RESEARCH ARTICLE

Cerebral dopamine neurotrophic factor promotes the proliferation and differentiation of neural stem cells in hypoxic environments

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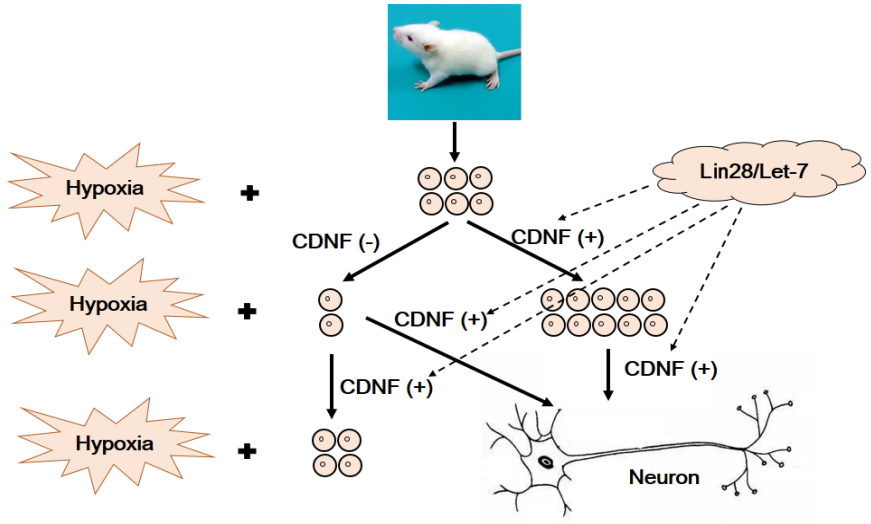
Graphical Abstract

Cerebral dopamine neurotrophic factor reverses the adverse effects on proliferation, differentiation, and apoptosis of neural stem cells in hypoxic environment



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Abstract

Previous research found that cerebral dopamine neurotrophic factor (CDNF) has a protective effect on brain dopaminergic neurons, and CDNF is regarded as a promising therapeutic agent for neurodegenerative diseases. However, the effects of CDNF on the proliferation, differentiation, and apoptosis of neural stem cells (NSCs), which are very sensitive to hypoxic environments, remain unknown. In this study, NSCs were extracted from the hippocampi of fetal rats and cultured with different concentrations of CDNF. The results showed that 200 nM CDNF was the optimal concentration for significantly increasing the viability of NSCs under non-hypoxic environmental conditions. Then, the cells were cultured with 200 nM CDNF under the hypoxic conditions of 90% N_2, 5% CO_2, and 5% air for 6 hours. The results showed that CDNF significantly improved the viability of hypoxic NSCs and reduced apoptosis among hypoxic NSCs. The detection of markers showed that CDNF increased the differentiation of hypoxic NSCs into neurons and astrocytes. CDNF also reduced the expression level of Lin28 protein and increased the expression of Let-7 mRNA in NSCs, under hypoxic conditions. In conclusion, we determined that CDNF was able to reverse the adverse proliferation, differentiation, and apoptosis effects that normally affect NSCs in a hypoxic environment. Furthermore, the Lin28/Let-7 pathway may be involved in this regulated function of CDNF. The present study was approved by the Laboratory Animal Centre of Southeast University, China (approval No. 20180924006) on September 24, 2018.

Key Words: *apoptosis; astrocyte; cerebral dopamine neurotrophic factor; differentiation; hypoxia; Let-7; Lin28; neural stem cells; neuron; proliferation*

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Introduction

Neural stem cells (NSCs) are self-renewing and multipotent cells that have the potential to differentiate into multiple cell types, such as neurons and astrocytes (Ruland et al., 2016; Zhang et al., 2019a). Generally, endogenous NSCs become active and participate in the neural repair process when a brain injury occurs (Huang and Zhang, 2019). Previous studies showed that the proliferation of NSCs decreased under severely hypoxic conditions, although mild hypoxia can improve the self-renewal and differentiation abilities of NSCs

(Plane et al., 2004; Hayashi et al., 2005; Ikeda et al., 2005; Yang and Levison, 2006). Furthermore, NSC apoptosis also increases in a low-oxygen environment (Ikeda et al., 2005). Some published studies revealed that NSCs were more sensitive to oxygen concentrations than other types of cells and that hypoxia had significant adverse effects for NSCs (Qi et al., 2017; McGrath et al., 2018). Therefore, exploring whether any interventions exist that can rescue the proliferation and differentiation abilities of NSCs under severely hypoxic conditions is important.

Neurotrophic factors, including proteins and peptides, are essential for the growth and survival of the central nervous system and may play potential roles in the protection and recovery of NSC viability (Tome et al., 2017). Among known neurotrophic factors, cerebral dopamine neurotrophic factor (CDNF), a newly-identified neurotrophic factor, has potential therapeutic benefits during Parkinson's disease and is regarded as a promising therapeutic agent for other neurodegenerative diseases (Tang et al., 2017). Airavaara et al. (2012) found that CDNF was able to protect midbrain dopaminergic neurons and restore motor functions in a Parkinson's disease rat model. In addition, CDNF has few side-effects and greater effects at low to medium concentrations compared with other potentially protective factors (Tang et al., 2017). However, no studies have investigated the relationship between CDNF and NSCs thus far. Therefore, exploring whether CDNF can contribute to improving the viability of NSCs is necessary.

Lin28, a highly conserved RNA-binding protein, plays an important role in cell metabolism, cell cycle regulation, and pluripotency (Ambros and Horvitz, 1984). A recent study showed that Lin28 can promote proliferation and differentiation during mammalian primordial germ cell formation, particularly spermatogonial stem cell self-renewal and differentiation (Wang et al., 2015). In addition, Viswanathan et al. (2008) discovered that Lin28 may be a negative regulator of microRNA (miRNA)-associated biological functions and play a key role in blocking the miRNA-mediated differentiation of stem cells. The lethal-7 (Let-7) miRNA is synthesized from primary miRNA by Drosha and Dicer enzymes in the nucleus and cytoplasm (Büssing et al., 2008). Farzaneh et al. (2017) found that Lin28 can regulate the self-renewal of mammalian embryonic stem cells by reducing the expression of Let-7. Because the overexpression of Let-7 may be harmful to neural progenitor cells, the negative regulation of Let-7 by Lin28 is considered to represent an important regulatory process for cell proliferation and differentiation during development (Farzaneh et al., 2017). Furthermore, Let-7 family miRNAs were found to be upregulated during brain development in a mouse model, and their expression levels were dramatically increased during neuronal differentiation and maturation (Wulczyn et al., 2007; Thornton and Gregory, 2012).

The purpose of the present study was to determine the effects of CDNF on the proliferation, differentiation, and apoptosis of NSCs in a hypoxic environment and to explore whether the Lin28/Let-7 pathway is associated with the CDNF-mediated regulation of NSC viability in a hypoxic environment.

Materials and Methods

NSC isolation and culture

The animal study was approved by the Laboratory Animal Centre of Southeast University, China (approval No. 20180924006) on September 24, 2018. Four 14-day-old specific-pathogen-free (SPF)-grade Sprague-Dawley (SD) rats (3 females and 1 male) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (License No. SYXK (Su) 2016-0014; Nanjing, Jiangsu Province, China), and were maintained with a 12-hour light/12-hour dark cycle and provided with sufficient food and water. Primary NSCs were extracted from the hippocampi of fetal rats (from pregnant female rats at 14 days), according to a previously established protocol (Peng et al., 2013). Cells were collected and resuspended in complete medium [Dulbecco's modified Eagle medium/nutrient mixture F-12 basal medium (Shanghai Yuanpei Biotechnology Co., Ltd., Shanghai, China) containing 20 ng/mL epidermal growth factor (GenScript, Nanjing, Jiangsu Province, China), 20 ng/mL basic fibroblast growth factor (GenScript), 2% B27, 2% N_2, 1% penicillin-streptomycin, 2 nM glutamate (Aladdin, Shanghai, China), and 2 mg/mL heparin sodium (Aladdin)] in a culture flask and maintained in a 37°C, 5% CO_2 incubator (ThermoFisher, Oakland, New Zealand). The medium was replaced with fresh medium every 3 days, and cells were passaged every 7 days. Then, single cells were dissociated from neurospheres with Accutase dissociation reagent (Sigma, Merck Darmstadt, Germany) and allowed to form new neurospheres.

Immunofluorescence assay for NSCs

To identify NSCs, cells were dissociated with Accutase dissociation reagent and plated at 1×10^5 cells per well, in a 24-well plate coated with poly-D-lysine polymers (Sigma), in complete medium, for 7 days. Cells were fixed in 4% paraformaldehyde, after being washed in phosphate-buffered saline, followed by Triton X-100 (Sigma). Then, chamber slides were blocked in 10% donkey serum solution (Jackson, Lancaster, PA, USA). Cells were subsequently incubated with rabbit anti-nestin primary antibody (1:1000; Abcam, Cambridge, UK), at 4°C overnight. Then, cells were incubated with donkey anti-rabbit-Alexa Fluor® 647 secondary antibody (1:500; Bioss, Beijing, China), at 25°C for 1 hour. After the addition of the nuclear marker 4′,6-diamidino-2-phenylindole, the slides were cover-slipped, and images were captured using a fluorescence microscope (Leica, Wetzlar, Germany).

Cell viability/proliferation cell counting kit-8 assay

A hypoxic environment was established by placing NSCs in a humidified chamber (StemCell Technologies, Vancouver, Canada) with a gas mixture containing 90% N_2, 5% CO_2 and 5% air, at 37°C. As described in a previous study (Zhao and Zheng, 2017), a hypoxic cell model was generated by exposing cells to hypoxic conditions for 6 hours.

To determine the optimal concentration of CDNF, 100 μL of NSCs (at a cell concentration of 2×10^5/L) were seeded and inoculated in 6 wells of a 96-well plate, in a non-hypoxic environment (5% CO_2 and 95% air), using fresh complete medium containing different concentrations of CDNF (0, 50, 100, 150, 200, and 250 nM; Meilunbio, Dalian, Liaoning

Province, China). Then, 10 μL cell counting kit-8 was added to each well (KARLBIO, Hangzhou, Zhejiang Province, China). After incubation 2 hours, the proliferation rate was colorimetrically determined. Proliferation rate (%) = [optical density $(OD)_{450}$ of cytokine group – OD_{450} of control group]/OD_{450} of control group × 100.

In addition, 400 μL of NSCs at a cell concentration of 2 × 10^5/L were equally divided into four groups, including non-hypoxic, non-hypoxic + CDNF, hypoxic, and hypoxic + CDNF groups, which were seeded into individual wells of a 96-well plate. The non-hypoxic and non-hypoxic + CDNF groups were cultured in a non-hypoxic environment (5% CO_2 and 95% air), whereas the hypoxic and hypoxic + CDNF groups were cultured in a hypoxic environment, as described above. After 6 hours, cell counting kit-8 was added to each well (Nanjing KeyGen Biotech, Co, Ltd., Nanjing, Jiangsu Province, China). After incubation, the proliferation rate was colorimetrically determined. Proliferation rate (%) = (OD_{450} of cytokine group – OD_{450} of control group)/OD_{450} of control group × 100.

Apoptosis assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to evaluate the apoptosis ratio of cells. Apoptotic cells were detected with TUNEL staining, according to the manufacturer's instructions (Roche, Shanghai, China). Cells with yellow-brown granules in the nuclei were regarded as apoptotic cells. The percentages of apoptotic neurons were calculated for each group and normalized against the percentage of apoptotic neurons under control conditions, using a fluorescence microscope (LSM700; Carl Zeiss, Oberkochen, Germany).

NSC differentiation experiment

NSCs were dissociated with Accutase dissociation reagent and plated at 5 × 10^5 per well, in a 6-well plate coated with poly-D-lysine polymers, in differentiation medium (Dulbecco's modified Eagle medium/nutrient mixture F-12 supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin) for 3 days. NSCs were cultured under different oxygen concentration conditions for one day and then cultured in a non-hypoxic environment for 2 days.

Western blot analysis

Total protein was extracted from cells with radioimmunoprecipitation assay buffer. Protein concentration was determined based using a bicinchoninic acid protein assay reagent kit (Bio-Rad, Santa Rosa, CA, USA), according to the manufacturer's instructions. The extracted protein sample was combined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample loading buffer (Beyotime, Shanghai, China), and incubated in boiling water for 10 minutes. A total of 10 μg protein was loaded in each well of a 10% acrylamide gel, and electrophoresis was performed, for 45 minutes at 100 mV. Then, the proteins were transferred to a polyvinylidene fluoride (Biosharp, Hefei, Anhui Province, China) membrane, for 60 minutes at 500 mA. Membranes were blocked with 5% nonfat dry milk, for 1 hour at room temperature, and were incubated, at 4°C overnight, with the following primary antibodies: monoclonal antibodies against Tuj1 (neuron marker; 1:1000; rabbit; Abcam), glial fibrillary acidic protein (GFAP, astrocyte marker; 1:1000; rabbit; Abcam), Lin28 (1:1000; rabbit; Abcam), Iba-1 (microglia marker; 1:1000; goat; Abcam), Nestin (NSC marker; 1:1000; rabbit; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2000; rabbit; Abcam). After washing four times with Tris-buffered saline containing Tween-20 (Seebio, Shanghai, China), for 15 minutes at room temperature, the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit/goat IgG; 1:10,000; Biosharp, Hefei, Anhui Province, China) was added to the polyvinylidene fluoride membrane and incubated for 1 hour at room temperature. Subsequently, the polyvinylidene fluoride membrane was washed with Tris-buffered saline containing Tween-20 (15 minutes × 4 times), and protein bands were detected using an enhanced chemiluminescence kit (Pierce, San Francisco, CA, USA). Protein bands were quantified using a LAS400 mini (GE Healthcare, Boston, MA, USA), using GAPDH as an internal reference. The optical density of each band was quantified using ImageJ software (http://rsbweb.nih.gov/ij/) and normalized against the intensity of the GAPDH band.

Quantitative real-time polymerase chain reaction

Total RNA from NSCs was isolated using TRIZOL reagent (Invitrogen, San Francisco, CA, USA), and cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, USA), according to the manufacturer's protocol. The quantity and quality of RNA were subsequently identified, using a NanoDrop 2000 (Thermo Scientific, San Francisco, CA, USA), following the manufacturer's instructions. Quantitative real-time polymerase chain reaction was performed using SYBR green (Thermo Scientific). The polymerase chain reaction primers were as follows: Let-7: forward primer, 5′-ACC GGA CCT GGT GGA GTA TTC-3′, reverse primer, 5′-GGT AGG GCT GTG GAT TTC TTC-3′; and GAPDH: forward primer, 5′-TTC CTA CCC CCA ATG TAT CCG-3′, reverse primer, 5′-CAT GAG GTC CAC CAC CCT GTT-3′. The relative mRNA expression levels of Let-7 were quantified using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) and normalized against endogenous GAPDH mRNA expression levels, under control conditions.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance, and Bonferroni's *post hoc* test was used for multiple comparisons. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant (two-sided).

Results

Identification of NSCs from primary fetal rat hippocampus

NSCs were grown as suspensions, and with the gradual fusion of proliferating cells, large nerve balls containing thousands of cells could be identified by the naked eye after 6 to 7 days (**Figure 1A**). Primary NSCs were neurosphere-like, *in vitro*, and were confirmed to express the NSC-specific marker

Nestin, using immunofluorescence (**Figure 1B**). Nestin was observed in our cell extracts, as assessed by western blot assay (**Figure 1C**), but GFAP and Iba-1 were not observed, indicating that the neurospheres were pure NSCs (data not shown).

Effects of different CDNF concentrations on the viability of NSCs under non-hypoxic conditions

NSCs were incubated with different concentrations of CDNF. Using the cell-counting kit-8 assay, we found that higher concentrations of CDNF (200 and 250 nM) significantly increased the cell viability of NSCs compared with lower concentrations (all $P < 0.01$); however, the viability of NSCs was not significantly different between the 200 and 250 nM CDNF groups (**Figure 2A**). Therefore, the 200 nM concentration of CDNF was used for subsequent research.

Effects of CDNF on NSCs in a hypoxic environment

Effects on the viability and apoptosis of NSCs

As shown in **Figure 2B**, compared with the non-hypoxic group, the viability of NSCs significantly decreased in the hypoxic environment ($P < 0.01$). However, 200 nM CDNF significantly improved the viability of NSCs in the hypoxic environment ($P < 0.05$). In addition, the apoptotic cell ratio in the hypoxic group significantly increased compared with that in the non-hypoxic group ($P < 0.01$). Compared with the hypoxic group, the number of apoptotic cells was significantly reduced in the hypoxic + CDNF group ($P < 0.05$; **Figure 2C**).

Effects on NSC differentiation

We found that the expression levels of GFAP and Tuj1 were significantly lower in the hypoxic group than in the non-hypoxic group (all $P < 0.01$). In addition, we also found that the protein expression levels of GFAP and Tuj1 in the hypoxic + CDNF group were significantly increased compared with those in the hypoxic group (GFAP: $P < 0.05$; Tuj1: $P < 0.01$; **Figure 3**).

Lin28/Let-7 pathway in NSCs with CDNF under hypoxic condition

Using western blot analysis, we found that the protein expression level of Lin28 was significantly higher in the hypoxic group than that in the non-hypoxic group ($P < 0.001$). Compared with that in the hypoxic group, the protein expression level of Lin28 significantly decreased in the hypoxic + CDNF group ($P < 0.01$). In addition, we also found that the relative expression level of Let-7mRNA was significantly decreased in the hypoxic group compared with the non-hypoxic group ($P < 0.001$) but was significantly increased after CDNF intervention ($P < 0.001$; **Figure 4**).

Discussion

In this study, we found that the co-culture of CDNF with NSCs in nondifferentiation medium was able to increase the survival of NSCs and reduce NSC apoptosis in a hypoxic environment. In addition, CDNF can reverse the insufficient differentiation of NSCs into neurons and astrocytes in a hypoxic environment. Finally, both Lin28 and Let-7 levels were significantly altered in NSCs under hypoxic conditions following CDNF treatment.

Although CDNF is a potential protective factor in many animal and cell models of nervous system diseases, the effective concentration for CDNF application has not been defined, and the mechanisms underlying these effects also not received much attention. In the present study, similar changes were detected in NSCs in both the non-hypoxic + CDNF and hypoxic + CDNF groups, suggesting that CDNF may improve the adverse effects associated with hypoxic conditions and restore normal proliferation and differentiation levels in NSCs. Additional strengths of the present study include the determination of an optimal concentration of CDNF, 200 nM, which was able to prevent the adverse pathological changes at similar levels as high-dose CDNF, and the identification of a potential protective mechanism, through which CDNF alters the expression of components in the Lin28/Let-7 pathway.

Since the concept of NSCs was first proposed by Reynolds in 1992, NSC transplantation has become a new approach to repairing nerve function after brain injury (Reynolds and Weiss, 1992; Nerhus et al., 2016; Fan et al., 2017). However, several uncertain factors during central nervous system injury may result in the transfer of a low proportion of NSCs, further decreasing the proliferation, differentiation, and migration of NSCs (Gao et al., 2013). Among these adverse factors, hypoxia is a common factor known to influence the normal functional activity of NSCs because NSCs are more sensitive to oxygen concentrations than other types of cells, and hypoxia easily induces the death of NSCs (Shao et al., 2009; Lee et al., 2016). In our study, obvious apoptosis and reduced levels of proliferation and differentiation were observed in NSCs under hypoxic conditions. Interestingly, we also found that CDNF can significantly reverse the hypoxia-mediated adverse effects in NSCs, suggesting that the protective function of CDNF may also apply to other populations of NSCs, in addition to dopaminergic neurons. Previous studies have revealed that in addition to nerve repair and protection functions, CDNF has anti-inflammation and anti-apoptotic activities, which can further significantly enhance cell survival by modulating the neuroinflammatory response and the apoptotic pathway (Mei and Niu, 2014; Zhao et al., 2014; Liu et al., 2015; Lindahl et al., 2017; Tang et al., 2017). Taken together, a suitable concentration (200 nM) of CDNF can significantly improve proliferation, differentiation, and apoptosis in NSCs, suggesting that CDNF may have potential clinical value for NSC transplantation to treat central nervous system injury.

The interplay between Lin28 and Let-7 is highly evolutionarily conserved and is crucial for the regulation of development (Lim et al., 2018; Zhang et al., 2019b). Previous studies have reported that the Let-7 family (Let-7a and Let-7b) plays important roles in NSC viability and that the Lin28/Let-7 pathway regulates cell growth and metabolism (Su et al., 2012; Jun-Hao et al., 2016). In our study, we found that Lin28 levels increased, whereas Let-7 levels decreased in NSCs under hypoxic conditions; however, the changes in Lin28 and Let-7 expression levels were significantly attenuated by CDNF intervention, suggesting that the Lin28/Let-7 pathway may be involved in the metabolism of NSCs and that CDNF can affect the regulation of Lin28/Let-7 expres-

sion when NSCs are exposed to hypoxic conditions.

The present study has certain limitations. Because the present study was performed *in vitro*, further explorations of the effects of CDNF on NSCs under hypoxic conditions *in vivo* remain necessary. Although the Lin28 and Let-7 expression levels were significantly changed in our study, the potential mechanism through which CDNF regulates the Lin28/Let-7 pathway in NSCs remains unclear, and further research is necessary to ascertain this process.

Conclusions

In the present study, the CDNF treatment of NSCs in a hypoxic environment was able to rescue viability, reduce apoptosis, and promote differentiation into neurons and astrocytes. In addition, the CDNF-mediated promotion of NSC differentiation may be associated with the Lin28/Let-7 regulatory pathway.

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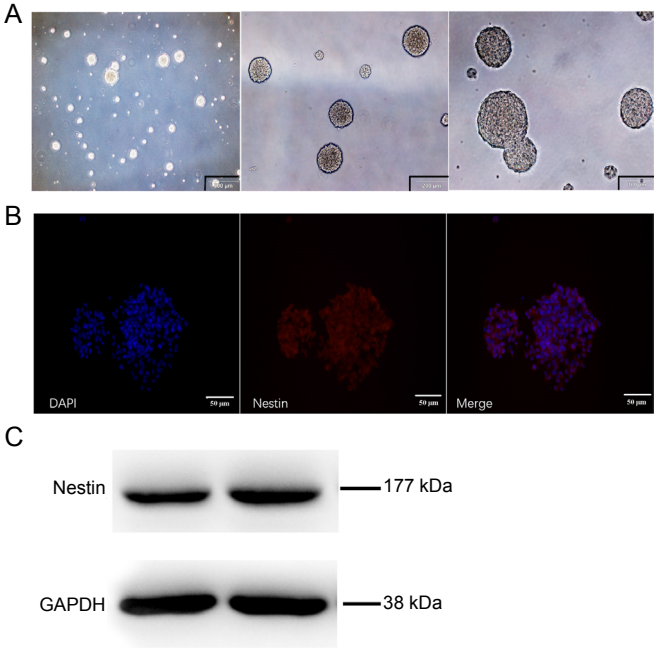

Figure 1 The characteristics of hippocampus-derived NSCs from fetal rats.
(A) Neurospheres were observed by light microscopy, *in vitro*. Scale bars: left, 500 μm; middle, 200 μm; right, 100 μm. (B) Neurospheres expressing an NSC-specific marker (Nestin, red). Scale bars: 50 μm. (C) Nestin was identified in NSCs by western blot assay (one duplicate). DAPI: 4′,6-Diamidino-2-phenylindole; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NSCs: neural stem cells.

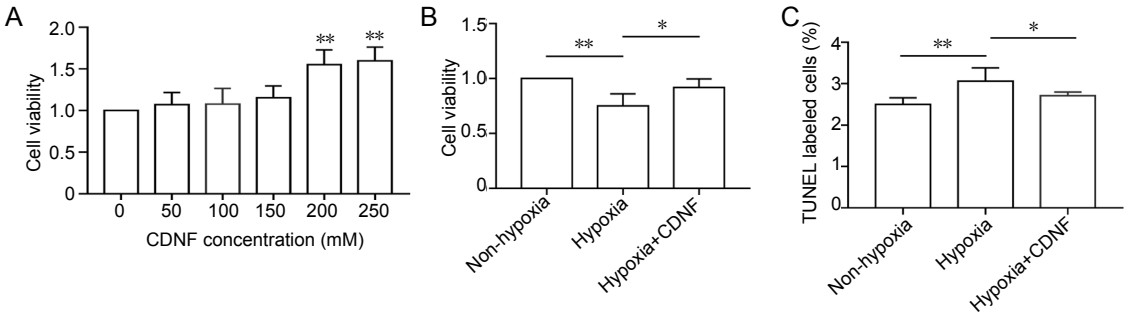

Figure 2 Effects of CDNF on NSC viability in a hypoxic environment.
(A) Effects of different concentrations of CDNF on the viability of NSCs in a non-hypoxic environment. A significant difference was identified when comparing the 200 and 250 nM concentrations with the lower concentrations. (B) Effects of CDNF on the viability of NSCs in a hypoxic environment. (C) Effects of CDNF on NSC apoptosis in a hypoxic environment. The experiment was repeated three times. Data are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$ (one-way analysis of variance, followed by Bonferroni's *post hoc* test). CDNF: Cerebral dopamine neurotrophic factor; NSCs: neural stem cells; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

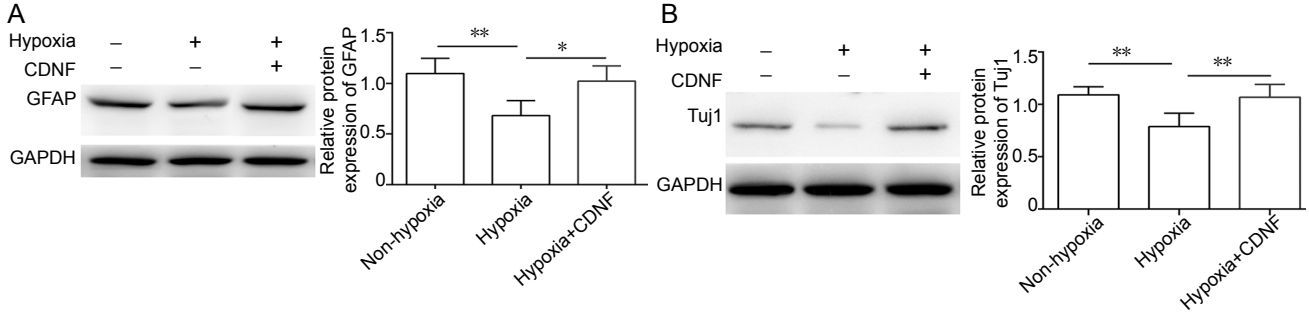

Figure 3 Effects of CDNF on NSC differentiation.
(A) Western blot analysis of GFAP protein expression levels, with or without CDNF intervention. (B) Western blot analysis of Tuj1 protein expression levels, with or without CDNF intervention. GAPDH was used as the loading control. The experiment was repeated three times. Data are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$ (one-way analysis of variance followed by Bonferroni's *post hoc* test). CDNF: Cerebral dopamine neurotrophic factor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; NSCs: neural stem cells.

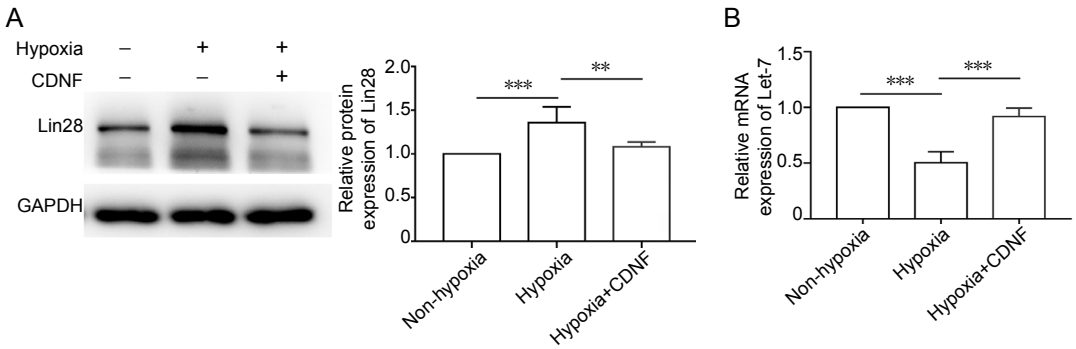

Figure 4 Effects of CDNF on the Lin28/Let7 pathway in a hypoxic environment.
(A) Western blot analysis of Lin28 protein expression levels, with or without CDNF intervention. (B) Relative mRNA expression of Let-7, as assessed by quantitative real-time polymerase chain reaction. The experiment was repeated three times. Data are expressed as the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way analysis of variance, followed by Bonferroni's *post hoc* test). CDNF: Cerebral dopamine neurotrophic factor; Let-7; lethal-7.

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
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