Available Online at http://www.journalajst.com

ASIAN JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN: 0976-3376

Asian Journal of Science and Technology
Vol. 5, Issue 10, pp.615-619, October, 2014

RESEARCH ARTICLE

DEVELOPING ARGININE AS AN INHIBITOR FOR A-SYNUCLEIN AGGREGATION: AN INNOVATIVE THERAPY TO COMBAT PARKINSON'S DISEASE

***Asthana Shrishti**

≠ 257, Sec.16 A, Chandigarh (UT)

ARTICLE INFO

Article History:
Received 25th July, 2014
Received in revised form
15th August, 2014
Accepted 08th September, 2014
Published online 27th October, 2014

Key words:
α-synuclein, Parkinson's disease, Arginine, Thioflavin T spectrofluorimetry, innovative, economically viable, preventive and prophylactic therapy

ABSTRACT

α-synucleinParkinson's disease is a neurodegenerative disorder and its root cause is the aggregation of the protein α-synuclein, which leads to neural cell death. There exists no technique through which the aggregation process can be reversed. A large no. of epidemiological studies has shown increased risk of Parkinson's in populations exposed to pesticides. I wish to investigate the role of arginine as an inhibitor in pesticide induced α-synuclein aggregation and develop this innovative therapy to combat Parkinson's disease. Arginine, a natural, readily available amino acid has been reported as an aggregation suppressor and protein structure stabilizer. This non-toxic compound has been used to inhibit the progression of Parkinson's disease. α-synuclein was expressed in E.coli cells and then purified. Gel electrophoresis was performed to confirm expression and purification of α-synuclein followed by the western blot analysis to detect and separate native protein. This was followed by incubation and aggregation of α-synuclein in the presence of rotenone (representative pesticide), arginine and rotenone+ arginine. Thioflavin T spectrofluorimetry was used to analyse the solutions. I discovered that arginine was able to arrest aggregation of α-synuclein in all stages. I plan to extend this study to monitor the effect of arginine addition at delayed time points of aggregation in a cell based system. This can be a promising therapeutic strategy to slow down aggregation of α-synuclein in this progressively neurodegenerative disorder. It is innovative, economically viable and easily available. It is a safer, preventive and prophylactic therapy and thus can be quite effective.

INTRODUCTION

Parkinson's Disease is a neurodegenerative disorder and its root cause is the aggregation of the protein α-synuclein, which is a very important protein for neuro transmission. The aggregation of this protein leads to neural cell death. There exists no technique through which the aggregation process can be reversed. It has been reported that pesticides accelerate the process of aggregation. α-synuclein is an intrinsically disordered protein which means that its structure is not known. And arginine is an amino acid that has been reported as an aggregation suppresor and protein structure stabilizer. There also exist other molecules that are known to slow down the process of aggregation of various proteins but I decided to use arginine because it is a natural and non-toxic compound for the human body and found in some quantities in the human body itself. The aim of my study was to develop an inhibitor for this process of pesticide-induced aggregation.

MATERIALS AND METHODS

Expression of α-synuclein protein in E. coli BL21 cells: *E. coli* BL21 cells (containing pRSETB-*α-synuclein*gene, gifted by Prof. R. Cappai, University of Melbourne, Australia) were grown at 37°C, 200 rpm in LB media containing ampicillin (0.6% w/v) till OD_{600} of 0.6. Expression of the protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the cells were further incubated for 3.5 h at 37°C, 200 rpm. After the completion of the induction period, the cells were centrifuged at 7000 g for 30 min at 4°C. The resulting pellets were stored overnight at -80°C.

These were then thawn on ice for 30 min and resuspended in lysis buffer (10 mM sodium phosphate monobasic, 40 mM sodium phosphate dibasic, 1 mM EDTA, pH 7.4) containing 0.5 mg/ml lysozyme and 1 mMphenylmethanesulphonyl fluoride (PMSF). After incubation for 45 min at 4°C, cells were sonicated on ice with three 15 s bursts with a time interval of 1 min, 40% amplitude and 0.5 cycle/min of sonication. The resulting lysed cells were centrifuged at 12,000 g for 1 h at 4°C. Supernatant now called as cell lysate (lysate 1) was heated at 95°C for 30 min. The heat treated lysate was cooled to room temperature and centrifuged at 15,000 g for 1 h. The supernatant obtained was called as heat treated lysate (lysate 2). Lysate 2 was treated with 1 N HCl to reduce the pH to 3.5, since α-synuclein is stable at low pH whereas other proteins precipitate out. After acid induced precipitation, the lysate was centrifuged at 15,000 g for 1 h. This heat and acid treated lysate (lysate 3) was used for anion

Corresponding author: Asthana Shrishti,
≠ 257, Sec.16 A, Chandigarh (UT)

exchange chromatography for further purification of the protein.

Purification of α-synuclein

The cleared supernatant of the lysate was incubated overnight at 4°C with 10 ml DEAE- Sepharose matrix Cappai *et al*. (2005) FASEB J 19, 1377] that has been washed and equilibrated with 20 mMTrisHCl buffer, pH 7.8. The suspension was shaken at 12 rpm on a rotary mixer. The matrix bound lysate was poured into a column and the flow through was collected. The matrix was washed with 20 mMTrisHCl buffer, pH 7.8 (3 times, 10 ml each) for removing the loosely bound protein. The bound α-synuclein protein was eluted with 10 ml of elution buffer (20 mMTrisHCl, pH 7.8 containing 0.5 M NaCl) for 3 h at 4°C with rotation at 12 rpm and the eluates were collected. The concentration of the protein in the various fractions like the flow through, washings and eluates was determined by the bicinchoninic acid assay, using bovine serum albumin (BSA) as a standard protein.

Gel electrophoresis

15% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed to confirm the expression and purification of α-synuclein protein. The protein samples including the lysate, and eluates were denatured by heating with sample buffer containing β-mercaptoethanol and SDS and loaded onto the gel [Laemmli *et al*. (1970) Nature 227, 680]. The samples were run along with a standard protein marker (lysozyme 14 kDa) at constant current (25 mA) in miniVE electrophoresis unit. The resolved protein bands were detected by Coomassie staining.

Western blot analysis

Western blotting was performed using the purified α-synuclein samples. The proteins were loaded on 15% SDS-PAGE gel. After completion of the run, the protein on the SDS-PAGE gel was transferred electrophoretically to nitrocellulose (NC) membrane (0.45 µm) with transfer buffer [25 mMTris, 20 mM glycine, and 10% (v/v) methanol, pH 8.3]. The transfer was carried out for 55 min, at 500 mA on a semi dry blotting assembly. The membrane was washed first with TBST (Tris-buffered saline and 1% v/v Tween 20, pH 7.6) and then TBS. In order to block non specific binding, the membrane was kept overnight in blocking solution (10% skimmed milk powder in TBS). After washing off the blocking solution with TBS, the membrane was incubated with mouse anti-α-synuclein monoclonal antibody (1:5000 dilution) for 6 h. After washing, the membrane was transferred to a solution of anti-mouse horseradish peroxidase (HRP) (1:5000 dilution)-conjugated monoclonal antibody for 1.5 h. Finally, the blot was washed and developed by adding 1% (v/v) of tetramethylbenzidine/ hydrogen peroxide (TMB/H_2O_2), which is a substrate of horseradish peroxidase.

Dialysis and concentration of protein

Eluate fractions were pooled and transferred into dialysis tubing. Dialysis of the eluted protein against water was performed in order to remove the NaCl completely. Dialysis was carried out for 4 cycles, each of 1 h. An ultrafiltration spin column, followed by lyophilization, concentrated the protein.

Aggregation of α-synuclein

Lyophilized protein was dissolved in 20 mMTrisHCl buffer, pH 7.8, and subjected to ultra centrifugation (1,00,000 g for 1 h) to remove any preformed aggregates. Samples were incubated at 37°C. At predefined time intervals, aliquots were withdrawn and studied by Thioflavin T spectrofluorimetry. a-Synuclein was also incubated in the presence of 100 mM rotenone and 0.5 M arginine and analyzed as described before.

Thioflavin T (ThT) fluorescence measurement

A stock solution of ThT (250 mM) was prepared in 20 mMTrisHCl buffer, pH 7.8. Final concentrations of 10 µM protein and 20 µMThT were used for the assay. Fluorescence emission was recorded immediately after addition of the aliquots to the ThT solution. Emission intensity was recorded at 485 nm with excitation at 440 nm, at slit width of 5 nm and 10 nm for excitation and emission, respectively for each sample. Emission intensity of ThT solution alone was subtracted as blank. The aggregation kinetics was followed by fitting the data using the formula

$$y = y_i + mx_i + [(y_f + mx_f) / (1 + e^{(x-x0)/\tau})]$$

where $y_i + mx_i$ is initial line, $y_f + mx_f$ is the final line and x_0 is the midpoint of maximum signal. The apparent rate constant (k_{app}) is 1/t and lag time is calculated to be $x_0 - 2t$.

RESULTS

Expression and purification of α –Synuclein

E. coli BL21 cells already transformed with the pRSETB vector containing the gene for α-synuclein expression, were used in these experiments. Expression of the target protein was carried out using IPTG as an inducer. The expression of the target protein was monitored by SDS PAGE (Figure 1). The expressed protein was isolated from the cells by lysis and subjected to purification using DEAE sepharose matrix based anion exchange chromatography. The target protein was eluted with elution buffer.

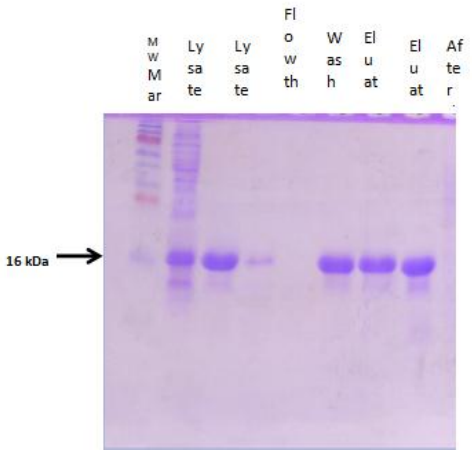

Figure 1. SDS PAGE analysis of purification of α-synuclein

α-Synuclein is a heat- and acid-stable protein. Hence, an additional step of acid treatment was carried out, as reported in the literature, which resulted in significant purification of d the protein from the crude cell lysate (Figure 1). Only a faint band was observed at the expected position of α-synucleinin the flow through, indicating that the binding of α-synuclein protein was almost complete. The eluate from the anion exchanger showed a single band (Figure 1), indicating the homogeneity of the purified protein. The concentration of the protein in different fractions was determined by bicinchoninic acid assay [Smith *et al*. (1985) Anal Biochem 150, 76] and the results are shown in Table 1.

Table 1. Yield of protein at different steps of purificationThe table shows the representative amount of protein recovered from 1 L culture

Fraction	Protein yield, mg
Lysate 1	156.8
Lysate 2	59.0
Flow through	7.2
Wash (pooled)	4.9
Eluate (pooled)	74.4
After dialysis	64.0

The total amount of target protein obtained was 64 mg from a 1 L culture in the above representative run. Western blotting with anti α-synuclein monoclonal (primary) antibody was performed to confirm the presence of α-synuclein. The band corresponding to the expected position of α-synuclein in case of the uninduced sample did not stain with anti α-synuclein monoclonal antibody. As can be seen (Figure 2), a single band, stained with anti α-synucleinmonoclonal antibody, was observed in the induced sample and after purification, confirming the identity of the protein.

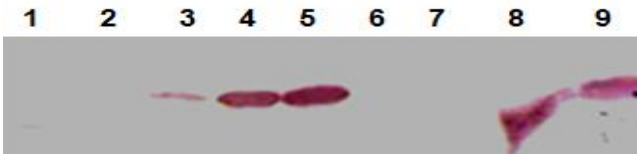

Figure 2. Western blot of human α-synuclein purified from *E. coli*

This eluted protein was used further for aggregation studies.

Aggregation of α-synuclein

Human α-synuclein, purified as above, was incubated at 37°C. Aliquots were withdrawn at different time intervals and analyzed for aggregate formation by Thioflavin T spectrofluorimetry. Thioflavin T is a cationic benzothiazole dye that has been used to identify amyloid aggregates since it was first demonstrated to increase in fluorescence upon binding to amyloid fibrils.

Upon binding with cross β-sheet structures in amyloids, Thioflavin T exhibits changes in both fluorescence emission and excitation spectra. As can be seen in Figure 3, the wavelength of maximum emission as well as emission intensity underwent significant changes when α-synuclein was subjected to aggregation for 96 h.

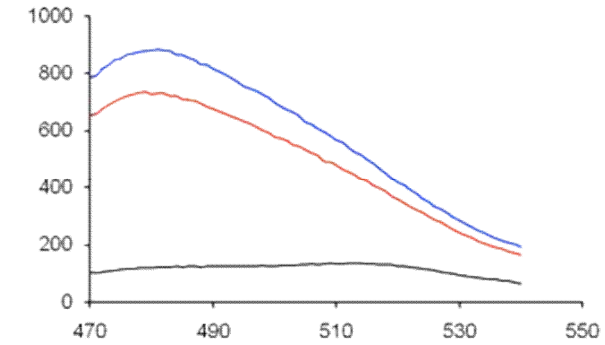

Figure 3. Thioflavin T fluorescence spectra of α-synuclein monomer (black line), incubated for 96 h (red line) and incubated for 96 h in the presence of 100 μM rotenone (blue line). Samples were excited at 440 nm

The kinetics of α-synuclein was also followed using the emission intensity of Thioflavin T. As α-synuclein was found to form amyloid type of aggregates, measurement of ThT fluorescence would be an important probe for characterization of nature of aggregates. A characteristic sigmoidal curve, with well-defined nucleation, growth and saturation stages, was observed (Figure 4).

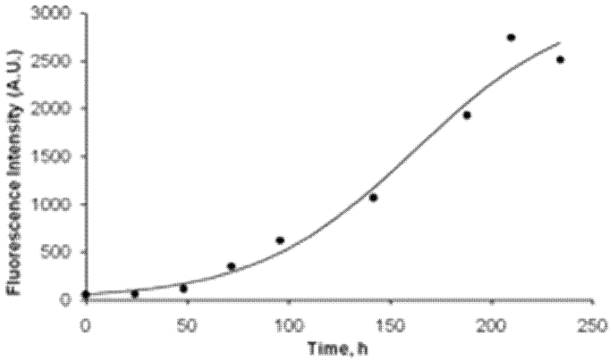

Figure 4. Kinetics of aggregation of α-synuclein

The apparent rate constant of fibrillization was calculated to be 0.025 h^{-1} for α-synuclein incubated alone, with a lag time of 83.7 h. In the presence of the pesticide rotenone, the extent of aggregation was more, seen from the Thioflavin T spectrum of the aggregated protein recorded in the presence of the neurotoxin (Figure 3). The aggregation kinetics was also measured (Figure 5). Lag time was reduced to 72.9 h as compared to the nucleation stage for α-synuclein alone.

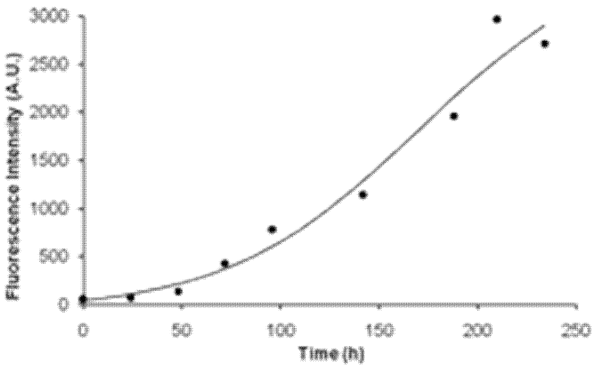

Figure 5. Kinetics of aggregation of α-synuclein in the presence of rotenone

This pattern matches with those of studies already reported in the literature [Uversky *et al*. (2001) FEBS Lett 500, 105].

Inhibition of aggregation of α-synuclein

Next, the effect of arginine, a well-known protein aggregation suppressor, on the fibrillation pattern of α-synuclein was studied. Arginine (0.5 M) was added to α-synuclein in the presence of rotenone and aggregation kinetics was studied by Thioflavin T spectrofluorimetry. A significant reduction in the emission intensity of the protein sample incubated in the presence of the amino acid was seen (Figure 6), demonstrating the ability of arginine to act as an inhibitor of aggregation.

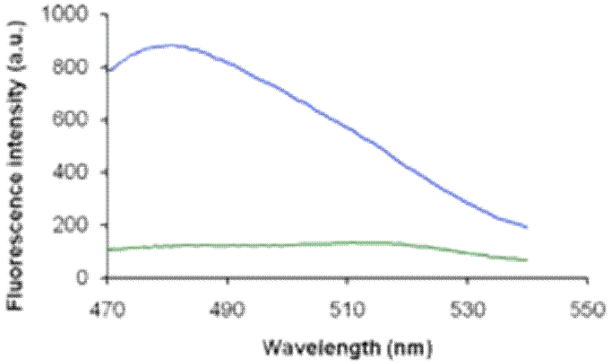

Figure 6. Thioflavin T fluorescence spectra of α-synuclein incubated for 96 h in the presence of 100 μM rotenone (blue line) and in the presence of 0.5 M arginine (green line). Samples were excited at 440 nm

Kinetic analysis did not show increase in the emission intensity of the dye at any time point (Figure 7)

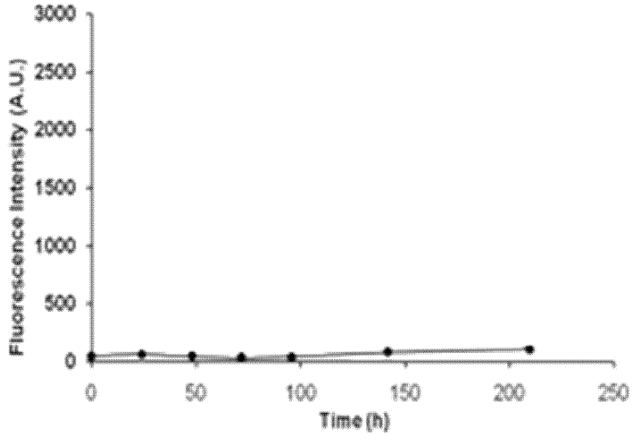

Figure 7. Kinetics of aggregation of α-synuclein in the presence of rotenone and arginine

Interestingly, arginine was also able to suppress aggregation once nucleation had been initiated. In order to monitor this, arginine was added to α-synuclein which had already been incubated for 48 h, i.e. midpoint of lag phase. Thioflavin T emission spectrum recorded after 96 h of incubation showed a significant decrease in the emission intensity of the dye as compared to the protein incubated in the presence of the pesticide alone (Figure 8).

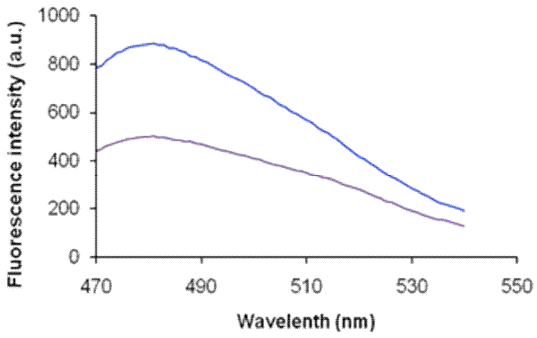

Figure 8. Thioflavin T fluorescence spectra of α-synuclein incubated for 96 h in the presence of 100 μM rotenone (blue line) and in the presence of 0.5 M arginine added after 48 h of incubation (purple line). Samples were excited at 440 nm

Kinetic analysis showed that addition of arginine at this stage (after lag phase had set in) did not allow aggregation to proceed any further and caused nucleation arrest (Figure 9).

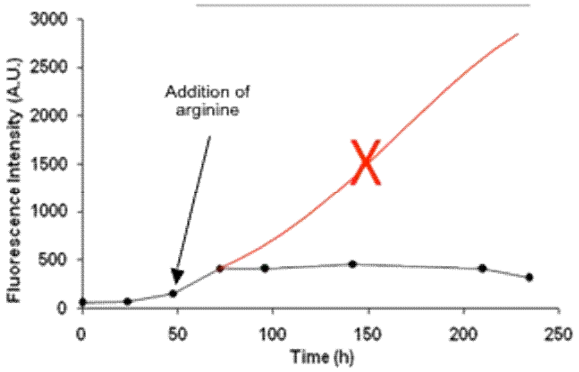

Figure 9. Kinetics of aggregation of α-synuclein in the presence of rotenone and arginine added after 48 h of incubation

The consolidated picture of the aggregation process is depicted in Figure 10.

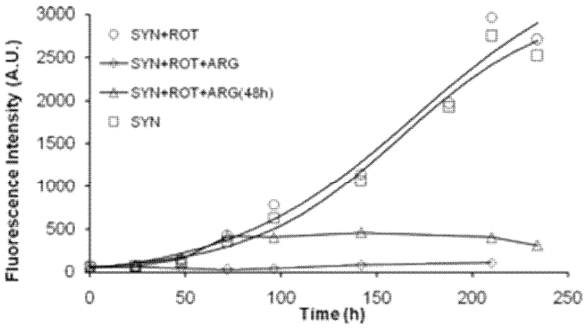

Figure 10. Kinetics of aggregation of α-synuclein under different conditions

As can be seen, fibrillation of α-synuclein is accelerated in the presence of the pesticide, rotenone. The presence of arginine is able to inhibit aggregation of the protein. Notably, and more interestingly, arginine is also able to suppress aggregation of α-synuclein, once nucleation/oligomerization has been initiated.

Conclusion

The major finding of the current report is the elucidation of the role of arginine as a suppressor of a-synuclein aggregation. As far as I am aware, this is the first such report of arginine-

induced inhibition of aggregation of a-synuclein, the major component of Lewy bodies, the pathological hallmark of Parkinson's disease. Inhibition of aggregation of a-synuclein has been shown to have therapeutic benefit in cells harbouring this protein and is acknowledged to be a viable strategy in slowing down the progress of the disease. Even more interestingly, I found that administration of arginine was able to inhibition aggregation even after it had been initiated. Thus, the strategy can prove to be potentially beneficial even in cases where oligomerisation of the protein had commenced.

Future Prospects

The study can be extended to monitor the effect of arginine addition at delayed time points of aggregation. This will establish the beneficial effect of the osmolyte at even advanced stages of aggregation. The efficacy of the additive will need to be established in a cell-based system. If successful, the remarkable inhibitory effect of arginine can establish it as a promising therapeutic strategy to slow down the aggregation of a-synuclein in this progressively neurodegenerative disorder.

Conflict of Interest Statement

It is hereby declared that this paper does not have any conflict of interest.

Acknowledgement

I am thankful to Dr. Ipsita Roy, Assistant Professor, Department of Biotechnology, National Institute of Pharmaceutical Research and Development (NIPER), Mohali for providing necessary facilities and valuable guidance.

REFERENCES

Baba, M. *et al*. Aggregation of á-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Amer*. *J*. *Pathol*., 1998.

Breakefield, X.O. and Nirenberg, M.W. Selection of neuroblastoma cells that synthesize certain transmitters. *Proc*. *Natl*. *Acad*. *Sci*., *USA*

Busciglio, J. and Yankner, B.A. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *in vitro*. *Nature,* 1995.

Campbell, B.C.V. *et al*. The solubility of á-synuclein in multiple system atrophy differs from that of dementia with Lewy bodies in Parkinson's disease. *J*. *Neurochem*., 2001.

Conway, K.A. *et al*. Acceleration of oligomerization, not fibrillization, is a shared property of both á-synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy. *Proc*. *Natl*. *Acad*. *Sci*., *USA* 2000.

Conway, K.A., Rochet, J.-C., Bieganski, R.M. & Lansbury, P.T. Kinetic stabilization of the á-synucleinprotofibril by a dopamine-á-synuclein adduct. *Science,* 2001.

da Costa, C.A., Ancolio, K. &Checler, F. Wild-type but not Parkinson's disease-related ala-53→Thr mutant á-synuclein protects neuronal cells from apoptotic stimuli. *J*. *Biol*. *Chem*., 2000.

Drukarch, B., Jongenelen, C.A.M., Schepens, E., Langeveld, C.H. and Stoof, J.C. Glutathione is involved in the granular storage of dopamine in rat PC12 pheochromocytoma cells: Implications for the pathogenesis of Parkinson's disease. *J*. *Neurosci*., 1996.

Feany, M.B. and Bender, W.W. A *Drosophila* model of Parkinson's disease. *Nature* 2000.

Giasson, B.I. *et al*. Oxidative damage linked to neurodegeneration by selective á-synuclein nitration in synucleinopathy lesions. *Science,* 2000.

Hashimoto, M., Rockenstein, E., Mante, M., Mallory, M. &Masliah, E. â-Synuclein inhibits á-synuclein aggregation: A possible role as an anti-parkinsonian factor. *Neuron* 2001.

Hastings, T.G., Lewis, D.A. and Zigmond, M.J. Role of oxidation in the neurotoxic effects ofintrastriatal dopamine injections. *Proc*. *Natl*. *Acad*. *Sci*., *USA* 1996.

Hsu, L.J. *et al*. á-synuclein promotes mitochondrial deficit and oxidative stress. *Am*. *J*. *Pathol*., 2000.

Iwata, A., Maruyama, M., Kanazawa, I. &Nukina, N. á-Synuclein affects the MAPK pathway and accelerates cell death. *J*. *Biol*. *Chem*., 2001.

Junn, E. & Mouradian, M.M. Human á-Synuclein over-expression increases intracellular reactive oxygen species levels and susceptibility to dopamine. *Neurosci*. *Lett*., 2002.

Kawamoto, Y. *et al*. 14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains. *J*. *Neuropathol*. *Exp*. *Neurol*., 2002.

Kirik, D. *et al*. Parkinson-like neurodegeneration induced by targeted over expression of á-synuclein in the nigrostriatal system. *J*. *Neurosci*., 2002.

Krüger, R. *et al*. Ala30 Pro mutation in the gene encoding á-synuclein in Parkinson's disease. *Nature Gen*., 1998.

Masliah, E. *et al*. Dopaminergic loss and inclusion body formation in á-synuclein mice: Implications for neurodegenerative disorders. *Science,* 2000.

Olanow, C.W. and Tatton, W.G. Etiology and pathogenesis of Parkinson's disease. *Ann*. *Rev*. *Neurosci*. 1999.

Ostrerova, N. *et al*. á-Synuclein shares physical and functional homology with 14-3-3 proteins. *J*. *Neurosci*., 1999.

Polymeropoulos, M.H. *et al*. Mutation in the á-Synuclein gene identified in families with Parkinson's disease. *Science,* 1997.

Shimura, H. *et al*. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nature Genet*., 2000.

Shimura, H. *et al*. Ubiquitination of a new form of á-synuclein by parkin from human brain: Implications for Parkinson's disease. *Science,* 2001.

Spillantini, M.G. *et al*. á-Synuclein in Lewy bodies. *Nature,* 1997.

Tabrizi, S.J. *et al*. Expression of mutant á-synuclein causes increased susceptibility to dopamine toxicity. *Human Mol*. *Gen*., 2000.

Tompkins, M.M. and Hill, W.D. Contribution of somalLewy bodies to neuronal death. *Brain Res*., 1997.

Yuan, J. and Yankner, B.A. Apoptosis in the nervous system. *Nature,* 2000.

Zhou, W., Hurlbert, M.S., Schaack, J., Prasad, K.N. and Freed, C.R. Over expression of human á-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells. *Brain Res*., 2000.

Zhou, W., Schaak, J., Zawada, W.M. and Freed, C.R. Overexpression of human á-synuclein causes dopamine neuron death in primary human mesencephalic culture. *Brain Res.,* 2002.

****