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Current Topics

Zeroing in on the Pathogenic Form of α-Synuclein and Its Mechanism of Neurotoxicity in Parkinson's Disease†

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ABSTRACT: Parkinson's disease (PD) is linked to mutations in the protein α-synuclein, which can exist in vitro in several aggregation states, including a natively unfolded monomer, a β-sheet rich oligomer, or protofibril, and a stable amyloid fibril. This work reviews the current literature that is relevant to two linked questions: which of these species is pathogenic, and what is the mechanism of neurotoxicity? The amyloid fibril, fibrillar aggregates, Lewy bodies, and the α-synuclein monomer, which is normally expressed at high levels, are all unlikely to be pathogenic, for reasons discussed here. We therefore favor a toxic protofibril scenario, and propose that the pathogenic species is transiently populated during the process of fibrillization. Toxicity may arise from pore-like protofibrils that cause membrane permeabilization. An approach to testing this hypothesis is discussed.

Parkinson's Disease

The clinical features of Parkinson's disease (PD),[1] which include tremor, rigidity, and bradykinesia (slowness of voluntary movement), were first systematically described by James Parkinson in *An Essay on the Shaking Palsy* (*1*). One to two percent of the population that is more than 65 years old has PD (*2*). While the majority of cases appear to be idiopathic (i.e., of unknown origin), in rare cases, the disease can be inherited in an autosomal dominant fashion (*3*). An Italian family that allowed the first such mutation to be identified carried a point mutation (A53T) in the gene encoding α-synuclein (*4*). Subsequently, a second PD-linked autosomal dominant mutation in α-synuclein (A30P) was found in a German family (*5*). Recently, other PD-linked mutations in genes encoding parkin (*6*), UCH-L1 (*7*), and DJ-1 (*8*) have been described. The interactions of these gene products with α-synuclein may be important in the pathogenesis of PD (*9*−*11*). This review will focus on α-synuclein, since it appears to be centrally important in the pathogenic pathway.

Since a detailed exploration of the pathological mechanism(s) of PD and the involvement of α-synuclein in the natural environment of PD (the living human brain) is not possible with current technology, one must gather evidence using either post-mortem brain, which offers a snapshot of late stage disease, or through the study of model systems. These are discussed below, in order of increasing biological "distance" from the PD brain.

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[1] Abbreviations: AFM, atomic force microscopy; EM, electron microscopy; LB, Lewy body; PD, Parkinson's disease; SN, *substantia nigra*; SNc, *substantia nigra pars compacta*; TH, tyrosine hydroxylase; WT, wild type.

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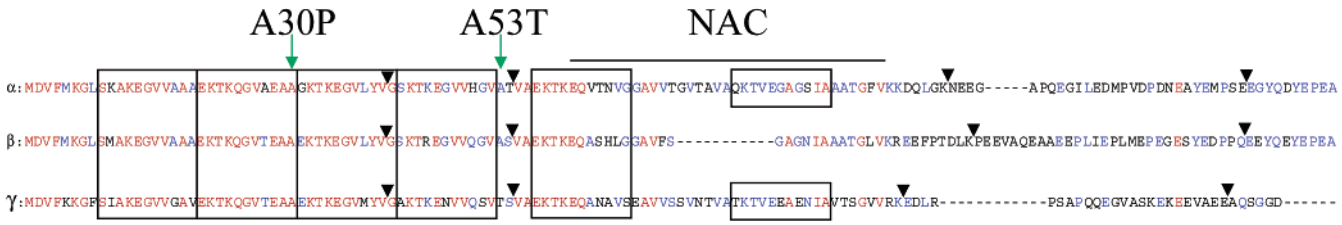

FIGURE 1: Sequence alignment of α-synuclein (NCBI entry NM_000345), *β*-synuclein (NCBI entry NM_003085), and *γ*-synuclein (NCBI entry NM_003087) using ClustalW (*117*). Residues were colored red and blue to indicate identity across three and two of the synuclein sequences, respectively. Boxes enclose 11-residue repeats; arrowheads denote exon−exon boundaries, and green arrows are used to denote the two PD-linked mutations in α-synuclein, A53T and A30P. The "NAC" sequence was first described by Saitoh et al. (*26*) and contains a sequence that is important in fibril formation (*118*). The sequences were displayed and colored using GeneDoc (*119*).

Parkinson's Disease Post-Mortem Brain

Neuropathology of Parkinson's Disease. It was in 1912 that the cardinal pathological feature of PD, intracytoplasmic inclusion bodies (Lewy bodies), was first described (*12*). Lewy bodies (LBs) are primarily composed of aggregated (fibrillar) α-synuclein (*13*), and are most predominant in the *substantia nigra pars compacta* (SNc) region of the PD brain. This area is rich in dopaminergic neurons that project into the neostriatum (the major input center to the movement-modulating basal ganglia), and the death and degeneration of a large proportion of these neurons are characteristic of PD and probably account for the majority of its clinical symptoms (*14*). Ehringer and Hornykiewicz (ca. 1960) found that this neuronal loss results in a severe dopamine deficiency in the neostriatum (*15*); this is the basis for the most common treatment of PD symptoms, dopamine replacement via oral administration of L-DOPA, a dopamine precursor. L-DOPA is converted to dopamine by dopaminergic neurons, through the action of the cytoplasmic enzyme DOPA decarboxylase. The supplement of dopamine is able to partially offset the dopamine deficiency, giving some symptomatic relief. However, patients normally become refractory and/or suffer side effects after several years of treatment.

Despite the anatomical correlation of LBs and neurodegeneration in PD, the LBs themselves may not *cause* the degenerative process. A comparison of neurons from this region with and without Lewy bodies has not shown any obvious differences in (i) cell body and nucleolar size (*16*), (ii) tyrosine hydroxylase (TH) protein levels (*17*), (iii) cellular light neurofilament subunit mRNA levels (*18*, *19*), and (iv) apoptotic-like changes (condensed and in situ end-labeling-positive chromatin) (*20*).[2] Furthermore, mutations in the parkin gene have been linked to early/juvenile-onset parkinsonism with SNc degeneration, a form of PD in which LBs are not typically found (*6*) [exceptional cases of parkin-linked PD in which LBs are found have been reported (*21*)]. This evidence and that discussed below suggest that LBs do not cause, but rather are correlated with, PD neurodegeneration.

Synuclein in the Normal and PD Brain. The function of α-synuclein (and its homologues *β* and *γ*; see below) is unknown (*22*, *23*). α-Synuclein [whose major splice form (*24*) is 140 amino acids, MW = 14 460] was originally characterized as a synaptic vesicle binding protein in *Torpedo californica* (*25*) and was predicted to have a structural and/or regulatory function, based on the positively charged 11-amino acid repeat sequences in the N-terminus (Figure 1) (*25*, *26*). These repeats have the potential to form amphipathic α-helices (*27*). The central portion of α-synuclein is hydrophobic [a fragment of α-synuclein containing a sequence, known as NAC (Figure 1), had been previously isolated from Alzheimer's disease brain (*26*)]. Finally, the C-terminal region is highly acidic and contains several proline residues (*25*, *28*). *β*- and *γ*-synucleins are highly homologous to α-synuclein in the N-terminal repeat region (Figure 1) (*29*, *30*), and have an acidic C-terminus. α-Synuclein (and probably *β*-synuclein) is expressed at high levels (*31*, *32*), predominantly in brain (*26*, *30*), and are localized presynaptically (*27*, *30*, *31*). In contrast, *γ*-synuclein is more prevalent in the peripheral nervous system (*33*). In normal SN, the α-synuclein mRNA levels have been reported by different groups to be particularly high (*34*) and particularly low (*32*) relative to levels in other regions of the brain. However, in the PD brain, it appears that α levels are increased and *β* levels are decreased, relative to non-PD controls (*32*).

A fraction of α- and *β*-synuclein purified from brain appears to be post-translationally modified, possibly at the N-terminus (*30*, *35*, *36*). An O-glycosylated form of α-synuclein (termed αSp22) has been extracted from human brain, and shown to be a substrate for parkin (*10*). Additionally, serine 129 was found to be phosphorylated in α-synuclein extracted from the brains of dementia with Lewy bodies (an Alzheimer's disease-like dementia in which LBs are abundant in the cortex) (*37*). Accumulations of phosphosynuclein are also found in the brains of transgenic *Drosophila* (see below) (*38*).

Model Systems: Animal Models of α-Synuclein Neurotoxicity

Since idiopathic PD has not been observed in animals, animal models are typically created by a genetic (via trangenesis, gene therapy, etc.) or chemical (drug, toxin, etc.) perturbation. An animal model that showed an age-dependent movement disorder phenotype and also recapitulated all of the known end-stage pathological features of PD would be extremely useful. Without that, a second type of model in which only some PD characteristics are reproduced can be nearly as valuable; differences between the observed animal phenotype and human PD can reveal information about PD pathogenesis.

[2] However, to our knowledge, characteristics of cells with and without Lewy *neurites* (LB-like inclusions within neuronal processes) have not been compared (*20*).

The first α-synuclein transgenic mouse was reported in 2000 by Masliah et al. (*39*). Over time, these mice, which express human wild-type (WT) α-synuclein at a level comparable to the level of endogenous mouse α-synuclein, developed *nonfibrillar* intraneuronal inclusions in several brain areas, including the SN. The mice also lost striatal tyrosine hydroxylase-positive nerve terminals (TH, which is involved in dopamine biosynthesis, is a marker for dopaminergic neurons), and performed poorly on a motor performance test relative to their nontransgenic littermates. Crossing these mice with a transgenic human *β*-synuclein mouse (this mouse had no obvious Parkinsonian symptoms or pathology) produced a bigenic mouse in which the severity of all three "Parkinsonian" phenomena were decreased (*40*). Another laboratory has produced transgenic mice (A53T and WT) that exhibited neurodegeneration outside the SN without fibrillar inclusions (*41*). Two other transgenic mouse models showed a severe movement disorder with fibrillar α-synuclein inclusions outside the SN (*42*, *43*). In these latter two cases, this phenotype was produced only by the A53T transgene, which was driven by the murine prion promoter, giving very high expression levels relative to the endogenous form. One A30P transgenic synuclein mouse showed a severe movement abnormality with brain gliosis, but without inclusions (*44*). The authors suggest that this phenotype may result from extreme overexpression of a presynaptic protein (in this case driven by the hamster prion promoter) and is not necessarily synuclein-specific; other lines which they produced with lower expression levels (including WT, A53T, and a second A30P mouse) displayed no motor phenotype (*44*).

Transgenic *Drosophila* models of PD produced by expression of WT, A53T, or A30P throughout the brain (*Drosophila* do not express an endogenous homologue of α-synuclein) (*45*, *46*) show progressive and selective degeneration of dopaminergic neurons with fibrillar inclusions (*46*). The transgenic *Drosophila* also develop a movement disorder that is reminiscent of human PD. Coexpression of the chaperone Hsp70 or pharmacological upregulation of heat-shock factors ameliorates dopaminergic cell loss, but does not affect inclusion formation (*45*, *47*). A phosphorylated form of α-synuclein (at Ser129) is deposited in the *Drosophila* model, but only after deposition of unphosphorylated α-synuclein (*38*).

In addition to transgenesis, viral expression systems have also been used to introduce the α-synuclein gene to specific regions of the midbrain. Lentiviral-based expression of human α-synuclein in rat SN resulted in selective dopaminergic toxicity with nonfibrillar inclusions (*48*). Overexpression of *rat* α-synuclein in this system also resulted in inclusions, but no cell loss was observed (*48*). Adeno-associated viral-based expression of α-synuclein in rat SN also produced inclusion formation with selective dopaminergic degeneration, although ultrastructural studies of the inclusions were not described (*49*). This system also caused nigral degeneration and inclusion formation in primates (*50*).

Toxin-induced PD models, such as those based on MPTP or rotenone, also produce specific dopaminergic degeneration (*51*). At the concentrations that were used, rotenone, a complex I inhibitor, does not directly inhibit ATP synthesis, but may kill cells by increasing oxidative stress (*51*). Dopaminergic neurons appear to be most sensitive to this type of insult, because their basal oxidative burden is already large as a result of catecholamine metabolism. Fibrillar LB-like inclusions develop in the rotenone model. MPTP also selectively kills dopaminergic neurons, possibly because it accesses these cells via the dopamine transporter. Interestingly, an α-synuclein knockout mouse is insensitive to MPTP toxicity (*52*), demonstrating that the MPTP toxicity pathway may require α-synuclein.

The cumulative evidence from studies of animal models further supports the notion that the LB does not cause [and may protect against (*53*)] PD. The α-synuclein-containing inclusions in some mouse models do not contain fibrils, and the fibril-containing inclusions of the fly can occur in the absence of neurodegeneration (*45*, *47*). Additional evidence from cell culture and *in vitro* systems regarding this issue is given below. Finally, these transgenic animal models suggest that α-synuclein-induced toxicity results from a gain of function (either a new toxic function or an endogenous process that becomes toxic when it exceeds a threshold) (*53*), as opposed to a loss of function (the α-synuclein knockout mouse does not develop PD characteristics) (*22*, *23*).

Model Systems: Cell Culture Models of α-Synuclein Neurotoxicity

An ideal cellular PD model will show selective dopaminergic degeneration in response to a PD-related stimulus (e.g., α-synuclein overexpression). Dopaminergic cells in rat or human primary mesencephalic neuronal cultures are selectively lost after α-synuclein overexpression in all neuronal types (*54*, *55*). Inclusion formation in this system was insignificant relative to cell death. Additionally, A53T was more toxic than the WT protein, which mirrors a key feature of human PD. This finding has been confirmed in mouse primary midbrain cultures (again, no inclusions were detected) (*11*) and was proposed to be due to proteasome inhibition by mutant α-synuclein (*11* and references therein). The α-synuclein neurotoxicity can be eliminated by overexpression of parkin, a PD-linked gene product that functions as an E3-ligase in the ubiquitin proteasomal system and is involved in the degradation of α-synuclein (*10*, *11*). Thus, the loss of parkin, which produces a recessive form of PD that can present in adolescence, may unmask the intrinsic toxicity of α-synuclein. α-Synuclein induces apoptosis in human primary neuronal dopaminergic (but not in non-dopaminergic cortical) cultures (*56*), again without detectable inclusion formation. α-Synuclein-induced apoptosis could be eliminated in this system by adding antioxidants or inhibiting the production of dopamine (*56*). The requirement of dopamine for α-synuclein toxicity explains the cell-type selectivity of PD and is consistent with *in vitro* studies (see below) that demonstrate that oxidized dopamine is capable of inhibiting α-synuclein fibril formation, resulting in accumulation of potentially toxic protofibrils (*57*). Additional evidence that protofibrils, not fibrils, are pathogenic was derived from overexpressing α-synuclein in COS-7 cells; nonfibrillar inclusions (detected by biochemical methods) were temporally associated with Golgi fragmentation and reduced cellular viability. These changes preceded the detection of larger fibrillar inclusions (*58*).

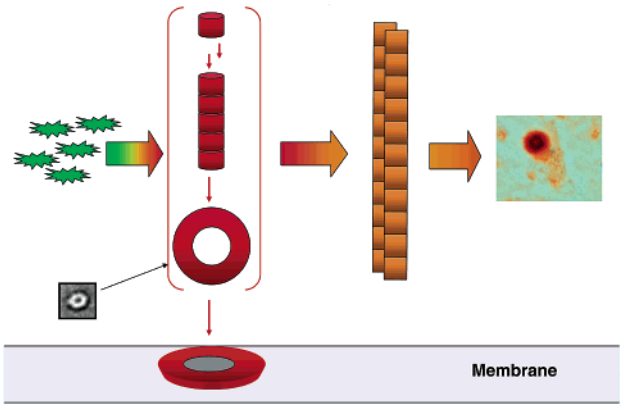

FIGURE 2: Working model of the fibrillization pathway for α-synuclein. Disordered monomeric (green starburst) α-synuclein oligomerizes to form a heterogeneous population of β-sheet rich protofibrils (red), which includes pore-like structures (electron micrograph, image-averaged, 10−12 nm outer diameter, courtesy of H. Lashuel). Insertion of the protofibril pore into a membrane, which is thought to account for protofibril permeabilizing activity, is shown schematically. The protofibril population dissipates as amyloid fibrils (orange rods) are formed. Eventually, the fibrils coalesce to form a Lewy body, as shown in the tissue section at the far right.

Model Systems: In Vitro Models of α-Synuclein (Fibrillization, Membrane Interactions, and Possible Toxicity)

An ideal *in vitro* PD model would reconstitute the essential reaction(s) of the neurodegenerative pathway, and be appropriately responsive to disease-associated mutations. In "physiological" buffer, α-synuclein populates a number of quaternary structures which can be divided into three classes: the natively unfolded monomer, structured protofibrils (transient, β-sheet rich oligomers), and the amyloid fibril (Figure 2) (*59*−*62*). Starting with the monomer (green starburst, Figure 2), the system transiently[3] populates a heterogeneous mixture of protofibrils (red cylinders, chains, and rings in Figure 2). The protofibrils seem to be consumed as the stable fibrillar state (orange rods, Figure 2) forms, but not all protofibrillar species are necessarily on the direct pathway leading to fibrils. Fibrils coalesce *in vivo* to form LBs, which are visible microscopically (Figure 2, right panel).

The α-Synuclein Monomer: Its Physical Properties and Possible Biological Activities. All three synuclein variants (α, β, and γ) are unfolded and monomeric in dilute physiological buffer, existing as a rapidly equilibrating mixture of conformers (*60*, *63*−*66*). The 100 N-terminal residues (especially residues 6−37) display a preference for α-helical structure, and transient helices probably form in this region (*65*). This helical propensity is decreased by the A30P mutation, and the A53T mutation has the more subtle effect of locally favoring extended backbone configurations, relative to WT (*67*). Monomeric α-synuclein binds to phospholipid vesicles containing acidic lipids and, upon doing so, undergoes a significant increase in N-terminal helical structure [the resulting amphipathic helices are probably responsible for membrane binding (*27*); see 11-mer repeats that are boxed in Figure 1] (*65*, *68*−*74*). The A30P mutation disrupts membrane binding and the associated α-helix formation (*67*, *68*, *71*, *75*, *76*). Under certain conditions, membrane binding may involve dimerization or trimerization of α-synuclein, but the structural consequences of this interaction have not been determined (*70*, *76*−*79*).

Although the function of α-synuclein has not been determined, some biochemical activities have been described. Studies of α-synuclein knockout mice have led to two proposals for its *in vivo* function: (i) it may negatively regulate activity-dependent dopamine neurotransmission (by inhibiting dopamine release, for example) (*22*), and/or (ii) it may play a role in the maintenance and/or mobilization of the reserve, or resting pool, of dopaminergic synaptic vesicles (*23*). In cell culture, α-synuclein inhibits dopamine biosynthesis by reducing tyrosine hydroxylase (TH) activity (*80*), and it may also inhibit proteasomal function (*11*). In addition, α-synuclein interacts with the dopamine transporter to enhance uptake of dopamine, apparently by promoting the translocation of the transporter to the plasma membrane (*81*). Finally, α-synuclein (and β-synuclein) inhibits phospholipase D2 *in vitro* (*82*). Whatever the function of α-synuclein, it may be regulated by phosphorylation. α-Synuclein can be phosphorylated on multiple serine residues, including serine 129, *in vitro* and in cell culture (*36*, *83*). α-Synuclein can also be phosphorylated on tyrosine 125 in cell culture or *in vitro* by members of the Src kinase family (*84*). Phosphorylated synuclein has been observed in human LBs and in *Drosophila* LB-like inclusions (*37*, *38*). Although it is important to elucidate the function of the α-synuclein monomer, this function is unlikely to be related to the disease, since PD seems to be driven by the gain of toxic function (as opposed to the loss of native function), linked to α-synuclein aggregation. It remains a formal possibility that the gained toxic activity may be due to a monomeric form of α-synuclein that is linked to fibrillization. However, we have chosen to initially focus our attention on the possibility that the protofibril is responsible (see below).

The α-Synuclein Fibril. α-Synuclein forms amyloid fibrils *in vitro* that are similar (by dye binding properties, antiparallel β-sheet structure, wound ultrastructure, and proteolytic resistance) to those extracted from human brain LBs (*61*). The rate of formation of these fibrils is accelerated by the A53T mutation (relative to WT) (*59*, *60*, *62*, *85*−*87*), while the A30P mutation slows fibrillization (*59*, *62*, *87*). Mixtures of WT and either of the two mutants (corresponding to the familial PD patients, all of whom are heterozygotes) form fibrils at rates intermediate between the WT and mutant rate (*62*). The lack of a correlation between the PD-causing mutations and the acceleration of fibril formation suggests that the fibril is not a pathogenic entity in PD.

γ-Synuclein fibrillizes at a slower rate than α-synuclein, and β-synuclein fibrillizes even more slowly than γ (no β-synuclein fibrils have been observed) (*63*, *66*, *88*). Furthermore, β-synuclein inhibits the fibrillization of α-synuclein (*40*, *63*, *66*).

α-Synuclein Protofibril. Protofibrils are transient β-sheet-containing oligomers (*72*) that are formed during fibrillization. Protofibrils are observed by AFM and EM as a heterogeneous mixture of morphologies, including spherical, annular, pore-like, tube-like, and chain-like structures (Figure 2) (*59*−*62*, *72*, *89*, *90*). Some of these morphologies may be on the direct pathway to fibrillization, while others may

[3] The appearance of fibrils *in vitro* is accompanied by the disappearance of protofibrils (*57*, *59*, *86*, *116*).

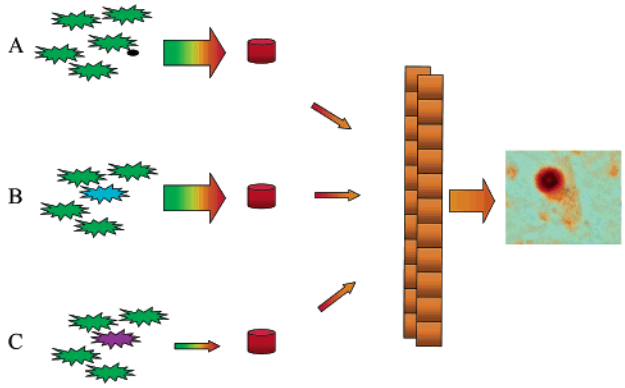

FIGURE 3: Kinetics of fibrillization of α-synuclein can be strongly affected by small amounts of homologous proteins. (A) In the presence of an abundance of α-synuclein, a small molar fraction of a dopamine−α-synuclein adduct (black dot with green starburst) promotes protofibril accumulation by inhibiting fibrillization (see the thin orange arrow) (*57*). (B) When the "impurity" is mouse α-synuclein (blue starburst), the amount of protofibril is increased, relative to that formed with pure WT α-synuclein, and fibrillization may be somewhat inhibited (*86*). (C) α-Synuclein with *β*-synuclein "impurity". A small quantity of *β*-synuclein (purple starburst), relative to α-synuclein, strongly inhibits protofibril formation, and also fibrillization (*66*).

be off the direct pathway. If protofibrils are pathogenic in PD, then phenomena linked to PD, including the mutations, may promote them, either thermodynamically (by stabilizing them) or kinetically (by accelerating their formation or slowing their conversion to fibrils). Detailed rate measurements of these interconversions are not yet possible, but crude studies suggest that this is the case. First, the PD-linked protein A30P appears to form more protofibrils than WT, and form them more rapidly (*60*, *87*). Since protofibril dissipation occurs via fibril formation,[3] A30P protofibrils may have a longer lifetime than WT protofibrils (*59*, *87*). Second, small amounts of proteins that are closely related to α-synuclein and may also be related to PD can have a large effect on the rate of protofibril and fibril formation (Figure 3). This effect is analogous to crystal poisoning by trace impurities (*91*). Incubation of α-synuclein with small relative amounts (ca. 1−5%) of an α-synuclein−dopamine adduct (formed by incubation of α-synuclein with dopamine under oxidizing conditions) slows fibril formation and increases the quantity and lifetime of protofibrils (Figure 3A) (*57*). This protofibril-stabilizing effect of dopamine offers a potential explanation for the dopaminergic selectivity of PD neurodegeneration. A similar effect is seen on incubation of mixtures of mouse α-synuclein [which fibrillizes faster than WT, A30P, or A53T (*59*, *86*)] and human α-synuclein; again, inhibition of fibril formation leads to the accumulation of protofibrils (Figure 3B) (*86*). This *in vitro* experiment may model the transgenic mouse, where the expression level of the human transgene was approximately equal to the level of the endogenous mouse α-synuclein (*39*). The relevance of studies of mouse α-synuclein and human/mouse mixtures to human disease is suggested by sequence similarity (there are seven amino acid differences between mouse and WT human α-synuclein, including a threonine at position 53) and similar *in vitro* properties (natively unfolded monomer, protofibril and fibril formation) (*75*, *86*) of the mouse and human homologues. As discussed above, only *nonfibrillar* inclusions were observed in the mouse brain, consistent with expectations based on the *in vitro* behavior (*39*). If this model is legitimate and protofibrils are pathogenic, one would predict that it may be difficult to generate a symptomatic mouse by expressing α-synuclein in a mouse in which endogenous α-synuclein had been knocked out. Crossing the symptomatic α-synuclein transgenic mouse with an asymptomatic *β*-synuclein transgenic mouse eliminates the neurodegenerative phenotype (*40*). Again, the *in vitro* model offers a consistent explanation: addition of small relative amounts of *β*-synuclein to α-synuclein incubations inhibits the formation of fibrils (discussed above) *and* the formation of protofibrils (Figure 3C) (*66*). This convergent evidence suggests that a mutation in the *β*-synuclein promoter that decreased its expression level or a mutation in the coding sequence that decreased its inhibitory activity would be pathogenic for PD.

Taken together, these *in vitro* models support a toxic protofibril hypothesis but do not specify any particular neurotoxic mechanism. The heterogeneity of the protofibril population makes it extremely difficult to measure the precise kinetics of the system. We have therefore attempted to separate protofibril subpopulations and to search for properties that are unique to particular subpopulations. Such activities are potentially toxic mechanisms.

The α-Synuclein Protofibril Population Includes Species with Pore-like Activity and Species with Pore-like Structure. The α-synuclein protofibril binds to synthetic phospholipid vesicles much more strongly than the monomer or the fibril (*59*, *72*). α-Synuclein (a mixture of monomer, small aggregates, and putative fibrils) disrupts planar bilayers (*69*). Finally, α-synuclein protofibrils (but not monomer or fibrils) cause leakage of synthetic vesicles, without affecting gross vesicle integrity (*72*, *75*). This permeabilizing activity, like that of a molecular pore, exhibits size selectivity; calcium and dopamine were released in the presence of protofibrils, but larger molecules, including cytochrome *c*, were not (*72*, *75*). Protofibrillar fractions comprising A53T or A30P had a greater specific permeabilizing activity (activity per mole of α-synuclein) than WT. This finding suggests that the PD mutations either directly affect the architecture of the pore or skew the heterogeneous protofibril population to favor the permeabilizing species. Both of these scenarios are consistent with the pore playing a role in PD pathogenesis. The pore-like activity is not always associated with protofibrils comprising other synuclein congeners; *β*-synuclein protofibrils (which are rich in *β*-sheet structure) do not permeabilize or bind acidic vesicles, but *γ*-synuclein protofibrils have these properties (*66*). Significantly, annular pore-like structures have been observed in α-synuclein protofibril preparations that have permeabilizing activity (*59*, *62*, *89*, *90*). These pore-like structures typically have outer diameters of 10−12 nm, and inner diameters of ca. 2 nm (*89*, *90*). Interestingly, the size selectivity studies discussed above set an upper limit on the inner diameter of the "pore" of 2.5 nm (*75*). The formation of these pores in cellular membranes could potentially disrupt ionic and/or metabolic homeostasis, leading to toxicity by numerous downstream mechanisms.

A Testable Hypothesis for Explaining Protofibril Neurotoxicity: The "Amyloid Pore"

Could the Amyloid Pore Be a Toxin in Other Degenerative Diseases that Are Also Characterized by Protein Fibrilliza-

tion? The hypothesis that pore formation is neurotoxic is not new. "Channel" hypotheses have been proposed to explain pathogenesis of other amyloid diseases. However, these were not widely accepted since the proteins that are involved are all normally produced at high levels and channel formation was not linked to fibril formation (*92*). The Aβ peptide of Alzheimer's disease permeabilizes phospholipid membranes (*93*−*99*), and forms channels in excised neuronal membrane (*100*) and in cells in culture (*101*−*103*). Furthermore, pore-like structures have been observed in Aβ preparations (*90*, *104*). Islet amyloid polypeptide (IAPP; deposited in pancreatic amyloid fibrils in type II diabetes) also forms channels in phospholipid and cell membranes (*101*, *105*−*107*). Our laboratory has characterized the structure of pore-like IAPP protofibrils (H. Lashuel et al., personal communication). Other amyloid proteins and fragments thereof form pores or channels, including a fragment of the prion protein (*101*, *108*), β-2-microglobulin (implicated in dialysis-associated amyloidosis) (*109*), serum amyloid A (*110*), a polyglutamine peptide (which is relevant in Huntington's and other triplet repeat diseases) (*111*, *112*), lysozyme (*113*), calcitonin (*114*), and α-atrial natriuretic peptide (*115*). Thus, the circumstantial evidence supporting the amyloid pore hypothesis is strong. However, it remains to be seen whether this shared property is relevant to disease.

Testing the Amyloid Pore Hypothesis. To test the amyloid pore hypothesis, one must attempt to disprove it in an animal and/or cellular model. The characterization of genetic enhancers and suppressors of the Parkinsonian phenotype in *Drosophila* will be extremely useful in this regard. If the protofibril is pathogenic, then enhancers should be found that decrease the amount of α-synuclein fibrils, while some suppressor mutations may *increase* the amount of fibrils. If such a relationship does not exist, then the toxic protofibril hypothesis is invalid. If the hypothesis is valid, then the dependence of disease onset and progression on the level of transgene expression in flies should be nonlinear and a critical concentration effect should be observed (*91*). The hypothesis would be strengthened by a direct demonstration of protofibril neurotoxicity, for example, by microinjection of purified α-synuclein protofibrils into dopaminergic neurons. However, the failure to observe toxicity would not disprove the hypothesis. Finally, sequence variants of α-synuclein that have altered structural and/or permeabilizing properties could be expressed in one or more of the cellular or animal models (Figure 4). If there is not a correlation between *in vivo* neurotoxicity and *in vitro* protofibril activity, the amyloid pore hypothesis is not valid. If a correlation does exist, then the circumstantial case becomes even stronger. To find a sequence variant with altered properties, we are screening a library of α-synuclein sequence variants, hoping to find variants with key *in vitro* physical properties (rate of fibrillization or protofibrillization, or specific permeabilizing activity) that differ significantly from those of WT. Those sequences will be expressed in *Drosophila*, and the resulting flies will be compared to WT transgenic flies. We are also taking the reverse approach, in which the sequence library is screened *in vivo* (in *Drosophila* or in cell culture) to select variants with altered *in vivo* properties. Those will be studied *in vitro* to determine if their altered pathogenicity corresponds to altered physical properties. Finally, we are screening small-molecule libraries to identify drug-like molecules that will be used as chemical probes in a parallel attempt to disprove the hypothesis. Should the amyloid pore hypothesis survive such scrutiny, such molecules may lead to a new generation of therapeutics aimed at the root cause of neurodegeneration.

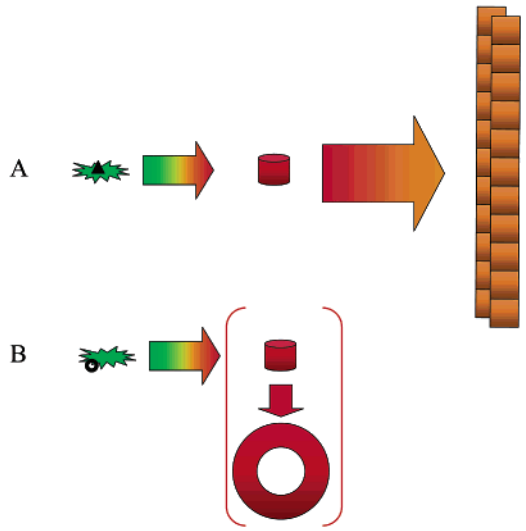

FIGURE 4: Schematic depiction of two unnatural α-synuclein sequence variants (denoted with a green starburst with a black triangle or circle) with properties that would aid in the testing of the amyloid pore hypothesis. (A) A sequence in which fibril formation is very rapid (relative to WT) and protofibrils do not accumulate. Such a sequence could produce a transgenic *Drosophila* model in which LB formation was rapid but neurodegeneration was very slow. (B) A sequence variant in which fibril formation is forbidden but pore formation is facile. Such a sequence could produce a transgenic *Drosophila* in which LB formation would not occur but neurodegeneration would be rapid.

NOTE ADDED AFTER ASAP POSTING

This paper was inadvertently posted on the Web on 06/06/03 with the word "that" missing between family and allowed in the fourth sentence of the text. The correct version of this paper was posted 06/11/03.

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