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RESEARCH ARTICLE

In vitro neuroprotective effects of ciliary neurotrophic factor on dorsal root ganglion neurons with glutamate-induced neurotoxicity

Shu-yun Wen[1, 2], Ai-min Li[3], Kuan-qing Mi[4], Rui-zheng Wang[4], Hao Li[5], Hua-xiang Liu[2], Yi Xing[1, *]
1 Department of Neurosurgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong Province, China
2 Department of Rheumatology, Qilu Hospital, Shandong University, Jinan, Shandong Province, China
3 Department of Rheumatology, Qingdao Fifth People's Hospital, Qingdao, Shandong Province, China
4 Department of Neurosurgery, Jinan Fifth People's Hospital, Jinan, Shandong Province, China
5 Department of Orthopedics, Qilu Hospital, Shandong University, Jinan, Shandong Province, China

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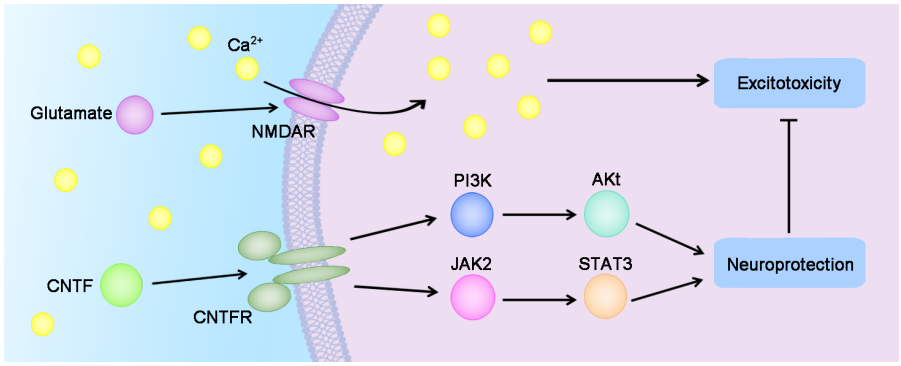
Graphical Abstract

Ciliary neurotrophic factor (CNTF) alleviates glutamate-induced excitotoxicity in cultured dorsal root ganglion



***Correspondence to:**
Yi Xing, M.D., Ph.D.,
xingyi2016@yahoo.com.

orcid:
0000-0001-7594-8398
(Yi Xing)

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Abstract

Ciliary neurotrophic factor has neuroprotective effects mediated through signal transducer and Janus kinase (JAK) 2/activator of transcription 3 (STAT3) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways. Whether ciliary neurotrophic factor is neuroprotective for glutamate-induced excitotoxicity of dorsal root ganglion neurons is poorly understood. In the present study, the *in vitro* neuroprotective effects of ciliary neurotrophic factor against glutamate-induced excitotoxicity were determined in a primary culture of dorsal root ganglion neurons from Wistar rat embryos at embryonic day 15. Whether the JAK2/STAT3 and PI3K/Akt signaling pathways were related to the protective effects of ciliary neurotrophic factor was also determined. Glutamate exposure inhibited neurite outgrowth, cell viability, and growth-associated protein 43 expression and promoted apoptotic neuronal cell death, all of which were reversed by the administration of exogenous ciliary neurotrophic factor. Additionally, preincubation with either JAK2 inhibitor AG490 or PI3K inhibitor LY294002 blocked the neuroprotective effect of ciliary neurotrophic factor. These data indicate that the two pathways JAK2/STAT3 and PI3K/Akt play major roles in mediating the *in vitro* neuroprotective effects of ciliary neurotrophic factor on dorsal root ganglion neurons with glutamate-induced neurotoxicity.

Key Words: *nerve regeneration; ciliary neurotrophic factor; JAK2/STAT3; PI3K/Akt; glutamate; neuron; excitotoxicity; neuroprotection; growth-associated protein 43; neurite outgrowth; dorsal root ganglion; neural regeneration*

Introduction

Ciliary neurotrophic factor (CNTF), belonging to the interleukin-6 cytokine family, is widely known as a neurocytokine that exerts multiple effects on a broad range of neurons in the central and peripheral nervous systems (Siegel et al., 2000; Sango et al., 2008; Lee et al., 2017). The upregulation of CNTF has a protective effect after spinal cord injury (Chen et al., 2015; Feng et al., 2017; Hodgetts and Harvey, 2017). CNTF expressed in local spinal nerves is a likely trigger of corticospinal tract axon sprouting (Jin et al., 2015). As a neurotrophic factor, CNTF promotes the long-distance regrowth of severed optic nerve fibers after intracranial injury (Vieira et al., 2009; Lee et al., 2016; LeVaillant et al., 2016; Yin et al., 2016; Beach et al., 2017; Cen et al., 2017; Jindal

et al., 2017; Zhou et al., 2017). Recently, CNTF has gained interest because of its key role in the regeneration of injured peripheral nerves (Barbon et al., 2016; Lee et al., 2017). CNTF is critically important for promoting the survival of postmitotic neurons and the genesis and differentiation of neurons in the immature dorsal root ganglion (DRG) by stimulating neurons to release mitogenic factors (Hapner et al., 2006). These are the key functions of CNTF to maintain the normal development of different subpopulations of neurons in DRG. Both small and large subpopulations of DRG neurons are supported by neurotrophic actions derived from CNTF (Sango et al., 2008). A previous *in vitro* study reported abundant CNTF mRNA and protein expressed in neurons in a dissociated DRG cell culture. CNTF was also expressed in DRG neuronal cell bodies and in regenerating neurites, suggesting that the synthesis site is at the neuronal cell body and that peripheral CNTF in neurites is transported from the neuronal cell bodies over time during cell culture (Sango et al., 2007). The upregulation of CNTF in DRG suggested it had a restorative effect after sciatic nerve crush injury in mice (Gan et al., 2014). Chemical acellular allogeneic nerve grafts combined with CNTF repaired sciatic nerve defects in rats. Both *in vitro* and *in vivo* experiments revealed that the induction of DRG axonal regeneration by CNTF was mediated by the upregulation of a target gene, collapsin response-mediator protein 4 (Jang et al., 2010; Selvaraj and Sendtner, 2013). CNTF was reported to have neuroprotective effects on DRG neurons under various conditions (Hapner et al., 2006; Liu et al., 2014). Several recent studies of CNTF targeting on developing (immature) and adult (mature) DRG neurons confirmed that CNTF is a critical neurotrophic factor that maintains normal DRG development and DRG neuronal regeneration after injury (Zhou et al., 2008; Gu et al., 2010; Kammouni et al., 2012; Saleh et al., 2013; Chowdhury et al., 2014).

Glutamate (Glu) is an excitatory neurotransmitter that plays a major role in the nervous system. The dysregulation of Glu may induce seizures (Takanashi et al., 2015). The Glu receptors are also related to ischemia induced neurotoxicity (Brassai et al., 2015). Glu is also shown to be important for primary afferent neurons (Brumovsky et al., 2011). Glu also causes excitatory neurotoxicity of cultured DRG neurons (Liu et al., 2011, 2012; Li et al., 2013a, b). Neuronal cell damage or cell death induced by excitatory excitotoxicity is caused by high permeability to calcium *via* activation of NMDA receptor subtypes (Del Río et al., 2008). Furthermore, CNTF had a neuroprotective role after neuronal injury through the activation of PI3K/Akt signaling and signal transducer and activator of transcription 3 (STAT3) signaling *in vitro* (Leibinger et al., 2014; Liu et al., 2014; Gu et al., 2016). However, whether the neurotrophic factor CNTF is neuroprotective for developing DRG neurons from later gestational age with Glu challenge *in vitro* is unclear. Based on the above research, we hypothesized that: (1) CNTF has neuroprotective effects on DRG neurons with Glu-induced excitotoxicity; and (2) the neuroprotective effects of CNTF are mediated through the activation of JAK2/STAT3 and PI3K/Akt signaling pathways. This is the first report to investigate the effects of CNTF and its downstream signaling pathways on primary sensory neuronal protection by establishing an *in vitro* model of dissociated DRG neurons. The data from this experimental research may offer novel therapeutic options on relieving Glu-induced excitotoxicity by the administration of CNTF under distinct neuropathological conditions.

Materials and Methods

DRG culture preparations

All animals used in this study were from the Experimental Animal Center of Shandong University of China (animal license No. SCXK (Lu) 20130009). The study protocol was approved by the Ethics Committee for Animal Experimentation of the Shandong University (approval No. 201402260001). The experimental procedure followed the *United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1986)*.

Male or female Wistar rat embryos at embryonic day 15 were dissected for DRG neuronal culture. To obtain a suspension of DRG cells, DRG explants were processed for digestion (0.25% trypsin; Sigma, St. Louis, MO, USA), centrifugation (1,000 r/min, 5 minutes), trituration, and then filtered with a 130-μm filter. Before plating, the number of cells in the cell suspension was counted to determine the cell density. Dissociated DRG cells were plated at a density of 5×10^5 cells/mL for PCR or western blot assay and 1×10^5 cells/well for fluorescence labeling in 24-well plates. DRG cells were plated at 5×10^4 cells/well in a volume of 0.1 mL in 96-well plates for cell viability measurement. After plating, all cells were maintained in Dulbecco's Modified Eagle's Medium/F-12 (HyClone Laboratories, Logan, UT, USA), containing 5% fetal bovine serum, 2% B-27 supplement (Gibco, Grand Island, NY, USA), and L-glutamine (0.1 mg/mL, Sigma), and incubated at 37°C in a 5% CO_2 incubator for 24 hours and then maintained in culture medium containing cytosine arabinoside(5 μg/mL; Solarbio Life Sciences, Beijing, China) for an additional 24 hours to inhibit the growth of non-neuronal cells. Then, the culture medium was removed and DRG cells were exposed to various agents for an additional 24 hours before final examination.

Incubation of DRG neurons under different experimental conditions

At 48 hours of culture, dissociated cultured DRG cells were treated for another 24 hours. The incubation paradigm in this study was as follows: (1) Glu group: Glu 200 μM incubation for 24 hours; (2) Glu + CNTF group: Glu 200 μM plus CNTF 25 ng/mL incubation for 24 hours; (3) Glu + CNTF + AG490 group: JAK2 inhibitor AG490 (10 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; (4) Glu + CNTF + LY294002 group: PI3K inhibitor LY294002 (20 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; and (5) Control group: neurons were cultured in culture medium alone without any other treat-

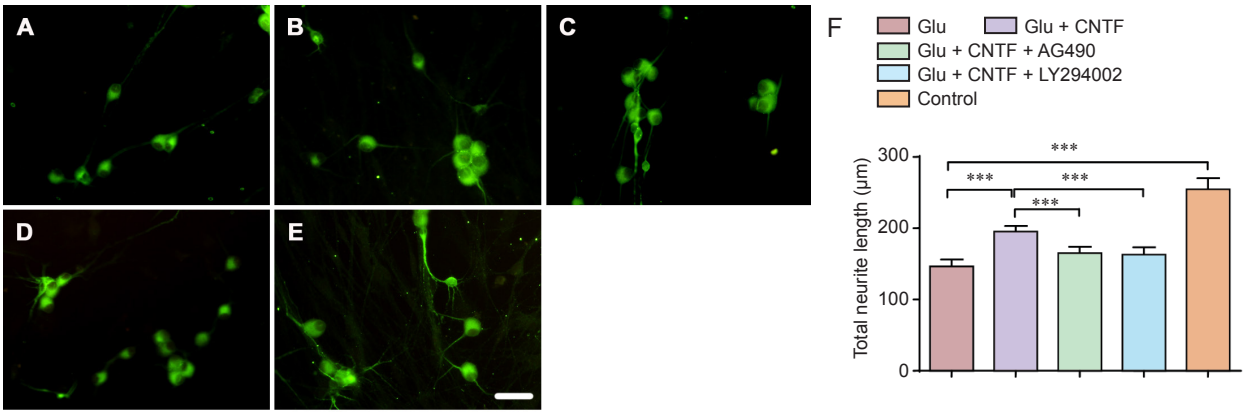


Figure 1 Effects of CNTF on the growth of dorsal root ganglion neurons induced by Glu.
(A–E) Total neurite length of Glu, Glu + CNTF, Glu + CNTF + AG490, Glu + CNTF + LY294002 and control groups (fluorescent microscopy). Scale bar: 50 μm. (F) Histogram of neurite length quantification. ***$P < 0.001$ (mean ± SD, $n = 5$, one-way analysis of variance and Student-Newman-Keuls test). Each condition was repeated three times. Glu group: Glu 200 μM incubation for 24 hours; Glu + CNTF group: Glu 200 μM plus CNTF 25 ng/mL incubation for 24 hours; Glu + CNTF + AG490 group: JAK2 inhibitor AG490 (10 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; Glu + CNTF + LY294002 group: PI3K inhibitor LY294002 (20 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; control group: neurons were cultured in culture medium alone without other treatments. Glu: Glutamate; CNTF: ciliary neurotrophic factor; AG: AG490; LY: LY294002.

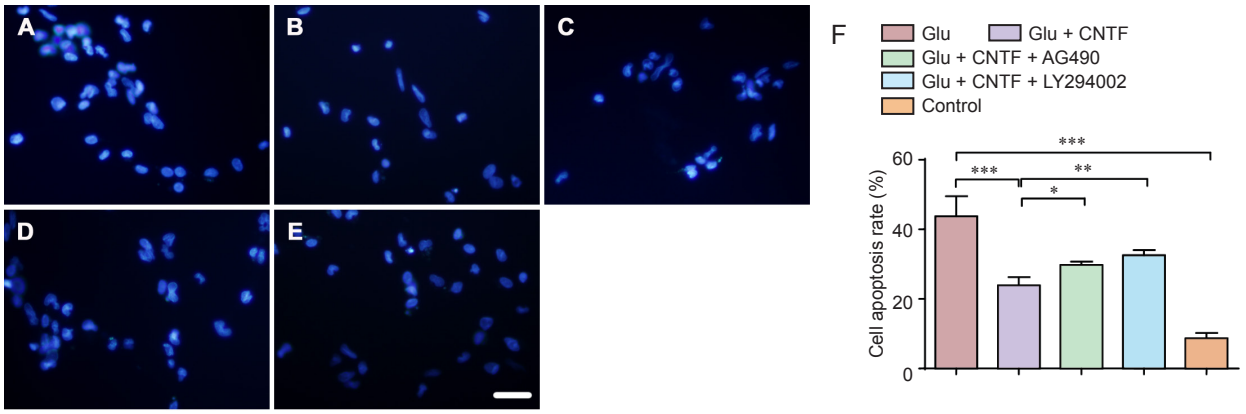


Figure 3 Effects of CNTF on cell apoptosis in dorsal root ganglion neurons induced by Glu.
(A–E) Neuronal apoptosis of Glu, Glu + CNTF, Glu + CNTF + AG490, Glu + CNTF + LY294002 and control groups (fluorescent microscopy). Condensed or fragmented nuclei were defined as apoptosis after Hoechst 33342 staining. Scale bar: 50 μm. (F) Histogram of the percentage of apoptotic neurons. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (mean ± SD, $n = 5$, one-way analysis of variance and Dunnett's T3 test). Each condition was repeated three times. Glu group: Glu 200 μM incubation for 24 hours; Glu + CNTF group: Glu 200 μM plus CNTF 25 ng/mL incubation for 24 hours; Glu + CNTF + AG490 group: JAK2 inhibitor AG490 (10 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; Glu + CNTF + LY294002 group: PI3K inhibitor LY294002 (20 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; control group: neurons were cultured in culture medium alone without other treatments. Glu: Glutamate; CNTF: ciliary neurotrophic factor; AG: AG490; LY: LY294002.

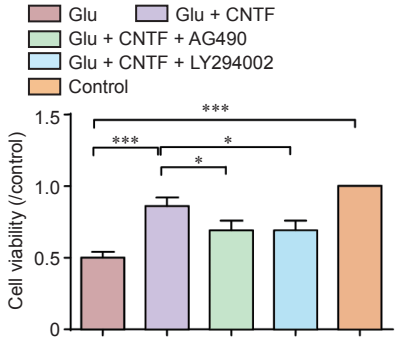


Figure 2 Effects of CNTF on cell viability of dorsal root ganglion neurons induced by Glu.
Cell viability was determined by the absorbance value in each group compared with the control group (standardized to 1). *$P < 0.05$, ***$P < 0.001$ (mean ± SD, $n = 5$, one-way analysis of variance and Dunnett's T3 test). Each condition was repeated three times. Glu group: Glu 200 μM incubation for 24 hours; Glu + CNTF group: Glu 200 μM plus CNTF 25 ng/mL incubation for 24 hours; Glu + CNTF + AG490 group: JAK2 inhibitor AG490 (10 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; Glu + CNTF + LY294002 group: PI3K inhibitor LY294002 (20 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; control group: neurons were cultured in culture medium alone without other treatments. Glu: Glutamate; CNTF: ciliary neurotrophic factor; AG: AG490; LY: LY294002.

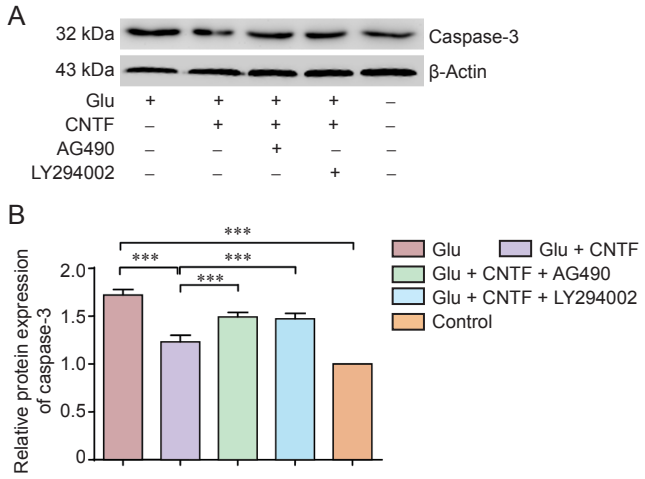


Figure 4 Effects of CNTF on caspase-3 level in dorsal root ganglion neurons induced by Glu.

Gray-scale values of immunoreactive bands of the target protein compared with the internal control protein β-actin to obtain a proportion for each group. The proportion value in each group was compared with the control group (standardized to 1). (A) Immunoblotting bands of caspase-3. (B) Histogram for caspase-3 levels. ***P < 0.001 (mean ± SD, n = 5, one-way analysis of variance and Student-Newman-Keuls test). Each condition was repeated three times. Glu group: Glu 200 μM incubation for 24 hours; Glu + CNTF group: Glu 200 μM plus CNTF 25 ng/mL incubation for 24 hours; Glu + CNTF + AG490 group: JAK2 inhibitor AG490 (10 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; Glu + CNTF + LY294002 group: PI3K inhibitor LY294002 (20 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; control group: neurons were cultured in culture medium alone without other treatments. Glu: Glutamate; CNTF: ciliary neurotrophic factor; AG: AG490; LY: LY294002.

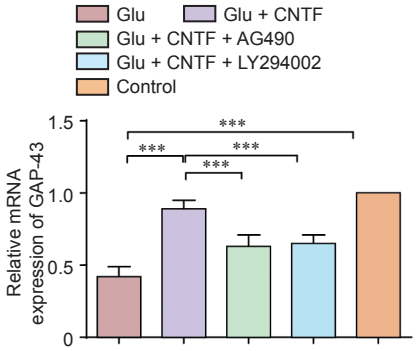
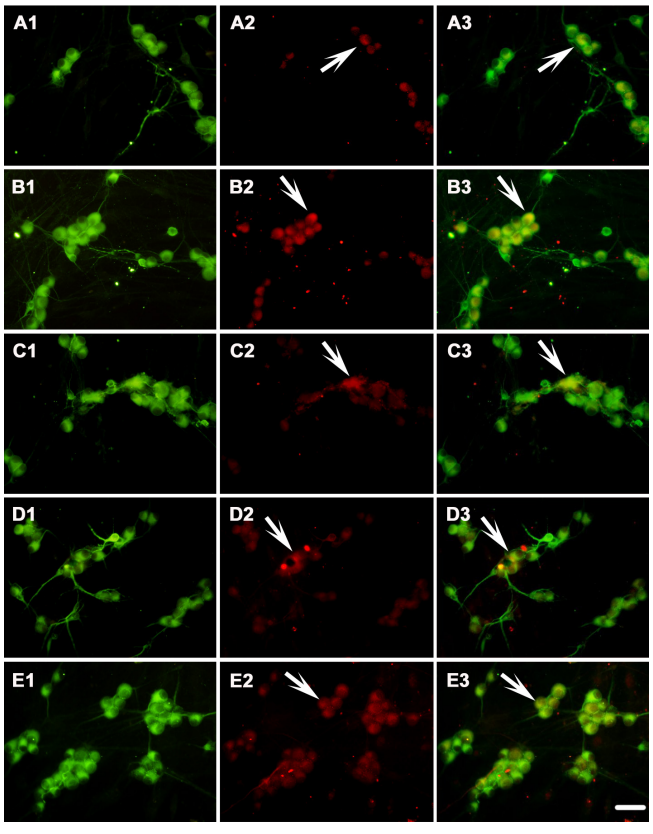


Figure 5 Effects of CNTF on GAP-43 mRNA levels in dorsal root ganglion neurons induced by Glu.

The relative transcript amount of the target gene was normalized to that of GAPDH using the $2^{-\Delta\Delta Ct}$ method as the fold change of each target gene. When the fold change of each target gene was obtained, the final results of real-time polymerase chain reaction were expressed as the ratio of fold change of target mRNA of that to the control group. ***P < 0.001 (mean ± SD, n = 5, one-way analysis of variance and Student-Newman-Keuls test). Each condition was repeated three times. Glu group: Glu 200 μM incubation for 24 hours; Glu + CNTF group: Glu 200 μM plus CNTF 25 ng/mL incubation for 24 hours; Glu + CNTF + AG490 group: JAK2 inhibitor AG490 (10 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; Glu + CNTF + LY294002 group: PI3K inhibitor LY294002 (20 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; control group: neurons were cultured in culture medium alone without other treatments. Glu: Glutamate; CNTF: ciliary neurotrophic factor; GAP-43: growth-associated protein 43; AG: AG490; LY: LY294002.



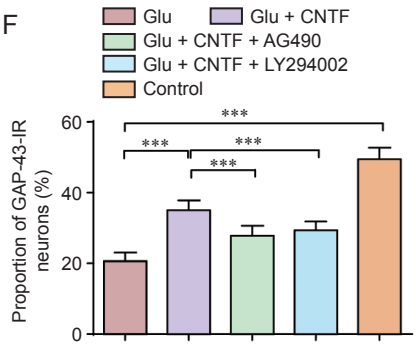


Figure 6 Effects of CNTF on the GAP-43 immunoreactivity of dorsal root ganglion neurons induced by Glu.

(A–E) GAP-43 immunoreactivity in DRG neurons in Glu, Glu + CNTF, Glu + CNTF + AG490, Glu + CNTF + LY294002 and control groups (fluorescent microscopy). Scale bar: 50 μm. (F) The percentage of GAP-43-immunoreactive (IR) neurons. ***P < 0.001 (mean ± SD, n = 5, one-way analysis of variance and Student-Newman-Keuls test). Each condition was repeated three times. Arrows represent GAP-43-IR neurons. Glu group: Glu 200 μM incubation for 24 hours; Glu + CNTF group: Glu 200 μM plus CNTF 25 ng/mL incubation for 24 hours; Glu + CNTF + AG490 group: JAK2 inhibitor AG490 (10 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; Glu + CNTF + LY294002 group: PI3K inhibitor LY294002 (20 μM) incubation for 30 minutes followed by treatment with Glu 200 μM plus CNTF 25 ng/mL for 24 hours; control group: neurons were cultured in culture medium alone without other treatments. Glu: Glutamate; CNTF: ciliary neurotrophic factor; GAP-43: growth-associated protein 43; AG: AG490; LY: LY294002.

ment. The vehicle solution used in all groups of this *in vitro* experiment was normal culture medium, which did not interfere the observation results. Five samples were examined in each group (n = 5). The concentrations and time point of the inhibitor administration (AG490 and LY294002) were based on previous studies (Li et al., 2013b; Liu et al., 2014) and slightly modified according to our preliminary experiment. All cultures mentioned above were incubated at 37°C in a humidified atmosphere of 5% CO_2-air.

Neurite length measurement

After incubation under different experimental conditions for 24 hours, microtubule-associated protein 2 (MAP2) fluorescence labeling was used as a marker to monitor the elongation or retraction of growing neurites. The primary antibody in this fluorescence labeling protocol was mouse monoclonal anti-MAP2 (1:400; Abcam, Cambridge, MA, USA) and the secondary antibody was goat anti-mouse conjugated to Cy2 (1:200; Abcam). Before incubation with the primary antibody, the cells were processed for fixation, non-specific site blocking, and permeabilization. Three washes with 0.1 M phosphate buffered saline were carried out between each step before covering with a coverslip. Slide observation and image capture were performed using a fluorescent microscope (BX63; Olympus, Tokyo, Japan) with a digital photo processing system (CellSens Dimension, version 1.6; Olympus). The neurite length of single neurons was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Twenty neurons in each coverslip (each group included three coverslips) were randomly selected for the measurement of neurite length. The average neurite length was calculated.

Assessment of neuronal cell viability

After incubation under different experimental conditions for 24 hours, neuronal cell viability was assessed using a WST-1 kit (Beyotime Institute of Biotechnology, Haimen, China). The reagent (10 μL) was applied to each well of 96-well plates. Before application of the reagent (10 μL), each well contained 100 μL culture medium. The incubation time for the reagent was 2 hours. Absorbance values were measured at 450 nm with a Microplate Photometer (Multiscam MK3, Thermo Labsystems, Waltham, MA, USA). The cell viability in other experimental groups was compared with the cell viability of the control group, which was used as a standard. The cell viability in each group was obtained relative to the control group value.

Measurement of DRG neuron apoptosis by Hoechst 33342 staining

After incubation under different experimental conditions for 24 hours, neuronal apoptosis was evaluated by staining with Hoechst 33342, which directly shows morphological alterations of the nucleus in each neuron. Nuclear DNA was labeled by Hoechst 33342 (Sigma). After termination of the culture procedure at the designed incubation time point, neurons were grown on the surface of coverslips fixed with 4% paraformaldehyde for 10 minutes. Then, the neurons were stained with Hoechst 33342 (5 μg/mL) for 5 minutes in the dark to preserve fluorescence intensity. Apoptotic neurons showed shrunken or condensed nuclei after Hoechst 33342 staining. Neurons with shrunken or condensed nuclei in five visual fields (400×) in the central part of each coverslip were counted as apoptotic neurons in each sample. Simultaneously, all neurons in the same five visual fields were also counted as the total number of neurons. The ratio of apoptotic neurons to total number of neurons was determined.

Measurement of DRG neuron apoptosis by caspase-3 expression

After incubation under different experimental conditions for 24 hours, neuron apoptosis was analyzed by detecting caspase-3 levels by western blot assay. The western blot protocol for caspase-3 was performed using rabbit anti-caspase-3 polyclonal IgG (1:1,000; Cell Signaling Technology, Danvers, MA, USA) or mouse anti-β-actin monoclonal IgG (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the primary antibodies. The secondary antibodies were goat anti-rabbit IgG-horseradish peroxidase (HRP) (1:6,000; Beijing Sequoia Jinqiao Biological Technology Co., Ltd., Beijing, China) or goat anti-mouse IgG-HRP (1:3,000; Beijing Dingguochangsheng Biotechnology Co., Ltd., Beijing, China). The gray-scale values of immunoreactive bands were obtained by ImageJ (National Institutes of Health). The value of the target protein was compared with the internal control protein β-actin. Each experimental group was compared relative to the control group value (1.00) to obtain a ratio.

Determination of growth-associated protein 43 (GAP-43) mRNA levels with real-time polymerase chain reaction (PCR)

After incubation under different experimental conditions for 24 hours, real-time PCR was performed to detect GAP-43 mRNA expression, using GAPDH mRNA expression as an internal control. Total RNA isolation was performed with TRIzol (Takara Biotechnology, Dalian, China). Synthesis of cDNA was performed using a cDNA synthesis kit (Thermo Scientific Molecular Biology, Lithuania, EU, USA) following the manufacturer's instructions. Synthetic oligonucleotide primer sequences for GAP-43 and GAPDH are shown in **Table 1**. A comparative cycle of the threshold fluorescence (Ct) method was used (Thermo Scientific Molecular Biology). The relative transcript amount of the target gene was normalized to that of GAPDH using the $2^{-\Delta\Delta Ct}$ method based on a previous study (Pfaffl, 2001). The fold change of each target gene was calculated. The final results of real-time PCR were expressed as a ratio of fold change of target mRNA of that to the control group.

Double fluorescent labeling to assess GAP-43 and MAP2 expression

After incubation under different experimental conditions for 24 hours, double fluorescence labeling for GAP-43 and MAP2 expression was performed using rabbit polyclonal anti-GAP-43 (1:500; Abcam, Cambridge, MA, USA) or mouse

Table 1 Primer sequences of target genes

Genes	Primer sequences	Product size (bp)
GAP-43	Sense: 5'-AAG AAG GAG GGA GAT GGC TCT-3' Antisense: 5'-GAG GAC GGC GAG TTA TCA GTG-3'	197
GAPDH	Sense: 5'-GGC ACA GTC AAG GCT GAG AAT G-3' Antisense: 5'-ATG GTG GTG AAG ACG CCA GTA-3'	143

GAP-43: Growth-associated protein 43; GAPDH: glyceraldehyde phosphate dehydrogenase.

monoclonal anti-MAP2 (1:400; Abcam) as the primary antibodies. The secondary antibodies were goat anti-rabbit conjugated to Cy3 (1:200; Abcam) or goat anti-mouse conjugated to Cy2 (1:200; Abcam). All procedures were performed in the dark to preserve fluorescence. Before incubation with the primary antibody, the cells were processed for fixation, non-specific site blocking, and permeabilization. Three washes with 0.1 M phosphate buffered saline were carried out between each step before covering with a coverslip. Slide observation and image capture were performed under a fluorescent microscope (Olympus BX63, Olympus Corporation, Tokyo, Japan) with a digital photo processing system (cellSens Dimension, version 1.6, Olympus Corporation, Tokyo, Japan).

Quantification of GAP-43-immunoreactive (IR) neurons

GAP-43-IR neurons were counted in a 200× visual field with red fluorescence. Five adjacent, but not overlapping, visual fields in the center of the coverslip were selected for GAP-43-IR neuronal counting. The same five visual fields with green fluorescence were used for MAP2-IR neuronal counting. Because each neuron expressed MAP2, the amount of MAP2-IR neuron was used as the total amount of DRG neurons. The proportion of GAP-43-IR neurons was calculated relative to the total number of neurons.

Statistical analysis

Quantitative data were expressed as the mean ± SD. Data were analyzed using SPSS 19.0 software (SPSS, Chicago, IL, USA). All quantitative data were verified by normality testing. All data were normally distributed and were analyzed by analysis of variance followed by Dunnett's T3 test (heterogeneity of variance) or Student-Newman-Keuls test (homogeneity of variance). A P value < 0.05 was considered statistically significant.

Results

Neurite outgrowth

After termination of the culture procedure at the designated incubation time point, the length of neurites in each neuron was quantified using ImageJ software after processing with MAP2 fluorescence labeling. MAP2 expression was visible by staining distinct growing neurites and neuronal cell bodies. MAP2 fluorescence labeling marked growing neurites. The length of neurites in each neuron was decreased significantly after treatment with Glu (P < 0.001). CNTF administration partially reversed the neurite retraction induced by Glu neurotoxicity (P < 0.001). Preincubation with either JAK2 inhibitor AG490 (P < 0.001) or PI3K inhibitor LY294002 (P < 0.001) blocked the CNTF effects on neurite outgrowth (**Figure 1**).

Neuronal cell viability

The cell viability under different experimental conditions was monitored using a WST-1 kit after termination of the culture procedure at the designated incubation time point. The cell viability was decreased significantly after treatment with Glu (P = 0.000). Treatment with exogenous CNTF partially reversed the Glu-induced decrease in cell viability (P = 0.000). Preincubation with the JAK2 inhibitor AG490 (P = 0.031) or PI3K inhibitor LY294002 (P = 0.031) blocked the promoting effects of CNTF on cell viability (**Figure 2**).

Apoptosis of DRG neurons

The apoptotic rate of neuronal cells was determined by Hoechst 33342 staining after termination of the culture procedure at the designated incubation time point. Glu exposure increased the neuronal cell apoptotic rate (P = 0.001), which was partially inhibited by CNTF treatment (P = 0.005). Preincubation with the JAK2 inhibitor AG490 (P = 0.023) or PI3K inhibitor LY294002 (P = 0.002) blocked the inhibitory effects of CNTF on apoptosis (**Figure 3**).

Caspase-3 levels in DRG neurons

To investigate the apoptosis of DRG neurons under each experimental condition, caspase-3 levels in DRG neurons were quantified by western blot assay. Glu exposure increased the caspase-3 levels in DRG neurons (P < 0.001), which was partially decreased by CNTF treatment (P < 0.001). Preincubation with the JAK2 inhibitor AG490 (P < 0.001) or PI3K inhibitor LY294002 (P < 0.001) blocked the inhibitory effects of CNTF on caspase-3 expression (**Figure 4**).

GAP-43 mRNA expression in DRG neurons

After various treatments, the mRNA levels of GAP-43 were analyzed by real-time PCR. The mRNA levels of GAP-43 were decreased after Glu treatment (P < 0.001). Application of exogenous CNTF in the cultures increased the mRNA levels of GAP-43 in the presence of Glu (P < 0.001). Preincubation with the JAK2 inhibitor AG490 (P < 0.001) or PI3K inhibitor LY294002 (P < 0.001) blocked the increase in GAP-43 mRNA expression by CNTF. These results suggested that CNTF regulated protein expression at the transcriptional level by activating the JAK2/STAT3 and PI3K/Akt signaling pathways (**Figure 5**).

GAP-43 expression in DRG neurons

To determine the GAP-43 expression *in situ* in DRG neurons, double fluorescence labeling of GAP-43 and MAP2 was performed to quantify the proportion of GAP-43 positive neurons under various experimental conditions. Glu exposure decreased the proportion of GAP-43-IR neurons (P

< 0.001). The application of exogenous CNTF in DRG cultures increased the proportion of GAP-43-IR neurons, which was decreased by Glu exposure ($P < 0.001$). Preincubation with the JAK2 inhibitor AG490 ($P < 0.01$) or PI3K inhibitor LY294002 ($P < 0.01$) blocked the increased proportion of GAP-43-IR neurons mediated by CNTF. These results suggest that CNTF partially rescues the neuronal regeneration capacity inhibited by Glu exposure and the effects of CNTF on the increased proportion of GAP-43-IR neurons by activating the JAK2/STAT3 and PI3K/Akt signaling pathways (**Figure 6**).

Discussion

CNTF is a multifunctional neurocytokine and is involved in the genesis, differentiation, and survival of various kinds of neurons, including primary sensory neurons in the central and peripheral nervous systems. CNTF is critical for promoting the survival of postmitotic neurons in DRG. CNTF also regulates earlier events in the developing DRG, related to the mitogenesis of DRG progenitor cells and the differentiation of immature DRG neurons. The JAK2/STAT3 and PI3K/Akt signaling pathways are involved in neurogenesis, proliferation, differentiation, and neuroprotection under various conditions. We determined the neuroprotective effect of CNTF against excitotoxicity in DRG neurons by administering CNTF to dissociated DRG cultures with Glu challenge. The present study is the first to investigate the neuroprotective effects of CNTF on primary sensory DRG neurons with Glu-induced neurotoxicity. CNTF administration partially reversed Glu-induced neurotoxicity by promoting GAP-43 expression, increasing cell viability, enhancing neurite outgrowth, and inhibiting apoptotic neuronal cell death. Furthermore, the inhibition of JAK2/STAT3 signaling by preincubation with AG490 or PI3K/Akt signaling by preincubation with LY294002 blocked the neuroprotective actions of CNTF. These data imply the capacity of CNTF to relieve excitotoxicity.

Glu killed oligodendrocytes and neurons in the rat spinal cord. It was proposed that the activation of apoptosis is inversely correlated with the concentration of intracellular calcium in immature DRG neurons in dissociated cell cultures. Glu exposure might cause neurite retraction by inhibiting GAP-43 expression in dissociated DRG cell cultures (Li et al., 2013b). In our present study, the inhibitory actions of Glu on neurite outgrowth and cell viability and the promotion of apoptosis were partially reversed by CNTF in DRG cultures, suggesting the neuroprotective effects of CNTF on Glu-induced neurotoxicity. To elucidate the mechanisms of the neuroprotective effects of CNTF, inhibitors that block JAK2 or PI3K signaling were used in DRG neuronal cultures. The JAK2 inhibitor AG490 or PI3K inhibitor LY294002 blocked the protective effect of CNTF on neurons. These data imply that the two signaling pathways are involved in the neuroprotective effects of CNTF in distinct DRG cell cultures.

To explore further the relationship between neurite elongation and nerve regeneration marker GAP-43, GAP-43 mRNA levels and GAP-43 expression *in situ* in DRG neurons were also evaluated. GAP-43 was used as a marker or indicator to assess regeneration, active axonal sprouting, and neuronal survival under *in vivo* (Gravel et al., 2011) and *in vitro* conditions (Anand et al., 2008) and neurite elongation was strongly associated with GAP-43 levels in DRG neurons *in vitro* (Tsai et al., 2007; Li et al., 2013b). The results of the present study showed CNTF increased the proportion of GAP-43-IR neurons. This suggests that the promoting effects of CNTF on neuronal regeneration might be mediated by initiating the neurite regeneration capacity inhibited by Glu exposure. Because Glu exposure significantly decreased the proportion of GAP-43-IR neurons, Glu-induced excitotoxicity might destroy the regeneration ability of neurons sensitive to Glu stimulation. Therefore, exogenous CNTF administration may rescue the regeneration ability of excitotoxic neurons.

In conclusion, CNTF relieved the Glu-induced neurotoxicity of DRG neurons by inhibiting apoptotic neuronal cell death and initiating GAP-43 expression to increase neuronal cell viability, promoting neurite outgrowth, and finally improving neuronal living status. The JAK2/STAT3 and PI3K/Akt signaling pathways play major roles in mediating the neuroprotective effects of CNTF on DRG neurons undergoing neurotoxicity caused by excessive Glu challenge. These data imply that CNTF might be a novel therapeutic option to relieve Glu-induced excitotoxicity under distinct neuropathological conditions.

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Research ethics: *The study protocol was approved by the Ethical Committee for Animal Experimentation of the Shandong University (approval No. 201402260001). The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1986).*
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