How does conserved dopamine neurotrophic factor protect against and rescue neurodegeneration of PC12 cells?

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Graphical Abstract

Abstract

Conserved dopamine neurotrophic factor protects and rescues dopaminergic neurodegeneration induced by 6-hydroxydopamine in vivo, but its potential value in treating Parkinson’s disease remains controversial. Here, we used the proteasome inhibitors lactacystin and MG132 to induce neurodegeneration of PC12 cells. Afterwards, conserved dopamine neurotrophic factor was administrated as a therapeutic factor, both pretreatment and posttreatment. Our results showed that (1) conserved dopamine neurotrophic factor enhanced lactacystin/MG132-induced cell viability and morphology, and attenuated alpha-synuclein accumulation in differentiated PC12 cells. (2) Enzyme linked immunosorbent assay showed up-regulated 26S proteasomal activity in MG132-induced PC12 cells after pre- and posttreatment with conserved dopamine neurotrophic factor. Similarly, 26S proteasome activity was upregulated in lactacystin-induced PC12 cells pretreated with conserved dopamine neurotrophic factor. (3) With regard proteolytic enzymes (specifically, glutamyl peptide hydrolase, chymotrypsin, and trypsin), glutamyl peptide hydrolase activity was up-regulated in lactacystin/MG132-administered PC12 cells after pre- and posttreatment with conserved dopamine neurotrophic factor. However, upregulation of chymotrypsin activity was only observed in MG132-administered PC12 cells pretreated with conserved dopamine neurotrophic factor. There was no change in trypsin expression. We conclude that conserved dopamine neurotrophic factor develops its neurotrophic effects by modulating proteasomal activities, and thereby protects and rescues PC12 cells against neurodegeneration.

Key Words: nerve regeneration; conserved dopamine neurotrophic factor; Parkinson’s disease; proteasomal inhibitor; 26S proteasome; alpha-synuclein; lactacystin; MG-132; glutamyl peptide hydrolase; chymotrypsin; trypsin; neural regeneration

Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder. It is primarily characterized pathologically by progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta, with accumulation of alpha-synuclein protein inclusions in surviving neurons. Despite this recognized pathology, its inherent pathogenesis remains elusive. Oxidative stress can cause neurodegeneration of DA neurons (Anusha et al., 2017; Fatkullina et al., 2017). However, numerous studies have demonstrated that ubiquitin-proteasomal system dysfunction plays a key role in PD pathogenesis, both in vitro and in vivo (Bentea et al., 2015; Wang et al., 2015; Chu et al., 2016).

The proteasome is a multienzyme complex that exhibits multienzymatic proteolytic activities of chymotrypsin- and trypsin-like hydrolases, and is implicated in degeneration of
abnormal proteins in eukaryotic cells. McNaught et al. reported impaired proteasomal function in the substantia nigra of PD patients in autopsy brain tissue (McNaught and Jenner, 2001; McNaught et al., 2006). Further studies found that proteasome inhibition by striatal and nigral injections or in vivo systemic administration causes abnormal behavior and neurodegeneration of substantia nigra DA neurons (McNaught et al., 2002, 2004; Bedford et al., 2008), which causes typical pathological features in vitro (Rideout et al., 2005; Nair et al., 2006). These studies have provided new insight into the rat model of PD after proteasome inhibition.

Conserved dopamine neurotrophic factor (CDNF) is a member of the mammalian mesencephalic astrocyte-derived neurotrophic factor family that significantly protects and prevents neurodegeneration of DA neurons from 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced damage, and has potential as a candidate neurotrophic therapy for PD (Lindholm et al., 2007; Voutilainen et al., 2011; Airavaara et al., 2012; Bäck et al., 2013). As far as oxidative stress is concerned, proteasomal dysfunction is a factor in PD pathogenesis, and consequently, it is of interest to determine similar effects of CDNF against DA neurodegeneration induced by proteasomal inhibitors.

In the present study, we aimed to determine whether CDNF exhibits comparable neuroprotective and reversal effects in proteasomal inhibitor-treated PC12 cells, and examine the underlying mechanism involved in proteasomal expression and its multienzymatic proteolytic activities.

Materials and Methods
CDNF extraction and cell culture
Human recombinant CDNF proteins were produced and purified as previously described (Lindholm et al., 2007). PC12 cells were directly obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and were maintained in Dulbecco's modified Eagle's medium (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) supplemented with 10% heat-inactivated newborn calf serum. Cells were cultured at 37°C in humidified air with 5% CO2. All experiments were performed within 24–48 hours after cell seeding.

Cell treatment
There were five groups in the study, with three groups designated as non-treated groups, and pretreated with CDNF before administration of lactacystin (Sigma-Aldrich Corp., St. Louis, MO, USA) or MG132 (Sigma-Aldrich Corp.), and post-treated with CDNF after administration of lactacystin or MG132. The five groups were: control group, MG132 group, lactacystin group, CDNF + MG132 group and CDNF + lactacystin group. PC12 cells were maintained in 10% heat-inactivated newborn calf serum and 100 U/mL penicillin/streptomycin. Cells were seeded at a density of 2 × 10^4 cells/mL onto 96-well plates and incubated for 24 hours. To investigate the protective effect of CDNF, cells were preincubated with 200 nM CDNF protein (extracted in-house) for 6 hours, and then exposed to 12.5 μM lactacystin or 5 μM MG132 for 24 hours. To investigate the reversible effect of CDNF, PC12 cells were pretreated with 12.5 μM lactacystin or 5 μM MG132 for 24 hours, and afterwards incubated with the same dose of CDNF protein for 24 hours. All experiments were performed in triplicate.

Viability of PC12 cells by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay
After 24 hours, cell viability was measured using the MTT assay. Cells were washed once with phosphate buffered saline (PBS) before adding 0.1 mL serum-free medium containing 1 mg/mL MTT (Sigma-Aldrich Corp.) to each well. After incubation for 3 hours, the supernatant was removed and obtained formazan product dissolved in 150 μL dimethyl sulfoxide (Sigma-Aldrich Corp.) per well, with stirring for 15 minutes on a microtiter plate shaker. Absorbance values were recorded at 570 nm.

Immunofluorescence staining for tyrosine hydroxylase (TH) and alpha-synuclein
After scheduled treatment for 24 hours, PC12 cells were permeabilized and fixed in 4% paraformaldehyde and 0.5% Triton X-100. Slides were blocked with 1% normal donkey serum (Merck, Darmstadt, Germany) in PBS for 30 minutes at room temperature. Cells were washed three times with gentle shaking in 0.1% bovine serum albumin (Beyotime Institute of Biotechnology, Shanghai, China) in PBS, and then incubated with primary antibodies diluted in 0.1% bovine serum albumin/PBS (TH, 1:500; and alpha-synuclein, 1:200; anti mouse monoclonal antibodies; Invitrogen, Paisley, UK) at 4°C overnight. Labeled donkey anti-rabbit immunoglobulin G (IgG) (1:1,000; Invitrogen) was used as the secondary antibody, with the solution incubated in the dark for 2 hours at room temperature. Specific antibody binding was detected by Alexa Fluor 488 (green label)-conjugated ExtrAvidin (Sigma-Aldrich Corp.). Fluorescence density was quantified. Fluorescence microscopy was performed using an Olympus BX51 200 M fluorescent microscope (Olympus, Tokyo, Japan).

Measurement of 26S proteasomal activity
After scheduled treatment for 24 hours, cells were harvested and homogenized in lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 2 mM adenosine 5′-triphosphate, and 1% Triton X-100). Lysates were centrifuged at 10,000 × g at 4°C for 20 minutes. Protein concentrations were assayed from the resulting supernatants using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). 26S proteasomal activity was quantified using a 26S proteasome activity assay kit (Chemicon International, Temecula, CA, USA), according to the manufacturer’s instructions. Assays were performed using 50 μg cell lysates and 50 μL enzyme reagent, with incubation at 37°C for 60 minutes. Chromogenic agent A (50 μL) and B (50 μL) were successively added and shaken, followed by incubation at 37°C for 15 minutes. Reactions were terminated with termination solution (50 μL). Fluorescence of released 7-amido-4-methyl coumaric acid was measured using a spectrofluorimeter (CytoFluor II, PerSeptive Biosystems, Framingham, MA, USA) at excitation/emission wavelengths of 380/460 nm.
Hydrolase enzymatic activity assays
Cells were lysed in 10 µM digitonin in Tris buffer (50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, and 10 mM ethylene glycol tetraacetic acid). Lysate supernatants were obtained by centrifugation at 14,000 × g for 5 minutes, and then incubated with 50 µL fluorogenic substrates (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for determination of activity of glutamyl peptide hydrolase, chymotrypsin, and trypsin (Sigma-Aldrich Corp.). Colored reagents A and B were added successively at 37°C for 15 minutes. Absorbance (optical density) values were measured at 450 nm using an enzyme-linked immunosorbent assay kits of glutamyl peptide hydrolase, chymotrypsin and trypsin (Chemicon International, Temecula, CA, USA).

Statistical analysis
All data are expressed as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance and Student-Newman-Keuls test. P-values of 0.05 or less were considered statistically significant.

Results
PC12 cell viability after pre- and posttreatment of CDNF
After exposure to 12.5 µM lactacystin or 5 µM MG132 for 24 hours, cell viability was decreased in PC12 cells, being 79.1% and 78.6%, respectively (Figure 1). Morphological analysis of TH-immunofluorescence staining demonstrated nuclear condensation and fragmentation, both typical hallmarks of apoptosis (Figure 1A–C). To investigate the protective effect of CDNF, cells were preincubated with 200 nM CDNF protein for 6 hours and then exposed to 12.5 µM lactacystin or 5 µM MG132 for 24 hours. Again, cell viability was decreased in PC12 cells, being down-regulated to 52.3% and 49.7%, respectively (Figure 1H). However, cell number increased compared with non-treated groups (Figure 1D, E). To investigate the reversible effect of CDNF, cells were first exposed to 12.5 µM lactacystin or 5 µM MG132 for 24 hours and then incubated with 200 nM CDNF protein for 24 hours. Cell viability was decreased in PC12 cells, being down-regulated to 59.5% and 56.2%, respectively (Figure 1I). Again, cell number also increased compared with non-treated groups (Figure 1F, G).

Expression of alpha-synuclein measured by immunofluorescence staining
Compared with controls, alpha-synuclein immunofluorescence was observed in numerous lactacystin- and MG132-treated PC12 cells with high fluorescence expression (Figure 2B, C). Fluorescence intensity in lactacystin- and MG132-treated PC12 cells was quantified as 2.1755 and 2.8990, respectively (Figure 2H, I). After pretreatment with 200 nM CDNF protein for 6 hours, the number of immunofluorescent PC12 cells was reduced, and fluorescence intensity faded (Figure 2D, E). Specifically, fluorescence intensity in lactacystin- and MG132-treated PC12 cells decreased to 1.3871 and 1.1710, respectively (Figure 2H). In samples posttreated with CDNF, the number of cells and fluorescence intensity also decreased (Figure 2F, G), with fluorescence intensity in lactacystin- and MG132-treated PC12 cells decreased to 1.4899 and 1.2958, respectively (Figure 2I).

26S proteasome activity results
After exposure to lactacystin and MG132, 26S proteasome activity was significantly decreased (P < 0.05) (Figure 3A, B). After CDNF pretreatment, 26S proteasome activity was upregulated in the lactacystin and MG132 groups (P < 0.05), with a greater effect in the MG132 group (P < 0.05) (Figure 3A). After CDNF posttreatment, upregulation was observed in the MG132 group (P < 0.05), but not the lactacystin group (P > 0.05) (Figure 3B).

Hydrolase enzymatic activity
Hydrolase enzymatic activity was determined, as described previously (Xie et al., 2010). After exposure to lactacystin and MG132, activities of glutamyl peptide hydrolase, chymotrypsin, and trypsin were significantly decreased (P < 0.05) (Figure 4A–F). After CDNF pretreatment, upregulation of glutamyl peptide hydrolase and chymotrypsin was observed in the MG132 group (P < 0.05) (Figure 4A–C). Only glutamyl peptide hydrolase expression was increased in the lactacystin group (P < 0.05). No statistical differences in activity were related to trypsin (P > 0.05). Finally, in the group pretreated with lactacystin and MG132, only glutamyl peptide hydrolase was reversely up-regulated compared with the other enzymes (Figure 4D–F).

Discussion
Here, we show for the first time that CDNF exhibits a neuroprotective and reversible effect on proteasomal inhibitor-treated (lactacystin and MG132) PC12 cells via upregulation of cell viability, reduction of alpha-synuclein protein, and upregulation of the 26S proteasome and its corresponding proteolytic activity.

Although PD pathogenesis encompasses multiple factors, the ubiquitin-proteasomal system is one of the major intracellular proteolysis systems, responsible for degradation of damaged or misfolded proteins including those involved in various cellular processes such as neurodegeneration. Emerging evidence has shown that failure of the ubiquitin-proteasomal system to degrade aberrant proteins plays a key role in PD pathogenesis (Jnombaptiste et al., 2002; McNaught et al., 2010; Xie et al., 2010). In our previous study, we found that accumulation of misfolded and aggregated alpha-synuclein after ubiquitin-proteasomal system impairment led to degeneration of DA neurons in the substantia nigra pars compacta of rats (Niu et al., 2009). Once proteasomal dysfunction has occurred, proteolytic activity of multi-hydrolases is likely to be inhibited, with no ensuing degradation of aberrant proteins detrimental to neuronal survival (McNaught, 2004). Some scholars revealed that chymotrypsin-, trypsin- and post-acidic-like hydrolyzing activities of the proteasome are remarkably impaired in the substantia nigra in autopsy tissue of PD patients (McNaught and Jenner, 2001; McNaught et al., 2006). As far as proteasome inhibitors are concerned, lactacystin (an irreversible
proteasome inhibitor) and MG132 (a reversible proteasome inhibitor) both lead to proteasomal dysfunction, and subsequently, preferential degeneration of DA neurons (Rideout et al., 2005; McNaught et al., 2010; Fan et al., 2016). In this study, we also observed the same damaging effects in lactacystin- and MG132-treated PC12 cells. Viability of PC12 cells was reduced after lactacystin/MG132 administration, which was accompanied by membrane shrinkage and rugosity, and accumulation of alpha-synuclein protein. Altogether, this is indicative of damaged proteasomal function, which is consistent with previous reports in other models (Lindholm et al., 2007; Airavaara et al., 2012; Ren et al., 2013; Mei and Niu, 2015a).

Nonetheless, CDNF has been shown to protect and restore DA neurons induced by 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in vivo (Lindholm et al., 2007; Voutilainen et al., 2011; Airavaara et al., 2012; Mei and Niu, 2015b). While the proteasome is responsible for degeneration of abnormal proteins, and plays a major protective role in DA neurons (McNaught et al., 2002; Aguileta et al., 2015). Hence, it is of interest to determine whether CDNF induces neurotrophic effects by modulating proteasome activity. Here, we first confirmed that CDNF exhibits protective and reversible effects on lactacystin- and MG132-induced PC12 cells, with no significant difference found between the groups. Furthermore, alpha-synuclein accumulation, the hallmark of PD, was attenuated by CDNF, similar to the effect of glial cell-derived neurotrophic factor and brain-derived neurotrophic factor (Sathiya et al., 2013; Böttner et al., 2015). Additionally, these previous studies found that CDNF provides protective and reversible effects on endoplasmic reticulum stress (Lindholm and Saarma, 2010; Voutilainen et al., 2015). Based on regulation of alpha-synuclein expression by CDNF, this also illustrates that CDNF may exert neurotrophic effects via regulation of proteasome activity. Bedford et al. (2008) confirmed that depletion of the 26S proteasome causes neurodegeneration of DA neurons in vivo. We also found that 26S proteasome activity is upregulated protectively and reversibly by CDNF in the MG132 group. In contrast, only protective upregulation of 26S proteasome activity was observed in the lactacystin

Figure 1 Protective and reversible effects of CDNF on degenerative PC12 cells. (A–G) TH immunofluorescence staining: (A) normal group: abundant cell number without reduced morphology; higher magnification image shown in the squared region. (B, C) Lac- and MG132-treated groups, respectively. Decreased cell number with membrane shrinkage and rugosity compared with (A); higher magnification images shown in the boxed regions. (D, F) CDNF + Lac (pretreated with CDNF and induced by Lac) and Lac + CDNF (pretreated with Lac and posttreated by CDNF) groups, respectively. (E, G) CDNF + MG132 (pretreated with CDNF and induced by MG132) and MG132 + CDNF (pretreated with MG132 and posttreated by CDNF) groups, respectively; higher magnification images shown in the boxed regions. (H, I) Cell viability and morphology in normal and Lac groups; higher magnification images shown in the boxed regions. Protective and reversible effect of CDNF by MTT assay. *P < 0.05, **P < 0.01 (mean ± SEM, n = 6, one-way analysis of variance and Student-Newman-Keuls test). CDNF: Conserved dopamine neurotrophic factor; TH: tyrosine hydroxylase; Lac: lactacystin; MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide. I: Control; II: MG132; III: Lac; IV: CDNF + MG132; V: CDNF + Lac.
Figure 2 CDNF effects on alpha-synuclein expression in degenerative PC12 cells.
(A) Normal group without alpha-synuclein immunofluorescence staining; higher magnification image shown in the boxed region. (B, C) Lac and MG132 groups, respectively. Compared with (A), alpha-synuclein immunofluorescence is clearly present in neurons; higher magnification images shown in the boxed regions. (D, F) CDNF + Lac (pretreated with CDNF and induced by Lac) and Lac + CDNF (preinduced with Lac and posttreated by CDNF) groups, respectively. Number and fluorescence staining intensity of alpha-synuclein positive cells in normal and Lac groups; higher magnification images shown in the boxed regions. (E, G) CDNF + MG132 (pretreated with CDNF and induced by MG132) and MG132 + CDNF (preinduced with MG132 and posttreated by CDNF) groups, respectively; higher magnification images shown in the boxed regions. Number and fluorescence staining intensity of alpha-synuclein positive cells in normal and MG132 groups. (H, I) Protective and reversible effect of CDNF by cell fluorescence intensity assay of alpha-synuclein.

*P < 0.05 (mean ± SEM, n = 6, one-way analysis of variance and Student-Newman-Keuls test).

CDNF: Conserved dopamine neurotrophic factor; Lac: lactacystin. I: Control; II: MG132; III: Lac; IV: CDNF + MG132; V: CDNF + Lac.

This may be because lactacystin achieves irreversible proteasome inhibition, in contrast with the reversible effect of MG132. When the 26S proteasome is inhibited by lactacystin, its function would not be reversed by CDNF, and may be related to translocation of proteolytically cleaved PKCdelta fragments to mitochondria by MG-132 (Sun et al., 2008). This may also explain why we did not observe a reversible CDNF effect after lactacystin pretreatment. As far as timely and adequate proteasomal processing of abnormal proteins, not only should the number of proteasomes be sufficient, but its enzymes are also likely to be essential (Wang et al., 2015).

In a previous study, enzymes (glutamyl peptide hydrolase, chymotrypsin, and trypsin) located in the 20S proteasome were visibly reduced in postmortem brain of PD patients (Bukhatwa et al., 2010; Alexopoulou et al., 2016). In this study, we were also interested to examine enzyme activities reflecting 20S proteasomal function. As with glutamyl peptide hydrolase, CDNF played a significant protective role in upregulation in the lactacystin and MG132 groups, and reversible overexpression in the CDNF-lactacystin and CDNF-MG132 groups, with a more pronounced effect in the latter. Regarding the protective and reversible effects on chymotrypsin and trypsin activities in the lactacystin and MG132 groups by CDNF, we found a protective effect on chymotrypsin activity in only MG132-induced PC12 cells. Moreover, no reversible role on chymotrypsin activity was observed between the two groups, although MG132 activity was statistically greater compared with the lactacystin group. More interestingly, no protective or reversible effects were observed on trypsin activity in the lactacystin and MG132 groups, although activity in the MG132 group was statistically greater compared with the lactacystin group. Of the three enzymes located in the 20S proteasome, CNDNF exerts protective and reversible modulation on only glutamyl peptide hydrolase, with no statistical effect on the other enzymes. Further research is needed on the underlying mechanism of this process. It is possible that proteasomal dysfunction may not be the sole factor in PD pathogenesis because previous
studies have confirmed that PD is also related to endoplasmic reticulum stress and apoptosis (Zhang et al., 2015; Fatkullina et al., 2017). Further, CDNF exerts a neurotrophic effect via modulation of Bcl-2/Bax and caspase-3 (Mei and Niu, 2014).

Based on our results, we have shown that CDNF plays neuroprotective and reversible roles in PC12 cells after proteasome inhibition, mainly via regulation of the 26S proteasome and glutamyl peptide hydrolase activity. For lactacystin, reversible proteasomal inhibition of MG132 appears to be reflected mainly in reversible inhibition of the 26S proteasome and glutamyl peptide hydrolase enzyme. While for reversible proteasomal inhibition of MG132, protection of the 26S proteasome and its enzymes by CDNF was greater in the MG132 group compared with the lactacystin group. Certainly, PC12 cells are a widely accepted cell model of PD, and not just DA neurons. Nonetheless, further studies are needed to identify the neurotrophic effect of CDNF in DA neurons. Overall, we have preliminarily demonstrated that CDNF exerts neurotrophic effects via regulation of proteasomal activities, although the mechanism needs further research.

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**Author contributions:** JMM and CSN designed this study. JMM analyzed and interpreted the data, wrote the paper, cooperated statistical analysis, and performed experiments in Anhui Key Laboratory of Brain Function and Brain Disease in China. CSN revised the paper. Both of these two authors approved the final version of the paper.

**Conflicts of interest:** None declared.
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