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Effects of neurotrophin-3 on the differentiation of neural stem cells into neurons and oligodendrocytes

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Abstract

In this study, cells from the cerebral cortex of fetal rats at pregnant 16 days were harvested and cultured with 20 μg/L neurotrophin-3. After 7 days of culture, immunocytochemical staining showed that, 22.4% of cells were positive for nestin, 10.5% were positive for β-III tubulin (neuronal marker), and 60.6% were positive for glial fibrillary acidic protein, but no cells were positive for O_4 (oligodendrocytic marker). At 14 days, there were 5.6% nestin-, 9.6% β-III tubulin-, 81.1% glial fibrillary acidic protein-, and 2.2% O_4-positive cells. In cells not treated with neurotrophin-3, some were nestin-positive, while the majority showed positive staining for glial fibrillary acidic protein. Our experimental findings indicate that neurotrophin-3 is a crucial factor for inducing neural stem cells differentiation into neurons and oligodendrocytes.

Key Words

neurotrophin-3; neural stem cells; differentiation; neuron; oligodendrocytes; stem cells; neural regeneration

Research Highlights

Neurotrophin-3 can promote the *in vitro* differentiation of neural stem cells into neurons and oligodendrocytes.

Abbreviations

GFAP, glial fibrillary acidic protein

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INTRODUCTION

The central nervous system (CNS) has a very limited ability for self-repair following injury. Although it is well-documented that neural stem cells exist in certain areas of the adult brain and are necessary for the spontaneous restoration of the lost neural function after injury, the mechanisms underlying this natural recovery of function remain controversial. Neural stem/ progenitor cells mainly differentiate into astrocytes after injury as the capacity for generating new functional neurons and oligodendrocytes is weak[1-2]. Therefore, newborn cells require an optimal micro-environment to differentiate and integrate into the CNS to replace lost nerve cells. Our previous studies confirmed that a number of neurotrophins are secreted by these neural stem cells[3]. Nerve growth factor[4], brain-derived neurotrophic factor[5], and ciliary neurotrophic factor[5] are reported to induce differentiation of neural stem cells into neurons; however, few studies have focused on the ability of neurotropin-3 to induce differentiation. Neurotrophin-3 is involved in the development of the nervous system through the growth induction of sensory neurons[6]. Previous studies in our group confirmed that

cultured neural stem cells secrete neurotrophin-3[5]. We hypothesized that neurotrophin-3 may promote neuronal-like differentiation of neural stem cells through auto- or para-secretion mechanisms. Thus, in this study, we aimed to investigate the effects of neurotrophin-3 on the differentiation of cultured neural stem cells.

RESULTS

Neurotrophin-3 promoted the adherence of neural stem cells cultured *in vitro*

Primary cultured neural stem cells formed spheres, which were strongly refractive over the first 24 hours, with two or more colony-forming cells present in the culture. On day 4 after primary culture, neural stem cells were placed in an inverted phase contrast microscope, where spheres of neural stem cell clones were observed (Figure 1A). With the prolongation of incubation time, the size and the number of colonies increased. On day 8, the remaining cells in suspension grew into colonies (Figure 1B). The cell bodies extended processes and adhered to the dishes after neurotrophin-3 stimulation (Figure 1C).

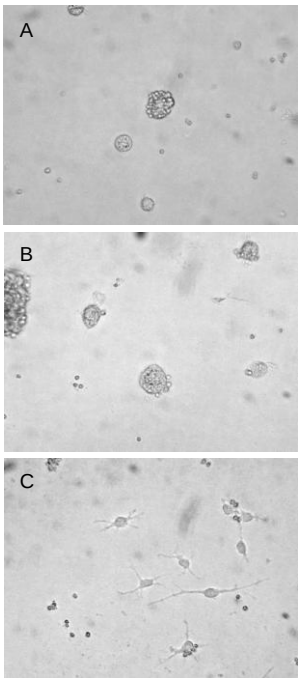

Figure 1 Primary culture of neural stem cells with and without neurotrophin-3 (NT-3) induction (phase contrast microscope, × 40).

(A) Primary cell culture without NT-3 on day 4, spheres of neural stem cells can be observed.

(B) Primary cell culture without NT-3 on day 8, the size and the number of colonies increased. Suspending cells grew into colonies.

(C) Primary cell culture with NT-3 on day 8. Cell bodies extended processes and adhered to the dishes after NT-3 induction.

Neurotrophin-3 promoted the differentiation of neural stem cells into neurons and oligodendrocytes

After 7 and 14 days of induced differentiation, neural stem cells were subjected to anti-nestin, anti-beta-III tubulin, anti-glial fibrillary acidic protein (GFAP) and anti-O_4 staining. On day 7, immunocytochemistry demonstrated that 22.4% of cells were still positive for nestin, 10.5% were positive for beta-III tubulin, 60.6% were positive for GFAP, but no cells were positive for O_4 (Figure 2).

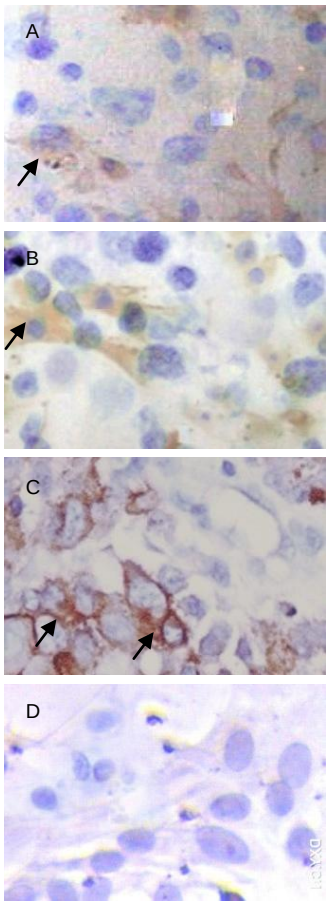

Figure 2 Differentiation of neural stem cells after 7-day induction with neurotrophin-3 (immunocytochemistry, × 400).

(A) Anti-nestin; (B) anti-beta III tubulin; (C) anti-glial fibrillary acidic protein; (D) anti-O_4. Arrows indicate the positive cells.

On day 14, there were 5.6% nestin-positive cells, 9.6% beta-III tubulin-positive cells, 81.1% GFAP-positive cells, and 2.2% O_4-positive cells (Figure 3). Control cells (untreated) were most GFAP-positive cells, with a few nestin-positive cells.

Neurotrophin-3-treated cells had more beta-III tubulin- and O_4-positive cells compared with the control cells ($P < 0.05$). The number of nestin-positive cells decreased with culture time ($P < 0.05$). At different time points, the counting of positive cells is summarized in Table 1.

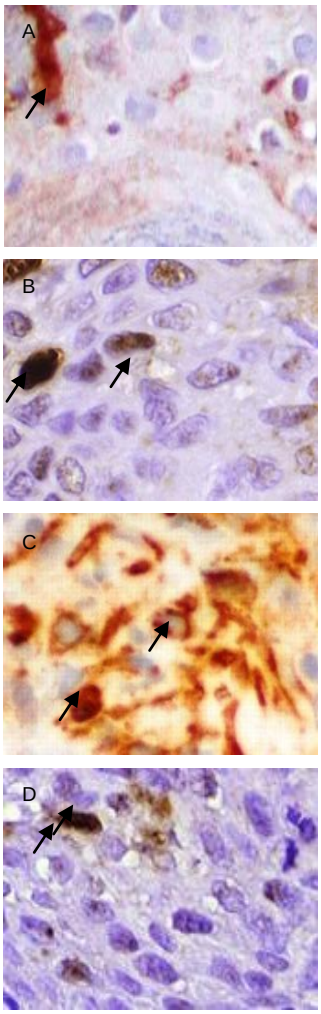

Figure 3 Differentiation of neural stem cells after 14-day induction with neurotrophin-3 (immunocytochemistry, × 400).

(A) Anti-nestin; (B) anti-beta III tubulin; (C) anti-glial fibrillary acidic protein; (D) anti-O_4. Arrows indicate the positive cells.

Table 1 Cell line markers (%) of NT-3-treated cells group and control cells group

Group	Nestin- positive cells	Beta III tubulin- positive cells	GFAP- positive cells	O_4- positive cells
7 d				
NT-3 group	22.4±4.5	10.5±3.3	60.6±5.3	0
Control group	28.1±6.5	0[b]	70.3±8.9	0
14 d				
NT-3 group	5.6±1.2[a]	9.6±3.4	81.1±6.6	2.20±0.5[a]
Control group	2.3±0.5[ab]	0[b]	96.4±7.6	0[ab]

Data are expressed as mean ± SD of 10 samples in each group, and the percentage of positive cells was calculated as the average of three randomly selected high-power fields (× 100).

[a]$P < 0.05$, *vs*. 7 d after induction in the same group; [b]$P < 0.05$, *vs*. NT-3 group at the same time point (Student's *t*-test). NT-3: Neurotrophin-3; GFAP: glial fibrillary acidic protein.

DISCUSSION

Our studies demonstrate the effects of neurotrophin-3 on the differentiation of neural stem cells. These findings may help provide greater insight into the mechanisms behind self-repair of the CNS. neural stem cells have the potential for self-renewal and differentiation into potential precursor cells[7]. In appropriate micro-environments, neural stem cells differentiate into neurons[8], astrocytes[9] and oligodendrocytes[10]. However, the majority of neural stem cells have the tendency for spontaneous differentiation into astrocytes *in vitro* [11]. In the CNS, neurons and oligodendrocytes are crucial for neural signal transduction. Studies on neural stem cell transplantation have focused on the differentiation of neural stem cells into mature functional neurons and oligodendrocytes, to promote repair after CNS injury. Since our previous study demonstrated that neurotrophin-3 is secreted from neural stem cells, which has not been fully documented in other reports, we hypothesized that neural stem cells may employ the auto-secretion of neurotrophin-3 to differentiate into mature functional neurons or oligodendrocytes. neurotrophin-3 may affect the survival of hippocampal, motor, ventral midbrain dopaminergic and GABAergic neurons, noradrenergic locus coeruleus neurons and cerebellar granule cells[12]. Neurotrophin-3 and Trk receptor combinations can activate the Ras/Erk pathway[13], which plays a key role in embryonic stem cell development[14].

In this study, neurotrophin-3 was discovered to be an inducer of neuronal and oligodendrocytic differentiation of neural stem cells. This study also found that in the culture of neural stem cells with neurotrophin-3 on day 7, there were still a considerable number of cells showing nestin-positive staining. Furthermore, with the culture of neural stem cells with neurotrophin-3 at a later phase (14 days), there were still nestin-positive cells present, indicating that these cells still have characteristics of stem cells. The mechanism behind this phenomenon still needs further investigation.

In the later phase of neurotrophin-3 culture of neural stem cells (14 days), although a small number of cells differentiated into O_4-positive oligodendrocyte-like cells (2.2%), beta-III tubulin-positive neuron-like cells were 9.6%, but GFAP-positive astrocyte-like cells were still the amin cell type present (81.1%). In recent years, studies for enhancing the differentiation of neuron-like cells from NBSCs has been a major hot spot. In addition to selecting the appropriate inducer, the topostructure of the culture environment also influences cellular differentiation[15]. Biological macromolecules can be coupled in the grid of large mole-

cules[16], where future research may focus on the coupling of neurotrophin-3 molecules to the polymer structure for its controlled release.

MATERIALS AND METHODS

Design

A comparative cytological experiment *in vitro*.

Time and setting

This experiment was performed in the Clinical Research Center in the Second Affiliated Hospital of Zhejiang University Medical College, China.

Materials

One pregnant Sprague-Dawley rat at day 16, weighing 438 g, was provided by Zhejiang University Laboratory Animal Center, China (license No. SYXK (Zhe) 2004-0052). All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[17].

Methods

Harvesting, culture and identification of neural stem cells

The pregnant Sprague-Dawley rat was anesthetized and killed by separating the spinal cord under sterile conditions, following which the embryos and womb were removed. The brain above the medulla oblongata, meninges and choroid plexus was removed and stripped at a low temperature. Brain tissue was cut into pieces and placed in a Petri dish with 0.25% trypsin (Zhongshan Jinqiao, Beijing, China) for digestion into a single cell suspension, using 200-mesh strainer filters and cultured with DMEM complete medium (Sigma, St. Louis, MO, USA), supplemented with B27 (Gibco, Carlsbad, CA, USA), 2% basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA) and 20 μg/L epidermal growth factor (PeproTech) and maintained at 37°C in a 5% CO_2 humidified culture. Cell growth was observed under a microscope (Olympus, Tokyo, Japan) every day and culture medium was changed every 3 days. Cells were passaged on day 8 as follows: neural stem cell clone balls were triturated into single cell suspension in the same medium and re-adjusted to a cell density of 1×10^9 cells/L. Undifferentiated neural stem cells were identified by anti-nestin (1:400 anti-nestin monoclonal antibody; Gibco) immunocytochemical staining.

Grouping and intervention

1×10^5/mL neural stem cells were divided into experimental and control groups, with 10 samples in each group. Experimental group was treated with 5% fetal bovine serum (Sijiqing, Hangzhou, China) and 20 μg/L neurotrophin-3 (Gibco), while the control group was cultured with 5% fetal bovine serum medium from the first passage.

Nestin, beta-III tubulin, GFAP and O_4 expressions by immunocytochemical staining

After culture with neurotrophin-3 or a control agent for 7 and 14 days, cells were incubated with rabbit anti-rat anti-nestin monoclonal antibody (1:400; Gibco), anti-beta III tubulin monoclonal antibody (1:250; Serotec, Oxford, UK), anti-GFAP monoclonal antibody (1:400; Serotec) and anti-O_4 monoclonal antibody (1:200; Serotec) at 4°C overnight, and with goat anti-rabbit IgG secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 2 hours. Three high-power fields (100 ×) were randomly selected for observation under a microscope (Olympus), to count the number of nestin, beta-III tubulin, GFAP, and O_4-positive cells relative to the total cell number.

Statistical analysis

The percentage of positive cells to the total population was expressed as mean ± SD. The rate of positive cells in each group and within the same group at different time points was compared using the Student's *t*-test. Statistical analysis was performed using SPSS 12.0 software (SPSS, Chicago, IL, USA), and $P < 0.05$ was considered statistically different.

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Author contributions: Guowei Zhu designed and conducted the experiments, collected and analyzed the data. Chongran Sun designed and conducted the experiments and wrote the manuscript. Weiguo Liu supervised the experiment and the manuscript.

Conflicts of interest: None declared.

Ethical approval: The animal experiment was carried out in accordance with all the documents issued by Animal Welfare Committee of Zhejiang University in China.

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