AD_____

Award Number: DAMD17-02-1-0171

TITLE: Role of Oligomeric a-Synuclein in Mitochondrial Membrane Permeabilization and Neurodegeneration in Parkinson's Disease

PRINCIPAL INVESTIGATOR: Seung-Jae Lee, Ph.D.

CONTRACTING ORGANIZATION: The Parkinson's Institute
Sunnyvale, CA 94089-1605

REPORT DATE: December 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2003	Annual (19 Nov 2002 - 18 Nov 2003)

4. TITLE AND SUBTITLE
Role of Oligomeric a-Synuclein in Mitochondrial Membrane Permeabilization and Neurodegeneration in Parkinson's Disease

5. FUNDING NUMBERS
DAMD17-02-1-0171

6. AUTHOR(S)
Seung-Jae Lee, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Parkinson's Institute
Sunnyvale, CA 94089-1605

E-Mail: sll@parkinsonsinstitute.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

A growing body of evidence suggests that aggregation of α-syn might be the fundamental cause of many neurodegenerative diseases. Several groups have developed cell culture models to study the cytotoxic effect of α-synuclein, and some of them indeed have observed enhanced cell death when α-syn, especially its mutant forms, was overexpressed. However, the link between α-syn aggregation and cell death has not been clearly addressed in these model systems, nor are the molecular mechanisms underlying the toxicity known. We have begun to address these issues in a COS-7 cell model, and found that α-syn aggregation/oligomerization is tightly associated with Golgi fragmentation and cell death. The fragmentation of the GA has been also confirmed in a neuronal cell model, and the mode of Golgi fragmentation appears identical to that caused by microtubule-disrupting agents; dispersed Golgi fragments are localized to the transitional endoplasmic reticulum. These findings indicate that α-syn aggregation leads to impairment of microtubule-dependent intracellular trafficking, which in turn causes Golgi fragmentation.

14. SUBJECT TERMS
Parkinson's disease, synuclein, fibril, protein aggregate, Golgi apparatus, inclusion body, microtubule

15. NUMBER OF PAGES
27

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

A. Introduction

Many human neurological disorders, including Parkinson's disease (PD), dementia with Lewy bodies, and multiple system atrophy, are characterized by amyloid-like fibrillar aggregates of α-synuclein (α-syn), such as Lewy bodies (LBs) and Lewy neurites (Hardy and Gwinn-Hardy, 1998; Trojanowski et al., 1998). While the direct role of fibrils and inclusion bodies in disease progression is the subject of intense debate, recent *in vitro* studies revealed various non-fibrillar species during the course of fibrillation and suggested a possibility that these metastable intermediate species, not the fibrils themselves, might elicit cytotoxicity (Conway et al., 2000; Conway et al., 2001). Elucidating which particular aggregate species possess the principal cytotoxic effect holds the key to understanding the etiologic role of protein aggregation in the disease pathogenesis. Study of this problem, however, has been hampered by the lack of experimental system in which intermediates of the endogenous fibrillation process can be biochemically defined and analyzed. We have recently established such an experimental cell system, and the goal of the current study is to elucidate the mechanisms of α-syn fibrillation and assess the effects of prefibrillar intermediates in the cytoplasm.

B. Body

In the first year of the project, we had shown that cellular α-syn aggregation process involves prefibrillar intermediates during the fibrillation. These intermediates are initially formed throughout the cytoplasm and transported through the microtubule system into the pericentrosomal region, where they are transformed into fibrils. We also reported that the formation of prefibrillar oligomeric α-syn aggregates is associated with Golgi fragmentation, suggesting the prefibrillar intermediates as being pathogenic species.

B.1. Cytotoxic effects of prefibrillar a-syn aggregates

A cytotoxic effect of α-syn has been reported in several mammalian cell systems (Ostrerova et al., 1999; Saha et al., 2000; Iwata et al., 2001; Zhou et al., 2002). However, the nature of toxic species has not been determined. Since the formation of prefibrillar aggregates correlates with the disruption of Golgi apparatus (GA), a vital organelle of eukaryotic cells, we measured cell viability as a function of the aggregate formation. Increase of monomer, without forming aggregates, did not affect the viability (MOI 0-20) (Fig. 1*A* and Fig. 2*A*, day 3). In contrast, cell viability was significantly reduced when aggregates formed without increasing the monomer level (Fig. 1*A* and Fig. 2*A*, day3). Tight correlation between reduction in cell viability and α-syn aggregation was also found in the time-course study. Aggregates were formed at slower rates at lower MOIs (MOIs 20 and 50), and when they became apparent on the fourth day, the viability was reduced correspondingly (Fig. 1*A* and *B*). Like the Golgi fragmentation, reduction in cell viability occurred before the appearance of the fibrillar inclusions (Fig. 2*A*, day 3 at the MOI of 70, and see also Fig. 1*B* and *C*), implicating a cytotoxic effect of prefibrillar aggregates. In contrast, overexpression of GFP did not affect the cell viability in the same time period (Fig. 1*C*). These results suggest that the cytotoxic effect of α-syn depends on its ability to form aggregates, and that prefibrillar aggregates, rather than the fibrillar inclusion bodies, may be responsible for the toxicity.

Although the causal relationship between Golgi fragmentation and cell death is not clear at the moment, following observations support that Golgi fragmentation is probably at the upstream of the cell death process, rather than a mere consequence of the latter. First, Golgi fragmentation

was found, without exception, in all dying cells that were stained with ethidium homodimer (EthD), whereas not all the cells with Golgi fragmentation were EthD-positive, though most were (Fig. 2*D*). This suggests that Golgi fragmentation precedes the membrane permeabilization in cell death process. Secondly, the same changes in the GA cannot be found in hydrogen peroxide-induced cell death, suggesting that the type of morphological changes in the GA found in association with α-syn aggregation is not a general consequence of cell death.

B.2. Golgi fragmentation in post-mitotic neuronal cells that produce prefibrillar α-synuclein aggregates

We showed in the previous report that in COS-7 cells, the GA is fragmented in cells that contain α-syn oligomeric aggregates, whereas an increase of monomer expression *per se* does not cause Golgi fragmentation. We also showed that Golgi fragmentation occurs prior to the appearance of fibrillar inclusion bodies. These data suggest that the accumulation of the oligomeric aggregates may cause Golgi fragmentation and, potentially, other forms of cellular impairment. Here, we present our recent data showing that α-syn oligomerization is also associated with Golgi fragmentation in post-mitotic neuronal cells.

To examine the effect of α-syn aggregation on the GA in post-mitotic neuronal cells, fragmentation of the GA was quantified at varying levels of α-syn overexpression in differentiated SH-SY5Y human neuroblastoma cells. Cells on coverslips were differentiated for 5 days with RA and then infected with adeno/α-syn. The cells were infected at four different MOI's (0, 30, 60, and 100) and incubated for 4 days. The cells were then fixed with 4% paraformaldehyde and immunostained to detect α-syn and GM130, a *cis*-Golgi marker, and then examined under a confocal microscope. Fluorescence images were obtained from randomly selected areas and assessed for the extent of Golgi fragmentation as described previously (Gosavi et al., 2002). All cells were placed into one of three categories: normal (GA is a compact, pericentriolar structure), intermediate (Golgi fragments are discernable, but still gathered in the pericentriolar region), and dispersed (GA is fragmented and scattered). Similar to our previous results in COS-7 cells, significant Golgi dispersion occurs only at high α-syn levels (Fig.3); the threshold α-syn expression level for Golgi fragmentation is approximately that required for aggregate formation. This result suggests that aggregation of α-syn may also lead to Golgi fragmentation in post-mitotic neuronal cells. Also, cells overexpressing α-syn exhibit lower viability than the control (Fig. 3C). Thus, this cell system can be used as a post-mitotic neuronal model to study the effects of α-syn aggregation.

B.3. Impaired microtubule function may be the underlying mechanism of Golgi fragmentation

It had been previously shown that microtubule-disrupting agents, such as nocodazole, cause Golgi fragmentation (Thyberg and Moskalewski, 1999). To compare patterns of Golgi fragmentation, COS-7 cells on coverslips were either infected with adeno/α-syn, or treated with 5 μg/ml nocodazole overnight. The cells were then fixed and immunostained to detect the *cis*-Golgi and *trans*-Golgi. Another set of cells was treated with 500 nM cytochalasin D, an agent that disrupts actin filaments, and stained for the *cis*-Golgi and the actin filaments. The GA appears normal with a compact, juxtanuclear morphology in the cytochalasin-treated cells, whereas dispersion of the GA into fragments that maintain *cis-trans* polarity is evident in the nocodazole-treated cells (Fig. 4A). Cells overexpressing α-syn showed similar Golgi dispersion

patterns (Fig. 4A), suggesting that α-syn-induced Golgi dispersion may be caused by the microtubule dysfunction.

It had been previously shown that dispersed Golgi components in nocodazole-treated cells localize to the transitional endoplasmic reticulum (tER) (Cole et al., 1996); Sec13 is a standard marker for these sites. To investigate whether α-syn-induced fragmentation shows a similar pattern of localization, COS-7 cells on coverslips were transfected with Sec13-YFP and either infected with adeno/α-syn, or treated with 5 μg/ml nocodazole. It was found that GM130 does indeed co-localize with the tER in both nocodazole-treated and α-syn-overexpressing cells, further bolstering the hypothesis that α-syn causes Golgi fragmentation via MT disruption (Fig. 4B). The same dispersion of GM130 to the tER was also found in differentiated SH-SY5Y cells overexpressing α-syn (Fig. 4B), indicating that the effects of α-syn extend to post-mitotic neuronal cells.

B.4. Trafficking defects in cells with α-syn aggregates

To further characterize the effects of microtubule dysfunction in α-syn-overexpressing cells, we investigated the effects of α-syn overexpression on the microtubule-dependent trafficking. The temperature-sensitive mutant form of the vesicular stomatitis virus G protein (VSV-G) is a widely-used model protein in studying trafficking through the biosynthetic pathway (Pepperkok et al., 1993; Scales et al., 1997; Seemann et al., 2000). Here, we studied the trafficking property of this protein using an adenoviral vector bearing the GFP-tagged VSV-G gene. As shown in Fig. 5, VSV-G fails to fold properly and accumulates in ER at the restrictive temperature of 39.5°C (t = 0). But after the cells are transferred to the permissive temperature of 31.5°C, VSV-G folds rapidly and is transported progressively from the ER through the GA (t = 20 min) to the plasma membrane (PM; t = 60 min) in a time-dependent manner.

To examine the effects of α-syn aggregation on VSV-G trafficking, we performed an experiment in which distribution of VSVG-GFP was visually observed. VSVG-GFP was expressed in COS-7 cells overexpressing α-syn at a restrictive temperature for 6.5 h, and then at a permissive temperature for 60 min, after which the cells were fixed, stained with α-syn antibody, and examined with confocal microscopy. The cells with diffuse α-syn expression without apparent aggregation often show typical VSV-G distribution; intense Golgi localization with clear cell surface expression (Fig. 6). On the other hand, the cells with noticeable spherical α-syn aggregates tend to show little VSV-G protein on the cell surface and dispersed cytoplasmic distribution (Fig. 6). This suggests that the trafficking of VSV-G may be impaired in cells with α-syn aggregates, thus supporting the conclusion that α-syn aggregation causes microtubule dysfunction.

C. Key Research Accomplishments

- Deleterious effects of α-syn aggregation on Golgi fragmentation and cell viability were confirmed in post-mitotic neuronal cells.

- Results of our study suggest that α-syn aggregation affects the microtubule function and the intracellular trafficking.

D. Reportable Outcomes

Publications

Lee S-J (2002) α-Synuclein aggregation: a link between mitochondrial defects and Parkinson's disease? *Antiox. Redox Signal.* 5, 337-348

Funding applied for based on work supported by this award

2003-2005 Michael J. Fox Foundation for Parkinson's Research (Awarded)
Title: Study of the metabolism and aggregation of α-synuclein in parkin-depleted neuronal cells (The fractionation of α-synuclein aggregates, that was established in year 1, is a key component of this proposal.)

2004-2006 Michael J. Fox Foundation for Parkinson's Research (Awarded)
Title: Intracellular trafficking dysfunction caused by alpha-synuclein aggregation (This project is to test the hypothesis that alpha-synuclein aggregation leads to microtubule dysfunction and intracellular trafficking defects. This hypothesis was formulated based on the results of the current study.)

E. Conclusions

A growing body of evidence suggests that aggregation of α-syn might be the fundamental cause of many neurodegenerative diseases. Several groups have developed cell culture models to study the cytotoxic effect of α-synuclein, and some of them indeed have observed enhanced cell death when α-syn, especially its mutant forms, was overexpressed (Ostrerova et al., 1999; Saha et al., 2000; Iwata et al., 2001; Zhou et al., 2002). However, the link between α-syn aggregation and cell death has not been clearly addressed in these model systems, nor are the molecular mechanisms underlying the toxicity known. We have begun to address these issues in a COS-7 cell model, and found that α-syn aggregation/oligomerization is tightly associated with Golgi fragmentation and cell death (Gosavi et al., 2002). More recently, fragmentation of the GA has been confirmed in a neuronal cell model, and the mode of Golgi fragmentation appears identical to that caused by microtubule-disrupting agents; dispersed Golgi fragments are localized to the transitional endoplasmic reticulum. These findings indicate that α-syn aggregation leads to impairment of microtubule-dependent intracellular trafficking, which in turn causes Golgi fragmentation.

Recent studies show that the microtubule transport system also plays a role in inclusion body formation, as part of the cellular response to the aggregation of misfolded proteins (Johnston et al., 1998; Garcia-Mata et al., 1999; Johnston et al., 2000). Aggregation of proteins could occur anywhere in the cytoplasm, resulting in many small aggregate particles scattered throughout the cell. These particles are deposited in the pericentriolar region, adjacent to the microtubule-organizing center, by retrograde transport on microtubules. These microtubule-dependent deposits of aggregates are called aggresomes (Johnston et al., 1998) and may explain the biogenesis of inclusion bodies found in neurological diseases, such as Lewy bodies in PD. The microtubule-dependent nature of inclusion formation suggests that extensive protein aggregation

in neurons may place a tremendous burden on the microtubule transport system, causing it to malfunction. In the first year of the project, we have shown that small α-syn oligomers are transported on the microtubules and deposit to form inclusion bodies (Lee and Lee, 2002). In addition, our current data suggest that one of the consequences of α-syn aggregation, Golgi fragmentation, might be the result of defective microtubule-dependent trafficking. These findings lead us to formulate the hypothesis that α-syn aggregation causes damage to the microtubule system and impairment of intracellular trafficking, which eventually leads to neuronal degeneration. Study of this problem will increase our understanding of the mechanism by which α-syn aggregation leads to neuronal dysfunction and degeneration.

F. References

Cole NB, Sciaky N, Marotta A, Song J, Lippincott-Schwartz J (1996) Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. Mol Biol Cell 7:631-650.

Conway KA, Rochet JC, Bieganski RM, Lansbury PT, Jr. (2001) Kinetic stabilization of the alpha-synuclein protofibril by a dopamine- alpha-synuclein adduct. Science 294:1346-1349.

Conway KA, Lee S-J, Rochet JC, Ding TT, Williamson RE, Lansbury PT, Jr. (2000) Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. Proc Natl Acad Sci U S A 97:571-576.

Garcia-Mata R, Bebok Z, Sorscher EJ, Sztul ES (1999) Characterization and dynamics of aggresome formation by a cytosolic GFP- chimera. J Cell Biol 146:1239-1254.

Gosavi N, Lee H-J, Lee JS, Patel S, Lee S-J (2002) Golgi Fragmentation Occurs in the Cells with Prefibrillar alpha - Synuclein Aggregates and Precedes the Formation of Fibrillar Inclusion. J Biol Chem 277:48984-48992.

Hardy J, Gwinn-Hardy K (1998) Genetic classification of primary neurodegenerative disease. Science 282:1075-1079.

Iwata A, Maruyama M, Kanazawa I, Nukina N (2001) alpha-Synuclein affects the MAPK pathway and accelerates cell death. J Biol Chem 276:45320-45329.

Johnston JA, Ward CL, Kopito RR (1998) Aggresomes: a cellular response to misfolded proteins. J Cell Biol 143:1883-1898.

Johnston JA, Dalton MJ, Gurney ME, Kopito RR (2000) Formation of high molecular weight complexes of mutant Cu, Zn- superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. Proc Natl Acad Sci U S A 97:12571-12576.

Lee H-J, Lee S-J (2002) Characterization of Cytoplasmic alpha -Synuclein Aggregates. FIBRIL FORMATION IS TIGHTLY LINKED TO THE INCLUSION-FORMING PROCESS IN CELLS. J Biol Chem 277:48976-48983.

Ostrerova N, Petrucelli L, Farrer M, Mehta N, Choi P, Hardy J, Wolozin B (1999) alpha-Synuclein shares physical and functional homology with 14-3-3 proteins. J Neurosci 19:5782-5791.

Pepperkok R, Scheel J, Horstmann H, Hauri HP, Griffiths G, Kreis TE (1993) Beta-COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. Cell 74:71-82.

Saha AR, Ninkina NN, Hanger DP, Anderton BH, Davies AM, Buchman VL (2000) Induction of neuronal death by alpha-synuclein. Eur J Neurosci 12:3073-3077.

Scales SJ, Pepperkok R, Kreis TE (1997) Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. Cell 90:1137-1148.

Seemann J, Jokitalo EJ, Warren G (2000) The role of the tethering proteins p115 and GM130 in transport through the Golgi apparatus in vivo. Mol Biol Cell 11:635-645.

Thyberg J, Moskalewski S (1999) Role of microtubules in the organization of the Golgi complex. Exp Cell Res 246:263-279.

Trojanowski JQ, Goedert M, Iwatsubo T, Lee VM (1998) Fatal attractions: abnormal protein aggregation and neuron death in Parkinson's disease and Lewy body dementia. Cell Death Differ 5:832-837.

Zhou W, Schaack J, Zawada WM, Freed CR (2002) Overexpression of human alpha-synuclein causes dopamine neuron death in primary human mesencephalic culture. Brain Res 926:42-50.

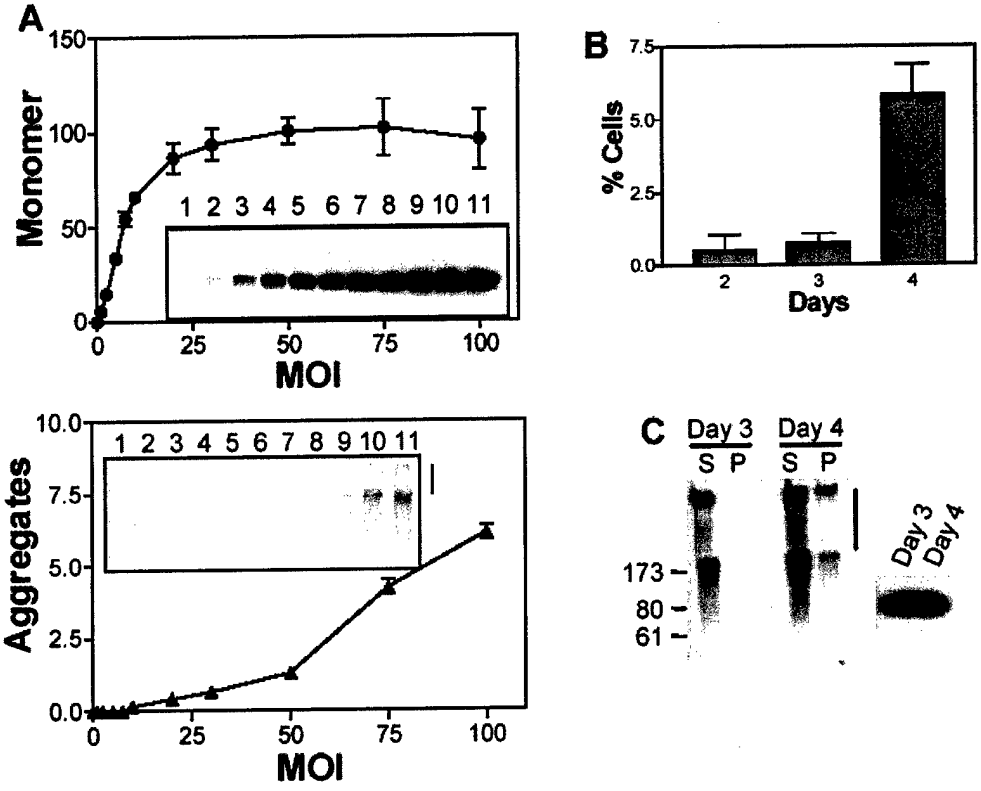

Fig. 1. Non-linear relationship between the production of monomeric α-synuclein and its aggregation. (*A*) COS-7 cells were infected with different amount of adenoviral vector (adeno/α-syn) carrying α-synuclein cDNA and incubated for three days, and the levels of monomers and aggregates were measure from detergent-soluble and insoluble fractions, respectively. Empty viral vector was added so as to make the total number of viral particles in each sample constant. Arbitrary optical density units are plotted in the graphs. Insets are the representative autoradiograms of the Western blotting using LB509 antibody and ^{125}I-labeled secondary antibody. Specific MOIs used in the Western analysis are: lane 1, 0; lane 2, 1; lane 3, 2.4; lane 4, 5; lane 5, 7.4; lane 6, 10; lane 7, 20; lane 8, 30; lane 9, 50; lane 10, 75; lane 11, 100. The vertical line on the right side of the inset indicates the stacking gel portion. (***B***) The percentages of cells that contain juxtanuclear inclusion bodies were calculated at the indicated time points. α-Synuclein was expressed at MOI of 75. Images were obtained from two optical sections to ensure the detection of inclusion bodies that are located in a different plane. (***C***) Fibrillar inclusions (P) and prefibrillar oligomers (S) were separated based on their sizes at day 3 and day 4, and analyzed by Western blotting. The left and right panels show the detergent-insoluble aggregates and soluble monomers, respectively. The vertical line indicates the stacking gel portion.

Seung-Jae Lee, Ph.D.
Principal Investigator

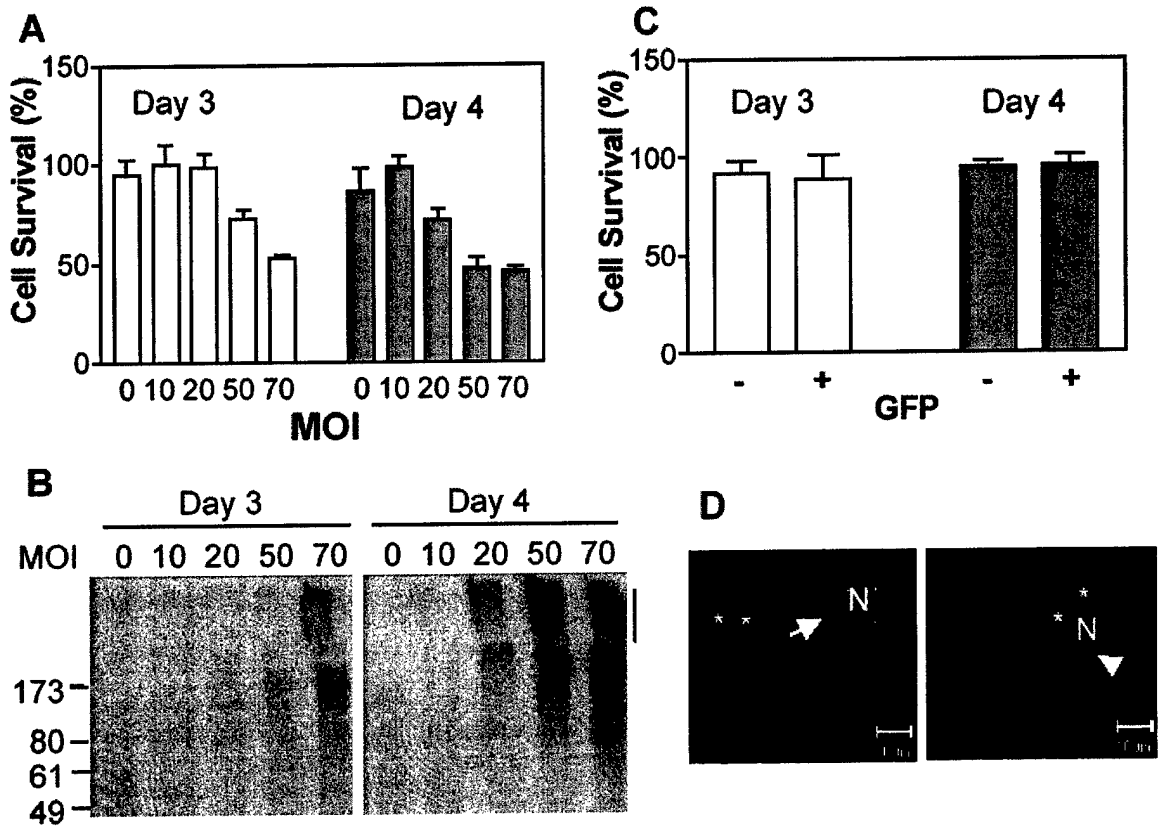

Fig. 2. Cytotoxic effect of α-synuclein is dependent on the aggregation and occurs before the formation of fibrillar inclusions. (*A*, *B*) COS-7 cells were infected with adeno/α-syn at given MOIs (the total number of viral particles was adjusted to be equivalent by adding appropriate amount of empty viral vector), and each sample was subjected to the trypan blue exclusion assay (*A*) and Western blotting (*B*). The percentage of live cell number was calculated, with the non-expressor at day 3 as being 100%. The Western blotting was performed with the detergent-insoluble fractions. (*C*) Cell viability was analyzed in control-transfectants (-) or GFP-transfectants (+). The percentage of live cells was obtained, with the control-transfectants at day 3 as being 100%. (*D*) COS-7 cells were infected with adeno/α-syn at the MOI of 75 and stained with EthD (red) and anti-GM130 antibody (green) at day 3. EthD stains the nucleus of dying cells. Left image shows a dying cell with EthD-positive nucleus (N) and fragmented Golgi. All the EthD-positive cells analyzed in this study showed Golgi fragmentation. The image on the right shows an example of the cell with fragmented Golgi and EthD-negative nucleus (N). Note that live cells have EthD-negative nucleoplasms [two nucleoli (*) are stained per cell] and normal Golgi morphology. Scale bars, 10 μm

Seung-Jae Lee, Ph.D.
Principal Investigator

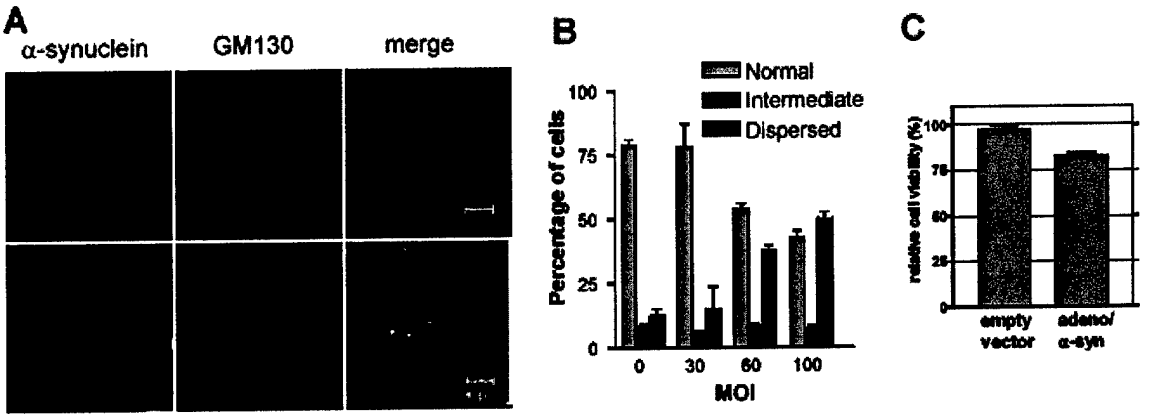

Fig. 3. Golgi fragmentation and reduced cell viability in differentiated SH-SY5Y cells overexpressing α-synuclein. (**A**) Cells were infected with either empty vector (top row) or adeno/α-syn (bottom row) at MOI 100, and stained for α-synuclein (*red*) and the GA (GM130, *green*). Cells infected with adeno/α-syn show dispersed GA. (**B**) Quantitative analysis of Golgi disruption at different levels of α-synuclein overexpression. At least 100 cells were analyzed per coverslip. Data were obtained from four independent experiments. (**C**) Cells infected as in (A) were subjected to the MTS reduction assay. Cells overexpressing α-synuclein show reduced viability.

Seung-Jae Lee, Ph.D.
Principal Investigator

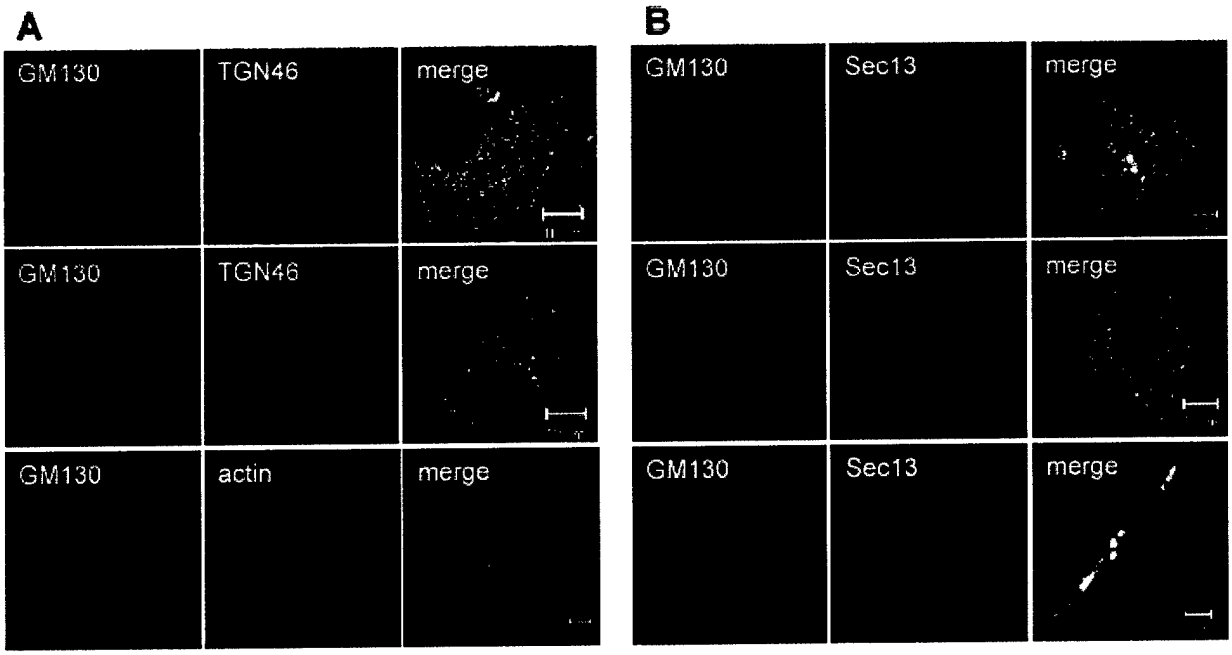

Fig. 4. Comparison of Golgi fragmentation in cells overexpressing α-synuclein and cells treated with nocodazole. (**A**) Golgi disruption in COS-7 cells caused by α-synuclein overexpression (top row) is similar to that induced by nocodazole (middle row), a microtubule-destabilizing agent. Depolymerization of actin filaments with cytochalasin D does not cause Golgi disruption (bottom row). GM130 marks the *cis*-Golgi, and TGN46 marks the *trans*-Golgi. Actin filaments are visualized with FITC-labeled phalloidin. (**B**) The scattered GM130 is found at the tER (marked by Sec13) in both COS-7 (top row) and differentiated SH-SY5Y (bottom row) cells overexpressing α-synuclein, which is also a characteristic of nocodazole-treated cells (middle row). *Blue*: nuclei

Seung-Jae Lee, Ph.D.
Principal Investigator

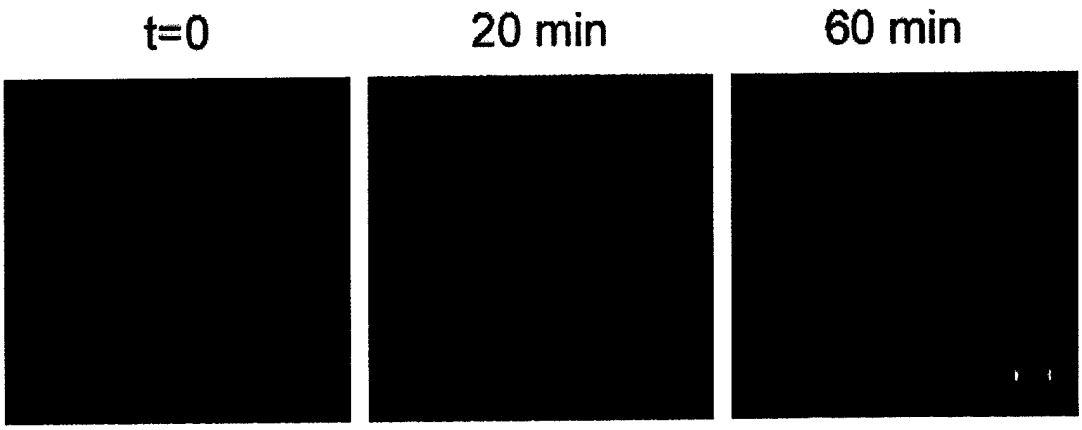

Fig. 5. Trafficking of VSVG-GFP in COS-7 cells. (**A**) Representative cells at three time points. At the permissive temperature, VSVG-GFP moves from the ER (t=0) to the GA (20 min) and then to the cell surface (60 min). *green*: total VSVG-GFP

Seung-Jae Lee, Ph.D.
Principal Investigator

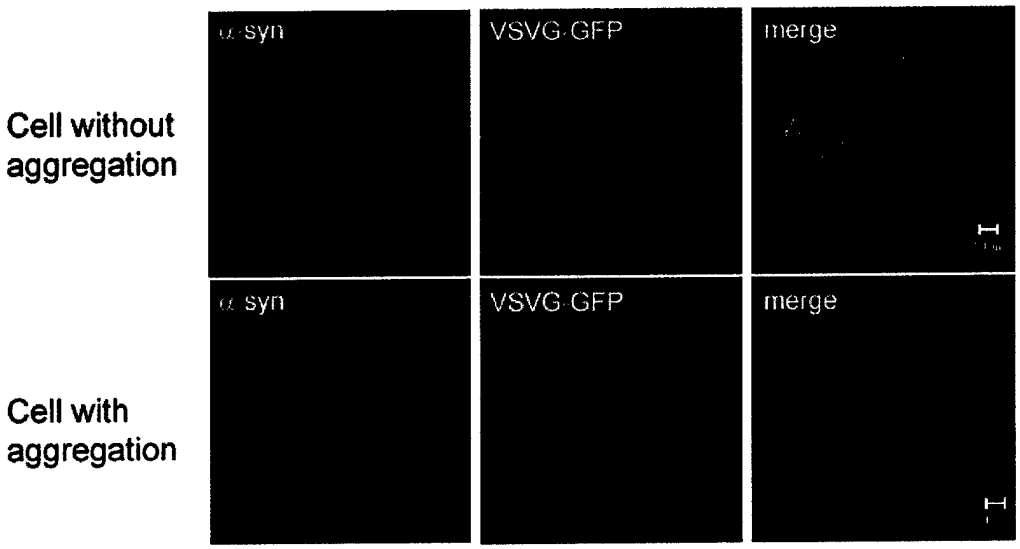

Fig. 6. Effect of α-synuclein aggregation on VSVG-GFP export in COS-7 cells overexpressing α-synuclein. Representative cell without apparent aggregation (top) exports VSVG-GFP to the cell surface, while a representative cell with spherical aggregates (bottom) shows little VSVG-GFP expressed on the cell surface. *red*: α-synuclein, *green*: VSVG-GFP

ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 3, 2003

Forum Review

α-Synuclein Aggregation: A Link Between Mitochondrial Defects and Parkinson's Disease?

SEUNG-JAE LEE

ABSTRACT

Protein aggregation is a shared feature of many human neurodegenerative diseases and appears to be an inevitable consequence of excessive accumulation of misfolded proteins. Recent studies suggest that accumulation of fibrillar α-synuclein aggregates is associated with Parkinson's disease and other Lewy body diseases. Furthermore, the missense mutations in α-synuclein that are responsible for some early-onset familial types of the disease promote the aggregation process of this protein. Therefore, the mechanism underlying the cellular α-synuclein aggregation is of great importance in understanding the pathogenic process of these diseases. This review summarizes recent advances in our understanding of the mechanisms underlying α-synuclein aggregation and how the mitochondrial dysfunction plays a role in this process. Protein misfolding and aggregation *in vivo* can be suppressed and promoted by several factors, such as molecular chaperones, protein degradation systems, and free radicals. Many of these factors are under the control of normal mitochondrial function, prompting the speculation that mitochondrial dysfunction might cause the accumulation of protein aggregates. Recent studies indeed show that mitochondrial defects can lead to the aggregation of α-synuclein. In addition, potentially toxic effects of α-synuclein have been linked to the aggregated forms rather than the monomers, both *in vitro* and in cultured cells. Therefore, it is postulated that aggregation of α-synuclein might be one of many possible links that connect mitochondrial dysfunction to neurodegeneration. *Antioxid. Redox Signal.* 5, 337–348.

INTRODUCTION

PROGRESSIVE IMPAIRMENT OF MITOCHONDRIAL FUNCTION and increased oxidative stress have been implicated in the pathogenesis of Parkinson's disease (PD) and other neurodegenerative diseases (7, 103). In idiopathic PD, complex I activity of the mitochondrial respiratory chain decreases by 30–40% in the substantia nigra (53, 81, 108). Furthermore, a selective reduction in the immunoreactivity for complex I subunits was reported in the substantia nigra of PD patients, whereas immunoreactivity for other electron-transport complexes was unaltered (46). Cybrid studies provided additional evidence for mitochondrial defects in PD. Cybrids that carried mitochondria derived from platelets of PD patients displayed reduced complex I activity (42, 121). In addition, several groups have reported complex I inhibition and reduction of mitochondrial ATP production by a potent and selective parkinsonian toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, further providing support for the notion that mitochondrial defects are involved in the pathogenesis of PD (92, 109, 117).

PD is pathologically defined by two hallmark features, dopaminergic neuronal loss and Lewy pathologies such as Lewy bodies (LBs) and Lewy neurites (30). Although several mechanisms have been proposed to explain the role of mitochondrial impairment in cell death (for reviews, see 10, 20, 68), the relationship between the Lewy pathologies and mitochondrial defects had not been studied until recently, when fibrillar aggregates of α-synuclein were discovered as major

The Parkinson's Institute, Sunnyvale, CA 94089.

components of LBs and Lewy neurites. This review will focus on our current knowledge of the mechanisms of α-synuclein aggregation and discuss the implications of recent findings in the role of mitochondrial impairment in this pathological protein deposition.

α-SYNUCLEIN AND PD

α-Synuclein is 140-amino acid protein that is enriched in neurons in the central nervous system. It is a member of the synuclein protein family, which includes two other members: β- and γ-synuclein (15). All three synucleins have highly conserved N-terminal domains that are composed of imperfect repeats of the consensus sequence KTKEGV. This region is important in the interaction with phospholipid membranes, and upon binding to the membranes, it undergoes a structural transition from random coil to α-helix (22, 25, 99). C-termini of synucleins are enriched with negatively charged amino acids and thought to be important in maintaining solubility of these proteins (123). The mid-region of α-synuclein contains a 12-amino acid motif that is absent in β-synuclein, and this sequence seems to be important for aggregation of this protein (38).

A body of evidence suggests that α-synuclein is normally involved in synaptic transmission. Immunohistochemical studies have shown that α-synuclein is primarily localized in presynaptic terminals (51, 61). In addition, the homozygous deletion of α-synuclein results in a decrease in the size of the "reserve" pools of presynaptic vesicles and impairments in electrophysiological response to a prolonged train of low-frequency repetitive stimulation (13). Finally, another set of α-synuclein knockout mice showed subtle, but clear, abnormalities in nigrostriatal synaptic transmission (1). At a molecular level, it has been suggested that α-synuclein might be involved in lipid trafficking and metabolism (16, 111), regulation of phospholipase D (2, 54), and "chaperoning" protein folding (64, 96, 119). Whether these activities are physiologically relevant and how they are related to the synaptic function remains unknown.

PD is the second most common neurodegenerative disease only to Alzheimer's disease. Clinical symptoms of PD include bradykinesia, rigidity, and resting tremor. Pathologically, the disease is characterized by a loss of nigrostriatal dopaminergic neurons and the presence of LBs (31). The first connection between α-synuclein and PD was made when a missense mutation that was linked to an early-onset type of the disease was identified in an Italian kindred and three Greek families (101). This mutation causes amino acid substitution from alanine to threonine at position 53 (A53T). A more extensive search of mutations in the α-synuclein gene led to the identification of another missense mutation, A30P, in a German family (69). Furthermore, it was quickly found that fibrillar wild-type α-synuclein is a major component of LBs in virtually all LB diseases, including idiopathic PD (6, 120). These findings established that α-synuclein is involved in at least some aspect of PD pathogenesis.

The role of α-synuclein in the pathogenic process has been studied using transgenic animal approaches. Expression of human α-synuclein (wild-type or one of the two mutants, A30P and A53T) in the neurons of transgenic flies produced an age-dependent PD phenotype including fibrillar α-synuclein-containing cytoplasmic inclusions, selective loss of dopaminergic neurons, and a motor deficit (29). In addition, neuronal expression of human α-synuclein in mice generated four transgenic models with neurodegenerative phenotypes. The first transgenic model, developed by Masliah and colleagues, exhibits abnormal pathological and behavioral phenotypes that resemble PD. These mice display nonfibrillar α-synuclein-positive inclusions as opposed to fibrillar ones in PD, loss of dopaminergic synapses, and a motor deficit (83). In another model, expression of either wild type or A53T in mice showed perikaryal and neuritic accumulations of α-synuclein, neuromuscular degeneration, and reduced rotor rod performance in both animals (128). On the other hand, some studies showed the mutant-specific pathological and behavioral phenotypes. Giasson *et al.* reported that mice expressing the A53T mutant, but not wild type, developed neuronal cytoplasmic LB-like inclusion bodies, axonal degeneration in motor neurons, and severe motor impairment (39). Mice generated by M.K. Lee *et al.* showed that overexpression of the A53T mutant, but not the wild type or A30P, causes α-synuclein aggregation and motor abnormality (76). On the contrary, Neumann *et al.* showed a drastic accumulation of proteinase K-resistant, hyperphosphorylated α-synuclein and progressive locomotor deficits in A30P transgenic mice (90). In addition to the transgenic mice models, viral vector-mediated overexpression of wild type or mutant forms of α-synuclein produced selective dopaminergic neuron loss and synucleinopathy lesions (65, 79). Although none of these models seems to recapitulate perfectly the clinical and pathological features of PD, each model provides tools to investigate the fundamental roles of α-synuclein in certain aspects of pathogenesis.

Although the mechanism by which α-synuclein is involved in PD pathogenesis remains elusive, the following lines of evidence support the hypothesis that α-synuclein causes neurodegeneration through a "gain-of-toxic-function" mechanism: (a) The mutations in the α-synuclein gene that are linked to rare cases of familial parkinsonism show an autosomal dominant, rather than recessive, inheritance (69, 101). (b) The pathological features of PD, dopaminergic neuronal loss and LB formation, can be replicated by simply overexpressing α-synuclein in transgenic fly (5, 29). The finding that drosophila does not have an α-synuclein homologue (32, 106) rules out a possible dominant-negative effect of transgene-derived proteins. And, as mentioned above, some transgenic mice overexpressing wild-type or mutant α-synuclein also show inclusion bodies and neurodegenerative phenotypes. (c) The lack of a neurodegenerative phenotype in knockout mice suggests that neurodegeneration is not a "loss-of-function" phenotype of α-synuclein (1, 13). These observations are consistent with the notion that α-synuclein exerts its role in PD pathogenesis by acquiring a toxic function rather than a loss of normal function. The fact that the insoluble inclusion bodies of α-synuclein are associated with neurodegeneration in both human and transgenic animal models prompted the hypothesis that α-synuclein gains its toxic function through the aggregation process.

α-SYNUCLEIN AGGREGATION PROCESS

In vitro aggregation process

Purified recombinant α-synuclein can spontaneously form fibrillar aggregates that show all classical properties of amyloid fibrils, such as cross β-structure in x-ray diffraction, typical electron microscopic morphology, and green birefringence by Congo red (18, 110). Like other amyloidogenic aggregation, fibrillation of α-synuclein is a nucleation-dependent process (134).

Various spectrophotometric and hydrodynamic analyses showed that in dilute solution, both wild-type and mutant monomeric α-synuclein possess characteristics of unfolded proteins (125, 132). However, small-angle x-ray scattering analysis suggests that the "natively unfolded" conformation of α-synuclein is more compact than a perfect random coil conformation (125). Consistent with this observation, some residual helical structure in the N-terminus of the protein has been detected by high-resolution characterization of conformational propensities using solution NMR spectroscopy (12). Interestingly, a region of this residual structure is disrupted by the A30P mutation, whereas the A53T mutant maintains the helical preference and shows slightly enhanced regional preference for β-sheet-like conformations around the mutation site (12).

Fibrillation of α-synuclein is initiated by the acquisition of a partially folded conformation (125), which is subsequently stabilized by self-association (124). Prior to the formation of fibril, several nonfibrillar oligomeric aggregates, or protofibrils, have been identified (40) (Fig. 1). These protofibrils are enriched with β-sheet motifs, suggesting that they may be structurally related to fibrils (130). *In vitro* studies showed that fibril formation is accompanied by a reduction in the level of protofibrils (17, 24), supporting the notion that the protofibrils are the on-pathway intermediates of the fibrillation process. The earliest and most common protofibrillar species are spherical with an average height of 4.2 nm (24). The spherical oligomers are thought to undergo head-to-tail associations to form elongated chain- (18) and ring-like (24) protofibrillar species.

In their search for a potential pathogenic property of α-synuclein protofibrils, Lansbury and colleagues demonstrated that only protofibrillar α-synuclein, but not monomers or fibrils, binds tightly to and permeabilizes synthetic lipid vesicles (130). The protofibril-mediated membrane permeabilization occurs preferentially for low-molecular-mass molecules, suggesting a pore-like mechanism (129). Furthermore, recent

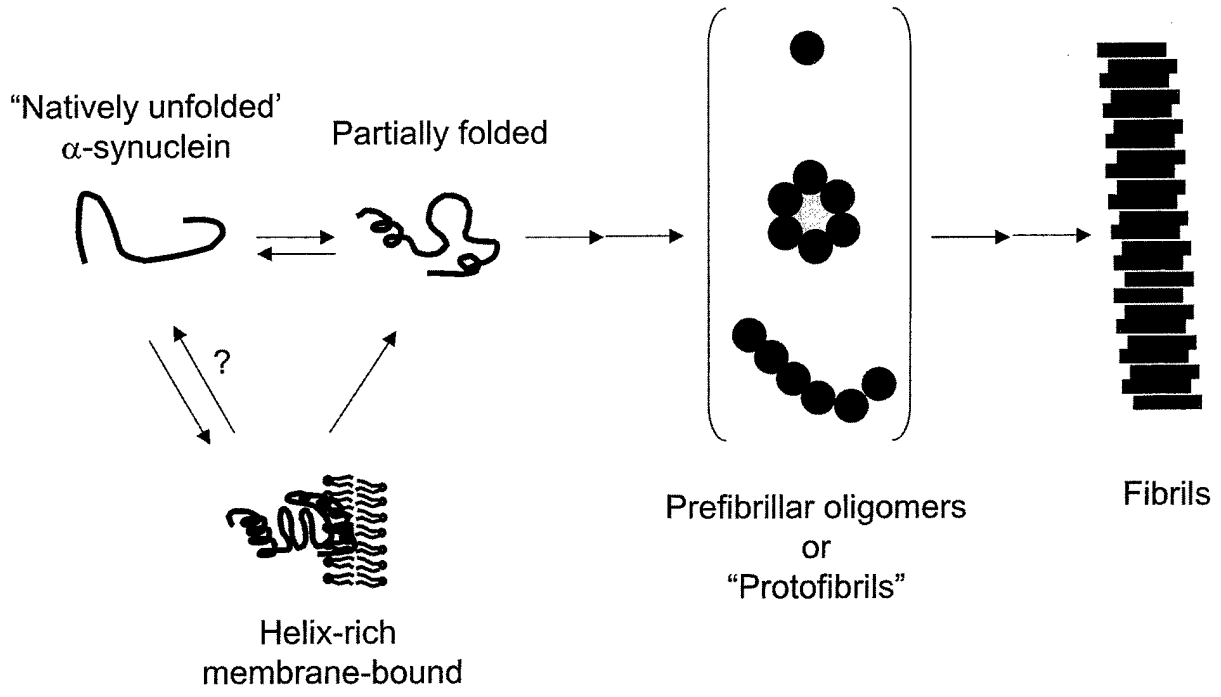

FIG. 1. α-Synuclein conformers in the fibrillation process. The native state of α-synuclein in solution is characterized by unfolded conformation. A partially folded conformation can be induced upon exposure to misfolding stresses and stabilized by self-associations to form dimers and oligomers. The oligomers with partial conformation gain high-density β-structure, as they become larger prefibrillar oligomers, or protofibrils. Protofibrils are believed to be "on-pathway" intermediates of fibrillation based on their conformational properties and kinetic behavior. The native state of membrane-bound α-synuclein is enriched with α-helical structure and can also undergo the aggregation process in the membranes. However, it remains unknown whether the binding of α-synuclein to the membrane is reversible or what controls the steady-state distribution of α-synuclein between membrane compartments and cytosol.

findings show that the two pathogenic mutations (A53T and A30P) promote the formation of annular pore-like protofibrils and result in an increased permeabilization activity relative to the wild-type protein (24, 71, 129). These results support the hypothesis that the intermediates (protofibrils), rather than the final product (fibrils) of the fibrillation process, are the pathogenic aggregate species that are responsible for neuronal cell death in PD. Although this idea needs to be tested in biological systems, a recent study provides a biochemical basis for the importance of protofibrils in dopaminergic neuron-selective degeneration (19). In this study, Conway *et al.* screened a compound library for fibrillation inhibitors and initially identified 15 compounds, 14 of which were catecholamines, including dopamine and its precursor L-Dopa. Further analysis showed that an oxidized metabolite of dopamine, dopamine quinone, stabilizes protofibrils by forming a dopamine–α-synuclein adduct, which slows the transition from protofibril to fibril. This study suggests that the stabilization of protofibrils may lead to the selective dopaminergic degeneration in PD, thus offering a mechanistic link between three well established pathological features of PD: oxidative stress, dopaminergic neuronal loss, and α-synuclein aggregation.

Factors influencing in vivo aggregation

In addition to the intrinsic conformational properties of individual proteins, there are several other considerations that influence protein folding and aggregation in cells.

Molecular crowding. Unlike the dilute solutions that are usually used in test-tube studies, the cytosolic environment is highly "crowded" with other macromolecules. This molecular crowding has significant quantitative effects on the rates and the equilibria of macromolecular interactions (27, 87). In fact, experimental evidence shows that the addition of crowding agents enhances both the physiological self-association of monomeric subunits (105) and the nonproductive aggregation of partially folded proteins (127). Addition of crowding agents also accelerates both α-synuclein protofibril formation and protofibril-to-fibril transition (116, 126). Therefore, the *in vivo* α-synuclein aggregation process is likely to be under the influence of the excluded volume effect of crowded cytosol.

Chemical modifications. Cellular proteins are subjected to various chemical modifications, such as phosphorylation, glycosylation, and oxidative modification of amino acid side chains. These modifications are likely to have direct effects on the normal conformation of the protein, thus affecting the aggregation propensity of the protein. α-Synuclein can be phosphorylated on multiple serine and tyrosine residues by several protein kinases in cultured cells and *in vitro* (26, 88, 89, 93, 102). In an effort to address the effects of phosphorylation on the aggregation of α-synuclein, Iwatsubo and colleagues showed that phosphorylation at Ser^{129} promoted fibrillation *in vitro*, and this residue was extensively phosphorylated in human synucleinopathy lesions (33). Oxidative and nitrative modifications of α-synuclein also affect the fibrillation process both *in vitro* and in cells (44, 97, 118), and nitrated α-synuclein has been observed in synucleinopathy lesions (37).

Molecular interactions. Cellular proteins are often engaged in physical interactions with other molecules, ranging from small molecules to lipid membranes. These interactions normally result in alterations in protein conformation and may change the aggregation propensity of the protein (8). A small fraction of cellular α-synuclein is associated with membranous compartments in the brain (36, 50, 61, 73). *In vitro,* recombinant α-synuclein binds to the negatively charged surface of phospholipid liposomes (22, 57, 130). Upon binding to liposomes, α-synuclein shows a dramatic increase in helical content (22, 25). When aggregation rates are compared, brain membrane-bound α-synuclein shows a higher propensity for aggregation than the cytosolic form. In addition, the aggregates formed in the membranes were capable of seeding aggregation of the cytosolic form (73). Interactions of α-synuclein with polyunsaturated fatty acids and cytoplasmic triglyceride droplets also appear to induce oligomerization of the protein (16, 100). In addition to lipids, α-synuclein interacts with several proteins (2, 3, 28, 54–56, 94, 98). Some of these proteins, such as tubulin and synphilin-1, seem to promote α-synuclein aggregation (3, 14).

Molecular chaperones. Molecular chaperones assist the folding of newly synthesized polypeptides and refolding of misfolded proteins, which otherwise tend to self-associate to form protein aggregates that often irreversibly lock the nonnative conformation (43). These folding catalyst proteins are particularly needed in the crowded environment in which the association constants for self-assembly of misfolded proteins are significantly increased. Interestingly, macromolecular crowding can also enhance the effectiveness of molecular chaperones in assisting proper folding and preventing aggregation (82, 127). Indeed, a recent study shows that overexpression of torsin A and the heat-shock proteins inhibits the formation of α-synuclein-positive inclusions (85). Furthermore, in a fruit fly model, hsp70 family chaperones alleviate neurodegenerative phenotypes caused by overexpression of α-synuclein (5), suggesting that molecular chaperones may play a role in preventing proteins from transforming into the pathogenic conformation.

Protein degradation. Proteins that fail to fold correctly are targeted for degradation systems, such as the ubiquitin-proteasome pathway and autophagy. Failure in these systems leads to the accumulation of misfolded proteins, which in turn results in protein aggregation. It is therefore noteworthy that two other genetic components that underlie the pathogenesis of familial PD are components of the protein degradation system: parkin and ubiquitin C-terminal hydrolase (UCH)-L1 (66, 77, 80). Parkin is an E3 ubiquitin ligase (48, 49, 114, 136), an enzyme that catalyzes the conjugation of ubiquitin to proteins that are to be degraded by the 26S proteasome complex (133). Recent evidence suggests that parkin might be involved in the metabolism and aggregation of α-synuclein (14, 115). UCH-L1 has been known to hydrolyze isopeptide bonds within the multiubiquitin chains to recycle the monomeric ubiquitin (70). In addition, a recent study showed that this protein also has a ligase activity that ubiquitinates α-synuclein–ubiquitin conjugates to produce polyubiquitinated proteins (78). The same

study also showed that the ligase activity of UCH-L1 forms polyubiquitin chains through Lys^{63}, instead of Lys^{48}, the ubiquitin acceptor residue that is involved in the proteasome degradation. Therefore, overexpression of UCH-L1 promotes the formation of nondegradable polyubiquitin conjugates of α-synuclein, leading to the accumulation of α-synuclein (78). Importantly, the PD-linked I93M mutant form of UCH-L1 has higher ligase activity than wild type, and this activity of I93M is inhibited by the S18Y variant (78), which was linked to a decreased susceptibility to PD.

Identification of mutations in these genes in familial PD patients raised the possibility that abnormal protein degradation due to failures in the ubiquitin-proteasome pathway may be the cause of PD. However, the role of the ubiquitin-proteasome pathway in α-synuclein degradation remains controversial. Some studies show that the ubiquitin-proteasome pathway is responsible for α-synuclein degradation (9, 86), and that inhibition of this pathway results in aggregation (84, 86, 104). However, other studies failed to find the effect of selective proteasome inhibitors on α-synuclein metabolism and aggregation (4, 97). In addition, there is also a report that α-synuclein is degraded by proteasome without undergoing ubiquitination (122).

Cellular aggregation process

Protein aggregation in cells often yields an end product of large intracellular foci termed inclusion bodies. Considering the high tendency of misfolded proteins to self-associate, and the reduced diffusion rate in the crowded cytoplasmic environment, one might predict multiple inclusion bodies in a single cell. In contrast to this prediction, cytoplasmic inclusion bodies in postmortem brains or in cultured mammalian cells are present in low number, usually only one per cell. Recent evidence shows that aggregation of proteins and formation of inclusion bodies are separate processes that are coordinated by the cytoplasmic transport apparatus (35, 58, 59). Aggregation of proteins occurs throughout the cytoplasm, resulting in a number of small aggregate particles. These particles are deposited in the pericentriolar region, adjacent to the microtubule organizing center, by retrograde transport on microtubules. These microtubule-dependent inclusion bodies are called aggresomes (58). The aggresome model explains that inclusion bodies are deposits of numerous individual protein aggregates.

LBs contain fibrillar aggregates of α-synuclein, implying that the fibrillation process *in vivo* is somehow integrated into the inclusion-forming process. In an attempt to understand α-synuclein fibrillation in the context of the inclusion-forming process, our group has established a cell system in which overexpression of α-synuclein results in inclusion bodies (72). In these cells, small punctate aggregates appear at early time points throughout the cytoplasm, followed by large pericentriolar inclusion bodies. Treating the cells with nocodazole, a microtubule-disrupting agent, results in the reduction in the number of inclusions and a concomitant increase in the number of small aggregate particles, suggesting that α-synuclein inclusions are formed by the aggresome mechanism. Using ultrastructural characterization and fibril-specific dye-binding analysis, we have demonstrated that the small aggregates are nonfibrillar spherical aggregates and that the inclusion bodies are filled with fibrillar aggregates that resemble the ones found in the LBs (72). These results suggest that *in vivo,* α-synuclein fibrillation is similar to the *in vitro* process, in which protofibrillar intermediates are involved in the fibrillation. Interestingly, nocodazole treatment leads to the accumulation of unusually large peripheral aggregates, presumably because of the inability to transport the small aggregates to the inclusion-forming site, which allows them to grow at the initial sites. Despite their large size, these peripheral aggregates maintained the characteristics of spherical protofibrils (72), suggesting that in cells, the protofibril-to-fibril transition is not a diffusion-driven process and requires microtubule-dependent deposition of protofibrils in the pericentriolar region (Fig. 2). This study clearly demonstrates that *in vivo,* α-synuclein fibrillation is tightly linked to the microtubule-dependent inclusion-forming process.

MITOCHONDRIAL DEFECTS AND α-SYNUCLEIN AGGREGATION

The most notable outcomes of mitochondrial dysfunction with regard to the protein folding and aggregation are the increase of free radical generation and the reduction in ATP production. Free radicals cause covalent modifications of amino acid residues, which subsequently destabilize the native conformation of proteins (23). Hydroxyl radicals produced by iron-catalyzed oxidation promote fibrillation of α-synuclein *in vitro* (44). Oxidative reactions from iron and free radical generators, such as dopamine and hydrogen peroxide, also stimulate the aggregation of α-synuclein in human neuroblastoma cells (95). In an independent set of studies, Ischiropoulos and colleagues showed that incubation of recombinant α-synuclein with nitrating agents stimulates nitrative modification and oligomerization of the protein (118). The oligomers and fibrils so formed were stabilized by intermolecular dityrosine cross-linking via oxidation of tyrosine. The same group confirmed the role of nitrative modification of α-synuclein in the aggregation in HEK293 cells stably expressing α-synuclein. Exposure of the cells to nitrating agents resulted in nitration of α-synuclein and the formation of α-synuclein-positive perinuclear inclusion bodies (97). Furthermore, nitration on the tyrosine residues of α-synuclein was found in all synucleinopathy lesions including LBs of PD and dementia with Lewy bodies and glial inclusion bodies of multiple-system atrophy (37), suggesting that oxidative and nitrative stresses are involved in the mechanisms underlying α-synuclein aggregation in the pathogenic process. Collectively, these studies support the hypothesis that increased generation of oxidative and nitrative radicals, often caused by mitochondrial dysfunction, results in covalent modification of α-synuclein, leading to conformational change and aggregation.

Considering the fact that ATP is required for the proper function of molecular chaperones, the ubiquitin-proteasome system, and the autophagic-lysosomal pathway (63, 113), it is predicted that a reduction of ATP production due to the mitochondrial defects would have a significant impact on the accumulation and aggregation of misfolded proteins. Indeed, some studies have shown a correlation between ATP deple-

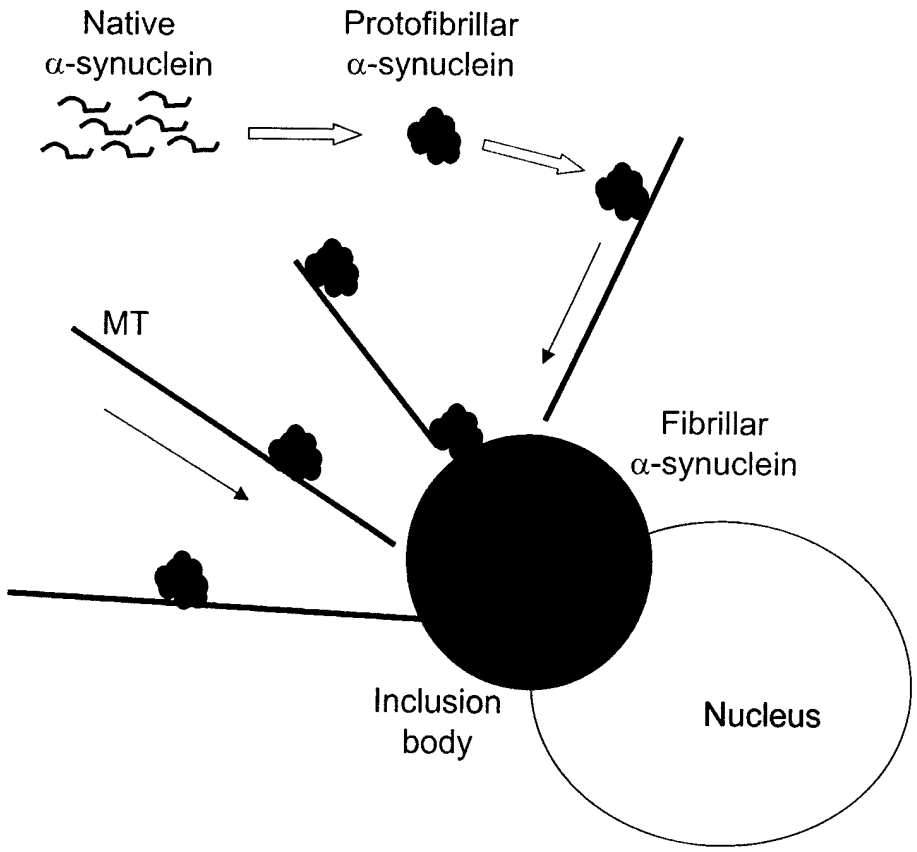

FIG. 2. Working model of α-synuclein fibrillation in cells. Small protofibrillar aggregates formed throughout the cytoplasm are transported into the pericentriolar region in a microtubule (MT)-dependent manner. Protofibril-to-fibril transition seems to occur after the protofibrils are accumulated in the pericentriolar region. See text for the details.

tion and cellular protein aggregation in cultured cells (34, 91). It seems reasonable that protein misfolding and aggregation can be induced by mitochondrial dysfunction due to the combined effects of increased levels of free radicals and decreased levels of ATP, with the former elevating the rate of protein misfolding and the latter reducing the ability to remove the misfolded proteins.

Direct evidence that mitochondrial dysfunction is responsible for α-synuclein aggregation and neurodegeneration was provided in a recent study by Greenamyre and colleagues (11). They showed that systemic administration of a complex I inhibitor, rotenone, to rats results in major clinical and pathological signs of human PD, including α-synuclein-positive inclusion bodies, selective dopaminergic loss in the substantia nigra, and reduced motor skills. This study supports the hypothesis that mitochondrial defects, especially complex I impairment, are sufficient to trigger the entire pathogenic process that includes α-synuclein aggregation and neurodegeneration. Similarly, in human neuroblastoma cells, chronic rotenone exposure (5 n*M*) increased α-synuclein protein levels in a week, and longer treatment (2–4 weeks) of rotenone induced oxidative damage and apoptotic cell death (112). Although this study showed an increase of detergent-insoluble α-synuclein after 4 weeks of rotenone treatment, whether this treatment indeed promoted α-synuclein aggregation was not clearly demonstrated.

Rotenone-induced α-synuclein aggregation was characterized in more detail in COS-7 cells using a higher dose of rotenone (100 n*M*) (74). In this study, rotenone treatment induced ubiquitin- and α-synuclein-positive fibrillar inclusion bodies within 3 days, whereas spontaneous aggregation was minimal at the same expression level. Small spherical α-synuclein aggregates formed throughout the cytoplasm before the appearance of inclusion bodies, and these inclusion bodies formed in the pericentriolar region, suggesting that the aggresome mechanism underlies the rotenone-induced inclusion formation. Interestingly, when rotenone was washed out with fresh medium, the small prefibrillar aggregates were progressively degraded, whereas the fibrillar inclusion bodies were relatively spared (74). The clearance of the aggregates paralleled the recovery of cellular ATP production. These results suggest that cells have the ability to protect themselves by degrading toxic aggregates, and this degradation activity may require mitochondrial energy production.

The accumulation of α-synuclein aggregates in cells is determined by a dynamic equilibrium between production and removal. The studies summarized here suggest that defects in mitochondrial function lead to an increase in α-synuclein aggregate production and a decrease in the ability to remove them, subsequently leading to the accumulation of the aggregates in the cytoplasm. Given the importance of mitochondrial function in α-synuclein aggregation dynamics, it is noteworthy that overproduction of α-synuclein results in mitochondrial dysfunction and increased levels of free radicals (47). Although the role of α-synuclein aggregation in causing mitochondrial

dysfunction was not clearly addressed in this study, the authors did show the formation of α-synuclein aggregates in their cell system. Furthermore, an *in vitro* study by Ding *et al.* showed that protofibrillar α-synuclein with membrane-permeabilizing activity binds to rat brain mitochondria much more avidly than the monomer (24). Although more studies are needed, there might be a positive feedback loop between mitochondrial defects and α-synuclein aggregation.

Although the studies described here effectively support the principle that impairment of mitochondrial respiratory chain function could lead to α-synuclein aggregation, the *in vitro* studies with acute complex I inhibition should be interpreted with caution. The most salient concern comes from the fact that the superoxide production from mitochondria requires ~90% inhibition of complex I in a preparation of brain mitochondria (131), which is much higher than the 30–40% inhibition found in PD (53, 81, 108). Also, in the rat rotenone model, pathologic and behavioral PD symptoms can be produced without significantly impairing respiration, thus without substantially reducing ATP production (11). Therefore, the acute *in vitro* model does not perfectly reflect the pathogenic cascade that links mitochondrial defects to α-synuclein aggregation and neurodegeneration. It is possible that the cumulative effect of the mild impairment of mitochondrial function over time may eventually cause increased production of free radicals and reduced production of ATP. It is also possible that there are additional components in cells, such as dopamine and iron, that can amplify the effects of minor changes in superoxide and ATP production (19, 95). Exposure to certain environmental toxins may also have synergistic effects with the subphenotypic deficits of mitochondrial function.

ARE α-SYNUCLEIN AGGREGATES CYTOTOXIC?

The cytotoxic effect of α-synuclein after the exogenous overexpression of the wild-type or mutant forms of α-synuclein has been investigated by several groups. These studies generated a wide spectrum of outcomes: some reported cytotoxic effects for all α-synuclein variants (52, 94, 107, 135, 138), whereas others found toxicity either only with the mutant forms (75, 137) or only in the presence of additional stress (60, 62, 67, 137). Some of these studies showed dopaminergic neuron-selective neurotoxicity with α-synuclein expression (135, 137, 138). Furthermore, overexpression of wild-type, but not mutant, α-synuclein showed protective effects against cytotoxic insults (21, 45, 75). One of the most important issues that may be relevant to these rather confusing results is whether the aggregation of α-synuclein is responsible for the toxicity of this protein.

In a recent study, we have shown that in COS-7 cells, cytotoxicity of α-synuclein correlated with the amount of aggregates, whereas an increase in monomer level did not affect cell viability (41). In addition, α-synuclein aggregation was also associated with fragmentation of the Golgi apparatus and impairment of protein trafficking through the biosynthetic pathway (41). Although it is not clear as yet whether Golgi fragmentation is linked to cell death, these results support the notion that α-synuclein gains pathogenic function through forming higher order quaternary structures to damage specific cellular targets. Interestingly, the same study showed that both cell death and Golgi fragmentation were tightly correlated with the production of prefibrillar aggregates, but not with the formation of fibrillar inclusions, suggesting that the prefibrillar aggregates might be responsible for the cellular impairment. Although this study shows a strong correlation between α-synuclein aggregation and cellular impairment, it is still not clear whether the aggregates themselves are toxic agents or whether the *process* of aggregation is actually responsible for the impairment.

As discussed above, normal mitochondrial function is thought to be important in keeping the cytoplasm free of α-synuclein aggregates by preventing misfolding/aggregation and by clearing of the preformed aggregates. With the recent evidence that certain aggregate forms (protofibril or other species) of α-synuclein rather than the monomers are associated with cell death and other functional impairments, it is speculated that α-synuclein aggregate formation is one of the routes through which mitochondrial defects lead to neurodegeneration.

CONCLUDING REMARKS

The fibrillation process of α-synuclein involves a series of conformational changes and several metastable prefibrillar intermediates both *in vitro* and *in vivo.* Mitochondrial dysfunction leads to cytoplasmic accumulation of α-synuclein aggregates by promoting the production of aggregates and by inactivating cellular mechanisms to remove the preformed aggregates. In addition, studies in cultured cells and animals show that the accumulation of α-synuclein aggregates, not the monomeric α-synuclein, is associated with cellular impairments and death. These findings suggest that α-synuclein aggregation might represent one of the principal mechanisms underlying the pathogenesis of PD and other LB diseases. However, the causative role of α-synuclein aggregates in neurodegeneration still remains speculative. The critical questions include whether, and to what extent, α-synuclein aggregation contributes to neuronal loss and the progress of LB diseases, and whether α-synuclein aggregation is the critical link that connects mitochondrial defects to neurodegeneration. One way to approach these problems is to develop genetic and chemical tools to manipulate the aggregation process. Recent progress in cell and animal models should provide useful systems for the screening of various genes and chemical compounds in the search for aggregation modulators.

ACKNOWLEDGMENTS

The author thanks Hans Lee, Smita Patel, Stephen Lee, and He-Jin Lee for the critical comments on the manuscript. The work was funded by the Abramson Family Foundation and the U.S. Army Medical Research Acquisition Activity.

ABBREVIATIONS

LB, Lewy body; PD, Parkinson's disease; UCH, ubiquitin C-terminal hydrolase.

REFERENCES

1. Abeliovich A, Schmitz Y, Farinas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Ryan A, Hynes M, Phillips H, Sulzer D, and Rosenthal A. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25: 239–252, 2000.
2. Ahn BH, Rhim H, Kim SY, Sung YM, Lee MY, Choi JY, Wolozin B, Chang JS, Lee YH, Kwon TK, Chung KC, Yoon SH, Hahn SJ, Kim MS, Jo YH, and Min DS. Alpha-synuclein interacts with phospholipase D isozymes and inhibits pervanadate-induced phospholipase D activation in human embryonic kidney-293 cells. *J Biol Chem* 277: 12334–12342, 2002.
3. Alim MA, Hossain MS, Arima K, Takeda K, Izumiyama Y, Nakamura M, Kaji H, Shinoda T, Hisanaga S, and Ueda K. Tubulin seeds alpha-synuclein fibril formation. *J Biol Chem* 277: 2112–2117, 2002.
4. Ancolio K, Alves da Costa C, Ueda K, and Checler F. Alpha-synuclein and the Parkinson's disease-related mutant Ala53Thr-alpha-synuclein do not undergo proteasomal degradation in HEK293 and neuronal cells. *Neurosci Lett* 285: 79–82, 2000.
5. Auluck PK, Chan HY, Trojanowski JQ, Lee VM, and Bonini NM. Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 295: 865–868, 2002.
6. Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VM, Trojanowski JQ, and Iwatsubo T. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am J Pathol* 152: 879–884, 1998.
7. Beal MF. Energetics in the pathogenesis of neurodegenerative diseases. *Trends Neurosci* 23: 298–304, 2000.
8. Becker M, Martin E, Schneikert J, Krug HF, and Cato AC. Cytoplasmic localization and the choice of ligand determine aggregate formation by androgen receptor with amplified polyglutamine stretch. *J Cell Biol* 149: 255–262, 2000.
9. Bennett MC, Bishop JF, Leng Y, Chock PB, Chase TN, and Mouradian MM. Degradation of alpha-synuclein by proteasome. *J Biol Chem* 274: 33855–33858, 1999.
10. Bernardi P, Scorrano L, Colonna R, Petronilli V, and Di Lisa F. Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur J Biochem* 264: 687–701, 1999.
11. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, and Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 3: 1301–1306, 2000.
12. Bussell R Jr and Eliezer D. Residual structure and dynamics in Parkinson's disease-associated mutants of alpha-synuclein. *J Biol Chem* 276: 45996–46003, 2001.
13. Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, and Nussbaum RL. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. *J Neurosci* 22: 8797–8807, 2002.
14. Chung KK, Zhang Y, Lim KL, Tanaka Y, Huang H, Gao J, Ross CA, Dawson VL, and Dawson TM. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat Med* 7: 1144–1150, 2001.
15. Clayton DF and George JM. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci* 21: 249–254, 1998.
16. Cole NB, Murphy DD, Grider T, Rueter S, Brasaemle D, and Nussbaum RL. Lipid droplet binding and oligomerization properties of the Parkinson's disease protein alpha-synuclein. *J Biol Chem* 277: 6344–6352, 2002.
17. Conway KA, Harper JD, and Lansbury PT. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat Med* 4: 1318–1320, 1998.
18. Conway KA, Harper JD, and Lansbury PT Jr. Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry* 39: 2552–2563, 2000.
19. Conway KA, Rochet JC, Bieganski RM, and Lansbury PT Jr. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine–alpha-synuclein adduct. *Science* 294: 1346–1349, 2001.
20. Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 341: 233–249, 1999.
21. da Costa CA, Ancolio K, and Checler F. Wild-type but not Parkinson's disease-related Ala-53 → Thr mutant alpha-synuclein protects neuronal cells from apoptotic stimuli. *J Biol Chem* 275: 24065–24069, 2000.
22. Davidson WS, Jonas A, Clayton DF, and George JM. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *J Biol Chem* 273: 9443–9449, 1998.
23. Dean RT, Fu S, Stocker R, and Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324: 1–18, 1997.
24. Ding TT, Lee SJ, Rochet JC, and Lansbury PT Jr. Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. *Biochemistry* 41: 10209–10217, 2002.
25. Eliezer D, Kutluay E, Bussell R Jr, and Browne G. Conformational properties of alpha-synuclein in its free and lipid-associated states. *J Mol Biol* 307: 1061–1073, 2001.
26. Ellis CE, Schwartzberg PL, Grider TL, Fink DW, and Nussbaum RL. Alpha-synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases. *J Biol Chem* 276: 3879–3884, 2001.
27. Ellis RJ. Macromolecular crowding: an important but neglected aspect of the intracellular environment. *Curr Opin Struct Biol* 11: 114–119, 2001.
28. Engelender S, Kaminsky Z, Guo X, Sharp AH, Amaravi RK, Kleiderlein JJ, Margolis RL, Troncoso JC, Lanahan AA, Worley PF, Dawson VL, Dawson TM, and Ross CA. Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions. *Nat Genet* 22: 110–114, 1999.

29. Feany MB and Bender WW. A *Drosophila* model of Parkinson's disease. *Nature* 404: 394–398, 2000.
30. Forno LS. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* 55: 259–272, 1996.
31. Forno LS and Langston JW. Lewy bodies and aging: relation to Alzheimer's and Parkinson's diseases. *Neurodegeneration* 2: 19–24, 1993.
32. Fortini ME, Skupski MP, Boguski MS, and Hariharan IK. A survey of human disease gene counterparts in the *Drosophila* genome. *J Cell Biol* 150: F23–F30, 2000.
33. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, and Iwatsubo T. Alpha-synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* 4: 160–164, 2002.
34. Gabai VL and Kabakov AE. Rise in heat-shock protein level confers tolerance to energy deprivation. *FEBS Lett* 327: 247–250, 1993.
35. Garcia-Mata R, Bebok Z, Sorscher EJ, and Sztul ES. Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J Cell Biol* 146: 1239–1254, 1999.
36. George JM, Jin H, Woods WS, and Clayton DF. Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. *Neuron* 15: 361–372, 1995.
37. Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, and Lee VM. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290: 985–989, 2000.
38. Giasson BI, Murray IV, Trojanowski JQ, and Lee VM. A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. *J Biol Chem* 276: 2380–2386, 2001.
39. Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, and Lee VMY. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* 34: 521–533, 2002.
40. Goldberg MS and Lansbury PT Jr. Is there a cause and effect relationship between alpha-synuclein fibrillization and Parkinson's disease? *Nat Cell Biol* 2: E115–E119, 2000.
41. Gosavi N, Lee HJ, Lee JS, Patel S, and Lee SJ. Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion. *J Biol Chem* 277: 48984–48992, 2002.
42. Gu M, Cooper JM, Taanman JW, and Schapira AH. Mitochondrial DNA transmission of the mitochondrial defect in Parkinson's disease. *Ann Neurol* 44: 177–186, 1998.
43. Hartl FU and Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295: 1852–1858, 2002.
44. Hashimoto M, Hsu LJ, Xia Y, Takeda A, Sisk A, Sundsmo M, and Masliah E. Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro. *Neuroreport* 10: 717–721, 1999.
45. Hashimoto M, Hsu LJ, Rockenstein E, Takenouchi T, Mallory M, and Masliah E. Alpha-synuclein protects against oxidative stress via inactivation of the C-jun N-terminal kinase stress-signaling pathway in neuronal cells. *J Biol Chem* 277: 11465–11472, 2002.
46. Hattori N, Tanaka M, Ozawa T, and Mizuno Y. Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. *Ann Neurol* 30: 563–571, 1991.
47. Hsu LJ, Sagara Y, Arroyo A, Rockenstein E, Sisk A, Mallory M, Wong J, Takenouchi T, Hashimoto M, and Masliah E. Alpha-synuclein promotes mitochondrial deficit and oxidative stress. *Am J Pathol* 157: 401–410, 2000.
48. Imai Y, Soda M, and Takahashi R. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J Biol Chem* 275: 35661–35664, 2000.
49. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, and Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* 105: 891–902, 2001.
50. Irizarry MC, Kim TW, McNamara M, Tanzi RE, George JM, Clayton DF, and Hyman BT. Characterization of the precursor protein of the non-A beta component of senile plaques (NACP) in the human central nervous system. *J Neuropathol Exp Neurol* 55: 889–895, 1996.
51. Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A, and Saitoh T. The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 14: 467–475, 1995.
52. Iwata A, Maruyama M, Kanazawa I, and Nukina N. Alpha-synuclein affects the MAPK pathway and accelerates cell death. *J Biol Chem* 276: 45320–45329, 2001.
53. Janetzky B, Hauck S, Youdim MB, Riederer P, Jellinger K, Pantucek F, Zochling R, Boissl KW, and Reichmann H. Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. *Neurosci Lett* 169: 126–128, 1994.
54. Jenco JM, Rawlingson A, Daniels B, and Morris AJ. Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins. *Biochemistry* 37: 4901–4909, 1998.
55. Jensen PH, Hager H, Nielsen MS, Hojrup P, Gliemann J, and Jakes R. Alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. *J Biol Chem* 274: 25481–25489, 1999.
56. Jensen PH, Islam K, Kenney J, Nielsen MS, Power J, and Gai WP. Microtubule-associated protein 1B is a component of cortical Lewy bodies and binds alpha-synuclein filaments. *J Biol Chem* 275: 21500–21507, 2000.
57. Jo E, McLaurin J, Yip CM, St George-Hyslop P, and Fraser PE. Alpha-synuclein membrane interactions and lipid specificity. *J Biol Chem* 275: 34328–34334, 2000.
58. Johnston JA, Ward CL, and Kopito RR. Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* 143: 1883–1898, 1998.
59. Johnston JA, Dalton MJ, Gurney ME, and Kopito RR. Formation of high molecular weight complexes of mutant Cu,Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 97: 12571–12576, 2000.
60. Junn E and Mouradian MM. Human alpha-synuclein over-expression increases intracellular reactive oxygen

species levels and susceptibility to dopamine. *Neurosci Lett* 320: 146–150, 2002.
61. Kahle PJ, Neumann M, Ozmen L, Muller V, Jacobsen H, Schindzielorz A, Okochi M, Leimer U, van Der Putten H, Probst A, Kremmer E, Kretzschmar HA, and Haass C. Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. *J Neurosci* 20: 6365–6373, 2000.
62. Kanda S, Bishop JF, Eglitis MA, Yang Y, and Mouradian MM. Enhanced vulnerability to oxidative stress by alpha-synuclein mutations and C-terminal truncation. *Neuroscience* 97: 279–284, 2000.
63. Kim J and Klionsky DJ. Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu Rev Biochem* 69: 303–342, 2000.
64. Kim TD, Paik SR, Yang CH, and Kim J. Structural changes in alpha-synuclein affect its chaperone-like activity in vitro. *Protein Sci* 9: 2489–2496, 2000.
65. Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, Mandel RJ, and Bjorklund A. Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *J Neurosci* 22: 2780–2791, 2002.
66. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, and Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605–608, 1998.
67. Ko L, Mehta ND, Farrer M, Easson C, Hussey J, Yen S, Hardy J, and Yen SH. Sensitization of neuronal cells to oxidative stress with mutated human alpha-synuclein. *J Neurochem* 75: 2546–2554, 2000.
68. Kroemer G and Reed JC. Mitochondrial control of cell death. *Nat Med* 6: 513–519, 2000.
69. Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, and Riess O. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease [letter]. *Nat Genet* 18: 106–108, 1998.
70. Larsen CN, Krantz BA, and Wilkinson KD. Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. *Biochemistry* 37: 3358–3368, 1998.
71. Lashuel HA, Hartley D, Petre BM, Walz T, and Lansbury PT Jr. Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature* 418: 291, 2002.
72. Lee HJ and Lee SJ. Characterization of cytoplasmic alpha-synuclein aggregates. Fibril formation is tightly linked to the inclusion forming process in cells. *J Biol Chem* 277: 48976–48983, 2002.
73. Lee HJ, Choi C, and Lee SJ. Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form. *J Biol Chem* 277: 671–678, 2002.
74. Lee HJ, Shin SY, Choi C, Lee YH, and Lee SJ. Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. *J Biol Chem* 277: 5411–5417, 2002.
75. Lee M, Hyun D, Halliwell B, and Jenner P. Effect of the overexpression of wild-type or mutant alpha-synuclein on cell susceptibility to insult. *J Neurochem* 76: 998–1009, 2001.
76. Lee MK, Stirling W, Xu Y, Xu X, Qui D, Mandir AS, Dawson TM, Copeland NG, Jenkins NA, and Price DL. Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 → Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proc Natl Acad Sci U S A* 99: 8968–8973, 2002.
77. Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, and Polymeropoulos MH. The ubiquitin pathway in Parkinson's disease. *Nature* 395: 451–452, 1998.
78. Liu Y, Fallon L, Lashuel HA, Liu Z, and Lansbury PT Jr. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell* 111: 209–218, 2002.
79. Lo Bianco C, Ridet JL, Schneider BL, Deglon N, and Aebischer P. Alpha-synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. *Proc Natl Acad Sci U S A* 99: 10813–10818, 2002.
80. Lucking CB, Durr A, Bonifati V, Vaughan J, De Michele G, Gasser T, Harhangi BS, Meco G, Denèfle P, Wood NW, Agid Y, and Brice A, for The European Consortium on Genetic Susceptibility in Parkinson's Disease and The French Parkinson's Disease Genetics Study Group. Association between early-onset Parkinson's disease and mutations in the parkin gene. *N Engl J Med* 342: 1560–1567, 2000.
81. Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AH, and Marsden CD. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. *Brain* 115: 333–342, 1992.
82. Martin J and Hartl FU. The effect of macromolecular crowding on chaperonin-mediated protein folding. *Proc Natl Acad Sci U S A* 94: 1107–1112, 1997.
83. Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, Sagara Y, Sisk A, and Mucke L. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 287: 1265–1269, 2000.
84. McLean PJ, Kawamata H, and Hyman BT. Alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons. *Neuroscience* 104: 901–912, 2001.
85. McLean PJ, Kawamata H, Shariff S, Hewett J, Sharma N, Ueda K, Breakefield XO, and Hyman BT. Torsin A and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation. *J Neurochem* 83: 846–854, 2002.
86. McNaught KS, Mytilineou C, Jnobaptiste R, Yabut J, Shashidharan P, Jennert P, and Olanow CW. Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. *J Neurochem* 81: 301–306, 2002.
87. Minton AP. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J Biol Chem* 276: 10577–10580, 2001.
88. Nakamura T, Yamashita H, Takahashi T, and Nakamura S. Activated Fyn phosphorylates alpha-synuclein at tyrosine

residue 125. *Biochem Biophys Res Commun* 280: 1085–1092, 2001.
89. Negro A, Brunati AM, Donella-Deana A, Massimino ML, and Pinna LA. Multiple phosphorylation of alpha-synuclein by protein tyrosine kinase Syk prevents eosin-induced aggregation. *FASEB J* 16: 210–212, 2002.
90. Neumann M, Kahle PJ, Giasson BI, Ozmen L, Borroni E, Spooren W, Muller V, Odoy S, Fujiwara H, Hasegawa M, Iwatsubo T, Trojanowski JQ, Kretzschmar HA, and Haass C. Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies. *J Clin Invest* 110: 1429–1439, 2002.
91. Nguyen VT and Bensaude O. Increased thermal aggregation of proteins in ATP-depleted mammalian cells. *Eur J Biochem* 220: 239–246, 1994.
92. Nicklas WJ, Vyas I, and Heikkila RE. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sci* 36: 2503–2508, 1985.
93. Okochi M, Walter J, Koyama A, Nakajo S, Baba M, Iwatsubo T, Meijer L, Kahle PJ, and Haass C. Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. *J Biol Chem* 275: 390–397, 2000.
94. Ostrerova N, Petrucelli L, Farrer M, Mehta N, Choi P, Hardy J, and Wolozin B. Alpha-synuclein shares physical and functional homology with 14-3-3 proteins. *J Neurosci* 19: 5782–5791, 1999.
95. Ostrerova-Golts N, Petrucelli L, Hardy J, Lee JM, Farer M, and Wolozin B. The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. *J Neurosci* 20: 6048–6054, 2000.
96. Park SM, Jung HY, Kim TD, Park JH, Yang CH, and Kim J. Distinct roles of the N-terminal-binding domain and the C-terminal-solubilizing domain of alpha-synuclein, a molecular chaperone. *J Biol Chem* 277: 28512–28520, 2002.
97. Paxinou E, Chen Q, Weisse M, Giasson BI, Norris EH, Rueter SM, Trojanowski JQ, Lee VM, and Ischiropoulos H. Induction of alpha-synuclein aggregation by intracellular nitrative insult. *J Neurosci* 21: 8053–8061, 2001.
98. Payton JE, Perrin RJ, Clayton DF, and George JM. Protein–protein interactions of alpha-synuclein in brain homogenates and transfected cells. *Brain Res Mol Brain Res* 95: 138–145, 2001.
99. Perrin RJ, Woods WS, Clayton DF, and George JM. Interaction of human alpha-synuclein and Parkinson's disease variants with phospholipids. Structural analysis using site-directed mutagenesis. *J Biol Chem* 275: 34393–34398, 2000.
100. Perrin RJ, Woods WS, Clayton DF, and George JM. Exposure to long-chain polyunsaturated fatty acids triggers rapid multimerization of synucleins. *J Biol Chem* 276: 41958–41962, 2001.
101. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, and Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045–2047, 1997.
102. Pronin AN, Morris AJ, Surguchov A, and Benovic JL. Synucleins are a novel class of substrates for G protein-coupled receptor kinases. *J Biol Chem* 275: 26515–26522, 2000.
103. Reichmann H and Janetzky B. Mitochondrial dysfunction—a pathogenetic factor in Parkinson's disease. *J Neurol* 247(Suppl 2): II63–II68, 2000.
104. Rideout HJ, Larsen KE, Sulzer D, and Stefanis L. Proteasomal inhibition leads to formation of ubiquitin/alpha-synuclein-immunoreactive inclusions in PC12 cells. *J Neurochem* 78: 899–908, 2001.
105. Rivas G, Fernandez JA, and Minton AP. Direct observation of the enhancement of noncooperative protein self-assembly by macromolecular crowding: indefinite linear self-association of bacterial cell division protein FtsZ. *Proc Natl Acad Sci U S A* 98: 3150–3155, 2001.
106. Rubin GM, Yandell MD, Wortman JR, Gabor Miklos GL, Nelson CR, Hariharan IK, Fortini ME, Li PW, Apweiler R, Fleischmann W, Cherry JM, Henikoff S, Skupski MP, Misra S, Ashburner M, Birney E, Boguski MS, Brody T, Brokstein P, Celniker SE, Chervitz SA, Coates D, Cravchik A, Gabrielian A, Galle RF, Gelbart WM, George RA, Goldstein LS, Gong F, Guan P, Harris NL, Hay BA, Hoskins RA, Li J, Li Z, Hynes RO, Jones SJ, Kuehl PM, Lemaitre B, Littleton JT, Morrison DK, Mungall C, O'Farrell PH, Pickeral OK, Shue C, Vosshall LB, Zhang J, Zhao Q, Zheng XH, Zhong F, Zhong W, Gibbs R, Venter JC, Adams MD, and Lewis S. Comparative genomics of the eukaryotes. *Science* 287: 2204–2215, 2000.
107. Saha AR, Ninkina NN, Hanger DP, Anderton BH, Davies AM, and Buchman VL. Induction of neuronal death by alpha-synuclein. *Eur J Neurosci* 12: 3073–3077, 2000.
108. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, and Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem* 54: 823–827, 1990.
109. Scotcher KP, Irwin I, DeLanney LE, Langston JW, and Di Monte D. Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on ATP levels of mouse brain synaptosomes. *J Neurochem* 54: 1295–1301, 1990.
110. Serpell LC, Berriman J, Jakes R, Goedert M, and Crowther RA. Fiber diffraction of synthetic alpha-synuclein filaments shows amyloid-like cross-beta conformation. *Proc Natl Acad Sci U S A* 97: 4897–4902, 2000.
111. Sharon R, Goldberg MS, Bar-Josef I, Betensky RA, Shen J, and Selkoe DJ. Alpha-synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins. *Proc Natl Acad Sci U S A* 98: 9110–9115, 2001.
112. Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR, and Greenamyre JT. An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J Neurosci* 22: 7006–7015, 2002.
113. Sherman MY and Goldberg AL. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* 29: 15–32, 2001.

114. Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, and Suzuki T. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet* 25: 302–305, 2000.
115. Shimura H, Schlossmacher MG, Hattori N, Frosch MP, Trockenbacher A, Schneider R, Mizuno Y, Kosik KS, and Selkoe DJ. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* 293: 263–269, 2001.
116. Shtilerman MD, Ding TT, and Lansbury PT Jr. Molecular crowding accelerates fibrillization of alpha-synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease? *Biochemistry* 41: 3855–3860, 2002.
117. Singer TP, Castagnoli N Jr, Ramsay RR, and Trevor AJ. Biochemical events in the development of parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Neurochem* 49: 1–8, 1987.
118. Souza JM, Giasson BI, Chen Q, Lee VM, and Ischiropoulos H. Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Biol Chem* 275: 18344–18349, 2000.
119. Souza JM, Giasson BI, Lee VM, and Ischiropoulos H. Chaperone-like activity of synucleins. *FEBS Lett* 474: 116–119, 2000.
120. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, and Goedert M. Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc Natl Acad Sci U S A* 95: 6469–6473, 1998.
121. Swerdlow RH, Parks JK, Miller SW, Tuttle JB, Trimmer PA, Sheehan JP, Bennett JP Jr, Davis RE, and Parker WD Jr. Origin and functional consequences of the complex I defect in Parkinson's disease. *Ann Neurol* 40: 663–671, 1996.
122. Tofaris GK, Layfield R, and Spillantini MG. Alpha-synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. *FEBS Lett* 509: 22–26, 2001.
123. Uversky VN and Fink AL. Amino acid determinants of alpha-synuclein aggregation: putting together pieces of the puzzle. *FEBS Lett* 522: 9–13, 2002.
124. Uversky VN, Lee HJ, Li J, Fink AL, and Lee SJ. Stabilization of partially folded conformation during alpha-synuclein oligomerization in both purified and cytosolic preparations. *J Biol Chem* 276: 43495–43498, 2001.
125. Uversky VN, Li J, and Fink AL. Evidence for a partially folded intermediate in alpha-synuclein fibril formation. *J Biol Chem* 276: 10737–10744, 2001.
126. Uversky VN, Cooper EM, Bower KS, Li J, and Fink AL. Accelerated alpha-synuclein fibrillation in crowded milieu. *FEBS Lett* 515: 99–103, 2002.
127. van den Berg B, Ellis RJ, and Dobson CM. Effects of macromolecular crowding on protein folding and aggregation. *EMBO J* 18: 6927–6933, 1999.
128. van der Putten H, Wiederhold KH, Probst A, Barbieri S, Mistl C, Danner S, Kauffmann S, Hofele K, Spooren WP, Ruegg MA, Lin S, Caroni P, Sommer B, Tolnay M, and Bilbe G. Neuropathology in mice expressing human alpha-synuclein. *J Neurosci* 20: 6021–6029, 2000.
129. Volles MJ and Lansbury PT Jr. Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism. *Biochemistry* 41: 4595–4602, 2002.
130. Volles MJ, Lee SJ, Rochet JC, Shtilerman MD, Ding TT, Kessler JC, and Lansbury PT Jr. Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry* 40: 7812–7819, 2001.
131. Votyakova TV and Reynolds IJ. DeltaPsi(m)-dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J Neurochem* 79: 266–277, 2001.
132. Weinreb PH, Zhen W, Poon AW, Conway KA, and Lansbury PT Jr. NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry* 35: 13709–13715, 1996.
133. Weissman AM. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2: 169–178, 2001.
134. Wood SJ, Wypych J, Steavenson S, Louis JC, Citron M, and Biere AL. Alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. *J Biol Chem* 274: 19509–19512, 1999.
135. Xu J, Kao SY, Lee FJ, Song W, Jin LW, and Yankner BA. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med* 8: 600–606, 2002.
136. Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, and Dawson TM. Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc Natl Acad Sci U S A* 97: 13354–13359, 2000.
137. Zhou W, Hurlbert MS, Schaack J, Prasad KN, and Freed CR. Overexpression of human alpha-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells. *Brain Res* 866: 33–43, 2000.
138. Zhou W, Schaack J, Zawada WM, and Freed CR. Overexpression of human alpha-synuclein causes dopamine neuron death in primary human mesencephalic culture. *Brain Res* 926: 42–50, 2002.

Address reprint requests to:
Dr. Seung-Jae Lee
The Parkinson's Institute
1170 Morse Avenue
Sunnyvale, CA 98089

E-mail: slee@thepi.org

Received for publication September 19, 2002; accepted March 5, 2002.