Human Molecular Genetics, 2001, Vol. 10, No. 9 **919–926**

Inducible expression of mutant α-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis

Yuji Tanaka[1], Simone Engelender[6], Shuichi Igarashi[1], Raghuram K. Rao[1], Tracy Wanner[1], Rudolph E. Tanzi[7], Akira Sawa[1,2], Valina L. Dawson[2,3,4,5], Ted M. Dawson[2,3,5] and Christopher A. Ross[1,2,5,+]

[1]Department of Psychiatry, [2]Department of Neuroscience, [3]Department of Neurology, [4]Department of Physiology and [5]Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, MD 21205, USA, [6]Departamento de Anatomia, Universidade Federal do Rio de Janeiro, Ilha do Fundao, Brazil and [7]Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA

Received 18 December 2000; Revised and Accepted 26 February 2001

ABSTRACT

Parkinson's disease (PD) is a common progressive neurodegenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra. Although mutations in α-synuclein have been identified in autosomal dominant PD, the mechanism by which dopaminergic neural cell death occurs remains unknown. Proteins encoded by two other genes in which mutations cause familial PD, parkin and UCH-L1, are involved in regulation of the ubiquitin-proteasome pathway, suggesting that dysregulation of the ubiquitin-proteasome pathway is involved in the mechanism by which these mutations cause PD. We established inducible PC12 cell lines in which wild-type or mutant α-synuclein can be de-repressed by removing doxycycline. Differentiated PC12 cell lines expressing mutant α-synuclein showed decreased activity of proteasomes without direct toxicity. Cells expressing mutant α-synuclein showed increased sensitivity to apoptotic cell death when treated with sub-toxic concentrations of an exogenous proteasome inhibitor. Apoptosis was accompanied by mitochondrial depolarization and elevation of caspase-3 and -9, and was blocked by cyclosporin A. These data suggest that expression of mutant α-synuclein results in sensitivity to impairment of proteasome activity, leading to mitochondrial abnormalities and neuronal cell death.

INTRODUCTION

Parkinson's disease (PD) is a common progressive neurodegenerative disorder, characterized by tremor, muscle rigidity and bradykinesia. Post-mortem examination shows loss of dopaminergic neurons in the substantia nigra and the presence of inclusions termed Lewy bodies (1–5). Mutations in α-synuclein (A30P and A53T) cause autosomal dominant PD (6,7), which shares many of the phenotypic findings seen in sporadic PD. In both autosomal dominant and sporadic PD, there is deposition of α-synuclein in Lewy bodies (8–10). In addition, it has been recently reported that transgenic mice and flies expressing human wild-type or mutant α-synuclein show neuronal dysfunction, degeneration and abnormal cellular accumulation of α-synuclein (11–14), indicating that α-synuclein may have a role in the pathogenesis of both familial and sporadic PD.

Several lines of evidence suggest a role for the ubiquitin-proteasome pathway in the pathogenesis of PD. Proteasome subunits colocalize in Lewy bodies (15). The causal gene product for autosomal recessive juvenile parkinsonism (AR-JP), parkin, acts as an E3 ubiquitin-protein ligase (16–18). Due to disruption of parkin's ubiquitination function, accumulation of target proteins normally degraded by the ubiquitin-proteasome pathway may contribute to the development of AR-JP. In addition, a missense mutation in the ubiquitin C-terminal hydrolase L1 (UCH-L1) has been identified in another family with PD (19). UCH-L1 is thought to produce ubiquitin by cleaving polymeric ubiquitin or releasing ubiquitin from small adducts such as glutathione and cellular amines (20). These findings suggest that dysregulation of the ubiquitin-proteasome pathway is involved in the pathogenesis of PD.

It has been postulated that mitochondrial dysfunction may also have a role in the pathogenesis of PD (21–25). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been reported to cause acute and irreversible parkinsonism in humans, and 1-methyl-4-phenylpyridinium (MPP^+), a metabolite of MPTP, inhibits mitochondrial complex I, one of the enzymes involved in oxidative phosphorylation (26–28). In addition,

[+]To whom correspondence should be addressed at: Department of Psychiatry, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Ross Research Building, Room 618, Baltimore, MD 21205-2196, USA. Tel: +1 410 614 0011; Fax: +1 410 614 0013; Email: caross@jhu.edu

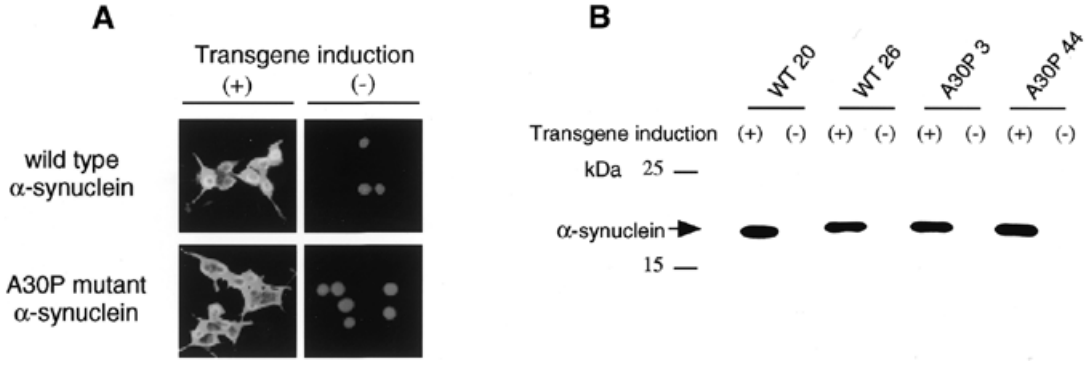

Figure 1. Inducible α-synuclein cell lines. (**A**) Representative immunofluorescent analyses of wild-type and A30P mutant α-synuclein Tet-Off inducible cell lines. The Tet-Off system is regulated through tTA, a fusion protein of the Tet repressor (TetR) and VP16 activation domain. Expression of the transgene is induced when Dox is removed [induced (+) conditions]. We detected cytoplasmic distribution of α-synuclein. Dark spots indicate DAPI nuclear staining. Conversely, in the presence of Dox, tTA is prevented from binding to the tet-responsive element and expression of the transgene is inhibited [uninduced (–) conditions], showing DAPI nuclear staining alone. (**B**) Confirmation of expression levels of α-synuclein by western blot analysis in induced (+) or uninduced (–) conditions. Left margin, protein molecular mass. We used wild-type α-synuclein cell lines 20 and 26 and A30P mutant α-synuclein cell lines 3 and 44 in each experiment.

cells overexpressing wild-type α-synuclein show mitochondrial dysfunction (29). However, it is not clear if there is a relation between PD-associated mutations of α-synuclein, cellular abnormalities involving proteasome dysfunction, mitochondrial changes and neuronal degeneration. To study how mutant α-synuclein is involved in the pathogenesis of PD, we have created inducible PC12 cell models in which expression of either wild-type or A30P mutant α-synuclein can be induced. We used PC12 cells for creating the cell models, because PC12 cells are known to synthesize and secrete dopamine, and can be differentiated into a neuron-like phenotype by nerve growth factor (30). We now report that expression of mutant α-synuclein decreases proteasome activity and renders cells vulnerable to mitochondria-dependent apoptosis.

RESULTS

Establishment of α-synuclein inducible cell lines

To study how α-synuclein is involved in the pathogenesis of PD, we have established inducible α-synuclein PC12 cell lines using the Tet-Off gene expression system. Regulation of the system is achieved through the tetracycline-controlled transactivator (tTA). The cells showed expression of the transgene in the absence of doxycycline (Dox) (induced condition), and no expression in the presence of Dox (uninduced condition) (Fig. 1A and B). We confirmed that there was no significant difference in the expression level of α-synuclein in each cell line using duplicate samples of cell cultures as measured by densitometer (data not shown). Two mutations (A30P and A53T) of α-synuclein cause autosomal dominant PD (6,7). PC12 cells are derived from rat pheochromocytoma, and the rat carries the T53 allele as its normal sequence. Because human A53T mutant α-synuclein may be better tolerated in rat cells, we chose to establish A30P mutant α-synuclein inducible cell lines. In addition, data from α-synuclein transgenic flies indicate that flies expressing A30P mutant α-synuclein show a significantly greater phenotype than those expressing A53T mutant α-synuclein (12).

Expression of mutant α-synuclein decreases proteasome activity and increases sensitivity to cell death by proteasome inhibition

We measured proteasome activities in wild-type and mutant α-synuclein inducible cell lines in both induced and uninduced conditions. We also used Tet-Off PC12 cells containing only pTet-Off plasmid, and a Tet-Off inducible cell line expressing full length huntingtin with a normal length polyglutamine tract (FL-23Q) as controls. In cells expressing mutant α-synuclein, chymotryptic-like, tryptic-like and postacidic activities of proteasome were significantly decreased by 22.1, 17.7 and 24.6%, respectively (Fig. 2). Although wild-type cell lines also showed decreased proteasome activities to some extent, there was no significant difference compared with control cell lines.

To test the toxicity of mutant α-synuclein, we used lactacystin, a proteasome inhibitor, since cells expressing mutant α-synuclein show proteasomal hypoactivity. Lactacystin is the most selective proteasome inhibitor and even at high concentrations and long incubation times, it does not strongly inhibit other proteases (31). In the absence of lactacystin, cell lines did not show significant differences in cell death in either induced or uninduced conditions. In contrast, in the presence of lactacystin, mutant cell lines show significantly more cell death (1.4- and 2.4-fold increases in the presence of 5 and 10 μM lactacystin, respectively) than wild-type cell lines in induced conditions as measured by the trypan blue assay (Fig. 3). Although wild-type cell lines showed increased toxicity in the presence of lactacystin, the amount of cell death was significantly less than in mutant cell lines. We also used Tet-Off inducible cells expressing truncated huntingtin with an expanded polyglutamine tract (N63-148Q) as an additional control expressing a toxic protein. N63-148Q alone caused increased cell toxicity in induced conditions compared with uninduced conditions. However, the cells expressing mutant huntingtin did not show increased toxicity in the presence of lactacystin (Fig. 3).

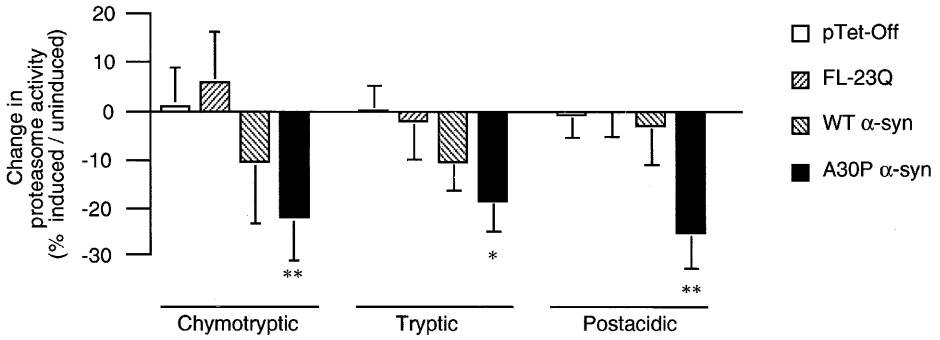

Figure 2. Decreased proteasome activity after induction of A30P mutant α-synuclein. Proteasome activities were measured 7 days after induction and differentiation, and also for uninduced conditions. The activities in induced conditions were normalized to those in uninduced conditions. Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$ when comparing mutant and pTet-Off cell lines. pTet-Off, PC12 cells containing only pTet-Off plasmid; WT α-syn, PC12 Tet-Off cell lines inducibly expressing wild-type α-synuclein (lines 20 and 26); A30P α-syn, PC12 Tet-Off cell lines inducibly expressing A30P mutant α-synuclein (lines 3 and 44); FL-23Q, PC12 Tet-Off cell line inducibly expressing full length normal polyglutamine (23Q) huntingtin.

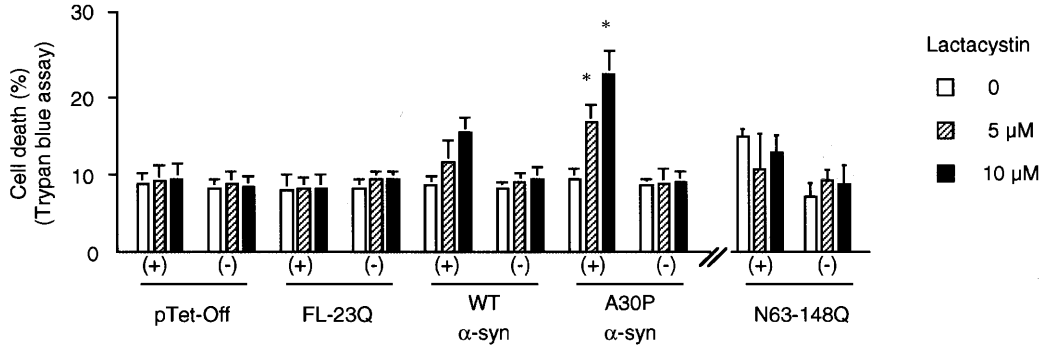

Figure 3. Cell toxicity after induction of A30P mutant α-synuclein. Cell viability measurements were based on the trypan blue exclusion assay. A30P mutant α-synuclein cell lines are more vulnerable to lactacystin-induced toxicity than pTet-Off, FL-23Q and wild-type α-synuclein cell lines in the induced conditions. The N63-148Q cell line does not show increased toxicity in the presence of lactacystin. N63-148Q, PC12 Tet-Off cell line inducibly expressing N-terminal truncated huntingtin with an expanded polyglutamine tract (148Q); +, induced condition; –, uninduced condition; open bars, without lactacystin; error bars represent SD. *, $P < 0.001$ when comparing mutant and wild-type cell lines.

Proteasome inhibition induces apoptotic cell death in cells expressing mutant α-synuclein

The manner in which cell death occurred was examined by nuclear morphology using 4′-6-diamidinophenylidole (DAPI) nuclear staining. We found that cell death was primarily apoptotic (Fig. 4A). Apoptotic cell death was substantially increased in induced mutant cell lines in a lactacystin concentration dependent manner (3.7- and 7.9-fold increases in the presence of 5 and 10 μM lactacystin, respectively) when compared with wild-type cell lines (Fig. 4B). Wild-type cell lines also showed an increase in the number of apoptotic cells in the presence of lactacystin, though the amount of apoptotic cells was significantly smaller than in mutant cell lines. As a further control for the effect of expression of a non-toxic protein, we examined a FL-23Q cell line. Toxicity in either induced or uninduced conditions were the same in pTet-Off and FL-23Q cell lines (Fig. 4B).

Caspase-3 and -9 are activated in cells expressing mutant α-synuclein in the presence of a protesome inhibitor

Apoptotic cell death is often associated with caspase-3 activation (32,33). After treatment with lactacystin, caspase-3 activity was much greater in induced mutant cell lines (2- and 2.4-fold increases in the presence of 5 and 10 μM lactacystin, respectively) compared with untreated cells (Fig. 5A). We also monitored caspases that are upstream of caspase-3. There were no significant differences between wild-type and mutant cell lines in caspase-8 activity (Fig. 5B). In contrast, caspase-9 was activated to a greater extent in mutant cell lines (2.6- and 3.2-fold increases in the presence of 5 and 10 μM lactacystin, respectively) compared with untreated cells (Fig. 5C).

Expression of mutant α-synuclein induces mitochondrial depolarization in the presence of a proteasome inhibitor and cyclosporin A (CsA) inhibits mitochondrial depolarization and apoptotic cell death

We studied mitochondrial depolarization using the fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 produces one of two emissions (red and green), depending on the mitochondrial membrane potential. The red and green signals represent polarized and depolarized mitochondria, respectively. We assessed the effect of lactacystin in pTet-Off, FL-23Q, wild-type and mutant α-synuclein cell lines. In the absence of lactacystin, there were no differences in mitochondrial membrane

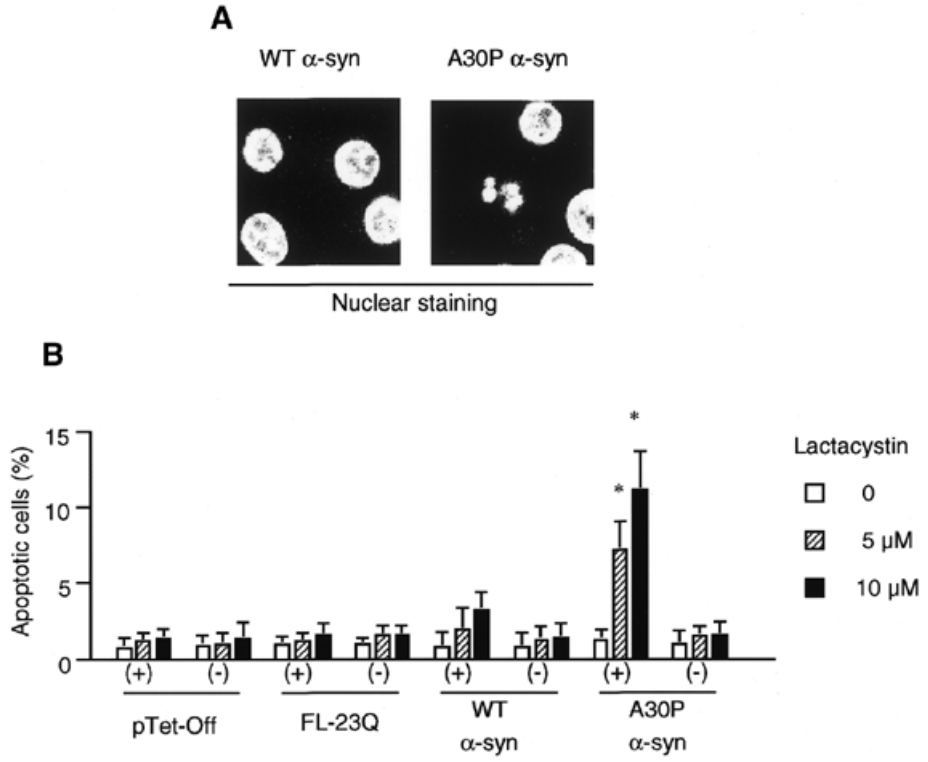

Figure 4. Apoptotic cell death after induction of A30P mutant α-synuclein. (**A**) Nuclear fragmentation characteristic of apoptosis. Nuclear morphology is shown by staining with DAPI in wild-type and mutant α-synuclein cell lines 7 days after induction and differentiation in the presence of 10 μM lactacystin. (**B**) Cells expressing mutant α-synuclein show increased apoptotic cell death 7 days after induction and differentiation in the presence of lactacystin. The difference between wild-type and mutant cells is statistically significant. Error bars represent SD.*, $P < 0.001$ when comparing mutant and wild-type cell lines.

potentials in all cells examined. In contrast, mutant cell lines showed significant increases in mitochondrial depolarization by 19.9 and 30.5% in the presence of 5 and 10 μM lactacystin, respectively (Fig. 6A and B). Wild-type cell lines showed an increase in mitochondrial depolarization in the presence of lactacystin, but the depolarization was significantly smaller than in mutant cell lines.

To assess the possibility of a causal role for mitochondrial dysfunction in cell death induced by mutant α-synuclein, we used CsA. CsA binds to a mitochondrial form of cyclophilin, and inhibits the mitochondrial permeability transition (MPT) (33,34). MPT induces mitochondrial depolarization, and is itself augmented by depolarization. MPT participates in the initiation of apoptosis (24,33,35,36). Treatment with CsA reduced mitochondrial depolarization (25.6 and 15.9% in the presence of 0.5 and 2.0 μM CsA, respectively) and apoptotic cells (9.3 and 6.0% in the presence of 0.5 and 2.0 μM CsA, respectively) in cells expressing mutant α-synuclein in the presence of lactacystin (Fig. 6C and D).

DISCUSSION

This report shows that mutant α-synuclein decreases proteasome activity, leading to increased sensitivity to mitochondria-dependent apoptosis. To study how mutant α-synuclein is involved in neuronal degeneration in PD, we have established inducible α-synuclein PC12 cell lines. Inducible cell lines are useful for cell toxicity models because expression of the toxic protein can be suppressed during clonal selection. After creating cell lines, expression of the transgene can be induced. Furthermore, cell toxicity in induced conditions can be compared with that in uninduced conditions. Here we compared proteasome activity and cell toxicity in induced conditions with those in uninduced conditions. After 7 days of induction and differentiation in the absence of a proteasome inhibitor, we found decreased proteasome activity in cells expressing mutant α-synuclein. Although the mechanism by which mutant α-synuclein decreases proteasome activity remains to be identified, it is possible that mutant α-synuclein directly affects the proteasome complex because it has been reported recently that α-synuclein interacts with a subunit of proteasome regulatory complexes (37).

Proteins encloded by other causal genes of familial PD, *parkin* and *UCH-L1*, are a ubiquitin-protein ligase and a deubiquitinating enzyme, respectively (16,19). In addition, proteasome subunits co-localize in Lewy bodies, and *gad* mice, which have an in-frame deletion of UCH-L1, show altered function of the ubiquitin system and neurodegeneration (15,38). Moreover, it has been reported recently that proteasomal function is impaired in the substantia nigra in sporadic PD, suggesting that dysregulation of the ubiquitin-proteasome pathway contributes to the pathogenesis of not only familial cases but also sporadic cases of PD (39). In the present study, our data suggest small but significant decreased proteasome activity as a result of mutant α-synuclein expression. In addition, we detected increased cell death in differentiated PC12 cells

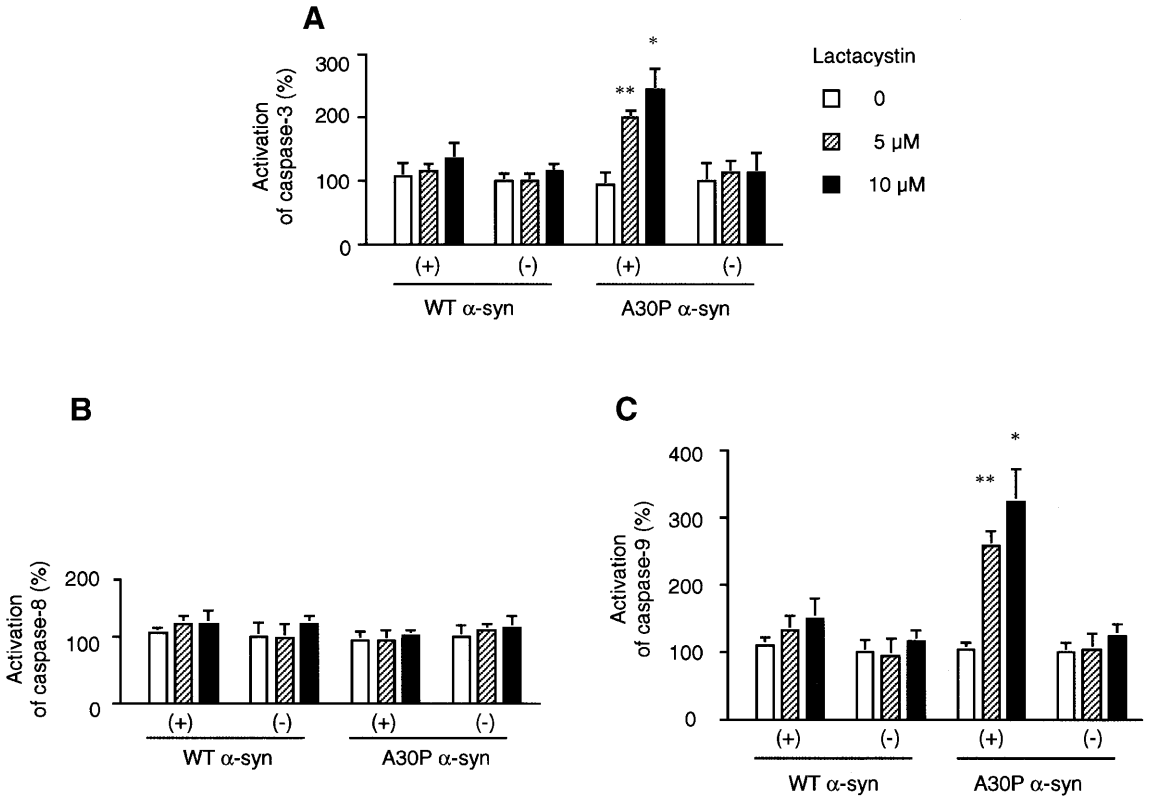

Figure 5. Selective activation of caspases after induction of A30P mutant α-synuclein. (**A**) Caspase-3 activation is significantly higher in mutant cell lines than in wild-type cell lines in induced conditions. (**B**) There is no significant difference in caspase-8 activation between wild-type and mutant cell lines. (**C**) Caspase-9 activation is significantly higher in mutant cell lines than in wild-type cell lines in induced conditions. Error bars represent SD. *, $P < 0.01$; **, $P < 0.001$ when comparing mutant and wild-type cell lines.

expressing mutant α-synuclein in the presence of a proteasome inhibitor. These data suggest that aberration in the proteolytic pathway plays a role in the pathogenesis of PD. In the absence of a proteasome inhibitor, we did not detect significantly increased cell death in cells expressing mutant α-synuclein. It has been reported that inducible expression of another type of mutant α-synuclein (A53T) did not increase cell toxicity unless cells were subjected to exogenous stress (40). To detect cell toxicity induced by mutant α-synuclein, it may be necessary to add an additional stress such as oxidative stress, administration of dopamine or proteasome inhibition.

Proteasome inhibition results in accumulation of molecules normally degraded by the ubiquitin-proteasome pathway such as p53, NFκB and Bax (41–43). These molecules appear to participate in apoptotic signaling. In cells expressing mutant α-synuclein, these or related molecules might tend to accumulate because of decreased proteasome activity compared with non-mutant cells, causing apoptotic cell death.

It has been reported that caspase-3 is a direct upstream activator of deoxyribonucleases responsible for DNA fragmentation during apoptosis (44,45). Caspase-3 activation is involved in both mitochondria-dependent and Fas-induced apoptosis. Caspase-9 is an upstream activator of caspase-3, and activation of caspase-9 is involved in mitochondrial-dependent apoptosis (46,47). Although caspase-8 is also an upstream activator of caspase-3, caspase-8 is involved in Fas-induced apoptosis (48–50). According to our data, caspase-9 was activated by expression of mutant α-synuclein, indicating mitochondria-dependent apoptosis induced by mutant α-synuclein.

It has been reported previously that mitochondrial dysfunction may have a role in the pathogenesis of neurodegenerative diseases such as PD (21–25), Alzheimer's disease (AD) (51–53) and Huntington's disease (HD) (33,54). Mutations in presenilin-1 increase the vulnerability of neural cells to mitochondrial toxins (52), and lymphoblasts from HD patients are abnormally susceptible to the induction of mitochondrial depolarization and apoptosis induced by apoptogenic stimuli (33). However, a relation between mutations of α-synuclein and mitochondrial dysfunction had not been shown. Here we report that cells expressing mutant α-synuclein showed a selective increase in mitochondrial depolarization and apoptotic cell death in the presence of a proteasome inhibitor. In addition, mitochondrial depolarization and apoptotic cell death were reduced by CsA, suggesting a causal role for mitochondrial dysfunction in cell death induced by mutant α-synuclein. Although treatment with CsA reduced the cell toxicity, the post-treatment values were not reduced to control levels. This finding suggests that other pathways beside CsA-sensitive apoptosis are involved in cell death induced by mutant α-synuclein. Wild-type cell lines showed decreased proteasome activity and cell viability, and increased mitochondrial dysfunction, consistent with previously published reports using transgenic mice, flies and stable cell lines (11,12,29). This result may be relevant to PD, since most patients with PD

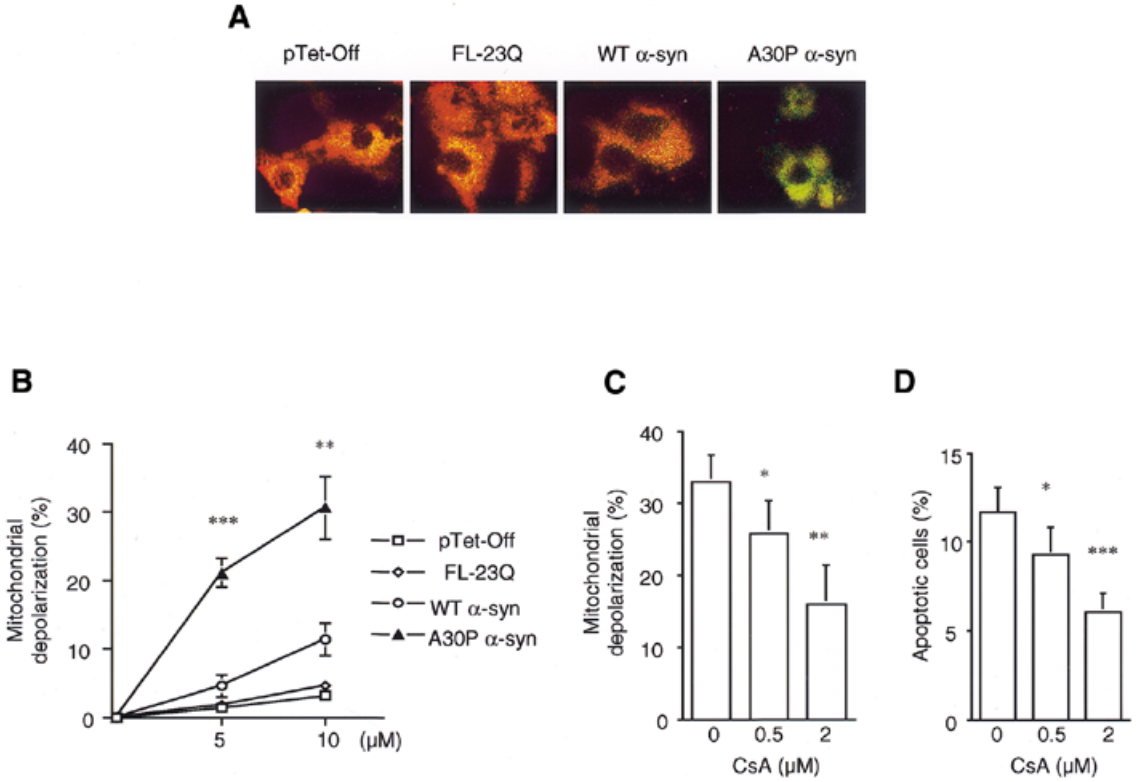

Figure 6. Mitochondria-dependent apoptosis. (**A**) Dual emission images for JC-1 fluorescence. Each panel shows typical examples of JC-1 images. Red, polarized mitochondria; green, depolarized mitochondria. (**B**) Cells expressing mutant α-synuclein show greater mitochondrial depolarization than pTet-Off, FL-23Q, wild-type cell lines. The percentage of mitochondrial depolarization was monitored by the green:red fluorescence ratio. Error bars represent SD. **, $P < 0.01$; ***, $P < 0.001$ when comparing mutant and wild-type cell lines. (**C**) CsA inhibits mitochondrial depolarization induced by 10 μM lactacystin in cells expressing mutant α-synuclein. Mitochondrial membrane potential was evaluated at 15 h after drug administration. (**D**) CsA inhibits apoptotic cell death induced by 10 μM lactacystin in cells expressing mutant α-synuclein. Cell viability was evaluated at 15 h after drug administration. Error bars represent SDs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with cultures treated with lactacystin alone.

do not have mutations in α-synuclein, and have wild-type α-synuclein incorporated into Lewy bodies. However, mutant α-synuclein was significantly more toxic in our experiments than wild-type.

Several lines of evidence suggest that apoptotic cell death contributes to the pathogenesis of PD (55–61). Expression of α-synuclein induces mitochondrial dysfunction (29). In addition, it has been reported that mutant forms of α-synuclein increase cell toxicity (62,63). Here we report that the mutant form of α-synuclein can decrease proteasome activity, leading to increased sensitivity to mitochondria-dependent apoptosis. These data suggest that dysregulation of the ubiquitin-proteasome pathway may be involved in cell death induced by mutant α-synuclein.

MATERIALS AND METHODS

Cell culture and inducible cell lines

Cells were grown in DMEM containing 10% FBS and 5% horse serum in a 5% CO_2 atmosphere. To create Tet-Off PC12 cell lines inducibly expressing either wild-type or A30P mutant α-synuclein, PC12 Tet-Off cells containing pTet-Off were purchased from Clontech. Tet-responsive α-synuclein expression constructs were made by cloning full-length cDNA of wild-type or A30P mutant α-synuclein into pTRE (Clontech). These α-synuclein constructs were cotransfected into PC12 Tet-Off cells with pTK-Hyg (Clontech) at a 10:1 molar ratio. Single colonies were obtained by limiting dilution with 100 μg/ml G418 and 200 μg/ml hygromycin B (Clontech) in the presence of 200 ng/ml Dox for 2–3 weeks. Clones were analyzed for the expression of α-synuclein by western blotting.

Immunocytochemistry and western blot

Tet-Off inducible cell lines were incubated with or without 200 ng/ml Dox for uninduced conditions or induced conditions, respectively. After 7 days of induction and differentiation, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 15 min at room temperature and processed as described (33). Cells were labelled with anti-α-synuclein antibody (1:100; Transduction Laboratories) and nuclei were stained with DAPI, and analyzed by confocal microscopy (Zeiss, LSM 410). To confirm the expression of α-synuclein by western blotting, an anti-α-synuclein antibody (1:1000; Transduction Laboratories) was used, and the blots were visualized by ECL western blotting detection reagent (Amersham Pharmacia Biotech).

Proteasome activity

Cells were grown in DMEM containing 1.0% FBS, 0.5% horse serum and 50 ng/ml nerve growth factor in a 5% CO_2 atmosphere. Proteasome activity was measured 7 days after induction and differentiation. The fluorogenic substrates, Suc-LLVY-AMC, Z-LLE-AMC and Boc-LRR-AMC, were used to measure the chymotryptic-like, tryptic-like and postacidic activities, respectively. The proteasome activities were monitored by the fluorescence activity (λ_{ex}: 380 nm; λ_{em}: 460 nm) (64).

Cell viability analysis

To assess cell viability, we used the trypan blue exclusion assay and DAPI nuclear staining. All cells with the exception of N63-148Q were incubated with or without 5 or 10 μM lactacystin for 15 h after 7 days of induction and differentiation. Because N63-148Q shows cell toxicity starting from 3 days after induction and differentiation, lactacystin was added to the cell line at 3 days post-induction/differentiation. We counted the percentage of blue-stained cells among total cells (2400–3600 cells in total) in the trypan blue exclusion assay. In addition, apoptotic nuclei were analyzed morphologically after staining with DAPI, and percentages of apoptotic cells were obtained among total cells (1200–1800 cells in total).

Caspase activity

Cells were incubated with or without 5 or 10 μM lactacystin for 15 h after 7 days of induction and differentiation. Caspase-3 activity was measured by a colorimetric kit (Clontech). The peptide substrate conjugated with *p*-nitroanilide was used. The extent of cleavage was monitored by the change in absorbance at 405 nm and normalized to total protein. Activities of caspase-8 and -9 were measured by similar methods using a colorimetric kit (Biovision).

Mitochondrial membrane potential

Cells were incubated with or without 5 or 10 μM lactacystin for 15 h after 7 days of induction and differentiation. In living cells, JC-1 (Molecular Probes) was used to monitor mitochondrial membrane potentials. Cells were incubated with JC-1 (10 μg/ml) for 10 min at 37°C according to the manufacturer's instructions. JC-1 exhibits membrane-potential-dependent accumulation in mitochondria and is indicated by a fluorescent emission shift from green to red. Mitochondrial depolarization is indicated by an increase in the green:red fluorescence intensity ratio. We monitored the green:red ratio using confocal microscopy and analyzed as described (33,65).

Statistics

Data were presented as mean ± NSD. For each treatment, three to six culture wells were used, and each experiment was repeated four to five times. Data were compared by analysis of variance.

ACKNOWLEDGEMENTS

We thank Paul McHugh and the Department of Psychiatry of Johns Hopkins University School of Medicine for support. This work was supported by USPHS NIH/NINDS grant NS38377. T.M.D. and V.L.D. are supported by the Edward O. and Mitchell Family Foundation. Y.T. was supported by the Welfide Medicinal Research Foundation (Japan).

REFERENCES

1. Forno, L.S. (1996) Neuropathology of Parkinson's disease. *J. Neuropathol. Exp. Neurol.*, **55**, 259–272.
2. Hardy, J. and Gwinn-Hardy, K. (1998) Genetic classification of primary neurodegenerative disease. *Science*, **282**, 1075–1079.
3. Gibb, W.R. and Lees, A.J. (1988) The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry*, **51**, 745–752.
4. Olanow, C.W. and Tatton, W.G. (1999) Etiology and pathogenesis of Parkinson's disease. *Annu. Rev. Neurosci.*, **22**, 123–144.
5. Dunnett, S.B. and Bjorklund, A. (1999) Prospects for new restorative and neuroprotective treatments in Parkinson's disease. *Nature*, **399**, A32–A39.
6. Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R. *et al.* (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*, **276**, 2045–2047.
7. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L. and Riess, O. (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nature Genet.*, **18**, 106–108.
8. Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R. and Goedert, M. (1997) Alpha-synuclein in Lewy bodies. *Nature*, **388**, 839–840.
9. Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M. and Goedert, M. (1998) Alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc. Natl Acad. Sci. USA*, **95**, 6469–6473.
10. Baba, M., Nakajo, S., Tu, P.H., Tomita, T., Nakaya, K., Lee, V.M., Trojanowski, J.Q. and Iwatsubo, T. (1998) Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am. J. Pathol.*, **152**, 879–884.
11. Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A. and Mucke, L. (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science*, **287**, 1265–1269.
12. Feany, M.B. and Bender, W.W. (2000) A *Drosophila* model of Parkinson's disease. *Nature*, **404**, 394–398.
13. van der Putten, H., Wiederhold, K.H., Probst, A., Barbieri, S., Mistl, C., Danner, S., Kauffmann, S., Hofele, K., Spooren, W.P., Ruegg, M.A. *et al.* (2000) Neuropathology in mice expressing human alpha-synuclein. *J. Neurosci.*, **20**, 6021–6029.
14. Kahle, P.J., Neumann, M., Ozmen, L., Muller, V., Jacobsen, H., Schindzielorz, A., Okochi, M., Leimer, U., van Der Putten, H., Probst, A. *et al.* (2000) Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. *J. Neurosci.*, **20**, 6365–6373.
15. Ii, K., Ito, H., Tanaka, K. and Hirano, A. (1997) Immunocytochemical co-localization of the proteasome in ubiquitinated structures in neurodegenerative diseases and the elderly. *J. Neuropathol. Exp. Neurol.*, **56**, 125–131.
16. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. *et al.* (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nature Genet.*, **25**, 302–305.
17. Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L. and Dawson, T.M. (2000) 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. USA*, **97**, 13354–13359.
18. Imai, Y., Soda, M. and Takahashi, R. (2000) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem.*, **275**, 35661–35664.
19. Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T. *et al.* (1998) The ubiquitin pathway in Parkinson's disease. *Nature*, **395**, 451–452.
20. Larsen, C.N., Krantz, B.A. and Wilkinson, K.D. (1998) Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. *Biochemistry*, **37**, 3358–3368.
21. Swerdlow, R.H., Parks, J.K., Davis, J.N., II, Cassarino, D.S., Trimmer, P.A., Currie, L.J., Dougherty, J., Bridges, W.S., Bennett, J.P.,Jr, Wooten,

G.F. *et al.* (1998) Matrilineal inheritance of complex I dysfunction in a multigenerational Parkinson's disease family. *Ann. Neurol.*, **44**, 873–881.
22. Mizuno, Y., Yoshino, H., Ikebe, S., Hattori, N., Kobayashi, T., Shimoda-Matsubayashi, S., Matsumine, H. and Kondo, T. (1998) Mitochondrial dysfunction in Parkinson's disease. *Ann. Neurol.*, **44**, S99–S109.
23. Schapira, A.H., Gu, M., Taanman, J.W., Tabrizi, S.J., Seaton, T., Cleeter, M. and Cooper, J.M. (1998) Mitochondria in the etiology and pathogenesis of Parkinson's disease. *Ann. Neurol.*, **44**, S89–S98.
24. Kroemer, G. and Reed, J.C. (2000) Mitochondrial control of cell death. *Nat. Med.*, **6**, 513–519.
25. Zhang, Y., Dawson, V.L. and Dawson, T.M. (2000) Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol. Dis.*, **7**, 240–250.
26. Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*, **219**, 979–980.
27. Nicklas, W.J., Vyas, I. and Heikkila, R.E. (1985) 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.
28. Tipton, K.F. and Singer, T.P. (1993) Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *J. Neurochem.*, **61**, 1191–1206.
29. Hsu, L.J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., Wong, J., Takenouchi, T., Hashimoto, M. and Masliah, E. (2000) Alpha-synuclein promotes mitochondrial deficit and oxidative stress. *Am. J. Pathol.*, **157**, 401–410.
30. Takashima, A. and Koike, T. (1985) Relationship between dopamine content and its secretion in PC12 cells as a function of cell growth. *Biochim. Biophys. Acta*, **847**, 101–107.
31. Fenteany, G. and Schreiber, S.L. (1998) Lactacystin, proteasome function, and cell fate. *J. Biol. Chem.*, **273**, 8545–8548.
32. Kroemer, G., Dallaporta, B. and Resche-Rigon, M. (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Annu. Rev. Physiol.*, **60**, 619–642.
33. Sawa, A., Wiegand, G.W., Cooper, J., Margolis, R.L., Sharp, A.H., Lawler, J.F.,Jr, Greenamyre, J.T., Snyder, S.H. and Ross, C.A. (1999) Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. *Nat. Med.*, **5**, 1194–1198.
34. Nicolli, A., Basso, E., Petronilli, V., Wenger, R.M. and Bernardi, P. (1996) Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel. *J. Biol. Chem.*, **271**, 2185–2192.
35. Marchetti, P., Castedo, M., Susin, S.A., Zamzami, N., Hirsch, T., Macho, A., Haeffner, A., Hirsch, F., Geuskens, M. and Kroemer, G. (1996) Mitochondrial permeability transition is a central coordinating event of apoptosis. *J. Exp. Med.*, **184**, 1155–1160.
36. Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309–1312.
37. Ghee, M., Fournier, A. and Mallet, J. (2000) Rat alpha-synuclein interacts with tat binding protein 1, a component of the 26S proteasomal complex. *J. Neurochem.*, **75**, 2221–2224.
38. Saigoh, K., Wang, Y.L., Suh, J.G., Yamanishi, T., Sakai, Y., Kiyosawa, H., Harada, T., Ichihara, N., Wakana, S., Kikuchi, T. *et al.* (1999) Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. *Nature Genet.*, **23**, 47–51.
39. McNaught, K.S. and Jenner, P. (2001) Proteasomal function is impaired in substantia nigra in Parkinson's disease. *Neurosci. Lett.*, **297**, 191–194.
40. Tabrizi, S.J., Orth, M., Wilkinson, J.M., Taanman, J.W., Warner, T.T., Cooper, J.M. and Schapira, A.H. (2000) Expression of mutant alpha-synuclein causes increased susceptibility to dopamine toxicity. *Hum. Mol. Genet.*, **9**, 2683–2689.
41. Scheffner, M., Huibregtse, J.M., Vierstra, R.D. and Howley, P.M. (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell*, **75**, 495–505.
42. Palombella, V.J., Rando, O.J., Goldberg, A.L. and Maniatis, T. (1994) The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell*, **78**, 773–785.
43. Chang, Y.C., Lee, Y.S., Tejima, T., Tanaka, K., Omura, S., Heintz, N.H., Mitsui, Y. and Magae, J. (1998) mdm2 and bax, downstream mediators of the p53 response, are degraded by the ubiquitin-proteasome pathway. *Cell Growth Differ.*, **9**, 79–84.
44. Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, **89**, 175–184.
45. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*, **391**, 43–50.
46. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479–489.
47. Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P. and Flavell, R.A. (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell*, **94**, 325–337.
48. Nagata, S. (1997) Apoptosis by death factor. *Cell*, **88**, 355–365.
49. Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S. and Dixit, V.M. (1998) An induced proximity model for caspase-8 activation. *J. Biol. Chem.*, **273**, 2926–2930.
50. Yang, X., Chang, H.Y. and Baltimore, D. (1998) Autoproteolytic activation of pro-caspases by oligomerization. *Mol. Cell.*, **1**, 319–325.
51. Cassarino, D.S., Swerdlow, R.H., Parks, J.K., Parker, W.D.,Jr and Bennett, J.P.,Jr. (1998) Cyclosporin A increases resting mitochondrial membrane potential in SY5Y cells and reverses the depressed mitochondrial membrane potential of Alzheimer's disease cybrids. *Biochem. Biophys. Res. Commun.*, **248**, 168–173.
52. Guo, Q., Fu, W., Holtsberg, F.W., Steiner, S.M. and Mattson, M.P. (1999) Superoxide mediates the cell-death-enhancing action of presenilin-1 mutations. *J. Neurosci. Res.*, **56**, 457–470.
53. Passer, B.J., Pellegrini, L., Vito, P., Ganjei, J.K. and D'Adamio, L. (1999) Interaction of Alzheimer's presenilin-1 and presenilin-2 with Bcl-X(L). A potential role in modulating the threshold of cell death. *J. Biol. Chem.*, **274**, 24007–24013.
54. Cooper, J.M. and Schapira, A.H. (1997) Mitochondrial dysfunction in neurodegeneration. *J. Bioenerg. Biomembr.*, **29**, 175–183.
55. Mochizuki, H., Goto, K., Mori, H. and Mizuno, Y. (1996) Histochemical detection of apoptosis in Parkinson's disease. *J. Neurol. Sci.,* **137**, 120–123.
56. Offen, D., Ziv, I., Sternin, H., Melamed, E. and Hochman, A. (1996) Prevention of dopamine-induced cell death by thiol antioxidants: possible implications for treatment of Parkinson's disease. *Exp. Neurol.*, **141**, 32–39.
57. Ziv, I., Barzilai, A., Offen, D., Nardi, N. and Melamed, E. (1997) Nigrostriatal neuronal death in Parkinson's disease—a passive or an active genetically-controlled process? *J. Neural Transm.*, **49** (suppl.), 69–76.
58. Tompkins, M.M., Basgall, E.J., Zamrini, E. and Hill, W.D. (1997) Apoptotic-like changes in Lewy-body-associated disorders and normal aging in substantia nigral neurons. *Am. J. Pathol.*, **150**, 119–131.
59. Tatton, N.A. and Kish, S.J. (1997) *In situ* detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience*, **77**, 1037–1048.
60. Ruberg, M., France-Lanord, V., Brugg, B., Lambeng, N., Michel, P.P., Anglade, P., Hunot, S., Damier, P., Faucheux, B., Hirsch, E. *et al.* (1997). *Rev. Neurol.*, **153**, 499–508.
61. Fall, C.P. and Bennett, J.P. (1999) Characterization and time course of MPP^+ -induced apoptosis in human SH-SY5Y neuroblastoma cells. *J. Neurosci. Res.*, **55**, 620–628.
62. Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Choi, P., Hardy, J. and Wolozin, B. (1999) α-Synuclein shares physical and functional homology with 14-3-3 proteins. *J. Neurosci.*, **19**, 5782–5791.
63. Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farer, M. and Wolozin, B. (2000) The A53T α-synuclein mutation increases iron-dependent aggregation and toxicity. *J. Neurosci.*, **20**, 6048–6054.
64. Keller, J.N., Huang, F.F., Dimayuga, E.R. and Maragos, W.F. (2000) Dopamine induces proteasome inhibition in neural PC12 cell line. *Free Radic. Biol. Med.*, **29**, 1037–1042.
65. Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A. and Nicotera, P. (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron*, **15**, 961–973.