Effect of stromal cell-derived factor-1/CXCR4 axis in neural stem cell transplantation for Parkinson's disease

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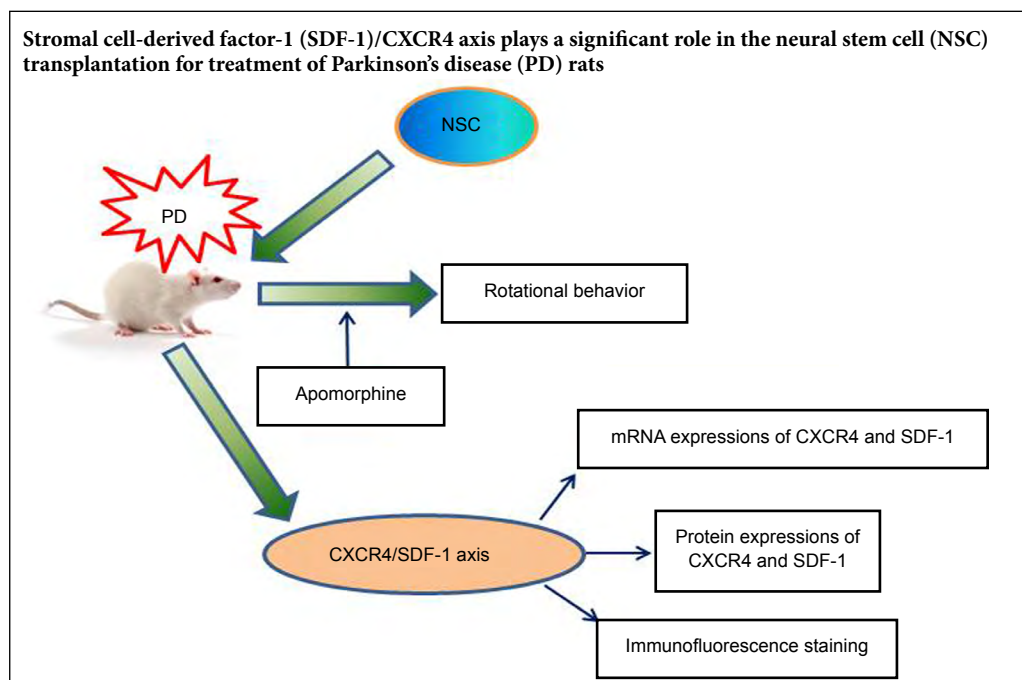2 Diagnosis Prenatal Unit, Department of Obstetrics, First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan Province, China
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Graphical Abstract

Stromal cell-derived factor-1 (SDF-1)/CXCR4 axis plays a significant role in the neural stem cell (NSC) transplantation for treatment of Parkinson's disease (PD) rats

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Abstract

Previous studies have shown that neural stem cell transplantation has the potential to treat Parkinson's disease, but its specific mechanism of action is still unclear. Stromal cell-derived factor-1 and its receptor, chemokine receptor 4 (CXCR4), are important regulators of cell migration. We speculated that the CXCR4/stromal cell-derived factor 1 axis may be involved in the therapeutic effect of neural stem cell transplantation in the treatment of Parkinson's disease. A Parkinson's disease rat model was injected with 6-hydroxydopamine *via* the right ascending nigrostriatal dopaminergic pathway, and then treated with 5 μL of neural stem cell suspension (1.5×10^4/L) in the right substantia nigra. Rats were intraperitoneally injected once daily for 3 days with 1.25 mL/kg of the CXCR4 antagonist AMD3100 to observe changes after neural stem cell transplantation. Parkinson-like behavior in rats was detected using apomorphine-induced rotation. Immunofluorescence staining was used to determine the immunoreactivity of tyrosine hydroxylase, CXCR4, and stromal cell-derived factor-1 in the brain. Using quantitative real-time polymerase chain reaction, the mRNA expression of stromal cell-derived factor-1 and CXCR4 in the right substantia nigra were measured. In addition, western blot assays were performed to analyze the protein expression of stromal cell-derived factor-1 and CXCR4. Our results demonstrated that neural stem cell transplantation noticeably reduced apomorphine-induced rotation, increased the mRNA and protein expression of stromal cell-derived factor-1 and CXCR4 in the right substantia nigra, and enhanced the immunoreactivity of tyrosine hydroxylase, CXCR4, and stromal cell-derived factor-1 in the brain. Injection of AMD3100 inhibited the aforementioned effects. These findings suggest that the stromal cell-derived factor-1/CXCR4 axis may play a significant role in the therapeutic effect of neural stem cell transplantation in a rat model of Parkinson's disease. This study was approved by the Animal Care and Use Committee of Kunming Medical University, China (approval No. SYXKK2015-0002) on April 1, 2014.

Key Words: *AMD3100; corpus striatum; CXCR4; neural stem cells; Parkinson's disease; stromal cell-derived factor-1; substantia nigra*

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Introduction

Parkinson's disease (PD) is a highly degenerative neurological disease with a worldwide prevalence of approximately 1% of the population over the age of 60 (Shen et al., 2016). The cardinal symptoms of PD include bradykinesia, disturbances in balance, and neuropsychiatric complications (Barker et al., 2015; Gao et al., 2019). The pathological hallmarks of PD are the loss of nigrostriatal dopaminergic neurons and the presence of intraneuronal proteinaceous cytoplasmic inclusions (Fjodorova et al., 2017). There is currently a lack of effective treatments or measures that can effectively improve patient symptoms in PD (Deng et al., 2013). Several advances have been reported recently in cell-based therapy that aim to replace lost dopaminergic neurons and to provide biological sources of therapeutic agents to the PD-affected brain (Staudt et al., 2016; Lee et al., 2018; Chen et al., 2019). Neural stem cells (NSCs) have the capacity to create new functional dopaminergic neurons, various glia cell types, and other neurons (Li et al., 2017b). Endogenous NSCs are located in two discrete brain regions in adults: the subgranular zone of the dentate gyrus and the subventricular zone of the lateral ventricle (Luo et al., 2014; Merino et al., 2015). In mouse models of traumatic brain injury, NSCs in the subventricular and subgranular zones are activated by astrocytes around the injured area and are promoted to migrate and differentiate into mature neurons in the striatum of adult mice (Zhang et al., 2004). However, the mechanisms by which donor cells are integrated into the host niche and migrate after transplantation remain unknown. Therefore, it is necessary to understand the mechanisms by which NSCs migrate and differentiate to be able to successfully design stem-cell-based therapeutic strategies.

Stromal cell-derived factor-1 (SDF-1), also known as chemokine CXCL12, is a small molecule cytokine belonging to the chemokine protein family. It is one of the most important chemokines in the mammalian brain (Cheng et al., 2017) and participates in maintaining homeostasis in adult brains (Liu et al., 2017). The chemokine receptor CXCR4 is widely expressed in cells of the central nervous system and the immune system, and is considered to be a receptor for SDF-1 (Addington et al., 2014). One previous study demonstrated that the SDF-1/CXCR4 axis may promote stem cell migration and homing (Zhang et al., 2016). Furthermore, the SDF-1/CXCR4 axis can be inhibited by AMD3100, a CXCR4 antagonist, thus attenuating the migration of neuroblasts (Robin et al., 2006; Peyvand et al., 2018).These previous studies demonstrate that the SDF-1/CXCR4 axis may have a significant effect on NSC transplantation in the treatment of a PD rat model.

In our study, behavioral improvement and the expression of CXCR4 and SDF-1 in the brain were observed after NSC transplantation, demonstrating a therapeutic effect of NSC transplantation in a PD rat model and suggesting that the SDF-1/CXCR4 axis plays a role in the migration of transplanted NSCs to the substantia nigra/striatum.

Materials and Methods

Animals

Animal experiments were conducted according to protocols approved by the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Fifty specific-pathogen-free male Sprague-Dawley rats weighing 180–220 g and aged 2 months were used for *in vivo* experiments, and five specific-pathogen-free fetal Sprague-Dawley rats were used for NSC isolation (pregnancy 12.5 days) at Kunming Medical University, China (license No. SCX-KK2015-0002). Rats were raised under a 12-hour light/dark cycle and had free access to food and water. Rats were fasted for 12 hours before surgery. This study was approved by the Animal Care and Use Committee of Kunming Medical University (approval No. SYXKK2015-0002) on April 1, 2014.

Ventral mesencephalic NSC cultures

NSCs were isolated from the ventral midbrains of fetal Sprague-Dawley rats at embryonic day 12.5 under sterile conditions, as previously described (Chen et al., 2018). The dissected tissue was digested and the cell suspension was transferred to serum-free Dulbecco's modified Eagle's medium/F-12 containing N2 supplement (10 μL/mL; HyClone Laboratories Inc., Logan, UT, USA), epidermal growth factor (20 ng/mL; Peprotech Inc., Rocky Hill, NJ, USA), and supplemented with basic fibroblast growth factor (20 ng/mL; HyClone Laboratories Inc.).

Ventral mesencephalic NSC differentiation culture

For *in vitro* differentiation, NSCs were induced in the same medium as in the previous paragraph, but epidermal growth factor and basic fibroblast growth factor were replaced with 10% fetal bovine serum. The neurosphere culture system and differentiated cells were determined using a fluorescence immunoassay. The primary antibodies included rabbit anti-rat Tuj1 (1:300, Abcam, Cambridge, UK), rabbit anti-rat tyrosine hydroxylase (TH, 1:500, Abcam), rabbit anti-rat CXCR4 polyclonal antibody (1:150, Abcam), rabbit anti-rat SDF-1β polyclonal antibody (1:300, Abcam), and rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:300, Abcam). The cells were incubated overnight with primary antibodies at 4°C followed by a 2-hour incubation at room temperature with fluorescence-labeled secondary antibodies; either Alexa Fluor 594 goat anti-rabbit IgG (1:200, Proteintech, Wuhan, China) or Alexa Fluor 488 goat anti-rabbit IgG (1:200, Proteintech). Nuclear counterstaining was achieved using 4′,6-diamidino-2-phenylindole (DAPI) (20 μg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA). Images were observed and captured using a fluorescence microscope (Olympus, Shibuya-ku, Tokyo, Japan).

PD modeling

Stereotaxic injections of 6-hydroxydopamine (6-OHDA, 2 μg/μL; Sigma-Aldrich Corp.) were given to healthy male Sprague-Dawley rats weighing 180–220 g. 6-OHDA with 0.2 mg/mL of ascorbic acid was prepared fresh in 0.2% ascorbate saline. Rats were intraperitoneally anesthetized with 50 mg/kg of sodium pentobarbital and mounted in a stereotaxic frame (RWD stereotaxic frame 68511, RWD Life Science Co., Ltd., Shenzhen, China). Two stereotaxic injections

of the selective catecholamine neurotoxin 6-OHDA were then unilaterally made into the right ascending nigrostriatal dopaminergic pathway using a Hamilton syringe at the following coordinates (Deng et al., 2013): 4 μL at anterior–posterior, –3.6 mm; lateral, –2.0 mm; vertical, –8.5 mm; 4 μL at anterior–posterior, +0.7 mm; lateral, –3.0 mm; vertical, –5 mm, from bregma. The injection was performed at a speed of 1 μL/minute, and the needle was left in place for an additional 5 minutes before withdrawal. The rats were then allowed to recover at room temperature (25°C). The success of the 6-OHDA lesion was verified using apomorphine (0.5 mg/kg, intraperitoneally; Sigma-Aldrich Corp.). Four weeks after surgery, rotational behavior of 6-OHDA-lesioned rats was tested over 30 minutes after receiving apomorphine. The number of complete (360°) turns in a 30-minute period was counted, and only severely lesioned rats were considered to be PD model rats (more than 7 full-body turns/minute on the lesioned side versus the unlesioned side) (Jiang et al., 2004; Deng et al., 2013). Grafted rats were also sequentially tested 4 weeks after transplantation using the same method. Behavioral testing was carried out at 1, 2, 3, and 4 weeks after transplantation. The pre-grafting/post-grafting revolutions per minute were considered as the rotation index for therapeutic effects.

Grouping and treatment

PD rats were randomly divided into three groups: NSC-grafted group (n = 12), NSC-grafted + AMD3100 group (n = 12), and AMD3100 group (n = 12). The untreated rats received saline instead of 6-OHDA as the control group (n = 12). The rats in the sham group (n = 12) received serum-free Dulbecco's modified Eagle's medium instead of NSCs and AMD3100. For the NSC-grafted group and the NSC-grafted + AMD3100 group, the NSC suspension was incubated in serum-free Dulbecco's modified Eagle's medium, and 5 μL of NSC suspension (1.5×10^4 cells/μL) were injected into the right substantia nigra of PD rats at the following coordinates: 5 μL at anterior–posterior, –3.6 mm; lateral, –2.0 mm; vertical, –8.5 mm; and 5 μL at anterior–posterior, +0.7 mm; lateral, –3.0 mm; and vertical, –5 mm from bregma. The injection speed was 1 μL/minute, and the needle was maintained in place for 15 minutes. The incisions were sutured and rats were intramuscularly injected with 100 kU of penicillin to prevent infection. The rats in the sham and AMD3100 groups received serum-free Dulbecco's modified Eagle's medium instead of cell suspensions. AMD3100 (Sigma-Aldrich Corp.) was dissolved in 1 mg/mL of sterile saline and intraperitoneally administered at a dose of 1.25 mL/kg once daily for 3 days (Saha et al., 2013) in the NSC-grafted + AMD3100 and AMD3100 groups.

Immunofluorescent staining

The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room temperature. After blocking unspecific binding with a 10% goat serum solution in PBS, cells were incubated with antibodies

according to antibody specification For experiments using brain tissue, rats were sacrificed and transcardially perfused with 500 mL sterile saline followed by 1.5% w/v paraformaldehyde in 0.1 M PBS (Fjodorova et al., 2017). The harvested brains were subsequently cryoprotected in 15%, 30%, and 40% sucrose solutions at 4°C. Coronal sections of substantia nigra were cut at 10 μm thickness on a freezing microtome (Leica) and stored at –20°C. After washing three times with 0.01 M PBS, sections were treated for 2 hours at room temperature with 15% goat serum solution in PBS to reduce nonspecific binding, and then incubated overnight with primary antibodies at 4°C. The primary antibodies were as follows: mouse monoclonal Nestin (1:300; Abcam) as a marker of NSCs, rabbit polyclonal TH (1:400; Abcam), rabbit polyclonal CXCR4 (1:500; Abcam), and rabbit polyclonal SDF-1 (1:400; SANTA, Delaware, CA, USA). Sections were then incubated for 2 hours at room temperature in fluorescence-labeled secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 488 donkey anti-mouse IgG, both diluted at 1:200) and nuclei were counterstained with DAPI (20 μg/mL; Sigma-Aldrich Corp.). Staining was visualized with a fluorescence microscope (Olympus Life Science, Waltham, MA, USA). Cell counting areas were mainly located in the central substantia nigra pars compacta region, and the number of neurons for each rat brain was calculated from the mean number counted from three sections.

Real-time quantitative polymerase chain reaction (PCR)

At 1 and 4 weeks after transplantation, fresh right nigral tissues were dissected out before total RNA was purified and homogenized in TRIzol reagent. For iScript reverse-transcription reactions (Bio-Rad Laboratories, Hercules, CA, USA), 1 μg of RNA was used as a template. The cDNA produced from this reaction was measured for gene expression levels of SDF-1/CXCR4 using the Eco Real-Time PCR System (Mx3005p, Agilent, Palo Alto, CA, USA). The primer sequences for producing PCR products are shown in **Table 1**. PCR amplifications were carried out under the following conditions: One cycle of initial denaturation for 10 minutes at 95°C; and then 30 cycles of denaturation for 15 seconds at 95°C, 30 seconds at 57°C, and annealing for 1 minute at 72°C. Relative changes in gene expression from control conditions were normalized using the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene.

Table 1 Primer sequences for producing PCR products

Gene	Sequence (5′–3′)	Product size (bp)
SDF-1	Forward: GAC AGA TTC CTT GCC GAG AG	283
	Reverse: GGC AAG CAG AGA TCA GAA C	
CXCR4	Forward: GGA TGG TGG TGT TCC AGT TC	326
	Reverse: TCC CCA CGT AAT ACG GTA GC	
GAPDH	Forward: AGA CAG CCG CAT CTT CTT GT	697
	Reverse: CTT GCC GTG GGT AGA GTC AT	

SDF-1: Stromal cell-derived factor-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Western blot assay

At 1 and 4 weeks after transplantation, the cells and rat brain tissue were homogenized in ice-cold RIPA lysis buffer (Solarbio, Beijing, China) supplemented with 1% phenylmethylsulfonyl fluoride (Solarbio), and then centrifuged at 12,000 × *g* for 3 minutes at 4°C to collect the supernatant according to the manufacturer's instructions. Protein concentrations were assessed using the bicinchoninic acid (Tiangen Biotech Co., Ltd., Beijing, China) assay. Protein was boiled for 20 minutes before electrophoresis was performed on 8% sodium dodecyl sulfate-polyacrylamide gels. The membranes were blocked with 5% non-fat milk and incubated at 4°C overnight with primary antibodies as follows: rabbit monoclonal anti-CXCR4 (1:800; Abcam), rabbit polyclonal anti-SDF-1 (1:800; Abcam), and rabbit polyclonal anti-β-actin (1:1000; Proteintech). The membranes were subsequently incubated for 1.5 hours with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, 1:8000; Proteintech) at room temperature. β-Actin was used as a loading control. Proteins were detected using enhanced chemiluminescent reagent (Millipore, Boston, MA, USA). The relative levels of immunoreactive protein were quantified using ImageJ software (NIH, Bethesda, MD, USA), and the data were normalized to β-actin before statistical analysis.

Statistical analysis

All data were analyzed using SPSS 19.0 software (IBM SPSS Inc., Chicago, IL, USA). Mean values were reported along with the standard error of the mean. Comparisons among multiple groups were made using one-way analysis of variance followed by Bonferroni *post hoc* tests. Statistical comparisons between two groups were made using Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

Results

Identification of CXCR4 and Nestin expression in mesencephalic NSCs and differentiation *in vitro*

Mesencephalic NSCs were isolated from rats at embryonic day 12.5, as previously described. After 5 days *in vitro*, cells formed large spherical colonies (**Figure 1A**). The NSC marker nestin and CXCR4 chemokine receptor were strongly expressed in these neurospheres (**Figure 1E–H**). Immunofluorescence staining results revealed that, with prolonged time, cells began to differentiate at 5 days *in vitro*, and were strongly immunoreactive for GFAP and SDF-1 at this time point (**Figure 1I–L**). After 7 days of cell culture, cells were immunoreactive for TH and GFAP (**Figure 1B**). TH-immunoreactive cells were detected in subpopulations of Tuj1-immunoreactive neuronal cells (**Figure 1C**), while CXCR4-immunoreactive cells were identified in subpopulations of TH-immunoreactive neuronal cells (**Figure 1D**).

NSC transplantation decreases apomorphine-induced rotational behavior in a PD rat model

According to a previous study (Deng et al., 2013), rotational asymmetry can be measured as a PD-like behavioral re-

sponse to the indirect dopamine agonist, apomorphine. Rats were assessed for their rotational response at 1, 2, 3, and 4 weeks after transplantation. Apomorphine-induced rotation behavior was reduced in the NSC-grafted group compared with other experimental groups after transplantation (*P* = 0.001; **Figure 2**). This difference occurred at the first week after transplantation and continued to the fourth week. There was no significant difference between NSC-grafted + AMD3100 or AMD3100 groups and the sham group at each time point (both *P* > 0.05; **Figure 2**).

NSC transplantation increases mRNA and protein expression levels of CXCR4/SDF-1 in the ipsilateral striatum in a PD rat model

To determine whether the effect of cell transplantation was associated with the CXCR4/SDF-1 axis in our PD model, we detected the expression of CXCR4/SDF-1. In culture, NSCs expressed CXCR4, a receptor for SDF-1 that is required for SDF-1-mediated chemotaxis (**Figure 1G**). The mRNA and protein expressions of CXCR4 and SDF-1 were also quantitatively assayed using real time fluorescence-based quantitative real-time PCR (qPCR) and western blot assays. The experimental data demonstrated that both mRNA and protein expression of CXCR4 were increased at 1 and 4 weeks after transplantation in the NSC-grafted and sham groups compared with the control group (*P* < 0.05; **Figures 3, 4A**, and **4B**). Furthermore, the mRNA and protein expression of CXCR4 was significantly higher in the NSC-grafted group than in the sham group (*P* < 0.05). This phenomenon disappeared with the addition of AMD3100, an inhibitor of CXCR4 receptors, in the NSC-grafted + AMD3100 and AMD3100 groups (**Figures 3, 4A**, and **4B**).

qPCR and western blot assay results revealed that SDF-1 levels were markedly higher in the NSC-grafted group than in the other groups at 1 and 4 weeks after transplantation. Additionally, SDF-1 expression was significantly greater in the experimental group than in the control group (*P* < 0.05). There was no significant change among the sham, NSC-grafted + AMD3100, and AMD3100 groups (*P* > 0.05; **Figures 3B, 4A**, and **4C**). Moreover, the expressions of CXCR4 and SDF-1 in the NSC-grafted group were significantly increased at 4 weeks compared with at 1 week after transplantation (*P* < 0.05; **Figures 3B, 4A**, and **4C**).

Immunofluorescence staining revealed that the number of TH-immunoreactive cells was significantly increased in the brains of PD rats in the NSC-grafted group compared with the sham, NSC-grafted + AMD3100, and AMD3100 groups at 1 and 4 weeks after transplantation. These results are in agreement with behavioral evaluations (*P* < 0.05). In addition, the numbers of SDF- and CXCR4-immunoreactive cells were significantly higher in the brains of PD rats in the NSC-grafted group compared with the other groups at 1 and 4 weeks after transplantation (*P* < 0.05). Compared with the control group, the number of SDF-1-immunoreactive cells was increased in the sham group (*P* < 0.05), NSC-grafted + AMD3100 group (*P* < 0.05), and AMD3100 group (*P* < 0.05) at 1 and 4 weeks. In contrast, the number of CXCR4-immu-
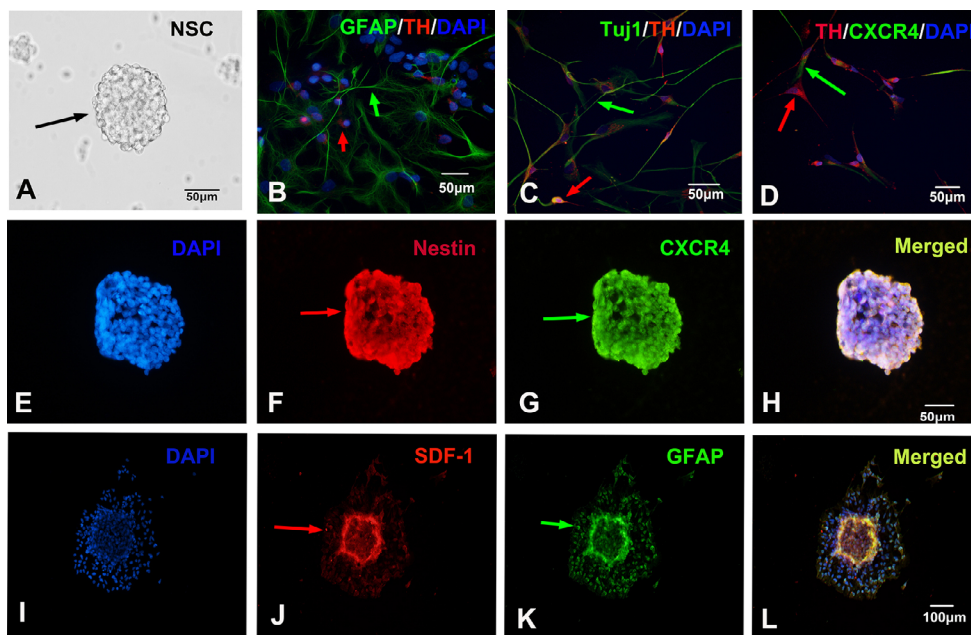
Figure 1 Cultivation and identification of NSCs *in vitro*.
(A, E–H) After 5 days of culture, the neurospheres isolated from ventral mesencephalic neural tissue (A, black arrow) were positive for nestin and CXCR4 (E–H, The red arrow points to Nestin/Alexa Fluor 594-goat anti-mouse and the green arrow points to CXCR4/Alexa Fluor 488-goat anti-rabbit). (J, K) After 5 days of differentiation culture, cells were strongly immunoreactive for SDF-1 (J) and GFAP (K); the red arrow indicates SDF-1/Alexa Fluor 594-goat anti-mouse and the green arrow indicates GFAP/Alexa Fluor 488-goat anti-rabbit. (B–D) After 7 days of differentiation culture. (B) GFAP- and TH-immunoreactive cells: the red arrow points to TH/Alexa Fluor 594-goat anti-mouse and the green arrow points to GPAP/Alexa Fluor 488-goat anti-rabbit. (C) TH-immunoreactive cells were detected in subpopulations of Tuj1-immunoreactive neuronal cells: the red arrow points to TH/Alexa Fluor 594-goat anti-mouse and the green arrow points to Tuj1/Alexa Fluor 488-goat anti-rabbit. (D) CXCR4-immunoreactive cells were detected in subpopulations of TH-immunoreactive neuronal cells: the red arrow points to TH/Alexa Fluor 594-goat anti-mouse and the green arrow points to CXCR4/Alexa Fluor 488-goat anti-rabbit. Scale bars: 50 µm in A–H, 100 µm in I–L.
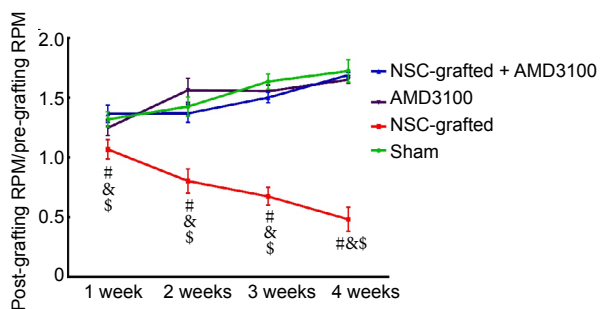
Figure 2 *In vivo* behavioral analysis after NSC transplantation in a Parkinson's disease rat model.
APO-induced rotation and the post-grafting RPM/pre-grafting RPM as a rotation index to measure therapeutic effects (Jiang et al., 2004). Data are expressed as the mean ± SD. #$P < 0.05$, *vs.* sham group; &$P < 0.05$, *vs.* NSC-grafted + AMD3100 group; $P < 0.05$, *vs.* AMD3100 group (one-way analysis of variance followed by Bonferroni *post hoc* test). APO: Apomorphine; NSC: neural stem cell; RPM: revolutions per minute.
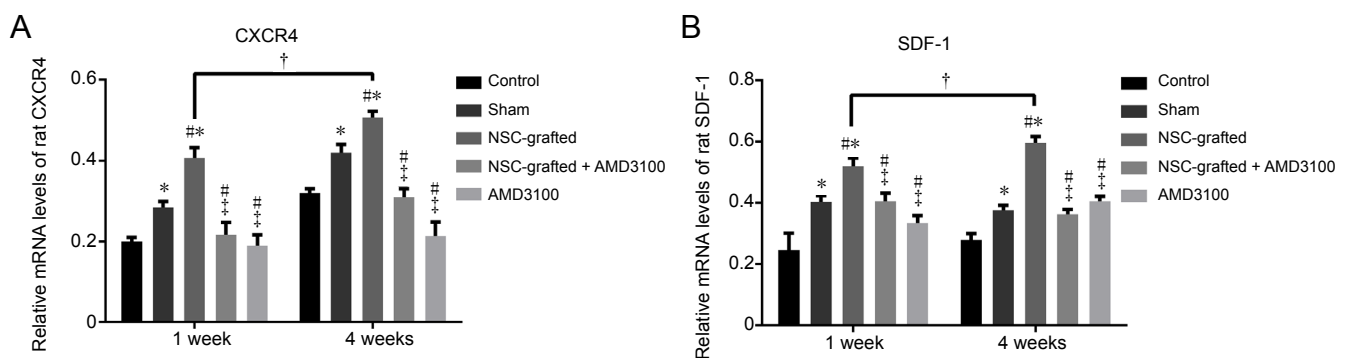

Figure 3 mRNA expression of CXCR4 (A) and SDF-1 (B) in the ipsilateral striatum in a Parkinson's disease rat model after NSC transplantation.
Reverse transcription-polymerase chain reaction analyses were performed to detect mRNA expression levels of CXCR4 and SDF-1 in the ipsilateral striatum at 1 and 4 weeks after transplantation. Data are expressed as the mean ± SD. *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* sham group; ‡$P < 0.05$, *vs.* NSC-grafted group; †$P < 0.05$ (one-way analysis of variance followed by Bonferroni *post hoc* test). SDF-1: Stromal cell-derived factor-1; NSC: neural stem cell.
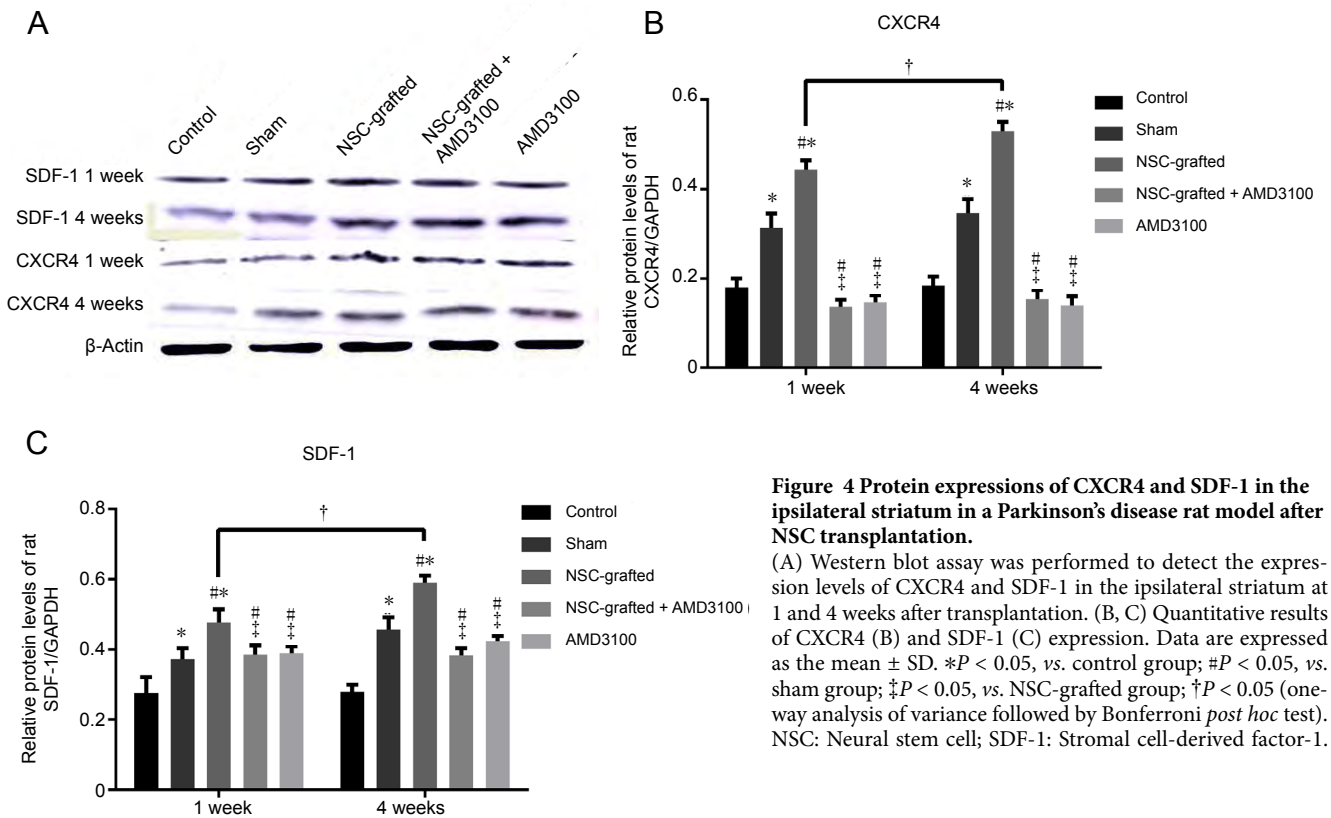
Figure 4 Protein expressions of CXCR4 and SDF-1 in the ipsilateral striatum in a Parkinson's disease rat model after NSC transplantation.

(A) Western blot assay was performed to detect the expression levels of CXCR4 and SDF-1 in the ipsilateral striatum at 1 and 4 weeks after transplantation. (B, C) Quantitative results of CXCR4 (B) and SDF-1 (C) expression. Data are expressed as the mean ± SD. $*P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* sham group; ‡$P < 0.05$, *vs.* NSC-grafted group; †$P < 0.05$ (one-way analysis of variance followed by Bonferroni *post hoc* test). NSC: Neural stem cell; SDF-1: Stromal cell-derived factor-1.
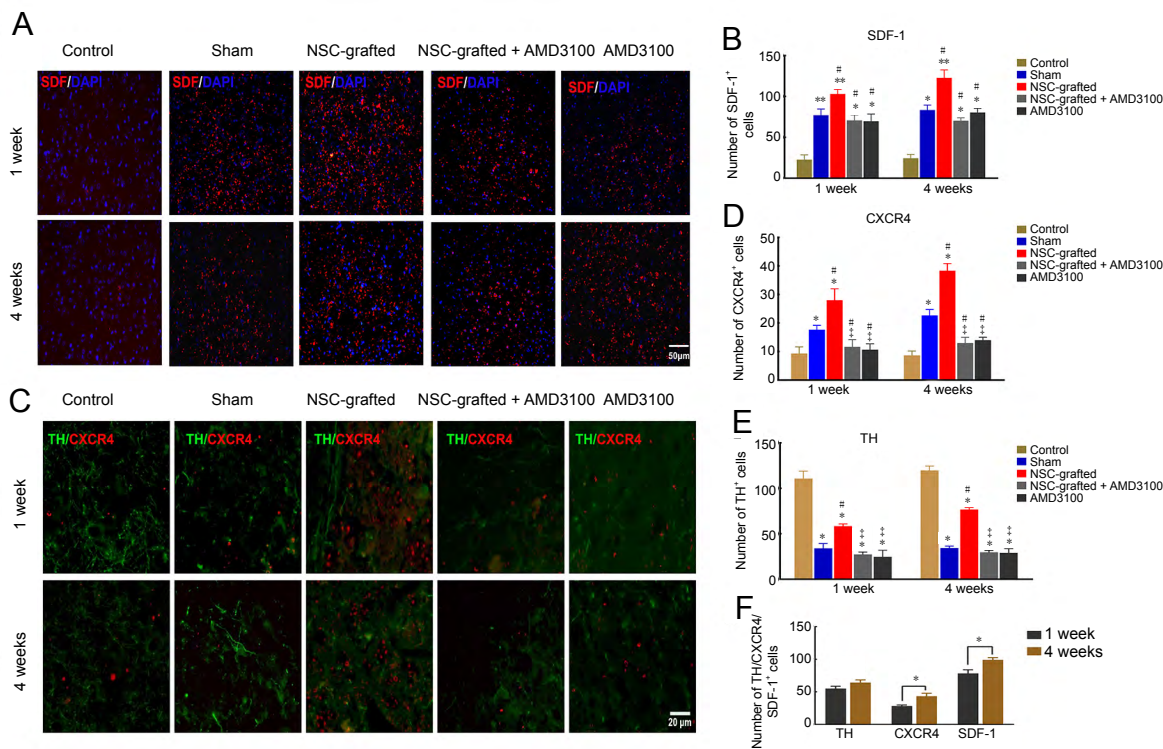
Figure 5 *In vivo* immunofluorescence analysis of TH[+], CXCR4^{+}, and SDF-1^{+} cells in the ipsilateral striatum of the Parkinson's disease rat model at 1 and 4 weeks after NSC transplantation.

The numbers of TH[+], CXCR4^{+} cells (C, the green arrow indicates TH/Alexa Fluor 488-goat anti-rabbit and the red arrow indicates CXCR4/Alexa Fluor 594-goat anti-rabbit), and SDF-1^{+} cells (A, the red arrow indicates SDF-1/Alexa Fluor 594-goat anti-rabbit) were significantly increased in the grafted striatum in the NSC-grafted group compared with the other groups. Data are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$, *vs.* control group; #$P < 0.05$, *vs.* sham group; ‡$P < 0.05$, *vs.* NSC-grafted group (one-way analysis of variance followed by Bonferroni *post hoc* test). A: Scale bar: 50 μm. C: Scale bar: 20 μm. NSC: Neural stem cell; SDF-1: Stromal cell-derived factor-1; TH: Tyrosine hydroxylase.

noreactive cells was higher in the sham group than in the NSC-grafted + AMD3100 and AMD3100 groups at 1 and 4 weeks ($P < 0.05$); and there was no significant difference among the NSC-grafted + AMD3100 group, AMD3100 group, and control group ($P > 0.05$). Moreover, the numbers of SDF- and CXCR4-immunoreactive cells were increased at 4 weeks compared with at 1 week in the NSC-grafted group; however, there was no such significant difference in the number of TH-immunoreactive cells (**Figure 5**).

Discussion

Progress in the development of regenerative cell replacement therapies provides a basis to potentially develop novel and robust therapeutic strategies for human neurological diseases such as PD (Barker et al., 2015). Previous studies have reported that transplanted NSCs can survive in the brain of PD rat models, and NSC proliferation and migration into the striatum and substantia nigra of PD rats has also been confirmed; thus, these cells retain the capacity to generate mature neurons when grafted into the rat brain (Gage et al., 1995; Deng et al., 2013). In a recent study, it was revealed that NSC transplantation could be used to treat neurological disorders because grafted cells differentiated into TH-positive cells in PD rats. In this process, however, NSCs must be directed to the sites of damage, and this depends on their correct migration (Makri et al., 2010; Gong et al., 2014). Current knowledge about how these cells migrate after transplantation remains very limited.

A series of chemotactic cytokines (chemokines) are known to be vital in the regulation of cell migration (Li et al., 2017a). Evidence showed that the SDF-1/CXCR4 axis continues to affect the migration and survival of oligodendrocytes (Dziembowska et al., 2005). Our results showed that CXCR4 was constitutively expressed in NSCs as well as in the TH-immunoreactive cells that differentiated from the NSCs. These discoveries are consistent with a recent finding that CXCR4 is present in dopaminergic neurons (Yang et al., 2013). In more recent studies, SDF-1 has been shown to be expressed in a variety of cells, including glial cells and vascular endothelial cells (Sun et al., 2013), fibroblasts (He et al., 2017), and inflammatory cells (Wang et al., 2015). Inflammatory cell infiltration increased SDF-1 expression (Mo et al., 2015; Huang et al., 2017). Our results showed that SDF was constitutively expressed in GFAP-immunoreactive cells that were differentiated from NSCs. These data are consistent with a previous report that SDF-1 is expressed in GFAP-positive cells in the damaged motor cortex of mice (Arimitsu et al., 2012). Moreover, HIF-1 activation promotes SDF-1 expression and ultimately promotes the migration of CXCR4-positive cells into damaged tissues after cell damage or inflammation activation (Zagzag et al., 2006). Our findings demonstrated that the expression of SDF-1 in the PD rat model was upregulated in comparison with the control rats. We speculated that SDF-1 expression was upregulated in the 6-OHDA-induced PD model in rats, as well as SDF-1 expression being upregulated in inflammatory cell infiltration, which is in accordance with a recent study (Bakogiannis et al., 2017). Furthermore, we found that SDF-1 expression was upregulated in the NSC-grafted group in comparison with the sham group at 1 and 4 weeks, as a result of NSC transplantation. It has been revealed that SDF-1, along with its corresponding receptor CXCR4, exerts a significant impact on the migration of mesenchymal stem cells to the injured site (Xiao Ling et al., 2016). In the present study, CXCR4 expression was significantly higher in the NSC-grafted group than in the sham group. In addition, CXCR4 expression was significantly increased in PD model rats compared with control rats. This phenomenon disappeared after the addition of AMD3100 treatment. These results indicated that the 6-OHDA-induced injury in the substantia nigra and the transplantation of NSCs into the host brain may lead to the increased expression of CXCR4.

NSC transplantation may be effective for treating PD rats, because many grafted cells differentiated into TH-positive cells in the substantia nigra of a PD rat model in a previous study (Parmar, 2018). *In vitro* investigations have also demonstrated that NSCs can successfully differentiate into TH- and Tuj1-positive cells. Moreover, immunofluorescence staining in the current study revealed that the number of TH-positive cells in the NSC-grafted group was significantly higher than in the sham group.

In the present study, the behavioral test results were consistent with the rest of our results. When AMD3100 blocked the SDF-1/CXCR4 axis, the related physiological effects described previously were significantly inhibited. These observations demonstrated that the SDF-1/CXCR4 axis is critical during the transplantation of NSCs into the nigra-striatum for the treatment of PD rat models.

This study has some limitations, however. First, PD is a neurodegenerative disease that mainly affects people who are over 60 years old, and the specific pathogenesis is still unclear (Pain et al., 2019). Therefore, the neuropathological characteristics of PD patients cannot be completely simulated by injecting 6-OHDA into the brain (Jakobs et al., 2019). Second, we observed a significant increase in the number of dopaminergic neurons in the substantia nigra/striatum region after NSC transplantation, but did not study whether dopamine levels increased in the brain (Eisinger et al., 2019). Future studies should take these limitations into account.

In summary, SDF-1/CXCR4 expression significantly increased after NSC transplantation. The SDF-1/CXCR4 axis plays an important role in the therapeutic effects of NSC-based cell replacement for PD, and this knowledge may contribute to the development of a new effective treatment strategy for PD. As a chronic degenerative disease of the central nervous system, PD is mainly treated by drugs and surgery (Sabino-Carvalho et al., 2019), both of which have shortcomings. If we can treat PD by transplanting NSCs into the brain, we will be able to improve the quality of life of patients (Valdez et al., 2019). In our study, which is of great significance for the treatment of PD by NSC transplantation.

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References

Addington CP, Pauken CM, Caplan MR, Stabenfeldt SE (2014) The role of SDF-1alpha-ECM crosstalk in determining neural stem cell fate. Biomaterials 35:3263-3272.

Arimitsu N, Shimizu J, Fujiwara N, Takai K, Takada E, Kono T, Ueda Y, Suzuki T, Suzuki N (2012) Role of SDF1/CXCR4 interaction in experimental hemiplegic models with neural cell transplantation. Int J Mol Sci 13:2636-2649.

Bakogiannis C, Sachse M, Stamatelopoulos K, Stellos K (2017) Platelet-derived chemokines in inflammation and atherosclerosis. Cytokine doi: 10.1016/j.cyto.2017.09.013.

Barker RA, Drouin-Ouellet J, Parmar M (2015) Cell-based therapies for Parkinson disease-past insights and future potential. Nat Rev Neurol 11:492-503.

Chen L, Hu L, Peng Y, Yang L, Shen H, Wang T, Zhao Z (2019) Differentiation of human pluripotent stem cells into dopaminergic neurons: security risk for heterogeneity. Zhongguo Zuzhi Gongcheng Yanjiu 23:118-124.

Chen XX, Qian Y, Wang XP, Tang ZW, Xu JT, Lin H, Yang ZY, Song XB, Lu D, Guo JZ, Bian LG, Li Y, Zhou L, Deng XL (2018) Nurr1 promotes neurogenesis of dopaminergic neuron and represses inflammatory factors in the transwell coculture system of neural stem cells and microglia. CNS Neurosci Ther 24:790-800.

Cheng X, Wang H, Zhang X, Zhao S, Zhou Z, Mu X, Zhao C, Teng W (2017) The role of SDF-1/CXCR4/CXCR7 in neuronal regeneration after cerebral ischemia. Front Neurosci 11:590.

Deng X, Liang Y, Lu H, Yang Z, Liu R, Wang J, Song X, Long J, Li Y, Lei D, Feng Z (2013) Co-transplantation of GDNF-overexpressing neural stem cells and fetal dopaminergic neurons mitigates motor symptoms in a rat model of Parkinson's disease. PLoS One 8:e80880.

Dziembowska M, Tham TN, Lau P, Vitry S, Lazarini F, Dubois-Dalcq M (2005) A role for CXCR4 signaling in survival and migration of neural and oligodendrocyte precursors. Glia 50:258-269.

Eisinger RS, Ramirez-Zamora A, Carbunaru S, Ptak B, Peng-Chen Z, Okun MS, Gunduz A (2019) Medications, deep brain stimulation, and other factors influencing impulse control disorders in Parkinson's disease. Front Neurol 10:86.

Fjodorova M, Torres EM, Dunnett SB (2017) Transplantation site influences the phenotypic differentiation of dopamine neurons in ventral mesencephalic grafts in Parkinsonian rats. Exp Neurol 291:8-19.

Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci U S A 92:11879-11883.

Gao H, Li YX, Wang D, Yang XL (2019) Protective effects and the mechanism of lipoic acid on cell model of Parkinson's disease induced by lipopolysaccharide. Zhongguo Zuzhi Gongcheng Yanjiu 23:447-452.

Gong J, Meng HB, Hua J, Song ZS, He ZG, Zhou B, Qian MP (2014) The SDF-1/CXCR4 axis regulates migration of transplanted bone marrow mesenchymal stem cells towards the pancreas in rats with acute pancreatitis. Mol Med Rep 9:1575-1582.

He G, Ma M, Yang W, Wang H, Zhang Y, Gao MQ (2017) SDF-1 in mammary fibroblasts of bovine with mastitis induces EMT and inflammatory response of epithelial cells. Int J Biol Sci 13:604-614.

Huang X, Wu P, Huang F, Xu M, Chen M, Huang K, Li GP, Xu M, Yao D, Wang L (2017) Baicalin attenuates chronic hypoxia-induced pulmonary hypertension via adenosine A2A receptor-induced SDF-1/CXCR4/PI3K/AKT signaling. J Biomed Sci 24:52.

Jakobs M, Fomenko A, Lozano AM, Kiening KL (2019) Cellular, molecular, and clinical mechanisms of action of deep brain stimulation-a systematic review on established indications and outlook on future developments. EMBO Mol Med 11:e9575.

Jiang C, Wan X, Jankovic J, Christian ST, Pristupa ZB, Niznik HB, Sundsmo JS, Le W (2004) Dopaminergic properties and experimental anti-Parkinsonian effects of IPX750 in rodent models of Parkinson disease. Clin Neuropharmacol 27:63-73.

Lee DJ, Dallapiazza RF, De Vloo P, Lozano AM (2018) Current surgical treatments for Parkinson's disease and potential therapeutic targets. Neural Regen Res 13:1342-1345.

Li H, Chen Y, Xu N, Yu M, Tu X, Chen Z, Lin M, Xie B, Fu J, Han L (2017a) AMD3100 inhibits brain-specific metastasis in lung cancer via suppressing the SDF-1/CXCR4 axis and protecting blood-brain barrier. Am J Transl Res 9:5259-5274.

Li XW, Gao HY, Liu J (2017b) The role of taurine in improving neural stem cells proliferation and differentiation. Nutr Neurosci 20:409-415.

Liu JM, Zhao K, Du LX, Zhou Y, Long XH, Chen XY, Liu ZL (2017) AMD3100 inhibits the migration and differentiation of neural stem cells after spinal cord injury. Sci Rep 7:64.

Luo J, Hu X, Zhang L, Li L, Zheng H, Li M, Zhang Q (2014) Physical exercise regulates neural stem cells proliferation and migration via SDF-1alpha/CXCR4 pathway in rats after ischemic stroke. Neurosci Lett 578:203-208.

Makri G, Lavdas AA, Katsimpardi L, Charneau P, Thomaidou D, Matsas R (2010) Transplantation of embryonic neural stem/precursor cells overexpressing BM88/Cend1 enhances the generation of neuronal cells in the injured mouse cortex. Stem Cells 28:127-139.

Merino JJ, Bellver-Landete V, Oset-Gasque MJ, Cubelos B (2015) CXCR4/CXCR7 molecular involvement in neuronal and neural progenitor migration: focus in CNS repair. J Cell Physiol 230:27-42.

Mo B, Li J, Wei J, Wang C, Zeng J, Wang J, Huang J (2015) The role of SDF-1/CXCR4 on airway inflammation and airway remodeling in a rat asthma model. Zhonghua Jie He He Hu Xi Za Zhi 38:39-44.

Pain S, Vergote J, Gulhan Z, Bodard S, Chalon S, Gaillard A (2019) Inflammatory process in Parkinson disease: neuroprotection by neuropeptide Y. Fundam Clin Pharmacol doi: 10.1111/fcp.12464.

Parmar M (2018) Towards stem cell based therapies for Parkinson's disease. Development 145:dev156117.

Peyvandi AA, Roozbahany NA, Peyvandi H, Abbaszadeh HA, Majdinasab N, Faridan M, Niknazar S (2018) Critical role of SDF-1/CXCR4 signaling pathway in stem cell homing in the deafened rat cochlea after acoustic trauma. Neural Regen Res 13:154-160.

Robin AM, Zhang ZG, Wang L, Zhang RL, Katakowski M, Zhang L, Wang Y, Zhang C, Chopp M (2006) Stromal cell-derived factor 1alpha mediates neural progenitor cell motility after focal cerebral ischemia. J Cereb Blood Flow Metab 26:125-134.

Sabino-Carvalho JL, Samora M, Teixeira AL, Daher M, Vianna LC (2019) Circulatory responses at the onset of handgrip exercise in patients with Parkinson's disease. Exp Physiol doi: 10.1113/EP087620.

Saha B, Peron S, Murray K, Jaber M, Gaillard A (2013) Cortical lesion stimulates adult subventricular zone neural progenitor cell proliferation and migration to the site of injury. Stem Cell Res 11:965-977.

Shen Y, Huang J, Liu L, Xu X, Han C, Zhang G, Jiang H, Li J, Lin Z, Xiong N, Wang T (2016) A compendium of preparation and application of stem cells in Parkinson's disease: current status and future prospects. Front Aging Neurosci 8:117.

Staudt MD, Di Sebastiano AR, Xu H, Jog M, Schmid S, Foster P, Hebb MO (2016) Advances in neurotrophic factor and cell-based therapies for Parkinson's disease: A Mini-Review. Gerontology 62:371-380.

Sun J, Li Y, Graziani GM, Filion L, Allan DS (2013) E-selectin mediated adhesion and migration of endothelial colony forming cells is enhanced by SDF-1α/CXCR4. PLoS One 8:e60890.

Valdez LB, Zaobornyj T, Bandez MJ, Lopez-Cepero JM, Boveris A, Navarro A (2019) Complex I syndrome in striatum and frontal cortex in a rat model of Parkinson disease. Free Radic Biol Med 135:274-282.

Wang L, Guo S, Zhang N, Tao Y, Zhang H, Qi T, Liang F, Huang Z (2015) The role of SDF-1/CXCR4 in the vasculogenesis and remodeling of cerebral arteriovenous malformation. Ther Clin Risk Manag 11:1337-1344.

Xiao Ling K, Peng L, Jian Feng Z, Wei C, Wei Yan Y, Nan S, Cheng Qi G, Zhi Wei W (2016) Stromal derived factor-1/CXCR4 axis involved in bone marrow mesenchymal stem cells recruitment to injured liver. Stem Cells Int 2016:8906945.

Yang S, Edman LC, Sánchez-Alcañiz JA, Fritz N, Bonilla S, Hecht J, Uhlén P, Pleasure SJ, Villaescusa JC, Marín O, Arenas E (2013) Cxcl12/Cxcr4 signaling controls the migration and process orientation of A9-A10 dopaminergic neurons. Development 140:4554-4564.

Zagzag D, Lukyanov Y, Lan L, Ali MA, Esencay M, Mendez O, Yee H, Voura EB, Newcomb EW (2006) Hypoxia-inducible factor 1 and VEGF upregulate CXCR4 in glioblastoma: implications for angiogenesis and glioma cell invasion. Lab Invest 86:1221-1232.

Zhang R, Zhang Z, Zhang C, Zhang L, Robin A, Wang Y, Lu M, Chopp M (2004) Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat. J Neurosci 24:5810-5815.

Zhang XQ, Mu JW, Wang HB, Jolkkonen J, Liu TT, Xiao T, Zhao M, Zhang CD, Zhao CS (2016) Increased protein expression levels of pCREB, BDNF and SDF-1/CXCR4 in the hippocampus may be associated with enhanced neurogenesis induced by environmental enrichment. Mol Med Rep 14:2231-2237.