Available online at www.sciencedirect.com
ScienceDirect
NeuroToxicology 27 (2006) 807–815
NeuroToxicology

Proteasome inhibitor MG-132 induces dopaminergic degeneration in cell culture and animal models

Faneng Sun, Vellareddy Anantharam, Danhui Zhang, Calivarathan Latchoumycandane, Arthi Kanthasamy, Anumantha G. Kanthasamy *

Parkinson's Disorder Research Laboratory, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, 2062 Veterinary Medicine Building, Ames, IA 50011-1250, USA

Received 4 January 2006; accepted 23 June 2006
Available online 29 June 2006

Abstract

Impairment in ubiquitin–proteasome system (UPS) has recently been implicated in Parkinson's disease, as demonstrated by reduced proteasomal activities, protein aggregation and mutation of several genes associated with UPS. However, experimental studies with proteasome inhibitors failed to yield consensus regarding the effect of proteasome inhibition on dopaminergic degeneration. In this study, we systematically examined the effect of the proteasome inhibitor MG-132 on dopaminergic degeneration in cell culture and animal models of Parkinson's disease. Exposure of immortalized dopaminergic neuronal cells (N27) to low doses of MG-132 (2–10 μM) resulted in dose- and time-dependent cytotoxicity. Further, exposure to MG-132 (5 μM) for 10 min led to dramatic reduction of proteasomal activity (>70%) accompanied by a rapid accumulation of ubiquitinated proteins in these cells. MG-132 treatment also induced increases in caspase-3 activity in a time-dependent manner, with significant activation occurring between 90 and 150 min. We also noted a 12-fold increase in DNA fragmentation in MG-132 treated N27 cells. Similarly, primary mesencephalic neurons exposed to 5 μM MG-132 also induced >60% loss of TH positive neurons but only a minimal loss of non-dopaminergic cells. Stereotaxic injection of MG-132 (0.4 μg in 4 μl) into the substantia nigra compacta (SNc) in C57 black mice resulted in significant depletion of ipisilateral striatal dopamine and DOPAC content as compared to the vehicle-injected contralateral control sides. Also, we observed a significant decrease in the number of TH positive neurons in the substantia nigra of MG-132-injected compared to the vehicle-injected sites. Collectively, these results demonstrate that the proteasomal inhibitor MG-132 induces dopamine depletion and nigral dopaminergic degeneration in both cell culture and animal models, and suggest that proteasomal dysfunction may promote nigral dopaminergic degeneration in Parkinson's disease.

Keywords: Proteasome activity; Ubiquitin; Nigral neurons; UPS dysfunction; Dopamine; Apoptosis; Parkinson's disease

1. Introduction

The ubiquitin–proteasome system (UPS) is the primary proteolytic complex responsible for the elimination of unwanted and misfolded intracellular proteins. The UPS is critical for various cellular functions including cell development, survival, apoptosis and intracellular signaling (Glickman and Ciechanover, 2002). The degradation of cellular proteins by UPS is tightly regulated by a system in which ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligase (E3) act sequentially to attach the polyubiquitin chain to the target proteins, and specify the degradation by 26S proteasome. Impairment in UPS function interferes with its proteolysis capacity, and leads to inadequate protein degradation.

Parkinson's disease (PD) is a major neurodegenerative disorder affecting over 1.5 million people in the US. The mechanisms underlying the selective and progressive loss of nigral dopaminergic neurons in PD are still unclear. Several studies have implicated UPS dysfunction in the pathogenesis of PD (Betarbet et al., 2005; Dawson and Dawson, 2003). Impairment of UPS is demonstrated by the decrease in proteasomal enzyme activities as well as decreased protein levels of the α-subunit of proteasome in the substantia nigra of

* Corresponding author. Tel.: +1 515 294 2516; fax: +1 515 294 2315.
E-mail address: akanthas@iastate.edu (A.G. Kanthasamy).

0161-813X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuro.2006.06.006

patients with idiopathic PD (Betarbet et al., 2005; McNaught and Jenner, 2001; McNaught and Olanow, 2003). Additionally, mutations in genes associated with protein processing and degradation, namely Parkin, α-synuclein and ubiquitin C-terminal hydrolase-L1 (UCH-L1), have been found in patients with familial PD (Dauer and Przedborski, 2003; Dawson and Dawson, 2003). In support of this view, dopamine (Keller et al., 2000), 6-OHDA (Elkon et al., 2004), and the mitochondria complex I inhibitors MPP^+ (Sawada et al., 2004) and rotenone (Hoglinger et al., 2003) have been shown to result in decreased proteasomal activity in various cell culture models including PC12 cells and primary mesencephalic cultures. Recently, we demonstrated that exposure to the environmental neurotoxin dieldrin inhibits proteasomal activity to induce α-synuclein aggregation and cell death in dopaminergic neuronal cells (Sun et al., 2005). However, some recent studies yielded inconsistent results regarding effects of proteasome inhibitors on dopaminergic degeneration. Systematic administration of the proteasomal inhibitor epoxomicin produces delayed and progressive neurological and neuropathological changes similar to those associated with PD in rodents (McNaught et al., 2004), whereas the proteasome inhibitors protected dopaminergic neurons in a rat 6-OHDA PD model (Inden et al., 2005). In this study, we examined the effect of the well known proteasome inhibitor MG-132 in dopaminergic neuronal cultures and animal models to determine the role of proteasomal inhibition in nigrostriatal dopaminergic degeneration.

2. Materials and methods

2.1. Cell cultures

The immortalized rat mesencephalic dopaminergic neuronal cells (N27) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C as previously described (Yang et al., 2004). We and others have extensively used N27 cells as a useful model to study the neurotoxic mechanisms pertaining to Parkinson's disease (Clarkson et al., 1999; Kaul et al., 2003, 2005a,b; Miranda et al., 2004; Peng et al., 2005).

2.2. Cytotoxicity assay with Sytox green

Assessment of cytotoxicity was conducted using Sytox green as described previously (Latchoumycandane et al., 2005). Membrane-impermeable DNA dye Sytox green can readily enter the cells with altered membrane permeability, resulting in increased fluorescence. The intensity of fluorescence is directly proportional to the amount of dead cells, and this method is more efficient and sensitive than other cytotoxic measurements (Kitazawa et al., 2004). Twenty-four hours after cells were grown in 24-well plates, cells were incubated with 1 μM Sytox simultaneously with 5.0 μM MG-132 or vehicle (0.1% DMSO) as a control. DNA bound Sytox green (Ex 485 nm and EM 538 nm) was detected using a fluorescence microplate reader (Molecular Devices Corporation, Gemini Plate Reader). Fluorescence intensity was monitored and normalized by the time-matched control to quantify cell death.

2.3. Proteasomal peptidase activity assay

Chymotrypsin-like proteasomal activity was assessed with the method described previously (Sun et al., 2005). After collection and lysis of cells with lysis buffer (10 mM HEPES, 5 mM $MgCl_2$, 10 mM KCl, 1% sucrose, 0.1% CHAPS) on ice for 20 min, 50 μl lysates were used for assay by incubation with 75 μM fluorogenic Suc-LLVY-AMC (Calbiochem, San Diego, CA) in the assay buffer (50 mM Tris–HCl, 20 mM KCl, 5 mM MgOAc and 10 mM DTT, pH 7.6) at 37 °C for 30 min. Fluorescence intensity of the enzymatically cleaved product was measured using a fluorescence plate reader (Gemini Plate Reader, Molecular Devices Corporation). Protein concentration was determined by the Bradford method. Enzymatic activity was normalized by protein concentration.

2.4. Caspase enzymatic activity assay

Assessment of caspase activation was conducted as described previously (Sun et al., 2005) using Ac-DEVD-AFC (Bachem Bioscience, King of Prussia, PA) as substrate for the enzymatic activity assay. The cleaved product by caspase-3 was measured (Ex 400 nm and Em 505 nm) using a fluorescence plate reader (Molecular Devices Corporation). Bradford protein assay was used for determination of protein concentration.

2.5. SDS-PAGE and Western blot

Western blot analysis was performed as described previously (Sun et al., 2005). Cells were collected and washed once with ice-cold PBS before lysis with buffer (protease inhibitors and 0.5% Triton-X 100 in PBS). The lysates were ultracentrifuged at 100,000 × g for 40 min. The resulting supernatants were collected for protein assay. Equal amounts of protein were resolved on 8% SDS-PAGE and transferred onto nitrocellulose membrane. A standard Western blot procedure was followed for immunoblot with polyclonal ubiquitin antibody (DAKO, Carpinteria CA, 1:500). An ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was used for detection of ubiquitinated proteins. The blot was reprobed with monoclonal β-actin antibody (Sigma Chemicals, St. Louis, MO, 1:5000) to confirm equal protein loading.

2.6. DNA fragmentation assay

Cell Death Detection ELISA Plus Assay Kit (Roche Molecular Biochemicals, Indianapolis, IN) was used for analysis of DNA fragmentation by quantification of histone-associated low molecular weight DNA in the cytoplasm of cells (Anantharam et al., 2002). Briefly, cell pellets were lysed with the lysis buffer provided in the assay kit. After being spun down at 200 × g, 20 μl of supernatant was incubated with the mixture

of HRP-conjugated antibody-recognizing histones and fragmented single- and double-stranded DNA. After unbound components were removed by washing, bound HRP-conjugates were assessed colorimetrically with ABTS as substrate using a spectrophotometer at 405 nm, and the optical density at 490 nm was used as reference. Protein concentration was determined by the Bradford protein assay.

2.7. Primary mesencephalic culture

Preparation of primary mesencephalic neuronal cultures was conducted as described previously (Yang et al., 2004). Ventral mesencephalon was first dissected out from 15- to 17-day-old mouse embryos, and cell dissociation was achieved by incubating dissected tissues in trypsin–EDTA (0.25%) for 20 min. The dissociated mesencephalic cells obtained from four litters were suspended in serum-free neurobasal medium supplemented with B-27, L-glutamine, penicillin, and streptomycin (Life Technologies) before cells were grown on poly-L-lysine coated coverslips in 24-well plates. The plating density was around 40,000 cells/ml of culture medium per well. Each well represents an experimental unit (n). Twenty-four hours after cells were in culture, 10 μM cytosine arabinoside was added to suppress glial cell proliferation. The cells were maintained in a humidified CO_2 incubator (5% CO_2, 37 °C) for approximately 6–7 days before treatments. Tyrosine hydroxylase (TH) immunostaining yields approximately 30–40 TH positive cells, accounting for less than 0.1% of the total population of cells in each culture well.

2.8. Immunocytochemistry

Immunostaining of the tyrosine hydroxylase (TH) marker of dopaminergic neurons was performed in primary mesencephalic neurons derived from C57 black mice. Briefly, after treatment, primary neurons grown on poly-L-lysine coated glass cover slips were double stained with TH antibody and Hoechst staining to determine the number of TH^+ and TH^- neurons and the experiments were blinded. Nuclei were counterstained with Hoechst 33342 at a final concentration of 10 μg/ml. Primary neurons were fixed with 4% paraformaldehyde, permeabilized, and non-specific sites were blocked with 5% normal goat serum containing 0.4% BSA and 0.2% Triton-X 100 in PBS for 20 min. Cells were then incubated with antibodies directed against TH (1:500 dilution) overnight at 4 °C followed by incubation with Cy3-conjugated (1:1000) secondary antibody for 1 h at RT. Then the cover slips containing cells were washed with PBS, mounted on a slide, viewed under a Nikon inverted fluorescence microscope (Model TE-2000U) and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

2.9. Stereotaxic injection of MG-132

C57 black mice were maintained in a temperature/humidity-controlled environment with free access to food and water. After mice were anesthetized, MG-132 (0.4 μg in 4 μl) and vehicle (1% DMSO in PBS) were stereotaxically injected into the substantia nigra at the target site (Bregma AP, −3.2 mm, ML, ±2.0 mm, DV, −4.7 mm) in the right and left sides, respectively (Hommel et al., 2003). Twelve days after injection, mice were either sacrificed for dissection of the striatum for dopamine and DOPAC measurement or perfused intracardiacally with 4% paraformaldehyde for immunohistological study.

2.10. Neurotransmitter analysis

Striata were dissected from mouse brain on an ice-cold glass platform, and the weight of each striatal tissue was measured. The samples were homogenized in buffer containing 0.2 M perchloric acid, 0.5 mg/ml Na_2EDTA and 1 μg/ml $Na_2S_2O_5$ and subjected to 13,200 × g centrifugation. The supernatant was analyzed for dopamine and DOPAC by HPLC–EC detection as described in our previous publication (Kitazawa et al., 2001). The HPLC system included a pressure module, a solvent delivery system (Rainin Instrument Co. Inc., Woburn, MA, USA), and an automatic AS-48 sampler (Bio-Rad Laboratories, Hercules, CA, USA) controlled by Rainin Dynamax HPLC method manager software (Version 1.4, Rainin Instrument Co. Inc.). A C-18 reversed-phase column (Rainin Instrument Co. Inc.) was used to separate neurotransmitters isocratically with the mobile phase (pH 3.1, 0.15 M monochloroacetic acid, 0.13 mM sodium octyl sulfonate, 0.67 mM disodium EDTA, 0.12 M sodium hydroxide, and 1.5% acetonitrile) at the flow rate of 1 ml/min. Measurement of the neurotransmitters was achieved with an electrochemical detection (EC) system consisting of an ESA coulochem model 5100A and a guard cell model 5020 (ESA Inc., Bedford, MA). Calibration of the HPLC–EC with DOPAC and dopamine was performed before each use. The sample injector was programmed to wash automatically after each injection with 50% acetonitrile in deionized water. The dopamine and DOPAC levels were normalized by the wet tissue weight, and normal levels of dopamine and DOPAC in control animals were approximately 15.0 and 5.0 ng/mg wet tissue, respectively.

2.11. Quantification of TH and non-TH cell count

We used Metamorph software (Universal Imaging, Version 5.0) for measurement of TH^+ neurons in primary cell culture and *in vivo* sections. The total number of TH^+ cells were counted in 5–7 cover slips obtained from two separate experiments for primary neurons. The total number of TH^+ cells averaged 35–40/coverslip in untreated controls. For *in vivo* sections, fixed brain tissues were cut into sections of 30 μm thickness using cryostat sectioning, and the free floating nigral sections were stained with tyrosine hydroxylase (Rabbit, 1:2000) and counterstained with nucleus dye Hoechst 33342. Quantification of TH positive neurons at nigral sections was performed with sections at the caudorostral level of the third cranial nerve as described previously (Kanthasamy et al., 1997). For measurement of TH and non-TH cell count, the images were first thresholded, and then neuronal count and volume were measured using the integrated morphometry

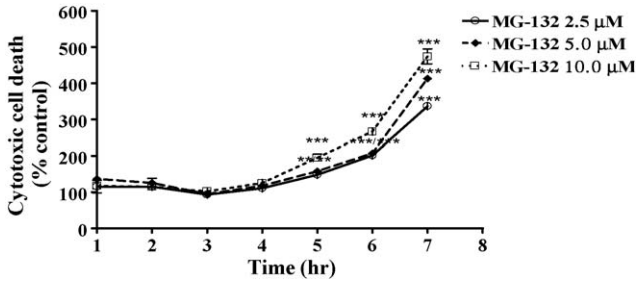

Fig. 1. Cytotoxicity of MG-132 in dopaminergic neuronal (N27) cells. N27 cells were treated with 5.0 μM MG-132. The neurotoxicity was assessed at various time points over a 7 h period using the Sytox green assay. Cell death was expressed as the percentage of the time-matched control groups. The results represent mean ± S.E.M. from six samples in each group ($^{**}p < 0.01$ and $^{***}p < 0.001$).

analysis (IMA) function in the Metamorph image analysis software (Molecular Devices, Downingtown, PA). The data were logged to an Excel spreadsheet with defined row and column positions and then analyzed. The data were exported to Graph Pad Prism 4.0 software and analyzed.

2.12. Data analysis

All data analysis was performed with Prism 4.0 software (GraphPad software, San Diego). One-way ANOVA was used for multiple comparisons. Single comparisons were made using the Student's *t*-test. A significant difference was accepted if $p < 0.05$.

3. Results

3.1. Exposure to proteasome inhibitor MG-132 induces cytotoxicity in dopaminergic neuronal cells

Immortalized mesencephalic neuronal cells (N27 cells) were exposed to MG-132 (2.5–10.0 μM) for 7 h and cell viability was monitored by staining with Sytox green dye, a membrane-impermeable DNA binding fluorescent dye which preferentially stains dead cells. Exposure to MG-132 induced a dose- and time-dependent increase in cytotoxic cell death (Fig. 1). Significant cell death was observed starting at 5 h and progressively increased up to 7 h.

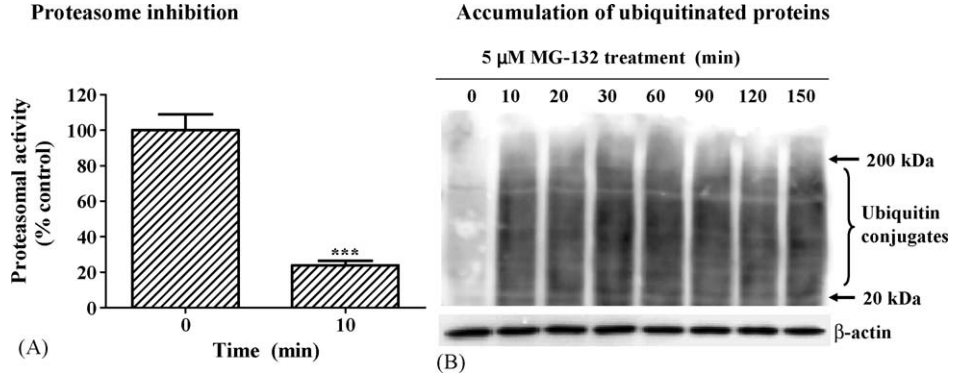

Fig. 2. MG-132 causes rapid proteasome inhibition and accumulation of ubiquitinated proteins: (A) proteasome inhibition. Proteasomal activity was measured 10 min after N27 cells were exposed to 5.0 μM MG-132 using the fluorogenic substrate Suc-LLVY-AMC. Enzymatic activity was normalized by protein concentration and expressed as the percentage of vehicle-treated cells. The data represent mean ± S.E.M. from six samples in each group ($^{***}p < 0.001$, Student's *t*-test) and (B) accumulation of ubiquitinated proteins. N27 cells were exposed to 5.0 μM MG-132 for various durations ranging from 10 to 150 min as indicated. Cytosolic fractions were prepared as described in Section 2, resolved on 8% SDS-PAGE and blotted with ubiquitin antibody. Membranes were also reprobed with β-actin antibody to ensure equal protein loading.

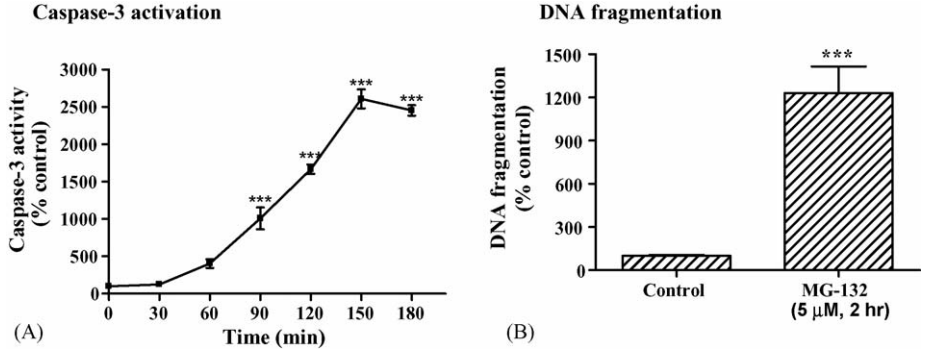

Fig. 3. Proteasome inhibition by MG-132 induces apoptosis in dopaminergic neuronal cells: (A) caspase-3 activity. Cells were treated with 5.0 μM MG-132 and then caspase-3 activity was measured with the fluorogenic substrate Ac-DEVD-AFC. The results represent mean ± S.E.M. from eight samples. Statistical significance between the control group and each treatment group was determined by ANOVA followed by Dunnett's post-test ($^{***}p < 0.001$) and (B) DNA fragmentation. DNA fragmentation was assayed using the ELISA assay in N27 cells treated with 5.0 μM MG-132 for 120 min. Data were expressed as the percentage of the control group. Values represent mean ± S.E.M. from eight individual samples ($^{***}p < 0.001$).

3.2. Inhibition of proteasomal activity and accumulation of ubiquitin-conjugated proteins in MG-132 treated cells

Since MG-132 is a proteasomal inhibitor, we examined the inhibitory effect of MG-132 on proteasomal activity in dopaminergic neuronal cells. We assayed the enzymatic activity of the 20S/26S proteasome using the specific fluorogenic substrate Suc-LLVY-AMC in N27 cells. Fig. 2A shows a rapid decrease in the proteasomal activity within 10 min of 5 μM MG-132 exposure. Less than 25% proteasomal activity remained after the 10 min exposure ($p < 0.001$), indicating that MG-132-induced proteasomal inhibition precedes cell death. Following inhibition of proteasomal enzymatic activity, the levels of ubiquitinated proteins increase in

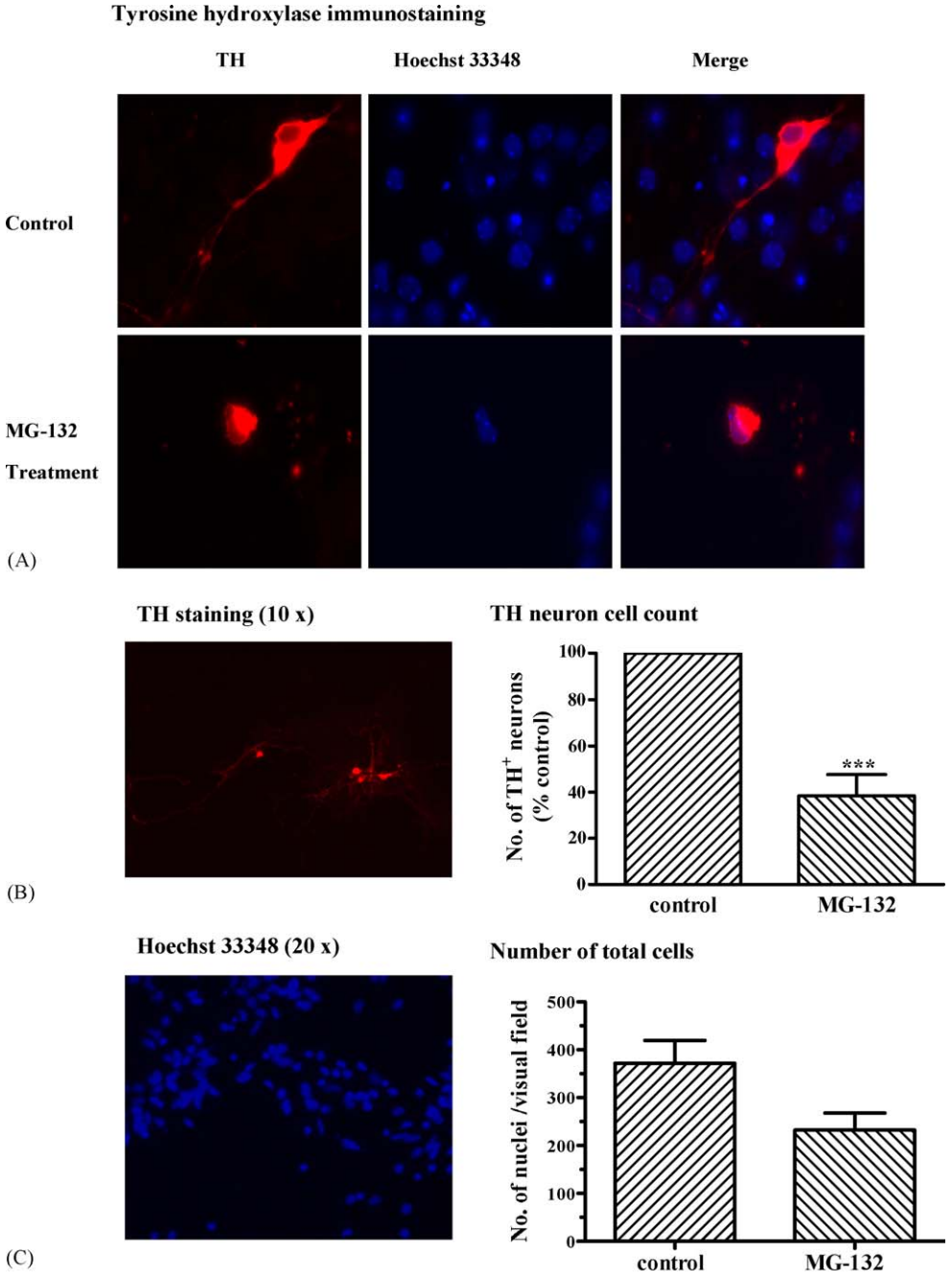

Fig. 4. MG-132 induced morphological changes and dopaminergic neuronal loss in primary mesencephalic culture: (A) immunostaining for tyrosine hydroxylase (TH). After 6–7 h in culture, the primary mesencephalic culture was treated with 5.0 μM MG-132 for up to 5 h. Immunocytochemistry was performed using mouse monoclonal TH primary antibody and Cy3-conjugated secondary antibody; (B) quantification of TH positive neurons (approximately 35–40 TH neurons observed per each well in control group). TH positive neurons present in control and MG-132 treated samples were visualized with Cy3 under 10× objective and quantified using Metamorph image analysis software. Data were expressed as the percentage of the control group. Values represent mean ± S.E.M. from 5 to 7 individual litter brains ($^{***}p < 0.001$); (C) quantification of mesencephalic culture. Nuclei in the mesencephalic culture were stained with Hoechst 33342, and the nuclei present in 12 randomly selected visual fields were quantified under 20× objective ($p < 0.05$).

the cytosol due to the reduced clearance of proteins by the UPS (Rideout and Stefanis, 2002) and therefore, we determined levels of high molecular weight poly-ubiquitinated proteins (200 and 20 kDa) following MG-132 treatment. As shown in Fig. 2B, MG-132 treatment resulted in rapid accumulation of poly-ubiquitinated proteins as determined by Western blot. Densitometric analysis of the level of ubiquitinated proteins (20–200 kDa) revealed the accumulation of the poly-ubiquitinated proteins within the first 10 min of MG-132 treatment and continued to increase over time. However, the percent increase was not statistically different from the 10 min time point. β-Actin was used as the internal control for equal protein loading (Fig. 2B).

3.3. MG-132 treatment induces caspase-3 activation and apoptotic cell death

To determine whether caspase mediated apoptotic cell death plays any role in MG-132-induced dopaminergic cell death, we measured caspase-3 enzyme activity and DNA fragmentation in MG-132-treated N27 cells. As shown in Fig. 3A, exposure to 5 μM MG-132 resulted in a time-dependent increase in caspase-3 enzyme activity, with significant activation occurring between 90 and 180 min (10- to 25-fold, $p < 0.001$). Exposure to 5 μM MG-132 for 2 h also resulted in a 12-fold increase in DNA fragmentation as measured by an ELISA-sandwich assay (Fig. 3B). These results clearly demonstrate that treatment

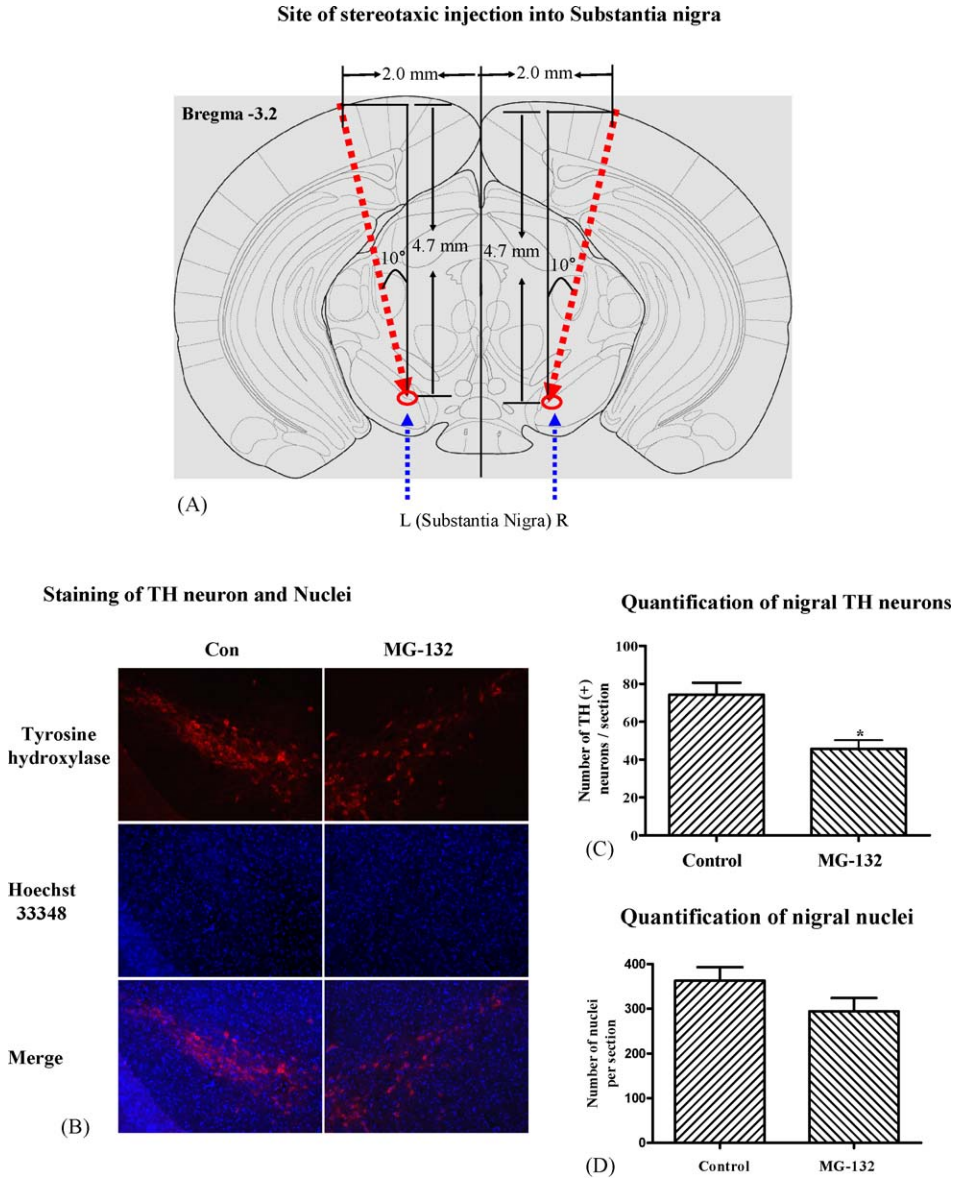

Fig. 5. Intranigral stereotaxic injection of MG-132 causes dopaminergic neuronal loss in a mouse model: (A) schematic diagram depicting the stereotaxic microinjection site into the left (L) and right (R) substantia nigra (Bregma AP, −3.2 mm, ML, ± 2.0 mm, DV, −4.7 mm). Red lines represent injection routes, red arrows indicate the needle distance, and the blue arrow indicates the site of injection (substantia nigra). Immunohistochemical analysis of nigral sections. MG-132 (0.4 μg in 4 μl) and vehicle were injected stereotaxically into mouse substantia nigra at right and left sides, respectively. Twelve days following the injection, brains were fixed and processed for TH immunohistochemical analysis as described in Section 2; (B) visualization of nigral TH neurons and nuclei under 10× objective; (C) quantification of TH neurons in nigral sections, $^{*}p < 0.05$, $n = 5$; (D) quantification of nuclei present in the nigral regions ($n = 4$).

with the proteasomal inhibitor MG-132 dramatically activates apoptotic cell death in dopaminergic neuronal cells.

3.4. MG-132 induces TH-positive neuronal loss in primary mesencephalic cultures

Next we extended our neurotoxic studies with N27 dopaminergic clonal cells to mouse primary neuronal cultures. We determined the effect of MG-132 on the survival of TH neurons in primary nigral dopaminergic neuronal cultures. Primary mesencephalic dopaminergic neuronal culture cells were exposed to 5 μM MG-132. After a 5 h exposure, primary neurons were fixed and stained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons. MG-132 treatment profoundly altered the morphology of dopaminergic neurons. As shown in Fig. 4A, cell bodies of TH-positive neurons shrunk following MG-132 exposure, indicating ongoing degeneration. Also, a significant loss of dopaminergic neurons was observed following MG-132 treatment in primary cultures. Quantitative analysis revealed about 60% loss of TH positive cell count in MG-132-treated cells as compared to untreated primary neurons (Fig. 4B). However, quantification of the total population of cells present in the mesencephalic culture showed only 37% reduction, which is less profound than the loss of TH neurons in the culture (Fig. 4C). These observations suggest that proteasomal inhibition can induce neurotoxic insult to dopaminergic neurons in primary mesencephalic cultures.

3.5. Stereotaxic injection of MG-132 causes striatal dopamine depletion and promotes dopaminergic neuronal loss in mouse substantia nigra

Finally, we examined whether inhibition of proteasomal function in the nigra promotes dopaminergic neuronal degeneration in animal models. Vehicle and MG-132 (0.4 μg) were stereotaxically injected into the left and right mouse substantia nigra as depicted in Fig. 5A, and after 12 days the brains were dissected from the animals and mid-brain sections were immunostained for TH. As depicted in Fig. 5B, a marked decrease was observed in the number of TH^+ neurons in the MG-132-injected sides of the substantia nigra as compared to vehicle-injected control sides. Quantitative analysis of TH positive neurons indicated that microinjection of MG-132 to substantia nigra led to significant reduction in the number of nigral TH neurons (Fig. 5C, $p < 0.05$), whereas the reduction in the number of Hoechst stained nuclei present in the same nigral regions was not statistically significant (Fig. 5D). Further, consistent with the nigral dopaminergic degeneration, HPLC analysis of striatal tissue revealed a significant depletion in striatal dopamine (Fig. 6A) and its metabolites DOPAC (Fig. 6B) in the MG-132 injected ipisilateral side as compared to the vehicle-injected contralateral side.

4. Discussion

Our studies in cell culture models demonstrate that the proteasome inhibitor MG-132 impairs ubiquitin–proteasome function (UPS) in dopaminergic neuronal cells and promotes degeneration of dopaminergic neurons in mouse mesencephalic primary culture. The time course study revealed that MG-132 induced inhibition of proteasomal activity and accumulation of ubiquitin conjugates before cell death, suggesting that the impairment in ubiquitin–proteasome-mediated protein degradation possibly triggers the neurotoxic response in dopaminergic neuronal cells. Activation of caspase-3 and DNA fragmentation during MG-132 treatment indicate that proteasomal dysfunction triggers the apoptotic cell death cascade. Our results from microinjection of MG-132 show a significant depletion of dopamine and DOPAC with concurrent loss of nigral dopaminergic neurons, and suggest that inhibition of nigral proteasome function can induce nigral dopaminergic degeneration similar to that in Parkinson's disease.

Several lines of recently generated evidence suggest that dysfunction of UPS is one of the causal factors of PD. Studies with postmortem brain samples revealed reduced proteasomal activities (McNaught et al., 2003) and selective loss of α-subunits of proteasome in the substantia nigra of PD patients (McNaught et al., 2002a,b). Mutation of some genes involved in the UPS degradation pathway, including parkin, Uch-L1, and α-synuclein, has been found in familial PD (McNaught and Olanow, 2003; McNaught et al., 2003; Moore et al., 2005). A pathogenic role of a dysfunctional ubiquitin–proteasome system in PD is supported by the inhibition of proteasomal

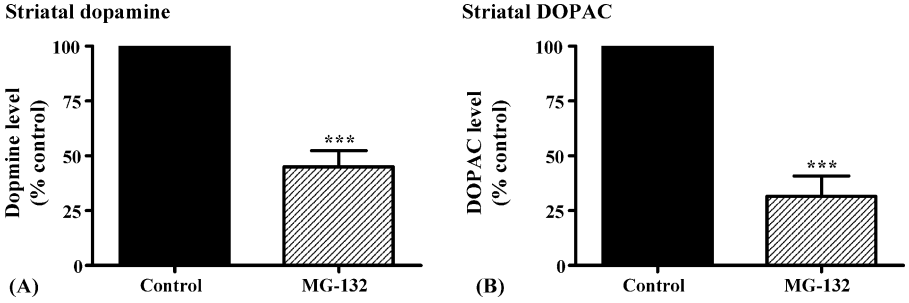

Fig. 6. Striatal dopamine DOPAC depletion following microinjection of MG-132 to substantia nigra. Mouse striatums were isolated from intranigral vehicle-injected or MG-132 injected sides and then analysis of (A) dopamine and (B) DOPAC were performed using HPLC. Dopamine and DOPAC levels were approximately 15 and 5.0 ng/mg weight nigral tissue, respectively. The data were expressed as the percentage of the vehicle control group. Data represent mean ± S.E.M., $n = 6$, $^{***}p < 0.001$.

activity in cell culture models of PD by dopamine (Keller et al., 2000), 6-OHDA (Elkon et al., 2004), MPP^+ (Sawada et al., 2004) and rotenone (Hoglinger et al., 2003), and wild-type and mutant human α-synucleins (Betarbet et al., 2005; Dawson and Dawson, 2003). Also, we recently showed that α-synuclein overexpression decreases proteasomal activity and sensitizes dopaminergic N27 neuronal cells to environmental neurotoxin-induced apoptotic cell death (Sun et al., 2005).

The relationship between UPS dysfunction and apoptotic cell death in dopaminergic neurons has not been clearly studied in detail. In the present study, we show that MG-132 inhibits proteasomal activity within 10 min, resulting in the accumulation of intracellular ubiquitinated proteins. This rapid inhibition of UPS triggers a dramatic activation of the key effector proapoptotic protease caspase-3 and DNA fragmentation. In a recent study, we observed about three-fold activation of caspase-3 and DNA fragmentation after 24 h of treatment with 300 μM MPP^+, a Parkinsonian toxin, in N27 cells (Kaul et al., 2003, 2005a). In comparison, in the present study, we observed an approximate 25-fold increase in caspase-3 activity and a 12-fold increase in DNA fragmentation following 2 h of 5 μM MG-132 treatment in N27 cells, suggesting that dopaminergic neuronal cells appear to be sensitive to proteasome inhibition. Also, the magnitude of MG-132-induced TH positive neuronal loss in a primary mesencephalic culture within 5 h is comparable to the neuronal loss observed following 10 μM MPP^+ treatment for 24 h, further supporting the increased vulnerability of nigral dopaminergic neurons to UPS dysfunction. It is still a controversy as to whether dopaminergic neurons are more susceptible to UPS dysfunction. Findings of the mutation of Parkin, UCH-L1 in familial PD, protein aggregation and accumulation of neurotoxic PaeR as Parkin substrate in dopamine neurons (Yang et al., 2003), as well as the increased sensitivity of dopaminergic neurons to oxidative stress upon proteasome inhibition (Mytilineou et al., 2004), suggests particular vulnerability of dopamine neurons to proteasome inhibition. The cellular mechanisms underlying the exacerbated toxicity from proteasomal inhibition in dopaminergic neurons are not currently known. A recent study demonstrated that mesencephalic dopaminergic neurons are particularly susceptible to proteasome inhibition-induced apoptosis due to failure to upregulate the expression of chaperone proteins HSP70 in response to proteolytic stress (Rideout and Stefanis, 2002). The HSP70 upregulation failure might underlie the susceptibility of dopamine neurons to proteasome inhibition, which has also been observed by others (McNaught et al., 2002a,b; Petrucelli et al., 2002). It is also possible that a number of other signaling proteins may play a role in the proteasome inhibitor induced cell death because the levels of proapoptotic and anti-apoptotic proteins are tightly regulated by UPS (Dawson and Dawson, 2003; Hattori and Mizuno, 2004; Ross and Pickart, 2004; Layfield et al., 2005).

Further examination of the effect of MG-132 on nigral dopaminergic degeneration *in vivo* indicated that stereotaxic injection of MG-132 into substantia nigra led to significant depletion of ipisilateral striatal dopamine and its metabolite DOPAC level, which is accompanied by profound loss of dopamine neurons at MG-132 injected substantia nigra regions (Fig. 5B, panel a). Alternatively, the quantitative analysis of the nuclei present in the nigral sections indicated that the percentage loss of nuclei (Fig. 5B and D) was less profound compared to that in TH neurons (Fig. 5B and C) indicating enhanced vulnerability of dopaminergic neurons to proteasomal dysfunction. Our data clearly shows that proteasomal inhibition by single injection of the proteasome inhibitor MG-132 can cause dopaminergic neuronal death in substantia nigra. Recently, McNaught et al. (2004) demonstrated that repeated systemic administration of the naturally occurring proteasome inhibitor epoxomicin and a synthetic proteasome inhibitor known as PSI in rats can replicate several features of PD including delayed motor deficits, a progressive nigrastriatal degeneration and protein aggregation (McNaught et al., 2004). This will be a highly useful animal model to study the pathogenic mechanisms of PD; however, the model is yet to be easily replicated in other laboratories. There are some inconsistencies observed between *in vitro* and animal studies with regard to the neurotoxic effect of proteasome inhibitors. A recent study showed that injection of proteasome inhibitors protected dopamine neurons from the neurotoxic effect of 6-OHDA in a rat model (Inden et al., 2005), while treatment with proteasome inhibitors in PC12 cells potentiated 6-OHDA toxicity (Elkon et al., 2004). In the present study, the *in vitro* results obtained in N27 cells and animal studies consistently showed that proteasome inhibition can promote dopaminergic degeneration.

In conclusion, our results demonstrate that proteasomal inhibition by MG-132 induces neurotoxicity in nigral dopaminergic neurons both in cell culture and animal models and that proteasome inhibition in dopaminergic neuronal cells activates the apoptotic cascade to induce cell death. Also, our results suggest that proteasomal dysfunction may play a key role in the dopaminergic degenerative processes associated with Parkinson's disease.

Acknowledgment

This work was supported in part by NIH grants NS45133 and ES10586. W. Eugene and Linda Lloyd Endowed Professorship to AGK is also acknowledged.

References

Anantharam V, Kitazawa M, Wagner J, Kaul S, Kanthasamy AG. Caspase-3-dependent proteolytic cleavage of protein kinase C delta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. J Neurosci 2002;22:1738–51.

Betarbet R, Sherer TB, Greenamyre JT. Ubiquitin–proteasome system and Parkinson's diseases. Exp Neurol 2005;191(Suppl. 1):S17–27.

Clarkson ED, Edwards-Prasad J, Freed CR, Prasad KN. Immortalized dopamine neurons: a model to study neurotoxicity and neuroprotection. Proc Soc Exp Biol Med 1999;222:157–63.

Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron 2003;39:889–909.

Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003;302:819–22.

Elkon H, Melamed E, Offen D. Oxidative stress, induced by 6-hydroxydopamine, reduces proteasome activities in PC12 cells: implications for the pathogenesis of Parkinson's disease. J Mol Neurosci 2004;24:387–400.

Glickman MH, Ciechanover A. The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002;82:373–428.

Hattori N, Mizuno Y. Pathogenetic mechanisms of parkin in Parkinson's disease. Lancet 2004;364:722–4.

Hoglinger GU, Carrard G, Michel PP, Medja F, Lombes A, Ruberg M, et al. Dysfunction of mitochondrial complex I and the proteasome: interactions between two biochemical deficits in a cellular model of Parkinson's disease. J Neurochem 2003;86:1297–307.

Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ. Local gene knockdown in the brain using viral-mediated RNA interference. Nat Med 2003;9:1539–44.

Inden M, Kitamura Y, Kondo J, Hayashi K, Yanagida T, Takata K, et al. Serofendic acid prevents 6-hydroxydopamine-induced nigral neurodegeneration and drug-induced rotational asymmetry in hemi-parkinsonian rats. J Neurochem 2005;95:950–61.

Kanthasamy AG, Kanthasamy A, Matsumoto RR, Vu TQ, Truong DD. Neuroprotective effects of the strychnine-insensitive glycine site NMDA antagonist (*R*)-HA-966 in an experimental model of Parkinson's disease. Brain Res 1997;759:1–8.

Kaul S, Kanthasamy A, Kitazawa M, Anantharam V, Kanthasamy AG. Caspase-3 dependent proteolytic activation of protein kinase C delta mediates and regulates 1-methyl-4-phenylpyridinium (MPP^+)-induced apoptotic cell death in dopaminergic cells: relevance to oxidative stress in dopaminergic degeneration. Eur J Neurosci 2003;18:1387–401.

Kaul S, Anantharam V, Kanthasamy A, Kanthasamy AG. Wild-type alpha-synuclein interacts with pro-apoptotic proteins PKC delta and BAD to protect dopaminergic neuronal cells against MPP^+-induced apoptotic cell death. Brain Res Mol Brain Res 2005a;139:137–52.

Kaul S, Anantharam V, Yang Y, Choi CJ, Kanthasamy A, Kanthasamy AG. Tyrosine phosphorylation regulates the proteolytic activation of protein kinase C delta in dopaminergic neuronal cells. J Biol Chem 2005b;280:28721–30.

Keller JN, Huang FF, Dimayuga ER, Maragos WF. Dopamine induces proteasome inhibition in neural PC12 cell line. Free Rad Biol Med 2000;29:1037–42.

Kitazawa M, Anantharam V, Kanthasamy AG. Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. Free Rad Biol Med 2001;31:1473–85.

Kitazawa M, Anantharam V, Kanthasamy A, Kanthasamy AG. Dieldrin promotes proteolytic cleavage of poly(ADP-ribose) polymerase and apoptosis in dopaminergic cells: protective effect of mitochondrial anti-apoptotic protein Bcl-2. Neurotoxicology 2004;25:589–98.

Latchoumycandane C, Anantharam V, Kitazawa M, Yang Y, Kanthasamy A, Kanthasamy AG. Protein kinase C delta is a key downstream mediator of manganese-induced apoptosis in dopaminergic neuronal cells. J Pharm Exp Ther 2005;313:46–55.

Layfield R, Lowe J, Bedford L. The ubiquitin–proteasome system and neurodegenerative disorders. Essays Biochem 2005;41:157–71.

McNaught KS, Jenner P. Proteasomal function is impaired in substantia nigra in Parkinson's disease. Neurosci Lett 2001;297:191–4.

McNaught KS, Olanow CW. Proteolytic stress: a unifying concept for the etiopathogenesis of Parkinson's disease. Ann Neurol 2003;53(Suppl. 3):S73–84 discussion S84-6.

McNaught KS, Bjorklund LM, Belizaire R, Isacson O, Jenner P, Olanow CW. Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. Neuroreport 2002a;13:1437–41.

McNaught KS, Mytilineou C, Jnobaptiste R, Yabut J, Shashidharan P, Jennert P, et al. Impairment of the ubiquitin–proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. J Neurochem 2002b;81:301–6.

McNaught KS, Belizaire R, Isacson O, Jenner P, Olanow CW. Altered proteasomal function in sporadic Parkinson's disease. Exp Neurol 2003;179:38–46.

McNaught KS, Perl DP, Brownell AL, Olanow CW. Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. Ann Neurol 2004;56:149–62.

Miranda M, Sorkina T, Grammatopoulos TN, Zawada WM, Sorkin A. Multiple molecular determinants in the carboxyl terminus regulate dopamine transporter export from endoplasmic reticulum. J Biol Chem 2004;279:30760–70.

Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of Parkinson's disease. Ann Rev Neurosci 2005;28:57–87.

Mytilineou C, McNaught KS, Shashidharan P, Yabut J, Baptiste RJ, Parnandi A, et al. Inhibition of proteasome activity sensitizes dopamine neurons to protein alterations and oxidative stress. J Neural Transm 2004;111:1237–51.

Peng J, Stevenson FF, Doctrow SR, Andersen JK. Superoxide dismutase/catalase mimetics are neuroprotective against selective paraquat-mediated dopaminergic neuron death in the substantial nigra: implications for Parkinson disease. J Biol Chem 2005;280:29194–8.

Petrucelli L, O'Farrell C, Lockhart PJ, Baptista M, Kehoe K, Vink L, et al. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. Neuron 2002;36:1007–19.

Rideout HJ, Stefanis L. Proteasomal inhibition-induced inclusion formation and death in cortical neurons require transcription and ubiquitination. Mol Cell Neurosci 2002;21:223–38.

Ross CA, Pickart CM. The ubiquitin–proteasome pathway in Parkinson's disease and other neurodegenerative diseases. Trends Cell Biol 2004;14:703–11.

Sawada H, Kohno R, Kihara T, Izumi Y, Sakka N, Ibi M, et al. Proteasome mediates dopaminergic neuronal degeneration, and its inhibition causes alpha-synuclein inclusions. J Biol Chem 2004;279:10710–9.

Sun F, Anantharam V, Latchoumycandane C, Kanthasamy A, Kanthasamy AG. Dieldrin induces ubiquitin–proteasome dysfunction in alpha-synuclein overexpressing dopaminergic neuronal cells and enhances susceptibility to apoptotic cell death. J Pharm Exp Ther 2005;315:69–79.

Yang Y, Nishimura I, Imai Y, Takahashi R, Lu B. Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in *Drosophila*. Neuron 2003;27:911–24.

Yang Y, Kaul S, Zhang D, Anantharam V, Kanthasamy AG. Suppression of caspase-3-dependent proteolytic activation of protein kinase C delta by small interfering RNA prevents MPP^+-induced dopaminergic degeneration. Mol Cell Neurosci 2004;25:406–21.