

Neuroprotective properties of ciliary neurotrophic factor on retinoic acid (RA)-predifferentiated SH-SY5Y neuroblastoma cells

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Abstract

Ciliary neurotrophic factor (CNTF) is a neurocytokine, which could promote survival and/or differentiation in many cell types. In this study, the biological effects of CNTF on retinoic acid (RA)-predifferentiated SH-SY5Y neuroblastoma cells and the underlying molecular mechanism of this effect were investigated for the first time. The results showed that RA was able to increase cells susceptibility to CNTF via regulating the expression levels of CNTF receptors. A further study revealed that CNTF could induce phosphorylation of STAT3, Akt and ERK1/2 in RA-predifferentiated SH-SY5Y neuroblastoma cells, while the promoting activity of CNTF on survival and neurite growth of cells was attenuated by co-treatment with JAK2 inhibitor AG490 (25 μ M), STAT3 inhibitor Curcumin (50 μ M), PI3K inhibitor LY-294002 (50 μ M), but not by co-treatment with MEK inhibitor PD98059 (50 μ M). These findings suggested that JAK2/STAT3, as well as PI3K/Akt, play important roles in mediating the survival and neurite growth response of RA-predifferentiated cells to CNTF. Our study may be useful to further understand the functional role of CNTF and offer a convenient model to explore the therapeutic potential of CNTF in neurodegenerative diseases.

Key words: CNTF, SH-SY5Y neuroblastoma cells, survival, neurite outgrowth, signalling pathway.

Introduction

Neurotrophic factors such as the ciliary neurotrophic factor (CNTF) are essential proteins for the maintenance and survival of neurons in both developing and mature nervous systems [14,16,21]. In particular, CNTF is responsible for neurotransmitter synthesis and neurite outgrowth in certain neuronal populations [22,26]. CNTF is a 25-kDa polypeptide that was originally isolated as a target-derived survival factor of parasympathetic ciliary ganglion neu-

rons [19]. It is believed that CNTF's physiological role only becomes apparent after tissue injury due to its lack of hydrophobic sequence. Known cell-surface receptors for CNTF include CNTF receptor α (CNTFR α), gp130, and LIF receptor (LIFR) [3,4]. Binding of CNTF to CNTFR α triggers heterodimerization of gp130 and LIFR, forming an active trimeric receptor complex and activates the downstream signalling pathway [25]. It has been established that JAK2/STAT3 pathway is mainly involved in survival of neurons in

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response to CNTF [11,12,26]. Phosphorylated STAT3 dimerizes and translocates to the nucleus to regulate target gene transcription [27]. In addition, CNTF can also trigger and activate PI3K/Akt or MEK/ERK pathways, either concomitantly or independently of JAK2/STAT3 signalling pathway [1,5,6,24].

In cell-culture experiments, CNTF has also been established to be an important neurocytokine for the survival of a variety of neuronal subpopulations, including dorsal root ganglia, sympathetic neurons, GABAergic septohippocampal neurons, and motor neurons [8,9]. A previous study conducted by Kazunori *et al.* has found that CNTF may promote the survival and neurite outgrowth of DRG neuron from an adult rat after it had been cultured for 7 days [24]. However, currently, there is little information available on the effects of CNTF on human neuroblastoma cells. Human neuroblastoma cells are currently used as an *in vitro* model of neuronal function and differentiation [20]. In particular, a human neuroblastoma dopaminergic neuronal cell line – SH-SY5Y is used as a model for neurodegenerative disorders as the cells can be converted to various types of functional neurons through the addition of specific compounds such as retinoic acid (RA), phorbol ester or staurosporine [2,17]. In many studies, SH-SY5Y neuroblastoma cells are often induced to differentiate by RA to obtain more neuron-like properties, including neurite outgrowth and morphological changes, this in turn allows the mimicking of responses of neurons [7]. A previous study has reported that SH-SY5Y neuroblastoma cells expressed functional CNTF receptors and the expression levels were found to increase several folds through continuous exposure to RA [10,18]. However, limited studies have been reported about the CNTF response of SH-SY5Y neuroblastoma cells following RA treatment.

In this study, we tried to elucidate the biological effects of CNTF on RA-predifferentiated SH-SY5Y neuroblastoma cells for the first time. Moreover, the underlying molecular mechanisms of such effects were also explored in regards to change of related signalling pathways in SH-SY5Y neuroblastoma cells.

Material and methods

Materials and chemicals

Recombinant human CNTF was produced in *Escherichia coli* by our laboratory. The purity of CNTF is higher than 95% by high-performance liquid chroma-

tography (HPLC) and the molecular weight of which is 24 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (data not shown). All cell culture reagents were purchased from Gibco (Grand Island, NY, USA). Retinoic acid, AG490, Curcumin, LY-294002 and PD98059 were purchased from Sigma-Aldrich (St Louis, Mo, USA). Trypan blue, Ribonuclease A (Rnase A), Polyvinylidene Fluoride (PVDF) membranes and enhanced chemiluminescence (ECL) detection kit were purchased from Beyotime (Nantong, China). Antibodies against CNTFR α , gp130, LIFR and β -actin were obtained from Santa Cruz Biotechnology (CA, USA). Antibodies against P-STAT3, STAT3, P-Akt, Akt, P-ERK1/2, ERK1/2 were obtained from Cell Signalling Technology (MA, USA). All other chemicals and reagents were of analytical grade.

Cell culture

The SH-SY5Y neuroblastoma cell line was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% of air and 5% of CO₂. The medium was changed every 3 days. For cell survival and neurite outgrowth, cells were plated in 35-mm-diameter (Corning, NY, USA) at an initial density of 5×10^4 cells per well.

Assay for cell survival and neurite outgrowth

The number of living cells was established by staining with trypan blue and counting in the hemocytometer (Bürker). Cells were treated with RA at 10 μ M for 5 days, washed three times with DMEM and switched to serum-free DMEM medium containing various concentrations of CNTF (0, 5, 50, 500 ng/mL) for 48 h. Before switching to CNTF-containing medium, the living cells were counted and the resulting value was defined as 100% survival. Results were therefore expressed as a mean \pm SEM percentage of this value. For evaluation of neurite outgrowth, cells treated with the indicated drugs were observed in a phase-contrast microscope (Olympus X51, Japan) and the cell bodies and neurites were counted. The ratio between neurites and cell bodies was calculated yielding the average of neurites per neuron.

Measurement of neurotrophic activities of CNTF in the presence of signalling pathway inhibitors

The effects of related signalling pathway inhibitors on the CNTF-induced survival and neurite outgrowth of RA-differentiated SH-SY5Y neuroblastoma cells were investigated. Cells were treated for 5 days with RA at 10 μ M and then cultured with CNTF (50 ng/mL) in the absence or presence of JAK2 inhibitor AG490 (25 μ M), STAT3 inhibitor Curcumin (50 μ M), PI3K inhibitor LY-294002 (50 μ M) and MEK inhibitor PD98059 (50 μ M) for 48 h, respectively. The survival and neurite outgrowth of cells were evaluated.

Immunocytochemistry

Cells were collected and fixed with 4% paraformaldehyde for 10 min at 4°C, and then treated with 0.1% Triton-X-100 in phosphate buffer saline (PBS) for 5 min at room temperature or with 100% methanol for 10 min at -20°C. The fixed cells were incubated overnight at 4°C with CNTFR antibody, gp130 polyclonal antibody and LIFR antibody, respectively. All of which were diluted with 20 mM of PBS containing 0.4% Block Ace. After rinsing with PBS, the cells were incubated for 1 h at 37°C with peroxidase-conjugated secondary antibodies. The immunoreaction was visualized as described above.

Western blot analysis

Cell extracts were prepared from cultured cells using lysis buffer (10 mM of Tris [pH 7.4], 150 mM of NaCl, 5 mM of ethylenediaminetetraacetic acid

[EDTA], 1% Triton-X-100, 1% Tergitol-type NP40, and the following protease inhibitors: aprotinin, benzamide, leupeptin, pepstatin A, and phenylmethanesulfonyl fluoride [PMSF]). The protein concentration of samples was determined with Bradford method [25]. The samples (50 μ g) were then applied to 10% SDS-PAGE and transblotted onto PVDF membranes. After blocking with 5% bovine serum albumin (BSA) in Tris-buffer saline (TBST) for 1 hour, membranes were incubated with the primary antibodies (all in 1 : 500 dilutions) overnight and followed by a secondary antibody (all in 1 : 200 dilutions) incubation for 1 hour at room temperature. Protein bands were visualized by ECL detection kit. The density of each band was normalized by β -actin.

Statistical analysis

All data are expressed as mean \pm SEM. Point-to-point comparisons were made by Student's *t*-test. Groups were compared by two-way ANOVA using the unpaired Tukey-Kramer method as post-test. All experiments were done in triplicates and the results were indicative of three independent studies. Results were considered significantly different if $P < 0.05$.

Results

Effects of RA treatment on the expressions of CNTF receptors

The immunocytochemical analysis confirmed that SH-SY5Y neuroblastoma cells expressed the CNTF receptor complex (CNTFR α , gp130 and LIFR) at the cell surface, which is consistent with previous studies

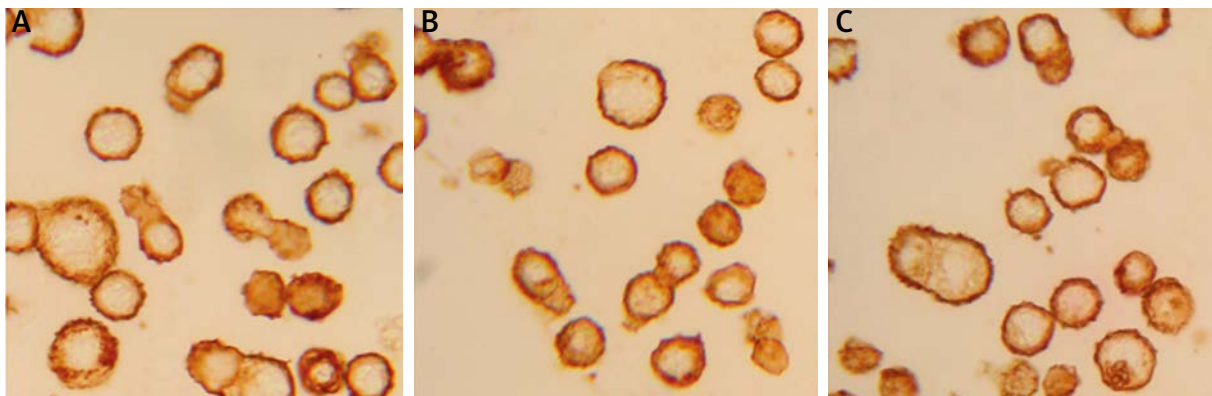


Fig. 1. Immunocytochemical localization of CNTFR α , gp130 and LIFR at the surface of cultured SH-SY5Y neuroblastoma cells. Cells were cultured for 24 h and the peroxidase reactions with antibodies to CNTFR α (A), gp130 (B) and LIFR (C) were performed by immunocytochemical analysis.

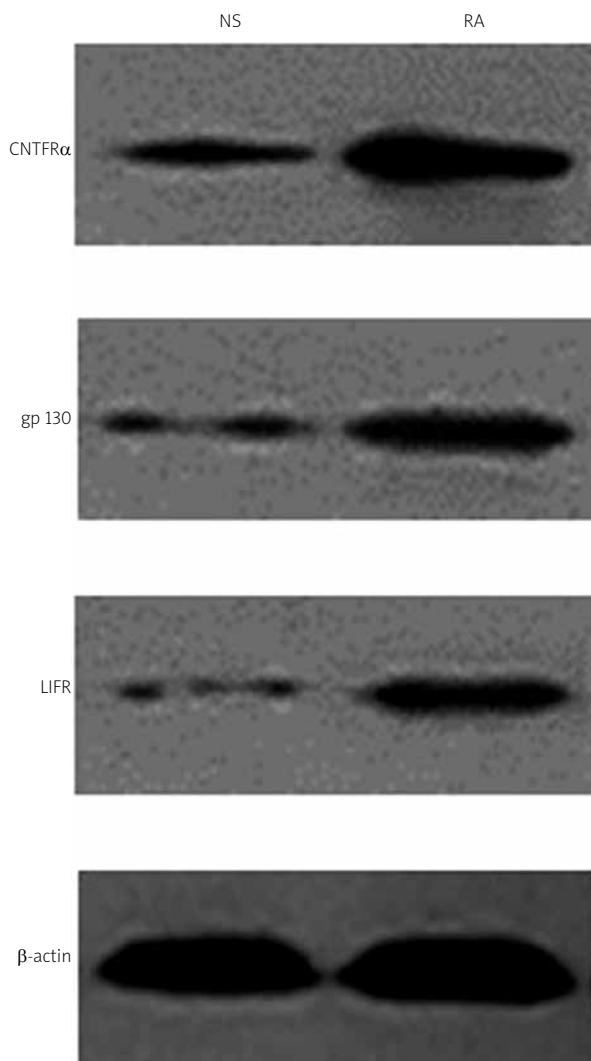


Fig. 2. Effects of RA treatment on the expressions of CNTF receptors (CNTFR α , gp130 and LIFR) on cultured SH-SY5Y neuroblastoma cells. Cells were treated for 5 days with 10 μ M RA and the reactions with antibodies to CNTFR α , gp130 and LIFR (all in 1 : 500 dilutions) were performed by western blot analysis. NS: non-stimulated cells.

(Fig. 1) [12,18]. Effects of exposure of RA on the up-regulation of the expression level of CNTF receptors were also examined. As shown in Fig. 2, a 2.2-fold increase for CNTFR α , 2.7-fold increase for gp130 and 3.2-fold increase for LIFR in band intensity after 5 days of RA treatment (10 μ M) as compared to those of non-stimulated controls were observed. The results suggested that RA might increase the responsiveness of CNTF via regulating the levels of CNTF receptors.

Effects of CNTF on survival and neurite growth of RA-pretreated cells

SH-SY5Y neuroblastoma cells were cultured for 5 days in the presence of 10 μ M RA and then switched to serum-free medium supplemented with CNTF at various concentrations. After an additional 48 hours in culture, cell survival was evaluated by means of the trypan blue staining. The results showed that CNTF had a significant effect on the cell survival of RA-pretreated cells (Fig. 3A). In addition, effects of CNTF on neurite growth were further investigated. After cultured with CNTF for 48 hours, RA-pretreated cells acquired rounded, phase-bright bodies and displayed long neurites and the ratio between neurites and cell bodies was increased in a dose-dependent relationship (Fig. 3B).

Effects of CNTF on signalling pathways of RA-pretreated cells

The activation status of CNTF receptors-related signalling pathways was assessed to elucidate the molecular mechanism of the biological effects of CNTF. Western blot analysis showed that cells treated by RA for 5 days were acutely stimulated by CNTF where the expressions of phosphorylated signalling molecules including P-STAT3, P-Akt and P-ERK1/2 were undetectable initially but were rapidly induced by co-treatment with CNTF for 15 min and still detectable after 4 h of exposure to CNTF (Fig. 4). These findings suggested that CNTF could induce phosphorylation of STAT3, Akt and ERK1/2 in RA-pretreated SH-SY5Y cells.

Effects of signalling pathway inhibitors on CNTF-induced survival and neurite growth of RA-pretreated cells

Based on previous results, we further investigated which pathways are involved in CNTF-induced survival and neurite outgrowth, using the inhibitors of individual pathways: JAK2 inhibitor – AG490, STAT3 inhibitor – Curcumin, PI3K inhibitor – LY294002 and MEK inhibitor – PD98059. The promoting activity of CNTF on survival and neurite growth of RA-pretreated cells was attenuated by co-treatment with AG490 (25 μ M), Curcumin (50 μ M), LY294002 (50 μ M), but not by co-treatment with PD98059 (50 μ M). These findings suggested that the JAK2/STAT3 pathway, as well as PI3K/Akt pathway, were the principal pathways in CNTF-induced survival and neurite growth of RA-pretreated cells.

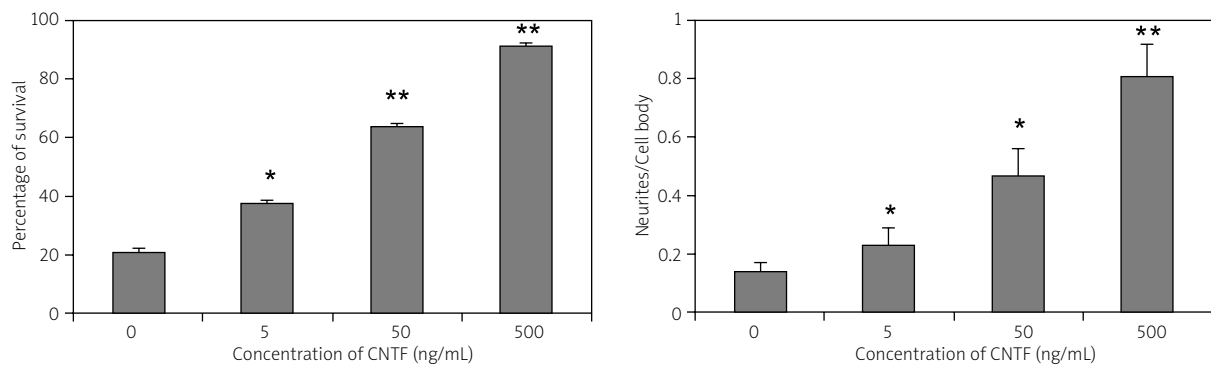


Fig. 3. Effects of CNTF on survival and neurite growth of RA-pretreated SH-SY5Y neuroblastoma cells. Cells were treated for 5 days with 10 μ M RA and then cultured in a serum-free medium containing the indicated concentrations of CNTF (0, 5, 50, 500 ng/mL) for an additional period of 48 h. **A)** Cell survival was assessed by trypan blue staining. **B)** Neurite outgrowth of cells was scored as described in Materials and methods. Data are mean \pm SEM values. * P < 0.05 vs. control, ** P < 0.01 vs. control.

Discussion

Until now, few studies have focused on the effects of CNTF on human neuroblastoma cells such as SH-SY5Y, which could be differentiated to neuronal-like cells using retinoic acid and used as a model for neurodegenerative disorders [15,22,23]. In this study, we examined the biological effects of CNTF on RA-predifferentiated SH-SY5Y neuroblastoma cells for the first time and further explored the molecular mechanisms of such effects.

Our data confirmed that SH-SY5Y cells express a functional CNTF receptor complex including CNTFR α , gp130 and LIFR at the cell surface and showed a stable increase in protein levels after treatment with RA for 5 days, which are consistent with those in previous studies [23]. We further investigated if RA pretreatment was able to enhance the cells sensitivity to CNTF via regulating the CNTF receptor levels. The results showed that CNTF exerted the most striking effects on cell survival and neurite growth of RA-pretreated cells compared with those lacking neurotrophic support, which indicated that high expressions of CNTF receptors induced by RA are biologically active.

The JAK2/STAT3 pathway is the main signalling cascade used by CNTF in many cell types, however, CNTF can also signal through PI3K/Akt and/or MEK/ERK pathways [13]. To clarify this, the signalling pathway of CNTF in RA-predifferentiated SH-SY5Y neuroblastoma cells was also investigated in this study. The results from our western blot analysis indicated that

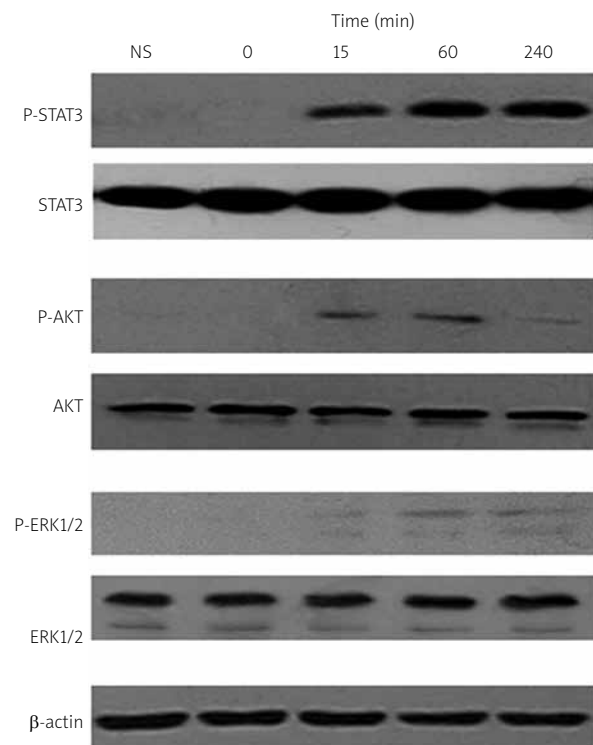


Fig. 4. Effects of CNTF on the expressions of signaling pathway molecules on RA-pretreated SH-SY5Y neuroblastoma cells. Cells were treated for 5 days with 10 μ M RA and cultured in serum-free medium containing the 50 ng/mL CNTF for an additional time. Then, the reactions with antibodies to P-STAT3, STAT3, P-Akt, Akt, P-ERK1/2, ERK1/2 (all in 1 : 500 dilution) were performed by western blot analysis. The density of each band was normalized by β -actin. NS: non-stimulated cells.

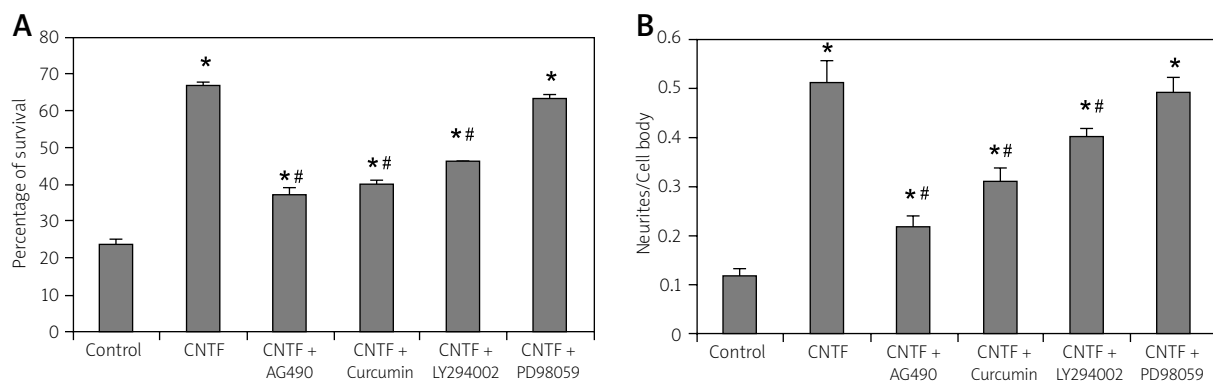


Fig. 5. Effects of signalling pathway inhibitors on CNTF-induced survival and neurite growth of RA-pretreated SH-SY5Y neuroblastoma cells. Cells were treated for 5 days with 10 μ M RA and then cultured in serum-free medium containing the CNTF (50 ng/mL) and inhibitors [AG490 (25 μ M), Curcumin (50 μ M), LY-294002 (50 μ M) and PD98059 (50 μ M)] for an additional period of 48 h. **A)** Cell survival was assessed by trypan blue staining. **B)** Neurite outgrowth of cells was scored as described in Materials and methods. Data are mean \pm SEM values. * P < 0.05 vs. control, # P < 0.05 vs. CNTF.

CNTF could induce the expressions of phosphorylated signalling molecules including P-STAT3, P-Akt and P-ERK1/2 in cells pretreated by RA. Further survival and neurite outgrowth assays showed that promoting activity of CNTF was attenuated by co-treatment with JAK2 inhibitor AG490, STAT3 inhibitor Curcumin, PI3K inhibitor LY294002, but not by co-treatment with MEK inhibitor PD98059. These findings suggested that the MEK/ERK pathway is unlikely to be a crucial pathway for CNTF-induced survival and neurite outgrowth activity of RA-predifferentiated SH-SY5Y neuroblastoma cells, while the JAK2/STAT3 pathway, as well as PI3K/Akt pathway, played important roles in mediating the neuroprotective function of CNTF. Further research is required to elucidate how these signalling pathways exert their effect during CNTF-induced survival and neurite outgrowth in RA-predifferentiated SH-SY5Y neuroblastoma cells.

In conclusion, our study has enhanced our understanding towards the functional role of CNTF on RA-predifferentiated SH-SY5Y neuroblastoma cells in promoting cell survival and neurite growth. We also found that the JAK2/STAT3 and PI3K/Akt signalling pathways are mainly involved in the CNTF-induced survival and neurite outgrowth activity of RA-predifferentiated SH-SY5Y neuroblastoma cells. These results may supplement the current understanding of how CNTF functions in diverse tissues and organisms, which offers a convenient model to explore the therapeutic potential of CNTF in neurodegenerative diseases.

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Disclosure

Authors report no conflict of interest.

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