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Survival and differentiation of transplanted neural stem cells in mice brain with MPTP-induced Parkinson disease

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KEY WORDS neural stem cells; cell transplantation; Parkinson disease; cell survival; cell differentiation

ABSTRACT

AIM: To determine survival and differentiation of cultured neural stem cells (NSCs) into viable and functional neurons upon transplantation into mice brain of MPTP-induced Parkinson disease (PD). **METHODS:** Mouse model of PD was established with two subcutaneous (sc) injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 40 mg/kg) twice, 16 h apart. NSCs isolated from rat embryo midbrain were cultured in clonal density. After labeled with 5-bromo-2'-deoxyuridine (BrdU), the NSCs were transplanted into the uni- or bi-lateral striatum of PD mouse. Tyrosine hydroxylase (TH) immunofluorescence was used to evaluate the toxicity of MPTP on the neural cells in the substantia nigra. Immunohistology and laser confocal microscope were used to detect the survival and differentiation of transplanted NSCs. **RESULTS:** The cultured NSCs generated neurospheres and differentiated into neuron and astrocyte. It indicated that the cultured NSCs were multipotent and self-renewal *in vitro*. TH-positive neural cells were significantly reduced in the substantia nigra. Immunohistology showed that the uni- or bi-lateral transplanted NSCs survived in the brain of PD model mouse. Laser confocal microscope indicated that some transplanted NSCs could properly differentiate into targeted TH-positive neural cells *in vivo*. **CONCLUSION:** The transplanted multipotent NSCs could survive and differentiate into functional dopamine neurons.

INTRODUCTION

Parkinson disease (PD) is a common and disabling neurodegenerative disorder characterized by tremor, bradykinesia, stiffness and poor balance[1]. These motor abnormalities are due to profound depletion in striatal dopamine (DA) content that results from the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc)[2,3]. Although there are therapies available to alleviate PD symptoms, they may produce debilitating side effects and lose efficacy over time as they do not modify the progression of the disease. Injecting 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin, seems to achieve a severe and irreversible PD-like syndrome in humans and nonhuman primates. Therefore, MPTP was used to develop animal model for elucidating the cellular mechanisms of the degenerative process in PD and for testing new therapeutic strategies.

Neural stem cells (NSCs) have been isolated from the adult[4,5] and embryonic[6] rodents central nervous system (CNS), as well as fetal[7,8] and adult[9,10] human CNS. They are pluripotent *in vitro*, having the potential to differentiate into neurons, astrocytes, and oligodendrocytes, generating exitory and inhibitory synapse[11,12],

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and active neuronal networks[13]. The mounting data demonstrated that transplanted neural stem cells could survive, differentiate into neurons and glia with precise regional specificity, and improve or repair the function[14-18].

In the present study, therefore, we transplanted NSCs from the midbrain of rat embryo into the striatum of PD mouse brain, detected their fate and observed whether they could survive, properly differentiate into functional neurons and replace the lost neurons *in vivo*.

MATERIALS AND METHODS

Materials Trypsin, DNase I, N2 medium supplement, fetal bovin serum, and human recombinant FGF-2 were purchased from GIBCO BRL(USA). DMEM/F-12 medium was purchased from Hyclone (USA). Monoclonal mouse anti-neuron specific enolase (NSE) antibody, monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody, FITC and rodamine labeled secondary antibody were purchased from Santa Cruz Inc (USA). Monoclonal mouse anti-nestin and polyclonal rabbit anti-tyrosine hydroxylase (TH) antibody were purchased from Chemicon (USA). 5-Bromo-2'-deoxyuridine (BrdU), monoclonal mouse anti-BrdU antibody, poly-*L*-lysine and MPTP·HCl were purchased from Sigma (USA). SABC kit was purchased from BoShi-de Co (Wuhan, China).

Timed pregnant Wistar rats and C57BL/6 mice were purchased from Institute of Animal Sciences, Chinese Academy of Medical Sciences.

Isolation and passage of NSCs from the rat embryo midbrain Timed pregnant Wistar rats were deeply anesthetized at E16 (the day when a vaginal plug was found was considered day E0), and embryos were aseptically removed and placed in cold D-Hanks' solution. Ventral midbrain was dissociated and incubated in 0.1 % trypsin-0.01 % DNase I for 15 min at 37 ºC. The trypsin activity was terminated with trypsin inhibitor. After counted with trypan blue, primary cells were cultured in uncoated 24-well culture plates (10 000 cells per well) at 37 ºC in 5 % CO_2 atmosphere[19]. The basal growth medium was DMEM/F-12 (1:1; Hyclone), supplemented with penicillin G (100 kU/L), streptomycin sulphate (100 mg/L), N2 (1:100), human recombinant FGF-2 (20 μg/L), and *L*-glutamate (2 mmol/L). Half of the medium was replaced every 3 d.

After cultured for 1 week, generated neurospheres were collected by centrifugation, then digested with 0.1 % trypsin for 15 min with slightly mechanical trituration. The cells were still plated into uncoated 24-well culture plates in 1×10^4 cells per well. It has been demonstrated previously that cultured cells at this density would result in clonal neurosphere colonies, as form in single-cell cultures, and that neurospheres did not arise as a result of cell aggregation at that density[19,20]. Some other cells were plated on poly-*L*-lysine coated coverslips for immunocytochemistry. The cells for BrdU immunocytochemistry were labeled with 5 mmol/L BrdU for 3 d. The used primary antibodies were as follows: mouse anti-NSE (1:500), mouse anti-GFAP (1:500), mouse anti-nestin (1:100), and mouse anti-BrdU (1:1000).

Animals Forty male C57BL/6 mice, aged 10 weeks and weighing 24-26 g at the beginning of the study, were randomly divided into four groups: control, MPTP-treated, MPTP-treated unilateral transplantation, and MPTP-treated bilateral transplantation. They were maintained five per cage with food and water *ad libitum* under controlled temperature and humidity conditions with a standardized light and dark cycle (lights on at 8 am and off at 8 pm).

Drug administration MPTP·HCl was dissolved with saline and the solution was injected sc, 40 mg/kg (8 mL/kg) twice, 16 h apart[21-23]. Control animals received saline (8 mL/kg).

Transplantation procedure One week recovery after MPTP injection, mice were anesthetized with 0.6 % pentobarbital (sc, 0.1 mL per 10 g). Transplantation surgery was performed as formerly described[24-26]. In brief, using an aseptic technique, a burr hole was made on the skull to expose the dura. A 10 μL Hamilton syringe containing semisuspended NSCs (200 000 in 2 μL) was inserted through the burr hole into the right striatum (unilateral, n=10) or both striatum (bilateral, n=10) at the coordinates of 2.0 mm lateral to the midline, 3.0 mm in vertical, and anterior-posterior at the zero point to the bregma. The cell suspension was injected in 3 min, and the syringe was raised 1 mm and left in place for 2 min to minimize cell diffusion up the needle track. Animals were killed at week 2, 4, 8 after the transplanta-tion. The animals in control and model group were received 2 μL DMEM/F-12 basal medium.

Immunohistology At the designed time, mice were deeply anesthetized and perfused with phosphate-buffered saline, pH=7.4(PBS), then perfused with 4 %

paraformaldehyde in PBS. Brains were removed and postfixed overnight in the same 4 % paraformaldehyde solution at 4 ºC; then they were equilibrated in sucrose (30 % in PBS), cut into 30 μm coronal sections on a freezing microtome, and collected on 3-aminopropyl-triethoxysilame-coated glass slide. To evaluate the PD model, alternate sections were processed for TH immunofluorescence

DNA denaturation of sections processed for BrdU immunodetection was carried out by (1) incubating sections in 50 % formamide/2×SSC at 65 ºC for 2 h followed by a wash in 2×SSC for 5 min, and (2) incubating sections in 2 mol/L HCl at 37 ºC for 30 min. Sections were neutralized with 0.1 mol/L borate buffer (pH 8.5) for 15 min. Sections were then washed in TBS (tris-buffer saline: 0.1 mol/L Tris-HCl pH 7.4/0.9 % NaCl), and incubated in blocking buffer (TBS containing 0.3 % TritonX-100 and 5 % normal goat serum, TBS++) for 1 h, and then transfered to blocking buffer containing mouse monoclonal antibody to BrdU. Sections were incubated overnight at 4 ºC and washed 3 times in PBS. Secondary antibodies were diluted in blocking buffer and applied for 2 h at room temperature. Sections were then treated with strept Avidin-Biotin-enzyme complex, developed with AEC (uni-lateral) or BCIP/NBT (bi-lateral). Negative control sections received identical preparations for immunohistology staining, except the primary antibody was omitted.

To detect if BrdU positive cells were also immunoreactive to TH, we performed double labeling for BrdU and TH[16,27,28]. After pretreated with BrdU, sections were incubated with a primary antibody cocktail, including mouse anti-BrdU (1:1000) and rabbit anti-TH(TH1: 1000), in TBS++ for 48 h at 4 ºC. The sections were then rinsed in TBS for 3×10 min and incubated with a cocktail of fluorochrome-labeled secondary antibodies for 4 h. Sections were washed again and coverslipped in glycerol. Fluorescent signals were detected and processed using a confocal laser scanning head on a Zeiss Axiovert microscope (MRC 1024,BIO RAD).

RESULTS

Self-renewal, proliferating capacity, and multipotential differentiation of cultured NSCs *in vitro*

Self-renewal, the capacity of a cell to generate progeny identical to itself, is one of defining characteristics of stem cells[29,30]. NSCs can proliferate and maintain undifferentiated state. In our study, it was observed that NSCs became hypertropic (Fig 1A) and could divide symmetrically and asymmetrically (Fig 1B,C) under the stimulation of bFGF, then generating new stem cells and progenitors. In our study, cells were cultured in clonal density to avoid the cell aggregation under the presence of bFGF. Individual bFGF-dependent cells from the primary culture (primary founder cells) gave rise to neurospheres (primary neurospheres) (Fig 1D, E).

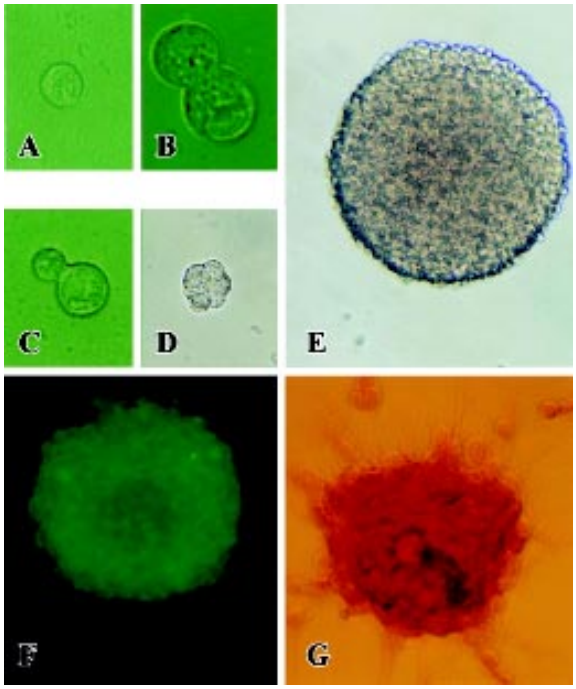

Fig 1. bFGF induced proliferation of neural stem cells (NSCs) isolated from the midbrain of rat embryo. (A) Cells isolated from rat embryo midbrain became hypertrophic by 2-3 days *in vitro* in the presence of bFGF 20 μg/L; (B, C) NSCs divided symmetricly and asymmetricly by 4-5 days *in vitro*; (D) After 5-6 days *in vitro*, neurospheres were generated from NSCs; (E) Phase-contrast photomicrograph of 21-d *in vitro* bFGF-generated sphere; (F) The sphere was immuno-reactive for nestin; (G) The sphere incorporated BrdU into their DNA when proliferating.

One of the other characters of neural stem cell is immuno-reactive to nestin, an intermediate filament which is expressed in neural progenitor /stem cell. In this study, generated neurospheres were immuno-reactive to anti-Nestin (Fig 1F)[31]. The cells dissociated from primary neurospheres could also proliferate into new neurospheres (second neurospheres) and the cells within second neurospheres could incorporate BrdU into their DNA (Fig 1G). We further investigated whether

undifferentiated cells within secondary neurospheres could differentiate into the major cell types found in the mature brain. After bFGF withdrawal and in the presence of serum, these neurospheres were capable of differentiating into GFAP-(Fig 2A), NSE-(Fig 2B) immunoreactive cells. It demonstrated that the cells dissociated from the neurospheres had multi-differentiation potential.

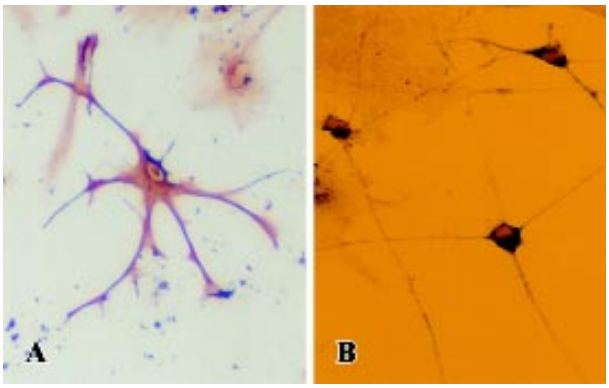

Fig 2. bFGF-dependent cells isolated from the midbrain of rat embryo were multipotent and generated cells displaying neural and astroglial markers after bFGF withdrawal and serum addition. (A) Astroglial (GFAP, ×200); (B) Neuron (NSE, ×200).

Histology MPTP could pass the blood-brain barrier and selectively damage dopaminergic neurons predominantly those originating in the substantia nigra pars compacta (SNpc). It produces the most clinical symptoms of PD patients. In this study, TH immunofluorescence was adopted to observe the neurotoxicity of MPTP and evaluate the model. TH is a rate-limiting enzyme in DA synthesis and the marker of DA neuron. The results showed that compared with the normal mice, the number of TH-positive cells were significantly decreased in the substantia nigra of all MPTP-treated mice at the designed time (Fig 3A, B).

The survival and differentiation of transplanted NSCs are the key step to the functional replacement. In the present study, BrdU-immunoreactive cells were found which demonstrated the survival of transplanted NSCs *in vivo*. These cells almost existed in the whole striatum and showed the long distant migration of NSCs (Fig 4A, B: unilateral; C, D: bilateral). To detect whether the survived BrdU positive cells properly differentiated, BrdU/TH double-labeling immunofluorescence was processed. The result of double-labeling showed that some BrdU immuno-reactive cells were also immuno-

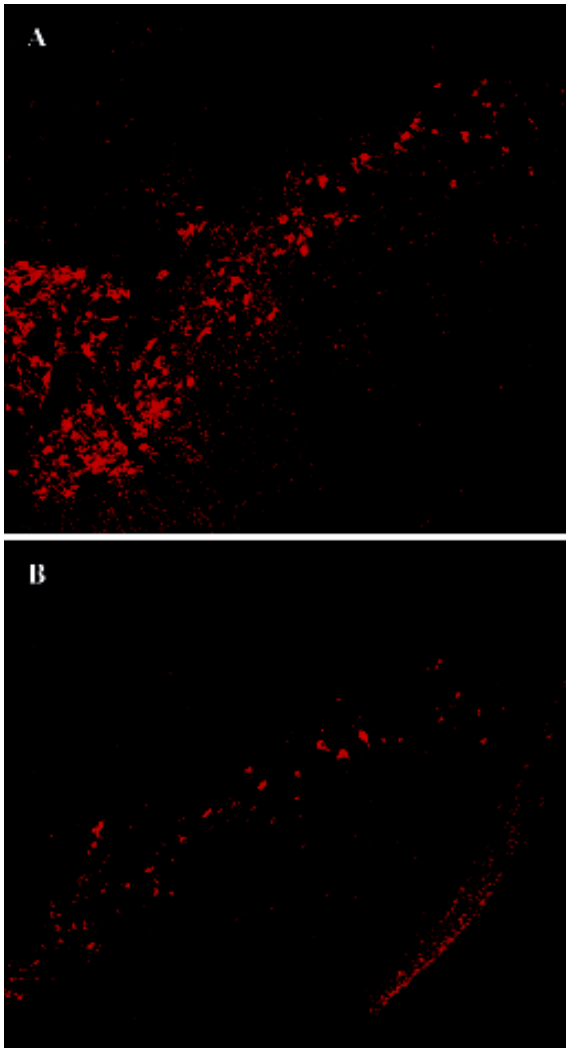

Fig 3. MPTP treatment (40 mg·kg^{-1}×2, sc) led to a significant decrease of TH-positive neural cells in the substantia nigra pars compacta compared with control group from 2 weeks to 2 months after MPTP injection. (A) Control animal; (B) Model animal. ×100.

reactive to TH (Fig 5 A, B, C). It indicated that the transplanted NSCs differentiated into targeted neuron under the control of local cues. Since TH is the marker of DA neuron, it suggested that some BrdU-positive cells could synthesize DA and repair the damaged DA system in some degree.

DISCUSSION

In this study, neural stem cells used for transplantation were isolated from the ventral midbrain (including substantia nigra) of rat embryo. They proliferated and expanded as neurospheres *in vitro* when they were cultured in uncoated task or plated with the serum-free

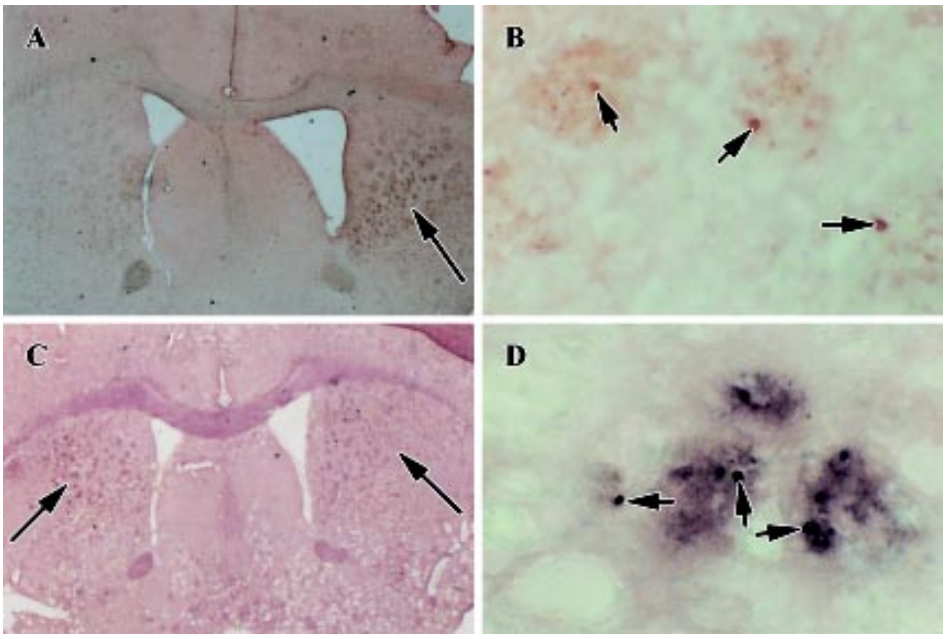

Fig 4. The survival and differentiation of both uni-and bi-lateral transplanted NSCs in the striatum of adult mice. Immunohistology demonstrated that transplanted NSCs could survive in the adult mice striatum. (A: ×15; B: ×200) Unilateral; (C: ×15; D: ×200) bi-lateral.

and mitogen-contained medium. The cells dissociated from primary neurospheres could generate new neurospheres. Of importance, the cells within secondary neurospheres expressed nestin, marker of neural stem/progenitor cell, and incorporated BrdU into DNA when proliferating. Neural stem cells could divide *in vitro*, and give rise to one stem and one committed daughter at steady state, so balance the pool of stem cell and the need of development[32]. They could also differentiate into astroglia and neuron after bFGF withdrawal and addition of fetal bovin serum. Moreover, bFGF-dependent cells also retained a normal karyotype over serial passaging: 40, XY[4]. Collectively, the data showed that bFGF-responsive cells derived from the midbrain of rat embryo possessed the fundamental properties that define stem cells.

In the present study, the primary cells were cultured in low density (1×10^4 cells per well) in uncoated 24-well plate. It has demonstrated previously that culturing cells at this density would result in clonal neurospheres, as form in single-cell cultures, and that neurospheres did not arise as a result of cells aggregation[19,20]. It not only purified the stem cells for the transplantation, but also provided the enough cell which had advantage of single cell subclone.

PD is suitable for cell transplantation therapy because the dopaminergic neurons are clearly circumscribed, the targeted striatum is anatomically defined and there are well-characterized animal models[33]. According to detailed data, C57BL/6 mouse was more sensitive to MPTP than other strains. MPTP passes the blood-brain barrier and is converted by monoamine oxidase B (MAO-B) to the metabolite MPP^+ in glial cells. The latter molecule has a high affinity for the dopamine transporter (DAT) and can easily enter dopaminergic cells. MPTP showed neurotoxicity by inhibiting mitochondrial complex I, producing oxidative stress, and disturbing intracellular calcium homeostasis. It induced many pathological changes, such as Lewy body like intraneuronal eosinophilic inclusions, markers of increased oxidative stress, and decrements in mitochondrial complex I activity[34-36]. The recent studies showed that reactive oxygen species, especially nitric oxide (NO), played an important role in the MPTP neurotoxic process[37-39]. Our study also demonstrated that MPTP (2×40 mg/kg, sc) could cause the obvious behavioral deficits in C57BL/6 mouse (unpublished data), so it successfully produced a Parkinson animal model for the experiment.

Cell transplantation in PD is designed to correct the impairment of striatal dopaminergic neurotransmission caused by degeneration neurons in the substantia nigra, so as to improve behavioral deficits. In this study, NSCs were transplanted into the striatum, not the sub-

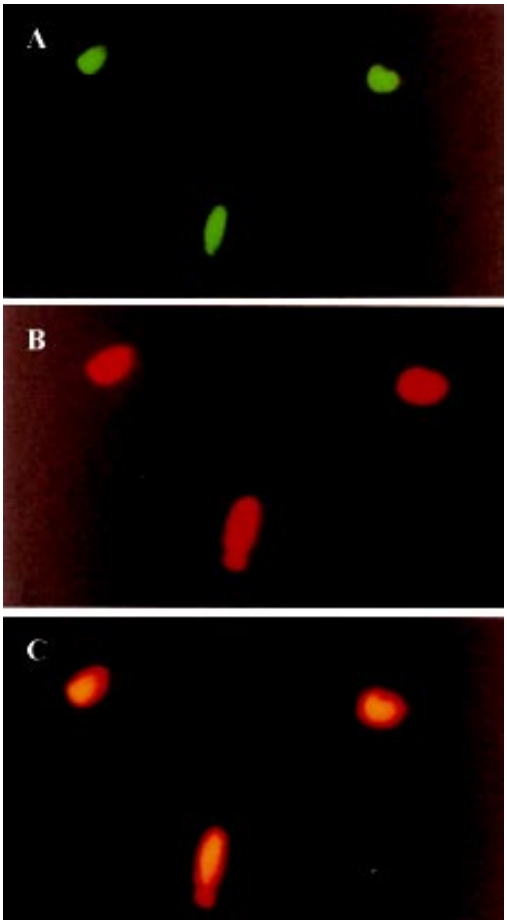

Fig 5. Transplanted NSCs differentiated in the striatum of adult mice under the control of located signals. Double labeling immunofluorescence demonstrated that transplanted NSCs generated new TH^+ neurons. (A) Anti-BrdU staining (green). (B) Anti-TH staining (red). (C) Merge image. (×1000).

stantia nigra. It demonstrated that expanded ventral midbrain NSCs derived from the rat embryo could survive, integrate into host brain and appropriately differentiate without any immunosuppressant. The results also showed that transplanted NSCs differentiated into TH-positive neural cells under the control of local environmental signals. It suggested that the fate of exogenous stem cells *in vivo* was very much influenced by local cues. Although the further studies should be carried out to assess the behavioral recovery after NSCs transplantation, our results showed that NSCs transplantation could become one hopeful method to treat the disorders of central nervous system, such as Parkinson disease.

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