Intriguingly, there is an obvious gene dosage effect, in that cases with the SNCA triplication versus duplication demonstrate a much higher, basically complete, penetrance, earlier age of onset, and enhanced disease severity, including prominent cognitive decline. This brings home the important point that excess levels of the normal AS protein are sufficient to lead, in a dose-dependent fashion, to the neuropathological and clinical manifestations of PD, establishing the importance of AS levels in PD pathogenesis.

The genome-wide association studies (GWAS) in populations of predominant Caucasian origin have collectively and cumulatively shown that the highest hit most strongly associated with sporadic PD is in the SNCA locus (Nalls et al. 2019). This has been recently confirmed in two large non-Caucasian ethnic groups in China and India (Pan et al. 2023; Andrews et al. 2024). Thus, irrespective of ethnic origin, genetic alterations within the SNCA locus significantly influence the risk of development of sporadic PD, proving that sporadic PD is linked genetically to SNCA and AS. The GWAS approach provided thus an unbiased platform to confirm prior targeted association studies (Mueller et al. 2005). The polymorphisms associated with the disease appear to be associated with higher SNCA mRNA and AS protein levels (Fuchs et al. 2008), but more work is needed to substantiate this. This genetic association, not surprisingly, extends to other synucleinopathies, such as dementia with Lewy bodies (DLB) (Guerreiro et al. 2018; Chia et al. 2021) or REM sleep behavior disorder (RBD) (Krohn et al. 2022), although the exact sites of association may not be identical. These studies overall clearly establish SNCA as a pleomorphic gene locus involved both in rare genetic and sporadic forms of PD and other synucleinopathies.

NEUROPATHOLOGICAL FINDINGS LINKING α -SYNUCLEIN TO PARKINSON'S DISEASE

Very soon after the genetic discovery of the p. A53T SNCA mutation, studies were performed

to assess whether deposition of AS could be discerned within neuronal Lewy bodies (LB) and Lewy neurites (LN), the characteristic neuropathological features of PD. This proved to be the case, not only in the rare genetic synucleinopathies, but also in the vast majority of iPD brains examined, and even in a range of related conditions, termed collectively LB diseases, such as DLB (Spillantini et al. 1997, 1998; Baba et al. 1998). AS antibodies (Abs) label the filamentous portion of LBs, consisting of a single protofilament, as identified by cryo-electron microscopy, thus having different properties from AS filaments identified in the oligodendrocytic synucleinopathy multiple system atrophy (MSA) (Yang et al. 2022). AS filaments may not be homogeneous across PD cases, suggesting different strains that may confer variable pathogenic effects, accounting partially for disease heterogeneity and distinct subtypes (Strohäker et al. 2019). Of note, nonfilamentous AS, that may be quite abundant, also exists within LB (Shahmoradian et al. 2019). There is likely also considerable astrocytic AS pathology in the spectrum of LB diseases, which is just beginning to be appreciated (Altay et al. 2022).

As immunostaining was used for the groundbreaking neuropathological study of Braak et al. (2003), that provided a basis for the staging of the disease. LN, mainly, and also LB, were present in various brain regions even in asymptomatic individuals. According to this staging scheme, aberrant AS deposition follows a stereotypical pattern from initial sites of involvement in the olfactory bulb and the dorsal motor nucleus of the vagus to more rostral areas of the brainstem, involving the substantia nigra pars compacta at a third stage, and eventually to higher order association cortical areas in stages 5 and 6 (Braak et al. 2003). This AS immunohistochemical staging scheme has been controversial but has been borne out by most subsequent studies (e.g., Coughlin et al. 2019). Alternative staging schemes have been proposed to account for more rostral AS deposition in the absence of obligatory brainstem involvement in initial disease stages (Beach et al. 2009; Borghammer et al. 2021). Overall, there is consensus that aberrant AS deposition, as assessed by traditional AS immunostaining, is associated with regional

brain dysfunction, that however is not always obvious clinically, as seemingly healthy individuals may harbor advanced stages of AS pathology (Parkkinen et al. 2008). Whether AS aberrant deposition is actually the cause of neuronal dysfunction is a question of debate, as some consider that such deposition could be secondary and incidental, and this notion cannot be excluded at this time (Espay et al. 2019). Of great interest is the newly developed technique of proximity ligation assay (PLA), which labels preferentially intermediate, oligomeric, and not fully fibrillar forms of AS (Roberts et al. 2015). In a recent application of this technique in PD brains, it was found that PLA-identified AS pathology in the hippocampus correlated much better with cognitive dysfunction than staining for classical Lewy pathology (Sekiya et al. 2022).

A related issue is that some genetic PD cases do not manifest Lewy pathology. In particular, biallelic *PRKN* mutation carriers rarely manifest such pathology (for review, see Madsen et al. 2021), while *LRRK2* mutation carriers manifest quite significant variability in this regard. Among *LRRK2* mutation carriers, those with the G2019S mutation most often show evidence of synucleinopathy, in 60%–70% of cases. Interestingly, there is an association between the existence of Lewy pathology and more widespread nonmotor disease manifestations, suggesting again that AS pathology is linked to brain dysfunction (Kalia et al. 2015).

Overall, the combination of genetic and neuropathological evidence, but also the evidence provided by the cell and animal models below, provides a strong argument for the pathogenicity of AS abnormal conformations in the context of PD and related synucleinopathies.

STRUCTURE, PHYSIOLOGICAL FUNCTION, AND SECRETION

Full-length AS is a small 140 aa protein primarily expressed in the presynaptic nerve endings of the adult brain in a region-specific manner. Except from the full-length protein, truncated AS fragments of 126, 112, and 98 aa are produced by alternative splicing (Beyer et al. 2006; McLean et al. 2012). The primary structure of AS consists

of three distinct well-characterized domains, each conferring different physicochemical properties to the protein (Fig. 1). The basic amino terminus (1–60 aa) carries seven 11 aa repeats containing the consensus KTKEGV sequence, which is well-conserved among species and among all the members of the synuclein family, α , β , and γ -synucleins (Bussell and Eliezer 2003). Due to this repeated motif, AS can adopt a helical secondary structure that can take the form of either two interconnected antiparallel α -helices in solution or one contiguous α -helix upon binding to acidic lipid membranes (Fusco et al. 2014). The highly hydrophobic nonamyloid component (NAC) core domain (61–95 aa) provides, at least to a great extent, the inherent property of AS to self-aggregate, generating high-order fibrillar or low molecular weight (LMW) oligomeric assemblies (Giasson et al. 2001). Last, the acidic carboxy-terminal tail (96–140 aa) carries most of the posttranslational modifications (PTMs) and is capable of Ca^{2+} binding (Oueslati et al. 2010). This domain underlies the flexible nature of the protein since it hosts the majority of the molecular interactions of AS with other proteins, metals, or small molecules (Uversky et al. 2001). Importantly, the carboxyl-terminus region can interact transiently with the amino-terminus domain forming compact structures that are resistant to aggregation (Hong et al. 2011; Burré et al. 2012). In addition, carboxy-terminally truncated AS (CT-AS) products tend to aggregate faster than the full-length protein, supporting a role of the C-end in preserving the normal structure of the protein (Hoyer et al. 2004; Li et al. 2005).

Despite extensive investigation the exact native secondary structure of AS remains largely unresolved. AS has been characterized as an intrinsically unfolded protein retaining minimal ordered structure in simple solutions (Uversky et al. 2001; Fauvet et al. 2012). In a cellular environment, it can physiologically adopt multiple conformations ranging from α -helical LMW multimers to β -sheet rich high molecular weight oligomers and aggregates (Uversky 2003). Even though it lacks a *trans*-membrane or a lipid-anchor domain, AS can peripherally associate with cellular membranes showing a preference to acidic or high curvature membranes. Membrane

binding drives a conformational change toward the α -helical structure and can either promote or prevent multimerization. In this context, previous studies have shown that the molecular crowding conditions in the cytosol encourage the endogenous formation of tetrameric assemblies, which are characterized by a helical conformation and appear resistant to further aggregation (Bartels et al. 2011). However, subsequent studies failed to confirm that the tetramer is a predominant conformation of AS in the cytosol, concluding that cytosolic AS remains mostly as a mobile monomeric protein constantly binding to various interactors (Binolfi et al. 2012; Theillet et al. 2016).

The primary physiological function of AS has not been fully elucidated, but it appears to have a key role in the regulation of synaptic transmission and dopamine synthesis. Even though it is not required for neuronal development, synapse formation, or neurotransmission *per se*, AS can potently modulate synaptic activity through different modes of action. A plethora of studies have elaborated on the role of AS in synaptic vesicle (SV) trafficking, particularly in SV clustering (Diao et al. 2013; Wang et al. 2014) and distribution (Scott and Roy 2012; Sun et al. 2019). Further elaboration on this role suggested that AS could modulate exocytosis in a dose-dependent manner through dilation of the exocytic fusion pore during the “kiss-and-run” process, a mechanism that applies to both regulated protein secretion and neurotransmission (Logan et al. 2017; Nellikka et al. 2021). AS can directly associate with the SV membrane via its interaction with the chaperone cysteine-string protein a (CSPa) and the vesicle SNARE protein synaptobrevin-2 (VAMP2) to either facilitate SNARE complex assembly or prevent the disassembly of the SNARE complex until neurotransmitter release is completed (Burré et al. 2010; Garcia-Reitboeck et al. 2010). Further supporting a chaperone-like activity, 14-3-3 chaperone protein and its binding partners can also bind to AS (Ostrerova et al. 1999; Williams et al. 2021). Finally, AS can act as a negative modulator of dopamine synthesis and recycling as suggested by its interaction with the dopamine synthesis enzymes, tyrosine hydroxylase (TH), and aro-

matic amino acid decarboxylase as well as with dopamine transporter (DAT) (Tehranian et al. 2006; Swant et al. 2011; Butler et al. 2015, 2017; Sivakumar et al. 2023).

Apart from these well-defined physiological functions, AS has also been implicated in suppression of apoptosis by inhibiting PKC activity (Jin et al. 2011; Guo et al. 2021), regulation of glucose levels (Rodriguez-Araujo et al. 2013; Wijesekara et al. 2021), regulation of calmodulin activity (Martinez et al. 2003; Ueda et al. 2023), maintenance of polyunsaturated fatty acid levels, and neuronal differentiation (Surguchov 2024).

Despite the lack of a signal sequence, AS is physiologically secreted in the extracellular milieu suggesting a yet unidentified paracrine role for this protein (Lee et al. 2005; Emmanouilidou et al. 2010a,b; Wu et al. 2023). In support for such a modulatory role, the mechanism of AS secretion is Ca^{2+} -dependent and seems to be precisely regulated by neuronal activity in the brain (Emmanouilidou et al. 2016; Yamada and Iwatsubo 2018). Still, our understanding about the mechanisms that regulate AS release is largely incomplete. Although initially proposed, passive diffusion cannot account for such release, as cell-produced AS cannot freely diffuse out from the cell interior (Lee et al. 2008). Instead, insights from cell culture systems indicate that AS follows an unconventional pathway of release that involves, at least in part, the externalization of exosomes, nano-sized extracellular vesicles (EVs) of endosomal origin that participate in targeted intercellular communication (Emmanouilidou et al. 2010a,b; Alvarez-Erviti et al. 2011; Fussi et al. 2018). Since exosome-associated AS is only a minor part of externalized AS, conventional ER-Golgi exocytosis could mediate AS export, as indicated by the association of the protein with secretory vesicles, although direct evidence that these vesicles are responsible for AS secretion is missing (Lee et al. 2005; Logan et al. 2017). In the context of the living brain, the secretion of AS from glutamatergic terminals in the striatum is tightly controlled by the levels of the neurotransmitter GABA through an intercellular mechanism that involves presynaptic Ca^{2+} channels, further suggesting that the maintenance of extra-

cellular AS levels in the brain parenchyma is critical for neuronal homeostasis (Emmanouilidou et al. 2016).

It is unclear whether the different conformations (normal, misfolded, or fibrillar) are released using common secretory pathways. Part of the misfolded cytoplasmic AS can escape cells using an unconventional pathway of release called misfolding-associated protein secretion (MAPS) that is mediated by the selective sorting of cargos to late endosomes and fusion of these endosomes with the plasma membrane (Lee et al. 2016). Acting as a co-chaperone of Hsc70, CSPa forms a high-order oligomer that captures AS upon palmitoylation and mediates its translocation to the late endosome lumen. The multivesicular body (MVB) that is subsequently generated carries soluble AS cargo, which is released upon fusion of the MVB with the plasma membrane (Wu et al. 2023). Alternatively, AS multimers that can accumulate within lysosomes can be released from neurons via SNARE-dependent lysosomal exocytosis (Xie et al. 2022).

Increased levels of AS oligomeric species have been observed to be associated with exosomes

Q1 (Delenclos et al. 2017; Guo et al. 2020). Furthermore, AS has been detected in exosomes from patients with synucleinopathies (Stuendl et al. 2016; Harischandra et al. 2019), and AS mutations have been reported to aid the packaging of aggregated protein in exosomes (Gustafsson et al. 2018). In this regard, exosomes derived from PD patient tissue or from inflamed cells induce AS aggregation and pathology in vitro and in vivo (Grey et al. 2015; Lee et al. 2016; Huang et al. 2022; Jin et al. 2023). Endogenous AS appears to be essential for the ability of exosomes to propagate pathology in vivo (Melachroinou et al. 2023). In general, research to date is confirming a role for exosomes in the transmission of AS pathology in synucleinopathies; however, the exact mechanisms related to their packaging, release, and uptake have not been elucidated yet.

α-SYNUCLEIN AGGREGATION STATES: FOCUS ON STRAINS

It is widely considered that the toxic potential of AS is linked to its propensity to assume under

certain circumstances abnormal conformations, such as intermediate soluble oligomers, termed also protofibrils, and eventually mature fibrils. The exact nature of the toxic species remains elusive. Importantly, fibrillar forms can transform soluble monomeric AS into an aggregated conformation. This forms the basis for the presumed disease propagation across brain regions (Lee et al. 2011) and the first wet biomarker for PD (see below).

So far, the findings from in vitro and in vivo experiments, and the observation in human tissue samples suggest that oligomers play a critical role in the initiation and progression of α-synucleinopathies (Kalia et al. 2013; Cremades et al. 2017). The oligomers that lead to fibril formation are known as “on-pathway” species. However, there are also “off-pathway” species that do not progress into fibrils (Miraglia et al. 2018). De Giorgi et al. (2020) demonstrated that, during fibril formation from monomeric AS, newly generated fibrils could be ThT-negative despite exhibiting a clear β-sheet structure in ssNMR. Interestingly though, injection of such fibrils into the SN of mice caused pS129 AS accumulation and spreading to other interconnected brain structures (De Giorgi et al. 2020). The different disease phenotypes observed in synucleinopathies suggest that each disorder may be caused by a different “strain” of AS conformations. Importantly, these different strains appear to affect specific cellular populations in the brain and maintain an ability to be serially transmitted, reminiscent of prions (Lau et al. 2020). This observation possibly suggests that strain-specific information is carried by the structure of the aggregated AS and can be transmitted, akin to prion molecules. It has also been proposed that the cellular environment drives one conformation over another (Woerman 2021). In this respect, Peng and colleagues demonstrated in a formative study that oligodendrocytes present a specific type of AS conformations, which differs from that in neurons (Peng et al. 2018). It is possible that different assemblies also exist between different neuronal types, which could in part explain their differential vulnerability in PD. In agreement with different conformers of AS having distinct biological and structural properties, distinct AS

strains named ribbons and fibrils could propagate in human iPSC (induced pluripotent stem cell)-derived neurons; ribbons were more potent in recruiting and seeding endogenous AS, and resulted in more p-S129 positive AS inclusions (Gribaldo et al. 2019).

Importantly, neurons from AS knockout mice exposed to AS fibrils do not develop intracellular inclusions and have intact neuronal function. Thus, endogenous AS templating to form insoluble fibrillar aggregates is crucial for pathology initiation and progression (Rey et al. 2018). Despite these major advances, a crucial unanswered question is whether these recombinant oligomers have different properties to the actual aggregates found in the brain in terms of heterogeneity and toxicity. Innovative approaches are urgently needed to detect and separate specific strains in the brains of individuals with synucleinopathies, as existing methods fall short in this regard.

CELLULAR AND ANIMAL PARKINSON'S DISEASE MODELS

Various cellular and animal PD models have been developed to investigate the pathological roles of the protein, including transgenic approaches, use of viral vectors, and, more recently, inoculation with recombinant preformed AS fibrils or LB extracts. However, none of these models recapitulates faithfully all aspects of PD pathophysiology and the choice of the appropriate model depends on the question being addressed.

Cellular models have been instrumental in the identification of the pathological roles of AS on various intracellular processes, such as mitochondrial function, oxidative stress, and proteasomal/lysosomal degradation pathways (for review, see Delenclos et al. 2019). Their main advantage is that they enable modeling of the mechanisms controlling the folding, oligomerization, aggregation, and cell-to-cell propagation of the protein, as well as the high-throughput screening of potential modifiers of these processes. From the first and simplest yeast models (Outschoorn and Lindquist 2003) to more complex cellular systems (for review, see Delenclos et al. 2019) using mammalian neuronal and nonneuronal

cell lines and, more recently, humanized iPSC-derived cultures generated from patient fibroblasts (Mohamed et al. 2019), these cell-based systems provide a unique opportunity to model the disease in a dish and test novel pharmacological interventions. The generation of fluorescent reporter lines enabled the dynamic monitoring of AS-AS interactions and subsequent aggregation, even though it is questioned whether the modified AS behaves similar to its nonmodified counterpart (Delenclos et al. 2019). The newest models that use patient-derived iPSCs allow investigation of the contribution of protein aggregation to early axonal dysfunction and provide novel mechanistic insights related to patient-specific risk factors or disease-specific mutations, thus paving the way for personalized treatments. Finally, several studies are now using iPSC-derived organoids or assembloids from PD patients to model disease pathophysiology in a more integrative manner that recapitulates better the brain's microenvironment (Bose et al. 2022; Calabresi et al. 2023a,b).

Q6

On the other hand, PD animal models offer the potential to model early alterations associated with AS overexpression and aggregation, that precede dopaminergic cell loss, such as synaptic dysfunction (synaptopathy) and nigrostriatal plasticity (Cenci and Björklund 2020). These models gradually develop Lewy body-like inclusions of aggregated AS, which usually leads to neuronal loss, thus recapitulating major aspects of PD pathology. The simplest invertebrate PD models are particularly useful for high-throughput screening applications, whereas mammalian models are required to explore complex motor/nonmotor features and behavioral alterations. Transgenic animal models involve the expression of wild-type (WT) or PD-linked mutant forms of AS through different promoters, thus enabling regional and temporal control of expression. Viral vector-mediated models, on the other hand, offer many advantages, including the targeted injection into selective brain areas, the capacity to transduce both neurons and glia depending on the serotype used, and the ability of injection at any age of the animal.

A major breakthrough in understanding the mechanisms underlying the cell-to-cell propa-

gation of AS-related pathology originated from studies where human (or mouse) recombinant AS preformed fibrils (PFFs) or PD brain extracts containing LBs or extracts from AS transgenic mice are injected into the brain (striatum, substantia nigra, and olfactory bulb), muscles, peritoneal cavity, or in the periphery of AS-over-expressing or WT rodents or nonhuman primates (for review, see Recasens et al. 2018). These studies are highly reproducible and are characterized by the presence of widespread Ser129-phosphorylated AS inclusions, mirroring aspects of the spread and staging of the human disease. Similar approaches have been also very fruitful in cellular, and in particular neuronal models, where it has been possible to model the maturation of seeded AS fibrils into LB-like structures, through the engagement of various compensatory, but also detrimental neuronal process (Mahul-Mellier et al. 2020).

α -SYNUCLEIN POSTTRANSLATIONAL MODIFICATIONS

AS exhibits a number of PTMs, including phosphorylation, ubiquitination, nitration, acetylation, truncation, SUMOylation, and O-GlcNAcylation. Of these, phosphorylated AS is thought to be the major pathological form (Fig. 1; Anderson et al. 2006).

Phosphorylation

Several studies have shown that AS phosphorylated at serine 129 (p-ser129) is a marker of mature AS aggregates. Examination of postmortem tissue from PD and MSA patients at different disease stages showed that p-ser129 AS is the major and earliest PTM along PD progression (Wakabayashi 2020; Sonustun et al. 2022). Nevertheless, p-ser129 seems to occur after the initial aggregation process (Pantazopoulou et al. 2021; Ghanem et al. 2022) and may have a physiological role at the synapse (see below). An additional caveat is that in models of AS overexpression, such as for example viral models, phosphorylated AS is not necessarily aggregated and should not be used as a sole readout of aggregation in these circumstances.

Whether p-ser129 is a driving force for AS aggregation and neurotoxicity remains a subject of debate. Mice inoculated with p-ser129 AS PFFs exhibited enhanced AS pathological deposition and dopaminergic cell loss associated with motor deficits (Karampetsov et al. 2017). The Lashuel and Li groups, using semisynthetic approaches to synthesize p-ser129 AS, showed that the phosphorylated fibers were toxic and less resistant to proteolysis by proteinase K compared to WT fibers, suggesting that S129 phosphorylation induces a distinct strain of AS species (Fauvet and Lashuel 2016; Ma et al. 2016). However, other studies have reported that p-ser129 phosphorylation does not influence AS aggregation and can reduce its toxicity (Weston et al. 2021; Ghanem et al. 2022).

The role of p-ser87 is controversial as this PTM falls within the NAC region of AS, which is crucial for its aggregation and fibrillogenesis. However, p-ser87 AS viral overexpression in the nigrostriatal system of rats caused reduced accumulation and no dopaminergic neuron loss or motor impairment, in contrast to WT AS overexpression (Oueslati et al. 2012). Collectively, data on the phosphorylation sites at S129, S87, and Y39 support the notion that phosphorylation decreases AS binding to membranes (Dikay et al. 2016; Reimer et al. 2022). Q7

Nitration, Acetylation

Nitrated AS has been reported in various in vivo and in vitro experimental models of PD and also in association with LB pathology (Przedborski et al. 2001; He et al. 2019; Manzanza et al. 2021; Magalhães and Lashuel 2022). Increased levels of nitrated AS have been detected in LBs and SN neurons, as well as in peripheral blood monocytes of PD patients (Prigione et al. 2010). Nitration of AS in mice was shown to elicit macrophage activation and T-cell responses that lead to exacerbated nigrostriatal degeneration (Benner et al. 2008). Interestingly, recent in vivo studies in an AAV-AS mouse model showed that AS nitration induces loss of neurons and increased cell-cell transfer of AS pathology (Barrett and Timothy Greenamyre 2015; Musgrove et al. 2019).

Studies using label-free single molecule detection methods, as well as recombinant acetylated AS, have shown that an amino-terminal acetylation has a protective effect, as it can significantly decrease oligomerization (Iyer et al. 2016; Bu et al. 2017). Bell et al. (2023) studied five amino-terminal acetylated familial variants (A30P, E46K, H50Q, G51D, and A53T) of AS and found that each variant responds to amino-terminal acetylation in unique ways, highlighting the great complexity of the behavior of AS and its high susceptibility to chemical modifications. In general, acetylation reduces the oligomerization capacity of AS, as well as the rate of fibril formation.

Truncation

Studies indicate a strong link between CT-AS and its aggregation. CT-AS is present in the brain and colon of PD patients and has increased ability to form fibrils and increased toxicity. Blocking carboxy-terminal truncation using antibodies to the carboxyl-terminus of the protein in an AS transgenic animal model reduced PD symptoms and reversed AS accumulation (Games et al. 2014). However, in vitro and in vivo studies with CT-AS fibrils have produced mixed results, with some reporting increased capacity to induce prion-like seeding of full-length AS by CT fibrils and others observing a decreased ability compared to WT AS fibrils (Sorrentino and Giasson 2020; Ohgita et al. 2022). It is possible that these controversies may stem from the different length of truncated forms used in the different studies.

Sumoylation, Ubiquitination

Impairment of AS SUMOylation in vitro by mutations of SUMO residues increased its aggregation propensity and neuronal toxicity. Interestingly, increased SUMOylated AS has been detected in PD brains (Rott et al. 2017; Rousseaux et al. 2018). Verma et al. (2020) showed in cellular models that SUMOylation is neuroprotective against MPP⁺ or AS PFFs. Similar results were obtained in vivo. Other reports show that SUMOylation competes with ubiquitination of AS, thus potentially blocking ubiquitin-dependent

degradation pathways. Increased SUMOylation also increased extracellular AS levels and its association with exosomes. It is, therefore, possible that SUMOylation may in this way affect the spreading of AS between cells (Kunadt et al. 2015; Stuendl et al. 2016). A number of studies have shown that ubiquitination by ligases such as Nedd4 enhanced the protein's clearance through an endosomal–lysosomal pathway (Liani et al. 2004; Tofaris et al. 2011); however, recent in vitro and in vivo ubiquitination studies have suggested that AS ubiquitination may promote the production of aggregated forms (Rott et al. 2017; Zhang et al. 2017; Wang et al. 2019). In contrast, the in vitro ubiquitination of WT AS at different sites was found to produce structurally different aggregates but with reduced aggregation ability (Moon et al. 2020).

O-GlcNAcylation

The O-GlcNAcylation of AS decreases its aggregation propensity and toxicity in cultured primary neurons without affecting its membrane binding affinity (Marotta et al. 2015; Levine et al. 2017). Moreover, O-GlcNAcylation hampers the cleavage of AS by calpain in vitro, a process involved in the formation of aggregates. It is possible that O-GlcNAc could similarly inhibit the cleavage of AS by as yet unidentified proteases that generate aggregation-prone protein fragments. In addition, pharmacological inhibition of glycoside hydrolase O-GlcNAcase (OGA) (which thus increases O-GlcNAcylation) blunts AS PFF cellular uptake (Tavassoly et al. 2021) and alleviates the degeneration and pathology in dopaminergic neurons caused by AS overexpression in an AAV mouse model (Lee et al. 2020). Similarly, small inhibitors to OGA after daily dosing improved motor impairment, reduced astrogliosis, and facilitated dopamine neurotransmission in mouse models of PD (Permanne et al. 2022).

PATHOGENIC EFFECTS OF α -SYNUCLEIN IN VARIOUS CELLULAR COMPARTMENTS AND FUNCTIONS

AS has a pathogenic potential within neurons and possibly astrocytes in the context of LB dis-

eases, and this may occur by aberrant effects at various cellular sites. Due to its predominant localization in presynaptic terminals and its physiological role at the synapse, major efforts have been undertaken to characterize its pathogenic role at synaptic terminals (Fig. 2); however, other aberrant cellular effects are also considered (Fig. 3).

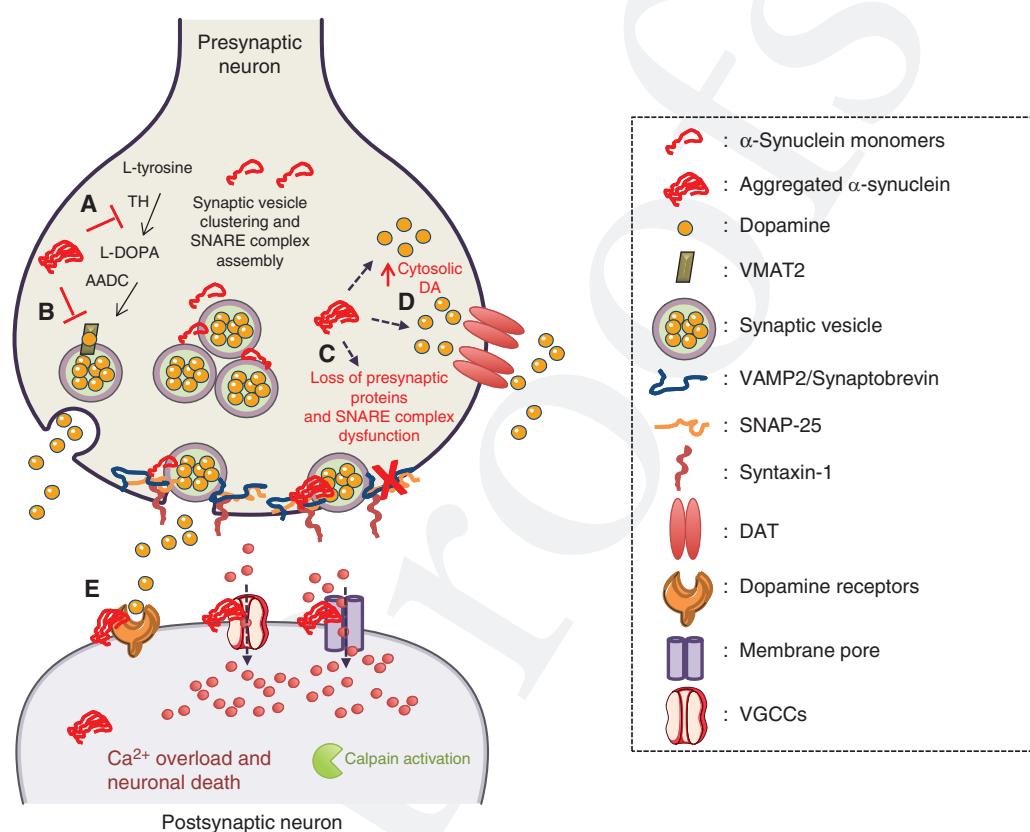


Figure 2. The pathological effects of α -synuclein (AS) at the synapse. (A) Aggregated AS reduces the activity of tyrosine hydroxylase (TH), the enzyme responsible for catalyzing the conversion of L-tyrosine to L-DOPA, thus impairing dopamine biosynthesis. (B) Increased levels of AS inhibit VMAT2, which is responsible for the uptake of monoamines transmitters (such as dopamine) into SVs; therefore, it modulates the neurotransmitter storage. (C) Disease-related AS conformations alter the levels of presynaptic proteins and evoke SNARE complex dysfunction, interfering with the SV fusion and dopamine release. (D) AS aggregates trigger dopamine transporter (DAT) recruitment to the plasma membrane, leading to increased entry of dopamine and increased cytosolic dopamine levels, which may be neurotoxic by facilitating further protein aggregation through the generation of dopamine-modified AS adducts. (E) Aberrant AS conformations may affect the activity of dopamine receptors and voltage-gated calcium channels (VGCCs), as well as promote the formation of ion-permeable pores in the plasma membrane; this may lead to intracellular Ca^{2+} overload and calpain activation, facilitating further protein aggregation and triggering a Ca^{2+} -dependent signaling cascade leading to neuronal demise.

α -Synuclein at the Synapse: a Love and Hate Affair

Among its multiple functions, the physiological role of AS in SV homeostasis and neurotransmitter release, as well as its aberrant effects on synaptic transmission and SNARE complex assembly, are the most well-documented (Scott

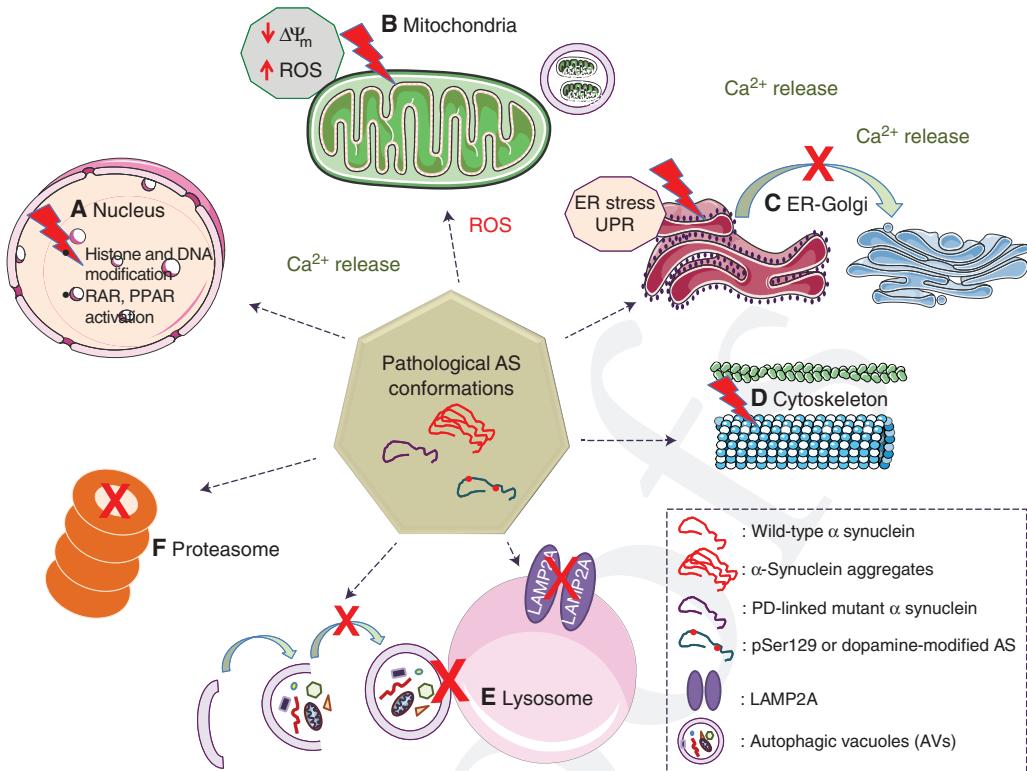


Figure 3. Aberrant effects of disease-related α -synuclein (AS) conformations (aggregated, dopamine modified, and phosphorylated) on the various cellular functions/compartments. (A) Within the nucleus, AS inhibits histone acetylation via its direct binding to histones or by inhibiting the action of histone acetyltransferase enzymes, thus interfering with the process of gene transcription. Through interaction with RA and peroxisome proliferator-activated receptors (PPARs), AS can alter Nurr1 transcription, thus affecting dopaminergic neuron survival. (B) AS mutations of aggregated species can transiently and dynamically engage with mitochondria, causing their depolarization, reduced energy production, fragmentation, and destruction through mitophagy. (C) Within the endoplasmic reticulum (ER), pathological AS assemblies can induce ER stress and unfolded protein response (UPR), impairment of Ca^{2+} homeostasis, and alterations in the vesicle-dependent protein trafficking, the latter affecting the ER-Golgi protein transport. (D) Aggregated AS may affect the structure and function of the neuronal microtubule cytoskeleton (through interaction with actin and tubulin), leading to axonal transport defects. (E) Disease-related conformations of AS may impair chaperone-mediated autophagy (CMA) activity through aberrant interaction with the LAMP2A receptor, as well as macroautophagic activity, affecting the formation of autophagosomes or their maturation and fusion with the lysosome. (F) Increased levels or pathological forms of AS may inhibit proteasomal function, thus leading to AS accumulation and formation of insoluble protein aggregates.

et al. 2010; Gao et al. 2023). PD-linked mutations, as well as aberrant AS conformations may exert pathological effects at the presynaptic terminal, including loss of presynaptic proteins (Chung et al. 2009), redistribution of SNARE proteins and impairment of neurotransmitter release (Garcia-Reitboeck et al. 2010), and inhibition of SV recycling pool size and mobility

(Nemani et al. 2010). At the presynaptic terminal, AS can exist both in a cytosolic and a membrane-bound form, and this localization may alter the propensity for aggregation. Membrane binding seems to exert a protective effect against aggregation, based on observations that some PD-linked AS missense mutations may inhibit this (Jo et al. 2002; Fares et al. 2014; Ghosh et al.

2014; Liu et al. 2021). Other reports, however, suggest the opposite (Lee et al. 2002; Perni et al. 2017; Limbocker et al. 2021). This controversy may reflect the coexistence of monomeric and oligomeric membrane-bound species that accelerate further the protein aggregation thus exerting a neurotoxic function. It has been also found that AS oligomers are preferably bound to synapsin 1 and VAMP2 (Betzer et al. 2015) and attenuate SNARE complex assembly. Intriguingly, recent findings support a rather physiologic role for the pathology-related pSer129 phosphorylated AS at synapses, advocating a model where activity-induced pSer129-AS triggers the interaction of AS with a network of synaptic proteins that eventually leads to physiologic attenuation of neurotransmitter release (Parra-Rivas et al. 2023).

Not only the conformation but also the protein dosage may alter the vesicle recycling and docking, as both gain- and loss-of-AS function can impair SV recycling. AS is expressed in all presynaptic terminals; however, in PD, dopaminergic neurons are the most vulnerable, possibly because AS regulates dopamine synthesis and turnover by altering the activity of critical components of the pathway, such as TH, DAT, and VMAT2 (Calabresi et al. 2023a,b). Differences between neuronal subtypes and neurotransmitter systems affected in early (norepinephrine, serotonergic, cholinergic) or late (dopaminergic) stages of the disease may also account for the differential vulnerability to AS-related synaptopathy. Common features between these neurons are prominent calcium currents, low intrinsic calcium buffering capacity, sustained spontaneous spiking, and broad spikes (Surmeier and Schumacker 2013).

Furthermore, a great wealth of data spanning from primary neuronal cultures, iPSC-derived neurons, and animal PD models, pinpoints a pathogenic effect of aggregated AS (established by overexpression of the WT or PD-linked AS mutations or PFF inoculation) on the levels of presynaptic proteins, such as SNAPs, VAMP2, Synapsins, Syntaxins, Synaptotagmins, Synaptophysin, SV2, PSD95, GAP42, Drebrin, Neurogranin, Rabphilin 3A, and neurotransmitter release (for review, see Murphy and McKernan 2022).

Animal studies using AS knockout (KO) and overexpression models cement further a critical role of the protein in the regulation of dopamine homeostasis. In particular, increased dopamine release and decreased reuptake, low striatal TH and DAT levels and a reduced number of nigral dopaminergic neurons have been reported in AS KO mice (Abeliovich et al. 2000; Chadchankar et al. 2011), although AS deletion was not shown to alter cytosolic dopamine levels (Mosharov et al. 2009). On the other hand, AS aggregates are reported to perturb dopaminergic neurotransmission and induce presynaptic and postsynaptic dysfunction, possibly through interactions with oxidized DA that facilitates further protein aggregation, through the generation of dopamine-modified protein adducts (Conway et al. 2001; Mor et al. 2017). Human AS overexpression Q8 evoked dopaminergic terminal loss (Masliah et al. 2000), deficient dopamine release, and altered SV distribution (Janezic et al. 2013), as well as defective DAT function (Lundblad et al. 2012). Exposure of neurons to AS oligomers increases Ca^{2+} intracellular levels resulting in mitochondria and ER stress, reactive oxygen species (ROS) production, and increased DA release, thus initiating a toxic cascade leading to neurodegeneration (Calabresi et al. 2023a,b). Finally, decreased levels of synaptic proteins and alterations in SNARE complex assembly correlating with duration of dementia have been also reported in human PD postmortem material (Mukaetova-Ladinska et al. 2013; Vallortigara et al. 2016), thus underscoring AS-mediated deregulation of synaptic neurotransmission.

Pathogenic Effects of α -Synuclein: Impact on the Nucleus

Following the first observation of AS localization in the nuclear envelope of the Torpedo electric organ (Maroteaux et al. 1988), studies have Q9 reported on the presence of AS in the nucleus, even though its function in this compartment is only partially understood. Nuclear AS is implicated in the regulation of gene transcription through direct binding either to naked DNA or to enzymes involved in transcription such as methyltransferases, histone deacetylases,

RNA-interacting proteins, and histones (Somayaji et al. 2021).

Chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq) confirmed that the ability of AS to directly bind to supercoiled DNA alters DNA conformation and stability and affects gene expression (Hegde et al. 2007; Pinho et al. 2019). Further, AS can directly interact with the nucleus-resident DNA methyl transferase 1 forcing its export in the cytosol, thereby causing DNA hypomethylation that increases the expression of various genes, Q10 including SNCA itself (Desplats et al. 2011). AS can also bind histone 3 (H3) reducing its acetylation, which in turn results in inhibition of gene expression through a disturbance in the balance between histone acetylation and deacetylation (Kontopoulos et al. 2006; Outeiro et al. 2007; Q11 Paiva et al. 2017). In addition, AS can interfere with histone methylation; for example, it can enhance histone lysine N-methyltransferase 2 (EHMT2) activity, decreasing the expression of genes regulated by the REST complex (Sugeno et al. 2016). Finally, AS can impact nuclear receptor-mediated transcription indirectly via its interaction with retinoic acid (RA). The AS-RA complex translocates to the nucleus, where it activates the RAR and PPAR nuclear receptors and down-regulates the orphan receptor, Nurr1, through mobilization of their respective response elements (Yakunin et al. 2012; Volakakis et al. Q12 2015; Davidi et al. 2020). These effects, given the role of Nurr1 in the development and maintenance of dopaminergic neurons, could be detrimental.

Pathogenic Effects of α -Synuclein at the Mitochondria

AS has a cryptic mitochondrial targeting sequence and has been reported to localize to mitochondria and influence mitochondrial dynamics (Devi et al. 2008; Parihar et al. 2008; Nakamura et al. 2011). However, other reports have demonstrated both in vitro and in brains that AS does not directly localize in mitochondria but rather associates with mitochondrial-associated ER membranes (MAMs). This interaction is reduced by pathogenic AS mutations, leading to mitochon-

drial fragmentation (Cooper et al. 2012; Guardia-Laguarta et al. 2014). AS monomers interact with mitochondria and regulate mitophagy events of fusion and fission, as well as transport and degradation of mitochondria (Lurette et al. 2023). In particular, AS promotes mitochondrial fission events and inhibits fusion through the activity of mitofusins. Treatment of isolated brain mitochondria with monomeric AS leads to an increase in ATP production through association of AS and the α -subunit of ATP synthase, suggesting a role of monomeric AS as a mitochondrial bioenergetic regulator (Ludtmann et al. 2018). AS oligomers interact with high affinity with important mitochondrial proteins like VDAC and TOM, proteins required for mitochondrial protein import, leading to their internalization and disruption of mitochondrial function (Guardia-Laguarta et al. 2014; Di Maio et al. 2016). In related research, Bérard et al. (2022) used an optogenetic system to manipulate and monitor AS aggregation in cells. They discovered that AS aggregates transiently and dynamically engage with mitochondria, causing their depolarization, reduced energy production, fragmentation, and destruction through mitophagy, which is dependent on cardiolipin externalization (Bérard et al. 2022). Furthermore, postmortem studies of PD brains showed that aggregated S129-phosphorylated AS preferentially binds to mitochondria (Wang et al. 2019). Treatment of primary neurons with AS fibrils caused the appearance of phosphorylated AS inclusions that appeared associated with the mitochondrial membrane, leading to cytochrome C release, and oxidative stress (Prots et al. 2018). Aggregates produced in iPSC-derived neurons bearing AS triplication were shown to promote the opening of osmotic transition pore, causing mitochondrial swelling, ultimately leading to neuronal death (Ludtmann et al. 2016). An additional point is that mitochondrial transport and function may be compromised in the process of the maturation of AS fibrils into LB-like structures (Mahul-Mellier et al. 2020). Cumulatively, these data support the idea that metabolism and function of healthy neurons may depend on the critical interplay between AS and mitochondria (Risiglione et al. 2021). Therefore, targeting the aberrant mitochondrial localization of AS aggreg-

gates may prove beneficial for α -synucleinopathies.

Pathogenic Effects of α -Synuclein: Impact on the ER, Golgi, and Relevant Trafficking

The pathogenic effects of AS on the endoplasmic reticulum (ER) include induction of ER stress and unfolded protein response (UPR), impairment of Ca^{2+} homeostasis, and alterations in the vesicle-dependent protein trafficking. The UPR can be initiated by three different ER-resident stress sensors, inositol-requiring enzyme 1 α (IRE1 α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Manie et al. 2014). In the absence of ER stress, these proteins remain in an inactive form through binding to BiP, a chaperone protein that acts as a detector of nonproperly folded proteins. Mutated or aggregated AS can penetrate the ER membrane, inducing morphological changes to the ER and binding to BiP (Bellucci et al.

Q14 2011; Colla et al. 2012; Gorbatyuk et al. 2012). This promotes the dissociation of BiP from IRE1 α , PERK, and ATF6, which then activate a series of cascade reactions directed to preserve cellular proteostasis; however, overreaction may lead to cell death. UPR activation seems to be a major contributor of AS-related cytotoxicity since ER stress markers, such as BiP or p-PERK, are increased in brain material from PD patients (Conn et al. 2004) and genetic or pharmacological targeting of UPR components are beneficial in preclinical models of PD (Colla et al. 2012; Martinez et al. 2019; Siwecka et al. 2023). The fact that BiP overexpression *in vivo* can resolve ER stress and protect from AS-induced cytotoxicity (Gorbatyuk et al. 2012) further highlights the importance of the BiP-AS interaction in the initiation and maintenance of the UPR and downstream detrimental effects. ER stress conditions can further potentiate AS aggregation feeding a vicious cycle linking AS pathology and ER dysfunction (Jiang et al. 2010; Bellucci et al. 2011; Carranza et al. 2012).

AS can also interact with vesicular traffic components within the ER affecting ER to Golgi protein transport. The first observation of abnormalities in ER-dependent vesicular traffic came

from studies in yeast where overexpression of AS-induced inhibition of vesicle docking to and fusion with the Golgi membrane, which was rescued by overexpression of Rab family members such as Rab1, Rab3A, and Rab8A, suggesting that, except from the ER-Golgi route, AS could impair other steps of the secretory pathway (Cooper et al. 2006; Gitler et al. 2008). Vesicular trafficking is finely orchestrated by intracellular calcium. Aggregated AS can directly bind to SERCA; this interaction distorts ER Ca^{2+} levels, interferes with intracellular Ca^{2+} homeostasis, and compromises vesicle targeting and fusion (Betzer et al. 2018; Kovacs et al. 2021). Importantly, alterations in ER to Golgi and vesicular trafficking occur in the process of LB-like structure maturation and may be responsible for the breakdown of cellular homeostasis (Mahul-Mellier et al. 2020).

Pathogenic Effects of α -Synuclein on the Cytoskeleton

It is notable that in the process of LB-like inclusion formation following AS PFF application and seeding in primary neuronal cultures, neuritic AS aggregates closely apposed to cytoskeletal elements are formed first (Mahul-Mellier et al. 2020). AS may affect the structure and function of the neuronal microtubule cytoskeleton, leading to axonal transport defects (Carnwath et al. 2018; Prots et al. 2018). High concentrations of AS can also alter the actin cytoskeleton when applied to hippocampal neurons, and can subsequently lead to disruption in neuronal functions, including axonal growth and migration. Overexpression of AS in fly neurons increased F-actin levels, promoted mislocalization of the mitochondrial fission proteins, and consequently led to mitochondrial and autophagic-lysosomal dysfunction (Ordonez et al. 2018; Sarkar et al. 2021). Recent data aided by superresolution imaging approaches, revealed the close association of neurofilaments and β -tubulin to pSer129 α -syn in LBs of PD postmortem tissue (Moors et al. 2021). So far, however, there is no evidence to support the hypothesis that AS is a true microtubule associated protein (i.e., a protein able to bind tubulin or microtubules and regulate their behav-

ior). In addition, it is not yet known whether AS binds tubulin directly or via as yet unidentified binding partners.

A Reciprocal Relationship: Pathogenic Effects of α -Synuclein on Protein Degradation Systems

Limiting intracellular AS levels, as well as its pathogenic effects on the function of the intracellular proteolytic machineries represents an obvious therapeutic approach for PD and related α -synucleinopathies. The manner of AS degradation still remains controversial, with both the proteasome (Bennett et al. 1999; Tofaris et al. 2001, 2011; Webb et al. 2003; Shabek et al. 2012) and the lysosome (Paxinou et al. 2001; Webb et al.

Q20 2003; Sevlever et al. 2008; Vogiatzi et al. 2008), contributing to AS clearance, in a conformation-, PTM-, cell-type-, and tissue-specific manner (Emmanouilidou et al. 2010a,b; Xilouri et al. 2013a, 2016a). Indicatively, soluble or relatively insoluble (but not fully aggregated) S129-phosphorylated AS appears to be cleared by the proteasome, whereas seeded aggregated AS is cleared by macroautophagy (Pantazopoulou et al. 2021). It has been suggested that AS is degraded via the proteasome under basal conditions *in vivo*, whereas under conditions where intracellular AS protein load is augmented, the lysosome takes over (Ebrahimi-Fakhari et al. 2011). More recently, a *de novo* K45, K58, and K60 ubiquitination of AS mediated by NBR1 binding and entry into endosomes in a process that involves ESCRT I-III for subsequent lysosomal degradation was identified, using diverse approaches in living or fixed cells (Zenko et al. 2023). This may be an important pathway for a pool of AS, which is rapidly turning over. We and others have found that WT-soluble AS, but not the A53T and A30P mutants, or phosphorylated or dopamine-modified AS, is degraded, at least partly, via the chaperone-mediated autophagy (CMA) lysosomal pathway (Cuervo et al. 2004; Martinez-Vicente et al. 2008; Vogiatzi et al. 2008; Xilouri et al. 2009; Mak et al. 2010). Further supporting the role of CMA on AS degradation are *in vivo* findings showing that overexpression of CMA's rate-limiting step, the LAMP2A receptor, concurrent-

Q21 over (Xilouri et al. 2013b) and in the *Drosophila* brain (Issa et al. 2018), was capable of mitigating AS levels and alleviating AS-related toxicity. Conversely, CMA deficiency in the rat substantia nigra through LAMP2A down-regulation led to the cytoplasmic accumulation of small AS aggregates, signifying that CMA is responsible for AS turnover within nigral dopaminergic neurons (Xilouri et al. 2016b). Within the lysosome, cathepsin D and cathepsin L have both been reported to clear AS aggregates (Cullen et al. 2009; Bae et al. 2014; McGlinchey and Lee 2015; Prieto et al. 2022).

An interrelated theme to AS degradation is the impact of increased WT- or PD-linked mutant protein load on the function of the proteasome and the lysosome. Initial studies proposed that the PD-linked A30P and A53T mutants evoke proteasomal impairment (Stefanis et al. 2001; Tanaka et al. 2001; Petrucci et al. 2002; Snyder et al. 2003), although other studies failed to detect such an effect (Martin-Clemente et al. 2004). In addition to the cell and animal data, reports in human postmortem material also suggest that proteasome function is impaired in sporadic PD patients (for review, see Cook and Petrucci 2009). Furthermore, many studies highlight a central pathogenic role of aberrant AS on endolysosomal function, focusing mostly on macroautophagy and CMA. Pathological conformations of AS (mutations, oligomeric/aggregated species) have been reported to interfere with different stages of autophagosome formation, maturation, trafficking, and fusion with the lysosome (Xilouri et al. 2016b; Sanchez-Mirasierra et al. 2022). Briefly, impaired macroautophagic activity has been initially reported in *in vitro* and *in vivo* synucleinopathy models, in a manner dependent on AS-Rab1a interaction and Atg9 mislocalization (Winslow et al. 2010). Early-stage autophagosome formation was impaired in the presence of both E46K- and A30P PD-linked mutants, in a manner dependent on JNK pathway (Yan et al. 2014; Lei et al. 2019), whereas the A53T mutant protein exerted contradictory effects on mitophagy (Chen et al. 2018; Obergasteiger et al. 2018). Multiple lines of evidence suggest that bulk AS aggregates disrupt

endolysosomal trafficking events, including those related to protein secretion partly via exosomes, that impede further AS clearance, thus permitting the persistence of pathological protein species within neurons or glia cells, ultimately leading to cell destruction (Klein and Mazzulli 2018). Intriguingly, it has been proposed that AS secretion via exosomes could act as a protective mechanism against the aberrant effects of AS on lysosomal function (Fussi et al. 2018). In addition, aberrant species or elevated levels of AS can have a detrimental effect on CMA function, likely through excessive binding to Lamp2a, impeding the access of other substrates to this rate-limiting component of the pathway, and thus setting the stage for a vicious cycle of pathogenicity (Cuervo et al. 2004; Xilouri et al. 2009, 2013a,b).

Q22 It is interesting to note here that, beyond AS, the aberrant actions of multiple PD-linked genetic defects, such as in LRRK2, VPS35, ATP13A2, and β -glucocerebrosidase (GCase), converge on the lysosome and are often accompanied by AS accumulation due to the ongoing lysosomal impairment (Klein and Mazzulli 2018). In particular, multiple cell- and animal-based studies propose the existence of a bidirectional loop underlying the relationship between *GBA1* mutations, AS, and the lysosome (Smith and Schapira 2022). It has been recently shown that mutant GCase contains a CMA-targeting motif and impairs the formation of the CMA lysosomal translocation complex required to translocate AS into CMA-active lysosomes for degradation, thus providing a new link between AS, CMA, and GCase (Kuo et al. 2022). Interestingly, the protein levels of the CMA markers LAMP2A and HSC70 are decreased in the human substantia nigra and amygdala of PD brains compared to controls (Alvarez-Erviti et al. 2010), and this reduction in LAMP2A levels correlated with increased AS accumulation selectively in regions harboring AS pathology (Murphy et al. 2014, 2015). Altered levels of LAMP2A and/or HSC70 can be detected in peripheral blood mononuclear cells of sporadic PD patients (Wu et al. 2011; Sala et al. 2014; Papagiannakis et al. 2015, 2019), suggesting that a systemic reduction in CMA activity may be present in PD patients.

α -SYNUCLEIN AS A BIOMARKER FOR SYNUCLEINOPATHIES

Many researchers have worked to establish AS-based tools for the evaluation of AS in early stage diagnosis, differential diagnosis of PD from other parkinsonian disorders, and assessment of disease progression (Chopra and Outeiro 2024). This topic has exploded in recent years, and a full accounting is beyond the scope of the current review.

The fact that AS is present in other tissues except the CNS complicates the interpretation of alterations in the levels of the protein. CSF total AS has been quantified using different immunoassays, and the results were controversial ranging from no difference (Mollenhauer et al. 2011; Toledo et al. 2013; Hansson et al. 2014) to a significant decrease observed in PD patients compared to controls (Hall et al. 2012; Kang et al. 2013; Parnetti et al. 2014). This decrease likely reflects the entrapment of soluble interstitial AS within LBs and related aggregates in a manner akin to β -amyloid deposition and related low CSF β -amyloid in AD. The magnitude of the decrease observed is quite low, ~10%–15%, weakening the ability of the assay of total AS in CSF to distinguish PD from controls. The measurement of CSF oligomeric and phosphorylated species has also been pursued. Both conformers were found to be significantly increased in the CSF of PD patients and the oligomer-to-total AS ratio could differentiate PD subjects from controls with higher sensitivity and specificity compared with total AS (Park et al. 2011; Wang et al. 2012; Parnetti et al. 2014; Stewart et al. 2015); yet, these assays have not provided enough separation between PD and controls to be useful as diagnostic biomarkers.

The levels and species of AS have also been examined in other more easily accessible body fluids or tissues. Even though the major source of AS in blood is erythrocytes, most of the work so far has focused on plasma or serum AS. The results from these studies are varied, showing either increased, unaltered, or even decreased levels of AS, highlighting the importance of technical confounders in biological samples with high constituent complexity such as plasma or serum (Duran et al. 2010; Foulds et al.

2013; Shah et al. 2017). In contrast, in studies where aggregated or total AS was quantified in erythrocyte membranes, the results have consistently shown a significant increase in PD samples compared to healthy controls or other related disease groups (Papagiannakis et al. 2018; Abd Elhadi et al. 2019; Li et al. 2021).

Considering that AS conformers are packaged in EVs, which can facilitate disease propagation in the brain, recent studies assessing neuronal cell-adhesion molecule LCAM1-positive EVs isolated from blood or saliva reported significantly higher levels of EV-associated total AS in PD (or even in prodromal PD) compared to controls, a difference that could not be observed in plasma samples from the same groups (Cao et al. 2019; Jiang et al. 2020; Yan et al. 2024). Assessment of pathological aggregated AS in this material may even lead to higher sensitivity and specificity (Kluge et al. 2022).

Another relatively easily accessible material is skin. Skin biopsies have been used mainly as material for immunohistochemistry with antibodies against altered conformations of AS, in particular phosphorylated AS. Such assays have shown high specificity and sensitivity and discriminatory ability compared to controls or non-LB-related parkinsonism (e.g., in Donadio et al. 2014), but results in other studies have been variable, largely likely due to methodological issues. This has culminated in a large multicenter study that clearly established the very high specificity and sensitivity (over 90% for both) of this qualitative assay for differentiating synucleinopathies from controls (Gibbons et al. 2024).

New technological advances have changed our perspective of measuring AS in biofluids. In vitro detection methods in biological material now include conformer-specific immunoassays and electrochemical biosensors (Chen et al. 2022), as well as complex methods based on the addition of recombinant AS and its seeding by relevant biological material, such as CSF. These latter assays that originated as separate methods termed protein-misfolding cyclic amplification (PMCA) (Jung et al. 2017; Nicot et al. 2019), and real-time quaking-induced conversion (RT-QuIC) (Fairfoul et al. 2016; Nakagaki et al. 2021; Huang et al. 2024), have converged on seed am-

plification assays (SAAs) (Concha-Marambio et al. 2023; Fernandes Gomes et al. 2023; Siderowf et al. 2023), that have tremendously increased the sensitivity and diagnostic accuracy of disease-related AS. Importantly, such assays in the CSF clearly differentiate LB disease-afflicted patients and related prodromal forms from controls and non-LB-related parkinsonism. Beyond the diagnostic utility, such assays have sparked a great degree of enthusiasm as they potentially reflect the ongoing nervous system pathogenetic process. However, the CSF SAA is difficult to implement and requires specialized equipment. At this point, it is qualitative and does not reflect disease progression; it is hoped that refinements of the assay may lead to its further development along these lines. In the meantime, efforts are underway to transfer the success of SAA to more accessible tissues, including skin and serum, among others. An intriguing publication by Okuzumi et al. (2023), in particular, suggests that serum AS SAA may be very promising.

Another landmark in the field would be the development of a PET tracer that could detect abnormal conformations of AS in living patients, as this would provide regional information, and could be followed longitudinally and presumably quantitatively, to provide, among others, meaningful end points for disease-modifying clinical trials. Converging information suggests that there is hope for significant developments in this area in the coming years. Already, Smith et al. (2023) reported specific aberrant AS-targeted PET tracer uptake in the cerebellum of MSA patients, and it is hoped that similar approaches may be successful in PD.

EMERGING CLINICAL TRIALS TARGETING α -SYNUCLEIN IN PARKINSON'S DISEASE

Based on the idea that removal of pathogenic AS species may be beneficial for PD and related synucleinopathies, a number of companies have embarked on clinical trials with the aim to decrease such levels or inhibit the effects of such species through the application of molecular strategies, immunization, or small compounds. Immunization strategies in particular entail both active and passive immunization, with the latter being more

advanced in terms of application in large clinical trials. In 2022, two large studies in early PD using different antibodies, with variable specificity toward aggregated AS conformations, failed to show a significant benefit (Lang et al. 2022; Pagano et al. 2022), even though the used antibodies bound completely monomeric AS in the periphery and had shown ameliorating effects in rodent animal models of synucleinopathy. These results raised questions about the validity of the immunization approach, and even the basic principle of targeting pathogenic AS for the treatment of PD (Whone 2022). As argued, however, by Jensen et al. (2023), notwithstanding the rigorous nature of the performed studies, various issues preclude a hasty abandonment of similar approaches: The execution of the animal studies on which the clinical trials were based could be improved, in particular regarding the temporal pattern of treatment, to more closely reflect the human situation; target engagement, in particular of the potentially noxious oligomeric species within neurons, was not demonstrated; it may be difficult to observe motor benefits with neuroprotective strategies even in early disease, as neurodegeneration is already quite advanced, while the progression of the disease is generally quite slow, possibly necessitating studies of longer duration. With these notions in mind, and taking into account the only very recent partial success of similar β -amyloid-targeting strategies in AD, after decades of failures, there is reason to believe that the conduct of further clinical trials targeting AS, possibly in earlier disease stages, such as prodromal or even presymptomatic, as in asymptomatic genetic synucleinopathies, will ultimately demonstrate efficacy (Jensen et al. 2023). In fact, a preordained analysis of a subset of PD patients with characteristics suggesting more rapid progression appears to show a very significant benefit with treatment in one of the two clinical trials mentioned above (Pagano et al. 2024), further reinforcing the notion that anti-AS therapies may prove to be successful in PD and related synucleinopathies.

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Queries

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