Accelerated Publications

Molecular Crowding Accelerates Fibrillization of α-Synuclein: Could an Increase in the Cytoplasmic Protein Concentration Induce Parkinson's Disease?†

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ABSTRACT: Parkinson's disease (PD) is one of many neurodegenerative diseases that are characterized by amyloid fibril formation. α-Synuclein is a primary component of the fibrillar neuronal inclusions, known as Lewy bodies, that are diagnostic of PD. In addition, the α-synuclein gene is linked to familial PD. Fibril formation by α-synuclein proceeds via discrete β-sheet-rich oligomers, or protofibrils, that are consumed as fibrils grow. Both FPD mutations accelerate formation of protofibrils, suggesting that these intermediates, rather than the fibril product, trigger neuronal loss. In idiopathic PD, other factors may be responsible for accelerating protofibril formation by wild-type α-synuclein. One possible factor could be molecular crowding in the neuronal cytoplasm. We demonstrate here that crowding using inert polymers significantly reduced the lag time for protofibril formation and the conversion of the protofibril to the fibril, but did not affect the morphology of either species. Physiologically realistic changes in the degree of in vitro crowding have significant kinetic consequences. Thus, nonspecific changes in the total cytoplasmic protein concentration, induced by cell volume changes and/or altered protein degradation, could promote formation of and stabilize the α-synuclein protofibril.

α-Synuclein is a 140-amino acid presynaptic protein that has been linked, by genetics and neuropathology, to Parkinson's disease (PD)[1] (*1*, *2*). Two point mutations in α-synuclein (A53T and A30P) are linked to autosomal dominant PD (FPD) (*3*, *4*). In addition, α-synuclein is a major fibrillar component of Lewy bodies, the cytoplasmic inclusions that are characteristic of FPD and idiopathic PD (*5*). In vitro, monomeric α-synuclein is disordered, or natively unfolded (*6*, *7*). However, at high concentrations, it slowly forms β-sheet-rich oligomers, designated protofibrils, that are consumed as amyloid fibrils appear (*1*, *8*, *9*). Both FPD mutations promote in vitro protofibril formation (*1*, *9*−*11*), suggesting that protofibrils, not fibrils, are the toxic species (*2*, *12*). However, the relevance of the ideal solutions used for these in vitro studies to the conditions at the site of in vivo α-synuclein fibrillization, the neuronal cytoplasm, is unclear. Although α-synuclein is an abundant neuronal protein, its cytoplasmic concentration is likely to be less than 30−60 μM (*13*), yet 300 μM solutions of α-synuclein can be incubated for months without significant fibrillization (*1*, *9*, *14*, *15*). Therefore, for α-synuclein fibrillization to occur in vivo, the effective cytoplasmic concentration of α-synuclein must somehow be elevated. One mechanism by which this could be achieved would be increased specific concentration of cytoplasmic α-synuclein, either by its overexpression (*16*) or by its inhibited degradation (*17*). An alternative mechanism by which its effective concentration could be elevated is nonspecific molecular crowding (*18*−*20*).

The neuronal cytoplasm is not an ideal solution. In fact, approximately 40% of its volume may be occupied by macromolecules, predominantly RNA and proteins (*18*−*22*). In this crowded environment, the volume accessible to any given protein is decreased; protein folding and protein−protein interaction equilibria are driven toward the lower volume (globular/oligomeric) species (*18*, *20*, *21*, *23*−*27*). This effect is nonspecific and can be sensitive to small fluctuations; one calculation predicts a 1 order of magnitude increase in the effective concentration based on an increase in the extent of crowding from 30 to 33% (*28*). Cell volumes fluctuate over a comparable range; a 7% decrease in neuronal volume has been observed in aged rats (*29*), and changes of 2−3-fold have been measured in Schwann cells progressing through the cell cycle (*30*). In addition to equilibrium constants, the rates of reactions for which the activation volume is negative are similarly increased. Both protofibril and fibril formation are known to involve structure formation and compaction and hence should be favored, both thermodynamically and kinetically, due to the decreased volume of each

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[1] Abbreviations: AFM, atomic force microscopy; CHO, Chinese hamster ovary; FPD, familial Parkinson's disease; MW, molecular weight; PBS, phosphate-buffered saline; PD, Parkinson's disease; PEG, polyethylene glycol; SEC, size-exclusion chromatography; ThioT, thioflavin T; UCH-L1, ubiquitin C-hydrolase L1 (neuronal); WT, wild-type.

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protein molecule in the protofibril and fibril relative to the monomeric protein. In the case of α-synuclein, which is unfolded in its monomeric state (*6*, *7*), the volume-related driving force is expected to be significant. An alternative explanation for crowding-induced fibrillization invokes an entropic driving force; the increase in translational entropy experienced by all of the other proteins more than compensates for the decreased entropy of the fibril-forming protein (*18*).

Crowding by cytoplasmic macromolecules has been experimentally modeled with "inert" polymers (*31*−*38*). The level of nonspecific crowding in a mammalian CHO cell cytoplasm has been approximated by solutions containing 20% PEG 8000, 20% dextran 11000, or 8−12% dextran T-70 (*39*−*41*). We demonstrate here that polymer-induced crowding increases the α-synuclein effective concentration and reduces the lag time for protofibril and fibril formation without altering their morphology. These data suggest that cell volume fluctuations or dysfunction of the protein degradative pathway during aging could promote PD.

EXPERIMENTAL PROCEDURES

Preparation of α-Synuclein. The protein was overexpressed in *Escherichia coli* and purified to >95% purity as previously described (*6*, *9*). Expression and purification were carried out at the Center for Biocatalysis and Bioprocessing at the University of Iowa (Iowa City, IA). A stock solution was produced by dissolving protein in PBS (pH 7.4) and 0.04% NaN_3 (Sigma, St. Louis, MO) and filtering the mixture through Microcon 100 kDa cutoff filters (Millipore, Pittsburgh, PA) to remove the oligomeric material. The filtrate was stored at −20 °C until the α-synuclein concentration was determined by quantitative amino acid analysis (the original protein concentration was generally greater than 600 μM).

Determination of Crowding Effects on Protofibril and Fibril Formation. The α-synuclein stock solution was diluted to 600 μM and mixed with an equal volume of a stock aqueous polymer solution [PEG 400, 3350, 6K, and 20K (Hampton Research, Laguna Niguel, CA), dextran T-70 (Amersham-Pharmacia, Piscataway, NJ), or ficoll 70 (Sigma)] to yield a solution of 300 μM α-synuclein in PBS, 0.02% NaN_3, and a chosen concentration of a crowding agent. Solutions were incubated (stationary at 37 °C in triplicate), and measured aliquots were drawn, removed, and analyzed by thioflavin T (ThioT) fluorescence (*42*, *43*) (LJL plate reader, LJL Biosystems, Sunnyvale, CA), size exclusion chromatography (SEC) [Waters 2690, Waters Corp., Taunton, MA; HR10/30 Superdex 200 column (Pharmacia)], and atomic force microscopy (AFM) (*9*, *15*). In some cases, a slow rotary platform (~60 rpm) was used to accelerate fibrillization; these are mentioned in the figure legends. Each experiment was repeated at least three times. Although lag times were difficult to reproduce between series due to the stochastic nature of nucleation (*44*), the observed trends remained constant. The representative data reported here are derived from a single experimental series. Error bars represent standard deviations. Data were analyzed using Microsoft Excel (Microsoft, Seattle, WA) for ThioT fluorescence and Millennium software (Waters Corp.) for SEC. Results were plotted using Igor Pro (Wavemetrics Inc., Lake Oswego, OR).

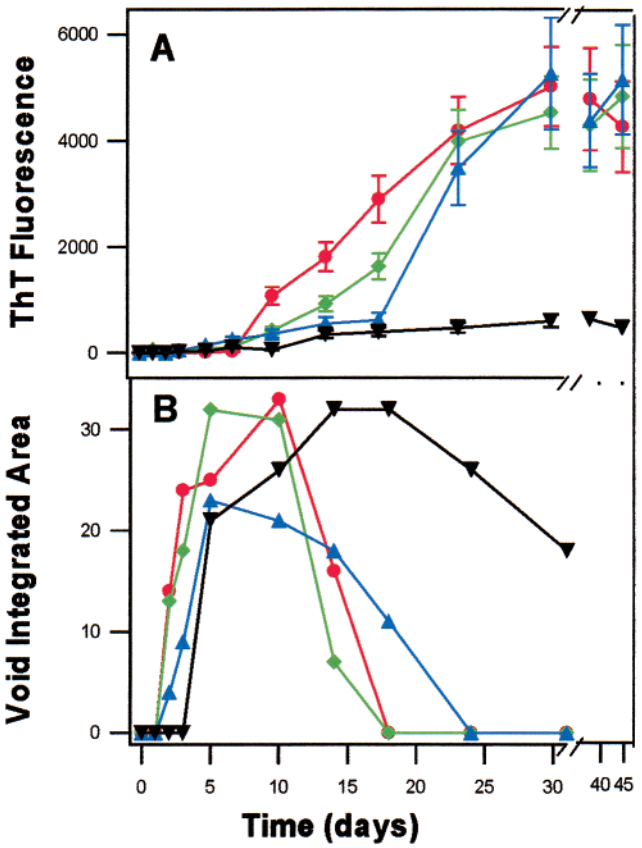

FIGURE 1: Fibril (A) and protofibril (B) formation by α-synuclein in the presence of crowding agents. α-Synuclein (300 μM) was incubated in PBS, at 37 °C, in the presence of 0% (black), 5% (blue), 7.5% (green), and 10% PEG 20K (red). In panel A, thioflavin T fluorescence (background subtracted), which is proportional to the amount of fibrils but insensitive to the protofibrillar species, is shown. Note the discontinuous *X*-axis. In panel B, the integrated area under the protofibril peak, as measured by SEC, is shown. Note that protofibrils are consumed as fibrils appear.

Concentration Dependence of Protofibril and Fibril Formation. An α-synuclein stock solution (600 μM) was diluted to lower concentrations with PBS and 0.04% NaN_3 and then mixed with a crowding polymer in water, as described above. Aliquots of equal volume were drawn from all tubes. For ThioT and SEC measurements, equal amounts of α-synuclein were analyzed, based on the α-synuclein concentration.

Atomic Force Microscopy of α-Synuclein Protofibrils and Fibrils. AFM analysis was accomplished using tapping mode imaging in air as reported in previous studies (*1*, *45*).

RESULTS

The Lag Time for α-Synuclein Fibril Formation Decreased as the Level of Crowding was Increased. α-Synuclein fibrillization is intrinsically slow, due to the kinetic barrier of nucleus formation. Under the conditions used here (300 μM protein in PBS and 0.02% NaN_3), the lag time for fibril formation (ThioT, Figure 1A) was on the order of 80−90 days (*1*, *15*). The lag time was strikingly reduced (to ~8−10 days, Figure 1A) in the presence of 10% PEG 20000, a level of crowding that is comparable to that of typical cell cytosol [20% PEG 8000 (*39*)]. The reduction in lag time was proportional to the degree of crowding, with 5% PEG 20000 still producing a significant acceleration (Figure 1A).

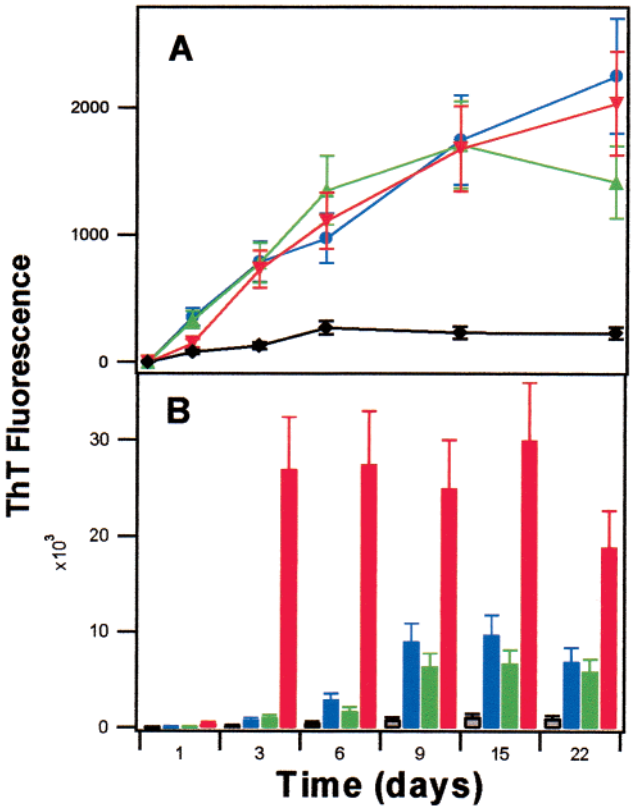

FIGURE 2: Reduction in fibrillization lag time depends on the molecular weight, but not the chemical nature, of the crowding agent. In panel A, three diverse crowding agents produced comparable effects: 15% PEG 3350 (red), 150 g/L dextran T-70 (blue), and 150 g/L ficoll (green) (buffer system shown in black). In panel B, the molecular weight of the background species was altered: 15% (weight/volume) each of PEG 400 (black), PEG 3350 (blue), PEG 6000 (green), and PEG 20000 (red). The effect demonstrates the importance of excluded volume interactions.

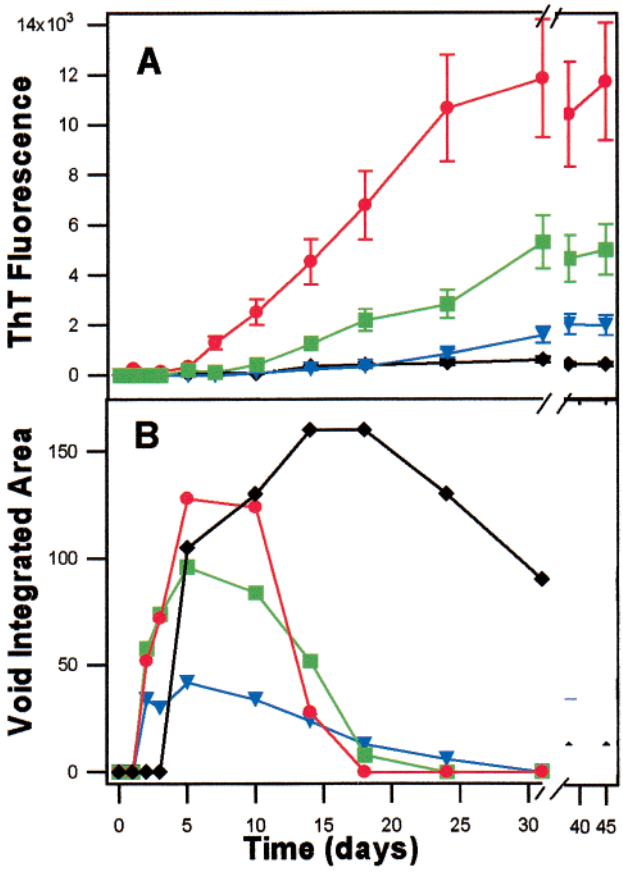

FIGURE 3: Crowding increases the effective α-synuclein concentrations for protofibril and fibril formation. In the presence of 7.5% PEG 20000, lower concentrations of α-synuclein [60 (blue), 120 (green), and 240 μM (red)] undergo fibrillization (A) and protofibrillization (B) more rapidly than 300 μM α-synuclein in a simple buffer system (black; the lag time for fibrillization was 80−90 days).

The Lag Time for α-Synuclein Protofibril Formation and for Protofibril-to-Fibril Conversion Decreased as the Level of Crowding Increased. A time course of α-synuclein protofibril formation (followed by SEC and quantitated by absorption at 276 nm, Figure 1B) shows that crowding accelerates protofibril formation (lag time reduced from 3 days to 1 day at 5% PEG 20000). As expected, the lifetime of the protofibrillar species is also decreased as fibrils consume these transient intermediates (*1*, *9*, *15*).

Acceleration of Fibrillization Depended on the Molecular Weight, but Not on the Subunit Structure of the Crowding Polymer. To confirm that the observed acceleration is due to molecular crowding, as opposed to a PEG-specific intermolecular interaction, the chemical structure of the crowding polymer was varied. Two different polysaccharides, dextran and ficoll, were used (Figure 2A). The crowding effect with these polysaccharides was qualitatively identical to that with PEG (Figure 2A). Next, PEGs of different molecular weights were used to separate excluded volume effects from osmotic stress effects (Figure 2B). Low-MW polymers have a higher surface area per volume and compete more strongly with the protein for solvent water and thus are better molecular osmolytes. Since PEGs form low-density random coils with the coil size proportional to the square root of the chain length, high-MW PEGs occupy a more significant fraction of total volume and produce a stronger volume exclusion effect. Consistent with acceleration being caused by molecular crowding, a greater reduction in lag time was observed for 15% (weight/volume) PEG 20000 (red) than for 15% PEG 3350 (blue) and 15% PEG 6000 (green). The effect of 15% PEG 400 (black) was significantly weaker (Figure 2B).

The Effective α-Synuclein Concentration Was Dramatically Increased in a Crowded Environment. Crowding was expected to increase the effective concentration of α-synuclein. The dependence of lag time on α-synuclein concentration was determined in a model system of a physiologically relevant viscosity (7.5% PEG 20000, Figure 3). Under these conditions, the lag time (*10*), which was ~80 days at 300 μM in PBS, was dramatically reduced, to ~20 days at 60 μM, 13 days at 120 μM, and 7 days at 240 μM. Thus, the concentration of α-synuclein required for timely fibrillization could easily be reduced by 1 order of magnitude in vivo. Protofibril formation also proceeded more rapidly at significantly lower α-synuclein concentrations under crowded conditions (Figure 3B). The lifetime of the protofibrillar state was the shortest at high α-synuclein concentrations, where fibril formation was rapid. While protofibril and fibril formation were affected by crowding to a different extent (the volume changes for the two processes are expected to be different), both processes are more favorable in a crowded environment.

Protofibrillar and Fibrillar Morphologies Were Not Significantly Changed by Crowding. To ensure that the assembly pathway under crowded conditions was identical

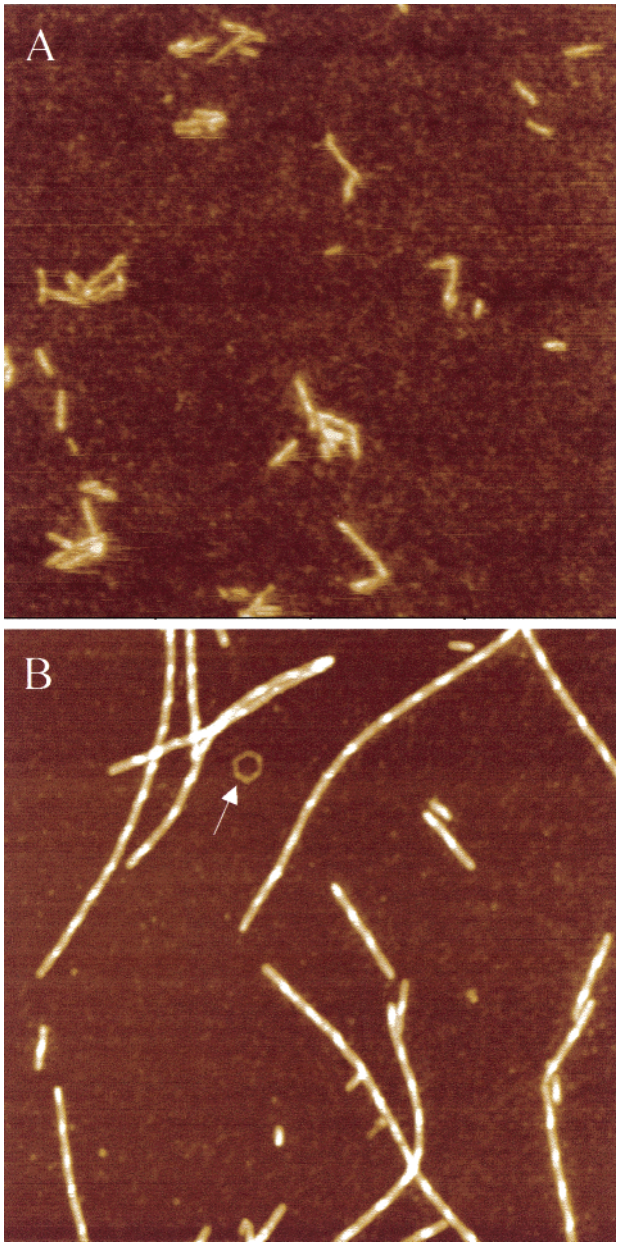

FIGURE 4: Crowding does not significantly change the protofibril or fibril morphologies. Chainlike α-synuclein protofibrils appeared rapidly in the presence of 10% PEG 3350 (24 h) (A). These species are ~4 nm in height. On prolonged incubation, these develop into mature, 7−8 nm high fibrils (B). Some annular protofibrils persist under these conditions (B, arrow; 3−4 nm in height) (72 h). Scan sizes: 1 μm (A) and 2 μm (B).

to that in ideal buffer solutions, morphological analysis of the process by AFM was undertaken (*1*, *9*, *14*, *46*). Aliquots taken from α-synuclein incubations in a crowded solution (10% PEG 3350 on a rotary platform) were imaged by AFM (Figure 4). The species observed in the crowded incubation were indistinguishable from those observed previously in simple buffers. Oligomerization proceeded rapidly through small spherical protofibrils (3−4 nm in height, data not shown) to chainlike protofibrils (3−4 nm in height, Figure 4A) to mature amyloid fibrils (~7 nm in height, Figure 4B). However, in contrast to the case of simple buffers, fibrillar species were found to coexist with annular protofibrils (3−4 nm in height, Figure 4B, arrow), suggesting that these two morphologies may have a similar "per monomer" volume.

DISCUSSION

Although the substantia nigra of a Parkinson's disease brain is characterized by fibrillar α-synuclein, accumulating evidence suggests that it is the protofibrillar intermediates, rather than the fibrils themselves, that are neurotoxic (*2*). First, both point mutations linked to early-onset PD promote protofibril formation, but not fibril formation, suggesting that the former is pathogenic (*1*, *11*). Second, expression of wild-type (WT) α-synuclein in mouse brain causes formation of nonfibrillar α-synuclein, along with loss of dopaminergic termini and motor dysfunction (*47*). This finding argues that fibril formation is not required for toxicity, consistent with the notion of a toxic protofibril. Crossing the α-synuclein transgenic mice with *β*-synuclein transgenic mice results in bigenic mice that have a decreased number of nonfibrillar deposits, less synaptic loss, and improved motor performance (*48*). Finally, modification of α-synuclein by dopamine *o*-quinone produces a covalent adduct that efficiently inhibits the protofibril-to-fibril transition and leads to accumulation of protofibrillar species (*49*). Thus, if α-synuclein's neurotoxicity was due to a protofibrillar species, it would be increased by dopamine. This proposal is consistent with the selective degeneration of substantia nigra dopaminergic neurons in Parkinson's disease.

Previous studies of in vitro α-synuclein fibrillization have been conducted in simple buffer systems, which do not simulate the crowded and viscous neuronal cytoplasm. Notably, the α-synuclein concentration required to observe fibrillization in vitro is much greater than the likely in vivo cytoplasmic concentration. We demonstrate here that molecular crowding is sufficient to increase the effective concentration of α-synuclein without qualitatively altering the monomer-to-protofibril-to-amyloid fibril pathway that has been observed under ideal solvent conditions (*1*, *9*, *14*). Furthermore, physiologically reasonable changes in the extent of in vitro crowding have a significant effect on the rates of these conversions, suggesting that factors that cause changes in in vivo crowding may be PD susceptibility factors.

Significant increases in the level of molecular crowding could result from two age-associated effects: a reduction in cell volume and the inhibition of protein degradation. Age-dependent cell volume changes have been measured in rat neurons; a 7% decrease in water content was detected by 30 months of age (*29*). Such a change in human brains would significantly increase the rates of α-synuclein protofibril and fibril formation. It is important to note that an increased level of crowding may not always promote disease; under certain conditions, an increase in the level of crowding may decrease the lifetime of the protofibril by promoting its conversion to the fibril. This may explain the fact that incidental Lewy body disease (i.e., Lewy bodies in the absence of any clinical phenotype) is more prevalent than Parkinson's disease [from ~4% in the seventh decade (as compared to ~0.4% for PD) to 10% in the eighth to 14% in the ninth decade (1−2% for PD) (*50*, *51*)].

A second mechanism by which the level of cytoplasmic crowding could be increased is by the inhibition of proteasomal protein degradation. Regardless of whether α-synuclein itself is degraded by the proteasome (*17*, *52*, *53*), the increase in the concentration of all of the other cytoplasmic proteins as a result of proteasome inhibition

could significantly increase the level of crowding. Several pathological findings, cell biological studies, and genetic findings support this idea. First, reduced proteasomal activity has been measured in PD brain (*54*). Second, overexpression of α-synuclein in PC12 cells decreases proteasome activity (*55*). In a separate experiment, it was demonstrated that proteasomal inhibition leads to the formation of α-synuclein inclusions without significantly increasing the level of α-synuclein (*56*). Finally, the importance of the ubiquitin-dependent degradative pathway in PD is supported by genetic evidence; the gene encoding UCH-L1, an enzyme implicated in the proteasome pathway, has been linked to familial (*57*) and sporadic PD (*58*, *59*). In addition, the E3 ubiquitin ligase parkin has been linked to juvenile onset Parkinsonism and idiopathic PD (*60*, *61*). The mechanism of the parkin mutations may involve compromised α-synuclein degradation (*17*).

The possible importance of molecular crowding in Parkinson's disease suggests several possible therapeutic strategies against PD, including the upregulation of parkin or other components of the proteasomal pathway or an increase in cellular volume. Finally, whether or not the crowding effect forms a basis for new treatments, there are important practical implications for this effect for the design of high-throughput assays designed to discover novel druglike molecules that inhibit α-synuclein oligomerization [the amount of α-synuclein required for a large screen can be greatly reduced (*49*)].

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