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# Influence of endogenous ciliary neurotrophic factor on neural differentiation of adult rat hippocampal progenitors<sup>☆</sup>

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## Abstract

Ciliary neurotrophic factor is the only known neurotrophic factor that can promote differentiation of hippocampal neural progenitor cells to glial cells and neurons in adult rats. This process is similar to spontaneous differentiation. Therefore, ciliary neurotrophic factor may be involved in spontaneous differentiation of neural stem cells. To verify this hypothesis, the present study isolated neural progenitor cells from adult male rats and cultured them *in vitro*. Results showed that when neural progenitor cells were cultured in the absence of mitogen fibroblast growth factor-2 or epidermal growth factor, they underwent spontaneous differentiation into neurons and glial cells. Western blot and immunocytochemical staining showed that exogenous ciliary neurotrophic factor strongly induced adult hippocampal progenitor cells to differentiate into neurons and glial cells. Moreover, passage 4 adult hippocampal progenitor cells expressed high levels of endogenous ciliary neurotrophic factor, and a neutralizing antibody against ciliary neurotrophic factor prevented the spontaneous neuronal and glial differentiation of adult hippocampal progenitor cells. These results suggest that the spontaneous differentiation of adult hippocampal progenitor cells is mediated partially by endogenous ciliary neurotrophic factor.

## Key Words

neural regeneration; stem cells; spontaneous differentiation; neural progenitor cells; endogenous neurotrophic factors; ciliary neurotrophic factor; regeneration; grants-supported paper; photographs-containing paper; neuroregeneration

## Research Highlights

- (1) Late stage neural stem cells expressed high levels of ciliary neurotrophic factor possibly because of active differentiation of late stage cells.
- (2) Spontaneous differentiation can promote endogenous ciliary neurotrophic factor expression in neural stem cells.
- (3) A neutralizing antibody against ciliary neurotrophic factor inhibited the spontaneous neuronal and glial differentiation of adult hippocampal progenitor cells, indicating endogenous ciliary neurotrophic factor participates in triggering and/or maintaining spontaneous differentiation of neural progenitor cells.

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## INTRODUCTION

In the early 1990s, it was discovered that neural stem cells could be isolated from two regions of the developing or adult mammalian brain<sup>[1-3]</sup>: the subgranular zone of the dentate gyrus of the hippocampal formation and the subventricular zone<sup>[4-8]</sup>. Neural stem cells are multipotent. *In vivo*, neural stem cells can differentiate into one of three neural cell types of the brain including neurons, astroglia and oligodendroglia. Following isolation from the brain, the cells can grow in various culture media, such as Dulbecco's modified eagle's medium/F12 and neurobasal (A)/B27, in the presence of mitogen fibroblast growth factor-2 or epidermal growth factor<sup>[1, 9]</sup>. Once the mitogen is removed from the culture system, neural stem cells will rapidly and spontaneously differentiate into mature neural cells, *i.e.* spontaneous differentiation of neural stem cells<sup>[10-13]</sup>. Previous studies have focused on the effect of exogenous growth factors, cytokines and biochemical reagents on the development of fetal and adult neural stem cells. Even though researchers have begun to realize the critical role of cellular secreted factors, such as endogenous neurotrophins, on the development of neural stem cells, the mechanism(s) of spontaneous differentiation remain unknown<sup>[14-18]</sup>.

Recently, it has been shown that endogenous ciliary neurotrophic factor can be expressed in fetal mouse neural progenitor cells<sup>[19]</sup>. Other cytokines, secreted factors and receptor subunits related to growth and differentiation are also expressed in embryonic cortical and spinal cord neurospheres<sup>[15-17]</sup>, and endogenously produced neurotrophins including brain-derived neurotrophic factor and neurotrophin-3 can mediate embryonic cortical progenitor cell survival and spontaneous neuronal differentiation (neurogenesis)<sup>[15-16]</sup>. It remains unclear how cellular factors secreted from adult neural progenitor cells mediate spontaneous differentiation.

Previous studies reported that exogenous ciliary neurotrophic factor induced fetal neural progenitor cells to differentiate into astrocytes<sup>[20-22]</sup>. Louis *et al*<sup>[23]</sup> also demonstrated that exogenous ciliary neurotrophic factor enhanced the maturation and survival of oligodendrocytes. However, we recently reported that exogenous ciliary neurotrophic factor dramatically enhanced neuronal determination in adult hippocampal progenitor cells<sup>[24]</sup>. Ciliary neurotrophic factor is the only neurotrophic factor that has been shown to induce both neuronal and glial cell fate determination in neural stem/progenitor cells *in vitro*. Thus, if endogenous ciliary

neurotrophic factor is also produced by adult hippocampal progenitor cells, we hypothesize that endogenous ciliary neurotrophic factor may be involved in spontaneous differentiation, especially the spontaneous neurogenesis of adult hippocampal progenitor cells.

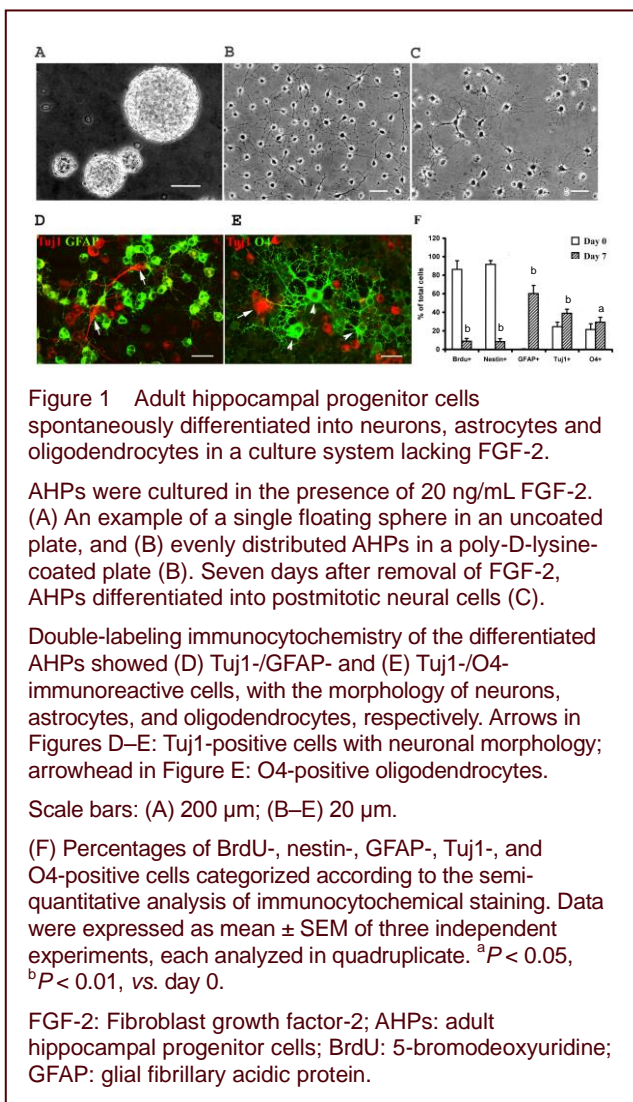
The present study used neural progenitor cells isolated from adult rat hippocampus to investigate the key role of endogenous ciliary neurotrophic factor in spontaneous neurogenesis and glial differentiation.

## RESULTS

### Adult neural progenitor cells spontaneously differentiated into neurons, astrocytes and oligodendrocytes in culture medium lacking mitogen

To address the characteristics of spontaneous differentiation in the development of adult hippocampal progenitor cells, we examined neural progenitor cells isolated from 3-month-old Wistar rat brain in the presence of 20 ng/mL fibroblast growth factor-2. Previously we showed that, even seeded on plates, most of these cells between passages 3 and 5 were dividing and nestin-positive<sup>[24-26]</sup>. Using an uncoated culture dish or flask, adult hippocampal progenitor cells could be cultivated for over 1.5 years as floating neurospheres (Figure 1A). In addition, these cells could also grow as adherent cells and the capacity for renewal and multipotency remained for at least 2 months as long as the concentration of fibroblast growth factor-2 in the medium was maintained at > 20 ng/mL (Figure 1B). After fibroblast growth factor-2 was removed from the culture system, adult hippocampal progenitor cells began to differentiate spontaneously and the proliferation sharply decreased during the following 7-day period. To investigate the details of spontaneous differentiation in adult hippocampal progenitor cells, we plated passage 4 cells on poly-D-lysine-coated dishes. One day after seeding, the culture medium was replaced with fresh medium without fibroblast growth factor-2. From the 3<sup>rd</sup> day, morphological changes characterized by long and thick processes were observed. Seven days later, some neuron-, astrocyte- and oligodendrocyte-like cells were observed throughout the plate (Figures 1C–E). Semi-quantitative analysis by immunocytochemical staining showed that on day 0, the percentage of nestin-positive cells was > 90%, Tuj1-positive and O4-positive cells were 24% and 21%, respectively, while the glial fibrillary acidic protein-positive cells were < 1%. On day 7, a marked increase of glial fibrillary acidic

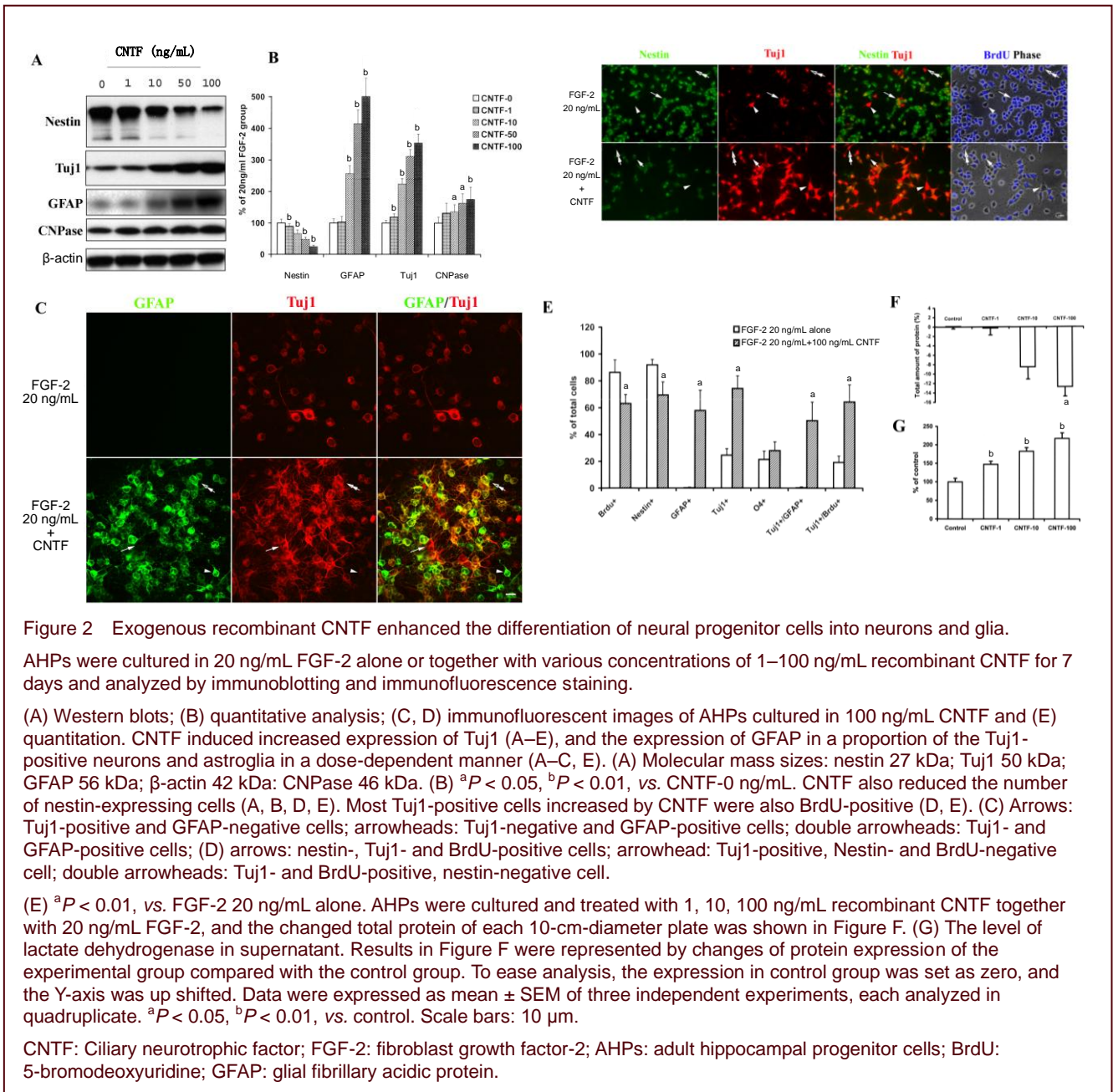
protein-positive cells from < 1% to 58% occurred, and the Tuj1-positive and O4-positive cells increased to 41% and 29%, respectively, while nestin-positive cells decreased to 7% of the total cells, and the signal was weak. The percentage of 5-bromodeoxyuridine-positive dividing progenitors also dramatically decreased from 86% to 9% (Figure 1F). Similar results were obtained using attached neural progenitor cells that had undergone multiple (more than 20) passages for 6 months, except there were 5% more neurons present after 1-week spontaneous development (data not shown).



### Exogenous recombinant ciliary neurotrophic factor enhanced the differentiation of neural progenitor cells into neurons and glial cells but compromised cell survival

Previous studies reported that ciliary neurotrophic factor induced astroglial differentiation *in vivo* [27] and in neural stem/progenitor cells isolated from various regions of the embryonic or adult brain [22, 28–29]. Ciliary neurotrophic factor can also maintain mammalian forebrain neural stem

cells [30] and the pluripotentiality of embryonic stem cells [31]. Moreover, ciliary neurotrophic factor might enhance forebrain neurogenesis indirectly *via* neighboring astroglia in adult mice [32]. To investigate the direct effect of ciliary neurotrophic factor on adult hippocampal progenitor cells, the cells were treated with various concentrations of exogenous recombinant ciliary neurotrophic factor (1–100 ng/mL) and the development of these cells was observed and analyzed by immunofluorescence and western blot. To maintain the cells immature phenotype, 20 ng/mL fibroblast growth factor-2 was added to the culture system during the experiments. As shown in Figures 2A and B, 7 days of ciliary neurotrophic factor treatment dose-dependently decreased the progenitor cell marker nestin and dramatically increased the expression levels of the neuronal marker Tuj1, as well as upregulating the astroglial marker glial fibrillary acidic protein and slightly increasing levels of the oligodendrocyte marker CNPase. Compared with the control group, 100 ng/mL ciliary neurotrophic factor induced a 4-fold expression increase in glial fibrillary acidic protein, 2.5-fold increase in Tuj1 and 75% more CNPase, while decreasing approximately 80% expression of nestin. Similarly, immunocytochemical staining showed that after 100 ng/mL ciliary neurotrophic factor treatment, approximately 60% of total cells expressed glial fibrillary acidic protein to some extent, and a few strongly glial fibrillary acidic protein-positive and Tuj1-negative cells were seen similar to radial type II astroglial cells, which have a neuron-like morphology [33]. Ciliary neurotrophic factor induced 74% of cells to express Tuj1, and some intensely-stained cells exhibited big cell bodies and thick, long processes compared with the control group, of which 25% of total cells expressed Tuj1. Interestingly, about 60% of Tuj1-positive cells co-expressed glial fibrillary acidic protein, which occurred exclusively in the ciliary neurotrophic factor treatment group. These glial fibrillary acidic protein- and Tuj1-positive cells might be described as neuronal-glial precursors (Figures 2C, E). In addition, ciliary neurotrophic factor decreased the nestin-positive cell population from 92% to 70%, and decreased the percentages of BrdU-positive dividing progenitors from 86% to 63% (Figures 2D, E). Finally, we observed that ciliary neurotrophic factor increased the percentages of 5-bromodeoxyuridine-positive neurons (Tuj1-positive) from < 20% to 64% (Figure 2E). However, we did not observe ciliary neurotrophic factor-induced increases in O4-positive oligodendroglia (Figure 2E). These data suggest that exogenous recombinant ciliary neurotrophic factor has a positive effect on the induction of neuronal and glial lineage determination in cultured adult hippocampal progenitor cells.



The effect of recombinant ciliary neurotrophic factor on the proliferation and cell survival was determined by analysis of total protein and lactate dehydrogenase assay, respectively. Adult hippocampal progenitor cells were treated with 1, 10, 100 ng/mL ciliary neurotrophic factor together with 20 ng/mL fibroblast growth factor-2 for 7 days. As shown in Figures 2F–G, recombinant ciliary neurotrophic factor dose-dependently decreased the total amount of protein and increased the supernatant levels of lactate dehydrogenase. These results suggest that, except for the induction of neuronal and glial cells, ciliary neurotrophic factor also inhibits the proliferation of cultured adult hippocampal progenitor cells probably by compromising the survival of adult hippocampal progenitor cells.

### Adult neural progenitor cells strongly expressed endogenous ciliary neurotrophic factor

Based on the above results and observations regarding the effect of neurotrophic factors on neural stem/progenitor cells, ciliary neurotrophic factor is the only neurotrophic factor to date that can induce both neuronal and glial cell fate commitment of neural progenitor cells. Coincidentally, spontaneous differentiation induces adult hippocampal progenitor cells to mature into one of three types of neural cells. To test the hypothesis that endogenous ciliary neurotrophic factor may play a key role in spontaneous differentiation, we investigated whether adult hippocampal progenitor cells produced endogenous ciliary neurotrophic factor. Immunoblot analysis showed that adult hippocampal progenitor cells

from floating neurospheres (passage 1), early (passage 4) to late stages (passage 22) expressed ciliary neurotrophic factor (Figure 3A), and the signal intensity of ciliary neurotrophic factor in late stage adult hippocampal progenitor cells was at least two-fold greater than in early adult hippocampal progenitor cells, and the neurospheres expressed very low ciliary neurotrophic factor levels. The existence of endogenous ciliary neurotrophic factor in adult hippocampal progenitor cells was also confirmed by immunocytochemical staining. As shown in Figure 3B, about 70–80% of early (passage 4) adult hippocampal progenitor cells exhibited an intense signal for ciliary neurotrophic factor in the cytoplasm, while many nuclear-stained cells could also be observed. In late stage (passage 22) adult hippocampal progenitor cells, almost all cells expressed high levels of ciliary neurotrophic factor in the cytoplasm and had thick processes. Next, the effect of spontaneous differentiation on the expression of ciliary neurotrophic factor and the co-localization of ciliary

neurotrophic factor with the neuronal marker  $\beta$ -tubulin and astroglial marker glial fibrillary acidic protein were investigated. As shown in Figure 3D, after depletion of fibroblast growth factor-2 for 1 week, the number of ciliary neurotrophic factor-positive cells (passage 4) significantly increased ( $P = 0.0072$ ) compared with controls. Moreover, spontaneous differentiation induced a marked increase of glial fibrillary acidic protein-positive adult hippocampal progenitor cells (Figure 1F), and promoted ciliary neurotrophic factor- and glial fibrillary acidic protein-positive adult hippocampal progenitor cells (from  $<1\%$  to  $90\%$ ). To our surprise, some glial fibrillary acidic protein-positive adult hippocampal progenitor cells were still ciliary neurotrophic factor-negative (Figure 3C upper panel, Figure 3E). Similarly, the number of ciliary neurotrophic factor- and Tuj1-positive adult hippocampal progenitor cells also significantly increased after spontaneous differentiation (from  $89\%$  to  $100\%$ ,  $P = 0.0016$ ).

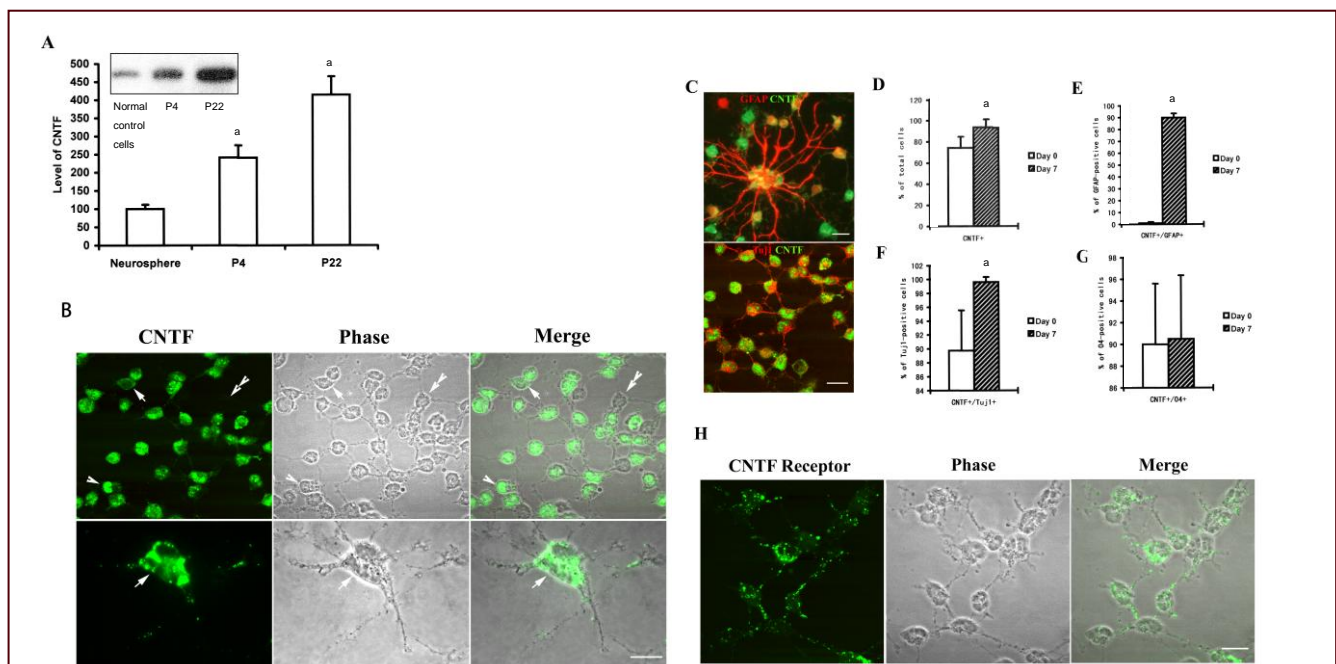


Figure 3 Adult rat hippocampal progenitor cells expressed endogenous CNTF and CNTF receptors.

Passage 1 floating neurospheres, passages 4 and 22 attached cells were analyzed by western blot (A) for the presence of CNTF protein. Passage 22 cells expressed 1.5- and 3-fold more CNTF than passage 4 attached cells and passage 1 neurospheres, respectively. The expression of endogenous CNTF in passage 4 (upper three panels) and passage 22 (lower three panels) AHPs was shown in Figure B. CNTF was widely distributed in the cytoplasm, nuclei and cell processes. Arrows: CNTF was mainly expressed in the cytoplasm; arrowhead: CNTF expressed in nuclei; double arrowhead: CNTF-negative cells. CNTF molecular mass in Figure A was 22.8 kDa, indicating CNTF expression in different passages of AHPs, expressed as the ratio of standardized normal control cells as 100% to passages 4 and 22.  $^aP < 0.01$ , vs. neurospheres 7 days after removal of FGF-2.

Immunocytochemical analysis of co-localization for CNTF and neural markers GFAP and  $\beta$ -tubulin (C), and semi-quantitative analysis for staining (D–G): the majority of astrocytic GFAP-positive cells stained by CNTF (upper panel, C) and a similar staining in all Tuj1-positive cells (lower panel, C). In addition, a large number of cells expressed CNTF receptor on the perikarya and neurites (H; confocal).

Data were expressed as mean  $\pm$  SEM of three independent experiments, each analyzed in quadruplicate.  $^aP < 0.01$ , vs. day 0. Scale bars: 10  $\mu$ m.

CNTF: Ciliary neurotrophic factor; FGF-2: fibroblast growth factor-2; AHPs: adult hippocampal progenitor cells; BrdU: 5-bromodeoxyuridine; GFAP: glial fibrillary acidic protein.

Interestingly, we observed that almost all Tuj1-positive cells were co-localized with strong ciliary neurotrophic factor signal after 7 days of differentiation (Figure 3C lower panel, Figure 3F). We failed to observe changes in O4-positive cell numbers after removal of fibroblast growth factor-2 for 7 days (Figure 3G).

Ciliary neurotrophic factor signals through the ciliary neurotrophic factor receptor  $\alpha$ /leukemia inhibitory factor receptor  $\beta$ /gp130 receptor complex to regulate neural stem/progenitor cell fate *in vivo* and *in vitro*<sup>[29]</sup>. Next, the ciliary neurotrophic factor-specific receptor, which is an essential element downstream of the ciliary neurotrophic factor signal transduction pathway, was detected by immunocytochemical staining. As shown in Figure 3H, most cells expressed this receptor specifically distributed in the perikarya and neurites of adult hippocampal progenitor cells.

#### Ciliary neurotrophic factor neutralizing antibodies inhibited the spontaneous differentiation of neural progenitor cells into mature neural cells

We hypothesized that the spontaneous differentiation of adult hippocampal progenitor cells was at least partly due to the presence of endogenous ciliary neurotrophic factor. To test this hypothesis, we investigated the effect of a ciliary neurotrophic factor neutralizing monoclonal antibody on endogenous ciliary neurotrophic factor-induced differentiation of adult hippocampal progenitor cells characterized by the change in levels of nestin, Tuj1, glial fibrillary acidic protein, and CNPase. Adult hippocampal progenitor cells were cultured for 7 days in the absence of fibroblast growth factor-2, culture media alone (control), 20 g/mL mouse IgG (IgG control), or 20 g/mL anti-ciliary neurotrophic factor mAb added into the culture medium. Western blot analysis of the treated cells revealed that addition of the antibody against ciliary neurotrophic factor inhibited the spontaneous differentiation of adult hippocampal progenitor cells, as determined by a 28% increase in the level of nestin, a 13% decrease in the level of Tuj1, and a 24% decrease in the level of glial fibrillary acidic protein. We also observed a slight but not significant ( $P > 0.05$ ) decrease in CNPase after the anti-ciliary neurotrophic factor treatment. The control mouse IgG did not have any effect on the levels of nestin, Tuj1 or any glial markers (Figure 4).

## DISCUSSION

Encouraging results using neuronal stem cells have

been obtained in animal models of neurodegenerative disease, including Parkinson's disease<sup>[34-35]</sup>, Alzheimer's disease<sup>[36-38]</sup>, stroke<sup>[35, 39-40]</sup>, multiple sclerosis<sup>[41-42]</sup>, and spinal cord injury<sup>[43-44]</sup>.

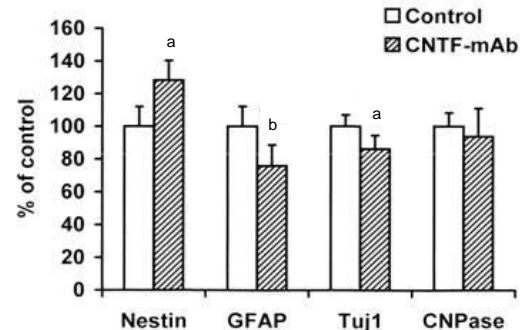


Figure 4 Functional neutralizing antibody against CNTF inhibited the spontaneous neuronal and glial differentiation in AHPs.

AHPs were cultured in the absence of FGF-2 (control), or in the presence of 20 g/mL mouse IgG or 20 g/mL CNTF-mAb for 7 days and then analyzed by western blot analysis. The levels of nestin, Tuj1, GFAP, and CNPase were normalized as 100% of control.

Treatment with CNTF antibodies caused a marked increase in nestin expression, a significant decrease in GFAP and Tuj1 expression, and had no effect on CNPase. The control mouse IgG did not have any effect on the levels of any neural markers. Data were expressed as mean  $\pm$  SEM of three independent experiments, each analyzed in quadruplicate. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs. control. Y-axis: ratio to internal reference; the ratio of control group was normalized as 100%.

CNTF: Ciliary neurotrophic factor; FGF-2: fibroblast growth factor-2; AHPs: adult hippocampal progenitor cells; BrdU: 5-bromodeoxyuridine; GFAP: glial fibrillary acidic protein; mAb: monoclonal antibody.

Several studies show that neural stem cells mainly differentiate into astrocytes after grafting<sup>[45]</sup>. Furthermore, neural stem cells need to be modified *in vitro* by directed-inducing differentiation before the treatment to assure their optimal growth and function in the specific injured or degenerative brain regions<sup>[46-49]</sup>. Spontaneous differentiation might be a native property of neural stem/progenitor cells that is critical in cell fate determination. To overcome this obstacle, most neural stem/progenitor cells-related studies have used fibroblast growth factor-2 as a mitogen to maintain the cells at an immature phenotype after the cells were isolated<sup>[13-14, 18]</sup>. However, the potential role and the related molecular mechanism of spontaneous differentiation are unclear. During development, growth factors provide important extracellular signals for regulating the proliferation and fate determination of neural stem/progenitor cells in the central nervous system<sup>[50-51]</sup>. These studies provided a better

understanding of exogenous mitogens such as fibroblast growth factor-2 and endogenous molecules that influence the spontaneous differentiation of neural stem/progenitor cells. In the present study, we reported two main conclusions. First, adult hippocampal progenitor cells start proliferating as adherent cells and attach to each other, eventually giving rise to spherical clusters that float in suspension and form the so-called “neurospheres” in the presence of fibroblast growth factor-2. When the cells were moved to a poly-D-lysine-coated culture plate, the neurospheres quickly attached to the plate and the adult hippocampal progenitor cells propagated outward from the middle of the spheres. On removal of fibroblast growth factor-2, the progeny of adult hippocampal progenitor cells promptly (within 1 week) differentiated spontaneously and expressed different antigenic markers. Nestin expression almost disappeared while glial fibrillary acidic protein was expressed intensively. Tuj1 expression increased markedly and oligodendrocyte marker O4 increased relatively mildly. These data indicate that in the absence of mitogens, adult hippocampal progenitor cells spontaneously differentiate into astrocytes, neurons, and oligodendrocytes. Endogenously produced molecules such as growth factors might be involved in these events, and may be responsible for the dramatic differentiation of adult hippocampal progenitor cells to astrocytes.

Recombinant ciliary neurotrophic factor is a strong inducer of glial lineage cells in cultured fetal precursors<sup>[52-53]</sup>. Ciliary neurotrophic factor was also found to be synthesized by fetal neural stem cells<sup>[19]</sup>. We hypothesized that ciliary neurotrophic factor might be involved in the spontaneous glial differentiation of adult hippocampal progenitor cells. First, the direct effect of exogenous recombinant ciliary neurotrophic factor on the adult hippocampal progenitor cells was investigated. We reported here that the role of ciliary neurotrophic factor on adult hippocampal progenitors was, to a certain extent, different from other reports. A few cells presumed to be previously uncommitted differentiated into astroglia, whereas the majority of cells strongly expressed Tuj1. In approximately 60% of the Tuj1-positive cells, ciliary neurotrophic factor treatment induced the expression of glial fibrillary acidic protein. This type of “neuronal-glial progenitor” might be derived from a developmentally less advanced population of progenitors possessing greater than the Tuj1-positive only cells. One report demonstrated that ciliary neurotrophic factor has potent survival and differentiation promoting effects *in vitro* on oligodendrocyte precursor cells isolated from adult spinal cord<sup>[54]</sup>, and acts on oligodendrocytes by favoring their

final maturation<sup>[23, 55]</sup>. These results suggest that, to date, ciliary neurotrophic factor is the only growth factor reported to induce adult neural progenitor cells to mature into one of three main types of neural cells.

The second conclusion of this analysis is that endogenous ciliary neurotrophic factor produced by adult hippocampal progenitor cells may be involved in spontaneous neuronal and glial differentiation. Late stage cells expressed high levels of ciliary neurotrophic factor, which implies this growth factor may play a critical role in late stage adult hippocampal progenitor cells with relatively limited multipotential and active differentiation. Similarly, spontaneous differentiation for 1 week caused an obvious increase in ciliary neurotrophic factor expression levels in adult hippocampal progenitor cells, suggesting that endogenous ciliary neurotrophic factor participates in the spontaneous development. Interestingly, spontaneous differentiation for 1 week promoted a marked increase in ciliary neurotrophic factor- and glial fibrillary acidic protein-positive cells, a relatively mild elevation of ciliary neurotrophic factor- and Tuj1-positive cells. Almost all neuronal (Tuj1-positive), and 90% of astroglial (glial fibrillary acidic protein-positive) cells co-localized with ciliary neurotrophic factor suggesting that endogenous ciliary neurotrophic factor probably plays a critical role in both spontaneous neurogenesis and astroglial differentiation, although this does not discount other unknown mechanisms that might be involved in spontaneous differentiation.

In addition, most oligodendrocytic O4-positive cells co-localized with ciliary neurotrophic factor. However, the proportion of O4- and ciliary neurotrophic factor-positive cells did not change significantly after 1 week of spontaneous differentiation, because of the relatively weak induction of endogenous ciliary neurotrophic factor on oligodendrocytic determination or an inadequate period of spontaneous differentiation. These results suggest that ciliary neurotrophic factor may play a role in cell fate determination to various degrees. With regard to the localization of ciliary neurotrophic factor in the nucleus previously reported, we obtained similar results. The significance of this finding is not clear and should be investigated in the future.

Treatment with a neutralizing antibody specific for ciliary neurotrophic factor counteracted the spontaneous neuronal and glial differentiation suggesting that endogenous ciliary neurotrophic factor contributes to the initiation and/or maintenance of spontaneous neural maturation in adult hippocampal progenitor cells cultured

in the absence of fibroblast growth factor-2. Intraventricular injection of neutralizing anti-ciliary neurotrophic factor antibodies were used to investigate the effect of ciliary neurotrophic factor on forebrain neurogenesis in adult mouse brain, and enhanced forebrain neurogenesis<sup>[32]</sup>. This study clearly showed a 13% decrease in neurogenesis and 24% decrease in astroglial differentiation as well a 28% increase in nestin expression on neural progenitor cells marker nestin. These changes were caused by administration of the neutralizing antibody against ciliary neurotrophic factor.

In the present study, the number of cells dramatically decreased after ciliary neurotrophic factor treatment as analyzed by measurement of the total amount of protein. Two or more possible mechanisms could explain the decreased number of adult hippocampal progenitor cells by ciliary neurotrophic factor. Either ciliary neurotrophic factor inhibits self-renewal of fibroblast growth factor-2-responsive adult hippocampal progenitor cells or the survival of cells is affected by ciliary neurotrophic factor. Even though the lactate dehydrogenase assay confirms that ciliary neurotrophic factor compromises the survival of cultured adult hippocampal progenitor cells, it is still possible that immature neurons and glial cells derived from the ciliary neurotrophic factor treatment cannot survive in the absence of specific neurotrophic factors or culture medium, *i.e.*, ciliary neurotrophic factor does not cause cell death directly. Previous studies reported that ciliary neurotrophic factor enhanced self-renewal of epidermal growth factor-responsive neural stem cells without affecting cell proliferation or survival<sup>[30]</sup>, and ciliary neurotrophic factor over-expression in neural progenitor cells increased cell proliferation<sup>[56]</sup>.

Taken together, these data obtained from a series of experiments using exogenous ciliary neurotrophic factor induction, endogenous ciliary neurotrophic factor expression and functional neutralization suggest that ciliary neurotrophic factor may play a critical role in the spontaneous neurogenesis and glial differentiation in cultured adult hippocampal progenitor cells.

In the adult rat brain, ciliary neurotrophic factor can induce the proliferation of hippocampal progenitors, followed by neurogenesis and enhancement of spatial memory<sup>[32]</sup>. Ciliary neurotrophic factor application in the neurogenic regions of brain did not have a remarkable effect on astrogliosis<sup>[32]</sup>, whereas it induced the activation of astroglial and microglial cells<sup>[57-58]</sup> when applied to other areas of brain. Glial activation, however, seems to be benign and does not interfere with the neuroprotective

activity of CNSase as demonstrated in animal models of Huntington's disease and spinal cord injury<sup>[59-61]</sup>. The generation of reactive oxygen species with the accumulation of oxidative damage is implicated in brain aging and neurodegenerative diseases, which inhibits ciliary neurotrophic factor activity in neurons<sup>[62]</sup>, indicating that ciliary neurotrophic factor is a growth factor with definite neuroprotective potential in many neural system diseases. However, the possible therapeutic potential of ciliary neurotrophic factor for conditions with extensive gliosis like Alzheimer's disease remains to be explored<sup>[24]</sup>.

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## MATERIALS AND METHODS

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### Design

Cell biology contrast observation.

### Time and setting

The experiment was performed in New York State Institute for Basic Research, Department of Neurochemistry and the Key Laboratory of Nervous System Disease, Huzhong University of Science and Technology, China in 2011.

### Materials

Three-month-old male Wistar rats (specific-pathogen free level) were used for all experiments. Animals were housed in standard cages and allowed free access to food and water. All protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China<sup>[63]</sup>.

### Methods

#### **Isolation and culture of progenitor cells from adult rat hippocampus**

Adult hippocampal progenitor cells were isolated as described previously<sup>[61]</sup>. Briefly, rats were euthanized with a lethal dose of nembutal administered intraperitoneally and the hippocampi were quickly dissected and chopped into 0.5 mm<sup>3</sup> pieces in Hibernate A containing 2% B27 supplement and 0.5 mM glutamine at 4°C (Invitrogen/Life Technologies, Grand Island, NY, USA). The tissue pieces were digested with 2 mg/mL papain (Worthington, Freehold, NJ, USA) in Hibernate A/B27 for 30 minutes at 30°C with shaking on a reciprocal shaker at 170 r/min. After washing with warm Hibernate A/B27 once and triturating 15–20 times with a siliconized Pasteur pipette in Hibernate A/B27, the progenitors were enriched on an Optiprep step density



gradient and plated onto a 0.01% poly-D-lysine (70–150 kDa; Sigma, St. Louis, MO, USA) -coated plate (Corning, Corning, NY, USA) in Neurobasal A containing 2% B27 supplement and 0.5 mM glutamine. After culture at 37°C in a CO<sub>2</sub> incubator for 1 hour, the medium was replaced with fresh medium containing 100 IU/mL penicillin, 100 g/mL streptomycin, and 10 ng/mL fibroblast growth factor-2 (Peprotec, Rockhill, NJ, USA). The medium was changed every other day, and the cells were passaged at confluence by trituration with a 1-mL Eppendorf pipette. After the first passage, the fibroblast growth factor-2 concentration was increased to 20 ng/mL. Early-stage cultures from the third to fifth passages and late stage cells after 20 passages (about 6 months culture time) were used for the present study.

Cells that had been cultured in 10-cm-diameter poly-D-lysine-coated dishes (Corning) were used to study the effect of various growth factors. Starting on day 0,  $2 \times 10^5$  cells/mL were seeded onto the plates, and the fibroblast growth factor-2 concentration was maintained at 20 ng/mL. After 1 day, the cells were treated with ciliary neurotrophic factor (Peprotec) or fibroblast growth factor-2 at different concentrations (0–100 ng/mL for ciliary neurotrophic factor and 0–100 ng/mL for fibroblast growth factor-2) in fresh medium. The medium was changed every other day. The experiments were terminated after 7 days of treatment (14 days for long-term observation), when the fastest growing cultures had reached 95% confluency.

#### ***Cytotoxicity assay by lactate dehydrogenase assay***

After culture for 5 weeks, a fluorometric assay (CytoTox-One Homogenous Membrane Integrity Assay Kit, Promega) was used for the measurement of lactate dehydrogenase 8 released from damaged or dying cells. Briefly, 100  $\mu$ L of conditioned media was aliquoted into a black 96-well plate. To each well, 100  $\mu$ L of the CytoTox-One reagent was added for 10 minutes. Following termination of the enzymatic reaction, resulting fluorescence was measured [560 nm (excitation)/590 nm (emission)] using a Viktor3 ELISA plate reader (Perkin Elmer, Boston, MA, USA). These values were subsequently normalized and expressed as a percentage of total lactate dehydrogenase release (assessed by treating cultures with 1% Triton X-100).

#### ***Determination of nestin, tubulin, glial fibrillary acidic protein, 5-bromodeoxyuridine, anti-O4 and IgG expression by immunofluorescence***

For immunofluorescence, the cells were cultured on 0.01% poly-D-lysine-precoated Lab-Tek II slides (Nunc;

Naperville, IL, USA) for 6–7 days. 5-bromodeoxyuridine (10 ng/mL; Sigma) was added to the cultures for the last 12 hours. Cells were then fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS for 5 minutes, blocked with 5% bovine serum albumin in Tris-buffered saline for 10 minutes, and incubated with a mixture of primary antibodies isolated from different species at 37°C overnight, followed by fluorescent secondary antibodies for 2 hours. The following antibodies were used as primary antibodies: mouse anti-rat nestin (1:500; BD Pharmingen San Jose, CA, USA), mouse anti-rat tubulin (Tuj1; 0.5 g/mL; BD Pharmingen), mouse anti-rat glial fibrillary acidic protein (1:2 000; BD Pharmingen); rat anti-5-bromodeoxyuridine (1:1 000; Accurate Chemical & Scientific Co.; Westbury, NY, USA); mouse anti-rat anti-O4 (11 g/mL; Chemicon; Temecula, CA, USA); mouse anti-rat IgG (1:5 000; ICN Pharmaceuticals UP Biochemical; Aurora, OH, USA). The following secondary antibodies were used: Alexa Fluor 488 goat anti-mouse IgG (H+L), Alexa Fluor 355 goat anti-rat IgG (H+L), Alexa Fluor 594 donkey anti-rabbit IgG (H+L), Alexa Fluor 594 donkey anti-mouse IgG (H+L), and Alexa Fluor 488 donkey anti-rat IgG (H+L). All fluorescent secondary antibodies were diluted at 1:1 000 and were purchased from Molecular Probes (Eugene, OR, USA). All antibodies were diluted in blocking solution. Samples were washed three times with Tris-buffered saline and cover-slipped in SlowFade Light Antifade solution (Molecular Probes; Eugene). For 5-bromodeoxyuridine staining, samples were incubated for 30 minutes in 2 M HCl at 37°C and rinsed for 10 minutes in 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> (Borax), pH 8.5, and six 15-minute rinses in Tris-buffered saline, pH 7.5, then permeabilized with Triton and incubated with antibodies. In some cases, cells were treated with 50 g/mL 4, 6-diamidino-2-phenylindole (Sigma) for 10 minutes. All images of immunostained cells were taken using a high-resolution digital camera set up for a Zeiss Axiophot equipped for epifluorescence or for a laser scanning confocal microscope (PCM 2000, Nikon, Sendai, Japan). Cells were quantified using a 63  $\times$  objective. Eight fields (a total of at least 500 cells) per well of eight-well chamber slides were counted, with at least two wells for each experimental point in each experiment. Cells of all staining intensities were included in the quantification.

#### ***Determination of nestin, tubulin, glial fibrillary acidic protein, ciliary neurotrophic factor, and CNPase expression by western blot***

Cells were cultured to 90% confluence. For harvesting,

the attached cells were rinsed once with cold (4°C) Neurobasal A, immediately followed by lysis buffer (1% SDS, 1%-mercaptoethanol, 1 mM freshly prepared phenylmethylene-sulfonylfluoride, 2 g/mL Aprotinin, 2 g/mL Leupeptin, and 1 g/mL Pepstatin A) for 5 minutes on ice. After the cells were fully lysed, the lysates were harvested using cell scrapers and were boiled for 5 minutes. To break the DNA, the lysates were mechanically triturated seven times using 1 mL insulin syringes (New Medical Tech; Livingston, Scotland) and then aliquoted and stored at -85°C until use. Protein concentrations were determined by the modified Lowry method of Bensadoun and Weinstein<sup>[64]</sup>. Protein samples were electrophoresed at least in triplicate on sodium dodecyl sulfate-polyacrylamide gels (8 cm × 6 cm × 0.75 cm or 20 cm × 10 cm × 0.75 cm; 7.5% or 10% acrylamide), transferred to Immobilon membranes (Millipore, Bedford, MA, USA), and probed with primary antibodies. The following primary antibodies were used: mouse anti-rat nestin (1:1 000; Developmental Studies Hybridoma Bank, Iowa City, IA, USA); mouse anti-rat β-tubulin monoclonal antibody and rabbit anti-β-tubulin polyclonal antibody (0.2 ng/mL; Covance, Berkeley, CA, USA); mouse anti-glial fibrillary acidic protein (ascites, SMI 22; 1:5 000; Sternberger); mouse neutralizing anti-ciliary neurotrophic factor (1 g/mL; R&D); mouse anti-CNPase (1:200; Chemicon, Temecula, CA, USA) at 37°C overnight. Generally, 20 g cell lysate protein per lane was applied, unless otherwise indicated. In some cases, bound antibodies were probed with <sup>125</sup>I-conjugated anti-mouse or anti-rabbit IgG. The radio immunoblots were scanned with a Fuji BAS 1500 Bio Image analyzer (Raytest USA Inc., Wilmington, DE, USA). Images were processed with the Tina software, and the intensity of immunostaining was expressed as pixels per square length.

### Statistical analysis

Data were expressed as mean ± SEM. Statistical comparisons were conducted by analysis of variance with *post hoc* Student-Newman-Keuls test when more than two groups were involved. Intergroup differences were determined using a *post hoc* Scheffer's test or paired *t*-test as appropriate. A value of *P* < 0.05 was considered statistically significant.

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**Ethical approval:** The study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, China.

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