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Glial Cell-Derived Neurotrophic Factor Protects Against Proteasome Inhibition-Induced Dopamine Neuron Degeneration by Suppression of Endoplasmic Reticulum Stress and Caspase-3 Activation

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Evidence has shown that ubiquitin proteasome system (UPS) impairment plays an important role in the dopamine (DA) neurodegeneration in Parkinson's disease (PD). It has been reported that application of proteasomal inhibitor lactacystin in ventral mesencephalon (VM) cultures can cause DA neurodegeneration, although the underlying mechanisms are not clear. Herein, we used the lactacystin-induced DA cell degeneration model to study the neuroprotection of glial cell-derived neurotrophic factor (GDNF) in VM cultures. We measured the expression of endoplasmic reticulum stress (ERS)-related genes, and determined the caspase-3 activation, apoptotic cell death, as well as α-synuclein-positive inclusions in DA neurons. We found that GDNF treatment significantly suppressed the expression of ERS-related genes and inhibited the activation of caspase-3 and apoptotic cell death without affecting α-synuclein-positive inclusions in DA neurons. Our study suggests that the protection of GDNF against DA neurodegeneration in the UPS impairment model is associated with ERS and caspase-3 suppression.

PARKINSON'S disease (PD) is a neurodegenerative disorder that affects about 1% of the population aged 65 years or older. The pathological hallmarks of this disease include progressive loss of nigral dopamine (DA) neurons and presence of inclusions known as Lewy bodies (LB), in which α-synuclein is a major component (1). Previous studies have suggested that both environmental and genetic factors may contribute to PD (1–3), but the convergent mechanisms underlying the neuropathogenesis of PD remained elusive until recent findings indicated that a ubiquitin proteasome system (UPS) impairment may play a role (1–4).

UPS is known to play a prominent role in the removal of short-lived, misfolded, or damaged proteins in eukaryotes. This process is implemented by a diverse group of proteases, among which the 20s proteasome is a pivotal protease complex (2,5). Several inhibitors acting on the 20s proteasome, such as lactacystin, have been used to study the role of UPS in neurodegenerative diseases (4,5). Although the study in vivo to recapitulate cardinal pathological features of PD by the systematic administration of proteasome inhibitors in rodent animals is controversial (6), it is well documented that the exposure of embryonic rat ventral mesencephalon (VM) cultures or DA cell lines to several proteasome inhibitors including lactacystin can cause DA neurodegeneration (4,6–9). This cellular model may provide a novel platform for investigating the mechanisms underlying the DA neurodegeneration induced by proteasome inhibition and for evaluating potential therapeutic agents such as neurotrophic factors for the treatment of PD.

It is known that endoplasmic reticulum stress (ERS) is involved in DA neuron injury under certain conditions (10–12) and that ERS can mediate apoptosis in carcinoma and fibroblast cell lines induced by proteasome inhibitors (13,14). Glial cell-derived neurotrophic factor (GDNF), a prospective drug for treating PD, has been proven effective in preventing DA neuron loss in several models of PD (15–17). This protective action is thought to be related to the inhibition of the caspase-3-mediated apoptotic pathway (18,19), which is associated with PD (20). However, it remains unknown whether ERS and caspase-3 are involved in the DA neurodegeneration initiated by UPS impairment, and whether and how GDNF is able to protect DA neurons against UPS impairment-induced neurodegeneration.

In the present study, we examined the role of ERS and caspase-3 activation in DA neurodegeneration induced by the proteasome inhibitor lactacystin and investigated the protective effects of GDNF on the ERS-related gene and protein expression, caspase-3 activation and apoptosis, as well as α-synuclein-positive inclusion formation in the DA neurons in VM cultures. We found that GDNF significantly protected DA cells against lactacystin-induced neurodegeneration through the suppression of both ERS and caspase-3 activation.

Table 1. Primers Used for Polymerase Chain Reaction

Gene Name	Sense	Antisense	Product Size (bp)	Cycle	GeneBank Accession No.
Bip	5′-ATCTGACTGGAATCCCTCCTGCTC-3′	5′-TTCTCGGCGTCATTGACCATC-3′	193	38	NM_013083
Chop	5′-TTCACTACTCTTGACCCTGCATC-3′	5′-CACTCTGTTTCCGTTTCCTAGTTC-3′	169	38	NM_024134
Xbp1	5′-AGCAAGTGGTGGATTTGGAAGAAG-3′	5′-CAACAGCGTCAGAATCCATGG-3′	296	38	NM_001004210 XM_214067
18s rRNA*	5′-CGGCTACCACATCCAAGGAA-3′	5′-GCTGGAATTACCGCGGCT-3′	187	32	X01117 K01593

Note: *For 18s ribosomal RNA (rRNA) primer, see Mouchel N, et al. *Biochem J*. 2004;378:909–901.

Materials and Methods

Primary Rat VM Cultures

Animals were treated in accordance with the guidelines on the care and use of laboratory animals at Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences. Primary mesencephalic neuron-enriched cultures were prepared as previously reported (4,21). Briefly, VM dissected from E14 Sprague-Dawley rat fetuses (Animal Center, Chinese Academy of Sciences, Shanghai) was digested with 0.025% trypsin (Sigma, St. Louis, MO) and dissociated by gentle trituration. Cells were plated on poly-D-lysine (20 μg/mL; Sigma)-coated 24-well culture plates or glass coverslips at a density of 1.5×10^5 cells/cm^2 in a complete medium containing DF12 (Sigma), 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), penicillin at 50 U/mL, and streptomycin at 50 μg/mL. After the cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C overnight, the medium was replaced with a serum-free one that contained DF12, 2% B27 supplement (Invitrogen), penicillin at 50 U/mL, and streptomycin at 50 μg/mL. The cells were allowed to grow in the serum-free medium for 7 days in vitro. No cytarabine was used in VM cultures. The composition of the cultures at the time of treatment was approximately 93% neurons as stained by microtubule-associated protein II (MAP2) antibody, 5% DA neurons as stained by tyrosine hydroxylase (TH) antibody, and $< 5\%$ astrocytes as stained by glial fibrillary acidic protein (GFAP) antibody.

VM Culture Treatment

Lactacystin (Calbiochem, Beeston, Nottingham, U.K.) dissolved in dimethyl sulfoxide (DMSO) was freshly prepared and added to the cultures, for a final concentration of 5 μM for the lactacystin-treated group (LC). A normal control group (NC) was treated with vehicle. The GDNF pretreatment group (GLC) was treated with recombinant rat GDNF (10 ng/mL or 100 ng/mL; Sigma) 4 hours before lactacystin addition, whereas GDNF control group (GC) was treated with GDNF and vehicle. Cells were harvested and underwent further analysis after lactacystin or vehicle treatment for 5 hours and 24 hours, according to experimental protocol.

RNA Extraction and Polymerase Chain Reaction

After 5 hours of the lactacystin, GDNF, or vehicle treatment, VM cells were washed once with phosphate buffered saline. The total RNA of VM cells was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol. The purified RNA was treated with RQ1 DNase (Promega, Madison, WI) at 37°C for 30 minutes. DNase was deactivated by adding 1 μL of RQ1 DNase Stop Solution and heating at 65°C for 10 minutes. A Superscript III first-strand synthesis system was used for the reverse transcription in a 20 μL reaction mixture using a random hexamer. The primers (Table 1) used for polymerase chain reaction (PCR) were purchased from Invitrogen Biotec (Shanghai, China). Bip (immunoglobulin heavy-chain binding protein), Chop (CCAAT/enhancer-binding protein-homologous protein), and Xbp-1 (X-box binding protein 1) were blasted using BLAST (www.ncbi.nlm.nih.gov/BLAST/). PCRs were performed on a Peltier Thermal Cycler (Bio-Rad, Hercules, CA) in a 25-μL reaction volume consisting of 1 μL of complementary DNA (cDNA), 2.5 μL of 10 X PCR buffer (QIAGEN, Hilden, German), 1.5 μL of 25 mM $MgCl_2$ (QIAGEN), 0.5 μL of 10 mM deoxyribonucleoside triphosphate (dNTPs; QIAGEN), 0.5 μL of 10 pmol forward and reverse primers, and 1 U of Hotstar Taq DNA Polymerase (QIAGEN). The program was set as follows: 20 seconds at 95°C, 30 seconds at 54°C, 30 seconds at 72°C for 32–38 cycles, with an initial denaturation at 95°C for 5 minutes and a final extension at 72°C for 10 minutes. The PCR products were run on a 2% agarose gel, and the relative expression level of the targeted genes was assessed semi-quantitatively with Quantity One software (Bio-Rad) after normalization to the internal control 18s ribosomal RNA (rRNA) amplified from the same cDNA.

Immunocytochemical and Immunofluorescent Staining

VM cells were fixed in 4% paraformaldehyde at 4°C for 20 minutes. For immunocytochemical staining, cells were treated with 1% H_2O_2 and then incubated at 4°C for 1 hour in a blocking solution that contained 4% horse serum and 0.3% Triton X-100. This was followed by an overnight incubation at 4°C with various primary antibodies (TH, 1:4000; MAP2, 1:2000; GFAP, 1:500; all were purchased from Sigma). After washing, the cells were incubated with a biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 2 hours at room temperature, and were visualized using the VECTASTAIN ABC kit (Vector Laboratories) and diaminobenzidine (DAB)–H_2O_2 reaction. For dual immunofluorescent staining, cells on coverslips were treated with a blocking solution at 4°C for 1 hour. This treatment was followed by an overnight incubation at 4°C with a combination of TH/α-synuclein antibodies (TH, 1:2000; α-synuclein, 1:200; Sigma), or TH/Chop antibodies (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), or TH/cleaved caspase-3 antibodies (1:200; Cell Signaling

Technology, Beverly, MA). After that, cells were incubated at room temperature for 2 hours with a mixture of fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) (1:200; Vector Laboratories) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated IgG (1:200; Sigma). The cells were counterstained with Hoechst 33258 (Beyotime Co., Hang Zhou, China) for 10 minutes. Finally, the coverslips were mounted with the antifade Aqua Poly/Mount (Polysciences, Warrington, PA) on glass slides, and then visualized and photographed using a confocal microscope (LCM510; Carl Zeiss, Oberkochen, Germany) or an inverted fluorescent microscope (Olympus IX 81; Tokyo, Japan) equipped with a DP70 CCD digital camera (Olympus).

Counting and Observation of Double-Stained Cells

The total TH- or MAP2-positive cells were chromostained with DAB and counted in triplicate at ×160 magnification (Olympus IX 81) from eight optical fields, which were randomly chosen within four strips across the center of each coverslip by an observer blinded to test group. The ratio of TH-positive neurons in each experimental group to those cells in control groups was used to evaluate survival. Data generated from three separate experiments were used for statistical analyses. The values of the data were expressed as the percentage of cell loss [(1 – the ratio of TH^+ cells in experiment group/TH^+ cells in control groups) × 100].

To assess the percentage of double-stained or apoptotic DA neurons, more than 100 TH-positive cells per coverslip were randomly chosen at a ×640 magnification, observed again by an observer blinded to test group, and photographed under a confocal microscope (oil immersion) or fluorescent microscope equipped with a DP70 CCD digital camera. Cells with condensed, fragmented nuclei were defined as apoptosis, and α-synuclein inclusions were defined as those discrete intracellular granules labeled with intense fluorescence. The experiments were repeated at least three times on cultures prepared from different rats.

Western Immunoblot Assay

Protein extracts were harvested from six wells in 24-well plates of the primary VM cultures treated with GDNF or vehicle for 24 hours. Cells were lysed on ice for 20 minutes in modified RIPA (50 mM Tris–HCl, pH 7.4; NaCl 150 mM; 1% Nonidet P-40 [NP40]; 0.25% sodium deoxycholate; 1 mM EDTA; 1 mM Na_2VO_4; 1 mM NaF; 1 mM phenylmethylsulfonyl fluoride [PMSF]; and aprotinin and leupeptin at 5 mg/mL each), followed by a centrifugation at 20,000 × *g*. The supernatants were collected and underwent protein concentration. A small amount of the supernatant (30 μg of protein per sample) was fractionated on a 10% gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The blots were incubated in a blocking solution containing 5% nonfat dry milk in 1 X Tris-buffered saline, then incubated overnight at 4°C with primary antibodies in 5% milk/Tris-buffered saline (α-synuclein antibody diluted at 1:1000; β-actin at 1: 2500; Sigma). The subsequent step for the Western blotting detection was performed with the SuperSignal West Dura Extended Duration Substrate Kit (Pierce, Rockford, IL), as described in the manufacturer's instruction.

Statistical Analysis

All data were expressed as means ± standard error of the mean (*SEM*). Statistical analysis was performed by independent *t* test or one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons using the Bonferroni test with the SPSS 10.0 software package (SPSS Inc., Chicago, IL). The criterion for statistical significance was set at $p < .05$.

Results

GDNF Prevented DA Neuron Loss and Apoptosis Induced by Lactacystin

An initial experiment was carried out to produce a UPS impairment cellular model in which the injury effect of lactacystin on DA neurons was tested. We treated VM cultures with lactacystin at a concentration of 5 μM for 24 hours, as previously reported (4,8). We examined the total neurons by MAP2 immunostaining and DA neurons by TH immunostaining. Through determination of the cell loss of the MAP2-postive neurons and that of TH-positive DA neurons by cell counting, we found that phenotype-defined DA neurons exhibited a more substantial loss after lactacystin treatment (MAP2, 25%, Figure 1A and B; TH, 41%, Figure 1C and D; $p < .05$, Figure 1E). We next investigated the possible protective effects of GDNF on DA neurons in the cultures by applying GDNF 4 hours prior to lactacystin treatment. After being normalized to the GC (Figure 1F, 10 ng/mL), and in comparison to vehicle pretreatment (LC), GDNF pretreatment (GLC) significantly ameliorated the lactacystin-induced DA neuron loss in a dose-dependent manner (25% at 10 ng/mL, Figure 1G; 19% at 100 ng/mL vs 40% of LC, Figure 1D and H, $p < .01$; $p < .05$ between two dosage groups).

We sequentially tested whether the protection of GDNF resulted from the suppression of the apoptosis in DA neurons. Through the observation of the nuclei morphology labeled with Hoechst 33258 in the remaining TH-positive cells, we assessed the apoptotic rate in the DA neurons. We found that lactacystin treatment (5 μM, 24 hours) significantly induced apoptosis in these neurons (19%, Figure 2A, i–iii, $p < .01$ vs NC). In comparison, GDNF pretreatment markedly attenuated the lactacystin-induced apoptosis in a dose-dependent manner (13% at 10 ng/mL, $p < .05$ vs LC; 10% at 100 ng/mL, Figure 2B, $p < .01$ vs LC; $p < .05$ between two dosage groups).

GDNF Suppressed the Lactacystin-Induced ERS and Caspase-3 Activation in DA Neurons

To verify whether ERS and caspase-3 activation were involved in the lactacystin-induced apoptotic event and to test whether GDNF exerted protection by suppressing these pathways, we examined the expression of the ERS-related genes (Bip, Chop, and Xbp1) in VM cultures after treatment

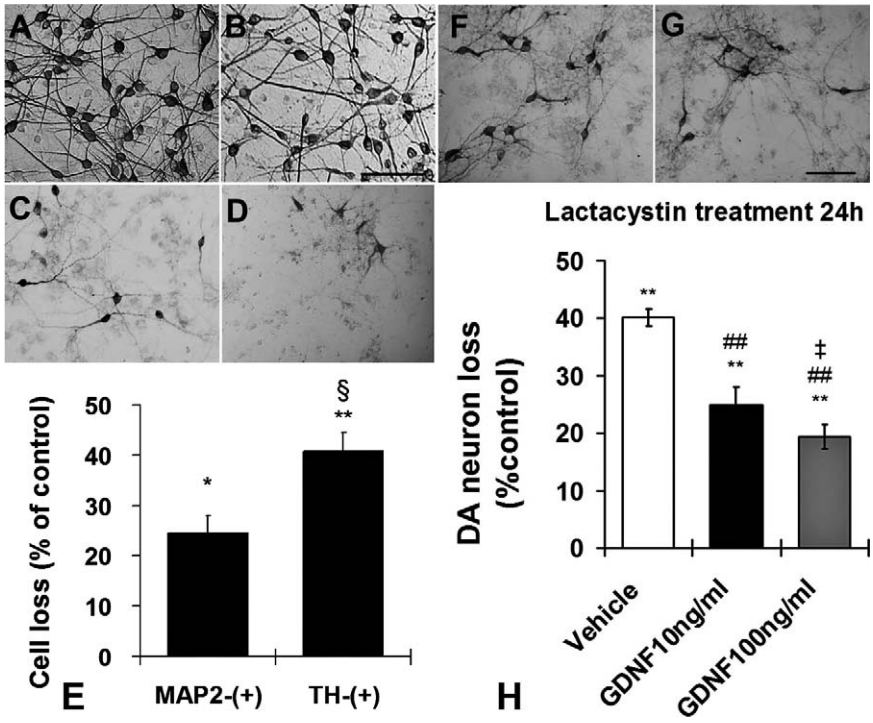

Figure 1. Glial cell-derived neurotrophic factor (GDNF) prevents lactacystin-induced dopamine (DA) neuron loss. After lactacystin treatment (5 μM) for 24 hours, the primary ventral mesencephalon (VM) cultures are immunostained for microtubule-associated protein II [MAP2-(+); **A** and **B**] or tyrosine hydroxylase [TH-(+); **C** and **D**] with diaminobenzidine (DAB) to determine the cell loss of the total neurons (**B**) and DA neurons (**D**), respectively. A more substantial impairment of DA neurons is shown (**E**). *$p < .05$, **$p < .01$ vs control; §$p < .05$ vs MAP2-(+) by independent *t* tests; GDNF treatment alone (GC) slightly increases the number of DA neurons (**F**). GDNF pretreatment attenuates DA neuron loss (GDNF 10 ng/mL, **G**; GDNF 100 ng/mL) in a dose-dependent manner in comparison to vehicle pretreatment (Vehicle) in the presence of lactacystin (**H**). **$p < .01$ vs controls; $^{\#}p < .01$ vs Vehicle; $^{\ddagger}p < .05$ vs GDNF 10 ng/mL group by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison. Data are from at least three experiments and are shown as mean ± standard error of the mean (*SEM*). The values presented in **E** and **H** are expressed as the percentage of cell loss. Scale bar = 50 μm.

with lactacystin (5 μM, 5 hours) in the presence or absence of GDNF. We found that lactacystin treatment increased the gene expression of Bip, Chop, and Xbp1 by 1.6-, 1.9-, and 2.2-fold over the controls, respectively, and the pretreatment with GDNF significantly suppressed the lactacystin-induced increase in the ERS-related gene expression in a dose-response manner (Figure 3A and B).

To determine whether ERS and caspase-3 activation in DA neurons were induced by lactacystin treatment, Chop protein and cleaved caspase-3 in the remaining DA neurons were examined by dual immunostaining. In contrast to the control groups in which Chop protein was only weakly stained in the cytoplasm, lactacystin induced a strong staining of Chop protein in the nuclei and significantly increased the percentage of the Chop protein-positive DA neurons (19%, $p < .01$ vs NC, Figure 3C and D). Similar to the effects on the ERS-related gene expression, GDNF pretreatment also significantly reduced the percentage of the Chop protein-positive DA neurons (10% at 10 ng/mL; 8% at 100 ng/mL; $p < .05$ vs LC, Figure 3C and D).

Coincident with the apoptosis and the upregulated Chop protein levels in DA neurons, lactacystin remarkably increased the percentage of cleaved caspase-3-positive DA neurons (32%, Figure 4G–I; $p < .01$ vs NC, Figure 4A–C). Pretreatment with GDNF significantly reduced the percentage of cleaved caspase-3-positive DA neurons (20% at 10 ng/mL, Figure 4J–L, $p < .05$ vs LC; 15% at 100 ng/mL, Figure 4M, $p < .01$ vs LC; $p < .05$ between two dosage groups).

GDNF Did Not Affect the Inclusion Body Formation in DA Neurons Induced by Lactacystin

The last set of experiments was designed to investigate the role of inclusions in both the lactacystin-induced DA neuron degeneration and the protection of GDNF in this model. An antibody against the C terminus of α-synuclein was used to locate inclusions in DA neurons by dual immunofluorescence staining. In the NC group (Figure 5A–C) and the GC group (Figure 5D–F), α-synuclein-positive granules were barely seen in VM cells. After lactacystin treatment for 24 hours, the percentage of DA neurons containing such inclusions increased dramatically (26%, Figure 5G–I; $p < .01$ vs NC). However, GDNF treatment did not lead to a significant change in the percentage of DA neurons containing inclusion (23% at 10 ng/mL, Figure 5J–L; 25% at 100 ng/mL, Figure 5M; $p > .05$ vs LC).

Because some α-synuclein-positive granules appeared in the nuclei of DA neurons (Figure 5N, i–ii), those neurons bearing this type of inclusion were also taken into account. The apoptotic rate between the inclusion-positive and inclusion-negative DA neurons was compared, but no significant difference was found (Figure 5O). In addition,

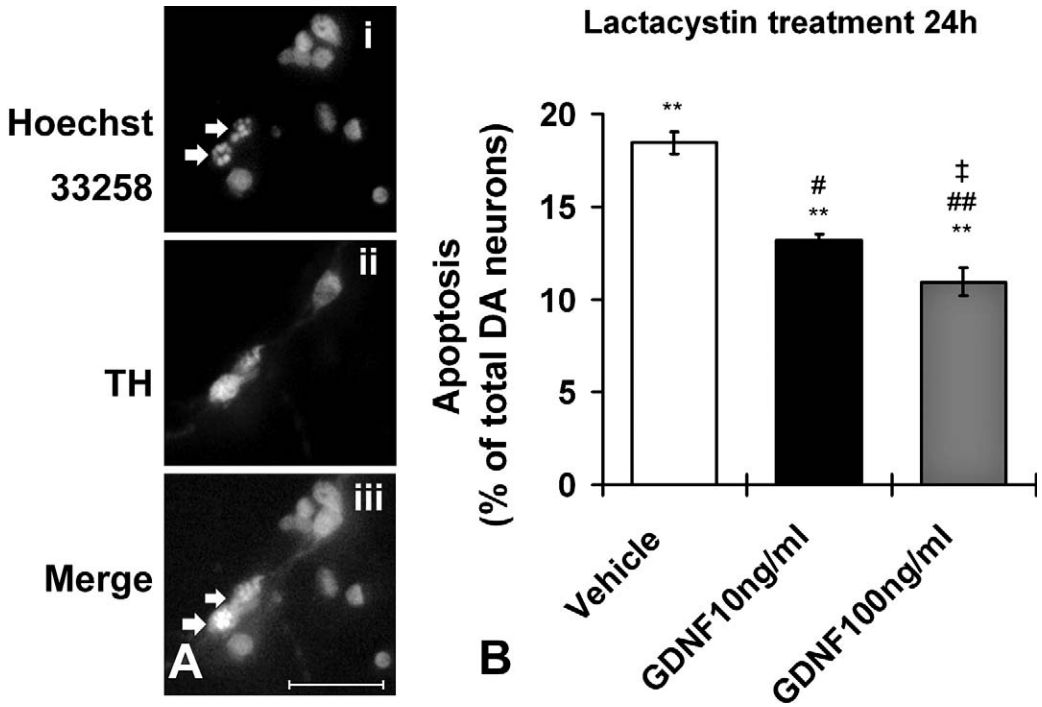

Figure 2. Glial cell-derived neurotrophic factor (GDNF) attenuates lactacystin-induced apoptosis in dopamine (DA) neurons. Primary ventral mesencephalon (VM) cultures are first treated with GDNF (10 or 100 ng/mL) or vehicle (Vehicle) in the presence of lactacystin (5 μM) for 24 hours. The percentage of apoptosis in DA neurons is then determined through the observation of nuclear morphology (counterstained with Hoechst 33258) in tyrosine hydroxylase [TH-(+)] cells. **A,** Lactacystin treatment elicits significant apoptotic death in DA neurons as demonstrated by the condensed, fragmented nuclei (i–iii; *arrow*). Nuclei labeled by Hoechst 33258 (A, i); TH immunolabeled by fluorescein isothiocyanate (FITC) (A, ii). **B,** Significant and dose-dependent blockade against the apoptosis in DA neurons is shown in the GDNF pretreatment groups. $^{**}p < .01$ vs controls; $^{\#}p < .05$, $^{\#}p < .01$ vs Vehicle; $^{\ddagger}p < .05$ vs GDNF 10 ng/mL group by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison. Data are from at least three experiments and are presented as mean ± standard error of the mean (*SEM*). Scale bar = 30 μm.

GDNF treatment alone did not affect the protein level of α-synuclein (Figure 5P).

Discussion

In the present study, we reported a protective effect of GDNF on DA neurodegeneration in the UPS impairment cellular model of PD. We demonstrated that GDNF exerted its protection against proteasome inhibition-induced apoptosis in DA neurons though the suppression of both ERS and caspase-3 activation. These results may provide useful information for our understanding of the pathophysiology of the UPS impairment-induced DA neurodegeneration and may also have implication for GDNF as a potential drug for the treatment of PD.

Lactacystin is a specific proteasome inhibitor that irreversibly inhibits the enzymatic activities of the 20s proteasome after being converted instantly into the active form (β-lactone) (5). Even though the failure of replicating nigral DA neurodegeneration in rodent animal models has been reported by a systematic administration of proteasome inhibitor Z-Ile-Glu(OtBu)-Ala-Leu-al (PSI) (6), several studies have indicated that lactacystin is able to induce DA neurodegeneration in VM cultures (4,8,9), and intracranial microinjection of lactacystin can cause sustained DA neurodegeneration (22). We found that lactacystin treatment in VM cultures impaired DA neurons and induced inclusion bodies, and DA neurons seemed more vulnerable than general neurons to lactacystin exposure. This finding is consistent with those in other reports (4,8,9,22), and supports the notion that UPS impairment is sufficient to cause DA neuron injury.

Furthermore, we demonstrated that lactacystin induced an increase in the ERS-related gene expression and protein level while activating caspase-3 and eliciting DA neuron injury. ERS-initiated apoptosis has been recognized as a new apoptotic pathway (10), which may play a role in the 6-hydroxydopamine-induced DA neuron injury in vivo and in vitro (11,12). A number of genes, such as Bip, Chop, and Xbp1, have been regarded as ERS markers (11,23), and Chop protein, the first protein identified to mediate the ERS-induced apoptosis (24), may participate in the process of several neurodegenerative diseases, including PD (12,24). In our study, both ERS-related genes in the VM cultures and the Chop protein level in the nuclei of the remaining DA neurons were increased after exposure to lactacystin, suggesting that DA neurons in this cellular model underwent ERS.

Apoptosis has been proposed to contribute to the DA neuron loss in PD (7,8), and caspase-3 may be a final effector (20). Fornai and colleagues (7) and Rideout and coworkers (8) documented that proteasome inhibition can induce apoptosis in DA neurons, whereas Rideout and colleagues (25) and Cheung and coworkers (26) reported that lactacystin activated caspase-3 in cortical neurons, cerebella granule neurons, and sympathetic neurons. Our study provided direct

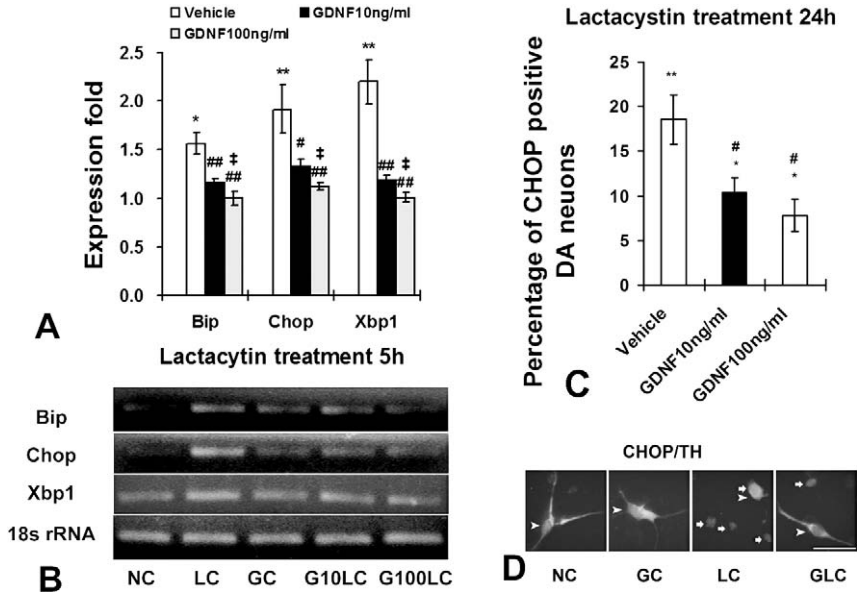

Figure 3. Glial cell-derived neurotrophic factor (GDNF) suppresses lactacystin-induced endoplasmic reticulum stress (ERS). **A** and **B,** Lactacystin treatment for 5 hours results in an increased expression of the ERS-related genes, such as Bip, Chop, and Xbp1. GDNF treatment suppresses the increased gene expression in a dose-dependent manner. NC, normal control; LC, lactacystin treatment; GC, GDNF control; G10LC, GDNF 10 ng/mL pretreatment in lactacystin; G100LC, GDNF 100 ng/mL pretreatment in lactacystin. **C,** The increase of Chop protein level in dopamine (DA) neurons after 24-hour treatment of lactacystin is also suppressed by GDNF treatment. **D,** Induced Chop protein is mainly located in the nuclei. *Arrows:* CHOP labeled by tetramethylrhodamine isothiocyanate (TRITC); *arrowheads:* tyrosine hydroxylase (TH) labeled by fluorescein isothiocyanate (FITC). GLC, GDNF pretreatment. $^{*}p < .05$, $^{**}p < .01$ vs controls; $^{\#}p < .05$, $^{\#}p < .01$ vs Vehicle; $^{\ddagger}p < .05$ vs GDNF 10 ng/mL group by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison. Data are from at least three experiments and are presented as mean ± standard error of the mean (*SEM*). Scale bar = 25 μm.

evidence showing that caspase-3 was activated in DA neurons in response to proteasome inhibition.

It is postulated that GDNF may protect neuron survival via several pathways, such as the activation of multiple signaling relevant to cell survival (15, 27), the inhibition of apoptosis (18,19), and the suppression of oxidative stress (17) and ERS (28). In this model, we observed that GDNF attenuated the increase in the ERS-related gene expression

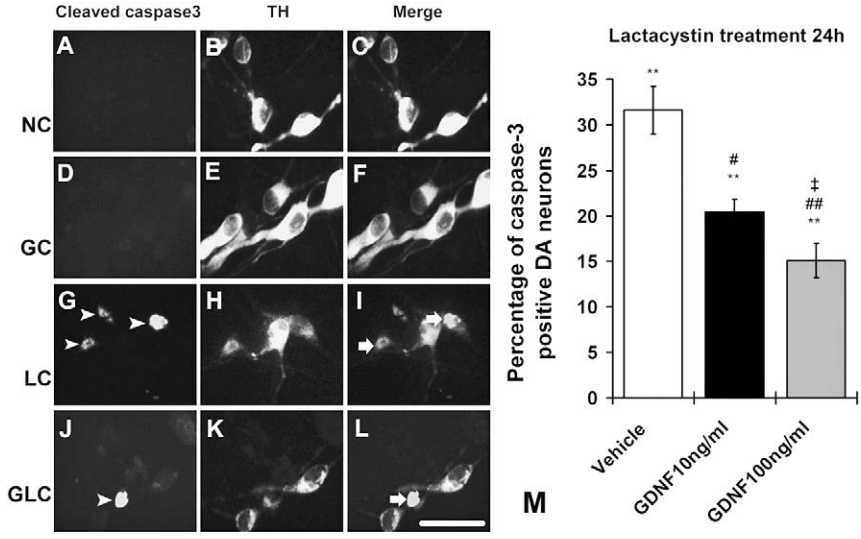

Figure 4. Glial cell-derived neurotrophic factor (GDNF) suppresses lactacystin-induced caspase-3 activation in dopamine (DA) neurons. Compared to the normal control group (NC) (**A–C**) and the GDNF control group (GC) (**D–F**), lactacystin treatment (LC) (5 μM, 24 hours) leads to a remarkable activation of caspase-3 in DA neurons (**G–I,** *arrow*). A significant blockade against the activation of caspase-3 is shown in the GDNF pretreatment group (GLC, **J–L,** GDNF 10 ng/mL) in a dose-dependent manner (**M**). Activated caspase-3 immunolabeled by tetramethylrhodamine isothiocyanate (TRITC) (**A, D, G, J,** *arrowheads*); tyrosine hydroxylase (TH) immunolabeled by fluorescein isothiocyanate (FITC) (**B, E, H, K**); $^{**}p < .01$ vs controls; $^{\#}p < .05$, $^{\#\#}p < .01$ vs Vehicle; $^{\ddagger}p < .05$ vs GDNF 10 ng/mL group by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison. Data are from three experiments and are presented as mean ± standard error of the mean (*SEM*). Scale bar = 25 μm.

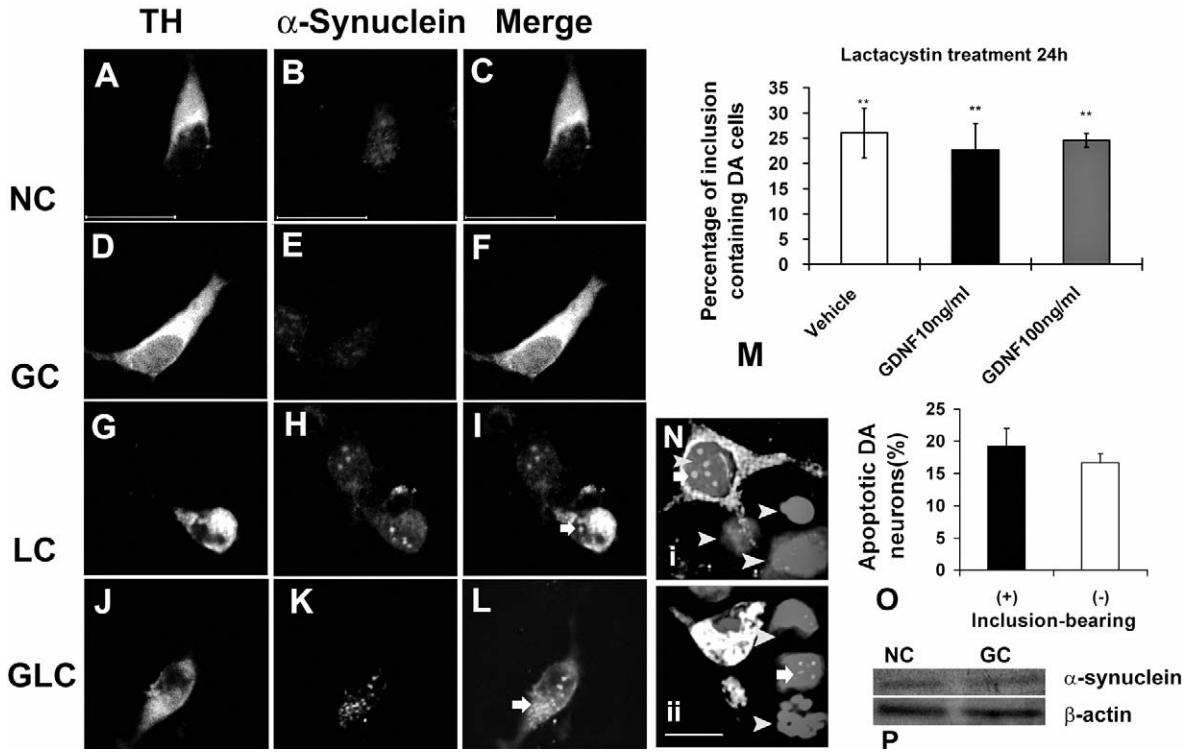

Figure 5. Glial cell-derived neurotrophic factor (GDNF) does not affect lactacystin-induced inclusion in dopamine (DA) neurons. Compared to the normal control group (NC; **A–C**) and the GDNF control group (GC) (**D–F**), lactacystin treatment (5 μM, 24 hours) induces inclusion-like bodies stained with α-synuclein in DA neurons (LC; **G–I,** *arrow* in I). The percentage of the inclusion-containing DA neurons is unaffected by GDNF pretreatment (GLC; **J–L,** *arrow* in L; **M**). **N,** α-synuclein-positive inclusion-like bodies also appear in the nuclei of some DA neurons (i, *arrow*) and non-DA cells (ii, *arrow*) following lactacystin treatment. Tyrosine hydroxylase (TH)-immunolabeled with fluorescein isothiocyanate (FITC) (A, D, G, J, N); α-synuclein immunolabeled with tetramethylrhodamine isothiocyanate (TRITC) (B, E, H, K, N); nuclei counterstained with Hoechst (N, *arrowheads*). **O,** There is no significant difference in the apoptotic rate between DA cells with and without inclusions by independent *t* test. **P,** GDNF treatment alone (GC; 10 ng/mL) for 24 hours does not affect the protein level of α-synuclein by independent *t* test. $^{**}p < .01$ vs control by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison. Data are from three experiments and are presented as mean ± standard error of the mean (*SEM*). Scale bar in (A–L) = 20 μm; in (N) =10 μm.

and Chop protein level as well as the activation of caspase-3 while preventing DA neuron loss. These lines of evidence strongly suggest that GDNF exerts its protection by inhibiting ERS and caspase-3 activation. However, GDNF only partially prevented DA neuron loss and the caspase-3 activation; this finding was similar to findings in a 6-hydroxydopamine–treated nigral cell injury model (19) and in a mutated α-synuclein gene-transfected mouse model (29). Two possible factors may contribute to the partial protection. First, lactacystin can cause the accumulation of α-synuclein and the over-translocation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which may disrupt those pathways crucial for GDNF-related protection (26,29,30). Second, proteasome impairment can inhibit protein synthesis (14), which is necessary for GDNF to carry out its protective action (31).

So far, the role of inclusions in DA neurodegeneration remains unclear (2,8). Several pathogenic factors have been linked to the formation of α-synuclein inclusions, including gene mutations (29), UPS impairment (2,4,6–8,22,25), oxidative stress, and endogenous DA (32). It has been reported that GDNF can inhibit oxidative stress (17) and up-regulate the endogenous DA levels (29,33). Therefore, we finally tested whether the lactacystin-induced α-synuclein inclusions were involved in neuron injury and whether GDNF interfered with the inclusion formation. Our findings indicate that inclusions in DA neurons did not have a significant impact on lactacystin-induced apoptosis, and GDNF treatment did not affect the α-synuclein protein levels and inclusions. These findings support the previous report that inclusion formation in vitro is independent of neuronal injury (8,25). Further study on animal models of PD may be helpful to clarify these findings.

Surprisingly, in the UPS impairment cellular model, we found α-synuclein inclusion-like granules in the nuclei of some VM cells. This result is similar to a recent report that an increased intranuclear α-synuclein was detected by an antibody against the C terminus of α-synuclein but not the antibody against the N terminus in oxidatively stressed DA neurons (34). The mechanisms underlying the formation of intranuclear inclusions and their role in the pathology of the UPS impairment model need further clarification.

Conclusion

Our results may provide useful information for identifying molecules and signal pathways relevant to DA

neurodegeneration and for assessing the value of GDNF as a therapeutic drug for the treatment of PD.

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