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Review

# The Function of α-Synuclein

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**Human genetics has indicated a causal role for the protein α-synuclein in the pathogenesis of familial Parkinson's disease (PD), and the aggregation of synuclein in essentially all patients with PD suggests a central role for this protein in the sporadic disorder. Indeed, the accumulation of misfolded α-synuclein now defines multiple forms of neural degeneration. Like many of the proteins that accumulate in other neurodegenerative disorders, however, the normal function of synuclein remains poorly understood. In this article, we review the role of synuclein at the nerve terminal and in membrane remodeling. We also consider the prion-like propagation of misfolded synuclein as a mechanism for the spread of degeneration through the neuraxis.**

## Introduction

α-synuclein was independently discovered on multiple occasions, providing important but still incompletely understood clues to its normal function as well as its role in disease. It was originally identified using an antibody to purified cholinergic vesicles of the *Torpedo* electric organ (Maroteaux and Scheller, 1991), providing the first evidence of a presynaptic role. In addition to localization at the synapse, the antibody detected expression at the nuclear envelope, accounting for the designation "synuclein" (Maroteaux et al., 1988). Subsequent work has confirmed the presence of α-synuclein in the nucleus (Gonçalves and Outeiro, 2013; McLean et al., 2000; Mori et al., 2002). However, synuclein is a small protein (140 amino acid residues) that falls below the molecular weight cut off of the nuclear pore (~40 kDa). Although the distribution of synuclein may be influenced by interaction with nuclear or cytoplasmic proteins (Goers et al., 2003; Kontopoulos et al., 2006; Specht et al., 2005), untagged, endogenous synuclein would thus be expected to enter the nucleus on the basis of simple diffusion. The discovery of α-synuclein in turn led to the identification of closely related β- and γ- isoforms (Maroteaux and Scheller, 1991).

Synuclein was also identified through the biochemical characterization of senile plaques in Alzheimer's disease (AD). Although not as abundant as the Aβ peptide, a fragment from the middle of α-synuclein (61–95) now termed the non-Aβ component (NAC) accumulates at high levels in plaques (Uéda et al., 1993). More recent work has shown that synuclein indeed contributes to the pathology of AD as well as of dementia with Lewy bodies (DLB) (Goedert, 1999; Trojanowski et al., 1998). However, this role appears to reflect cytoplasmic deposition rather than accumulation in extracellular plaques. Nonetheless, subsequent analysis of the NAC precursor (α-synuclein) helped to establish its primarily presynaptic localization (Iwai et al., 1995).

Third, α-synuclein mRNA transcripts were found to change specifically within regions of the zebra finch brain involved in control of song. Relative to other brain regions where synuclein remains at high levels through development and maturity, specific regions implicated in bird song show large, sustained reductions in synuclein expression during song acquisition (George et al., 1995). The regulated expression of synuclein within cell populations that participate in bird song has thus suggested a specific role for the protein in synaptic plasticity, but this role remains poorly understood.

Fourth, synuclein was purified as an inhibitor of phospholipase D2 (PLD2), identifying a specific biochemical function for the protein through a presumably unbiased experimental approach. PLD enzymes cleave the headgroup of phosphatidylcholine (PC) to release choline and phosphatidic acid (PA) and have been implicated in membrane trafficking, particularly regulated exocytosis (Hughes et al., 2004; Humeau et al., 2001; Vitale et al., 2001; Zeniou-Meyer et al., 2007). In contrast to the PLD1 isoform, which acts downstream of an ADP ribosylating factor (ARF) GTPase (Caumont et al., 1998; Cockcroft et al., 2002; Colley et al., 1997), PLD2 has constitutive activity. To understand how PLD2 might be regulated, factors that inhibit its function were purified, resulting in the isolation of α- and β-synuclein (Jenco et al., 1998). Additional work demonstrated a requirement for multiple domains in synuclein to inhibit PLD2 (Payton et al., 2004), but the physical interaction has not been documented. Originally, genetic studies in yeast supported a role for synuclein in PLD inhibition (Outeiro and Lindquist, 2003), but subsequent work has not borne this out (Rappley et al., 2009a). Although the initial purification of synuclein as a PLD inhibitor suggested a specific biochemical function of potentially profound significance, the biological relevance of this finding has thus remained uncertain.

Fifth, point mutations in α-synuclein were found to cause an autosomal dominant form of Parkinson's disease (PD) (Krüger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). The clinical phenotype resembles idiopathic PD, with typical tremor, rigidity, and bradykinesia, and the pathology shows cytoplasmic Lewy body inclusions characteristic of PD (Golbe et al., 1996), strongly suggesting relevance for the sporadic disorder. Indeed, mutations in α-synuclein account for only a tiny fraction of PD in the general population, but the Lewy bodies and dystrophic neurites observed in idiopathic PD label strongly for α-synuclein (Galvin et al., 1999; Spillantini et al., 1997, 1998b). Immunostaining for α-synuclein subsequently revealed abundant inclusions not previously detected using standard histological methods (Jellinger, 2011). In fact, many

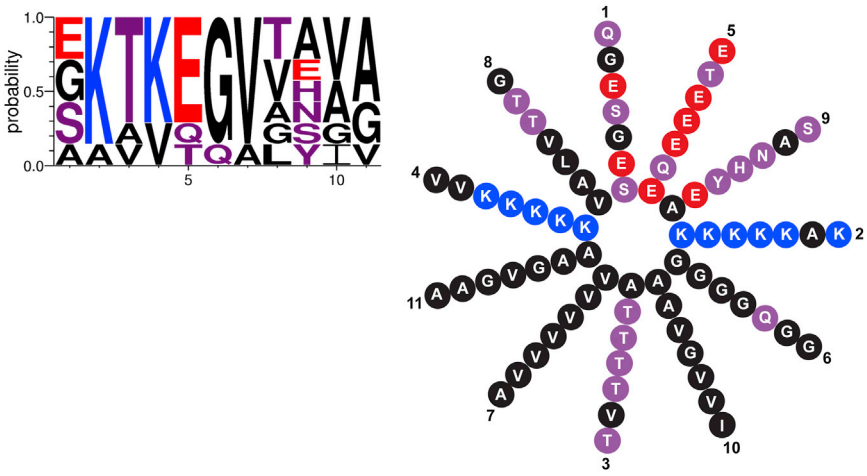


**Figure 1. The N Terminus of Synuclein Contains Seven 11 Residue Repeats that Form a Helix on Membrane Binding**

The consensus sequence of the repeats is shown to the left and helical wheel to the right. The height of the single letter amino acid code indicates the probability of finding that particular residue in the repeats of human α-synuclein. Blue indicates basic, red acidic, purple polar uncharged, and black nonpolar residues.

of the monoclonal antibodies previously raised against Lewy bodies recognize α-synuclein (Giasson et al., 2000b), supporting the impression that although other proteins may also accumulate in the inclusions of PD, α-synuclein predominates. Taken together, the genetic evidence for a causative role and the neuropathologic evidence for accumulation in essentially all patients with PD indicate a central role for synuclein in the idiopathic disorder.

## The Structure of Synuclein

The N terminus of α-synuclein contains seven 11 residue repeats that are predicted to form an amphipathic alpha-helix (Figure 1). The repeats are very highly conserved, both across species and among the three different isoforms. The motif is also unique, with no similar sequence identified outside the synuclein family. In addition, this sequence has been detected only in vertebrates, including the lamprey (Busch and Morgan, 2012). Remarkably, all of the mutations associated with PD—A53T, A30P, and E46K as well as the more recently described G51D and H50Q (Appel-Cresswell et al., 2013; Krüger et al., 1998; Lesage et al., 2013; Polymeropoulos et al., 1997; Proukakis et al., 2013; Zarranz et al., 2004)—cluster within this N-terminal domain. It is also interesting to note that rodent synuclein normally contains a threonine at position 53, which causes PD in humans. The A53T mutation thus appears pathogenic specifically within the human context. Model genetic organisms such as worms, flies, and yeast do not contain identifiable homologs, indicating that the synucleins are not required for synaptic transmission or membrane trafficking more generally. On the other hand, the apolipoproteins and a set of plant proteins that accumulate during desiccation and seed formation also contain amphipathic α helices with 11 residue repeats (George et al., 1995).

Repeats of this size enable the polypeptide to make exactly three turns of the helix and thus interact directly with the surface of a membrane through multiple repeats. However, the sequence of apolipoprotein and plant seed proteins bears little if any obvious similarity to the synucleins.

Purified, recombinant synuclein behaves like a natively unfolded protein in vitro (Bertoncini et al., 2005; Weinreb et al., 1996) but, as predicted from the sequence, forms an α-helix on binding to artificial membranes (Davidson et al., 1998). Shown initially by circular dichroism, the conformational change associated with membrane binding requires acidic phospholipid headgroups, suggesting an interaction of the membrane with lysines found on opposite sides of the helix (Figure 1). There is minimal specificity for a particular acidic headgroup, with phosphatidylserine recognized as well as phosphatidic acid and phosphatidylinositol (Zhu and Fink, 2003). Nuclear magnetic resonance (NMR) studies of synuclein on SDS micelles also reveals an α-helix but bent, presumably due to the small size of the micelle (Eliezer et al., 2001; Ulmer et al., 2005). On membranes, which have a larger diameter than micelles, the analysis of spin-labeled protein shows that synuclein adopts the extended 11/3 helix predicted from the sequence (Jao et al., 2004). Synuclein also lies along the surface of the membrane, at least half-buried in the bilayer (Bussell et al., 2005; Jao et al., 2008; Wietek et al., 2013).

Despite the original description as a natively unfolded protein, recent work has suggested that α-synuclein may in fact remain helical in solution, with important implications for its normal function and its susceptibility to aggregation. The evidence for intrinsic disorder has depended primarily on the analysis of bacterially expressed recombinant protein, and a denaturation step used by some groups in the purification has been suggested to account for the unfolded state (Bartels et al., 2011; Wang et al., 2011). Consistent with a lack of folding, synuclein behaves like a much larger protein by size exclusion chromatography, but multimerization is another possibility. To assess the multimeric state of native synuclein, a recent study from the Selkoe laboratory used a combination of crosslinking and analytical ultracentrifugation to determine the molecular weight of mammalian synuclein isolated from red blood cells and cell lines. In contrast to previous studies, this work found that native α-synuclein behaves as a folded, helical tetramer (Bartels et al., 2011). Electron microscopy (EM) reconstruction and NMR of protein purified under nondenaturing conditions further suggested a multimeric state that resists aggregation (Wang et al., 2011). However, subsequent work using recombinant synuclein has confirmed that even the nondenatured recombinant protein is intrinsically disordered and loses its α-helical conformation after dissociation from membranes (Fauvet et al., 2012). Loss of helicity could thus reflect the dilution inherent in preparing an extract, with the helical state maintained at higher concentrations (Dettmer et al., 2013; Wang et al., 2011), but NMR studies in *E. coli* have in fact suggested that macromolecular crowding

maintains the disordered state of synuclein (McNulty et al., 2006). It is also possible that synuclein folds to form a multimer only in mammalian cells, but the analysis of native brain synuclein has recently confirmed its almost entirely monomeric state (Burré et al., 2013). Recently, it has also been shown that synuclein can assemble into an oligomer (possibly tetramer) on nanoparticles (Varkey et al., 2013), but this phenomenon seems to differ from the ability of a preformed tetramer to interact with membranes (Wang et al., 2011). At this point, it remains possible that α-synuclein adopts a helical tetrameric state in solution, but the evidence is not definitive. The unavoidable dilution that accompanies purification of native synuclein complicates the analysis, but it is perhaps more important to acknowledge that despite extensive biochemical studies in vitro, the conformation of synuclein in cells remains poorly understood.

In contrast to the N-terminal membrane binding domain, the C terminus of human α-synuclein is polar, with a higher proportion of charged residues. This domain undergoes phosphorylation at multiple sites (Oueslati et al., 2010; Sato et al., 2013), suggesting a mechanism for regulation, but the function of the C terminus remains unclear, and it is the least conserved domain across species as well as among α-, β-, and γ- isoforms. The C terminus may affect membrane binding under particular conditions (Shvadchak et al., 2011), but phosphorylation toward the end of the N-terminal repeats, at Ser-87, more clearly affects membrane binding in vitro than phosphorylation at the other, more C-terminal sites (Paleologou et al., 2010). The observations thus suggest a potential biological role for Ser-87 phosphorylation, although this again remains to be identified in the context of the cell.

## Membrane Interactions and the Presynaptic Location of Synuclein

The presynaptic location of α-synuclein has been recognized since its original identification as a protein associated with synaptic vesicles (Maroteaux et al., 1988). In contrast to many proteins involved in neurodegeneration that are distributed throughout the neuron, however, α-synuclein localizes specifically to the nerve terminal, with relatively little in the cell body, dendrites, or extrasynaptic sites along the axon (George et al., 1995; Iwai et al., 1995). Several nonneural tissues including red blood cells also express α-synuclein (Barbour et al., 2008), but the protein is relatively specific to the nervous system (Iwai et al., 1995). In addition, α-synuclein is widely expressed by many neuronal populations within both central and peripheral nervous systems, suggesting a general role in neuronal function. However, α-synuclein appears to be one of the last proteins that localizes to developing synapses, arriving after integral membrane proteins of the synaptic vesicle and the peripheral membrane synapsin proteins (Withers et al., 1997). Consistent with its restriction to the vertebrate lineage, its accumulation at the synapse thus does not appear essential for synapse development or function.

Similar to α-synuclein, the β- isoform also exhibits a presynaptic location (Jakes et al., 1994; Mori et al., 2002; Quilty et al., 2003). Indeed, α- and β- isoforms colocalize at many but not all presynaptic boutons. However, γ-synuclein is expressed by glia and only specific neuronal populations, in particular dopamine neurons (Brenz Verca et al., 2003; Galvin et al., 2001). γ-synuclein is also expressed by a variety of cancers (breast, colon, pancreas) in which it apparently contributes to tumor progression through a number of potential mechanisms (Hua et al., 2009; Inaba et al., 2005; Ji et al., 1997; Pan et al., 2002).

Despite the original association with synaptic vesicles, it has been unclear how α-synuclein localizes to the nerve terminal. In the absence of an obvious transmembrane domain or lipid anchor, synuclein presumably relies on the N-terminal repeats for membrane binding in cells, similar to the observations with artificial membranes made in vitro. However, fractionation of brain extracts reveals a very weak association with synaptic vesicles, and the vast majority of synuclein behaves as a soluble protein (Fortin et al., 2004; Kahle et al., 2000). These observations suggest that the association with native synaptic vesicles is weak, or disrupted, by the procedures required for biochemical fractionation: dilution alone could result in the loss of synuclein from synaptic vesicles. To examine the mobility of synuclein in intact cells, cultured hippocampal neurons were therefore transfected with GFP-tagged synuclein and individual presynaptic boutons subjected to photobleaching. The synaptic fluorescence recovered quite rapidly (within seconds) after photobleaching, indicating that the protein is highly mobile (Fortin et al., 2004). More recently, this approach has been extended in vivo, to cortical neurons of transgenic mice expressing α-synuclein-GFP (Unni et al., 2010). In this case, recovery occurred more slowly (over minutes) but this presumably reflects the altered geometry in vivo, with adjacent synapses (and unbleached synuclein-GFP) simply further away from the bleached boutons. However, synapses with higher expression of synuclein also showed less complete recovery, raising the possibility that aggregation contributes. Not surprisingly, fluorescence recovery after photobleaching in a *C. elegans* model of inclusion formation by synuclein in muscle has shown a substantial immobile fraction (van Ham et al., 2008). The results in neurons with more physiological levels of expression thus indicate that synuclein interacts weakly with elements of the nerve terminal.

Despite its weak interaction with cellular membranes, synuclein nonetheless recovers more slowly after photobleaching than GFP (Fortin et al., 2004), and the N-terminal membrane-binding domain of synuclein seems likely to mediate the interaction. The A30P mutation associated with familial PD in fact disrupts both the association of synuclein with brain membranes and the presynaptic location of synuclein in cultured neurons and accelerates the rate of recovery after photobleaching to that of GFP (Fortin et al., 2004; Jensen et al., 1998). The A30P mutation also impairs the interaction of purified, recombinant synuclein with artificial membranes (Jo et al., 2002). Although less dramatic in vitro than in cells, the effect of the A30P mutation strongly supports a role for membrane binding by the N terminus in presynaptic localization.

How then does synuclein localize specifically to presynaptic boutons rather than other cell membranes? Acidic headgroups are found on the cytoplasmic leaflet of many intracellular membranes, but synuclein has a preference for membranes with high curvature (Jensen et al., 2011; Middleton and Rhoades, 2010), and synaptic vesicles are among the smallest biological

membranes described. Consistent with this, synuclein disperses from presynaptic boutons with stimulation (Fortin et al., 2005), suggesting that it dissociates from the membrane upon delivery to the relatively flat plasma membrane by synaptic vesicle exocytosis.

What confers the specificity of synuclein for membranes with high curvature? Interestingly, the hydrophobic face of the N-terminal α-helix contains a series of threonines at position 3 in the repeat (Figure 1). Although this polar residue might be expected to disrupt hydrophobic interactions with the membrane, threonine is in fact less polar than serine, and the precise positioning of this residue in repeats 2–5 and 7 is highly conserved among all synuclein isoforms. It is therefore possible that threonine at these positions weakens the interaction of synuclein with membranes precisely so that it can acquire specificity for high curvature. To test this possibility, the threonines were replaced by large, nonpolar residues (leucine and phenylalanine) and the recombinant mutant protein indeed loses its specificity for both acidic membranes and small vesicles (Pranke et al., 2011). When expressed in yeast, the mutant also localizes to the plasma membrane rather than to intracellular vesicles, consistent with stronger membrane interaction interfering with the preference for high curvature.

In addition to acidic phospholipid headgroups and high curvature, synuclein appears to have a preference for specific membrane microdomains. Since the large proportion of soluble synuclein makes it difficult to detect a membrane-bound fraction by morphological techniques in most cells other than neurons, digitonin was used to permeabilize selectively the plasma membrane of HeLa cells expressing human α-synuclein, releasing the unbound cytosolic protein (Fortin et al., 2004). The remaining synuclein appeared punctate but failed to colocalize with markers for many organelles. Rather, it colocalized with components of lipid rafts, a membrane microdomain with reduced fluidity that is enriched in cholesterol and saturated acyl chains (Fortin et al., 2004). The PD-associated A30P mutation abolished this localization, supporting the specificity of the interaction, and the biochemical analysis of detergent-resistant membranes by flotation gradient confirmed the localization to rafts. Importantly, the disruption of lipid rafts also prevents the accumulation of synuclein in presynaptic boutons (Fortin et al., 2004), supporting the relevance of this interaction for neurons. In addition to the requirement for acidic phospholipid, biochemical studies in vitro have indicated that synuclein requires a combination of phospholipid with oleoyl as well as polyunsaturated acyl chains (Kubo et al., 2005), suggesting that it may specifically recognize the phase boundary that arises between membranes that differ in fluidity. Remarkably, there was an apparent requirement for the acidic headgroup on the polyunsaturated acyl rather than oleoyl chain (Kubo et al., 2005), raising the possibility of a distinct and previously unknown microdomain in neurons. Further, recent work has found that synuclein can influence lipid packing within raft-like domains containing cholesterol (Leftin et al., 2013), suggesting that synuclein may not simply be recruited by these structures but also contributes to their formation, very similar to other peripheral membrane proteins such as caveolin (Parton and del Pozo, 2013).

It has also been suggested that synuclein might act as a fatty acid binding protein (Sharon et al., 2001). Synuclein promotes the uptake of polyunsaturated fatty acids into cells, and polyunsaturated fatty acids promote the oligomerization of synuclein (Assayag et al., 2007; Perrin et al., 2001; Sharon et al., 2003a, 2003b). Supporting a role for this activity in vivo, the analysis of α-synuclein knockout mice has shown remarkable changes in brain cardiolipin, including acyl chain composition (Ellis et al., 2005). Fatty acid uptake and metabolism also appear affected (Golovko et al., 2005, 2006, 2007), although with only modest changes in other brain phospholipids (Barceló-Coblijn et al., 2007; Rappley et al., 2009b). γ-synuclein knockouts also show only modest brain region-specific changes in both phospholipid headgroup and acyl chain composition (Guschina et al., 2011). Nonetheless, even small changes in acyl chain composition may influence membrane fluidity and trafficking, and the expression of α-synuclein appears to promote clathrin-dependent endocytosis (Ben Gedalya et al., 2009).

## The Role of Synuclein in Neurotransmitter Release

The presynaptic location of synuclein and its interaction with membranes have strongly suggested a role in transmitter release. Several of the original publications indeed reported that α-synuclein promotes release (Cabin et al., 2002; Murphy et al., 2000), but others suggested an inhibitory role. Dopamine release by α-synuclein knockout mice recovers faster from repetitive stimulation than in wild-type animals, and the knockouts show a mild reduction in striatal dopamine stores consistent with increased release (Abeliovich et al., 2000). The analysis of dopamine and norepinephrine release by knockout mice in vivo also shows more rapid facilitation than in wild-type and reduced depression after multiple bursts of stimulation (Yavich et al., 2004, 2006). These effects on dopamine release in vivo are among the most dramatic reported for α-synuclein knockout mice and suggest a major disturbance in the mobilization of synaptic vesicles. However, the findings at glutamate synapses are much less striking (Abeliovich et al., 2000; Chandra et al., 2004).

To mimic the increase in expression that causes PD in families with a duplication or triplication of the gene, α-synuclein has also been overexpressed both in culture and in transgenic mice. Although the overexpression of PD-associated or C-terminal truncation mutants can produce a degenerative process in vivo (Giasson et al., 2002; Gispert et al., 2003; Gomez-Isla et al., 2003; Lee et al., 2002b; Shults et al., 2005; Yazawa et al., 2005), nonviral overexpression of the wild-type protein usually produces little toxicity (Matsuoka et al., 2001). Transgenic mice overexpressing the wild-type human protein do show a number of behavioral abnormalities relating to olfaction, gastrointestinal motility, and motor activity (Fleming et al., 2008; Kuo et al., 2010; Noorian et al., 2012; Wang et al., 2008), suggesting that these animals may reproduce the prodromal phase of PD, but there is little if any detectable degeneration.

To understand how overexpression of synuclein might affect behavior and cause PD, the analysis was extended to synaptic physiology and a direct analysis of the release mechanism. First studied in chromaffin cells, overexpression of the wild-type human protein was found to inhibit the exocytosis of dense core vesicles as measured by direct amperometric recordings

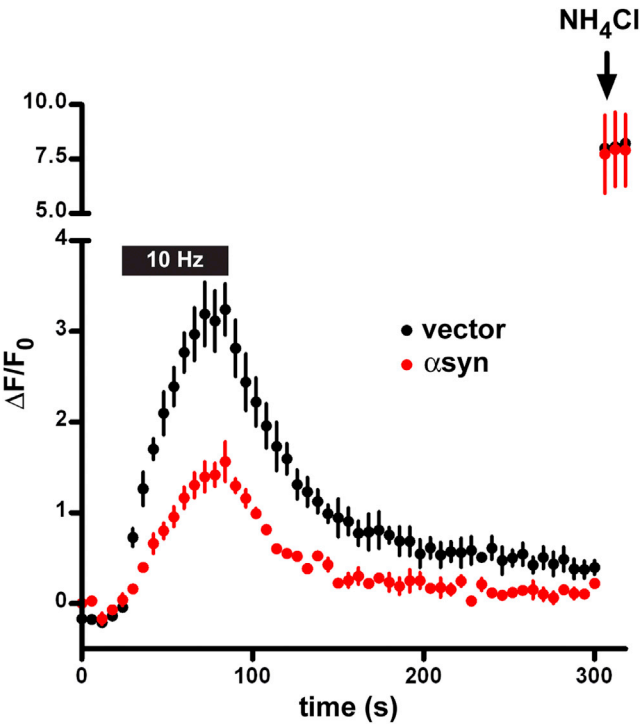


**Figure 2. Overexpression of α-Synuclein in Rat Hippocampal Neurons Inhibits Synaptic Vesicle Exocytosis**

Embryonic rat hippocampal neurons were transfected with VGLUT1-pHluorin and either wild-type human α-synuclein or empty vector. After growth in culture for 2 weeks, the cells were stimulated at 10 Hz for 60 s and the response of VGLUT1-pHluorin monitored. Quenched at the low pH of synaptic vesicles, the pHluorin (a modified form of GFP shifted in its pH sensitivity) becomes more fluorescent when exposed to the external pH during exocytosis. The reacidification that follows endocytosis results in the quenching of fluorescence. $NH_4Cl$ is used to alkalinize all of the intracellular VGLUT1-pHluorin pool and demonstrates that synuclein overexpression does not reduce expression of the reporter. Overexpression of synuclein impairs the exocytosis of synaptic vesicles but has no apparent effect on endocytosis after normalization to peak stimulated fluorescence. (Reproduced from Nemani et al., 2010.)

of quantal catecholamine release (Larsen et al., 2006). This did not involve a change in calcium sensitivity, quantal size, or the kinetics of individual quantal events but rather a reduction in the number of events. The PC12 cell granules also appear to accumulate at the plasma membrane, suggesting a specific defect at or close to the fusion event, and the A30P mutant had an effect surprisingly similar to wild-type human synuclein considering its defect in membrane interactions (Larsen et al., 2006).

In cultured neurons, overexpression of the wild-type human protein at levels that do not produce deposits or obvious toxicity causes an inhibition of synaptic vesicle exocytosis as measured by optical imaging of both hippocampal and midbrain dopamine neurons (Nemani et al., 2010) (Figure 2). Modest overexpression in transgenic mice produced a similar defect in neurotransmission measured by postsynaptic recording at hippocampal CA1 synapses (Nemani et al., 2010). It is also important to note that there was no change in quantal size. Several reports have shown that multimeric synuclein can form pores in artificial membranes in vitro (Rochet et al., 2004; Tsigelny et al., 2007; Volles et al., 2001). This should dissipate the $H^+$ electrochemical gradient that drives neurotransmitter uptake into vesicles; however, the lack of change in quantal size argues further against pore formation by multimers, at least in these cells. Although previous work on the role of synuclein in transmitter release had identified major defects only in monoamine neurons, these findings indicated that the disturbance with overexpression is more general.

Imaging further demonstrated a specific defect in exocytosis, with no change in the endocytosis of synaptic vesicle membrane despite the effects on clathrin-dependent endocytosis observed in other cells (Ben Gedalya et al., 2009). In contrast to LDCV release by chromaffin cells (Larsen et al., 2006), the A30P mutation abolishes the effect of synuclein overexpression on synaptic vesicle exocytosis (Nemani et al., 2010). Presumably, the specific accumulation of synuclein at release sites (disrupted by the A30P mutation) is more important for neurons, with many long processes, than for small, compact chromaffin cells. However, electron microscopy in the transgenic mice overexpressing synuclein also showed a dispersion of synaptic vesicles away from the active zone and into the axon (Nemani et al., 2010), and it is more difficult to reconcile this observation with the accumulation of secretory granules at the plasma membrane in chromaffin cells that overexpress synuclein (Larsen et al., 2006). Recent ultrastructural analysis of a different transgenic mouse line has shown enlargement of boutons and convoluted internal membranes connected to the cell surface (Boassa et al., 2013).

The precise nature of the defect in synaptic vesicle exocytosis remains unclear. Interestingly, the transgenic mice show a reduction in synapsins and complexin, consistent with a change in exocytosis. Subsequent work has also shown a defect in transmitter release with overexpression of synuclein in hippocampal cultures. However, this study described "vacant synapses" that were devoid of synaptic vesicle proteins and contained abnormal membranes that were not observed in other systems, suggestive of toxicity (Boassa et al., 2013; Scott et al., 2010). Importantly, defective endocytosis often accompanies toxicity, and no such "vacant synapses" were observed in another study (Nemani et al., 2010). It remains possible that even in the absence of aggregates and overt injury, toxic oligomers account for the inhibition of release by synuclein observed by multiple groups. However, the ability of the PD-associated A30P mutation to block the inhibition of release argues against this possibility. In addition, truncation of the C terminus, which promotes aggregation of synuclein in vitro and in vivo (Crowther et al., 1998; Hoyer et al., 2004; Li et al., 2005; Wakamatsu et al., 2008), had no effect on the inhibition of release, supporting a specific effect of synuclein independent of toxicity.

Although the phenotype of the single α-synuclein knockout is relatively modest, the animals show a remarkable resistance to the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) (Dauer et al., 2002). Exposure to MPTP results in the loss of substantia nigra dopamine neurons due to uptake of the active metabolite N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine ($MPP^+$) by the reuptake transporters for monoamines (Javitch et al., 1985), followed by apoptosis triggered by inhibition of the respiratory chain (Krueger et al., 1990). The vesicular monoamine transporter (VMAT) protects against

$MPP^+$ toxicity by sequestering the toxin inside secretory vesicles, away from mitochondria, and selection in $MPP^+$ was used to isolate the cDNA encoding VMAT (Liu et al., 1992a, 1992b). Subsequent work has confirmed the protection against MPTP toxicity conferred by loss of α-synuclein (Drolet et al., 2004; Fornai et al., 2005; Fountaine et al., 2008; Robertson et al., 2004; Thomas et al., 2011), but strains apparently differ in the magnitude of this effect (Schlüter et al., 2003). In α-synuclein knockout mice, mitochondria are not affected by MPTP administration, suggesting a defect in access, but the activities of monoamine transporters known to control access of the toxin appear no different from wild-type (Dauer et al., 2002). Thus, resistance to MPTP toxicity is one of the more robust aspects of the α-synuclein knockout phenotype, but the mechanism remains unknown. Although MPTP toxicity differs in important ways from PD, the ability of the α-synuclein knockout to protect against the toxin suggests a role for the normal function of synuclein in the pathogenesis of degeneration, particularly since overexpression of synuclein does not increase vulnerability to MPTP (Thomas et al., 2011).

The existence of three synuclein isoforms, in many cases expressed by the same cells, has raised the possibility that redundancy accounts for the modest phenotype of α-synuclein knockout mice. However, the analysis of α-/β-synuclein double knockout mice also showed no major change in synapse structure or excitatory transmission measured electrophysiologically in hippocampal region CA1 (Chandra et al., 2004). On the other hand, the α-/β- double knockout does show a modest reduction in striatal dopamine levels. In addition, α-/γ- double and synuclein triple knockouts show a substantial increase in striatal dopamine release in vivo not observed with the single knockouts (Anwar et al., 2011; Senior et al., 2008). These mutants did not exhibit a change in dopamine transporter activity or tissue dopamine levels, implicating a specific alteration of dopamine release. The mechanism remains unknown, but the α-/β- double knockout shows an increase in complexin (Chandra et al., 2004). Interestingly, synuclein overexpression reduces complexin levels (Nemani et al., 2010), suggesting that overexpression can increase the normal activity of synuclein and that an increase in the normal function of synuclein contributes to the degeneration produced by its upregulation.

At hippocampal synapses, the effect of the triple knockout has been controversial. According to one report from the Südhof laboratory, there was no change in baseline transmitter release (Burré et al., 2010). However, an independent report by a former member of the same group showed an increase in transmitter release in the triple knockout (Greten-Harrison et al., 2010). The increase was small, possibly accounting for the failure to detect a change by others and raising the possibility that any change in release might be secondary. Indeed, the loss of all three synuclein genes results in smaller presynaptic boutons (Greten-Harrison et al., 2010), suggesting an alternative role for these proteins.

Previous work has shown a strong genetic interaction between synuclein and the degeneration produced by loss of the presynaptic chaperone cysteine string protein (CSPα) (Chandra et al., 2005). Knockout of CSPα does not affect synaptic transmission shortly after birth but eventually results in rapidly progressive synaptic degeneration and death within 2 months (Fernández-Chacón et al., 2004). CSPα thus does not itself appear required for transmitter release but rather serves to maintain the function of the nerve terminal over a longer time frame. Work from the Südhof laboratory has now suggested that synuclein may have a similar role in maintenance of the nerve terminal, rather than transmitter release.

Remarkably, the overexpression of α-synuclein greatly delays the degeneration due to loss of CSPα, and the loss of synuclein exacerbates the CSPα knockout phenotype (Chandra et al., 2005), suggesting that synuclein may have a role as chaperone, very similar to CSPα. CSPα appears particularly important for the levels of t-SNARE SNAP-25 (Sharma et al., 2011, 2012). As might be anticipated for a chaperone of the transmitter release machinery, the resulting perturbations of SNARE complex assembly are activity dependent. Since synuclein overexpression inhibits transmitter release, the resulting decrease in activity might account for rescue of the CSPα phenotype. However, synuclein overexpression rescues the reduction in SNARE complex, not the decrease in SNAP-25 protein levels. The results suggest that whereas CSPα has a specific role with SNAP-25 that secondarily affects SNARE complex levels, synuclein has a specific role in SNARE complex formation and can bypass the defect in SNAP-25. The original work did not detect biochemical evidence of α-synuclein associating with the presynaptic SNARE complex (Chandra et al., 2005), but a subsequent study did identify a direct biochemical interaction (Burré et al., 2010). In particular, the hydrophilic C terminus of α-synuclein appears to interact with v-SNARE synaptobrevin 2 (Burré et al., 2010). Consistent with a requirement for the C terminus of α-synuclein to interact with synaptobrevin, γ-synuclein, which diverges in sequence from α- at the C terminus, does not rescue the loss of CSPα (Ninkina et al., 2012). In contrast to the role of CSPα as chaperone for SNAP-25, α-synuclein thus appears to have a role in SNARE complex formation.

How can a putative chaperone for the SNARE complex either have no effect on or inhibit transmitter release? The number of SNARE complexes may not be rate limiting for transmitter release, and rescue of the degeneration in CSPα knockout mice does not require an increase in SNAP-25. Regardless of mechanism, SNARE complex levels correlate more closely with the degenerative process than with transmitter release. However, the levels of SNARE complex have not been studied extensively in animals with other defects in transmitter release and may simply reflect changes in another process more directly affected by synuclein. Indeed, we do not know what comprises the total pool of SNARE complexes in the brain—*cis* complexes on synaptic vesicles or the plasma membrane, *trans*-complexes made by docked vesicles or some other pool? Recent work in vitro has also found that synuclein can inhibit membrane fusion independent of the SNARE proteins and failed to detect an interaction of synuclein with synaptobrevin (DeWitt and Rhoades, 2013). The mechanism by which synuclein rescues the loss of CSPα thus remains uncertain.

The synuclein triple knockouts do die prematurely but at around 1 year, a phenotype much milder than the CSP knockout (Fernández-Chacón et al., 2004; Greten-Harrison et al., 2010). In addition to smaller presynaptic boutons, the synuclein triple

knockout also produces an axonal defect in older animals but no obvious synapse loss. The ability to rescue loss of CSPα thus remains perhaps the most dramatic effect of synuclein observed in vivo, with a very modest degenerative phenotype in synuclein triple knockout mice alone.

Synuclein has also been reported to interact biochemically with a large number of proteins that might regulate its activity. One of the first identified, synphilin appears to promote the aggregation of synuclein (Engelender et al., 1999; McLean et al., 2001; Ribeiro et al., 2002). Synphilin also localizes to the nerve terminal in a synuclein-sensitive manner, but the physiological role of synphilin and its interaction with synuclein remain unclear. Tubulin appears to interact with a multimeric form of synuclein, and synuclein can influence the microtubule cytoskeleton (Lee et al., 2006). However, the functional ramifications of this interaction seem more relevant for the toxicity associated with synuclein than for its normal function (Alim et al., 2002; Chen et al., 2007; Kim et al., 2008; Lee et al., 2006). Since synuclein binds to membranes in an α-helical conformation, one interesting approach has been to use membrane-bound synuclein as a probe for conformation-specific interacting proteins (Woods et al., 2007). This again resulted in the isolation of tubulin but also other proteins associated with the cytoskeleton. In addition, this approach identified one novel protein that is natively unfolded until membrane bound (Boettcher et al., 2008). More recently, the small GTPase rab3a has been proposed to regulate the membrane association of α-synuclein in a GTP-dependent manner (Chen et al., 2013), suggesting functional integration of synuclein into the cycling of this synaptic vesicle rab and hence into the synaptic vesicle cycle. However, the role of these potential regulatory mechanisms remains unclear, largely because we do not understand the normal function of synuclein.

## The Synucleinopathies

Although the normal function of synuclein remains elusive, the protein has a central role in multiple neurodegenerative processes. Indeed, the identification of mutations in α-synuclein has shifted the focus of work on the pathogenesis of PD from a specific defect in dopamine neurons to a more widespread disturbance in the behavior of this protein. Previously, Lewy bodies had been detected by staining with hematoxylin and eosin and with somewhat more sensitivity by immunostaining for ubiquitin. However, immunostaining for α-synuclein revealed much more widespread deposits in dystrophic neurites as well as Lewy bodies of cell populations not previously known to be affected (Galvin et al., 1999; Spillantini et al., 1997, 1998b). In addition to demonstrating the relevance of synuclein for the idiopathic disorder, these observations have suggested a basis for the nonmotor manifestations of PD (Ahlskog, 2007; Dickson et al., 2009; Jellinger, 2011). Constipation, hyposmia, depression, and rapid eye movement (REM) behavior disorder, which involves the loss of muscle atonia during REM sleep and hence unsuppressed motor activity while dreaming, can precede the onset of characteristic parkinsonian motor symptoms by up to two decades, consistent with the deposition of α-synuclein in the enteric nervous system, olfactory bulb, dorsal motor nucleus of the vagus, and glossopharyngeal nerves, as well as other brainstem nuclei (Postuma et al., 2012). Additional autonomic problems (e.g., orthostatic hypotension, incontinence) become more symptomatic in advanced disease, and synuclein deposits in autonomic ganglia (Iwanaga et al., 1999; Orimo et al., 2008). Cortical deposits of synuclein that occur late in the disease presumably contribute to cognitive problems. Certain nonmotor manifestations of PD can respond to dopamine replacement, raising questions about the significance of synuclein deposition outside the nigrostriatal projection. However, many symptoms do not respond, and the widespread accumulation of synuclein presumably accounts for many of the dopamine-resistant symptoms.

It is important to note that the relationship between α-synuclein deposition and neuronal dysfunction remains unclear. In the substantia nigra, substantial cell loss occurs before symptoms develop, suggesting that protein deposition is not as important as cell loss. However, cell loss may not accompany synuclein deposition elsewhere. In the enteric nervous system, Lewy pathology is indeed not associated with cell loss (Annerino et al., 2012), raising the possibility of a functional rather than anatomic disturbance. On the other hand, synuclein deposition itself may not even produce dysfunction, and pathologic investigation of many older individuals (up to 30% of centenarians) reveals extensive synucleinopathy (incidental Lewy body disease) with no clear neurological symptoms (Ding et al., 2006; Markesbery et al., 2009). Indeed, synuclein aggregation may represent a neuroprotective response, with a different species of synuclein responsible for dysfunction. Although synuclein deposition has thus superseded cell loss as evidence of degeneration, its actual role in the degenerative process remains unknown.

α-synuclein has also been implicated in at least two other disorders, multiple system atrophy (MSA) and dementia with Lewy bodies (DLB). Interestingly, these conditions also produce clinical parkinsonism but involve the deposition of α-synuclein in different cells from those affected by typical PD. MSA can begin with parkinsonism, autonomic failure, or cerebellar ataxia but usually progresses to involve one or both of the other components, resulting in the recognition that these initially disparate conditions reflect a common disorder. However, the parkinsonism observed in MSA does not generally respond well to dopamine replacement, presumably because the pathology affects many cell populations in addition to dopamine-producing cells of the substantia nigra, including postsynaptic medium spiny neurons in the striatum (Papp and Lantos, 1994; Sato et al., 2007). In contrast, PD affects preferentially the dopamine neurons, with spared postsynaptic cells still responsive to dopamine replacement.

In MSA, α-synuclein deposits in glial (generally oligodendroglial) cytoplasmic inclusions (GCIs) (Spillantini et al., 1998a; Tu et al., 1998) rather than in the neuronal Lewy bodies or dystrophic neurites more characteristic of PD. The expression of α-synuclein in oligodendrocytes reproduces the MSA phenotype, suggesting that the protein there is causative (Shults et al., 2005; Yazawa et al., 2005). Remarkably, α-synuclein is not normally expressed by oligodendrocytes (Miller et al., 2005), and a fundamental question remains about the origin of this protein: is it taken up from neurons, or does the pathological process activate expression by glia? In fact, the pathology shows relatively little deposition of synuclein in neurons, with

only occasional nuclear and cytoplasmic inclusions (Farrer et al., 2004; Jellinger and Lantos, 2010; Nishie et al., 2004b). At the same time, oligodendrocytes do not seem to upregulate expression of α-synuclein even in MSA (Miller et al., 2005). Regardless of its source, α-synuclein accumulates to particularly high levels in MSA, suggesting a process distinct from Lewy pathology. In addition, GCI lesions are widespread in MSA but generally correlate with neuron loss in the substantia nigra, pons, cerebellum, and intermediolateral cell columns of the spinal cord, suggesting that the glial process may be primary.

MSA is rarely familial (Soma et al., 2006) and mutations in α-synuclein have not been observed (Ozawa et al., 2006). However, polymorphisms in the synuclein gene may influence susceptibility to MSA (Al-Chalabi et al., 2009; Scholz et al., 2009). In addition, the analysis of familial MSA has recently identified mutations in COQ2, a protein required for the synthesis of coenzyme Q10 (Multiple-System Atrophy Research Collaboration, 2013). The degenerative process in MSA may thus reflect a primary lesion in mitochondria.

DLB more closely resembles idiopathic PD in terms of Lewy body pathology. Although DLB appears to be a distinct syndrome, with early cognitive impairment, fluctuating alertness, and visual hallucinations in addition to progressive parkinsonism, the distribution of Lewy pathology appears remarkably similar to that observed in PD, with a brainstem-predominant form and others involving the cortex as well (McKeith et al., 2005). Like PD and MSA, DLB also involves primarily the deposition of α-synuclein.

Lewy pathology was originally considered to involve only α-synuclein, but β- and γ- can deposit in both PD and DLB (Galvin et al., 1999). Similar to α-synuclein, β- accumulates presynaptically in PD, but γ- forms axonal spheroids. β-synuclein has been suggested to ameliorate the toxicity of α-synuclein by reducing either its aggregation or its expression (Fan et al., 2006; Hashimoto et al., 2001). However, polymorphisms in β-synuclein predispose to DLB (Ohtake et al., 2004), and transgenic mice overexpressing the variant develop degeneration and behavioral abnormalities (Fujita et al., 2010). These animals do not develop typical Lewy pathology, but they do accumulate both α- and β-synuclein in axonal spheroids (Fujita et al., 2010). Indeed, β-synuclein appears as toxic as α-synuclein to cultured neurons (Taschenberger et al., 2013). Mutations in γ-synuclein have not been identified as a cause of familial PD or related disorders, and it has generally been considered nontoxic, but recent overexpression of even this isoform has produced degeneration in transgenic mice (Ninkina et al., 2009), and a polymorphism has also been linked to DLB (Nishioka et al., 2010). Rather than contribute to disease simply through a decline in their protective function (Li et al., 2004; Rockenstein et al., 2001), which nonetheless remains a possibility, β- and γ-synuclein may thus cause degeneration.

α-synuclein also deposits in other neurodegenerative disorders. Alzheimer's disease shows Lewy pathology in up to 60% of cases but is more often restricted to the amygdala than in PD or DLB (Hamilton, 2000; Leverenz et al., 2008; Uchikado et al., 2006). Neurodegeneration with brain iron accumulation due to mutations in pantothenate kinase type 2 also exhibit Lewy pathology labeling for α-synuclein and neuroaxonal spheroids labeling for β- and γ- (Galvin et al., 2000; Wakabayashi et al., 2000). Thus, synucleins accumulate in a variety of neurodegenerative processes, suggesting either that they are sensitive reporters for specific cellular defects or that they participate in the response to injury.

## Expression and Clearance

In addition to point mutations, duplication and triplication of the chromosomal region surrounding the α-synuclein gene have been found to produce dominantly inherited PD (Ahn et al., 2008; Singleton et al., 2003). The affected chromosomal region contains several other genes as well, but the neuropathology reveals deposition of synuclein (Seidel et al., 2010; Yamaguchi et al., 2005), and the phenotype most likely reflects multiplication of the α-synuclein gene. In this case, the sequence of synuclein is wild-type, making the important prediction that a simple increase in the protein rather than a change in its properties suffices to produce PD. The duplication produces a form of PD similar in onset and symptoms to the sporadic disorder, but the triplication causes an exceptionally severe phenotype, with much earlier onset and prominent cognitive as well as motor impairment (Ahn et al., 2008; Ibáñez et al., 2004; Ross et al., 2008).

The more global neurologic and behavioral deficits observed with gene multiplication and point mutation presumably reflect a generalized increase in synuclein by all of the neurons that normally express the gene, and α-synuclein is very widely expressed under normal conditions (Iwai et al., 1995). In contrast, the preferential involvement in sporadic PD of particular systems such as the nigrostriatal projection presumably reflects the upregulation of synuclein within specific cells. Indeed, genome-wide association studies of risk in idiopathic PD reveal the largest contributions from the synuclein gene itself (as well as the microtubule-associated protein tau) (Simón-Sánchez et al., 2009).

Consistent with a role for the amount of α-synuclein expressed in the degenerative process, the polymorphisms implicated in PD lie outside the protein-coding region of the gene and thus presumably affect mRNA expression. Indeed, one polymorphism has been shown to influence expression of a reporter gene in vitro (Chiba-Falek and Nussbaum, 2001). In addition, the most common inherited form of PD, due to mutations in leucine-rich repeat kinase 2 (LRRK2), generally involves Lewy pathology that may also reflect upregulation of α-synuclein gene expression (Carballo-Carbajal et al., 2010). Further, α-synuclein has been repeatedly identified as a gene responsive to toxic insult and growth factors. Injection of the toxin quinolinic acid directly into the striatum upregulates α-synuclein in the substantia nigra (Kholodilov et al., 1999), and oxidative stress due to insecticide or the loss of oxidant defenses also increases α-synuclein (Gillette and Bloomquist, 2003; Gohil et al., 2003). MPTP, rotenone, and paraquat produce or exacerbate synuclein deposition, and synuclein can protect against some agents (paraquat) but not others (MPTP) (Fornai et al., 2005; Manning-Bog et al., 2002, 2003; Przedborski et al., 2001). Synuclein may thus upregulate in response to many forms of injury but help to alleviate only some and exacerbate others. Perhaps consistent with a protective role, nerve growth factor induces α-synuclein expression in PC12 cells and basic fibroblast growth factor in midbrain

dopamine neurons (Rideout et al., 2003; Stefanis et al., 2001). Despite these in vitro observations, however, the mechanisms that regulate synuclein expression in vivo remain poorly understood, particularly under physiological circumstances. Interestingly, microRNA-7, which downregulates α-synuclein expression, itself decreases during MPTP toxicity, providing a mechanism for the upregulation of synuclein in response to injury (Junn et al., 2009).

In addition to production, clearance can regulate the levels of α-synuclein. Like other natively unfolded proteins, synuclein was originally thought to be degraded by the proteasome without a requirement for ubiquitination (Bennett et al., 1999; Rideout and Stefanis, 2002; Tofaris et al., 2001). However, it was subsequently found that monoubiquitination apparently promotes the degradation of synuclein by the proteasome, and this modification can be bidirectionally controlled by a specific ubiquitin ligase (SIAH-2) and deubiquitinase (USP9X) (Liani et al., 2004; Rott et al., 2011). In addition, considerable evidence has also accumulated to suggest the clearance of synuclein at the lysosome. Initially thought to promote the clearance of synuclein aggregates by macroautophagy, degradation in the lysosome also contributes to the turnover of soluble oligomers and even apparently monomeric synuclein under physiological conditions (Lee et al., 2004; Mak et al., 2010; Rideout et al., 2004). Indeed, chaperone-mediated autophagy (CMA), a process that targets individual, soluble proteins to the lysosome for proteolysis, contributes to the clearance of synuclein, and synuclein can in turn disrupt CMA, altering the turnover of other CMA-dependent proteins (Cuervo et al., 2004; Vogiatzi et al., 2008). Interestingly, ubiquitination by the ligase Nedd4 has also been shown to target synuclein for degradation in the lysosome, rather than by the proteasome (Tofaris et al., 2011).

Although we do not know how changes in the expression of synuclein may actually influence the development of human PD, recent work has suggested that changes in clearance may promote degeneration. In particular, idiopathic PD has been found to associate with mutations in the glucocerebrosidase (*GBA1*) gene. Mutations in *GBA1* are responsible for Gaucher's disease, a recessive lysosomal storage disorder. However, the spectrum of phenotypes in Gaucher's disease is very broad, with type 2 dying within the first 2 years of life and type 1 surviving longer. Indeed, some of the so-called nonneuropathic type 1 patients eventually develop parkinsonism among other neurological problems (Alonso-Canovas et al., 2010; Neudorfer et al., 1996). In addition, it has now become clear that heterozygotes with no overt symptoms of Gaucher's disease develop PD at higher rates than controls. *GBA1* mutations have been found in ~7% patients with idiopathic PD, and up to ~30% of Ashkenazi Jewish patients, with only 1.3% in the general population (Sidransky and Lopez, 2012; Sidransky et al., 2009). Mutations in GBA1 have also been reported in DLB but not MSA (Farrer et al., 2009; Segarane et al., 2009), supporting the difference in mechanism between MSA and Lewy related pathology.

*GBA1* mutations presumably increase susceptibility to PD by blocking the lysosomal degradation of α-synuclein (Manning-Boğ et al., 2009), but it has been difficult to understand how a modest reduction in enzyme activity could impair lysosomal function enough to produce a degenerative disorder. Recent work has indeed suggested that α-synuclein accumulates in both a mouse model of Gaucher's disease and induced pluripotent (iPS) cells from patients with Gaucher's disease but attributed the increase to aggregation in the presence of increased membrane glucocerebroside (Mazzulli et al., 2011). Consistent with the localization of synuclein to lipid rafts (which are enriched in sphingolipids such as glucocerebroside) (Fortin et al., 2004), and its preference for particular lipid acyl chains as well as head groups (Davidson et al., 1998; Kubo et al., 2005), the mechanism by which *GBA1* mutations confer susceptibility to PD may involve specific effects on cell membranes rather than a more general disturbance in lysosomal function that simply upregulates the normal protein. Indeed, much of the work on synuclein has focused on its misfolding and aggregation. Since many publications have addressed the pathways to misfolding of α-synuclein, including several recent reviews (Breydo et al., 2012; Goedert et al., 2013; Lashuel et al., 2013), we will now summarize the most salient observations.

## Misfolding

In contrast to its helical conformation on membranes, synuclein adopts a β sheet structure in aggregates. Indeed, Lewy bodies and neurites contain 5–10 nm filaments that appear to be composed primarily if not exclusively of α-synuclein (Spillantini et al., 1998b). In brainstem-type Lewy bodies, the pale-staining halo, which contains filaments by electron microscopy, labels more strongly for α-synuclein than the acidophilic core (Goedert et al., 2013). Dystrophic neurites and the less discrete cortical-type Lewy bodies contain similar filaments (Marui et al., 2002). Although Lewy bodies were originally considered by some an artifact of the degenerative process, the identification of α-synuclein mutations in familial PD demonstrated a causative role for the major component of Lewy-related pathology. However, it is important to remember that this is not the same as establishing a causative role for Lewy pathology in the degenerative process.

Recombinant synuclein also forms filaments after incubation in vitro for a protracted period (Conway et al., 1998). By X-ray diffraction, these filaments adopt a cross-beta structure characteristic of amyloid (Sawaya et al., 2007; Serpell et al., 2000). Recent solid-state NMR has also begun to analyze fibrils at high resolution, identifying the repeated units that underlie this structure (Comellas et al., 2011). Since aggregation has been considered the critical event in the pathogenesis of PD, the in vitro assay has received considerable attention. The point mutations originally identified in familial PD (A53T, A30P, and E46K) were originally proposed to accelerate aggregation, but the A30P mutant appears to form fibrils more slowly than the wild-type, although oligomerization may be enhanced (Conway et al., 2000; Giasson et al., 1999; Li et al., 2001; Narhi et al., 1999). β-synuclein does not fibrillize and both β- and γ- can inhibit the aggregation of α-synuclein in vitro and in vivo (Hashimoto et al., 2001; Uversky et al., 2002), but as noted above, β- and γ- can still contribute to disease (see Synucleinopathies above), suggesting that tendency to aggregate may not correlate closely with potential to cause degeneration.

Many other putative pathogenic factors have also been tested for their ability to influence the aggregation of synuclein, either through direct modification of the protein or indirectly through

effects on its environment. α-synuclein does not contain any cysteines but can undergo nitration and methionine oxidation in response to oxidative stress (Breydo et al., 2012; Giasson et al., 2000a). However, these modifications do not appear to promote aggregation. Similarly, the α-synuclein that deposits in Lewy bodies appears more heavily phosphorylated at Ser-129 than the soluble protein (Fujiwara et al., 2002; Nishie et al., 2004a). Phosphorylation indeed appears to promote synuclein aggregation (Smith et al., 2005b), but it remains unclear whether phosphorylation at this site actually promotes degeneration, with divergent effects in different models (Azeredo da Silveira et al., 2009; Chen and Feany, 2005). Deletion of the C terminus promotes both aggregation of synuclein in vitro and pathological changes in vivo, suggesting an important role for proteolysis in cells (Li et al., 2005; Murray et al., 2003; Periquet et al., 2007; Tofaris et al., 2006; Ulusoy et al., 2010). Environmental factors may also predispose to synuclein aggregation, and heavy metals appear to promote deposition of the protein in cells as well as in vitro (Breydo et al., 2012; Paik et al., 1999).

It also remains unclear whether synuclein fibrils promote toxicity. First, as noted above, the A30P mutation causes familial PD but does not promote fibrillization (Conway et al., 2000). Second, protein aggregation is not always accompanied by cell loss in a viral model of PD (Lo Bianco et al., 2002). In a *Drosophila* model, aggregates can occur in the absence of toxicity—the chaperone hsp70 can ameliorate the toxicity of α-synuclein without affecting inclusions (Auluck et al., 2002). The recently identified PD-associated α-synuclein mutant G51D also oligomerizes more slowly than wild-type α-synuclein but produces a severe form of degeneration, with early onset and pyramidal as well as extrapyramidal deficits (Lesage et al., 2013). In addition, dopamine has been suggested to promote the aggregation of α-synuclein but not the formation of amyloid (Bisaglia et al., 2010; Herrera et al., 2008; Rekas et al., 2010). Indeed, dopamine appears to stabilize synuclein aggregation at the stage of protofibrils, and oligomers of synuclein appear more toxic than fibrils (Norris et al., 2005; Rochet et al., 2004). There are multiple cellular mechanisms that regulate the cytosolic concentration of monoamines, from vesicular monoamine transport to feedback inhibition of tyrosine hydroxylase (Fon et al., 1997; Mosharov et al., 2003, 2009), and a change in any of these may thus increase the interaction with synuclein to produce degeneration.

Taken together, these results suggest that soluble, oligomeric forms of α-synuclein rather than fibrils may be responsible for toxicity. However, it is even possible that the monomeric form contributes. Indeed, gene multiplication causes a substantially more severe form of PD than the point mutations, and the amount of synuclein rather than its altered properties may be the principal factor that increases susceptibility to degeneration. It is also important to note that although many publications report the formation of aggregates in transfected cells, often in response to toxic insult, α-synuclein in fact rarely forms aggregates detectable by immunofluorescence in transfected cells (R.H.E., unpublished data). The principal form of synuclein in cells thus appears to be monomer or soluble oligomer.

Although the normal function of synuclein has often been considered irrelevant for its role in degeneration, the interaction of synuclein with membranes, which presumably involves a helical conformation and reflects its normal function, has been reported to influence misfolding and aggregation. As anticipated from the structural studies, one report has shown that membrane association inhibits synuclein oligomerization (Zhu and Fink, 2003), but others have suggested that oligomerization occurs on membranes (Jo et al., 2000; Lee et al., 2002a) and can be promoted by polyunsaturated fatty acids (Perrin et al., 2001). It is important to recognize that the oligomers formed on membranes may be helical, as suggested by the recent work using nanoparticles (Varkey et al., 2013); however, recent NMR and EM have shown directly that anionic phospholipid membranes can convert helical α-synuclein into fibrils (Comellas et al., 2012). It will now be important to determine how membranes influence the conformation and oligomerization of synuclein in cells.

## Propagation and Prions

Careful neuropathologic examination of synuclein deposition in brains with Lewy pathology (from incidental Lewy body disease to end-stage PD) has suggested that the degenerative process advances through the nervous system along specific anatomic pathways (Braak et al., 2003). The first synuclein deposits arise in either the dorsal motor nucleus of the glossopharyngeal and vagal nerves or the olfactory bulb (stage 1). In stage 2, the medulla and pontine tegmentum develop Lewy pathology and only in stage 3 does synuclein deposition occur in the midbrain as well as amygdala. At this point, the typical motor manifestations of PD generally appear. In stage 4, α-synuclein deposits in temporal cortex, and in stages 5 and 6 in neocortex, presumably contributing to the cognitive deficits observed in LBD and advanced PD. A minority of cases do not fit this pattern, and isolated Lewy pathology can arise in the amygdala of patients with AD, but the progression otherwise appears quite stereotyped (Dickson et al., 2010). The Braak staging of Lewy pathology presumably accounts for the development of symptoms such as hyposmia and REM behavior disorder up to decades before the onset of typical parkinsonism. It has also suggested a portal of entry for the disease in either the olfactory mucosa or the gastrointestinal tract. Indeed, the retrospective analysis of routine colon biopsies has recently shown synuclein deposits in the enteric nervous system years before the clinical onset of PD, suggesting a useful and accessible biomarker (Shannon et al., 2012). However, it remains unclear whether the process originates in the gut, spreading to the CNS through the vagal nerves rather than the spinal cord, or arises independently at multiple sites in sympathetic as well as parasympathetic nerves (Bloch et al., 2006; Iwanaga et al., 1999; Orimo et al., 2008). How then does misfolded synuclein spread between neurons—directly, or through effects on the cell that indirectly promote misfolding?

Several observations have suggested that misfolded synuclein may propagate through a prion-like mechanism (Lee et al., 2010b; Olanow and Prusiner, 2009). First, fetal dopamine cells transplanted into the striatum of patients with PD were found to develop Lewy pathology when examined neuropathologically one to two decades later (Kordower et al., 2008; Li et al., 2008). The clear implication is that the normal synuclein expressed by these cells begins to misfold and aggregate after exposure to the abundant misfolded α-synuclein of the host. This has indicated limits to the therapeutic potential of grafts

but also suggested a key feature of prions, the ability of misfolded protein to act as a template for conversion of the normal species to an abnormal conformation. Like the form of α-synuclein associated with membranes, the normal cellular form of the prion protein PrP(c) indeed appears predominantly helical, whereas the pathogenic form Prp(Sc), like the α-synuclein in Lewy pathology, is mostly β sheet (Colby and Prusiner, 2011). In the absence of spread between organisms, PD clearly differs from typical prion disorders such as Jakob-Creutzfeldt disease, scrapie, and bovine spongiform encephalopathy but may use a similar mechanism to amplify the pathogenic species at the level of the protein, without a need for nucleic acid (Prusiner, 2001). Second, the apparent inability of oligodendrocytes to make α-synuclein under either normal or pathologic circumstances (Miller et al., 2005; Spillantini et al., 1998a; Tu et al., 1998) requires a mechanism for transfer from the site of production, presumably in neurons, to the GCIs of MSA.

It was not initially clear how a cytosolic protein like synuclein might spread between cells—PrP is a lipid-anchored protein facing the cell exterior. However, it was recognized even before recent interest in the prion hypothesis for PD that small amounts of α-synuclein can undergo secretion through a vesicular mechanism (Lee et al., 2005). More recently, it has become apparent that synuclein release can involve exosomes, the luminal membranes of multivesicular bodies (mvbs) normally targeted for degradation by the lysosome (Emmanouilidou et al., 2010). This is particularly plausible because mvbs form through the invagination of endosomal membranes and would thus be expected to trap cytosolic proteins such as synuclein. Of course, this would also imply the regulated release of other cytosolic proteins, and the full extent of this mechanism for release remains unclear. It is also possible that oligomeric forms of synuclein, perhaps enriched on the pathway to degradation by the lysosome, become particularly susceptible to release. In addition, this release appears capable of calcium-dependent regulation (Lee et al., 2005; Paillusson et al., 2013), providing an activity-dependent mechanism for propagation that may be relevant for spread along synaptically connected pathways. However, it remains unclear how much of the synuclein released occurs through exosomes as opposed to other, less well-characterized pathways.

Several reports have also documented the uptake of fibrillar synuclein by cells and its ability to produce aggregates composed primarily of the endogenous, host cell protein. Initially, propagation involved either cell extracts including proteins other than α-synuclein or required transduction of preformed recombinant fibrils into cells overexpressing synuclein (Desplats et al., 2009; Luk et al., 2009). It was shown subsequently, however, that fibrils of recombinant synuclein can enter neurons directly by endocytosis and seed the formation of aggregates resembling Lewy pathology in cells that express only endogenous levels of synuclein (Volpicelli-Daley et al., 2011). The mechanism of uptake remains poorly understood, but glia can also take up synuclein derived from neurons, suggesting a mechanism for the formation of GCIs in MSA (Lee et al., 2010a), although it remains unclear how the process could propagate in the absence of any endogenous glial α-synuclein.

Synuclein also appears capable of spread between cells in vivo. Similar to the human transplants described above, cells transplanted into a transgenic animal model can acquire misfolded synuclein from the adjacent tissue and form aggregates (Desplats et al., 2009). Direct injection of fibrillar recombinant synuclein into transgenic mice overexpressing the PD-associated A53T mutant also promotes aggregate formation and disease, with knockouts protected against any pathologic changes (Luk et al., 2012b). However, these transgenic animals would develop degeneration even without injection. More recently, it has been possible to inject fibrils of recombinant mouse α-synuclein into the striatum of wild-type mice, resulting in synuclein aggregates in the substantia nigra, dopamine cell loss, and parkinsonian deficits (Luk et al., 2012a), and this model of propagation has come the closest yet to demonstrating propagation of the misfolded protein under relatively normal circumstances in vivo. Nonetheless, it still involves injection of extremely large amounts of fibrillar synuclein, and the involvement of dopamine neurons requires only uptake of the fibrils in the striatum, not actually propagation between neurons. Deposits were described in other brain regions such as the cortex and thalamus (Luk et al., 2012a), but at least some of these also project directly to the dorsal striatum and do not require spread between neurons. Regardless, a prion-like mechanism of transmission suggests that improved clearance of synuclein with circulating antibodies has considerable therapeutic potential (Bae et al., 2012).

Although the data are thus far consistent with a prion-like mechanism for the transmission of misfolded synuclein between cells, there are several important differences between PD and known prion disorders. First, although the incubation time can be long, most prion disorders result in a subacute clinical decline over weeks to months, and PD progresses over decades, particularly since certain features can long precede the onset of typical parkinsonian symptoms. Second, it is important to note that although Lewy pathology was recognized in a few cells of some human transplants, many of the grafts and indeed most of the transplanted cells even in affected grafts appeared entirely normal (Mendez et al., 2008). The process thus does not seem very efficient. Third, the misfolded state in typical prion disorders is quite stable and, indeed, heritable—different strains of the same misfolded protein reproducibly produce distinct forms of degeneration. However, very recent work has suggested that the conformation of misfolded synuclein can change over time and indeed promote the aggregation of an entirely distinct protein (tau) (Figure 3) (Guo et al., 2013). Considering the importance of tau for neurodegenerative disease as a whole and PD in particular (Simón-Sánchez et al., 2009), this work expands the relevance of synuclein aggregation but suggests important differences from typical prion disorders.

Fourth and perhaps most important, sporadic prion disorders presumably involve a very rare misfolding event, which then propagates through the prion mechanism. Consistent with this, overexpression of wild-type PrP does not by itself usually suffice to produce prion disease. In the case of human PD, however, overexpression of wild-type α-synuclein due to gene triplication produces more severe disease than the point mutations, even

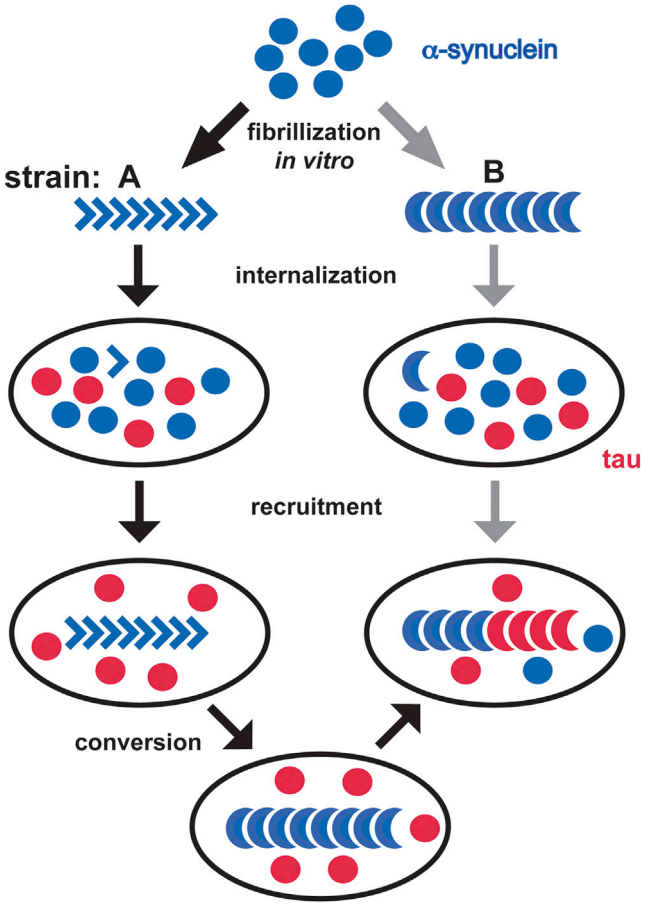


**Figure 3. Synuclein Exhibits Prion Strain-like Properties**
Fibrils of recombinant synuclein were taken up by primary neurons and the derived fibrils used for repetitive seeding of additional primary cultures. Early passages yield fibrils capable of forming only inclusions of synuclein (strain A). Fibrils derived from subsequent passages produced robust tau pathology with less deposition of synuclein (strain B). Both strains may derive from the same initial fibrillization reaction (gray arrows), but it seems more likely that strain A converts into strain B. (Adapted from Guo et al., 2013.)

though several of these apparently increase the propensity to aggregate. For PD, the amount of protein expressed thus appears particularly important, suggesting differences from the prion disorders. PD may simply reflect an increase in monomeric, rather than misfolded or oligomeric, synuclein. In addition, the particular sensitivity to expression may reflect the enhancement of a less rare misfolding event by increased protein. Alternatively, wild-type synuclein may misfold at such a high rate that its concentration is more important than any small difference in aggregation tendency. Interestingly, the recent overexpression of wild-type bank vole PrP in mice has been found to produce degeneration and prions, but only one variant does this and bank vole PrP appears unusually susceptible to prion formation (Watts et al., 2012). Rather than a rare misfolding event that requires propagation to cause disease, the misfolding of α-synuclein (and possibly bank vole PrP) might therefore originate at multiple sites, with fewer requirements for transmission between cells.

## Toxicity: Membranes

How does synuclein cause toxicity? The analysis of synuclein in multiple systems has suggested a role for its interaction with membranes. As noted above, synuclein oligomers can permeabilize membranes in vitro (Rochet et al., 2004; Tsigelny et al., 2007; Volles et al., 2001), but the relevance of this observation for cells has remained unclear. To study its toxicity in vivo, overexpression of human α-synuclein in yeast has provided an experimentally tractable system (Outeiro and Lindquist, 2003). Although yeast do not contain any sequences resembling synuclein, overexpression of the human protein appears to interfere with transport through the early secretory pathway, and genes that modify the toxicity of synuclein in yeast also tend to involve lipid metabolism and membrane trafficking (Willingham et al., 2003). The small GTPase rab1 that operates early in the secretory pathway rescues synuclein toxicity, both in yeast and in mammalian cells overexpressing a PD-associated mutant (Cooper et al., 2006; Gitler et al., 2008). This might be considered a nonspecific effect, but additional work has suggested an interaction of synuclein with rabs (Chen et al., 2013; Dalfó et al., 2004; Lee et al., 2011; Rendón et al., 2013). In the absence of a clear rab-related defect in synuclein knockout mice, the physiological significance remains unclear, but it may have a role in degeneration.

In yeast, overexpressed α-synuclein localizes to punctate structures. EM has shown that these accumulations are in fact clusters of vesicles rather than proteinaceous deposits, and synuclein appears to act by inhibiting membrane fusion (Gitler et al., 2008; Soper et al., 2008), similar to what has been reported in chromaffin cells (Larsen et al., 2006) (see Role in Neurotransmitter Release above). Human synuclein can also produce lipid droplets in yeast (Outeiro and Lindquist, 2003). Regardless of mechanism, a mutational analysis of synuclein has also shown that toxicity in yeast correlates with the strength of membrane interactions rather than the tendency to aggregate (Volles and Lansbury, 2007). However, the behavior of synuclein in mammalian cells differs in many respects from that observed in yeast, with less obvious membrane association and toxicity, particularly with the wild-type protein. In addition, human synuclein cannot form lipid droplets in mammalian cells but does coat and stabilize the fat droplets formed by feeding with oleic acid (Cole et al., 2002). Perhaps most dramatically, the γ-synuclein knockout shows resistance to obesity and increased lipolysis in white adipose tissue, apparently through increased access of lipolytic enzymes to fat droplets (Millership et al., 2012). The effect of this knockout on brain phospholipids is modest (Guschina et al., 2011), but the effect on adipose tissue strongly supports a role for the other isoforms as well in membrane access and modification.

In recent years, structural studies in vitro have suggested that when synuclein binds to membranes, it can remodel them (Bodner et al., 2009; Diao et al., 2013). The analysis of mitochondrial morphology has now corroborated this possibility in cells. Implicated in the pathogenesis of Parkinson's disease by the MPTP model and the role in mitochondrial autophagy of recessive PD genes parkin and PINK1 (Narendra et al., 2012), mitochondria have been found to interact with synuclein in several ways. In addition to protection against MPTP toxicity

by inactivation of the α-synuclein gene (Dauer et al., 2002; Fornai et al., 2005; Fountaine et al., 2008; Klivenyi et al., 2006), both overexpression and loss of synuclein have been reported to impair mitochondrial function (Ellis et al., 2005; Martin et al., 2006; Smith et al., 2005a; Stichel et al., 2007). Synuclein also binds to mitochondria (Cole et al., 2008; Devi et al., 2008; Li et al., 2007; Liu et al., 2009; Nakamura et al., 2008). More recently, however, overexpression of α-synuclein in a variety of cell types including neurons has been found to fragment mitochondria (Kamp et al. 2010; Nakamura et al., 2011). The change in mitochondrial morphology appears specific, with minimal morphological change in other organelles (such as the Golgi complex), and precedes any deterioration in mitochondrial function (Nakamura et al., 2011).

How does synuclein overexpression fragment mitochondria? Using a direct assay, synuclein does not prevent mitochondrial fusion but rather promotes fission and acts independently of the major fission protein Drp1 (Nakamura et al., 2011). The fragmentation also requires access of synuclein to mitochondria, suggesting a direct effect of synuclein on mitochondrial membranes. Indeed, recombinant synuclein can fragment artificial membranes containing the mitochondrial lipid cardiolipin but not membranes without an acidic phospholipid headgroup (Nakamura et al., 2011). Consistent with sequence conservation of the N-terminal membrane-binding domain, β- and γ-synuclein can also affect mitochondrial morphology (Nakamura et al., 2011).

The Langen group has independently provided compelling evidence that synuclein can remodel membranes (Jao et al., 2008; Varkey et al., 2010). Dramatically, high concentrations of recombinant synuclein can turn an opaque solution of artificial membranes clear (Figure 4A). At intermediate concentrations, synuclein produces massive tubulation, very similar to Bin-amphysin-Rvs (BAR) domain proteins that bend membranes (McMahon and Boucrot, 2011; Mim and Unger, 2012) (Figure 4B). However, synuclein seems to act through a distinct mechanism that may involve electrostatic interaction with the phospholipid headgroups to bind membranes but requires insertion into the bilayer for membrane bending (Jensen et al., 2011). In particular, synuclein appears to insert in an extended helical conformation parallel to the long axis of the tubule (Mizuno et al., 2012). The preference of synuclein for highly curved membranes and the ability to tubulate membranes may indeed derive from the same ability to recognize defects in the lipid bilayer (Ouberai et al., 2013; Pranke et al., 2011), similar to the amphipathic lipid packing sensor motifs desribed in ArfGAP1 (Antonny, 2011). Synuclein can also cluster artificial membranes and, consistent with previous work, the A30P mutation disrupts membrane tubulation (Diao et al., 2013; Nakamura et al., 2011; Zigoneanu et al., 2012).

The analysis of knockout mice further supports a role for synuclein in membrane bending. A proteomic analysis of the triple knockouts shows reciprocal changes in BAR domain proteins, in particular endophilin (Westphal and Chandra, 2013). This work also demonstrates the effect of synuclein on membrane curvature in vitro. In contrast to another study suggesting that a multimeric form of synuclein was responsible for tubulation (Nakamura et al., 2011), this report indicated a requirement for the monomeric protein (Westphal and Chandra, 2013).

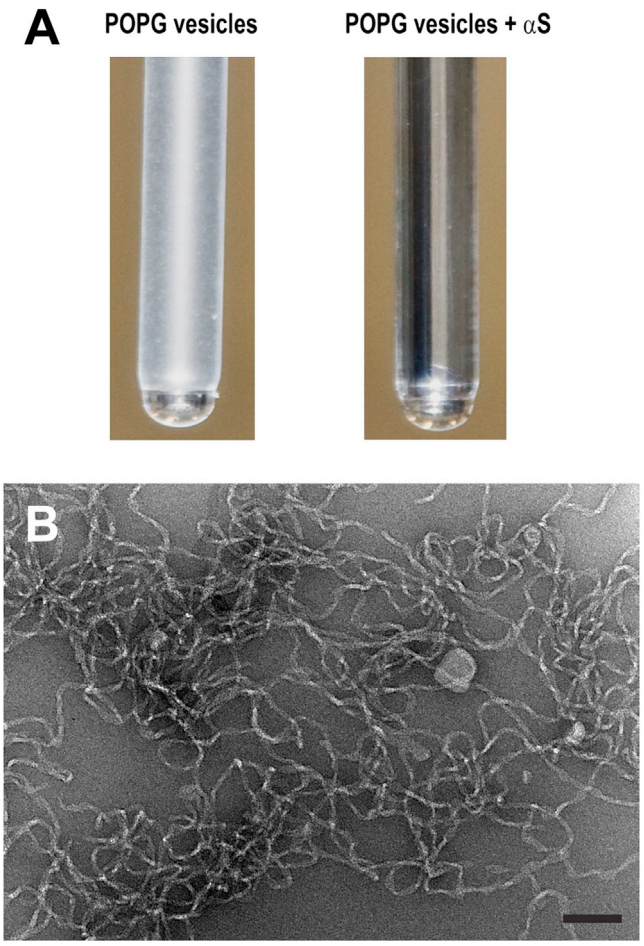


**Figure 4. α-Synuclein Tubulates Membranes In Vitro**
(A) Recombinant α-synuclein (600 μM) clarifies an opaque solution containing phosphatidylglycerol with palmitoyl and oleoyl side chains (60 μM) (Varkey et al., 2010). (Republished with permission from the *Journal of Biological Chemistry*.)
(B) Twenty μM recombinant α-synuclein was added to nonextruded, ~0.4 μm membranes containing a 1:1 mixture of dioleoyl phosphatidyl choline and dioleoyl phosphatidic acid (400 μM) and examined by negative staining electron microscopy (T.P.L. and R.H.E., unpublished data). Size bar indicates 100 nm.

Despite these observations, synuclein normally resides at presynaptic boutons, and most mitochondria localize to the cell body and dendrites. How then can synuclein influence mitochondrial behavior in neurons? We hypothesize that synuclein localizes to mitochondria only when upregulated. The high presynaptic concentration of synaptic vesicles with high curvature presumably accounts for the normal localization of synuclein to this site. If expression is increased, however, synuclein may then also associate with other membranes such as the mitochondrial inner membrane, which has high curvature at particular sites and is exceptionally rich in the acidic phospholipid cardiolipin. Indeed, the level of expression correlates with mitochondrial fragmentation (Nakamura et al., 2011).

## Conclusions

Recent work now indicates the potential for propagation of misfolded synuclein between cells, through a prion-like mechanism. However, the events that trigger misfolding of synuclein in the first place remain poorly understood. A simple increase in the amount of α-synuclein appears sufficient, but its interaction with membranes probably has a crucial role. Lower levels of synuclein contribute to its physiological role at the nerve terminal, influencing the amount of SNARE complex either directly, as a chaperone, or indirectly through other effects on the synaptic vesicle cycle. When upregulated through physiological or pathological mechanisms, synuclein may target other membranes such as mitochondria, and this presumably accounts for the toxicity observed in human PD. The interaction with membranes thus appears central to both the normal function of synuclein and its role in degeneration. Determining how this interaction influences the conformation of synuclein will help to understand the misfolding that occurs in PD. Similarly, understanding how the interaction affects membrane behavior will illuminate the normal function of the protein, provide a biological context to understand its regulation, and indicate mechanisms responsible for toxicity. However, all of these questions await better methods to understand the behavior and activity of synuclein within the cell.

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