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REVIEW ARTICLE

Interactions Among α-Synuclein, Dopamine, and Biomembranes

Some Clues for Understanding Neurodegeneration in Parkinson's Disease

Jean-Christophe Rochet,*[*,1,3] Tiago Fleming Outeiro,[2] Kelly A. Conway,[1,4] Tomas T. Ding,[1] Michael J. Volles,[1] Hilal A. Lashuel,[1] Robert M. Bieganski,[1] Susan L. Lindquist,[2] and Peter T. Lansbury, Jr.[1]

[1]Center for Neurologic Diseases, Brigham and Women's Hospital, Department of Neurology, Harvard Medical School, Cambridge, MA 02139; and [2]The Whitehead Institute for Biomedical Research, Cambridge, MA 02142

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Abstract

Parkinson's disease (PD) is a neurologic disorder resulting from the loss of dopaminergic neurons in the brain. Two lines of evidence suggest that the protein α-synuclein plays a role in the pathogenesis of PD: Fibrillar α-synuclein is a major component of Lewy bodies in diseased neurons, and two mutations in α-synuclein are linked to early-onset disease. Accordingly, the fibrillization of α-synuclein is proposed to contribute to neurodegeneration in PD. In this report, we provide evidence that oligomeric intermediates of the α-synuclein fibrillization pathway, termed protofibrils, might be neurotoxic. Analyses of protofibrillar α-synuclein by atomic force microscopy and electron microscopy indicate that the oligomers consist of spheres, chains, and rings. α-Synuclein protofibrils permeabilize synthetic vesicles and form pore-like assemblies on the surface of brain-derived vesicles. Dopamine reacts with α-synuclein to form a covalent adduct that slows the conversion of protofibrils to fibrils. This finding suggests that cytosolic dopamine in dopaminergic neurons promotes the accumulation of toxic α-synuclein protofibrils, which might explain why these neurons are most vulnerable to degeneration in PD. Finally, we note that aggregation of α-synuclein likely occurs via different mechanisms in the cell versus the test tube. For example, the binding of α-synuclein to cellular membranes might influence its self-assembly. To address this point, we have developed a yeast model that might enable the selection of random α-synuclein mutants with different membrane-binding affinities. These variants might be useful to test whether membrane binding by α-synuclein is necessary for neurodegeneration in transgenic animal models of PD.

Index Entries: Parkinson's disease; synuclein; fibril; protofibril; membrane; dopamine; yeast.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that affects approx 0.2% of the population (Dunnett and Bjorklund, 1999; Dawson and Dawson, 2002). The symptoms, which include difficulty initiating movements, resting tremor, and rigidity, result from the loss of dopaminergic neurons from the substantia nigra (Pollanen et al., 1993; Forno, 1996; Dunnett and Bjorklund, 1999). The pathogenic mechanisms leading to this degeneration of nigral neurons are thought to involve mitochondrial

*Author to whom all correspondence and reprint requests should be addressed. E-mail: rochet@pharmacy.purdue.edu
Present addresses: [3]Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907; [4]Johnson & Johnson Pharmaceutical Research and Development, Spring House, PA 19477.

impairment and oxidative stress (Orth and Schapira, 2002; Sherer et al., 2002). In addition, a neuropathological hallmark of PD is the presence in some surviving neurons of cytosolic inclusions named Lewy bodies (Takahashi and Wakabayashi, 2001).

Several key observations have led to the hypothesis that the protein α-synuclein plays a role in the pathogenesis of PD. Not only do Lewy bodies consist primarily of fibrillar α-synuclein (Spillantini et al., 1997, 1998; Wakabayashi et al., 1997; Arima et al., 1998; Baba et al., 1998), but two mutations in α-synuclein, A53T (Polymeropoulos et al., 1997) and A30P (Kruger et al., 1998), have also been linked to rare, early-onset forms of the disease. In addition, the production of human α-synuclein in transgenic mice (Masliah et al., 2000), rats (Kirik et al., 2002), or flies (Feany and Bender, 2000) leads to a Parkinsonian phenotype, including the degeneration of dopaminergic neurons, the presence of α-synuclein-containing inclusions, and the onset of motor deficits. Finally, the chronic exposure of rats to rotenone, a mitochondrial complex I inhibitor, reproduces key features of PD, including cytoplasmic, fibrillar inclusions enriched with α-synuclein (Betarbet et al., 2000; Sherer et al., 2003).

α-Synuclein is a small (14-kDa) cytosolic protein that is abundant in presynaptic nerve terminals in the brain (Maroteaux et al., 1988; Jakes et al., 1994; Iwai et al., 1995; Clayton and George, 1999). Even in benign buffer, the protein has a primarily random-coil secondary structure and therefore, is referred to as natively unfolded (Weinreb et al., 1996). The N-terminal region of α-synuclein contains six imperfect repeats of the amino acid sequence KTK(E/Q)GV, which is similar to the characteristic repeats of exchangeable apolipoproteins. The repeat region of α-synuclein is predicted to adopt an amphipathic α-helical structure upon the reversible association of the protein with synthetic vesicles or membranes (Davidson et al., 1998; Perrin et al., 2000; Bussell and Eliezer, 2003; Chandra et al., 2003).

Wild-type (WT) α-synuclein and the two PD mutants, A53T and A30P, form fibrils in vitro that are similar to the fibrils extracted from Lewy bodies (Conway et al., 1998; El-Agnaf et al., 1998; Hashimoto et al., 1998; Giasson et al., 1999; Narhi et al., 1999). However, whereas A53T forms fibrils more rapidly than WT α-synuclein, A30P forms fibrils less rapidly than WT α-synuclein (Conway et al., 2000). In contrast, both A53T and A30P form prefibrillar oligomers (protofibrils) more rapidly than WT α-synuclein (Conway et al., 2000; Li et al., 2001, 2002). These results suggest that the early-onset disease associated with A53T and A30P is attributable not to accelerated α-synuclein fibrillization but, rather, to accelerated protofibril formation. Consistent with this hypothesis, at least two neuropathological observations suggest an imperfect correlation between Lewy bodies and disease. First, dopaminergic neurons of PD patients that contain Lewy bodies appear to be healthier than adjacent neurons (Tompkins and Hill, 1997). Second, Lewy bodies have been observed in postmortem brain samples from elderly individuals who were asymptomatic during their lifetime (van Duinen et al., 1999). From all of these observations, we infer that α-synuclein protofibrils (rather than fibrils) might be the toxic species underlying PD pathogenesis.

An important goal of current research is to provide evidence that supports a link between α-synuclein protofibrils and PD. To this end, we have attempted to identify toxic properties (e.g., membrane permeabilization) specifically associated with protofibrillar but not monomeric or fibrillar α-synuclein. In addition, we have screened small-molecule libraries to identify compounds that stabilize protofibrils by preventing their conversion to mature fibrils. These compounds might then be used in studies of α-synuclein-transgenic flies or mice to test whether an increased accumulation of protofibrils in these transgenic animals produces a more severe form of the disease. Finally, we have developed a yeast model that might enable the isolation of random, unnatural variants of α-synuclein with different propensities to bind to biological membranes. These mutants might be useful to test whether membrane binding is essential for the nucleation event(s) leading to the formation of α-synuclein protofibrils.

α-Synuclein Protofibrils Form β-Sheet-Rich Rings and Can Permeabilize Membranes

To begin to address why protofibrillar α-synuclein might be neurotoxic, we compared the structural and functional properties of the monomer and protofibrils. The two forms of the protein were isolated by gel filtration and analyzed by far-UV circular dichroism (CD) and atomic force microscopy (AFM) (Volles et al., 2001). The monomer, which eluted in the included fraction from the gel-filtration

column, exhibited a CD spectrum characteristic of a primarily random-coil secondary structure. Monomeric α-synuclein produced no significant signal when analyzed by AFM, as predicted for a small, natively unfolded protein. In contrast, the protofibrils, which eluted in the void-volume fraction from the gel-filtration column, exhibited a CD spectrum characteristic of a primarily β-sheet secondary structure. In addition, the protofibrils were detected as spheres with a height of 3–4 nm when analyzed by AFM.

To understand how the formation of α-synuclein spheres is related to other steps on the fibrillization pathway, we carried out further analyses by AFM and electron microscopy (EM). In some AFM images (Conway et al., 2000), we observed that spheres anneal in a linear fashion to form chains. As one possibility, these chains might undergo winding interactions to form rigid fibrils, via a mechanism similar to that described for the fibrillization of the amyloid-β peptide associated with Alzheimer's disease (Harper et al., 1997a, 1997b; Walsh et al., 1997). In addition, we observed rings, or annular protofibrils, which might result from the annealing of spheres in circular fashion or from the circularization of the protofibrillar chains (Fig. 1A,B). Rings derived from WT α-synuclein, A53T, and A30P have a diameter of 32–180, 10, or 55 nm, respectively (Ding et al., 2002). In addition, ring-like species were observed by EM analysis of α-synuclein from the void-volume fraction (Lashuel et al., 2002). Single-particle analysis enabled us to divide the rings into groups of related structures. For each group, a representative class-average structure was determined (Fig. 1C). The diameter of the rings observed by EM was 10–12 nm (A53T) or 8–24 nm (A30P).

Several groups have shown that monomeric α-synuclein binds reversibly to synthetic vesicles (Davidson et al., 1998; Jo et al., 2000; Perrin et al., 2000; Eliezer et al., 2001). Given this information, and because the structure of the α-synuclein ring was suggestive of a pore, we examined whether the annular protofibrils could bind and permeabilize lipid membranes. Vesicles were purified from the microsomal fraction of a rat brain lysate, incubated with monomeric or protofibrillar α-synuclein, and analyzed by AFM (Ding et al., 2002). Vesicles treated with the protofibrils contained pore-like structures with a diameter of 18–28 nm (Fig. 2A). In contrast, such structures were not associated with vesicles treated with the monomer. To address whether the protofibrils permeabilize membranes, acidic synthetic vesicles were loaded with the dye Fura-2 and treated with monomeric or protofibrillar α-synuclein in a buffer that contained calcium (Volles et al., 2001). In this assay, an increase in fluorescence indicates that a complex has formed between the calcium ions and Fura-2 because of the permeabilization of the vesicles. Incubation of the Fura-2-loaded vesicles with α-synuclein protofibrils led to a substantial increase in fluorescence, whereas no fluorescence increase was observed compared to the buffer control upon mixing the vesicles with monomeric or fibrillar α-synuclein (Fig. 2B; not shown). From these results, we concluded that α-synuclein protofibrils, but not monomer or fibrils, permeabilize lipid membranes. This membrane permeabilization activity could potentially account for the proposed toxicity of α-synuclein protofibrils in PD.

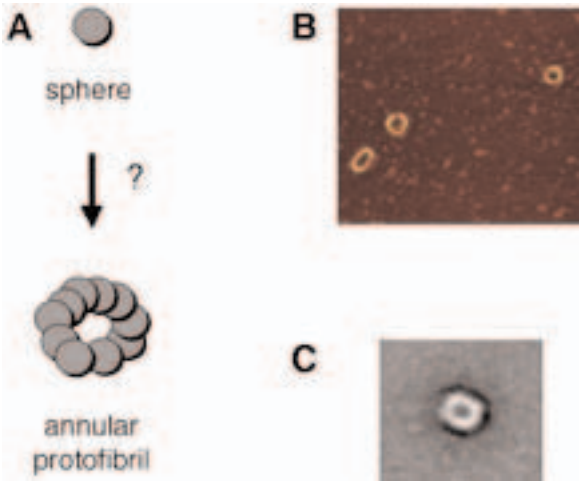

Fig. 1. α-Synuclein protofibrils consist of spheres and rings. (**A**) Schematic diagram depicting a possible route for the conversion of spherical α-synuclein protofibrils to annular protofibrils (rings). (**B**) AFM image (800 × 800 nm), showing rings from an equimolar mixture of WT α-synuclein and A53T (200 μ*M* total). (**C**) Example of a class-average structure of an α-synuclein ring, calculated from single-particle analysis of A53T protofibrils imaged by negative-staining EM.

Catecholamines Inhibit α-Synuclein Fibrillization but Promote Accumulation of Protofibrils

A current goal is to design in vivo experiments to address whether the toxic protofibril hypothesis accounts for the pathogenesis of PD. We have

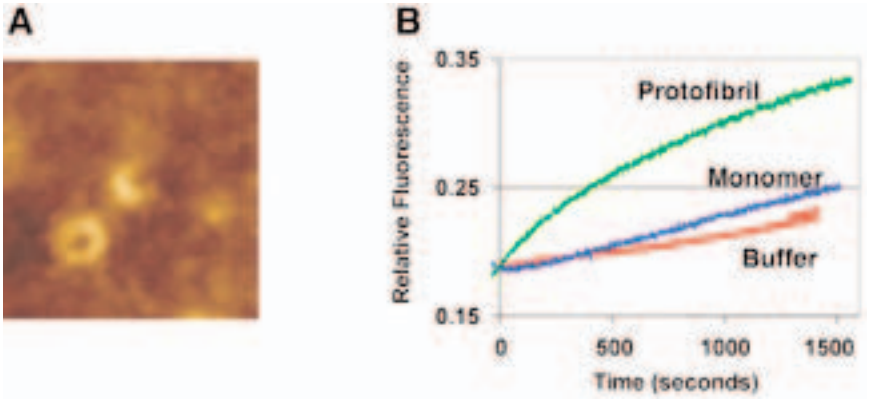

Fig. 2. α-Synuclein protofibrils bind and permeabilize lipid vesicles. (**A**) AFM image (200 × 200 nm) showing membrane-bound, ring-like structures formed by mixing protofibrillar A53T with brain-derived vesicles. (**B**) Graph showing the increase in fluorescence with time upon incubating Fura-2-loaded, synthetic vesicles in a calcium buffer in the absence or presence of α-synuclein monomer (26 μ*M*) or protofibrils (4.2 μ*M*). Only the protofibrils produce a substantial increase in fluorescence, which indicates membrane permeabilization.

considered two complementary strategies. First, one might identify a small molecule that binds specifically to α-synuclein protofibrils and stabilizes them by preventing their conversion to mature fibrils. Second, one might generate random, unnatural variants of α-synuclein that accumulate as protofibrils but do not form fibrils. If the toxic protofibril hypothesis were correct, then an α synuclein-transgenic animal treated with a drug that stabilizes protofibrils should develop a more severe Parkinsonian phenotype than an untreated control. Similarly, an α-synuclein-transgenic animal that produces high levels of a random mutant that forms protofibrils but not fibrils should develop a more pronounced form of the disease.

To identify small molecules that inhibit α-synuclein fibrillization, we screened a small-molecule library using the thioflavin-T assay (Conway et al., 2001). Because thioflavin T binds to mature fibrils, hits identified by this screen are expected to inhibit fibrillization by one of two mechanisms: (1) They might prevent the early assembly of protofibrils from monomeric α-synuclein, or (2) they might prevent the subsequent conversion of protofibrils to fibrils. These possibilities are distinguishable by quantifying the void-volume oligomer in each sample via gel filtration, in parallel with the thioflavin-T measurements.

We screened 160 compounds from the LOPAC library (Research Biochemicals, Inc./Sigma). Of these, 15 inhibited α-synuclein fibrillization over a 3-d assay period. Remarkably, 14 of these hits were catecholamines, including dopamine, L-DOPA, epinephrine, and norepinephrine (Fig. 3A). The catechol moiety was essential for inhibition, as molecules in which either catechol hydroxyl group had been modified had no effect on fibrillization (Fig. 3B). In addition, the inhibitory activity of the catecholamines was suppressed by antioxidants such as sodium metabisulfite or desferrioxamine combined with *N*-acetyl-cysteine (not shown). In parallel experiments, samples of α-synuclein incubated in the absence or presence of dopamine or L-DOPA were analyzed by gel filtration. After 3 d, a broad peak of absorbance at 276 nm (A_{276}), which included both void-volume and early included-volume fractions, was associated with the samples treated with either catecholamine but not with the untreated protein. Western blot analysis of α-synuclein treated with dopamine indicated that a ladder of α-synuclein-immunoreactive bands appeared and became progressively more intense during the incubation (Fig. 4A). A smear at the top of this ladder was similar to that reported for protofibrillar α-synuclein in earlier studies (Volles et al., 2001; Ding et al., 2002). From these results, it was inferred that the inhibition of α-synuclein fibrillization by catecholamines leads to the accumulation of a mixture of oligomers, some of which correspond to the protofibrils observed previously.

We also determined that the A_{276} signal corresponding to monomeric α-synuclein increased in the samples treated with either catecholamine during the course of the incubation. In addition, monomeric

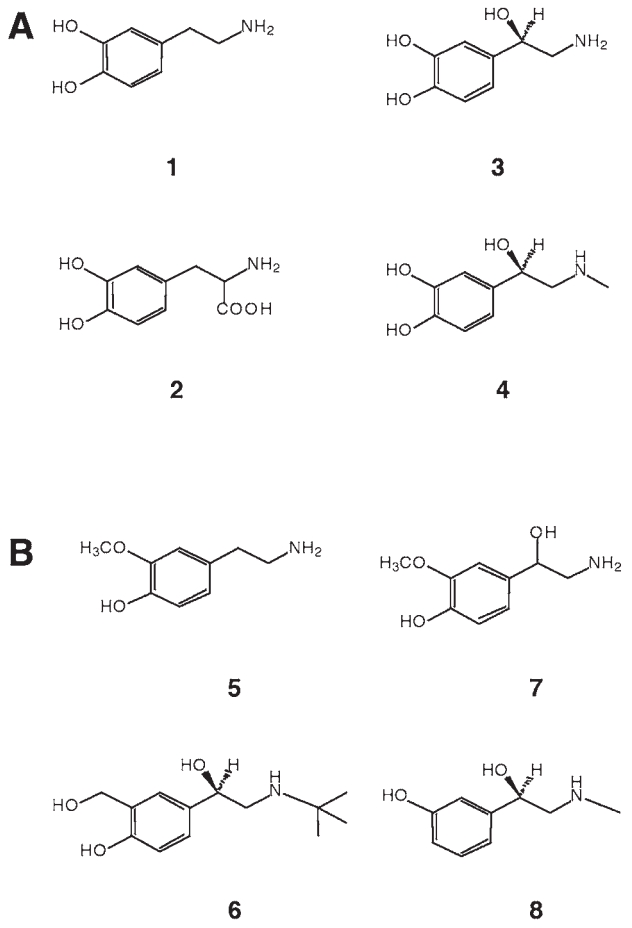

Fig. 3. Catechol hydroxyl moieties are essential for the inhibition of α-synuclein fibrillization by catecholamines. (**A**) Structures of fibrillization inhibitors, including dopamine (1), L-DOPA (2), norepinephrine (3), and epinephrine (4). (**B**) Structure of noninhibitors, including 3-methoxy-4-hydroxyphenethylamine (5), albuterol (6), normetanephrine (7), and phenylephrine (8).

α-synuclein treated with dopamine and then purified by gel filtration exhibited a fluorescence emission (λ_{max} = 468 nm) when excited with light at a wavelength of 360 nm. The untreated monomer did not exhibit this fluorescence emission. From these observations, we considered that α-synuclein might be modified by oxidized dopamine. To address this hypothesis, samples of α-synuclein were incubated for various times in the presence of [^{3}H]dopamine and analyzed by gel filtration. The eluted fractions were monitored by measuring A_{276} and by scintillation counting. After a 5-min incubation, a significant level of radioactivity was associated with monomeric α-synuclein (Fig. 4B). After a longer incubation (11 d), a much higher level of radioactivity was associated with both the monomer and the oligomer (Fig. 4B). Moreover, the amount of radioactivity associated with the purified monomer was not affected by boiling the sample prior to gel filtration. Therefore, it was concluded that α-synuclein is modified by dopamine to form a covalent adduct.

Next, we hypothesized that the inhibition of α-synuclein fibrillization by dopamine might be attributable to the formation of the adduct, which might interfere with the conversion of protofibrils to fibrils. To address this hypothesis, monomeric α-synuclein that had been modified by unlabeled dopamine was purified and mixed in various proportions with unmodified monomer. The mixtures were then incubated at 37°C, and the formation of fibrils and protofibrils was monitored by the thioflavin-T fluorescence assay and by gel filtration, respectively. Fibrillization occurred less rapidly, whereas protofibrils accumulated to a greater extent and for a longer time in mixtures that contained higher amounts of dopamine–α-synuclein adduct. From these results, we concluded that the dopamine–α-synuclein adduct slows the conversion of protofibrils to fibrils. As one possibility, this inhibition might be attributable to an unfavorable interaction between the dopamine–α-synuclein adduct and the unmodified protein.

A Yeast Model to Isolate Random α-Synuclein Mutants

As part of our ongoing effort to validate the toxic protofibril hypothesis in vivo, we established a yeast model to identify random mutants with varying propensities to aggregate. Our goal at the outset was to identify α-synuclein variants that readily form protofibrils but do not convert to fibrils. If the hypothesis were correct, then transgenic animals that produced high levels of such mutants should develop a more severe Parkinsonian phenotype.

Our yeast model was designed so that the extent of α-synuclein aggregation would be reflected by a macroscopic property of the yeast, using an approach similar to that used to study the yeast prion protein Sup35 (Chernoff et al., 2002). Sup35 is a translation termination factor that consists of amino (N)-, middle (M)-, and carboxy (C)-terminal domains. If Sup35 is soluble and abundant, then it interacts via its C-domain with a second subunit, Sup45, to terminate translation at a stop codon. However, if Sup35 is aggregated via interactions involving its

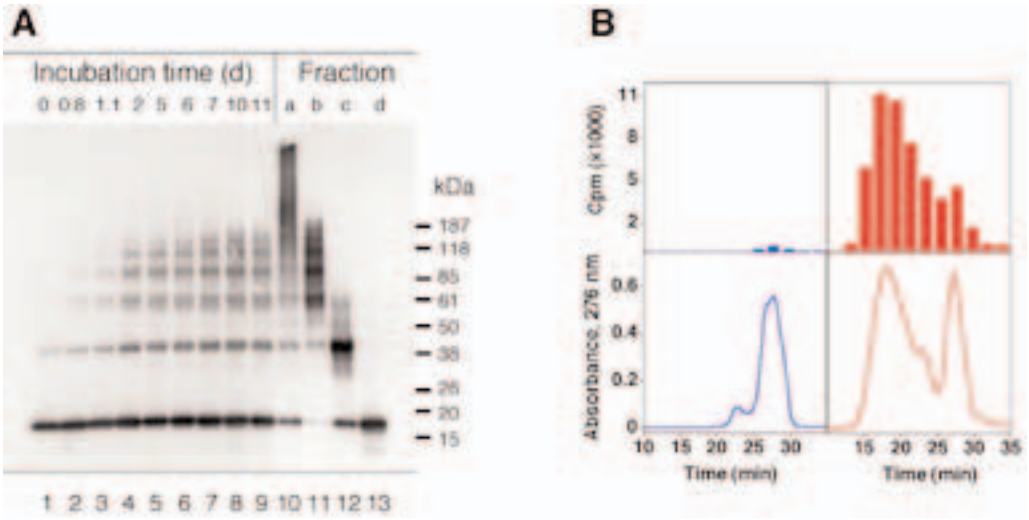

Fig. 4. Treatment of α-synuclein with dopamine leads to the formation of a covalent adduct and the accumulation of oligomers. (**A**) Western blot analysis of A53T (1 m*M*) incubated with dopamine (2 m*M*) at 37°C. (Lanes 1–9) Analysis of samples incubated for the indicated times; (lanes 10–13) analysis of fractions recovered after gel filtration of A53T incubated with dopamine for 11 d. (Fraction a) Void-volume oligomer; (fraction b) included-volume oligomer; (fraction c) dimer; (fraction d) monomer. SDS-PAGE was carried out with a 10–20% polyacrylamide, tricine gel. The primary antibody was Syn-1 (Transduction Labs). (**B**) Gel-filtration chromatogram of A53T (1 m*M*) treated with [2,5,6-^{3}H]dopamine (2 m*M*, 5 mCi/mmol), monitored by scintillation counting (top) and A_{276} (bottom). The solution was analyzed immediately (left) or after incubation for 11 d at 37°C (right). Oligomers eluted between 15 and 22 min, a dimer of α-synuclein eluted between 22 and 24 min, and the monomer eluted between 25 and 30 min.

N-terminal prion domain, then it can no longer associate productively with Sup45, and translation termination is partially suppressed. The normal and suppressed translation termination phenotypes are conveniently distinguished in yeast cells that contain a nonsense mutation in the *ade1-14* gene, which encodes an enzyme of the adenine biosynthesis pathway. Yeast cells in which translation termination occurs normally (referred to as [*psi*–]) produce a truncated, nonfunctional version of the adenine biosynthetic enzyme, and they are therefore not able to grow in the absence of adenine. In contrast, yeast cells in which translation termination is partially suppressed (referred to as [*PSI*+]) produce the full-length biosynthetic enzyme and are able to grow in the absence of adenine.

Using a similar screen, we examined whether translation termination was suppressed in yeast that produce a fusion protein in which the N-domain of Sup35 is replaced by α-synuclein. Our initial goal was to compare the growth phenotypes of yeast strains in which the endogenous Sup35 gene was replaced with DNA encoding WT α-synuclein-, A53T-, or A30P-Sup35MC. However, we were unable to isolate yeast in which the Sup35 gene was substituted with DNA encoding WT α-synuclein- or A53T-Sup35MC, suggesting that these two fusion proteins failed to carry out the translation termination function of full-length Sup35 (Outeiro et al., unpublished observations). As an alternate strategy, each α-synuclein-Sup35MC DNA was expressed in [*psi*–] yeast that contained the endogenous Sup35 gene. We found that translation termination was partially suppressed in yeast that produced WT α-synuclein- or A53T-Sup35MC, but not A30P-Sup35MC (Fig. 5A). Subsequently, we observed that yeast producing β-synuclein-Sup35MC, but not γ-synuclein- or [A30P+A53T]-Sup35MC, exhibited a partially suppressed translation-termination phenotype. Importantly, synuclein variants that caused suppression when fused to Sup35MC are reported to have a relatively high affinity for lipid membranes, whereas the synuclein variants that did not interfere with translation termination when fused to Sup35MC have a low affinity for membranes (Jensen et al., 1998; Perrin et al., 2000; Cole et al., 2002; Jo et al., 2002). In contrast, some forms of synuclein that caused suppression when fused to Sup35MC (e.g., β-synuclein) aggregate poorly in vitro (Giasson et al., 2001), whereas other forms of synuclein that did not cause

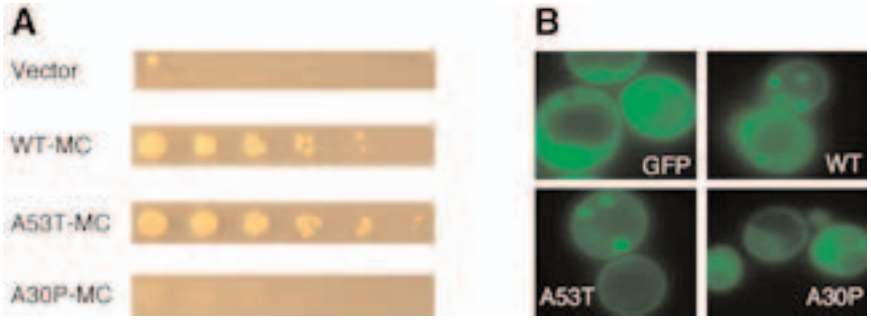

Fig. 5. α-Synuclein variants have different propensities to bind membranes in a yeast model. (**A**) [*psi*–] *ade1-14* yeast cells that produce WT α-synuclein or A53T fused to Sup35MC (WT-MC or A53T-MC, respectively) have a partially suppressed translation termination phenotype. In contrast, [*psi*–] *ade1-14* yeast cells that contain the control vector (Vector) or produce A30P fused to Sup35MC (A30P-MC) have a normal translation termination phenotype. Yeast cells were plated on synthetic media lacking uracil (to select for yeast carrying expression constructs with a Ura marker) and adenine (to select for yeast with a partially suppressed translation termination phenotype). Translation termination is partially suppressed in yeast cells that produce α-synuclein variants with high membrane-binding affinities. (**B**) Green fluorescent protein (GFP) fluorescence is punctate in yeast cells that produce WT α-synuclein or A53T fused to GFP (WT or A53T, respectively), whereas it is diffuse in yeast cells that produce unfused GFP (GFP) or A30P fused to GFP (A30P). Punctate fluorescence might reflect the binding of α-synuclein to organellar or vesicular membranes.

suppression (e.g., the [A30P+A53T] mutant) aggregate efficiently in the test tube. When produced as fusions, with green fluorescent protein (GFP) variants of synuclein that had caused suppression (when fused to Sup35MC) were found to localize to the plasma membrane or form a punctate pattern of fluorescence (perhaps because of association with organelles and/or vesicles) (Fig. 5B) (Outeiro and Lindquist, 2003). Conversely, the forms of synuclein that did not interfere with translation termination produced a diffuse pattern of fluorescence when fused to GFP (Fig. 5B). Finally, attempts to isolate α-synuclein-Sup35MC aggregates specifically from partially suppressed yeast, using ultracentrifugation protocols that have been shown to pellet aggregated Sup35 from [*PSI*$^+$] yeast (Chernoff et al., 2002), were unsuccessful.

From all of this evidence, we infer that the α-synuclein-Sup35MC fusions that cause suppression in [*psi*–] yeast might do so by binding to cellular membranes. As one possible mechanism, membrane binding by α-synuclein-Sup35MC might lead to the mislocalization of Sup45 to the membrane, because of the interaction between Sup45 and the Sup35C-domain of the fusion protein. In turn, this mislocalization would be expected to interfere with translation termination by endogenous Sup35. The results of preliminary experiments, which suggest that excess Sup45 partially rescues suppression from α-synuclein-Sup35MC, provide some supporting evidence for this mechanism (not shown). Importantly, the results imply that we have generated a yeast model for which the growth of colonies reflects the binding of α-synuclein to cellular membranes—not the aggregation of the protein, as observed in the test tube. Although this model differs from that which was anticipated at the start of this project, it will nevertheless be invaluable to studies that address how α-synuclein membrane interactions play a role in the pathogenesis of PD (*see* below).

Discussion

We have shown that α-synuclein protofibrils are enriched with β-sheet and exist as individual spheres, elongated chains, or rings. In contrast to the monomer or fibril, protofibrils bind and permeabilize lipid vesicles, perhaps via a pore-like mechanism similar to β-sheet-rich, membrane-permeabilizing toxins (Volles et al., 2001; Ding et al., 2002). Under oxidizing conditions, α-synuclein reacts with dopamine (possibly in its quinone form) to yield a covalent adduct that promotes the accumulation of protofibrils. Our results suggest the following model to explain the susceptibility of dopaminergic neurons in PD (Fig. 6). After a sufficiently long lag time, corresponding to years in a patient prior to disease onset, α-synuclein might form protofibrils via a nucleation-dependent mechanism. The protofibrils might be toxic because of

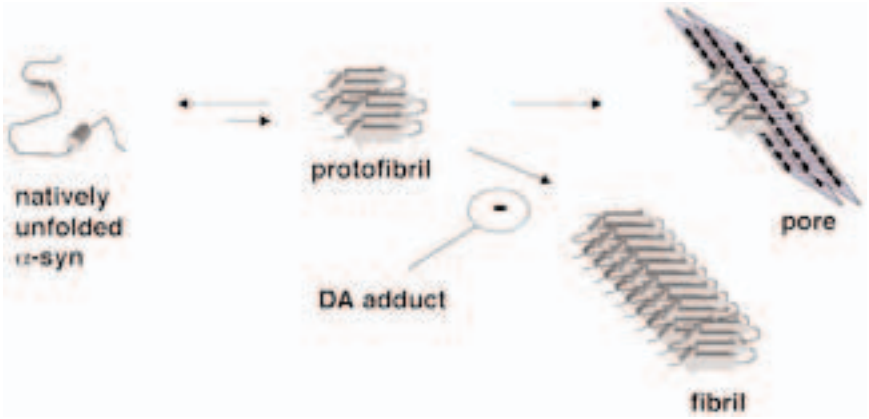

Fig. 6. The degeneration of dopaminergic neurons in PD might result from the aggregation and oxidation of α-synuclein, leading to membrane permeabilization. The conversion of natively unfolded, monomeric α-synuclein (left) to protofibrils (middle) is an unfavorable process (longer arrow in the reverse direction) that only occurs after an extended lag phase. The protofibrils might form toxic, pore-like structures (upper right) that permeabilize vesicles or organelles. Alternatively, α-synuclein protofibrils might assemble to form potentially less toxic fibrils (lower right). The highly oxidizing conditions in the cytosol of dopaminergic neurons might favor the formation of a dopamine–α-synuclein adduct, which promotes the accumulation of toxic protofibrils by inhibiting their conversion to fibrils. Therefore, this model provides a possible explanation for dopaminergic neurons being highly susceptible to the toxic effects of α-synuclein.

their ability to permeabilize membranes, which in turn might lead to the inappropriate redistribution of vesicular or organellar contents (*see* below). The accumulation of toxic protofibrils might be favored by the intracellular conditions expected of a dopaminergic neuron, namely, oxidative stress and the presence of cytosolic dopamine. Under these conditions, dopamine and α-synuclein might react to form an adduct that slows the conversion of protofibrils to less toxic fibrils. However, in non-dopaminergic neurons with relatively low levels of oxidative stress or cytosolic dopamine, the dopamine–α-synuclein adduct is not expected to be formed readily. In this case, toxic protofibrils would be rapidly consumed by fibrillization, and, therefore, the neurons should be relatively spared.

This model predicts that the presence of high levels of cytosolic dopamine is critical to the selective degeneration of dopaminergic neurons in PD. Consistent with this prediction, dopaminergic neurons of the substantia nigra are more vulnerable than those of the ventral tegmental area, in which higher levels of vesicular monoamine transporter (VMAT2) efficiently sequester dopamine out of the cytosol and into synaptic vesicles. In addition, protofibrillar α-synuclein might trigger a vicious cycle by permeabilizing dopamine-containing vesicles, which would lead to further increases in the levels of cytoplasmic dopamine and dopamine– α-synuclein adduct. In support of this mechanism, the production of α-synuclein in differentiated mesencephalic neurons leads to increased levels of dopamine in the cytosol (Lotharius et al., 2002). Our model also addresses the question of why age is a risk factor in sporadic PD: Not only does the nucleation of protofibrils occur only after a sufficient lag time, but the oxidizing conditions of dopaminergic neurons that lead to protofibril stabilization also become more pronounced with age. Finally, the fact that the two α-synuclein mutants, A53T and A30P, form potentially toxic protofibrils more rapidly than WT α-synuclein might account for these mutants being associated with an early-onset form of the disease.

A current goal is to achieve a more complete understanding of how α-synuclein protofibrils are linked to pathogenesis in PD. As one approach, we have begun to identify which species among the total population of protofibrils have the highest levels of specific membrane-permeabilizing activity. Once purified, the structure-function relationships of these species can be elucidated in greater detail. We are also conducting experiments to identify the site(s) on α-synuclein that react with dopamine to form the covalent adduct. Once the targeted residues are identified, it might be possible to test the importance of the dopamine–α-synuclein adduct in vivo using mutants in which the modification sites are disrupted. We also aim to develop a screen for compounds that inhibit the formation of the dopamine–α-synuclein adduct in dopaminergic neurons. To this end, we

have begun to develop a protocol to monitor formation of the adduct in SH-SY5Y and PC12 cells.

In addition to studies of the structure and function of α-synuclein protofibrils in the test tube, experiments were conducted to assess the effects of producing α-synuclein in yeast. Initially, the goal was to develop a genetically tractable model that would facilitate the identification of random α-synuclein mutants with different propensities to aggregate. However, our findings from analyses of yeast cells that produce α-synuclein-Sup35MC suggest that this model might provide a macroscopic readout for the binding of intracellular membranes by α-synuclein, rather than the formation of α-synuclein protofibrils or fibrils. Nevertheless, this model might still be a useful tool in addressing an important question in the field of PD research: namely, does α-synuclein form toxic aggregates when bound to cellular membranes or when free in the cytosol? Results of experiments in isolated brain fractions and mammalian cells suggest that the binding of monomeric α-synuclein to intracellular membranes might be an initiating event that leads to the formation of toxic aggregates (Lee et al., 2002). To address this hypothesis, we propose that our yeast model could be used to identify random α-synuclein mutants with different membrane-binding affinities. In turn, these mutants could be tested for their effects on the Parkinsonian phenotype of α-synuclein-transgenic mice or flies. The results of these studies would provide insight into whether membrane binding by monomeric α-synuclein is necessary to trigger neurodegeneration. Clearly, this information is critical for the optimal design of screens to identify drugs that block the toxic effects of α-synuclein in PD.

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