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Alpha-Synuclein Function and Dysfunction on Cellular Membranes

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Alpha-synuclein is a small neuronal protein that is closely associated with the etiology of Parkinson's disease. Mutations in and alterations in expression levels of alpha-synuclein cause autosomal dominant early onset heredity forms of Parkinson's disease, and sporadic Parkinson's disease is defined in part by the presence of Lewy bodies and Lewy neurites that are composed primarily of alpha-synuclein deposited in an aggregated amyloid fibril state. The normal function of alpha-synuclein is poorly understood, and the precise mechanisms by which it leads to toxicity and cell death are also unclear. Although alpha-synuclein is a highly soluble, cytoplasmic protein, it binds to a variety of cellular membranes of different properties and compositions. These interactions are considered critical for at least some normal functions of alpha-synuclein, and may well play critical roles in both the aggregation of the protein and its mechanisms of toxicity. Here we review the known features of alpha-synuclein membrane interactions in the context of both the putative functions of the protein and of its pathological roles in disease.

Key words: alpha-synuclein, Parkinson, amyloid, aggregation, neurotransmission, synucleinopathy

INTRODUCTION

Alpha-synuclein is a soluble, 140-residue, predominantly presynaptic protein that is highly conserved in vertebrates and has been implicated in Parkinson's Disease as well as other eponymously named "synucleinopathies" such as dementia with Lewy bodies and multiple system atrophy [1-7]. Several rare mutations in the alpha-synuclein gene (*SNCA*), have been identified in cases of familial Parkinson's Disease (A53T, A30P, E46K, and most recently H50Q and G51D), and *SNCA* gene duplications and triplications similarly lead to familial PD [8-15]. Lewy Bodies and Lewy Neurites, pathological hallmarks of the synucleinopathies, are composed largely of beta-sheet rich alpha-synuclein amyloid fibrils [3]. Alpha synuclein's contribution to such disorders could in principle result either from a toxic gain of function resulting from synuclein oligomerization and/or aggregation, or from a loss or perturbation of normal synuclein function (or possibly from a combination of the two). Unfortunately, the normal functions of alpha-synuclein remain elusive, though in general it has been implicated in synaptic plasticity [16] and learning [17], neurotransmitter release [18,19], and synaptic vesicle pool maintenance [2,20,21].

Alpha synuclein is intrinsically disordered when free in solution [22-24]. The N-terminal ~100 residues of the protein constitute a lipid-binding domain that contains 7 imperfect 11-residue repeats, each centered on a variation of a KTKEGV core consensus sequence. Similar repeat sequences are found in the exchangeable apolipoproteins, and as for many apolipoproteins, the N-terminal lipid-binding domain of alpha synuclein adopts an amphipathic helical structure upon binding to detergent micelles

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or phospholipid vesicles. Residues 61-95 of the N-terminal domain constitute a hydrophobic region referred to as the NAC domain (for non-Aβ component of senile plaques) that may contribute critically to synuclein oligomerization and aggregation [1]. The acidic C-terminal ~40 residues of the protein, often referred to as the C-terminal domain or tail, remain disordered even in the presence of membranes, although evidence exists for limited interactions of this region with membranes [1,2,25-29]. The membrane-induced disorder-to-order transition of the N-terminal lipid-binding domain is considered functionally important and has been characterized in a wide variety of contexts. Several helical membrane-bound conformations have been observed, featuring amphipathic helices that lie along the surface of the membrane with their apolar face embedded as deep as the C3 or C4 acyl chain carbons [30-32], and interfacial lysine residues may "snorkel" from the membrane interior to interact with negatively charged lipid headgroups [33,34]. An extended-helix conformation binds to the membrane surface via an ~100 residue long amphipathic alpha helix [32,35-39] with an unusual 11/3 periodicity [25,30-32]. A broken-helix conformation has also been observed in which the extended-helix is broken into two distinct helices separated by a non-helical linker region spanning residues 39-45 [25,40,41]. Both conformations have been observed in the context of both detergent micelles and lipid vesicles [25,30-32,35,37,41-46]. Additional binding modes observed on phospholipid vesicles involve a shorter helix at the N-terminus of the lipid-binding domain with the remainder of the domain remaining unbound. These include an SL1 binding mode involving the 25 N-terminal residues [28,47] and a binding mode where residues up to 19 are bound, but residues beyond 69 are not [48]. Structures comparable to these partly helical binding modes have also been observed in mixtures of organic and aqueous solvents [49] and on *n*-octyl-β-glucopyranoside (BOG) detergent micelles [50], and one of the most recently reported PD-associated mutations also seems to favor such binding modes [51].Though such conformational states have been posited to contribute to synuclein's putative functions, the detailed relationship between synuclein membrane binding, structure, and function remains an important open avenue of investigation [2]. Likewise, membrane interactions may mediate pathological roles of alpha synuclein, either through membrane effects on synuclein aggregation or through synuclein's effects on membrane structure and integrity [1,2,52]. Below we delineate possible normal functions of synuclein at the membrane and discuss a number of possible membrane-associated targets; we then consider the role that membranes might play in synuclein dysfunction and how this ultimately contributes to disease.

RECENT DEVELOPMENTS IN SYNUCLEIN/MEMBRANE BIOPHYSICS AT THE SYNAPSE

A detailed mechanistic understanding of synuclein function will ultimately require additional characterization of its structure and dynamics at the membrane surface as well as of the membrane determinants of synuclein binding. These will be critical for generating and verifying hypotheses regarding mechanisms of action and relevant *in vivo* binding target(s) – ie. specific cellular membrane(s) at which synuclein exerts its functions. Synaptic vesicles are considered the "classic" cellular membrane binding target for alpha synuclein. Synuclein localizes to the presynaptic terminal and specifically to synaptic vesicles, to which it can directly bind [17,53-58]. It has become clear, however, that alpha-synuclein may in fact interact with a wider variety of cellular membranes than previously expected and that these interactions may contribute to alpha synuclein function, pathology, or both. Efforts to characterize the membrane properties that favor synuclein binding indicate that both electrostatic interactions and hydrophobic interactions contribute to binding [1,59]. Membrane curvature also plays a key role, with enhanced binding to membranes of increased curvature [60-63]. This likely results from an increased size and number of so-called packing defects in more highly curved membranes [64,65]. Packing defects are regions where the hydrophobic acyl chain interior of the membrane is transiently exposed, and they likely act as effective protein binding sites [60-62,64,66-68]. Lipid headgroup composition, which can influence both charge and curvature, also modulates synuclein binding. An increased percentage of conical lipids such as phosphatidylethanolamine (PE) increases binding, perhaps through enhanced formation of packing defects [66]. Finally, electrostatic interactions with positively charged synuclein residues (in particular the many lysines) are enhanced by increasing the membrane negative charge density [27,60,61,66,67,69-71]. Notably, synaptic vesicles present a highly curved, negatively charged membrane surface [72], making them an optimal target for synuclein binding.

Synuclein's preference for more highly curved membranes has led to its classification as a "curvature sensing" protein [34,62,64]. In addition to sensing membrane curvature, alpha-synuclein is also able to actively alter membrane shape/curvature [28,36,73,74]. Such direct manipulation of the membrane could play a role in synaptic vesicle homeostasis and/or exocytosis, though whether, when, and how synuclein might actively model membranes *in vivo* remains unclear.

Multiple post-translational modifications of alpha-synuclein have been reported, including N-terminal acetylation [75],

serine/threonine and tyrosine phosphorylation [75-79], tyrosine nitration [80], ubiquitination [75,81,82], sumoylation [83], transglutamination [84-86], and methionine oxidation [87]. Many of these impact synuclein's interaction with membranes, indicating that such modifications will influence synuclein behavior in ways that must be characterized. As an example, it was recently reported that alpha-synuclein is N-terminally acetylated [75], probably by the acetyltranferase NatB [88,89], and that this modification increased the transient helical propensity of the N-terminal ~10 residues in the free state [50,89-91]. Conflicting results were initially reported regarding the effects of N-terminal acetylation on membrane binding, with enhanced binding observed in some cases and a negligible impact in others [89,90]. These differences were likely due to the differing liposome compositions and sizes used by the different groups, and a more recent study examining a larger set of liposome sizes and compositions showed that N-terminal acetylation has a pronounced effect on binding to highly curved membranes of moderate charge, but less of an effect on more highly charged membranes [50]. Importantly, such highly curved vesicles of moderate negative charge approximate the properties of synaptic vesicles; thus, the impact of N-terminal acetylation appears greatest for liposomes most closely comparable to a known synuclein binding target *in vivo* [50].

Alpha-synuclein can be phosphorylated at multiple serine, threonine and tyrosine residues in vivo (including Y39, S87, Y125, and S129) [75-79,92]. The structural and functional consequences of such modifications have begun to be addressed but remain incompletely understood, in part because generating phosphorylated proteins for *in vitro* studies remains a challenging task [93-95]. Several recent studies have examined the impact of phosphorylation on synuclein membrane binding. Phosphorylation of S87 was found to reduce binding to membranes and alter the detergent micelle bound conformation, as well as expand the free state of the protein and increase its conformational flexibility [94]. Phosphorylation of S129 in contrast has little effect on membrane association by wild type alpha-synuclein [95], although some effect on the membrane-binding of PD-linked synuclein mutants was reported [96]. This modification was also shown to have little impact on the SDS-micelle bound conformation [95]. Phosphorylation of other residues is only just beginning to be characterized and further work is clearly needed to more completely elucidate how phosphorylation impacts membrane binding, and how its perturbation of synuclein structure, dynamics, and membrane binding might contribute to both function and pathology.

Other post-translational modifications that have been shown to influence membrane binding of alpha-synuclein include methionine oxidation [87,97] and tyrosine nitrosylation [29], but as with phosphorylation, the effects of these and other modifications remain to be more fully characterized. Interestingly, lysine acetylation was recently shown to occur in the Alzheimer's protein tau and to be associated with tau pathology [98-100], and it may be interesting to see whether this or other less commonly reported types of modification may be discovered to occur on synuclein as well.

FUNCTIONAL IMPLICATIONS OF SYNUCLEIN INTERACTIONS WITH OTHER PROTEINS AT PRESYNAPTIC MEMBRANES

Alpha-synuclein structure/function relationships remain enigmatic but are perhaps best characterized in the context of presynaptic function and, more specifically, synaptic vesicle homeostasis. An area of focus is the role that synuclein/membrane and synuclein/protein interactions play in synuclein's contribution to synaptic vesicle docking, priming, clustering, fusion, and/or recycling. Importantly, it is likely that synuclein/membrane interactions at the synapse are modulated by additional proteins. Indeed, it has been shown that dissociation of membrane-bound synuclein depends on brain-specific cytosolic proteins [101], though none were specifically identified in this particular study. Synuclein also binds to calmodulin [102-104], a protein that is thought to regulate secretory processes at the synapse in a variety of ways, including by interacting with protein targets such as calcium-CaM-dependent kinase II and by inhibiting SNARE-mediated membrane fusion. Binding to calmodulin is mediated by the N-terminal amphipathic helical region of alpha synuclein (ie. the membrane binding domain), and an NMR structure of N-terminally acetylated synuclein bound to Ca-bound calmodulin has been recently described [104]. Membrane-bound synuclein is released upon interacting with calmodulin, suggesting competition for synuclein between membranes and calmodulin [105]. This leads to a model in which calcium bound calmodulin mediates presynaptic depolarization-dependent dissociation of alpha-synuclein from the membrane surface. Conversely, GTP-Rab3a may stabilize synuclein on synaptic vesicles, as antibodies to Rab3a and RabGDI abrogated synuclein membrane binding, while inhibition of Rab3a recycling increased synuclein sequestration on intracellular membranes. Rab3a is a presynaptic Rab that interacts with the synapse-organizing complex of RIMalpha/Munc13/α-liprin, and so its contribution to synuclein membrane interactions is intriguing, but the functional consequences of such interactions are as of yet unknown [106]. Rab8a, a Rab GTPase that modulates post-Golgi vesicle trafficking, also interacts with synuclein in a Ser129-phosphorylation dependent manner

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[107]. As discussed below, Rab-mediated cellular trafficking is often perturbed by synuclein; thus, aberrant synuclein/Rab interactions could in principle contribute to neurodegeneration in synucleinopathies.

Synaptic vesicle fusion is mediated by three SNARE proteins – syntaxin-1, SNAP-25, and synaptobrevin-2 – whose SNARE motifs zipper into a four-helix bundle. Recent evidence suggests a contribution of alpha-synuclein to SNARE complex assembly through a direct interaction between alpha-synuclein and synaptobrevin-2 [18,19]. A potential role for synuclein in SNARE assembly first came from the observation that expression of synuclein rescues CSPalpha deficient mice in a phospholipid-binding dependent fashion [18]. CSPalpha is an abundant presynaptic chaperone, and deletion of CSPalpha inhibits SNARE complex assembly. Synuclein was subsequently reported to directly bind to synaptobrevin-2 and was proposed as a potential nonclassical chaperone facilitating SNARE complex assembly [19]. This raised the question of how synuclein might affect SNARE-mediated synaptic vesicle docking, priming, and/or fusion, and whether membrane binding could play a role in this function. Indeed, it has been proposed that the broken helical conformation of synuclein could span both the synaptic vesicle and plasma membranes and so help stabilize docked vesicles at the presynaptic membrane [2,108], and it was recently shown that synaptosomal preparations of plasma-membrane associated docked synaptic vesicles are enriched for synuclein when compared with preprations of unattached undocked vesicles [109]. Synuclein overexpression in cultured chromaffin cells inhibits catecholamine release by blocking a late step in the exocytosis process and, specifically, by inhibiting the fusion of docked vesicles [110]. In addition, synuclein has been implicated to function in maintenance of SV pool size [111], and it may enhance vesicle clustering, again perhaps through a membrane-bridging mode. This hypothesis is suggested by initial observations in yeast [112-115], and by more recent work showing that synuclein induces clustering of synaptic vesicle mimics [116]. In the latter study, clustering depended on synuclein interactions with both synaptobrevin-2 and with anionic lipids in phospholipid bilayers. Synuclein also inhibits SNARE-mediated vesicle fusion in *in vitro* lipid mixing assays, likely through inhibition of docking rather than of the fusion step itself [117,118]. Inhibition requires membrane binding by synuclein and may or may not require SNARE binding; it is possible that the requirement for SNARE-binding may in fact depend on synuclein's oligomeric state [109,118,119]. Indeed, in some contexts, synuclein fails to interact with SNARE proteins and instead might modulate SNARE-mediated exocytosis through the more indirect sequestration of arachinoic acid, which can itself stimulate SNARE complex formation and exocytosis [120]. Synuclein additionally appears to promote clathrin-mediated endocytosis [121], and it is required for the fast kinetics of SV endocytosis through some impact on the early steps of SV endocytosis [122]. Finally, synuclein reportedly contributes to SV mobilization through its inhibition of SV reclustering after endocytosis [123]. All together, these observations point to some direct role for synuclein/membrane and synuclein/protein interactions in multiple steps of the synaptic vesicle cycle; however, open questions remain, particularly given the multitude of steps and apparent discrepancies observed across multiple studies.

As mentioned above, synuclein may directly generate membrane curvature and so remodel membranes, an activity that could also have a impact on synaptic vesicle fusion with the plasma membrane. Additional evidence for some role for synuclein in membrane remodeling processes comes from the early observation that synuclein may interact with and inhibit phospholipase D2, which catalyzes the hydrolysis of phophatidylcholine to phosphatidic acid [112,124,125]. The synuclein/PLD2 interaction has been contested as well, however [126]. In vitro, PLD binding requires both the helical membrane binding N-terminal domain and the disordered C-terminal tail and can be modulated by synuclein phosphorylation [125,127]. PLD2 likely acts on the plasma membrane and is implicated in the regulation of secretory vesicle budding and/or fusion: phosphatidic acid may mediate processes involved in vesicular transport and changes in cell morphology by modulating membrane curvature and by regulating phosphatidylinositol-4-phosphate 5 kinase activity [128]. Interestingly, synuclein also displays higher affinity for membranes rich in PA [59,60,129], and so a feedback mechanism could be envisioned in which high levels of PA recruit synuclein, which then inhibits PLD2 and so reduces PA levels. The enzyme CTP:phosphocholine cytidyltransferase (CCT) seems to represent an interesting parallel to synuclein in this regard. Similarly to alpha-synuclein, CCT also contains 11-residue repeats that are capable of binding to membranes in a helical conformation. CCT catalyzes the rate-limiting step in phosphatidylcholine synthesis. Further, it may preferentially bind membranes deficient in PC lipids, insofar as PC deficiency increases the relative proportion of negatively charged (PS) headgroups and conical (PE) headgroups, both of which could enhance binding. Though the CCT amphipathic helix is covalently attached to its relevant enzymatic domain, a similar feedback cycle emerges wherein PC deficiency enhances CCT binding, which catalyzes PC synthesis [130-139].

Finally, beyond potential contributions to the synaptic vesicle cycle, alpha-synuclein may also play a role in dopamine synthesis.

First, synuclein reportedly binds to and inhibits tyrosine hydroxylase, the rate-limiting enzyme in dopamine biosynthesis [140]. In addition, synuclein binds to a 14-3-3 protein that binds to and activates tyrosine hydroxylase [141]; homology between synuclein and 14-3-3 proteins has also been noted [140,141]. Importantly, synuclein is also reported to bind directly to the human dopamine transporter hDAT [142] and to inhibit its reuptake of dopamine from the synapse [142]. Decreased uptake required neither the N-terminal half of the membrane binding domain nor the C-terminal region, but was absolutely dependent on the NAC region of synuclein [143,144]. hDAT represents another potential functional target for synuclein at the membrane, though how membrane binding per se contributes to hDAT regulation remains unclear. More generally, synuclein is also reported to regulate other monoamine transporters, including those for serotonin and norepinephrine [143,145,146]. Finally, synuclein has been reported to interact with the signaling proteins ARPP16/19 in a membrane dependent fashion [147]. ARPP16/19 belong to the same family as DARPP32, a phosphoprotein involved in signaling networks that mediate responses to the binding of dopamine (and other neurotransmitters) to the post-synaptic receptors. Thus, synuclein-ARPP16/19 interactions may be involved in regulation of dopamine signaling pathways.

SYNUCLEIN MAY BIND TO AND FUNCTION AT OTHER CELLULAR MEMBRANES

Alpha-synuclein clearly localizes to synaptic terminals, contributes to synaptic vesicle homeostasis and synaptic plasticity, and binds to synaptic vesicles. However, a number of other potential cellular membrane targets have been proposed and studied, including the plasma membrane, lipid rafts, the inner nuclear membrane, and mitochondrial membranes (Fig. 1); further, synuclein binds fatty acids and may contribute to fatty acid metabolism. These interactions, which may be functional, pathological, or both, are included here because until greater certainty is achieved regarding synuclein's precise normal functions, all such observations should be considered as potentially relevant.

The plasma membrane represents a target that could cooperate with synaptic vesicle binding in facilitating synuclein's function at the presynapse. As discussed above, it has been proposed that synuclein may span the synaptic vesicle and plasma membranes through its broken helical conformation [2,108] and recent data supports this possibility [109]. Interestingly, synuclein has been reported to associate specifically with lipid rafts and caveolae [148,149], and membrane association is specifically enhanced by gangliosides including, among others, GM1 and GM3 [149-153]. GM1 also seems to specifically enhance the binding and helical folding of N-terminally acetylated relative to non-acetylated synuclein [154]. The synuclein/GM1 association is likely due to a specific interaction between helical alpha synuclein and both the sialic acid and carbohydrate moieties of GM1, and it may contribute to synuclein's presynaptic localization [149,150]. Notably, gangliosides are considered to reside on the outer leaflet of the plasma membrane, making it unclear how a cytosolic protein like synuclein could interact with them. However, recent interest in cell-to-cell transmission of synuclein pathology has suggested a role for extracellular synuclein in disease (see below), and this context may provide an opportunity for synuclein to interact with outer leaflet lipids and proteins.

Synuclein was initially discovered as a protein localized to both the presynaptic nerve terminal and to the nucleus, and indeed its name is derived from this observation [53]. Its nuclear localization has remained contentious, however, and it remains unclear whether synuclein really is enriched in the nucleus, whether it might function there, and whether aberrant nuclear localization might contribute to pathology. Later studies have also observed nuclear localization, though this appears to be antibody-dependent and could in part result from non-specific binding by certain antibodies [53,155-163]. Synuclein has, however, additionally been noted to interact with histones and with DNA, though these interactions may be pathologic rather than functional [155,164]. Thus, the nuclear membrane could represent an additional membranous target for alpha-synuclein, though synuclein-nuclear membrane interactions have not, to our knowledge, been clearly established in any study to date.

Synuclein has also been reported to interact with mitochondria, which, combined with strong evidence that oxidative damage contributes to Parkinson's disease, has spurred interest in the interplay between alpha-synuclein and lipid oxidation. Parkinson's Disease is characterized by selective degeneration of dopaminergic neurons of the substantia nigra. These neurons may be particularly sensitive to oxidative stress, as dopamine metabolism generates a number of toxic species; eventually these cells could lose the capacity to handle continuously generated reactive oxygen species [165]. Interestingly, red blood cells contain large amounts of alpha-synuclein and have a high oxygen/oxidative load [166]. One emerging theory suggests that alpha synuclein functions as an antioxidant that prevents oxidation of unsaturated membrane lipids. Monomeric synuclein has in fact been shown to prevent lipid oxidation, and membrane binding is required for this function [97,167]. Further, increased alpha-synuclein content is associated with neuroprotection from oxidative stress [168]. The N-terminal methionine residues of synuclein (particularly M1

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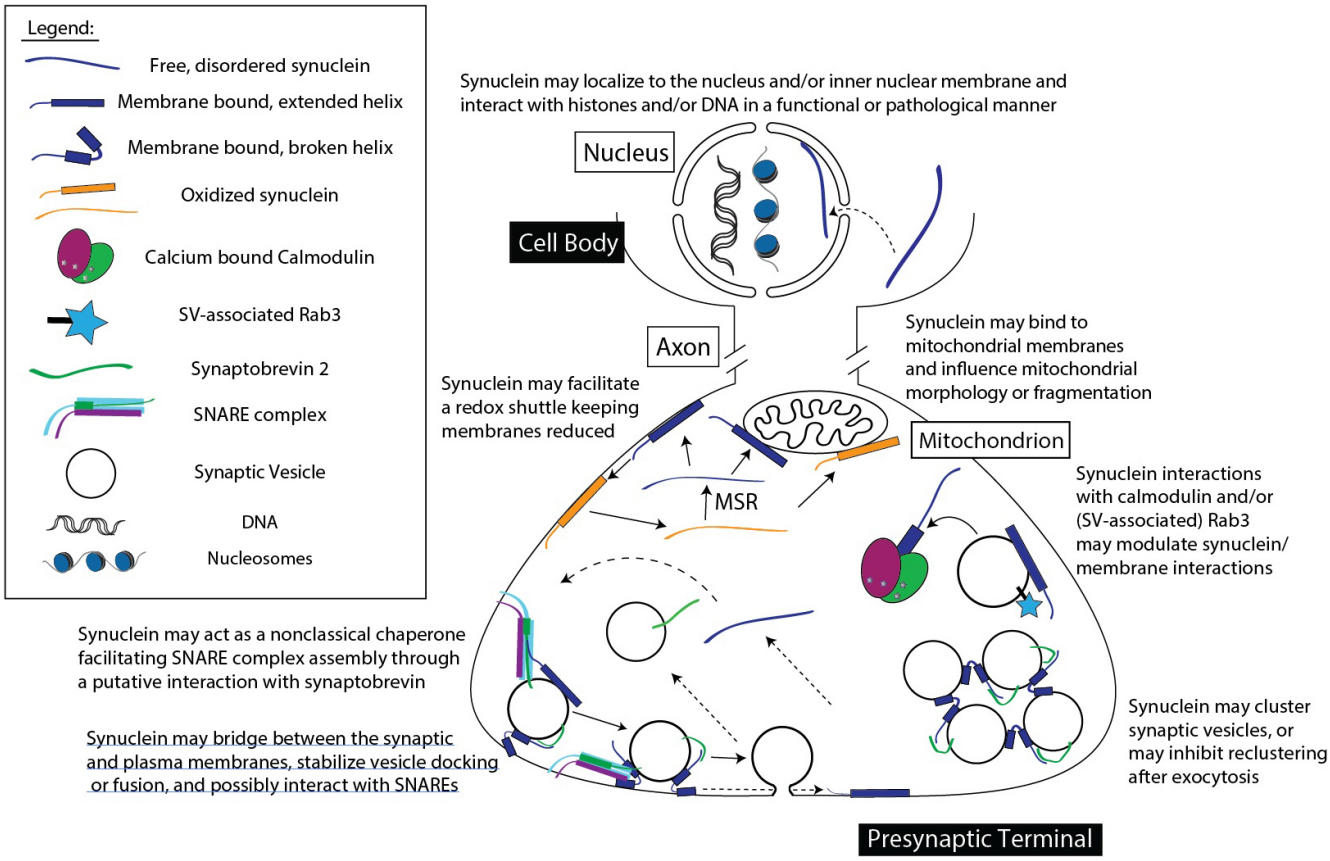

Fig. 1. Cellular membranes, targets and pathways potentially involved in the normal, physiological functions of alpha-synuclein.

and M5) become oxidized upon binding to vesicles containing peroxidized lipids, and these methionine residues have been established as substrates for methionine sulfoxide reductase when synuclein is in its free, soluble form. Interestingly, methionine sulfoxide reductase A (MsrA) protects dopaminergic cells from toxic, disease-related insults, including expression of mutant alpha-synuclein, by repairing methionine-oxidized proteins [169]. MsrA's participation in these cycles of methionine oxidation and reduction serves to ultimately consume reactive oxygen species [169]. From these observations, a cycle of synuclein membrane binding, methionine oxidation, release from membrane, and methionine reduction by Msr has been proposed [97,167]. It is not currently clear on which cellular membrane(s) such a cycle might optimally occur, or even whether this might be a general or membrane specific function, and the effects of methionine oxidation on synuclein binding to membranes of differing biophysical properties remain incompletely characterized.

Mitochondria represent a particularly intriguing potential binding partner for synuclein, given the established roles of mitochondrial dysfunction and oxidative stress in the pathogenesis of Parkinson's Disease. Oxidative stress associated with high levels of dopamine and/or mitochondrial dysfunction leads to elevated levels of lipid peroxides in the neuronal tissue of PD patients [97,170]. Synuclein has also been associated with mitochondrial function [171], and is reported to localize and bind to mitochondria [172-178] as well as to vesicles that mimic mitochondrial membranes [179]. Notably, cardiolipin – an inner mitochondrial membrane lipid - appears able to enhance synuclein membrane binding and to alter its behavior on and at the membrane [178,179].

There are thus clear links between alpha-synuclein and lipid oxidation, and between oxidized lipids, oxidative damage and neurodegenerative diseases. Polyunsaturated fatty acids (PUFAs) normally serve as both an energy reservoir and as intra-and extracellular second messengers that contribute to signaling pathways. Their unsaturated acyl chain bonds, however, represent a target for lipid oxidation in disease states [180]. The effects of PUFAs on alpha-synuclein properties and the effect of synuclein on PUFA metabolism have thus received considerable attention. Alpha-synuclein was noted to have homology to fatty acid binding proteins, and it reportedly interacts with free fatty acids (although it does not bind them like a classical fatty acid binding protein)

as well as phospholipid bilayers [180,181]. Binding to free PUFAs such as arachidonic acid or docosahexaeonic acid (but not to saturated fatty acids) is mediated by the N-terminal lipid-binding domain, which adopts a helical conformation upon such binding; binding to PUFAs reportedly prevented their micellar formation [182,183]. It was initially suggested that synuclein may transport fatty acids between cytosolic and membrane bound cellular compartments [180-184]. It was later proposed that synuclein and PUFAs may act together to enhance clathrin-mediated endocytosis and thus play a role in SV recycling after neuronal stimulation [121]. Indeed, synucleins were very recently shown to be required for the fast kinetics of SV endocytosis [122].

Alpha-synuclein also appears to contribute to fatty acid uptake and metabolism. Alpha-synuclein deficiency leads to: (1) disrupted astrocyte fatty acid uptake and trafficking, with increased trafficking to cholesteryl esters and triacylglycerols (ie. neutral lipid pools) and decreased trafficking to phospholipids [185]; (2) increased (whole brain) neutral lipid mass [186]; (3) decreased (whole brain) incorporation rate and fractional turnover of 16:0 acyl chains in a number of phospholipid classes albeit without direct binding to 16:0 (but note that synuclein deficiency led to increased incorporation rate and fractional turnover of 16:0 acyl chains in choline glycerophospholipids [187]); (4) reduced (brain) arachidonate (20:4n-6) turnover through modulation of endoplasmic reticulum-localized acyl-CoA synthetase, possibly because synuclein may play a role in substrate presentation to acetyl coa synthetase (and not substrate removal) [188]; and (5) a likely compensatory increase in 22:6n-3 incorporation and turnover, with a low level of synuclein/22:6n-3 binding. Such compensation makes sense insofar as 20:4n-6 and 22:6n-3 are two major PUFAs in the brain [189].

SYNUCLEIN/MEMBRANE INTERACTIONS IN THE MULTIFACETED PATHOLOGY OF PD

Alpha-synuclein represents the primary component of Lewy Bodies and Lewy Neurites, which are pathological hallmarks of Parkinson's Disease and, more generally, the synucleinopathies [3,6,7]. Synuclein is genetically linked to Parkinson's Disease as well: the synuclein point mutants A53T, A30P, E46K, H50Q and G51D have been linked to rare familial cases of Parkinson's Disease, as have *SNCA* gene duplications and triplications [8-15]. Intense effort has focused on the aggregation propensity and properties of synuclein because of the clear and extensive accumulation of mature beta-sheet rich amyloid fibrils in the brains of synucleinopathy patients. In theory, alpha-synuclein's contribution to the neuronal degeneration observed in the synucleinopathies could arise from a toxic gain of function, or from a loss of synuclein's normal function – the latter occurring either as a consequence of synuclein modification through e.g. familial mutations or post-translational modifications, or from sequestration into non-functional oligomeric or fibrillar aggregated forms. The alpha-synuclein knockout phenotype in mice is mild with only moderate electrophysiological anomalies, perhaps due to compensation by beta- and gamma-synucleins [21]. While alpha/beta synuclein double knockout mouse phenotypes are similarly moderate [190], a synuclein triple knockout mouse does displays age-dependent neurological impairments, including decreased SNARE-complex assembly and decreased life span [19]. It may be worth nothing that because gamma synuclein expression is largely orthogonal to that of alpha- and beta-synuclein, closer reexamination of the alpha/beta knockout mouse may be warranted.

Systematic examination of the role of distinct regions of the synuclein primary sequence on its physiological vs. pathological activities suggested that the normal and neuropathogenic effects of synuclein may be molecularly distinct and separable. The N-terminal and C-terminal sequences were required for synuclein function as a SNARE complex chaperone but were dispensable for its toxic function; conversely, the central NAC region was more essential for synuclein neurotoxicity but played a negligible role in SNARE-complex assembly [191]. These data favor a model in which some toxic gain of function primarily contributes to neurodegeneration in the synucleinopathies, particularly given the potential toxicity of synuclein oligomeric aggregates (see below). However, some caution is warranted as the model for toxicity used in these studies relied on overexpression and such models have not succeeded in completely capturing the features of human disease.

If a toxic gain of function causes neurodegeneration, this raises the critical question: what are the toxic species? Intense study has focused on defining the specific mechanisms and pathways of synuclein aggregation and on defining the toxic contributions of any and all species along such a pathway, from monomeric synuclein, to intermediate oligomeric or prefibrillar aggregates, and ultimately to mature amyloid fibrils. Oligomeric synuclein intermediates are increasingly favored as the key contributor to cellular dysfunction and death. Oligomeric or protofibrillar synuclein species have been reported to permeabilize membranes by acting as a pore or channel [192,193]. One or more molecules of monomeric synuclein, too, may at times form membrane permeabilizing channels [194]. Such pore formation could clearly alter membrane potential and ion distribution across the membrane and thus contribute to cellular toxicity [194]. Interestingly, membrane binding modulates synuclein aggregation,

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though results are conflicting as to whether membrane-bound synuclein is more or less prone to aggregation; membrane composition and relative concentration of protein vs. lipid are likely key factors determining the contribution of the membrane to synuclein aggregation [195-199]. Indeed, depending on protein:lipid (P:L) ratios, membrane binding could either protect against aggregation by isolating monomeric synuclein (at lower P:L ratios), or favor self-association and aggregation by raising the effective local concentration of synuclein on a reduced dimensionality (2D) surface [70,108,200,201].

The behavior and properties of the PD-linked synuclein mutants have also been intensely studied in the hopes that recurring themes might emerge that would shed light on PD pathogenesis. Notably, there is considerable variety in the effects of the mutants on aggregation. A53T, E46K and H50Q [202-204] enhance mature fibril formation [202-206], A30P enhances oligomer formation but retards mature fibril formation [205-207], while G51D retards aggregation [51,204], suggesting different mechanisms of toxicity. Different mutants also have differing effects on membrane binding affinity: Of the original three PD mutations, A30P clearly perturbs binding [47,55,59,66,69], A53T has no effect [47,59,69,129], and E46K shows enhanced binding [2,47,61,108,208]. Despite differing effects on overall membrane affinity, the membrane binding behavior of these mutants was unified by the observation that they all increase the population of the aggregation prone partly helical SL1 binding mode in which the N-terminal 25 residues are bound, while the remainder of the protein remains free. Enhanced concentration, due to the reduced dimensionality of the membrane surface, of an exposed hydrophobic NAC region in the SL1 binding mode could help nucleate synuclein aggregation [47]. A conformation similar to the SL1 binding mode has also been observed on BOG micelles [50]. The H50Q and G51D mutations have emerged much more recently and so have not yet been as extensively studied. G51D appears to decrease membrane binding affinity, but promotes the formation of partly helical states [51], while H50Q did not alter binding affinity nor obviously alter the bound-state structure [202].

Cellular studies of synuclein overexpression, oligomerization, and aggregation have also proven informative. Studies in yeast suggested that synuclein overexpression could contribute to trafficking defects [113,114]. In light of synuclein's potential interactions at the membrane with Rab and SNARE proteins during synaptic vesicle cycling and homeostasis, it is plausible that these interactions may go awry in disease states. Upon overexpression, synuclein accumulates in yeast cells, leads to ER stress and cytotoxicity, and blocks ER to golgi trafficking. Synuclein toxicity can be rescued by the Rab GTPase Ypt1p, which also functions at this trafficking step. Rab1, the mammalian YPT1 homolog, protected against synuclein-induced dopaminergic cell loss [113]. The effects of synuclein on trafficking result from a direct effect on the transport machinery, as vesicles bud efficiently from the ER but fail to dock and/or fuse to the Golgi membrane [113,114]. Cytoplasmic synuclein accumulations are associated with clusters of vesicles originating form the ER-Golgi transport step of the secretory pathway, further implying that synuclein expression impairs vesicular transport [115]. Rab3a and Rab8a – localized to presynaptic termini and post-Golgi vesicles, respectively – also suppress toxicity in synuclein-based neuronal models of PD, implying that synuclein overexpression can affect multiple membrane trafficking steps [114]. Overexpression of synuclein in mammalian kidney and neuroendocrine cells similarly delayed ER to Golgi transport, and this was rescued by expression of SNARE proteins, implying that the overexpressed synuclein antagonized SNARE function [209]. Purified A53T synuclein inhibited COPII vesicle docking and fusion at a pre-Golgi step, and soluble A53T bound ER/Golgi SNAREs to inhibit SNARE complex assembly [209]. This particular observation represents a possible toxic perturbation of synuclein's normal function as a putative SNARE complex chaperone contributing to SV exocytosis [19].

Aggregation of synuclein in yeast has also been associated with defects in endosomal trafficking and phospholipid biosynthesis. Synuclein aggregation was enhanced in the presence of higher levels of acidic phospholipids, colocalized with yeast membranes enriched for phosphatidic acid, and induced the aggregation of many yeast Rab GTPase proteins [210]. Synuclein expression further induced sensitivity to perturbations in retrograde endosome-Golgi transport [210]. Finally, direct synuclein/Rab interactions have been observed in disease or disease-related situations. Abnormal rab3a/synuclein interactions have been observed in brains of patients with multiple system atrophy, Parkinson's Disease [211], and Lewy Body Disease [212]. Rab8a interacts with synuclein in a Ser129 phosphorylation dependent manner [107] and Rab8a enhanced synuclein aggregation and reduced synuclein induced cellular toxicity [107]. Rab3a, Rab5, and Rab8 are associated with synuclein aggregates in transgenic mice overexpressing wild type or A30P synuclein [213]. These results suggest that synuclein aggregates may sequester a subset of Rab proteins, and that synuclein overexpression could perturb neuronal vesicular trafficking (particularly at docking and fusion steps) and so contribute to cellular toxicity.

Mitochondrial dysfunction has been extensively linked to PD pathogenesis. First, mitochondrial toxins such as MPTP, 6-OHDA, and others have been used to mimic PD symptoms in the absence

of Lewy Body formation. MPTP can arise as an accidental impurity in heroin manufacture and produces PD-like symptoms in people exposed to the toxin. MPP+, the active metabolite of MPTP, can enter neurons via the dopamine transporter and inhibit mitochondrial complex I. Inherited mitochondrial DNA mutations can cause familial Parkinson's disease, and a number of proteins with roles in mitochondrial function have been linked to PD, including Parkin (a ubiquitin E3 ligase that functions in mitophogy-related pathways), and the mitochondrial kinase PINK1 (which functions together with Parkin). The PD-linked kinase LRRK2, and the oxidative stress response protein DJ-1 have also been linked to mitochondria. Mitochondrial dysfunction could enhance cellular reactive oxygen species (ROS), which can exert toxicity in many ways. An interesting example is that ROS may alter membrane fusion and transmitter exocytosis by impacting the SNARE proteins [180]. Thus, synuclein/mitochondria interactions are of particular interest in the context of PD pathophysiology. Synuclein can directly interact with mitochondria [177] and overexpression of synuclein increases cellular susceptibility to mitochondrial toxins and inhibits mitochondrial complex I activity [214]. Synuclein can increase intra-mitochondrial ROS, nitric oxide, and Ca^{2+} levels and thereby lead to cytochrome c release and apoptosis [174,180,215,216]. Synuclein has been shown to inhibit mitochondrial fusion and drive mitochondrial fission in a cardiolipin-dependent manner [178,217]. Synuclein thus has a direct effect on mitochondrial morphology, and synuclein-associated mitochondrial fragmentation is followed by a decline in cellular respiration and ultimately neuronal death. These mitochondrial effects depend on the direct interaction between synuclein and mitochondrial membranes and so establish a role for this interaction in PD-associated mitochondrial toxicity [178]. It should be noted, however, that synuclein has also at times been shown to have an anti-apoptotic, protective role against mitochondrial-mediated cell death [180]. Interestingly, the G51D mutant was found to enhance mitochondrial fragmentation [51].

Increased oxidative stress can reportedly lead to the translocation of alpha-synuclein from the cytoplasm into the nucleus and perhaps generate a devastating positive feedback cycle: oxidative stress could disrupt the nuclear membrane, lead to synuclein translocation into the disrupted nucleus (specifically of a C-terminal fragment of synuclein), and then enhance cellular susceptibility to further oxidative stress (although the specific mechanisms for this last step are not presently clear) [218,219]. Iron has also been shown to alter mitochondrial morphology, disrupt the nuclear membrane, and cause the translocation of synuclein from the perinuclear region into the disrupted nucleus [220]. Synuclein may somehow mediate neurotoxicity in the nucleus, perhaps through some effect on histones, or on DNA itself [155,164]; synuclein can directly bind both DNA and histones, and was reported to reduce the level of acetylated histone H3 while also inhibiting histone acetylation [155,164,221]. It has also been suggested that nuclear synuclein accumulation is mediated by importin alpha, and that this promotes neurotoxicity through cell cycle acceleration [222]. Finally, the PD-linked G51D mutant was found to be enriched in the nuclear compartment [51].

As discussed above, synuclein may interact with a wide variety of cellular membranes, and so a number of membrane properties and membrane lipid components may contribute to synuclein/membrane binding and to synuclein dysfunction on the membrane surface (Fig. 2). First, synuclein may bind to lipid rafts, and this interaction may localize synuclein to synapses, which contain cholesterol rich lipid microdomains [148]. Synuclein also binds directly to artificial lipid raft membrane mimetics; this binding requires acidic phospholipids - particularly phosphatidylserine(PS) and, more specifically, a combination of PS with oleic (18:1) and polyunsaturated (20:4 or 22:6) fatty acyl chains. Binding was particularly enhanced with PS on the polyunsaturated fatty acyl chain (vs. the oleoyl chain), suggesting that synuclein membrane interactions are subject to a strict "combinatorial code" [223]. Interestingly, synuclein redistributed away from synapses upon raft disruption [148]. The membrane-binding impairing A30P synuclein mutation similarly exhibited impaired localization at the synapse [148]. If lipid rafts mediate synuclein localization in some functionally requisite fashion, perturbation of lipid rafts or of this association could lead to synuclein mislocalization and so contribute to disease [148]. In yeast, synuclein binds to lipid rafts [88] and inhibition of sterol synthesis led to decreased plasma membrane association by synuclein, increased (aberrant) vesicular association, and increased cellular toxicity [224]. Thus, higher membrane sterol concentrations favor plasma membrane binding of synuclein, though whether this is of functional or pathological relevance remains to be determined [224]. Finally, given the interactions between synuclein and fatty acids discussed above, it is of interest that PUFAs are able to directly promote synuclein oligomerization both *in vitro* and *in vivo* [180,198]. In each of these cases a contribution of specific lipids, membrane properties, and membrane compositions to synuclein function vs. dysfunction has been established yet remains incompletely understood.

Thus far, discussion of synuclein function and toxicity has focused on the view that the effects of alpha-synuclein will be cell autonomous. Recently, it has become apparent that this may not be the case, as cell-to-cell spread of synuclein oligomers/aggregates

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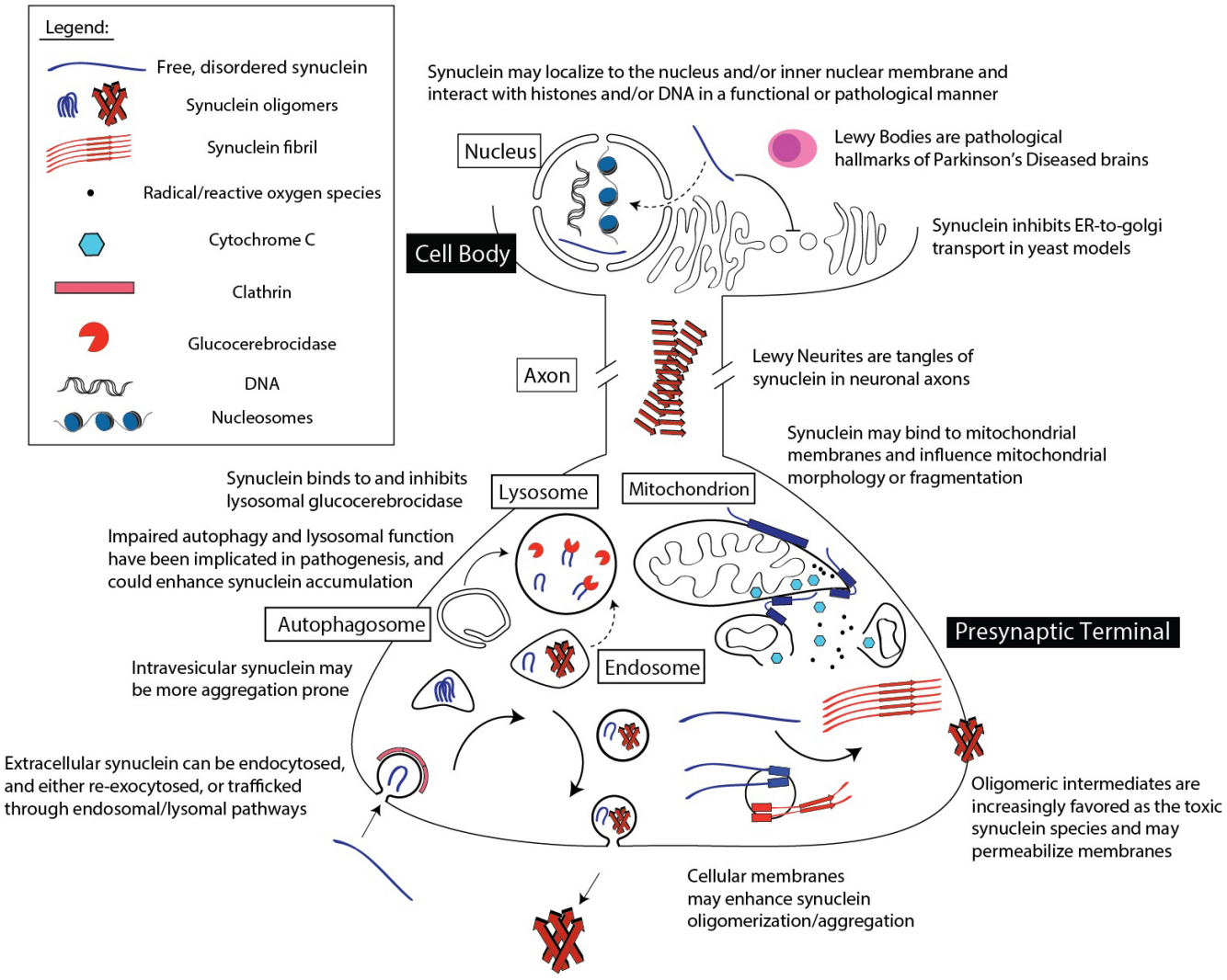

Fig. 2. Cellular membranes, targets and pathways potentially involved in alpha-synuclein dysfunction and its role in disease states in the synucleinopathies.

has become an attractive model for how neurodegeneration may spread through the synucleinopathy-diseased brain [5,225-227]. First, synuclein has been detected in extracellular biological fluids of both healthy and PD subjects, including the CSF and blood plasma [153,228-230]. A key observation resulted from an experimental Parkinson's Disease treatment in which patients received fetal ventral mesencephalic tissue transplants. Lewy body-like inclusions were found in these exogenously grafted nigral neurons in the brains of multiple patients with Parkinson's Disease; this implies spread of synuclein from endogenous neurons into disease-free, grafted neurons in a potentially prion-like manner [231-233]. Experimentally, both synuclein exocytosis and endocytosis have been observed. A portion of cellular synuclein is present in the lumen of vesicles, and this intravesicular synuclein is more aggregation prone than cytosolic protein [234]. Also, a small percentage of newly synthesized synuclein is rapidly secreted via ER/Golgi-independent exocytosis, and aggregated synuclein is also secreted [234]. Interestingly, both monomeric and aggregated synuclein secretion and transmission are elevated upon proteasomal and mitochondrial dysfunction [235], suggesting that synuclein exocytosis may increase in synucleinopathy patients. In general, extracellular synuclein is cytotoxic in culture media [236], and aggregated extracellular synuclein can induce microglial activation, dopaminergic neurotoxicity [237], and production of pro-inflammatory factors from astrocytes and astrocytoma cells [238,239]. Released synuclein acts as an endogenous agonist for Toll-like receptor 2, which activates microglial inflammatory responses [240]. Exogenous synuclein further induced neuronal cell death through Rab5A-dependent endocytosis [236], though the mechanism of synuclein internalization appears to be

synuclein assembly-state specific: aggregated fibrils or oligomers display receptor mediated endocytosis, while monomeric synuclein passively diffuses across the plasma membrane [241]. Some exogenous synuclein can also be resecreted by recycling endosomes; this process is regulated by rab11a. Hsp90 interacts with rab11a and is critical for the toxicity of exogenous synuclein [242]. Alternately, endocytosed synuclein aggregates can be degraded by lysosomes [241], which is particularly interesting given that impaired autophagy and lysosomal function have been implicated in Parkinson's pathogenesis [226,243]. Impaired autophagy could enhance synuclein accumulation, and increased exophagy of both synuclein monomers and aggregates has been observed upon manipulations that perturb autophagosomes (either by increasing the pool of autophagosomes/amphisomes through e.g. lysosomal disturbance, or by altering the polarity of vesicular transport of autophagosomes on microtubules) [244]. Finally, a dysfunctional interaction between alpha-synuclein and the Gaucher's Disase linked lysosomal glucocerebrosidase (GCase) has recently emerged. Functional loss of GCase causes synuclein accumulation, and synuclein inhibits the lysosomal activity of GCase [245]. This suggests that GCase and synuclein form a bidirectional pathogenic loop in the synucleinopathies and that GCase depletion contributions to synucleinopathy pathogensis. Further, the substrate of GCase, glucosylceramide, stabilized soluble synuclein oligomeric intermediate species [245]. Reduced GCase with synuclein accumulation has been observed in PD brain tissue as well [246]. Failure of cellular protein quality control systems (particularly lysosomes) also promotes the accumulation of transmitted synuclein (i.e. that spread from adjacent cells) and inclusion formation [235]. Indeed, synuclein aggregates appear to spread from cell to cell through a cycle of external aggregate uptake, co-aggregation with endogenous synuclein, and coaggregate exocytosis [247]. GCase depletion promotes this propagation of synuclein aggregates [247], and the enhanced spread of synuclein could further contribute to mechanism(s) by which GCase mutations contribute to PD and to increased cognitive impairment [247]. Clearly, then, as synuclein monomers and aggregates are secreted, endocytosed, and trafficked through endosomal, autophagosomal, or lysosomal compartments, they will contact a wealth of proteins and cellular membranes whose relevance to synuclein function or dysfunction was not previously considered or appreciated. The putative contribution, if any, of synuclein/membrane interactions within such compartments has not been extensively considered or examined, though it now appears likely that these pathways and interactions could contribute to the synucleinopathies.

The newly established cell-to-cell spread and endosomal/lysosomal trafficking of synuclein requires consideration of an increasing number of extracellular and intravesicular binding targets, including both proteins – as in the case of glucocerebrosidase - and novel cellular membrane binding partners such as the outer plasma membrane leaflet or inner endosomal and lysosomal leaflets. In this context, synuclein/sphingolipid and, more specifically, synuclein/gangliocide interactions become particularly relevant, as gangliocides are found primarily exposed to the cell exterior. Of note, gangliocides are negatively charged and so would be expected to interact favorably with synuclein [150]. Extracellular synuclein is internalized into microglia via the monosialoganglioside GM1 in a lipid raft-dependent (but clathrin-, caveolae, and dynamin-independent) manner [153]. Gangliocides also appear to have interesting effects on the biophysical behavior of synuclein at the membrane. Residues 34-50 of synuclein have been identified as a putative ganglioside-binding domain [152]. It has thus been proposed that synuclein first interacts with a cell surface glycophingolipid such as GM3 (in astrocytes) or GM1 (in neurons) through these residues; tyrosine 39 appears particularly critical for this interaction [150,151]. This binding then induces the helical folding of synuclein, including of residues 67~78, which then form a so-called "tilted peptide" with high affinity for cholesterol. The tilted geometry of the cholesterol/synuclein complex then facilitates the formation of an oligomeric channel with potential dysfunctional consequences [150]. It has been shown that GM1 (and to a lesser extent GM2 and GM3) can induce the formation of helical synuclein oligomers yet inhibit amyloid fibrillation [152].

CONCLUSION

While both the normal functions of alpha-synuclein and the specific mechanisms by which it leads to cell death and disease remain elusive, it is clear that the interactions of alpha-synuclein with membranes play an important role in both synuclein biology and synuclein pathology. Here we have covered much of the information currently available regarding the structural and biophysical aspects of synuclein-membrane interactions, how these are influenced by post-translational modifications, how they relate to synuclein's interactions with other proteins, which organelles they may involve and how they may influence synuclein aggregation and dysfunction. Much remains to be learned in each of these areas, but it is hoped that this review will help to provide both current and future investigators in this topic area with a snap shot of some of the most promising directions to pursue in order to fill in critical gaps in our knowledge. It is clearer than ever that alpha-synuclein is perhaps the most important single protein in

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the etiology of Parkinson's disease, and we posit that advances in our understanding of synuclein-membrane interactions will help bring us closer to improved treatments.

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