ANNUAL REVIEWS

Annual Review of Pathology: Mechanisms of Disease

The Membrane Interactions of Synuclein: Physiology and Pathology

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Annu. Rev. Pathol. Mech. Dis. 2021. 16:465–85

The *Annual Review of Pathology: Mechanisms of Disease* is online at pathol.annualreviews.org

https://doi.org/10.1146/annurev-pathol-031920-092547

Keywords

synuclein, synaptic vesicle, neurotransmitter release, Parkinson's disease, Lewy pathology

Abstract

Specific proteins accumulate in neurodegenerative disease, and human genetics has indicated a causative role for many. In most cases, however, the mechanisms remain poorly understood. Degeneration is thought to involve a gain of abnormal function, although we do not know the normal function of many proteins implicated. The protein α-synuclein accumulates in the Lewy pathology of Parkinson's disease and related disorders, and mutations in α-synuclein cause degeneration, but we have not known its normal function or how it triggers disease. α-Synuclein localizes to presynaptic boutons and interacts with membranes in vitro. Overexpression slows synaptic vesicle exocytosis, and recent data suggest a normal role for the endogenous synucleins in dilation of the exocytic fusion pore. Disrupted membranes also appear surprisingly prominent in Lewy pathology. Synuclein thus interacts with membranes under both physiological and pathological conditions, suggesting that the normal function of synuclein may illuminate its role in degeneration.

INTRODUCTION

In chronic disease, a sequence of events unfolds to result in a clinically recognizable syndrome. In most if not all cases, a change in normal physiology sets this process in motion and the pathology makes sense as a response. For instance, dysregulation of the normal mechanisms that regulate blood pressure causes hypertension, and modern medicine takes advantage of these changes to manipulate a major risk factor for cardiovascular disease. In the case of neural degeneration, however, we do not know how disease begins.

What we know about neurodegeneration is that characteristic misfolded proteins accumulate in specific brain regions. Along with clinical features, this pathology has come to define the different forms of degeneration. Originally thought only to reflect the degenerative process, pathogenic mutations have been identified in many of the genes encoding proteins that aggregate, indicating a causal role. However, the mechanisms responsible still remain unknown.

Misfolding has suggested that these proteins cause disease through the acquisition of an abnormal function. In the case of Alzheimer's disease (AD), processing of the amyloid precursor protein (APP) yields the Aβ peptide that accumulates in the senile plaques of Alzheimer's disease (1). Supporting a role in causation, mutations in APP produce rare familial forms of AD and affect processing to Aβ. However, the physiological role of APP processing remains unknown because we do not know the role of APP. Loss of APP alone produces a modest phenotype, but simultaneous loss of the related APP-like proteins (APLPs) causes early lethality (2). The mechanism may involve a presynaptic defect at the neuromuscular junction (3), but how APP contributes to this remains unknown. The microtubule-associated protein tau aggregates in the neurofibrillary tangles of AD and other disorders (4). Mutations in the tau gene have also been linked to multiple neurodegenerative diseases, but its biological role at microtubules remains unclear (5). Similarly, both the Lewy pathology of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) as well as the glial cell inclusions of multiple system atrophy (MSA) (6) contain aggregates of α-synuclein (7), but the normal function of this protein has also remained mysterious.

In the case of α-synuclein, point mutations produce a rare but penetrant autosomal dominant form of PD (8–10), indicating that synuclein has a causative role. However, most patients with sporadic PD inherit synuclein genes with no mutations. Thus, PD usually involves the wild-type protein. Indeed, duplication and triplication of the wild-type α-synuclein gene cause a severe, early onset form of PD (11), strongly suggesting that an increase in expression is sufficient to trigger at least some forms of the idiopathic disorder. In addition, genome-wide association studies of the sporadic disorder have shown linkage to polymorphisms in noncoding regions of the α-synuclein gene (12). Comparing the two alleles has indeed shown that one of the untranslated 3′ polymorphisms associated with PD confers increased expression (13). Thus, α-synuclein appears to have an important role in sporadic as well as familial PD.

In support of a role for misfolding, Lewy pathology appears to spread during the course of PD, from the gut or olfactory epithelium to the brain (14). The symptomatic spread of aggregates after injection of preformed α-synuclein fibrils (PFFs) in vivo supports this possibility (15). In contrast to MSA, however, extracts from patients with PD do not propagate easily (16–18), raising questions about the relationship of PFFs to the human disorder. In addition, the known PD mutations do not consistently promote synuclein aggregation (19, 20). From the genetic perspective, it thus remains unclear whether the PD mutations act directly at the level of protein folding and aggregation. Alternatively, they may influence the function of synuclein with downstream effects on folding that do or do not lie on the pathway to degeneration. With regard to sporadic disease in particular, it is difficult to identify factors that promote misfolding when we do not understand the normal function of α-synuclein or the biological context in which it operates.

CIRCUMSTANTIAL EVIDENCE: PRESYNAPTIC LOCATION AND REGULATED EXPRESSION

The synucleins were independently identified through multiple lines of investigation that provided clues to their function. Synuclein was first purified in association with cholinergic vesicles from the *Torpedo* electric organ (21), suggesting a presynaptic location. It was subsequently identified as an abundant phosphoprotein in bovine brain, again in association with nerve terminals (22). Both α- and β-synuclein were also identified presynaptically in human brain (23). In addition, isolation of the non-Aβ component (NAC) of senile plaques from AD revealed its origin as a 35-residue proteolytic fragment from the middle of α-synuclein (24). The role of NAC in Aβ aggregation has remained unclear and the significance of this finding has largely been dismissed (25), but the NAC precursor (α-synuclein) was again shown to localize presynaptically (26).

Synuclein has also been identified as a gene transcriptionally upregulated in a range of different circumstances. The expression of α-synuclein is transiently increased in specific nuclei important for the acquisition of song in birds (27), suggesting a role in neural plasticity. In addition, α-synuclein responds to injury, with the mRNA increasing in substantia nigra after excitotoxic injury to the striatum and protein aggregates appearing after injury to the lamprey spinal cord (28, 29). These findings suggest a specific role for synuclein in the response to injury, although what that might be has remained unknown. Endogenous α-synuclein also protects against RNA virus infection, specifically within the nervous system (30). Infection induces α-synuclein, and loss of endogenous α-synuclein results in massively higher viral titers in the brain. Interestingly, the α-synuclein knockout also shows a baseline increase in endoplasmic reticulum stress and infection modulates this response (30), suggesting that a role in trafficking through the early secretory pathway may secondarily influence virus replication.

MEMBRANE ASSOCIATION: FROM BIOCHEMISTRY TO NEURONS

Despite the apparent association with synaptic vesicles, the synucleins lack a transmembrane domain or lipid anchor. Indeed, they belong to a growing number of so-called intrinsically disordered proteins that do not have a well-defined structure in solution. However, considerable work has shown that α-synuclein (and by extension the other isoforms) adopts a helical conformation when in the presence of highly curved membranes with acidic phospholipid headgroups (31–34). Thus, synuclein is a peripheral membrane protein, forming an amphipathic α-helix that burrows into the lipid bilayer.

Synucleins are small proteins (α-synuclein contains 140 residues) that bind to membranes through an N-terminal domain with seven 11-amino-acid repeats. The repeats show high conservation among the three synuclein isoforms (α, β, and γ) and across vertebrates. The length of the repeats and their amphiphilic nature resemble those in apolipoproteins, but the synucleins show no primary sequence similarity to other proteins except for weak similarity to the lipid droplet protein perilipin 4, which contains 29 33-mer (3 × 11-mer) repeats (35). In the case of synuclein, basic residues on the outer face of the helix presumably interact with the required anionic phospholipid headgroups. Remarkably, there is no gap between any of the repeats except for four residues inserted between repeats 4 and 5 (**Figure 1**). In *Torpedo* and the lamprey, this insert appears to shift, but there is invariably a single such insert in the protein. In addition, exactly eight residues precede the first repeat in all species. The precise arrangement of repeats thus appears extraordinarily conserved. In contrast, the C terminus of synucleins is acidic and less conserved across both isoform and species. The synucleins are also restricted to vertebrates. Model organisms

Figure 1

Domain structure of the synucleins. The N-terminal membrane-binding repeats (alternating *yellow* and *green*) of the synucleins begin precisely eight amino acids after the initial methionine and continue uninterrupted with no break except for the four residue linkers (*blue*) between repeats 4 and 5. *Torpedo* (*Tetronarce californica*) α-synuclein contains two repeats in place of the single second repeat in other species. Human β-synuclein contains a single hybrid repeat instead of repeats 6 and 7 in other species, but the repeats are nonetheless precisely aligned in every case, illustrating the importance of a continuous α-helix. The residues highlighted in pink indicate the positions of PD-associated mutations in human α-synuclein. The residue numbers refer to the sequence of mouse and human α-synuclein. Abbreviation: PD, Parkinson's disease.

such as yeast, *Drosophila melanogaster*, and *Caenorhabditis elegans* contain no recognizable homologs. Despite their presynaptic location, the synucleins thus cannot be required for transmitter release.

In neurons, α-synuclein localizes to the axon terminal, as noted above (22, 26, 36). Since synaptic vesicles are among the smallest membranes in biology, this location may reflect the preference of synuclein for highly curved membranes. Consistent with this, α-synuclein shows no specific association with membranes in non-neural cells, exhibiting a cytosolic distribution like the green fluorescent protein (GFP) (36). Similarly, in brain extracts, α-synuclein does not remain associated with synaptic vesicles through their isolation by differential centrifugation and gradient fractionation. Synuclein also localizes to developing synapses after other presynaptic proteins (37). Using a fluorescence resonance energy transfer–based assay, we found that α-synuclein can interact directly with synaptic vesicles (38), and other work has suggested a role for Ca^{++} in regulating this interaction (39). The dilution inherent in biochemical fractionation may therefore result in dissociation from membranes to which synuclein binds in cells.

Although synuclein was originally identified in association with synaptic vesicles, it has been surprisingly difficult to demonstrate a direct interaction with membranes in neurons. Light microscopy cannot resolve the synaptic vesicles within a single bouton. The size of antibodies also limits the ability of immunoelectron microscopy to discriminate between expression on adjacent membranes. α-Synuclein has thus been fused to the singlet oxygen-generating miniSOG that can generate an electron-dense deposit, but overexpression of this fusion protein in neurons has

suggested localization to multiple membranes, and the generation of free radicals also appears to perturb presynaptic structure (40). More recently, loss of synuclein has been demonstrated to increase tethering of synaptic vesicles to the active zone and reduce the fibers that interconnect synaptic vesicles (41). However, we still do not know whether synuclein corresponds to any of the connectors visualized.

Despite the persistent questions about localization, the behavior of α-synuclein in living neurons has provided important information about the nature of presynaptic interactions. We used fluorescence recovery after photobleaching of individual boutons to show rapid, essentially complete recovery of α-synuclein (36). α-Synuclein-GFP behaves almost like a soluble protein but recovers slightly more slowly than does GFP. The A30P mutation associated with PD also accelerates the recovery to that of GFP, consistent with the reduced membrane association of this mutant (42). Expressed in vivo, α-synuclein-GFP recovers more slowly and less completely than in vitro (43), presumably reflecting slower diffusion through the complex, three-dimensional geometry of axonal processes in brain. These results indicate a weak interaction with presynaptic elements but do not identify a mechanism for the specific interaction with membranes of the secretory pathway or synaptic vesicles.

The weak association of synuclein with membranes has in turn limited our ability to study the mechanism, regulation, and biological role in cells—a large soluble pool obscures the fraction interacting with membranes. To circumvent this problem, we first used permeabilization with digitonin to release the soluble protein expressed in non-neural cells, leaving only that bound to membranes. The punctate residual α-synuclein colocalizes with components of lipid rafts, a finding confirmed by flotation gradients of brain extracts (36). Pharmacological inhibition of lipid rafts also blocks the presynaptic localization of α-synuclein in neurons (36). Further, the PD-associated A30P mutation also disrupts its localization to lipid rafts. Additional work has supported the association of α-synuclein with lipid rafts in neurons (44). Using an in vitro assay to recapitulate the interaction of synuclein with lipid rafts, we subsequently found a requirement for fatty acyl chains with both oleic acid and polyunsaturated fatty acids and for the acidic headgroup specifically on the phospholipid with polyunsaturated fatty acyl chains (45). The significance of this requirement remains uncertain other than to indicate the association of α-synuclein with specific membrane microdomains.

The effect of fatty acids has also provided circumstantial information about the membrane association of synuclein in neurons. Synuclein coats the fat droplets produced by feeding HeLa cells with oleic acid, protecting them from lipolysis (46). Although γ-synuclein did not show the same behavior in HeLa cells, it is expressed by adipocytes and the knockout shows resistance to obesity (47), suggesting that both isoforms behave similarly. Although fat droplets are much larger than synaptic vesicles, the interaction of synuclein with these membranes is presumably related. α-Synuclein has also been suggested to bind fatty acids in solution, with multimerization promoted by polyunsaturated fatty acids (48) as well as lipid droplets (46).

Synuclein also influences membrane composition. Overexpression increases the proportion of polyunsaturated acyl chains in membrane phospholipid, increasing membrane fluidity (49, 50). The knockout of α-synuclein shows a major reduction in total brain cardiolipin and the proportion with polyunsaturated fatty acyl chains (51). More recently, α-synuclein expression has been shown to increase dramatically the di- and triglycerides in yeast, with the toxicity of synuclein attenuated by lipid droplet formation and promoted by feeding with oleic acid (52). Presumably, lipid droplets sequester the toxic species of synuclein, which may require oleic acid for their toxicity. Although the interaction with fatty acids is more dramatic in yeast, which do not normally express synuclein, than in mammalian cells, which do, inhibition of the desaturase that produces oleic acid apparently

protects against synuclein toxicity in multiple models (52). The mechanisms by which synuclein exerts these effects and responds to changes in lipid composition nonetheless remain unclear.

How does synuclein respond to neural activity? Regulated exocytosis involves changes in the distribution of many presynaptic proteins. Synaptic vesicle proteins introduced into the plasma membrane by membrane fusion disperse into the axon before recycling by endocytosis. The Ca^{++} entry responsible for exocytosis also triggers phosphorylation of synapsin 1a and its displacement from synaptic vesicles before membrane fusion (53–55). In contrast, tetanus toxin, which inhibits membrane fusion, blocks the dispersion of α-synuclein but not synapsin (56). Thus, synuclein disperses into the axon after exocytosis, and electron microscopy confirms the redistribution by activity (57). These results provide some of the strongest evidence that synuclein associates with synaptic vesicles. The requirement for high curvature suggests that α-synuclein dissociates from the membrane as it flattens into the plasma membrane and hence predicts a role for membrane-bound α-synuclein at or before, but not after, exocytosis. Circumstantial evidence thus suggests a role for synuclein in exocytosis rather than endocytosis.

Since α-synuclein aggregates as a β sheet, membrane association might be predicted to prevent its misfolding, but the results have conflicted (46, 58–61), with membranes either promoting or inhibiting multimerization. Expressed in yeast, α-synuclein clusters membranes (62, 63), perhaps through multimerization. Multimerization may also enable the N-terminal repeats to disrupt membranes (64). However, oligomerization of α-synuclein may occur in physiological as well as pathological settings. Indeed, α-synuclein has been proposed to form an α-helical tetramer off as well as on membranes (65, 66). The existence of a multimeric state off membranes remains particularly controversial considering the behavior of synuclein as an intrinsically disordered protein (67) even in cells (68), but evidence for a multimeric state at the nerve terminal has begun to emerge (69, 70). In general, identification of an oligomeric state requires cross-linking, raising questions about its physiological relevance. Oligomeric α-synuclein has been suggested to promote SNARE complex assembly (69) but also to inhibit membrane fusion (71–73). Supporting a physiological role for the tetramer, point mutations associated with PD shift α-synuclein away from this state (74).

OVEREXPRESSION OF α-SYNUCLEIN: PHYSIOLOGY OR TOXICITY?

To understand how α-synuclein contributes to degeneration, the human protein has been overexpressed in transgenic mice. Overexpression of the A53T mutant associated with PD results in spinal cord degeneration (75, 76) even though threonine is the normal residue at this position in rodents. However, dopamine neurons do not degenerate in these mice. Transgenic overexpression of C-terminally deleted α-synuclein and viral expression of the wild-type protein both produce inclusions and impair dopamine release (77, 78), suggesting that the physiological consequences reflect toxicity. Triplication of the α-synuclein gene causes particularly severe parkinsonism in humans (11), but transgenic overexpression of wild-type human α-synuclein to similar levels produces a relatively modest phenotype. Originally reported to show both intranuclear and cytoplasmic deposits with loss of dopamine terminals and motor impairment, subsequent lines showed more modest behavioral effects with no degeneration (79–81). On the other hand, overexpression of wild-type α-synuclein in oligodendrocytes produces a model of MSA (82, 83), consistent with the accumulation of synuclein in glial cell inclusions characteristic of this disorder. More recently, a mutant engineered to disrupt the tetrameric state of synuclein produces a parkinsonian form of degeneration when overexpressed in mice (84). The phenotype of these animals is striking, but the effect of these mutations and the tetrameric state in the normal function of synuclein remain unclear.

Although locomotor behavior and pathology correlate in several of these models, overexpression of the A30P mutant defective in membrane association impairs movement and reduces dopamine release without any overt degeneration (85). In addition, transgenic mice containing a bacterial artificial chromosome with the human α-synuclein gene show an age-dependent loss of dopamine neurons without inclusions (86). Thus, physiological defects and even cell loss may not require protein aggregation.

To characterize the immediate physiological effect of α-synuclein overexpression, we and others have studied transmitter release. Overexpression of the wild-type human protein in chromaffin cells and the related PC12 cells inhibits the regulated exocytosis of large dense core vesicles (LDCVs) (87). Even though the A30P mutation disrupts synaptic localization of synuclein in neurons, it does not impair the ability of synuclein to inhibit release from these endocrine cells, due presumably to their small size. As seen with electron microscopy, LDCVs accumulate at the plasma membrane (87), suggesting that synuclein acts at a step relatively late in the pathway to exocytosis. In hippocampal neurons, overexpression of wild-type human α-synuclein reduces the postsynaptic response to a single action potential and slows recruitment of the synaptic vesicle recycling pool (88). In these experiments involving both transfected neurons in culture and brain slices from transgenic mice, the neurons did not show any protein aggregation or overt toxicity, although another study has reported empty boutons without synaptic vesicles (89). In the case with apparently normal morphology, what then causes the impaired release? Specific isoforms of synapsin and complexin, other peripheral membrane proteins of the synaptic vesicle, decrease in the transgenic mice overexpressing synuclein (88). The synapsins have also been suggested to downregulate in response to oligomeric α-synuclein (90), although we did not observe any oligomeric forms of synuclein in these mice. Recent work has suggested that the inhibition of synaptic vesicle exocytosis by synuclein requires the synapsins (91). However, loss of all three synapsin isoforms disrupts the accumulation of synaptic vesicles at presynaptic boutons (92), suggesting that the effect of α-synuclein may depend on normal presynaptic organization rather than specifically the synapsins.

Several studies have suggested effects of overexpressed synuclein on synaptic vesicle endocytosis and recycling as well as exocytosis. In the lamprey, the synuclein-3 isoform accumulates at the reticulospinal synapse after injury, and microinjection of recombinant protein arrests recycling at the step of either fission from the plasma membrane (in the case of dimeric synuclein) or subsequent clathrin uncoating (in the case of monomeric synuclein) (29, 93). The defects appear only with stimulation at higher frequency, and mutations that disrupt membrane interactions of α-synuclein block the effect (94). More recently, direct injection of recombinant wild-type human α-synuclein into the calyx of Held synapse was also found to disrupt endocytosis, and the A30P mutation abolishes this effect, providing an important negative control and implicating membrane interactions (95); the PD-associated A53T mutation had a less consistent effect (96). Although microtubules have generally been considered to have little role in transmitter release, the microtubule-depolymerizing agent nocodazole rescues the impairment due to wild-type synuclein overexpression (95). The acute addition of α-synuclein is a strength of this work since it suggests a direct effect on endocytosis or recycling. However, it is important to note that the recombinant protein introduced into either lamprey or the calyx of Held may cause toxicity not evident with expression by the cell, perhaps due to the absence of cellular modifications. Indeed, several observations provide circumstantial evidence against a role of synuclein in synaptic vesicle recycling: Synuclein moves away from presynaptic boutons after fusion with the plasma membrane; LDCVs do not recycle as synaptic vesicles do, but still show inhibition of exocytosis by synuclein overexpression. In addition, live imaging of transfected hippocampal neurons shows no defect in synaptic vesicle endocytosis with overexpression of α-synuclein (88).

THE FUNCTION OF α-SYNUCLEIN

One concern with overexpression is that it may produce toxicity, with the potential for pleotropic indirect effects, and the severe phenotype produced by gene triplication supports this possibility even for wild-type α-synuclein. To focus on the normal function of endogenous synucleins, we and others have therefore studied knockout mice. Mice lacking α-synuclein appear healthy, show normal survival, and show no evidence of parkinsonism (97). They have been reported to show a reduction in midbrain dopamine neurons during development, but this did not progress after birth (98). Loss of α-synuclein was also found to cause a mild reduction in dopamine levels and faster recovery (of dopamine release) from paired pulse depression in striatal slices, with no effect on excitatory neurotransmission (97, 99). Subsequent work in vivo has further characterized the changes in dopamine release in the absence of α-synuclein. In wild-type mice, stimulation releases more dopamine in response to the first burst and less to subsequent bursts, but with greater facilitation within the subsequent bursts (100). In contrast, mice lacking α-synuclein release similar amounts of dopamine in first and later bursts, but with strong facilitation in each (100). Since wild-type mice show facilitation in subsequent bursts similar to that observed in the first burst of knockout mice, the dispersion of α-synuclein in response to stimulation may be responsible (56). In this case, α-synuclein would seem to inhibit facilitation, possibly by increasing release probability and thereby depleting vesicle pools. The mechanism remains unclear, but loss of α-synuclein upregulates synapsin III, a synapsin isoform that accumulates in Lewy pathology (101) but reduces rather than increases dopamine release (102).

Loss of both α- and β-synuclein further reduces dopamine levels, but also with no clear effect on excitatory neurotransmission or overall health (103). However, dopamine neurons express unusually high levels of γ-synuclein (104), and loss of both α- and γ-isoforms increases dopamine release (105). The triple knockout also increases dopamine release despite even lower tissue dopamine than the α-/β-synuclein double knockout (106), suggesting a great increase in release probability. Surprisingly, the triple knockout shows no change in the number of midbrain dopamine neurons (106), despite the reduction observed in α-synuclein single knockouts (98). Synuclein triple knockout mice may die prematurely in the second year (107, 108), but this has not been a consistent finding (106). One of the most striking findings is a reduction in the size of presynaptic boutons later in life (108).

The synuclein knockout mice show relatively mild changes in behavior. The single α-synuclein knockout mouse shows a moderately reduced locomotor response to psychostimulant (104), consistent with the reduced dopamine, but increased intracranial self-stimulation (109), suggesting a predisposition to drug abuse. The triple knockout mouse exhibits hyperactivity, not parkinsonism (106), but does develop visual problems and axonal degeneration (108). Thus, loss of all three synucleins produces degeneration but not in the pattern characteristic of PD.

The synuclein triple knockout also has no clear effect on glutamatergic transmission (107, 108). It does show a reduction in the number of SNARE complexes (107), and α-synuclein overexpression redistributes SNARE proteins (110). α-Synuclein apparently interacts with the C terminus of v-SNARE VAMP2, but if so, why does it not influence release? If produced in excess, SNARE complexes might be able to sustain a reduction without impairment of release. Alternatively, the number of SNARE complexes may indirectly reflect a distinct presynaptic role for the synucleins. Recent work has confirmed interaction of VAMP2 with the α-synuclein C terminus and identified residues that are involved (111). Mutation of these residues blocks the ability of overexpressed synuclein to inhibit transmitter release, but we do not know what else these mutations might disrupt. Elimination of VAMP2 would provide a stringent test of this hypothesis, but VAMP2 is required for evoked release. Synuclein has indeed been suggested to interact with many proteins,

but evidence for effects on function have relied on heterologous expression or overexpression rather than the loss of endogenous proteins. For example, α-synuclein influences actin polymerization and cell migration when overexpressed (112), but, to our knowledge, a relevant phenotype in the knockout mice has not thus far been documented. These interactions may nonetheless be important; however, the interaction of synuclein with membranes appears to be of primary significance.

SYNUCLEIN CAN DRIVE MEMBRANE CURVATURE

Despite the early evidence that synuclein associates with membranes, it has become clear only recently that synuclein also influences membranes. Several groups have found that wild-type α-synuclein overexpression can fragment mitochondria (113, 114). The mechanism has been controversial, with evidence for an increase in fission (114) as well as a decrease in fusion (113). However, tethering synuclein to the plasma membrane blocks the effect, suggesting that it requires direct contact (114). Consistent with this possibility, synuclein appears to act downstream of the mitochondrial fission protein Drp1 (114). Indeed, α-synuclein has more recently been shown to localize at mitochondrially associated membranes, and PD-associated mutants fragment mitochondria independent of Drp1 or the fusion protein Mfn2 (115). In a *Drosophila* model of synuclein toxicity, overexpression affects mitochondrial morphology but makes the mitochondria larger rather than smaller (116). The mechanism also differs from that observed in mammalian cells: Overexpressed synuclein interacts with spectrin and the actin cytoskeleton, resulting in sequestration of Drp1 (116). In this case, Drp1 can rescue the toxicity of synuclein. The interaction with mitochondria is thus indirect in the *Drosophila* model, in contrast to the direct effects observed in mammalian cells. However, it is important to note that synuclein knockout mice have not been reported to show a defect in mitochondrial dynamics, so the effects on mitochondria presumably require overexpression.

Consistent with the effects of overexpression in mammalian cells, α-synuclein promotes the tubulation of artificial membranes in vitro. This requires the same acidic phospholipids with which synuclein is known to interact (114, 117). Presumably, the preference of synuclein for high curvature then drives the deformation of membrane (118–120). Synuclein thus appears to promote as well as sense membrane curvature, suggesting roles in the membrane bending that accompanies the exocytosis and recycling of synaptic vesicles. Endocytosis requires considerable membrane bending, and BAR (Bin-Amphiphysin-Rev) domain proteins such as endophilin both tubulate artificial membranes in vitro and contribute to endocytosis (121). A major defect in synaptic vesicle recycling has indeed been reported for the synuclein triple knockout mice (122). However, a role in endocytosis is difficult to reconcile with the dispersion of synuclein into the axon on membrane fusion (56).

Membrane bending also occurs during exocytosis, when the secretory vesicle fuses with the plasma membrane, establishing a pore to the external solution through which cargo is released. The fusion pore shows negative Gaussian curvature, with both negative curvature at the neck and positive curvature circumferentially around the base of the pore. In addition, the fusion pore regulates peptide release from LDCVs (123, 124), and since LDCV cargo vary in the rate of release, the fusion pore can control both what is released and how quickly. We therefore tested the role of synuclein in behavior of the fusion pore. This requires the analysis of individual exocytic events, so we imaged the regulated exocytosis of individual peptidergic LDCVs (chromaffin granules) from adrenal chromaffin cells. As with synaptic vesicles (88), overexpression of wild-type α-synuclein reduces the number of exocytic events, but the triple knockout has no effect (87, 125).

THE ROLE OF SYNUCLEIN AT THE FUSION PORE

To assess effects on the fusion pore, we examined the rate of peptide release from chromaffin granules. Overexpression slightly increases the rate of peptide release (126), but loss of all three synuclein isoforms more dramatically slows the rate of peptide secretion during individual exocytic events (125), consistent with the slowing of fusion pore dilation. However, the rate of peptide release provides only an indirect measure of the fusion pore. To monitor the pore directly, we then used the accessibility of a pH-sensitive lumenal fluorophore to low external pH. As a membrane protein, the vesicular monoamine transporter VMAT2 persists at the site of exocytosis for seconds after fusion. Fused to a lumenal domain of the transporter, the pH-sensitive GFP ecliptic pHluorin is quenched at the low pH of secretory vesicles and increases in fluorescence with exocytosis and exposure to the higher external pH (127). Since VMAT2 persists at the site of fusion after exocytosis, quenching by low external pH indicates that the vesicle membrane is still accessible to the external solution; conversely, lack of quenching indicates closure of the fusion pore (kiss-and-run exocytosis) (128). Indeed, α-synuclein overexpression increases the proportion of events still accessible to low external pH (125). Synuclein thus prevents kiss-and-run exocytosis, indicating a direct effect on the fusion pore. In neurons, which express much higher levels of the synucleins than do chromaffin cells, the exocytosis of LDCVs shows a similar effect of both overexpressed and endogenous synuclein to accelerate peptide release and prevent kiss-and-run exocytosis (125). β- and γ-synuclein appear to have similar effects, suggesting a role for the highly conserved N-terminal membrane-binding domain.

The fusion pore has been shown to limit the release of large polypeptides to a greater extent than the release of small molecules such as classical neurotransmitters. The minimal effect of the synuclein triple knockout on glutamate release is consistent with this (107, 108). Synaptic vesicles have indeed been thought to collapse immediately at membrane fusion, without the need for additional factors. Rapid-freeze electron microscopy reveals omega structures that are difficult to capture during the process of exocytosis (129). However, rapid (high-microsecond) fluctuation in dopamine release has suggested a flickering fusion pore (130), and coreleased glutamate and dopamine have been shown to differ in the frequency dependence of release (131), suggesting mechanisms that might also differ in the requirement for synuclein. Consistent with this, loss of α- and γ-synuclein increases the release of dopamine (105, 106) despite the minimal effect on glutamatergic neurotransmission. Independent of effects on transmitter release, the synucleins may also contribute to the rapid synaptic vesicle collapse that follows transmitter release in wild-type animals. Given the preference for high curvature, it would also be surprising if synuclein did not influence the collapse of synaptic vesicles, which are considerably smaller than LDCVs.

Synuclein could affect the fusion pore through a variety of mechanisms. The low levels of endogenous synuclein and the overexpressed protein generally show a diffuse distribution in adrenal chromaffin cells (125), raising the possibility of indirect effects. However, one synuclein antibody specifically labels LDCVs in wild-type but not synuclein triple knockout mice, suggesting that it may recognize specifically membrane-bound protein (125, 126). Many cells with secretory granules use actin-based mechanisms to drive pore opening and cargo extrusion (132–134). Dynamin has also been shown to promote pore closure (135, 136). In principle, synuclein could act through these mechanisms.

The proposed role for α-synuclein in SNARE complex formation may also influence the fusion pore. If synuclein promotes the formation of more SNARE complexes, they may simply exert more force to accelerate vesicle collapse. However, the physiological role for α-synuclein in SNARE complex formation remains unclear: It is certainly not required for release. In addition, the site of VAMP2 interaction at the C terminus of synuclein shows little conservation among

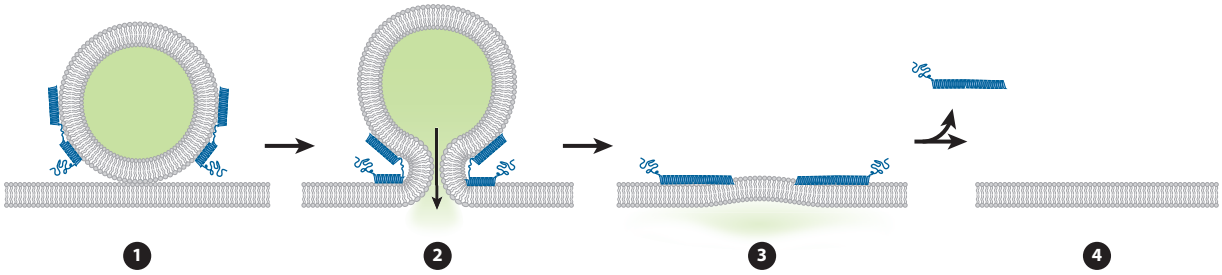

Figure 2

The role of synuclein in pore dilation. (❶) A synaptic vesicle filled with neurotransmitter (*green*) docks at the plasma membrane, with the broken helix of synuclein (*blue*) accommodated on the vesicle. (❷) At or before fusion, when transmitter exits the synaptic vesicle (*downward arrow*), the C-terminal synuclein helix transfers to the plasma membrane, and (❸) extension of the full helix pulls the fusion pore open. (❹) Synuclein then dissociates from the flat membrane.

isoforms. Alternatively, α-synuclein may act after fusion in a way that regulates SNARE complex disassembly, thereby influencing complex accumulation.

Synuclein may or may not use any of the mechanisms demonstrated to influence behavior of the fusion pore, but the highly conserved N-terminal membrane-binding domain seems very likely to play a role. Structural studies suggest a potential mechanism. Nuclear magnetic resonance shows that α-synuclein forms a bent α-helix on sodium dodecyl sulfate micelles due to their small size (137–139). On membranes, however, the synuclein helix extends, and the transition between bent and extended states has the potential to remodel membranes (139–142). If at the fusion pore the bent helix binds to the synaptic vesicle on one side of the bend and to the plasma membrane on the other, extension would drive pore dilation (**Figure 2**). This mechanism requires a specific configuration of the protein at the fusion pore, but we do not know the location of synuclein during exocytosis or the potential role for multimerization in this process.

The role of synuclein at the fusion pore predicts effects on the mobilization of new synaptic vesicles during stimulus trains (**Figure 3**). At wild-type synapses, synaptic vesicle collapse promoted by synuclein leaves many release sites unoccupied and hence available for the docking of a new synaptic vesicle. Previously unoccupied sites will also be available for docking, and the recruitment of new vesicles to previously occupied and unoccupied sites should facilitate transmitter release in response to stimulus trains. In synapses lacking synuclein, however, uncollapsed vesicles will remain for hundreds of milliseconds at release sites, preventing the recruitment of new synaptic vesicles at these sites. Unoccupied sites will still be able to recruit new vesicles, but the uncollapsed vesicles will reduce the number of sites available for subsequent stimulation. It will be particularly interesting to test these effects in vivo, where dopamine release exhibits less short-term depression than in vitro and hence may depend to a greater extent on the clearance of release sites initiated by vesicle collapse.

THE MEMBRANE INTERACTIONS OF SYNUCLEIN IN NEURODEGENERATION

Is the normal function of α-synuclein relevant for PD? The mutations that cause PD provide a way to test this possibility, at least for one familial form of the disorder. The A30P mutation clearly disrupts the synaptic localization in neurons as well as the membrane association of α-synuclein in vitro (36, 42), suggesting a disturbance in normal function. However, this mutation has been considered an outlier because the others, in particular A53T, localize normally to presynaptic

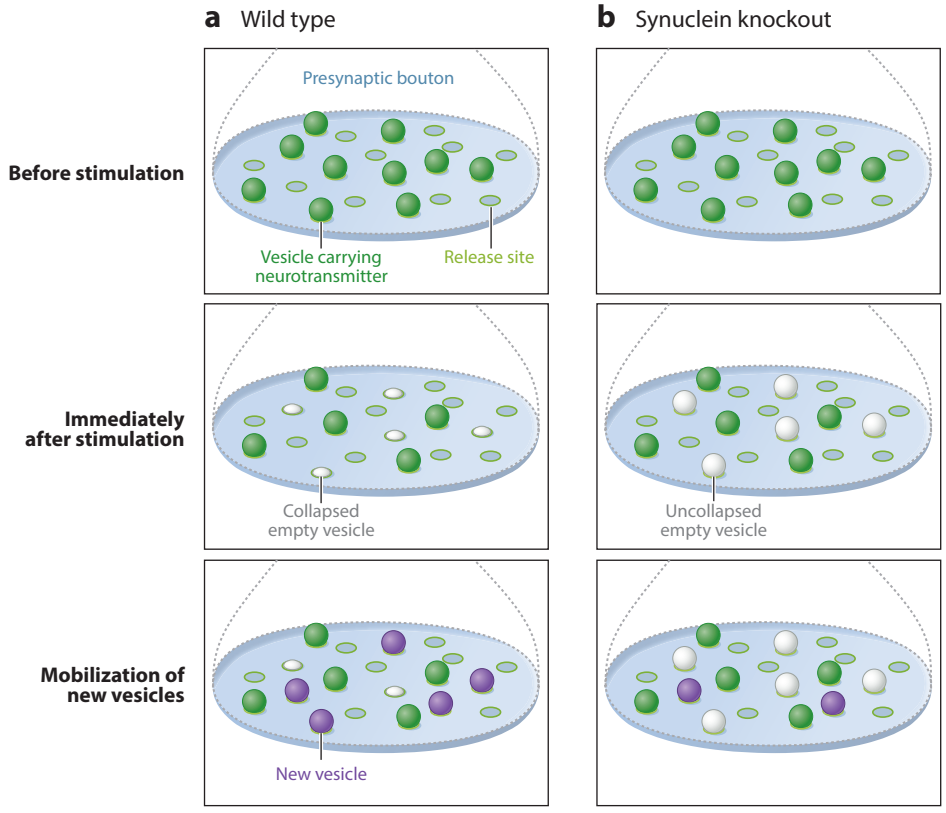

Figure 3

Predicted role for α-synuclein in the mobilization of new vesicles. (*a*) In wild-type neurons, synaptic vesicles filled with neurotransmitter (*dark green*) are recruited to a subset of release sites. With stimulation, many but not all of these vesicles undergo membrane fusion, releasing neurotransmitter (*white*). After stimulation, new vesicles (*purple*) are mobilized both to sites that have undergone fusion and subsequent vesicle collapse and to sites previously unoccupied. (*b*) Mice lacking all three synuclein genes show the same extent of membrane fusion but less vesicle collapse, which should prevent the mobilization of new vesicles to these sites but not to those previously unoccupied. Figure adapted from Reference 143 (CC BY 4.0).

boutons. Indeed, threonine is the normal residue at this position in rodents. On the other hand, the other PD-associated mutations (E46K, H50Q, and G51D), along with A53T but not A30P, all reside within a tight cluster near the end of the fourth N-terminal repeat (8, 10, 144, 145). This highly nonrandom distribution suggests a specific effect, but what that might be remains unclear. The A53T mutant aggregates slightly more than the wild-type protein, but A30P has no increased propensity to aggregate (19). In mammalian cells, all the mutants have the same tendency to oligomerize as the wild-type protein but differ in the extent of aggregation (146). In yeast, they all show toxicity similar to the wild-type protein, except for the A30P mutant, which shows less. A systematic comparison of α-synuclein mutations in yeast indeed suggests that toxicity requires membrane association, not aggregation (20). Further, a recent report of deep mutational scanning shows that toxicity in yeast is sensitive to mutations every third residue along the hydrophobic face of the amphipathic helix without interruption through all of the repeats, supporting the role of membrane association in toxicity (147). The twist of the helix also predicts that the hydrophobic surface will prefer negative Gaussian curvature, which occurs at the fusion pore. In addition, both

A30P and A53T mutants interfere with chaperone-mediated autophagy, presumably by competing with other substrates for degradation (148). Multiple mutants also fail to complement the defect in polyunsaturated fatty acyl CoA synthase activity observed in α-synuclein knockout mice (149), but how synuclein contributes to this activity remains unclear, and the effect may be indirect.

Do the PD-associated mutations influence the inhibition of synaptic vesicle exocytosis by overexpressed α-synuclein? The A30P mutant fails to inhibit synaptic vesicle exocytosis because it does not accumulate presynaptically (88), but it does inhibit the release of LDCVs (87), presumably due to the small size of chromaffin cells. Indeed, the A53T mutant, like wild-type synuclein, inhibits synaptic vesicle exocytosis (88), suggesting that the mutations have no effect on this property. However, overexpression of a C-terminal deletion mutant also inhibits synaptic vesicle exocytosis (88). Since this activity does not appear to reflect the normal function of endogenous synuclein, it may therefore not require domains that are required for normal function at the fusion pore.

In wild-type chromaffin cells, both A30P and A53T human α-synuclein inhibit LDCV exocytosis (125), as reported previously (87). This activity suggests normal expression, which was confirmed by immunofluorescence. In contrast to all three wild-type isoforms, however, neither mutant accelerated the release of peptide cargo (125). In this assay, A53T thus does not differ from A30P, suggesting that loss of function may cause disease, and it will be very interesting to determine whether the other PD-associated mutations behave similarly.

Even if the PD mutations cause disease by impairing the normal function of α-synuclein, why would knockout mice show no degeneration? Disease may reflect the accumulation of abnormal protein that acts through either membrane toxicity or misfolding. However, duplication and triplication of the α-synuclein gene cause particularly severe degeneration. How can excess, presumably wild-type, α-synuclein produce disease if disease were due to a loss of function? It is possible that the excess production of wild-type α-synuclein exceeds the capacity of the synaptic vesicle membrane to sequester the protein presynaptically. In this case, the gene multiplication would effectively increase the pool of extrasynaptic synuclein, an effect similar to that produced by the A30P mutation.

Although these mechanisms involve downstream misfolding of α-synuclein, is it possible that loss of normal function (through mutation or aggregation) contributes to degeneration? Although triple knockout mice do not show parkinsonian degeneration and survive for many months with no obvious synaptic impairment, the requirement for synuclein at the fusion pore predicts that normal ongoing neural activity results in the accumulation of uncollapsed synaptic vesicles. Remarkably, synuclein overexpression slows degeneration due to loss of the presynaptic chaperone cysteine string protein (CSP), and the A30P mutation blocks this effect, indicating a role for the membrane interactions of synuclein in preventing degeneration (150). CSP knockout mice do not show parkinsonian degeneration, and mutations in CSP cause human ceroid lipofuscinosis (151), not parkinsonism, but loss of synuclein also accelerates degeneration in the mice. This effect has been attributed to the property of synuclein as a chaperone for the SNARE complex, but how this rescues degeneration remains unknown. Synuclein may indeed interact with multiple predisposing factors to cause PD.

Human genetics has indicated an important role for mutations in the lysosomal enzyme glucocerebrosidase in what appears to be sporadic PD (152). Since the mutations are heterozygous and vary in the extent of functional impairment, it remains unclear whether they impair overall lysosomal function. Glucocerebrosidase has been implicated in the turnover of α-synuclein (153, 154), consistent with a pathogenic role for increased wild-type protein, but also in the exacerbation of synuclein pathology (155). Dopamine metabolism may impair glucocerebrosidase activity, contributing to PD in the absence of inherited glucocerebrosidase mutations (156). It nonetheless remains unclear why a lipid-modifying enzyme would affect relatively specifically the clearance of

α-synuclein. Alternatively, membrane remodeling by glucocerebrosidase may influence the interaction of synuclein with membranes.

Neuropathology has also begun to support a role for the membrane interactions of synuclein in PD. Classically, brain-stem-type Lewy pathology involves characteristic ultrastructural features, such as a halo of filaments surrounding an amorphous core. Immunostaining for α-synuclein has revealed much more extensive deposits, including aggregates within dystrophic neurites (157, 158). However, there has been a significant gap between the extensive synuclein-immunoreactive lesions observed by light microscopy and the proteinaceous aggregates observed by electron microscopy. To bridge this gap, a recent study used correlative light and electron microscopy, first to identify the immunoreactive lesions by light microscopy and then to determine their ultrastructure, avoiding the bias inherent in identifying lesions by electron microscopy. Although mitochondria have previously been reported to surround Lewy bodies, Lewy lesions defined in this way show widespread, pronounced membrane pathology (159) more prominent than classical inclusions. This does not exclude a role for the aggregation of α-synuclein in Lewy pathology—the lesions were all identified as synuclein immunoreactive—but suggests an interaction of membranes with aggregated as well as helical synuclein. This work on the neuropathology of PD thus unites the membrane inclusions and toxicity observed in yeast with the presynaptic role of synuclein in mammalian cells and indicates the importance of membrane interactions for pathology as well as physiology.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 NS062715 and R01 NS109295, the Weill Institute for Neuroscience, and the Michael J. Fox Foundation.

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