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Neurobiology of α-Synuclein

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Abstract

α-Synuclein is an abundant neuronal protein that has been linked both to normal synaptic function and to neurodegeneration. Most significantly, mutations in the gene encoding for α-synuclein are responsible for Parkinson's disease (PD) in rare familial cases, and the aggregated protein is a major component of Lewy bodies found in sporadic PD. Here we review recent data regarding the structure, the regulation at the transcriptional and posttranslational level, and the physiologic and aberrant functions of α-synuclein. We focus in particular on the fibrilization potential of α-synuclein and on its link with defects in protein degradation.

Index Entries: Parkinson's disease; Lewy body; fibrilization; aggregation; proteasome; neurodegeneration; synaptic transmission; vesicle.

Introduction and Elementary Genetics

α-Synuclein is part of a family of highly conserved proteins consisting of α-, β-, and γ-synucleins *(1,2).* It was originally cloned and identified as a synaptic protein in the torpedo ray *(3),* later as a protein that was regulated during song-learning in the zebra finch *(4),* and later as NACP, the precursor protein for the nonamyloid component (NAC) of Alzheimer's disease senile plaques *(1).* Even though this latest discovery created some interest in α-synuclein as a protein involved in neurodegeneration, subsequent studies have shown that this finding was likely spurious *(5,6).* The major finding that securely connected α-synuclein with neurodegeneration was the discovery that certain families in Italy, comprising the Contursi kindred, and in Greece, with an autosomal dominant mode of inheritance of Parkinson's disease (PD), harbored a mutation, A53T, in the gene encoding for α-synuclein on chromosome 4 *(7).* Shortly thereafter, α-synuclein was identified as a

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major constituent of Lewy bodies (LB) and the associated neuritic abnormalities referred as Lewy neurites, the pathological hallmarks of PD *(8–10).* α-Synuclein pathology is not restricted to PD, but rather is a prominent feature of a spectrum of diseases termed "α-synucleinopathies" such as dementia with Lewy bodies and LB variants of Alzheimer's disease. α-Synuclein is also found in glial cytoplasmic inclusions in brains of patients with multiple system atrophy (MSA). A major component of α-synuclein in these lesions is in an insoluble, aggregated conformation *(11,12).*

Despite these findings, some doubt still existed about the pathogenic role of α-synuclein, because T53 is actually the normal amino acid in rodents *(13).* However, a second missense mutation, A30P, was later found in another PD family from Germany with an autosomal mode of inheritance, further securing the central role of α-synuclein in the pathogenesis of PD *(14).* It should be noted, however, that there are some differences, both clinical and pathological, between sporadic-PD patients and those harboring the A53T mutant form *(15–17).* Therefore, although clearly very relevant to sporadic PD, insights from the way such mutant forms of α-synuclein lead to PD may not be immediately transferable to sporadic disease. Despite intensive search, no further mutations in α-synuclein have been identified to date, and their presence within a given patient with PD is extremely rare. From a genetic point of view, the two heterozygous missense mutations would be expected to lead to a gain of function, which could be either an excess of the normal function of α-synuclein, or a gain of an unrelated function.

The extreme rarity of α-synuclein mutations has led investigators to the examination of polymorphisms that may be linked to PD. There is some evidence that a dinucleotide-repeat polymorphism located at a significant distance upstream in the promoter region of the α-synuclein gene may be associated with increased risk of PD in Caucasians *(18–20).* Another study showed that this region may play a role in transcriptional regulation of α-synuclein *(21).* Similar observations concerning the variability of these repeats and allelic length as a risk for PD have been obtained by independent studies in an Asian population *(20).* Such studies suggest that, apart from the obvious rare cases with the mutations, α-synuclein may also play a role in PD pathogenesis through alterations in its transcriptional control.

Recently, Singleton et al. *(22)* reported a triplication in a region of chromosome 4 (PARK4) that includes the α-synuclein locus and an estimated 17 other genes. Carriers of this triplication are predicted to have four functional copies of α-synuclein, arguing for an increased gene dosage as a cause for Parkinson's disease. However, the possibility that other genes in the triplicated region may also be responsible cannot be excluded.

Structure/Function Insights: Fibrillization and Other Posttranslational Modifications

α-Synuclein is a relatively abundant 140-residue neuronal protein of uncertain function *(3,23)* (Fig. 1). It is an intrinsically unfolded, or natively unfolded, protein, meaning that in the purified form at neutral pH it lacks an ordered secondary or tertiary structure *(24).* The feature of this molecule that has attracted the most attention is its ability to aggregate in vitro, through a sequence of conversion from a natively unfolded monomeric form to an oligomeric prefibrillar form, also called protofibril, and finally to a fibrillar form. These fibrils of β-pleated sheet structure have morphologies and staining characteristics similar to those extracted from disease-affected brains *(25–28).* The amino acid sequence has seven imperfect repeats of KTKEGV with an apolipoprotein lipid-binding motif, which are predicted to form amphiphilic helices *(4,29,30).* This is followed by the NAC region, a hydrophobic stretch between residues 61 and

95. It is also characterized by the presence of acidic stretches within the C-terminal region (residues 96–140) *(31)* that has been suggested to be responsible for the metal binding and oxidative oligomerization in the presence of hydrogen peroxide *(24,32,33).*

Aa 61–95 of α-synuclein comprise the hydophobic core of the protein, which has been shown to be important in fibril formation *(27).* In another study a 12-aa peptide, residues 71–82, located in the NAC region was shown to be capable of self-aggregation *(34).* A modified α-synuclein lacking this domain or β-synuclein, which lacks it naturally, have a reduced propensity to fibrilize. Recently, it was also reported that a region between aa 31 and 109 is not prone to proteinase K proteolysis *(35).* Similarly, site-directed spin labeling (SDSL) and electric paramagnetic resonance studies *(36)* identified a core region between aa 34 and 101 that in the fibrillar state is packed in a highly ordered parallel fashion with the same residues from different strands in exact register. The same study showed that the N-terminus of fibrillar α-synuclein is more heterogeneous and that the C-terminus of the protein remains unfolded even within fibrillar structures. Consistent with a negative role of the acidic C-terminus in the fibrilization process, Murray et al. reported that C-terminal truncated forms (aa 1–102 or 1–110) of α-synuclein fibrilize more rapidly than the full-length molecule *(37).*

Apart from fibrilization, a number of other posttranslational modifications have been described for α-synuclein, to influence its fibrilization potential. In cultured cells overexpressing α-synuclein, oxidation by oxidizing agents in the presence of iron *(38)* or combined administration of nitrative and oxidative insults *(39)* led to α-synuclein aggregation. Exposure to oxidative and/or nitrative species caused dimerization and stabilization of α-synuclein filaments in vitro *(40–42).* Mutation of all tyrosine residues to phenylalanine in recombinant protein and in cultured cells showed that tyrosine residues are required for α-synuclein cross-linking and filament stabilization induced by nitrating agents, but not by metal oxidation agents *(43).* Adding physiological relevance to the above, Giasson et al. *(44)* showed that nitrated α-synuclein is a component of LBs. In addition, the neurotoxin MPTP led to selective nitration of α-synuclein in vivo and in vitro *(45),* and incubation of recombinant α-synuclein in the presence of pesticides, such as the herbicide paraquat, resulted in a dramatic acceleration of protein fibrilization *(46,47),* indicating that environmental factors related to PD pathogenesis influence α-synuclein conformation.

Constitutive phosphorylation of α-synuclein was first shown in cultured human HEK-293 and rat PC-12 cells by Okochi et al. *(48).* The main phosphorylation site was identified at serine 129, and casein kinase 1 and 2 were suggested as the responsible kinases. Subsequently, phosphorylation at this residue was demonstrated to increase the potential of α-synuclein to fibrilize, and specific antibodies to this phosphorylated form labeled LBs in diseased brains, arguing for a role for phosphorylation of α-synuclein in the formation of aggregates and inclusions *(49).* As with the nitrated α-synuclein species, it is unclear whether the presence of this phosphorylated species is causative in the formation of the inclusions or is an epiphenomenon. In a separate study, G protein-coupled receptor kinases were also shown to phosphorylate α-synuclein at the same serine residue *(50).* Phosphorylation of α-synuclein at tyrosine residues has also been reported. Ellis et al. *(51)* and Nakamura et al. *(52)* reported phosphorylation of α-synuclein at tyrosine residue 125 by the Src family of tyrosine kinases. Subsequently, the kinase Pyk2/RAFTK, an upstream regulator of the Src family kinases, was reported to be responsible for stress-induced α-synuclein phosphorylation at this site *(53).* Interestingly, phosphorylation at multiple tyrosine residues by p72syk (Syk) prevented α-synuclein fibrilization *(54).* It appears, therefore, that depending on the particular sites involved, phosphorylation may enhance or diminish the fibrilization potential of α-synuclein.

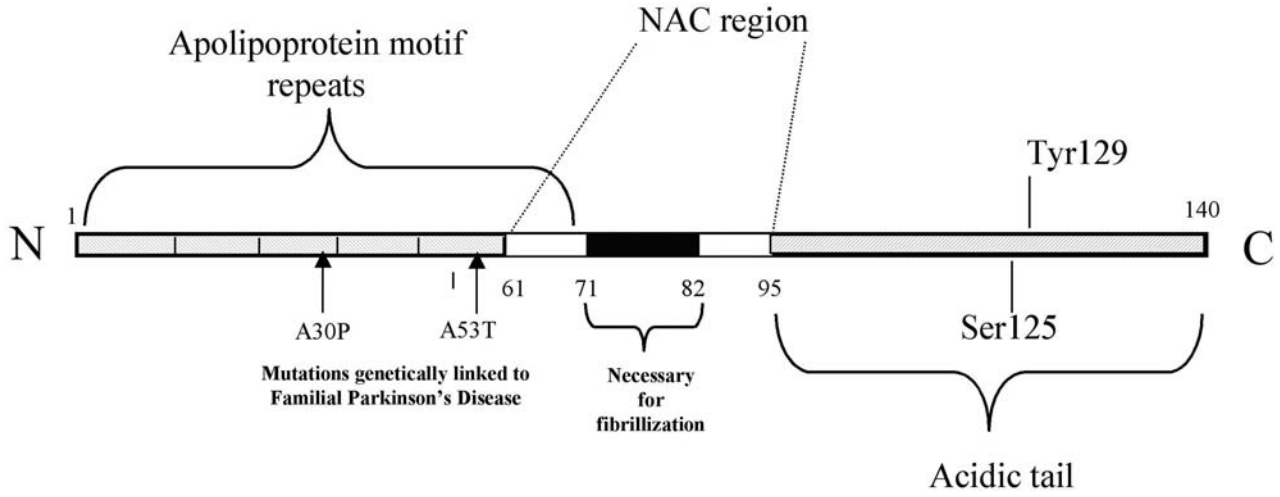

Fig. 1. Schematic diagram of α-synuclein structure.

Localization/Function Insights: Lipid Binding

The original name for α-synuclein was derived from the fact that it was identified in synapses and the nucleus in the torpedo ray *(3).* It is expressed predominantly in neurons *(55–57),* but expression has also been reported in a variety of other cell types, such as glia, platelets, and chromaffin cells *(58–61).* In the last case, α-synuclein was identified in the Golgi apparatus and vesicles *(61).* Its exact localization within neuronal cells remains unclear, especially given the fact that such localization may change, for example during neuronal development *(62).* In the mature CNS of mammalian species α-synuclein is clearly localized predominantly to presynaptic terminals. It is enriched in presynaptic vesicles, but has not been conclusively isolated within vesicular fractions *(55,56).* We have noted that, at least in cultured neuronally differentiated PC12 cells and neonatal sympathetic or embryonic cortical neurons, α-synuclein can also localize prominently to the nucleus *(63,64).* In vivo studies have shown prominent cytoplasmic staining for α-synuclein in a subset of neurons in the peripheral *(44)* and central nervous systems *(58,65).* In the latter case, nuclear labeling was clearly identified using a number of different specific antibodies targeted against α-synuclein *(65).* What the function of α-synuclein may be in the nucleus is unknown, but a more recent study showed binding of α-synuclein with histones in the nucleus of mouse dopaminergic neurons following paraquat administration *(66).*

The proximity or existence of α-synuclein within vesicular structures and the presence of the apolipoprotein-like repeat motif have led to studies of its interaction with lipids. In vitro studies suggest that α-synuclein binds acidic phospholipid vesicles in a way that markedly alters its secondary structure *(67).* Moreover, binding and oligomerization of α-synuclein were enhanced when acidic phospholipid vesicles were enriched with long-chain polyunsaturated fatty acids (PUFAs) *(68).* In cells treated with high fatty-acid (FA) concentrations, wild-type (WT) and A53T, but not A30P, α-synuclein accumulated on phospholipid monolayers surrounding triglyceride-rich lipid droplets *(69).* It was suggested that α-synuclein may protect stored triglycerides from hydrolysis. Recently, Sharon et al. (70) obtained evidence that α-synuclein shares some properties with

the family of fatty-acid binding proteins (FABP) and suggested that it may play a role in membrane biogenesis. Membranes have been reported to accelerate the fibrilization of α-synuclein and a recent study suggests that aggregation of α-synuclein may occur on membrane surfaces *(69,71).* It has also been reported that in the presence of lipid vesicles (synthetic or brain-derived) α-synuclein undertakes a helical conformation *(72).* Exposure of α-synuclein to lipid vesicles leads to preferential binding to the membrane, induction of a helical structure, and prevention of protein self-association *(73).* The effect of the vesicles on α-synuclein fibrilization was shown to be dependent on the ratio of lipid to protein with higher rates of fibrilization obtained with low mass ratios of lipid vesicles to α-synuclein. When the lipid content increased the α-synuclein conformation was largely α-helical. This observation suggests that if under normal conditions α-synuclein is mainly bound to membranes in dopaminergic neurons this would minimize its chances of aggregation.

Physiological Function

Apart from its potential involvement in lipid metabolism, α-synuclein has been proposed to have a number of other functions, but none of these has been firmly established as its physiological function. The predominant theory is that α-synuclein participates in synaptic functions such as neuronal plasticity and neurotransmission, consistent with its localization in presynaptic nerve terminals in the central nervous system, including dopaminergic neurons of the SNc *(55,56).* Moreover, association of the protein with synaptic vesicles supports its involvement in synaptogenesis *(62).* However, delayed expression of α-synuclein after synapse formation *(74,75),* together with a lack of abnormalities in synaptogenesis in KO mice *(76,77),* argue against a critical role for the protein in synaptogenesis. Targeted disruption of the α-synuclein gene caused a relative enhancement of a paired pulse response in the nigrostriatal system of mice, suggesting a role in the negative control of neurotransmission, at least within the dopaminergic system. Consistent with this idea, these animals also demonstrated an altered response to methamphetamine-induced rotations *(76).* An antisense oligonucleotide targeted to α-synuclein caused a significant drop in the size of the distal vesicular pool in cultured hippocampal neurons *(75).* Cabin et al. *(77)* have confirmed and expanded on this finding. They report that hippocampal slices from α-synuclein knockout mice show a depletion of the undocked vesicular pool, without an effect on the number of docked vesicles. Electrical stimuli that affected only docked vesicles, such as a brief train of stimulation, had no differential effect, whereas those stimuli that affected undocked vesicles, such as a prolonged train of repetitive stimulation, showed a significant impairment in synaptic response. These data argue for a function of α-synuclein in vesicle generation and/or maintenance, consistent with its interactions with lipids, and in positive control of neurotransmission. The apparent discrepancy with the study of Abeliovich et al. *(76)* could be due to the different neuronal systems studied.

There is also some indication that α-synuclein could play a role as a molecular chaperone. Studies have described binding with multiple proteins, including PLD2, tau, BAD, protein kinase Ca, synphilin, tubulin, and calmodulin *(71,78–82).* α-Synuclein may be a chaperone protein based on its ability to inhibit thermally induced protein precipitation *(83,84),* a hypothesis that is further supported by the finding that α-synuclein has structural homology with and binds to 14-3-3 proteins, a family of molecular chaperones *(78).* The homologous domains of α-synuclein and 14-3-3 contain residues that for 14-3-3 mediate protein-binding interactions *(78).* Thus, identifying proteins to which α-synuclein binds may help elucidate α-synuclein function.

Due to its relationship to PD, special attention has been paid to the possibility that α-synuclein may have a function that is specific to dopaminergic neurons. Perez et al. *(85)* showed that in a transformed CNS dopaminergic cell

line α-synuclein interacted directly with tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis. This interaction was associated with a reduction of TH activity, without an effect on total TH levels. This finding has yet to be confirmed, especially in an in vivo setting, but it suggests that α-synuclein may control not only neurotransmitter release, but also dopamine biosynthesis, thus acting at multiple levels of neurotransmission. Another interaction that may be important is that reported with the dopamine transporter (DAT), which acts as a dopamine uptake mechanism at the level of the cell membrane. Two different groups have shown that α-synuclein co-immunoprecipitates with DAT. One group reported that overexpression of α-synuclein led to increased clustering of the transporter and increased dopamine uptake *(86),* whereas the other reported that it led to decreased transporter activity *(87,88).* The physiological relevance of these observations remains unclear, especially given the fact that in α-synuclein knockout mice no difference in DAT activity was observed *(89).*

Another function that has been attributed to α-synuclein is an anti-apoptotic function. In a variety of cell culture systems overexpression of WT α-synuclein has been associated with survival-promoting effects against a variety of insults, including oxidative stress and serum deprivation *(71,90–92).* It has been proposed that such effects may be mediated through an interaction of α-synuclein with protein IB1, which in turn inhibits the activity of c-jun-N-terminal kinase (JNK), a known mediator of apoptotic cell death *(92).* Another possible survival mechanism, activated only when intracellular α-synuclein levels are below a certain threshold, is the activation of the PI3 kinase/Akt pathway *(91).* It should be noted, however, that in many cases detrimental effects of WT α-synuclein overexpression have been observed (see below). In our own experience, overexpression of WT α-synuclein in PC12 cells was not associated with protection against a classic paradigm of apoptosis, that induced by serum deprivation *(63).*

Aberrant Functions of α-Synuclein: Insights From In Vitro and Cell-Culture Models

Because α-synuclein is a main constituent of LBs even in sporadic cases of PD, it is thought that the WT form may have a potential aberrant function, which is accentuated in the case of the mutations. This hypothesis is reinforced by the recent finding of the triplication of the gene locus of α-synuclein in a family with autosomal dominant PD *(22).* Although the exact aberrant function of α-synuclein that links it to neurodegeneration is not known, the weight of the evidence suggests that the process of its conversion to soluble, then insoluble, oligomers and eventually to insoluble aggregates and inclusions is involved. As mentioned, in vitro studies have shown that WT recombinant α-synuclein can form insoluble fibrils and aggregates *(26,28).* The A53T, but not the A30P, mutant forms such aggregates faster than WT α-synuclein *(26).* This cast doubt on the idea that the end-product of this in vitro reaction, the fibrillar form, could be toxic. Later studies have shown that both mutant forms have a greater propensity to form protofibrils, a precursor of the mature fibrils, compared to the WT form *(93,94).* The idea that this protofibrillar form could be the toxic species was further reinforced by the finding that dopamine and other catecholamines inhibited the conversion of α-synuclein from a protofibrillar to a fibrillar form *(95).* It has been proposed that this toxic effect may be due to the formation of pores on vesicles. α-Synuclein mutants have a greater propensity to form such pores on synthetic vesicles, but whether such pores can be formed in a more physiologic setting is not known *(96,97).* In contrast to studies using recombinant α-synuclein, most studies have failed to find evidence for α-synuclein aggregation when it is overexpressed in mammalian cell culture systems, even though toxicity is often demonstrated. It is possible that endogenous inhibitors of α-synuclein aggregation exist within cultured cells. In some

cases, even the WT form is toxic *(98–100)*, whereas in others only the mutants are detrimental to cell viability *(63,71,101,102)*. In many reported cases, overexpression of α-synuclein, especially the mutant forms, leads to no difference in baseline viability, but to an increased sensitivity to a variety of insults, in particular oxidative stress and proteasomal dysfunction *(71,101,103–106)*. To reconcile the previously mentioned protective effects of α-synuclein overexpression in cell-culture systems with these findings, Seo et al. *(91)* have proposed that the levels of expression are critical. Only when intracellular levels of α-synuclein are above a certain threshold does the aberrant function emerge. This idea receives support from another study, in which the toxicity of the WT form approached that of the mutants with increased gene dosage *(107)*.

A cellular system in which the temporal sequence of recombinant α-synuclein oligomerization and eventually fibrillar inclusion formation can be recapitulated is that created by Lee and Lee *(108)*, based on the expression of an adenovirus encoding WT α-synuclein. In these studies the protofibrillar forms were those associated with cellular toxicity, in particular Golgi fragmentation *(109)*. Exposure to the mitochondrial complex I inhibitor rotenone led to an accelerated rate of protofibril and fibril formation *(110)*. Xu et al. *(107)* reported that overexpression of α-synuclein in human dopaminergic cells caused cellular toxicity that was dependent on the presence of dopamine, and was associated with the generation of soluble oligomeric forms. Like the study with the recombinant protein mentioned above *(95)*, such work provides a potential explanation for the selective vulnerability of dopaminergic neurons in PD. A further link to the dopaminergic system is provided by studies that show that A53T α-synuclein inhibits dopamine release, thus potentially leading to increased levels of cytosolic dopamine, which may be cytotoxic *(63,111)*.

A related theory about the aberrant function of α-synuclein posits that it is related to proteasomal dysfunction. Proteins encoded by two other genes in which mutations cause familial PD, parkin and UCH-L1, are involved in the regulation of the ubiquitin-proteasome system (UPS), suggesting that dysfunction of this pathway is involved in the pathophysiology of PD *(112)*. Consistent with this notion are findings of reduced proteasomal activity and reduced levels of α-subunits of 20S proteasome in the SN of sporadic PD patients *(113,114)*. Furthermore, proteasomal inhibition in a variety of cell-culture systems and in vivo can lead to the formation of LB-like cytoplasmic inclusions *(115–118)*. Three different groups have now reported that the mutant forms of α-synuclein can selectively cause proteasomal dysfunction in neuronal cell culture systems *(63,102,103)*. Recently, it has been shown that aggregation and inclusion formation can lead to secondary proteasomal dysfunction *(119)*. Whether proteasomal dysfunction following mutant α-synuclein overexpression is due to protein aggregation is unclear. In our study we found no obvious α-synuclein aggregates *(63)*. However, we cannot exclude the possibility that soluble oligomers of α-synuclein present in small amounts could be directly involved in proteasomal dysfunction.

Another mechanism through which α-synuclein could inhibit the proteasome is through a direct interaction. Two groups have reported that α-synuclein binds to Tat-binding protein 1, a component of the 19S regulatory subunit of the proteasome *(120,121)*. In the latter study, fibrillar α-synuclein had a much stronger inhibitory effect on the activity of the proteasome compared to the monomeric protein, and this effect appeared to be mediated at the level of the 19S proteasome. Arguing further for a relationship of α-synuclein toxicity with dysfunction of the UPS, Petrucelli et al. *(102)* demonstrated that expression of the E3 ligase parkin was protective both against pharmacological proteasomal inhibition and overexpression of A53T α-synuclein in primary ventral midbrain dopaminergic neurons. In our study we also noted a marked disruption of lysosomal acidification in cells expressing A53T, but not WT, α-synuclein, suggesting that the lysosomal system, the other

major component of cellular protein degradation, may also be affected *(63).* Whether these are independent effects or the result of cross-talk between these two degradation pathways is unknown.

A variety of other toxic effects have been attributed to α-synuclein based on cell-culture studies. A potential toxic function is that related to the ERK/MAP kinase pathway. α-Synuclein was found to interact with the transcription factor Elk-1, and to thus lead to an inhibition of the ERK/MAP kinase pathway, which is involved in cell proliferation and survival *(122,123).* Potentiation of cell death pathways was proposed as a mechanism by Seo et al. *(91),* who found an increase in pro-apoptotic Bax and a decrease of anti-apoptotic bcl-2 in cells overexpressing α-synuclein. α-Synuclein overexpression has also been reported to induce oxidative stress through unclear mechanisms *(100,105).* In one study, application of antioxidants prevented oxidative stress and mitochondrial impairment in cells expressing α-synuclein *(100).* The authors argued for a potential primary effect at the level of the mitochondria.

Animal Models of α-Synucleinopathies

A number of groups have generated transgenic mice expressing wild-type or mutant α-synuclein under the control of different neuronal promoters. Masliah et al. *(124)* were the first to report such a model. They expressed WT or A53T α-synuclein under the control of a platelet-derived growth factor β (PDGFβ) promoter, which induced expression mostly in the neocortex and limbic regions, but also in the substantia nigra (SN). Some neurons showed accumulation of cytoplasmic α-synuclein, sometimes in the form of granular, but not fibrillar, inclusions, and at later time points the mice showed a dopaminergic deficit, without overt cell loss *(124).* Although initially no difference was found between WT and A53T forms in terms of toxicity, subsequent studies by the same group have shown that this mutant form is more toxic. Significantly, this enhanced toxicity was associated with fewer inclusions, but with an increase in oligomeric forms compared to the WT-expressing mice *(125),* suggesting again that oligomeric, pre- or proto-fibrillar forms may be the toxic species. Expression of α-synuclein under the Thy-1 promoter led to more extensive expression throughout the CNS, but not the SN *(126).* There was marked toxicity with axonal and synaptic degeneration, especially in the brain stem and the motor neurons. No difference was observed between WT and mutant forms. Another group reported similar findings with expression of A30P α-synuclein under the same promoter. In affected regions of these animals, insoluble, proteinase K-resistant, and hyperphosphorylated α-synuclein was identified *(127,128).* Two other groups, using the prion promoter for α-synuclein expression, reported that only the A53T form was toxic, whereas expression of the WT or A30P form exerted no deleterious effects *(110,129).* The distribution and severity of the pathology was similar to that of the studies with the Thy-1 promoter. Despite expression in dopaminergic neurons of the SN, there was a paucity of dopaminergic pathology. The reported inclusions had a somewhat more fibrillar nature, although did not mimic LB formation exactly. Another study however, also using the prion promoter, found toxicity only in an A30P-expressing line, which had the highest levels of transgene expression. Gliosis and α-synuclein accumulation in the cell soma, but no LB-like inclusions, were identified. The dopaminergic system again was not affected, despite high levels of α-synuclein expression *(130).* To complicate matters further, a number of studies have reported no phenotype or pathology with α-synuclein expression driven specifically in dopaminergic neurons *(131,132).* Such neurons were not more sensitive to MPTP *(132).* In contrast, Richfield et al. *(133)* reported that expression of a double mutant form of α-synuclein under the control of a tyrosine hydroxylase promoter did confer dopaminergic toxicity.

The amino acid sequences of the murine and human α-synucleins differ at seven positions. As mentioned in the Introduction, the mouse protein contains a T residue at position 53 similar to the mutant human A53T. It has been demonstrated in vitro that the mouse protein is more fibrilogenic than the WT and mutant human forms, and that its fibrilization is delayed by the human WT and A53T proteins *(134).* This inhibition of fibrilization was shown to cause the accumulation of possibly toxic α-synuclein protofibrils. Therefore, possible interactions between the overexpressed human and the endogenous mouse α-synuclein need to be taken into account when interpreting the results derived from transgenic mice. In particular, the absence of frank fibrillar inclusions in most mouse models may be due to this effect.

The conclusion from studies in transgenic mice is that overexpression of all forms of α-synuclein can lead to neuronal axonal degeneration and aggregate/inclusion formation. Neuronal death has not been convincingly demonstrated. The lack of consistent correlation between fibrillar α-synuclein and toxicity further suggests that the oligomerized or prefibrillar form of α-synuclein may be the toxic species. Dopaminergic neurons appear relatively resistant to the toxic effects of α-synuclein overexpression. This may be related to the levels of the transgene achieved in dopaminergic neurons, or to some intrinsic, perhaps species-specific, protective element. Overall, the A53T form appears to be the most toxic, consistent with certain studies in cell models. Differences in levels of expression, the promoter, or the genetic background of the mouse strain utilized likely account for some of the differences observed across studies.

A significant finding related to mouse models of synucleinopathy is that double-transgenic progeny of mice that coexpress the nonfibrilizing homolog of α-synuclein, β-synuclein, show significant amelioration of behavioral and pathological PD phenotypes *(135).* This is consistent with in vitro studies, which have shown that α- and β-synucleins interact and that β-synuclein inhibits the fibril formation by α-synuclein *(136).* Recently, it was reported that β-synuclein inhibits A53T protofibril formation, and that although β-synuclein can form structural oligomers these do not have the propensity to form pores on vesicles like α- or γ-synuclein *(137).*

Aggregation of α-synuclein in the form of inclusions has been described in rats exposed to chronic rotenone *(138),* and in mice injected with paraquat *(47,139).* Less convincingly, aggregation of α-synuclein has been described in nigral neurons of mice and monkeys treated with MPTP *(140).* Whether such aggregates or other prefibrillar forms of α-synuclein play a role in the toxicity induced by these agents is unclear.

Studies with virus-mediated overexpression of α-synuclein in the ventral midbrain of rats or primates have yielded a more consistent picture *(141–143).* There is selective degeneration of dopaminergic neurons, despite the transduction of other neuronal subtypes, and formation of intracellular aggregates and inclusions. The animals show locomotor deficits commensurate with the degree of dopaminergic loss. There does not appear to be any difference between WT or mutant forms. Significantly, overexpression of the rat isoform caused inclusion formation, but no dopaminergic deficit, again suggesting a dissociation between inclusion formation and death *(141).* Species differences or more optimal levels of expression of α-synuclein in dopaminergic neurons using this technology may contribute to its greater success in mimicking aspects of PD. Consistent with the first possibility, Dong et al. *(144)* found no pathology in mice with successful viral transduction of dopaminergic neurons with A53T α-synuclein.

Overexpression of α-synuclein in flies has also yielded a very interesting model. Flies expressing α-synuclein specifically in dopaminergic neurons show loss of these neurons over time. Rather typical LB inclusions are also formed. When α-synuclein is expressed more widely in the CNS, there is a severe locomotor deficit. All forms of α-synuclein caused a similar degree of

toxicity *(145).* This model has already been used to demonstrate the protective effect of molecular chaperones *(146,147),* although the exact mechanism through which this was achieved remains unclear, given the fact that inclusion formation was not affected.

More recently, a *Caenorhabditis elegans* model has also been reported *(148).* Overexpression of all forms of α-synuclein specifically in dopaminergic neurons caused a moderate degree of cell loss. Ill-defined inclusions were present in a small minority of neurons. α-Synuclein was also toxic when expressed specifically in motor neurons or pan-neuronally. Motor deficits were observed when more widespread extradopaminergic cell loss occurred. This model may also be very useful, due to the ease of genetic manipulation in this system

Regulation of α-Synuclein Production

Both the data with the α-synuclein polymorphisms in the promoter region and those from the cell-culture and animal models, where overexpression of WT α-synuclein can cause toxic effects in certain circumstances, argue that alterations in the production of α-synuclein may have detrimental effects. The predominant idea, based on these studies, is that excess production of α-synuclein may lead to oligomerization and subsequent fibrilization and potentially further modifications that would be damaging to the cell. This idea is on much firmer ground following the recent report of α-synuclein gene triplication in familial PD *(22).* Increased α-synuclein mRNA levels have been reported in the temporal cortex of patients with diffuse Lewy body disease (DLBD) *(149).* It should be noted however that some evidence also exists for the contrary: Lymphoblasts of patients harboring the A53T mutation show very low levels of α-synuclein mRNA *(150),* and mRNA levels of α-synuclein in the substantia nigra of PD patients were reported to be decreased *(151).* In further studies, the decrease in mRNA expression of both mutant alleles in lymphoblastoid cell lines was found to occur only in advanced cases, suggesting that it was related to disease progression, but not initiation *(152).* In addition, it has been hypothesized that the fibrilization and sequestration of α-synuclein within LBs may lead to a decrease of its soluble pool *(85).* The studies mentioned earlier, supporting a survival effect of WT α-synuclein, also suggest that loss of this effect may be deleterious. It is therefore still possible that low levels of α-synuclein may be detrimental, even though no such detrimental effects have appeared in any of the null mice developed so far *(76,77,89).* In any case, regulation of α-synuclein at the transcriptional and translational levels is of the utmost importance, yet very little is known about its mechanisms. α-Synuclein is upregulated during late embryonic and early postnatal development in the rodent CNS *(62,153, 154).* It is also induced in a variety of injury models of the rodent substantia nigra pars compacta (SNpc): striatal injection of an excitotoxin *(155),* MPTP *(156),* and paraquat *(47).* However, the role of α-synuclein upregulation in these injury models is not understood. It is unclear whether it represents a response to maintain neuronal viability or a plastic/adaptive response to injury, or whether it is directly involved in dopaminergic neuron degeneration, perhaps through aggregate formation. It is also unclear, given the nature of these in vivo experiments, whether these effects are mediated directly on dopaminergic neurons by the injurious stimuli. Animal models that have been generated to answer such questions have given conflicting results. For example, in the MPTP model, α-synuclein null mice show a remarkable resistance to the toxic effects of MPTP, suggesting that α-synuclein may be playing a role in the toxicity induced by MPTP *(89).* In apparent contradiction, overexpression of α-synuclein in dopaminergic neurons of the substantia nigra caused resistance to the neurotoxin paraquat *(157),* suggesting that the induced expression of α-synuclein in this model may have been a protective response. One strategy to address these issues is to study

the expression of α-synuclein in simplified cell-culture systems, and to identify critical factors that regulate this expression. Using PC12 cells and primary sympathetic neurons, we *(158)* previously found that nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) induce a specific upregulation of the rodent homolog of α-synuclein, synuclein-1. It is known that NGF-treated PC12 cells release less dopamine in response to depolarizing stimuli compared to naive cells *(159).* Based on these findings, we hypothesized that α-synuclein upregulation in neuronally differentiated PC12 cells represents a growth-factor-mediated plasticity response that negatively controls neurotransmitter release *(158).*

We have recently extended our observations of regulation of α-synuclein by growth factors to embryonic ventral midbrain cultures. bFGF induced a specific upregulation of α-synuclein in dopaminergic neurons in these cultures *(64).* As in PC12 cells, this upregulation appeared to be transcriptionally mediated, because it was blocked by application of actinomycin D. We also observed a marked upregulation of α-synuclein in dopaminergic neurons with time in culture, consistent with findings in other cell-culture systems and with developmental regulation of α-synuclein in vivo *(62,74,75,155).* In contrast, GABAergic neurons had very low levels of α-synuclein, and showed no induction with time in culture or bFGF, consistent with studies in developing rats *(153,155)* and in humans *(160),* which show little α-synuclein expression in SN pars reticulata. Application of MPP+ to the cultures did not lead to an increase in the levels of α-synuclein, despite inducing dopaminergic neuron cell death, raising the possibility that the effects seen in vivo *(156)* may have been indirect. We hypothesize that such in vivo effects may in fact be due to bFGF, which is known to be upregulated by this regimen of MPTP in striatal glia *(161,162),* and could thus have an impact on intact nigrostriatal projections following MPTP administration.

It has recently been reported that in cocaine users α-synuclein mRNA was elevated in the substantia nigra and ventral tegmental area compared with age-matched drug-free control subjects *(163).* The functional relevance of this increase was confirmed by robust increases in the levels of protein. Canales and Graybiel *(164)* have suggested that different neural circuits become activated in response to cocaine as a result of repeated administrations and involve DA and glutamate as key co-players in regulating basal ganglia loops that affect both locomotion and stereotypy. Thus, changes in the expression of α-synuclein protein may be an adaptive response to cocaine in reward-related neurons of the nigral/ventral tegmental area complex.

Regulation of α-Synuclein Degradation

Regulation of the amount of protein in a cell occurs at many levels, including that of degradation. An issue that remains unresolved is that of α-synuclein degradation. This has special significance, given the potential involvement of the UPS in PD. In a number of studies α-synuclein levels accumulate in cells when the proteasome is inhibited, suggesting that the UPS normally degrades α-synuclein *(165,166).* It is significant that in most of these cases α-synuclein degradation was studied in transiently transfected cells, and thus may not reflect the steady state of the protein. A separate study showed that although α-synuclein had a very long half-life and was not ubiquitinated, it was still targeted and degraded by the proteasome, consistent with findings with other natively unfolded proteins that do not need ubiquitination for proteasomal degradation *(167).* A number of other studies, including ours, that have used stable cell lines or cells normally expressing α-synuclein have failed to replicate the finding of accumulation of α-synuclein with proteasomal inhibition *(39,102, 115,116,168).* In one of these studies, the lysosomal inhibitor ammonium chloride, but not the proteasomal inhibitor lactacystin, led to an increase of α-synuclein levels, suggesting that degradation is, at least in part, lysosomal *(39).*

In a more recent study, both proteasomal and lysosomal degradation of α-synuclein were found to occur in inducible cell lines *(169).* Interestingly, lysosomal degradation of α-synuclein in this study occurred through the mechanism of macro-autophagy, a mechanism through which the cell encircles organelles or parts of the cytoplasm in double-membrane structures, which are subsequently converted to autophagolysosomes and then mature lysosomes. It is likely that, depending on the situation and, in particular, posttranslational modifications, α-synuclein degradation may occur through either the proteasomal or the lysosomal pathway.

Recently, it has been shown quite convincingly that α-synuclein, and in particular the phosphorylated form, in LB diseased brains is at least mono- and di-ubiquitinated *(170,171).* This would not be sufficient to target α-synuclein for proteasomal degradation, but it does argue for some involvement of ubiquitination in an in vivo setting. Sampathu et al. *(172)* also reported ubiquitinated species of α-synuclein in brains of patients with LB dementia and in an A53T mutant human α-synuclein transgenic mouse model. Furthermore, in vitro ubiquitination of α-synuclein fibrils was shown to recapitulate the pattern of α-synuclein ubiquitination observed in human disease and in the A53T α-synuclein mouse model, suggesting that ubiquitination of α-synuclein is not required for inclusion formation and follows the fibrilization of the protein. Another posttranslational modification that may be important for α-synuclein degradation is glycosylation. Shimura et al. *(173)* reported that a glycosylated, but not the native, form of α-synuclein accumulated in brains of patients with parkin mutations, arguing for involvement of parkin, through its E3 ligase function, in the degradation of this modified form of α-synuclein. These results, which have yet to be confirmed independently, also argue that this glycosylated form may be the toxic α-synuclein species.

Other proteolytic systems have recently been proposed to play a role in α-synuclein cleavage and degradation. Mishizen-Eberz et al. *(174)* reported that the protease calpain I can cleave α-synuclein in vitro. Both the fibrillar and the A53T mutant forms were differentially cleaved compared to the native WT protein, arguing for a potential involvement of calpain in the pathogenesis of PD. Because calpain is dependent on calcium for its activity, it may play a role in α-synuclein cleavage during synaptic transmission. This finding has not been confirmed in a cellular context. In addition, kallikrein-6, a neuronal serine protease also called neurosin, was found to cleave α-synuclein in the cytoplasm upon cellular stress. Downregulation of neurosin led to an accumulation of α-synuclein, suggesting a physiological role of this protease in α-synuclein degradation, at least under the conditions studied *(175).*

Concluding Remarks

The normal and aberrant functions of α-synuclein are still unclear, but experimental evidence suggests that its physiological function is related to the control of neuronal transmission, likely through interaction with lipids on vesicular membranes, and its toxicity (Fig. 2) to the process of fibrilization, in which prefibrillar forms are likely to be more toxic. Posttranslational modifications, such as those induced by phosphorylation and oxidative or nitrative insults, influence the fibrilization potential. The relationship of the expression of mutant forms to proteasomal dysfunction is particularly interesting, because it leads to a unifying theory of PD pathogenesis. The regulation of α-synuclein production and degradation may be critical for PD pathogenesis. Diverse factors, including neurotoxins and trophic factors, upregulate α-synuclein expression through unclear mechanisms. It is likely that, depending on the conformation of α-synuclein, the proteasomal or the lysosomal system may be responsible for its degradation. Thus, dysfunction of these systems could lead to accumulation of aberrant species of α-synuclein.

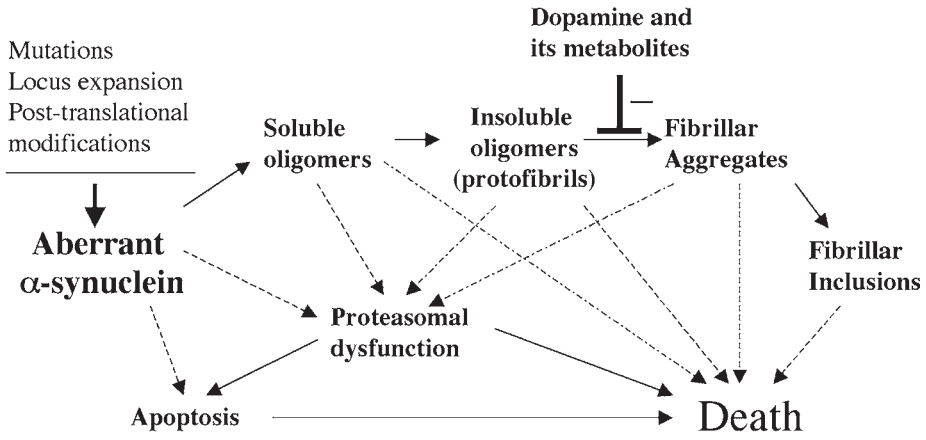

Fig. 2. Postulated mechanisms of α-synuclein toxicity. Solid arrows indicate more established pathways, whereas dotted arrows indicated largely hypothetical mechanisms.

Note

A third mutation in the *α-syn* gene in a family with autosomal dominant LBD has been identified by Zarranz et al. Interestingly, this mutation results in a radical amino acid substitution (E46K).

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