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ORIGINAL ARTICLE

Comparing neuroprotective effects of CDNF-expressing bone marrow derived mesenchymal stem cells via differing routes of administration utilizing an in vivo model of Parkinson's disease

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Abstract The potential value of cerebral dopamine neurotrophic factor (CDNF) in treating Parkinson's disease (PD) remains controversial. To evaluate the therapeutic effects of CDNF-expressing bone marrow derived mesenchymal stem cell (CDNF-MSCs) injections in a rat model of Parkinson's disease, we chose three different routes of CDNF-MSC administration, including intra-striatal, intraventricular, and intravenous pathways. Parkinsonism was induced by intra-striatal unilateral injection of 6-OHDA and then rats were subsequently randomized into three groups for either intra-striatal, intra-ventricular or intravenous injection for CDNF-MSC grafting. Therapeutic effects were evaluated by observing dopaminergic (DA) neurons both in the substantia nigra compacta (SNc) and within the striatum and by monitoring apomorphine-induced rotational behavior (circling). Data show that one intra-venous administration of CDNF-MSCs was ineffective for treating Parkinson's disease-like neurodegeneration. Conversely, intra-striatal grafts can reduce loss of DA neurons both in the SNc and striatum with improvement of Parkinson's-related behaviors, compared to intra-ventricular injections. Thus, intra-striatal grafts composed of CDNF-MSCs may provide a strategy for therapeutic delivery to treat PD.

Keywords Cerebral dopamine neurotrophic factor · Bone marrow stromal cells · Dopaminergic neurons · Parkinson's disease

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Introduction

Parkinson's disease (PD) is characterized by progressive loss of dopaminergic (DA) neurons within the substantia nigra compacta (SNc) accompanied by behavioral dyskinesia [1]. Therapeutic goals include relieving movement-related symptoms more so than delaying the onset of progressive DA neuronal degeneration. An ideal therapy would prevent pathological neuronal degeneration with the simultaneous salvage of dying neurons in a localized vicinity.

Myriad studies suggest that neurotrophic factors can protect against degenerative progression of DA neurons from various insults both in vitro and in vivo [2–4]. Conserved dopamine neurotrophic factor (CDNF) has been reported to protect DA neurons from injury and rescue dying DA neurons after 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatments. CDNF can also facilitate recovery of Parkinson's-like behavioral deficits [5, 6]. Continuous striatal infusion of CDNF has been reported to significantly reduce amphetamine-induced is pilateral turning behavior and rescue dopaminergic tyrosine-hydroxylase-positive cells in rat models of PD, but this lasted for 2 weeks [5, 7], suggesting that continuous CDNF administration is required to sustain this effect. Therefore, cell-mediated gene therapy may have the potential to improve CDNF therapeutic efficacy.

Cell replacement strategies offer an effective way to treat PD and bone marrow mesenchymal stem cells (MSCs) are ideal gene therapy vehicles for treating neurodegenerative disease, by virtue of their preferential migration to damaged brain areas while the loaded gene differentiates into homologous cells in injured areas [3, 8, 9]. In fact, neurotrophic factors delivered by MSCs

have shown promise for PD gene therapy in animal studies and clinical trials [4, 10]. Glial cell line-derived neurotrophic factor (GDNF) delivered by MSCs significantly halted DA neuronal degeneration and improved behavioral aspects of PD [4]. Intrastriatal injection was the most common grafting route for MSCs, and transplants have also been given through the lateral ventricular and intravenously [11–14]. To determine the best therapeutic effect for treating PD, we engineered MSCs to overexpress CDNF and injected them through three different routes: intra-striatal, intra-ventricular, and intravenous pathways.

Materials and methods

CDNF over-expression plasmid construct and transfected MSCs

Total RNA of heart and muscle tissues was isolated from rat with Trizol reagent to clone the *CDNF* gene. An amplified fragment was subcloned into the pEGFP-N1 vector creating a pEGFP-N1-CDNF construct and transformed into *E. coli* DH5α competent cells. Purified pEGFP-N1-CDNF recombinant plasmid was transfected to MSCs using Lipofectamine 2000 as previously described [15].

Animals and surgical procedures

First, 48 female Sprague–Dawley rats (200–250 g) were obtained from Anhui Provincial Hospital Research Center (Hefei, China). Rats were treated in accordance with *The Guidelines for Animal Care and Use of the National Institutes of Health.*

All surgical operations were performed under chloral hydrate anesthesia (300 mg/kg, ip) in a Kopf stereotaxic apparatus (Narishige, Japan). The coordinates (in mm) of surgical locales were as follows: anteroposterior (AP), +0.48; mediolateral (ML), ±3.0; dorsoventral (DV), −5.6/−4.3/−3.5 by injection pt aequ 6-OHDA (total 20 μg/6 μl). CDNF-MSCs diluted in PBS (2×10^5 cells/μl) were injected into new locations on striatal lesions, lateral ventricles, and the jugular vein.

Experimental design

Three separate groups of rats were randomly selected for the transplantation study and PD was modeled by 6-OHDA lesioning at the left striatum 1 week later (Fig. 1). Group 1: ipsilateral intrastriatal transplantation (CDNF-MSCs $n = 12$, saline vehicle $n = 4$); Group 2: ipsilateral lateral ventricle transplantation (CDNF-MSCs $n = 12$, saline vehicle $n = 4$), Group 3 intravenous transplantation (CDNF-MSCs $n = 12$, saline vehicle $n = 4$).

Rotational behavior

At 2, 4, and 6 weeks after the lesioning procedure, rats were assessed for rotational asymmetry induced with apomorphine (APO; Sigma-Aldrich, St. Louis, MO) for 30 min. Rotational behavior ("circling") was monitored in automated rotameter bowls after APO administration. The number of circles made to the ipsilateral side was counted for 30 min after APO administration (0.5 mg/kg, ip).

Histology

Immunohistochemistry and morphological analysis

After 6 weeks, the paraffinized brains of rats were sectioned coronally to detect TH+ immuopositive cells in the SNc and striatum. Alternate sections were then incubated in anti-TH (1:500; Sigma-Aldrich) overnight at 4 °C, and visualized using 3,3′-diaminobenzidine (DAB; Sigma-Aldrich). Samples were then cleared in Histoclear, and coverslipped with DPX.

DA cells were counted based on TH immunoreactivity. Cells in the midbrain were counted from 10 random sections in each case (1 section, every 5 sections from midbrain in sequence). Total cells in 10 sections in each animal were counted.

Optical densities of TH-immunoreactive fibers in the striatum were assessed with an image processing system (Olympus BX51). For each animal, optical density was measured at rostro-caudal levels according to the Atlas of Paxinos and Watson over the entire striatum.

Statistical analysis

All data are expressed as mean ± SEM of n separate experiments. Independent sample t tests or ANOVA were performed (p-values of 0.05 or less were considered significant).

Results

Generation of CDNF-expressing MSCs for transplantation

Mouse CDNF cDNA of the 564 bp amplicon was amplified by RT-PCR and confirmed by DNA sequencing. Then cDNA was cloned into the pEGFP-N1 vector to express CDNF. MSC primary cultures were established by fusiform

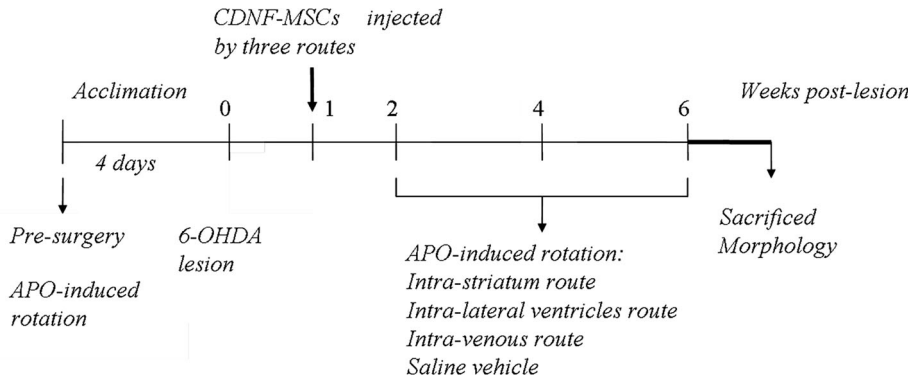

Fig. 1 Experimental assessments of CDNF-MSC treatments using multiple grafting routes. All rats without APO pre-induced rotations were eligible for induction of PD by 6-OHDA. After 1 week, rats were divided into three administration groups for transplantation of CDNF-MSCs. Then, effects of CDNF-MSCs were measured with behavioral and morphological observations

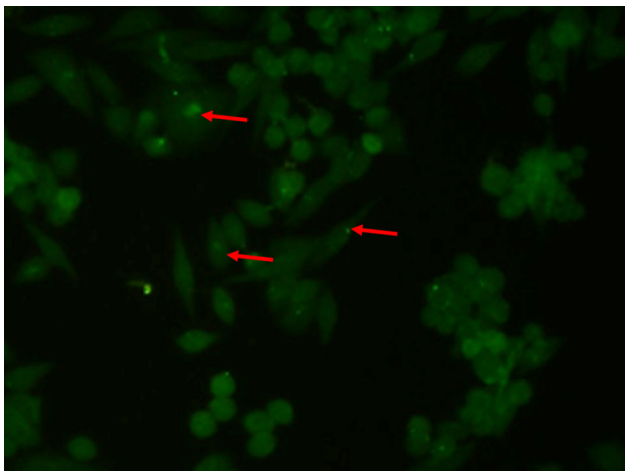

Fig. 2 Recombinant CDNF plasmid expression in MSCs. CDNF-plasmids with strong fluorescent dot material plasmid (*arrow*) were observed in cultured MSCs with fusiform shape and colony spread

shape and colony spread. Recombinant plasmid construction of pEGFP-N1-CDNFs were transfected into MSCs with Lipofectin. CDNF-plasmids were identified for transduction to the cultured MSCs according to fusiform shapes and colony spread as visualized with fluorescent microscopy (Fig. 2).

Behavioral testing

Behavioral testing is described in Table 1 and Fig. 3. APO-induced rotations were not decreased in lateral ventricle and intravenous administration route groups at 2 weeks compared to saline groups. After 4 weeks, APO-induced rotations reduced significantly in the lateral ventricle administration group ($p = 0.016$) and in the intrastriatal administration group ($p = 0.000$), compared to controls. At week 6, APO-induced rotations in the intrastriatal group were 69.84 % less than control group ($p = 0.000$); 14.41 % less than vehicle controls in the lateral ventricle group ($p = 0.000$); and only 0.03 % less than controls in the intravenous group ($p = 0.054$).

TH immunohistochemistry in SNc and striatum

After 6 weeks, TH positive fibers in the striatum were analyzed (Fig. 4a–e and Table 2). Those for intrastriatal administration route animals were 2.14 times greater than saline controls ($p = 0.000$), and the lateral ventricle group was only 1.09 times greater than the saline control ($p = 0.024$). The intrastriatal route group was greater than the lateral ventricle route ($p = 0.000$). However, no statistically significant difference existed between the intravenous group and controls ($p = 0.072$).

TH-positive cells in the SNc (Fig. 5a–e and Table 2) in the intrastriatal transplantation route group was approximately 3.64 times greater than those in saline controls ($p = 0.000$), and the lateral ventricle group had approximately 1.14 times more TH-positive cells than the saline control ($p = 0.013$), and the intrastriatal group had more TH-positive cells than the lateral ventricle group ($p = 0.000$). However, differences were not statistically significant between the intravenous group and controls regarding TH-positive cells in the SNc ($p = 0.159$).

Discussion

Here, we describe the neurotrophic therapeutic effects of CDNF-overexpressing MSCs transplanted via three distinct injection routes in an established 6-OHDA-induced rat model of PD. We observed that single intravenous administration of CDNF-MSCs was ineffective for treating PD-like neurodegeneration symptoms in the rat model. In contrast, intra-striatal and intra-lateral ventricular transplantation routes could significantly salvage affected DA neurons from 6-OHDA-induced neurotoxicity. Animals treated by these routes also had improved behavior and more TH staining in the midbrain and striatum, indicating that intrastriatal injections were superior to intra-lateral injections. These data indicate that intrastriatal transplantation of CDNF-MSCs may hold promise as an adjunctive therapy for patients suffering from PD.

Table 1 The apomorphine-induced rotations after CDNF-engineered MCSs transplantation by alernate routes in 6-OHDA-lesioned rats

The route of CDNF-MSCs transplantation	Apomorphine-induced rotations (per 30 min)		
	2 week	4 week	6 week
Group 1			
Intrastriatial routing	$27.92 \pm 5.42^{\blacktriangle ※}$	$34.00 \pm 7.98^{\blacktriangle\blacktriangle ※※}$	$66.17 \pm 5.57^{\blacktriangle\blacktriangle\blacktriangle ※※※}$
Vehicle (saline)	33.33 ± 5.03	115.08 ± 13.68	216.83 ± 5.44
Group 2			
Lateral ventricle routing	$32.50 \pm 3.09^{\#}$	$107.25 \pm 12.22^{\#\#}$	$188.08 \pm 12.94^{\#\#\#}$
Vehicle (saline)	34.42 ± 3.78	118.58 ± 8.84	219.75 ± 10.92
Group 3			
Intra-jugular vein routing	$32.33 \pm 4.27^{*}$	$112.75 \pm 7.61^{**}$	$211.083 \pm 8.13^{***}$
Vehicle (saline)	34.83 ± 3.46	119.25 ± 7.90	216.83 ± 5.44

Compared with vehicle: * $p = 0.129$, ** $p = 0.052$, *** $p = 0.054$, $^{\#}p = 0.187$, $^{\#\#}$ $p = 0.016$, $^{\#\#\#}$ $p = 0.000$, $^{\blacktriangle}$ $p = 0.019$, $^{\blacktriangle\blacktriangle}$ $p = 0.000$, $^{\blacktriangle\blacktriangle\blacktriangle}$ $p = 0.000$

Compared with Group 2: $^{※}$ $p = 0.018$, $^{※※}$ $p = 0.000$, $^{※※※}$ $p = 0.000$

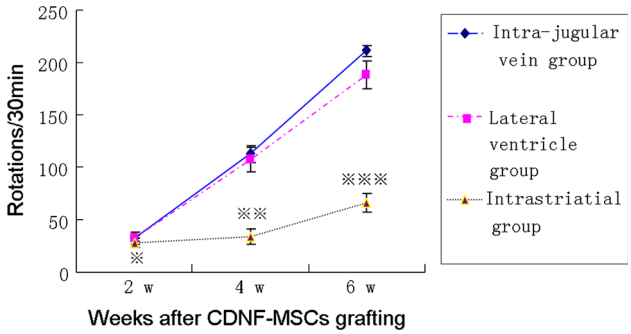

Fig. 3 Effects of CDNF-MSC grafting on behavioral testing. Rats implanted with CDNF-MSCs intrastriatally had significantly reduced APO-induced rotations at 2, 4, and 6 weeks ($p = 0.012$, ANOVA), compared with the lateral ventricle administration group ($^{※}p = 0.018$)

Treatment options for patients with PD are limited, but neurotrophic factors are standard treatment for neurorestorative therapy in patients with significantly impaired nigrostriatal DA systems [1, 16]. Previously, GDNF [17, 18] and neurturin (NRTN) [19] were shown to be neurorestorative in 6-OHDA-induced rats. However, preliminary clinical trial data were modest and alternative treatment options to combat this clinical dilemma are needed [20]. Alternatively, CDNF, a novel DA neurotrophic factor, has been reported to simultaneously protect and repair the DA system in 6-OHDA- and MPTP-induced rat models of PD [8, 10]. Also, CDNF gene delivery by adeno-associated viral (AAV) vectors not only induced significant neurorestoration of TH-stained neurons in the SNc, but also increased TH-stained fiber density in the striatum and these effects were accompanied by reversals of behavioral deficits [21]. AAV2-mediated CDNF was also shown to have therapeutic effects that were maintained from the second week after therapy to more than 1 year post-treatment. This strategy may lower health care costs and reduce the need for continuous brain infusions. Also, these studies suggest that the route of delivery of neurotrophic factors for PD is pivotal to treatment success, Although MSCs can differentiate into TH^{+} DA cells [22], these cells were expanded for autotransplantation, and rejection was not observed [6, 23].

Numerous studies suggest that MSCs are suitable delivery vehicles for GDNF, vascular endothelial growth factor (VEGF), neurturin, and tyrosine hydroxylase. These compounds were neuroprotective after striatal grafting in PD animal models [1, 3, 4, 10]. MSCs also preferentially migrate to sites of brain injury including areas of ischemia in rats [24], suggesting that MSCs are promising vehicles for therapeutic delivery of genes to the diseased brain.

In the present study, we transfected CDNF cDNA into MSCs and transplanted them via intrastriatal infusion into brains of rats with PD induced with 6-OHDA. Rats intrastriatally grafted with CDNF-MSCs had significantly fewer PD-like behavioral rotations. DA neuron and fiber degeneration was also attenuated in the SNc and striatum. CDNF-MSCs grafting was more effective than single MSCs transplantation in 6-OHDA-lesioned rats in our previous study.

Previous studies suggest that MSC migration in the brain could be accomplished via lateral ventricle and systemic intravenous grafting [25]. Thus, we evaluated the differences in therapeutic efficacy of CDNF-MSCs transplanted through the three aforementioned routes of administration. Intra-striatal and lateral ventricular transplantation of CDNF-MSCs significantly decreased 6-OHDA-induced rotations, and elevated the number of DA neurons. Thus, intrastriatal and lateral ventricular grafting of CDNF-MSCs was the most effective strategy for eradicating PD-like symptoms.

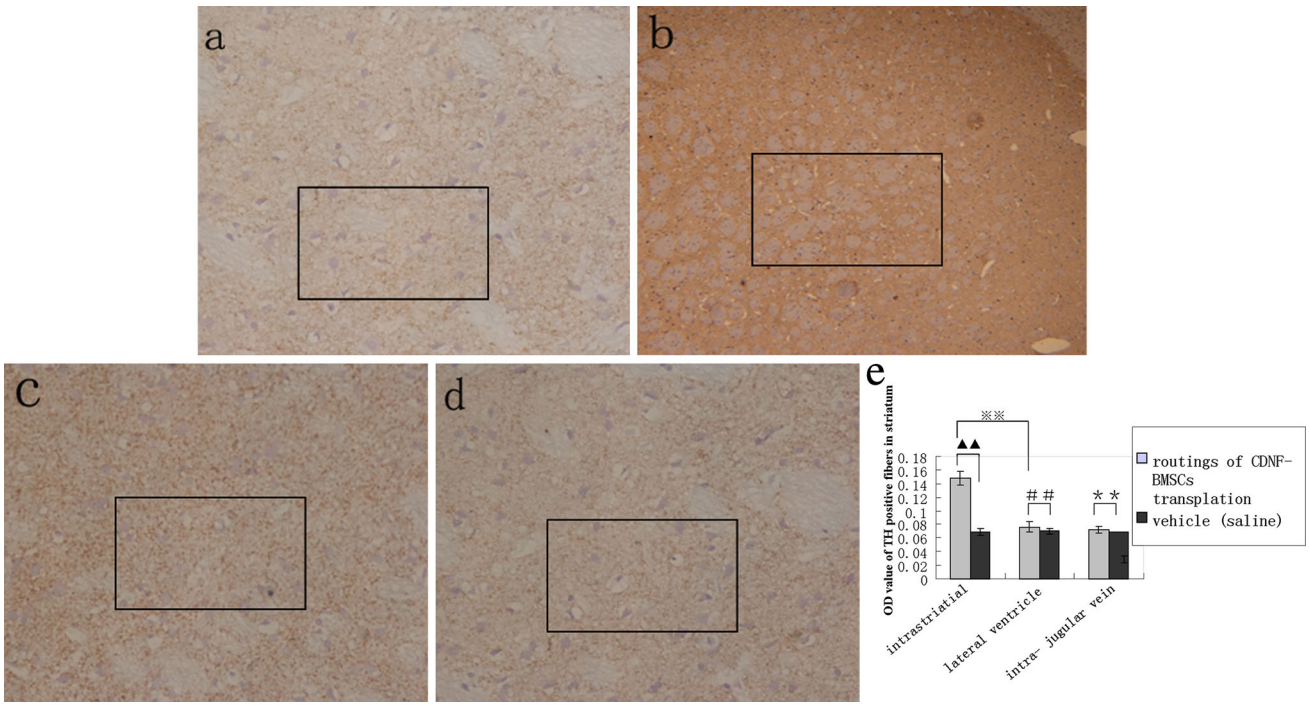

Fig. 4 TH immunohistochemistry in the striatum. TH^+ fiber density (*black frame*) in the striatum was significantly elevated in intrastriatal and lateral ventricle administration groups, and the former were better than the latter. **a** 6-OHDA; **b** 6-OHDA + Intrastriatal; **c** 6-OHDA + Lateral ventricular; **d** 6-OHDA + Intra-jugular; **e** data obtained from quantitative densitometry are presented as mean ± standard deviations. Compared with vehicle: $^{\#\#}p = 0.024$, $^{\blacktriangle\blacktriangle}p = 0.000$; compared with lateral ventricle group: $^{\divideontimes\divideontimes}p = 0.000$

Table 2 The TH+ immunopositive expressions in SNc and striatum after CDNF-engineered MCSs transplantation by alternate routes in 6-OHDA-lesioned rats

The routing of CDNF-BMSCs transplantation	6 weeks after transplantation	
	Number of TH+ immunostaining positive DA neurons in SNc	Optical density of TH+ immunostaining positive DA fibers in Striatum
Group 1		
Intrastriatal routing	$496.00 \pm 30.45^{\blacktriangle\divideontimes}$	$0.1477 \pm 0.0104^{\blacktriangle\blacktriangle\divideontimes\divideontimes}$
Vehicle (saline)	136.17 ± 10.97	0.0690 ± 0.00498
Group 2		
Lateral ventricle routing	$163.25 \pm 20.03^{\#}$	$0.0764 \pm 0.0079^{\#\#}$
Vehicle (saline)	143.00 ± 16.65	0.0700 ± 0.00471
Group 3		
Intra-jugular vein routing	$142 \pm 16.50^{*}$	$0.0729 \pm 0.0053^{**}$
Vehicle (saline)	134 ± 12.03	0.0691 ± 0.0045

Compared with vehicle: $^{*}p = 0.159$, $^{**}p = 0.072$, $^{\#}p = 0.013$, $^{\#\#}p = 0.024$, $^{\blacktriangle}p = 0.000$, $^{\blacktriangle\blacktriangle}p = 0.000$

Compared with Group 2: $^{\divideontimes}p = 0.000$, $^{\divideontimes\divideontimes}p = 0.000$

Although previous data indicate that grafting cells via an intra-lateral ventricular route might be beneficial for lesioned brains [11, 12], our work suggests that intra-lateral ventricular routes were not as effective as intrastriatal transplantations. Perhaps, transplanted cells were directed to the cerebrospinal fluid and eventually blocked by the blood brain barrier, thus decreasing cell migration. DA axon terminals in the striatum originated from DA neurons in the SNc. Thus, CDNF-MSCs grafted by intrastriatal injections may have migrated by retrograde axonal transport.

There was no obvious improvement in behavioral deficits and degeneration of TH^+ cells and fibers in rats intravenously treated. However, several reports suggest that more cells are needed for intravenous grafts to be therapeutic in lesioned brains. Shah and Jindal [14] reported that 10 million hematopoietic stem cells could be observed to localize to the brain after intravenous injection. Meanwhile, intravenous grafting of adipose-derived mesenchymal stem cells (ADMSCs) up to 2×10^6 cells/μl was observed to limit brain infarction areas and improve neurological status in an acute ischemic stroke rat model [13].

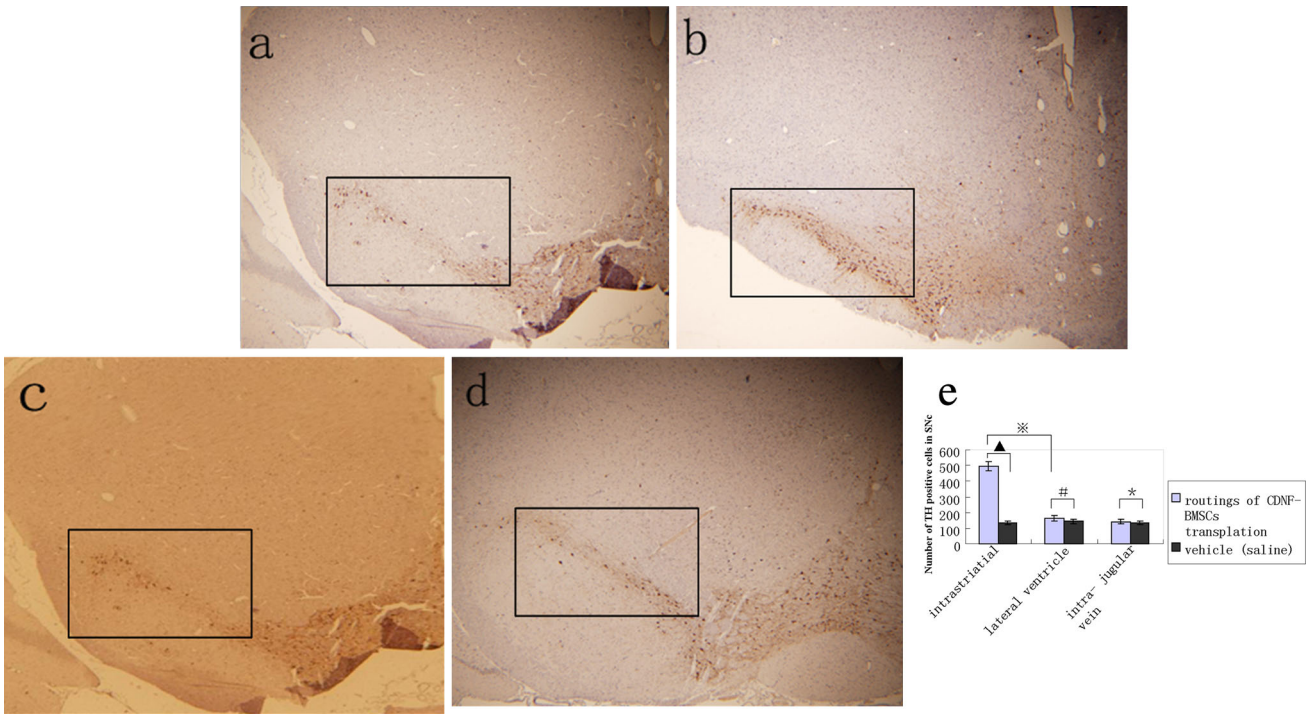

Fig. 5 TH immunohistochemistry in the SNc. TH+ cells in the SNc (*black frame*) were significantly increased with intrastriatal and lateral ventricular administration routes, the former were better than the latter. **a** 6-OHDA; **b** 6-OHDA + Intrastriatal; **c** 6-OHDA + Lateral ventricular; **d** 6-OHDA + Intra-jugular; **e** Data obtained from quantitative densitometry are presented as means ± standard deviations. Compared with vehicle: $^{\#}p = 0.013$, $^{\blacktriangle}p = 0.000$; compared with lateral ventricle group: $^{※}p = 0.000$

However, the number of transplanted cells was 10 times greater than used in the present experiments. Perhaps, a sufficient number of grafted CDNF-MSCs may have neurotrophic effects in PD-like rats.

In conclusion, CDNF-MSCs transplantation exerted neurotrophic effects on PD-like neurodegeneration by intra-striatal and intra-lateral ventricular transplantation routes. Elaborate migrating mechanisms underlying CDNF-MSCs would be appropriate future studies to extend these preliminary findings.

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