CNTF protects neurons from hypoxic injury through the activation of STAT3<sub>pTyr<sup>705</sup></sub>

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Abstract. The aim of the present study was to investigate whether ciliary neurotrophic factor (CNTF) plays its neuroprotective role following hypoxic injury through the activation of signal transducer and activator of transcription 3 (STAT3) signaling. Firstly, to determine whether CNTF exerts its effects via STAT3 following hypoxic injury, cultured neurons from the cerebral cortex of mice were prepared and a neuronal model of hypoxia was then established. The neurons exposed to hypoxia were then pre-treated with CNTF and transfected with small interference RNA (siRNA) targeting STAT3 (STAT3 siRNA) using Polybrene, or with STAT3<sup>pTyr<sup>705</sup></sub> mutant or STAT3<sup>Ser<sup>727</sup></sub> mutant using an electroporation system. The survival, proliferation and neurite outgrowth of the neurons subjected to different treatments were also determined. RT-qPCR and western blot analysis were employed to examine the expression levels of STAT3, p-STAT3<sup>pTyr<sup>705</sup></sub> and p-STAT3<sup>Ser<sup>727</sup></sub> following treatment with CNTF and other treatments. Our results revealed that treatment with CNTF: i) protected neurons from hypoxic injury by promoting survival and neurite growth; ii) induced a significant increase in the levels of STAT3, STAT3<sup>pTyr<sup>705</sup></sub> and the STAT3<sup>pTyr<sup>705</sup></sub>/STAT3 ratio; it did not however, significantly affect the levels of STAT3<sup>Ser<sup>727</sup></sub> in the hypoxic cerebral cortex neurons. Transfection of the hypoxic neurons pre-treated with CNTF with STAT3 siRNA or STAT3<sup>pTyr<sup>705</sup></sub> neutralized the protective effects exerted by CNTF. The findings of our study thus demonstrate that CNTF protects neurons from hypoxic injury through the activation of STAT3<sup>pTyr<sup>705</sup></sub>.

Introduction

Neonatal hypoxia/ischemia and subsequent brain damage continue to be an alarming socio-sanitary problem, being considered the single-most important cause of acute mortality and chronic disability in newborns worldwide (1-3). Although improved obstetric and neonatal intensive care practices have led to increased survival, infants born very preterm are prone to disorders of cerebral development, including impaired cognition and behavior, epilepsy and cerebral palsy (4-6). Similarly, infants who suffer brain injury from hypoxia/ischemia during critical developmental periods of cerebral circuit formation are also at an increased risk of developing seizures, neuropsychiatric conditions and cognitive disorders (7). The severity of neonatal encephalopathy depends on the intensity, duration and location of the insult (8,9). Approximately 15-20% of affected newborns will die in the post-natal period and an additional 25% will develop severe and permanent neuropsychological sequelae (10). Only are a small percentage of infants with severe injury survive without any handicaps (10,11).

Ciliary neurotrophic factor (CNTF) is a member of NTF family originally isolated from chick embryo ciliary neurons, which: i) promote survival and/or differentiation in many cell types; and ii) have been demonstrated to have therapeutic potential in neurodegenerative diseases and the injured central nervous system (CNS) (12). CNTF exerts its biological functions by binding to high or low affinity receptor complexes consisting of CNTFR·gp130·LIFR or IL-6R·gp130·LIFR, respectively (13). Recent data indicate that distinct intracellular signaling pathways mediate diverse neuroprotective processes in response to CNTF. There is evidence to indicate that Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinase (MAPK)-extracellular signal-regulated protein kinase (ERK)1/2, as well as phosphatidylinositol-3-kinase (PI3K)/Akt, play important roles in promoting neuronal survival and process the outgrowth response to CNTF (14-17). STAT3 is known to modulate injury following an imbalance between pro- and anti-inflammatory cytokines in peripheral and CNS injury, rendering it a potential molecule for study. It has been demonstrated that CNTF plays a role in neural stem/progenitor cell (NSP) cell responses to hypoxia/ischemia (18). As a major transducer of CNTF-mediated neuroprotective activity, the activation of the JAK2/STAT3 axis by CNTF has been demonstrated to be...
responsible for the neuroprotective effects against the pathogenesis of Alzheimer’s disease (AD) (14). It also points towards a significant role of STAT3 signaling following micro- and astrogliosis in the pathophysiology of neonatal hypoxia-related brain injury (19). However, whether the JAK/STAT3 axis mediated by CNTF is responsible for the neuroprotective effects in hypoxic injury remains unknown.

The aim of this study was to investigate whether CNTF plays its neuroprotective role following hypoxic injury through the activation of STAT3 signaling. Firstly, to determine whether CNTF exerts its effects via STAT3 following hypoxia, cultured neurons from the cerebral cortex of mice were prepared and a neuronal model of hypoxia was established. The neurons exposed to hypoxia were pre-treated with CNTF and transfected with small interference RNA targeting STAT3 (STAT3 siRNA) using polybrene, or with STAT3

\[ p\text{-STAT3}^{\text{Tyr705}} \] mutant or STAT3

\[ p\text{-STAT3}^{\text{Ser727}} \] mutant using an electroporation system. The survival, proliferation, and neurite outgrowth of neurons subjected to different treatments were also determined. Reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis were employed to examine the expression levels of STAT3, p-STAT3

\[ p\text{-STAT3}^{\text{Tyr705}} \] and p-STAT3

\[ p\text{-STAT3}^{\text{Ser727}} \] following treatment with CNTF and other treatments.

**Materials and methods**

**Ethics statement.** Animal use and care were carried out in accordance with the animal care guidelines, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). The Ethics Committee of Harbin Medical University specifically approved this study (Permit number: 2014ME1028). All efforts were made to minimize animal suffering.

**Neuronal culture.** Pregnant mice were housed with free access to food and water and exposed to a 12-h light/dark cycle at the Animal Central of Harbin Medical University, Harbin, China. A total of 20 neonatal C57BL/6J mice (postnatal 24 h), which were purchased from Harbin Medical University, were then employed in the present study for cell culture. The mice were sacrificed by decapitation and disinfected in 75% ethanol. The hippocampus was then completely removed following craniootomy. The hippocampus was dissected into slices at a volume of 1 mm³ using an anatomic microscope in PBS and then digested for 30 min at 37°C incubator with 2 mg/ml papain (Roche Diagnostics GmbH, Mannheim, Germany) containing 2 µl/ml DNase. Termination of the digestion was performed by the addition of an equal amount of DMEM including 10% FBS and 1% penicillin-streptomycin solution. The cell suspension was centrifuged at 1,000 rpm for 10 min at 4°C. After discarding the supernatant, the cells were re-suspended in the same medium by gently pipetting up and down and seeded on a 24-well plate at a density of 2x10⁵ cells/ml. The medium was replaced with neurobasal medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 2% B27 and 1% penicillin-streptomycin solution.

**Neuronal model of hypoxia.** Hypoxia was achieved by placing the neurons in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) that was then flushed for 5 min with a gas mixture of 90% N₂, 5% CO₂ and 5% O₂. These conditions are reported by the manufacturer to render the hypoxia chamber completely purged.

**Experimental grouping.** The cultured neurons were divided into different groups as follows: i) the normal untreated neurons; ii) the hypoxia group (the neurons were exposed to hypoxia as described above); iii) the hypoxia + CNTF-treated group [CNTF (recombinant human CNTF; Novoprotein Scientific, Inc., Summit, NJ, USA) was added to the culture exposed to hypoxia at a concentration of 100 ng/ml (20)]; the iv) si-STAT3 group [neurons were exposed to hypoxia, treated with CNTF and transfected with STAT3 siRNA (hypoxia + CNTF + STAT3 siRNA)]; v) the si-STAT3 control group [neurons were exposed to hypoxia, treated with CNTF and transfected with control siRNA (hypoxia + CNTF + control STAT3 siRNA; STAT3-si-Non)]; vi) the STAT3

\[ p\text{-STAT3}^{\text{Tyr705}} \] mutant group [neurons were exposed to hypoxia, treated with CNTF and transfected with STAT3

\[ Y705F \] mutant (hypoxia + CNTF + STAT3

\[ p\text{-STAT3}^{\text{Tyr705}} \] mutant); vii) the STAT3

\[ p\text{-STAT3}^{\text{Ser727}} \] mutant group [neurons were exposed to hypoxia, treated with CNTF and transfected with STAT3

\[ S727A \] mutant (hypoxia + CNTF + STAT3

\[ p\text{-STAT3}^{\text{Ser727}} \] mutant)]; and viii) STAT3 mutant control group [neurons were exposed to hypoxia, treated with CNTF and transfected with the blank pcDNA3 vector (hypoxia + CNTF + blank vector pcDNA3)]. The STAT3

\[ Y705F \] and S727A mutants, and pcDNA3 vector were purchased from Shenggong Biotechnology, Co. (Shanghai, China).

**STAT3 gene knockdown.** According to the CDS of STAT3 recorded in Nucleotide, we pre-designed siRNA targeting the mouse STAT3 gene (GenBank accession no. U06922.1) using the online system RNAiDesigner (http://RNAiDesigner.invitrogen.com). The siRNA sequences targeting STAT3 were as follows: si-1, 5'-CCACGTTTTGTTTTTCTAAA-3'; si-2, 5'-GGGTGAAATTGACCAGCAA-3'; si-3, 5'-GCAGATGGTACGGAGAATT-3'. A scrambled non-target siRNA was also used as a control (STAT3-si-Non). The lentivirus was packaged in PC12 cells (purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) and viral titers were determined. The interference efficiency of si-1-4 targeting STAT3 in the PC12 cells was determined by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. The target siRNA-2 was selected for further investigation as it had the highest interference efficiency. The neuronal cells exposed to hypoxia were then infected with 1x10⁶ recombinant lentivirus-transducing units containing the target siRNA or non-target siRNA in the presence of 6 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA), respectively.

**RT-qPCR.** The expression levels of STAT3 in the neuronal cells in each group were detected by RT-qPCR. RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies). Total RNA (2 µg) was used for cDNA synthesis using moloney murine leukemia virus reverse transcriptase (MMLV-RT; Takara Bio, Inc., Otsu, Japan), and the reverse transcript was used as the template for RT-qPCR using a Tower qRT-PCR system (Analytik Jena, Jena, Germany). qPCR was
conducted using 2X Mix SYBR-Green I (Biosea Biotechnology, Co., Ltd., Beijing, China) (10 μl), primer (0.25 μl, 10 pmol/l), template DNA (1 μl) and sterile water (8.5 μl). All PCR reactions included initial denaturation and multiple cycles at (95°C for 3 min); 37 cycles at 95°C for 10 sec, 54°C for 10 sec, and 72°C for 30 sec; followed by 95°C for 10 sec, 65°C for 5 sec and a final 95°C for 15 sec. The primer for each gene was synthesized by Invitrogen Life Technologies. The qPCR primers used to quantify GAPDH expression were as follows: forward, 5'-CGAGATCCCTCAGAAATCAA-3' and reverse, 5'-TTCCACCCCATGGAACAT-3'; and for STAT3 forward, 5'-TCAGTGGAACAGCTGCA-3' and reverse, 5'-AGAATC AAGCATTTCTG-3'. The expression of STAT3 was normalized to endogenous GAPDH expression. The Ct value was defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceed the background level). The correlation between the Ct value and the DNA copy number was calculated as follows: Ct = -3.347424 x log copy number + 35.885406, as previously described (21).

Transfection of neurons with STAT3mutant and STAT3mutant+35.885406 mutant. The Neon™ electroporation transfection system (Invitrogen, Eugene, OR, USA) was used to transfect the STAT3 mutant-pcDNA vector into the cultured neurons. Approximately 10 million cells were harvested, pelleted at 800 rpm and washed with 1X Dulbecco's phosphate-buffered saline (DPBS containing NaCl, Na2PO4, and KCl, but not Ca2+ and Mg2+) prior to re-suspending in resuspension buffer R, provided by the manufacturer. STAT3 mutants (100 nM) were then mixed with the suspended neurons and loaded into a 100 μl Neon tip. The neurons were then transfected with the STAT3 mutant-pcDNA vector via the Neon electroporation system at 1150 V/30 msec for 2 pulses. The cells were then cultured at 37°C/5% CO2/95% humidity for 48 h prior to harvesting and further analysis.

Western blot analysis. The STAT3 and p-STAT3 levels in the different groups were determined by western blot analysis. Briefly, the cells were lysed for 30 min in Cytobuster protein extraction buffer (Novagen, Madison, WI, USA) and centrifuged at 12,000 rpm. The supernatant was collected, total protein was measured, and 50 μg were used for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred to a nitrocellulose (NC) membrane and sealed with Tris-buffered saline and Tween-20 (TBST) containing 5% non-fat milk powder. The membrane was subsequently incubated with goat anti-rat STAT3 (1:1,000) and rabbit anti-p-STAT3 (Tyr705, rabbit mAb no. 9145 and Ser727, mouse mAb no. 9136) antibodies (both from Cell Signaling Technology, Inc., Danvers, MA, USA), and mouse anti-rat GAPDH (1:500; sc-81545; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibody at 4°C overnight. After washing in TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000; A0208; Beyotime Institute of Biotechnology, Shanghai, China) at 25°C, and the protein quantity was determined using electrochemiluminescence (ECL) technique (Bestbio Biotechnology, Co., Ltd., Shanghai, China). The results were photographed using the JS gel imaging system and the grey density was calculated using SensiAnsys software (both from Shanghai Peiqing Science and Technology, Co., Ltd., Shanghai, China).

Statistical analysis. Data are presented as the means ± SD. The comparisons of the mRNA levels and protein concentrations in the different groups were analyzed by one-way analysis of variance (ANOVA). Five independent experiments were performed. Statistical analyses were performed using GraphPad Prism, version 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Transfection with specific siRNA targeting STAT3 suppresses STAT3 expression. The stable transfection of 4 siRNAs targeting STAT3 (si-1, si-2, si-3 and si-4) in PC12 cells resulted in the inhibition of STAT3 expression by >80% (Fig. 1A). Considering the highest expression inhibition rates observed for STAT3, si-2 was selected as the target siRNA for use in the following experiments.
The cultured neurons were then stably transfected with STAT3 si-2 (named STAT3-si). Negative control neurons were transfected with non-target siRNA (recorded as STAT3-si-Non). The STAT3 mRNA levels, as detected by RT-qPCR, were significantly lower (as indicated by the higher Ct value) in the STAT3 siRNA-transfected neurons than in the control siRNA-transfected ones (P=0.00013, P<0.01; Fig. 1B). Western blotting found that the level of immunoreactive protein was significantly downregulated in STAT3-si-2 transfected neurons relative to the controls (P=0.00002, P<0.01; Fig. 1C).

Effects of CNTF on survival and neurite growth of neurons exposed to hypoxia. Exposure to hypoxia decreased the survival rate (vs. normal, P=0.00012, P<0.05; Fig. 2A) and neurite length of neurons (vs. normal, P=0.00012, P<0.05; Fig. 2B). The results revealed that CNTF had a significant protective effect on
neuronal survival under hypoxic conditions (Fig. 2A). In addition, the effects of CNTF on neurite growth were investigated. Following culture with CNTF for 48 h, the hypoxia-exposed neurons (hypoxia + CNTF) displayed outgrowth in the form of neurite extensions, when compared with that of the hypoxia-exposed neurons not treated with CNTF (P=0.0003, P<0.05; Figs. 2B and 3). 

Transfection of the hypoxia-exposed neurons with STAT3 siRNA or STAT3 Tyr705 mutant neutralized the protective effects induced by treatment with CNTF (compared with the si-STAT3 control group, P=0.0015, P<0.05; or compared with the STAT3 mutant control group, P=0.00025, P<0.05) (Figs. 2 and 3).

There was no significant difference between the STAT3 mutant control group and the si-STAT3 control group (P>0.05). Neither the STAT3 mutant control group nor the si-STAT3 control group exhibited a significant difference compared with the hypoxia + CNTF group (P>0.05). Transfection with STAT3 Ser727 mutant did not exert any effect on survival or neurite outgrowth compared to the hypoxia-exposed neurons or to the neurons transfected with the control siRNA or mutant (Fig. 2 and 3).

Thus, our results suggest that CNTF protects neurons from hypoxic injury through STAT3 Tyr705, but not through STAT3 Ser727. CNTF exerts protective effects against hypoxic injury to neurons through STAT3. As previously demonstrated, STAT3 pTyr705 is an indicator of its transcriptional activation (22-24). In this study, to gain further insight into the association between STAT3 activation and CNTF treatment, recombinant human CNTF was used to treat neurons and the activation state of STAT3 (STAT3 pTyr705) was monitored by western blot analysis. The level of STAT3 pSer727 was also detected.

Treatment with CNTF induced a significant increase in the levels of STAT3 and STAT3 pTyr705, and in the STAT3 pTyr705/STAT3 ratio, but not in the levels of STAT3 pSer727 in the cerebral cortex neurons under hypoxic conditions (hypoxia + CNTF group vs. hypoxia group, P=0.00017, P<0.05) (Figs. 4 and 5).

Moreover, the blocking of STAT3 signaling by STAT3 siRNA in the neurons exposed to hypoxia and treated with CNTF (si-STAT3 group) prevented the CNTF-induced increase in the levels of STAT3 pTyr705 (Fig. 4). Conversely, transfection with STAT3 Tyr705 mutant suppressed STAT3 signaling which was activated in the neurons treated with CNTF (STAT3 pTyr705 mutant group vs. STAT3 mutant control group, P=0.0006, P<0.05). However, the above-mentioned suppressive effects were not observed in the neurons transfected with STAT3 Ser727 mutant (Fig. 4).

There was no significant difference between the STAT3 mutant control group and the si-STAT3 control group (P>0.05). Neither the STAT3 mutant control group nor the si-STAT3 control group exhibited a significant difference compared with the hypoxia + CNTF group (P>0.05).

**Discussion**

The present data revealed that treatment with CNTF: i) protected neurons from hypoxic injury by promoting survival and neurite growth; ii) induced a significant increase in the
levels of STAT3 and STAT3\(^{pTyr705}\), and in the STAT3\(^{pTyr705/pSer727}\) STAT3 ratio, but not in the levels of STAT3\(^{pSer727}\) in hypoxic cerebral cortex neurons. The blocking of STAT3 signaling using STAT3 siRNA prevented the CNTF-induced increase in the levels of STAT3\(^{pTyr705}\). Transfection of the hypoxic neurons treated with CNTF with STAT3 siRNA or STAT3\(^{pTyr705}\) mutant neutralized the protective effects exerted by CNTF. These results demonstrated that CNTF exerted neuroprotective effects against hypoxic injury through the activation of STAT3\(^{pTyr705}\).

Our data demonstrated that treatment with CNTF protected the neurons from hypoxic injury by promoting survival rate and neurite growth. Hypoxia-associated brain damage results in immediate neuronal injury and in the exhaustion of cellular energy stores, which lead to a multi-faceted cascade of biochemical events, biological injury and neuronal death (25,26). NTFs are essential proteins for the maintenance and survival of neurons in both developing and mature nervous systems (27,28). Currently, CNTF is the only known factor which shows direct trophic effects on muscle and nerve system, and may have therapeutic effects on motor neuron diseases, nerve damage and muscular atrophy (29). Our data confirmed the fact that CNTF is an important neurocytokine for the survival and neurite growth of neurons following hypoxic injury.

Further experiments revealed that CNTF induced the phosphorylation of STAT3 in neurons under hypoxic conditions; however, the PROMo effects of CNTF on survival and neurite growth of neuron was attenuated by transfection with STAT3 siRNA or STAT3\(^{pTyr705}\) mutant, but not by transfection with STAT3\(^{pSer727}\) mutant. These data demonstrated that CNTF exerted neuroprotective effects under hypoxic conditions through the activation of STAT3/STAT3\(^{pTyr705}\). It has been demonstrated that the cellular response to CNTF is mediated by a receptor complex consisting of the signal transducers glycoprotein 130 (gp130) and LIF receptor \(\beta\) (\(\beta\)-receptor components) and CNTFR\(\alpha\) (30-32). The dimerization of the \(\beta\)-receptor components results in the phosphorylation of JAK (33) followed by signal transduction, including the STAT proteins (32,33). The JAK/STAT pathway is considered to be the primary cytokine signaling pathway among other pathways, such as the Ras-mitogen-activated protein (Ras-MAP) kinase pathway, including ERK1 and ERK2 (MAPK/ERK kinase system) and the cell line-dependent PI3K pathway (PI3K/Akt system) (32,34). It has been established that the JAK2/STAT3 pathway is mainly involved in the survival of neurons in response to CNTF (35,36). It has also been demonstrated that the STAT3 and PI3K/Akt pathways, but not the MEK/MAPK signaling play a major role in mediating the survival response of neurons by cytokines (37) with STAT3, specifically activated by CNTF, leading to increased neuronal survival (38). Phosphorylated STAT3 dimerizes and translocates to the nucleus to regulate target gene transcription (39). In addition, CNTF can also trigger and activate the PI3K/Akt or MEK/ERK pathways, either concomitantly or independently of the JAK2/STAT3 signaling pathway (40,41). Moreover, STAT3 is phosphorylated at Tyr705 upon the activation of cytokine and growth factor receptors, resulting in its homodimerization and nuclear translocation to activate the transcription of downstream responsive genes (22,24). Once activated, STAT3 mediates multiple biological functions, including the promotion of cell proliferation, angiogenesis and metastasis, the inhibition of differentiation and antitumor immune responses (42-44). Given these, the present study indicated that the protective roles of CNTF were dependent on STAT3/STAT3\(^{pTyr705}\)-mediated neuronal survival and proliferation under hypoxic conditions.

In conclusion, the findings of our study demonstrated that the treatment of neurons exposed to hypoxia with CNTF: i) protected cultured neurons from hypoxic injury by promotion survival and neurite growth; ii) induced the phosphorylation of STAT3. However, the promoting effects of CNTF on survival and neurite growth of neurons were suppressed by transfection with STAT3 siRNA or STAT3\(^{pTyr705}\) mutant, but not by transfection with STAT3\(^{pSer727}\) mutant. Taken together, the findings of the present study demonstrate that CNTF-mediated neuron survival and proliferation under hypoxic conditions is mediated by the activation of STAT3/STAT3\(^{pTyr705}\).

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References


