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ORIGINAL RESEARCH REPORT

Protective Effects of Neurotrophic Factor–Secreting Cells in a 6-OHDA Rat Model of Parkinson Disease

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Stem cell-based therapy is a promising treatment for neurodegenerative diseases. In our laboratory, a novel protocol has been developed to induce bone marrow-derived mesenchymal stem cells (MSC) into neurotrophic factors-secreting cells (NTF-SC), thus combining stem cell-based therapy with the NTF-based neuroprotection. These cells produce and secrete factors such as brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor. Conditioned medium of the NTF-SC that was applied to a neuroblastoma cell line (SH-SY5Y) 1 h before exposure to the neurotoxin 6-hydroxydopamine (6-OHDA) demonstrated marked protection. An efficacy study was conducted on the 6-OHDA-induced lesion, a rat model of Parkinson's disease. The cells, either MSC or NTF-SC, were transplanted on the day of 6-OHDA administration and amphetamine-induced rotations were measured as a primary behavior index. We demonstrated that when transplanted posterior to the 6-OHDA lesion, the NTF-SC ameliorated amphetamine-induced rotations by 45%. HPLC analysis demonstrated that 6-OHDA induced dopamine depletion to a level of 21% compared to the untreated striatum. NTF-SC inhibited dopamine depletion to a level of 72% of the contralateral striatum. Moreover, an MRI study conducted with iron-labeled cells, followed by histological verification, revealed that the engrafted cells migrated toward the lesion. In a histological assessment, we found that the cells induced regeneration in the damaged striatal dopaminergic nerve terminal network. We therefore conclude that the induced MSC have a therapeutic potential for neurodegenerative processes and diseases, both by the NTFs secretion and by the migratory trait toward the diseased tissue.

Introduction

Stem cell-based therapy offers promise for future treatment of neurodegenerative disease. There are 2 main potential approaches to stem cell-based therapy. One strategy is aimed at simply replacing the lost cells by transplanting stem cells. The second approach is to utilize the cells as vectors that contain and secrete neuroprotective agents in order to preserve the surviving neurons or to induce renewal of axonal sprouting.

Parkinson's disease (PD) is a debilitating neurodegenerative disorder that mainly results from a specific loss of nigral dopaminergic neurons. This particular cellular loss has made it a common target for stem cells-based replacement therapy [1–3]. Several potential cells sources have been investigated to replace the degenerated dopaminergic neurons, including embryonic stem (ES) cells [4,5] and adult stem cells. The later are of various origins, whether induced pluripotent stem cells (iPS) [6], multipotent mesenchymal stromal cells (MSC) [7–14], or neural stem cells [15,16]. Additionally, clinical trials for cell replacement using human fetal mesencephalic tissue have been reported [17,18]. Not only that clinical benefit was not observed, several issues were raised, such as graft-induced dyskinesias, appearance of Lewy bodies in the grafted cells [19,20], and some immune response against the graft. Apart from these difficulties, probably the main obstacle of fetal midbrain grafts is the low availability

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of dopaminergic neurons. The ES cells and iPS raise another important issue, that of their safety and potential tumor development [21].

Neurotrophic factors comprise of a family of proteins that are essential for neuronal development and survival. Specific factors are essential for subpopulations of cells. Brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) are 2 important examples relevant to PD, since they mediate dopaminergic neuronal survival and induce reinnervation of tissue deafferented following exposure to specific toxins [22]. Conditioned knockout of GDNF results in a progressive hypokinesia and a specific degeneration of the catecholaminergic neurons [23]. Trophic factors such as GDNF [24] and insulin-like growth factor 1 (IGF-1) [25] have also shown efficacy for their restorative effect on the dopaminergic fibers of animal models.

Clinical trials in which GNDF was administered chronically by infusion into the striatum via implanted catheters indicated positive effects in preliminary trial, but they were not reproduced in a subsequent controlled experiment [26,27]. Failure of clinical experiments could be due to inefficient mode of delivery of these potentially therapeutic agents into the brain [28], suggesting that better and more efficient long-term delivery strategies should be looked for.

Bone marrow contains 2 major populations of stem cells: hematopoietic and MSC [29,30]. Originally, MSC were shown to give rise to cells of mesenchymal lineage such as osteocytes, chondrocytes, and adipocytes. We have recently demonstrated that MSC possess neural predisposition and express neural markers [31]. Several studies have indicated that, following exposure to different factors in vitro or after injecting them in vivo into rodent brain, MSC change their phenotype and demonstrate neuronal and glial markers [7,8,11,12,32–34]. In addition to the suspected neural disposition, MSC have other advantages as compared to ES cells in the therapeutic context of neurodegenerative diseases: safety (nontumorogenic), the possibility of autotransplantation [35,36], their migratory capabilities, [37–39] and the lack of ethical restriction.

We developed a method to induce MSC of rat bone marrow origin into neurotrophic factors-secreting cells (NTF-SC). These cells also express several astrocytic markers [38]. The present study shows the results of the induction process in human bone marrow-derived MSC in terms of morphology, protein expression, and NTFs secretion. Furthermore, we present here better beneficial effects achieved by intrastriatal transplantation of these cells as compared with undifferentiated MSC on behavioral, biochemical, and histological parameters in the 6-hydroxydopamine (6-OHDA)-lesioned rats.

A major limitation of stem cell-based therapy is the assessment of the fate of the cells following transplantation. A noninvasive method that can be potentially applied for such purpose in vivo is magnetic resonance imaging (MRI), while using specific contrast agents labeling [40,41]. In this study we also examined indication for cell survival by following the cellular migratory route by employing MRI.

Materials and Methods

Isolation and proliferation of human MSC

Adult human bone marrow samples were collected from the posterior iliac crest of adult human donors, undergoing bone marrow aspiration, after obtaining informed consent (approved by the Helsinki Committee of the Laniado Medical Center, Israel). Bone marrow aspirates were diluted 1:1 with Hanks' Balanced Salt Solution (HBSS; Biological Industries, Beit-Haemek, Israel) and mononuclear cells were separated by density centrifugation, over UNI-SEP$_{MAXI}$/ UNI-SEP+ (Polysucrose–Sodium Metrizoate; NovaMed, Jerusalem, Israel) containing tubes. The mononuclear cell fraction was collected, washed in HBSS, and centrifuged. Cells were resuspended in Growth medium 1, counted, and seeded at a concentration of 250,000–350,000 cells/cm^2 in 75-cm^2 tissue culture flasks (Corning, NY). Flasks were incubated in a 37°C humidified incubator with 5% CO_2.

Growth medium 1 consisted of Dulbecco's Modified Eagle's Medium (DMEM; Biological Industries, Beit Haemek, Israel), supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 12.5 units/mL nystatin (SPN; Biological industries), 2 mM L-Glutamine (Biological industries), 2 IU/mL Heparin (Trima, Kibutz Maabarot, Israel), 0.001% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 1% MEM nonessential amino acid solution (Biological Industries), and 10% platelet lysate. Platelet lysate was processed from frozen-thawed human platelet-rich plasma (PRP) as previously described [42]. After 24 h, Growth medium 1 was aspirated to remove nonadherent cells from the flask. Human MSC were allowed to proliferate in Growth medium 1, which was replaced twice weekly. After 12–18 days the cells were trypsinized (trypsin from Biological Industries), centrifuged, counted, resuspended in Growth medium 2, and seeded in CellStacks (Corning, NY) at a density of 500–3000 cells/cm^2. Growth medium 2 consists of DMEM supplemented with SPN, glutamine, and heparin as in Growth medium 1 and 5% PRP. MSC cultures were passaged approximately every 2 weeks. Experiments on the cells were performed after 2–7 passages.

Induction of human MSC into NTF-SC

Human MSC (12,000 cells/cm^2) were first placed in DMEM supplemented with SPN, 2 mM L-Glutamine (Biological industries), 20 ng/mL human epidermal growth factor (hEGF), 20 ng/mL human basic fibroblast growth factor (hbFGF) (R&D Systems, Minneapolis, MN), and N2 supplement (Invitrogen, Carlsbad, CA). After 72 h, the medium was replaced with DMEM supplemented with 1 mM dibutyryl cyclic AMP (dbcAMP), 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO), 5 ng/mL human platelet-derived growth factor (PDGF), 50 ng/mL human neuregulin 1-β1/HRG1-β1 EGF domain, and 20 ng/mL hbFGF (all from R&D Systems) for 3 more days.

Immunocytochemistry

Human MSC and NTF-SC were fixed with 4% paraformaldehyde and stained with rabbit anti-glial fibrillary acidic protein (GFAP; 1:200, DAKO, Carpinteria, CA), rabbit anti-glutamine synthetase (GS; 1:100; Sigma-Aldrich, St. Louis, MO), rabbit anti-GDNF (1:100; Santa Cruz, Santa Cruz, CA), rabbit anti-BDNF (1:100; Santa Cruz), rabbit anti-IGF-1 (1:100; Santa Cruz). Secondary antibodies were goat anti-rabbit Alexa-488 (1:200; Molecular Probes, Invitrogen). For GDNF staining, secondary antibodies were biotinylated goat anti-rabbit (1:200; Jackson Laboratories, Bar Harbor, ME) and streptavidine-

Alexa-488 (1:200; Molecular Probes). Nuclear DNA was stained by 4,6-diamino-2-phenylindole (DAPI) (1:1,000; Sigma).

In vitro neuroprotection assay

Neuroblastoma cell line SH-SY5Y cells (ATCC, Manassas, VA) were grown in basal media consisting of DMEM with 10% fetal calf serum (FCS), 2 mM L-glutamine, and SPN (Biological industries). The SH-SY5Y cells were plated in 96-well plates. Each well was applied with either human MSC- or NTF-SC-conditioned media or with serum-free medium (DMEM, Glutamine and SPN), and immediately exposed to oxidative insult by 6-OHDA (Sigma-Aldrich) for 24 h. Cell viability after treatments was analyzed by adding 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution to each well followed by incubation at 37°C for 3 h. Absorbance was determined at 564 nm in a microplate reader. Cell viability was evaluated in sextuplets for each treatment and compared to the serum-free-treated cells.

ELISA-based measurements of NTFs secretion

At the end of the NTF-SC induction process, the cell culture supernatant was measured for human GDNF and BDNF concentrations by a sandwich ELISA procedure according to the manufacturer's instructions (DuoSet, R&D System for human BDNF and GDNF). The absorbance at 450 and 570 nm was recorded on a Microplate Reader (Labsystems Multiscan MS). The results were calculated for 1 million cells.

6-OHDA-induced striatal lesion

A total of 63 male Sprague-Dawley rats weighting 260–300 g (Harlan, Jerusalem, Israel) were used in this experiment. They were placed under 12 h light/12 h dark conditions and grown in individual ventilated cages (IVC) with *ad libitum* access to food and water. All experimental protocols were approved by the University Committee of Animal Use for Research and Education. Every effort was taken to reduce the number of animals used and to minimize their suffering.

The 7 μg/2.5 μL/site of 6-hydroxy dopamine (6-OHDA; Sigma-Aldrich, St. Louis, MO) was injected into 2 sites (total of 14 μg 6-OHDA) in the right striatum according to the rat brain atlas [43] in 56 animals. Under chloral hydrate anesthesia the rats were placed in a digital stereotactic frame (Stoelting, Wood Dale, IL) and 6-OHDA was injected to the following coordinates (relative to the bregma and dura): anterior-posterior (AP) AP + 0.5 mm, medial-lateral (ML) ML 2.5 mm, dorsal-ventral (DV) DV-6.0 mm & AP–0.5 ML 4.2 DV-6.0 at a rate of 1 μL/min using a Hamilton 701N syringe. The inserted needle was withdrawn from each location after 5 min.

Stem cells transplantation

Human MSC grown in serum-free conditions for 6 days or induced NTF-SC were used. On treatment day, the cells were trypsinized, washed with phosphate-buffered saline (PBS), and counted. Two concentrations of cells were injected (50,000 cells/μL or 150,000 cells/μL in PBS). A total of 150,000 or 450,000 cells/3 μL were injected per site at a rate of 1 μL/min, and cells were transplanted into 2 sites along the same DV axis: AP-1.8, ML 4.6, DV-5 and -7. Cells viability was assessed by Trypan blue (Sigma-Aldrich, St. Louis, MO) after each transplantation session.

Study design

The in vivo experiment was performed on the 6-OHDA-induced striatal lesion model. Cells, or PBS as control, were transplanted on the same day of the 6-OHDA injections, 50 min later, posterior to the lesion within the treated striatum. The experiment consisted of the following groups: the control group was treated with 6-OHDA and with PBS instead of cellular treatment ($n = 10$); MSC-treated groups were treated with either a high dose (450,000 cells, $n = 10$) or a low dose (150,000 cells, $n = 11$) of serum-free medium-treated MSC; NTF-SC group was treated with either high dose (450,000 cells, $n = 10$) or low dose (150,000 cells, $n = 11$) of induced NTF-SC. Another group of untreated animals ($n = 7$) were used as controls for the open field test.

For cell tracking purposes 3 different time points were analyzed by using histology-based study or in vivo MRI. At the first time point, 7 days post-cellular treatment, 4 animals that were treated with high-dose NTF-SC were sacrificed for histological evaluation only. For the second time point, on the 35th day, we conducted an in vivo MRI study on selected animals. These animals ($n = 3$ from the control group and $n = 3$ from the low-dose NTF-SC-treated group) were treated with cells that were pre-labeled with super-paramagnetic iron oxide particles (SPIOs, 5 μg/mL; Feridex, Advanced magnetic, Cambridge, MA). SPIOs were incubated with poly-L-lysine (1 μg/mL medium, 70–150 kDa; Sigma-Aldrich, St. Louis, MO) for 1 h before adding to the medium on the last day of stage 1 medium treatment. Cultures were washed with stage 2 medium of induction after 24 h. The control group was treated with 1 μg of SPIOs in 6 μL of PBS (the same volume of the cell suspension). The last time point for cell tracking was at the end of the experiment, at 50 days after treatment day.

Immunosuppression was induced by daily subcutaneous administration of 15 mg/kg cyclosporine A (Novartis, Basel, Switzerland), starting 1 day prior to cellular treatment and continued throughout the experiment. Animals received prophylactic antibiotic treatment with Enrofloxacin (50 mg/kg, Bayer, Germany) for 5 days from the first day of the experiment.

Behavioral tests

D-Amphetamine-induced rotational behavior was measured for 90 min following i.p. administration of 2.5 mg/Kg (Sigma-Aldrich, St. Louis, MO) using an automated Rotameter device (San Diego Instruments, San Diego, CA). The net ipsilateral rotations were measured at 14, 28, and 42 days post-cell transplantation.

Open field test was conducted at 7 days posttreatment by introducing the animals into a 50 cm^2 arena and videotaping the spontaneous behavior of the rats for 30 min. The images were analyzed by EthoVision 3 software (Noldus, The Netherlands).

MRI

Anesthesia was induced with 4% isoflurane in 95% O_2 and maintained with ~1%–2% isoflurane (Vetmarket Ltd., Petah-Tikva, Israel) at a flow rate of ~1 L/min. Respiratory rate was monitored throughout all the experiments. Body

temperature was maintained by circulating water at 37°C. MRI scans were performed on a 7.0 T/30 cm Bruker Biospec equipped with a gradient system capable of producing gradient pulses of up to 400 mT/m (Bruker Biospin, Karlsruhe, Germany). A body coil was used as the transmit coil, and a rat quadurature coil was used as the receiving coil. MRI experiments were performed on the 35th day post-transplantation and 6-OHDA injection. Scans included: T_2-weighted images (WI) RARE8 (TR/TE = 3500/75 ms). The field of view (FOV) was 2.56 × 2.56, the matrix size was 256 × 128 zero-filled to 256 × 256, and a slice thickness of 700 μm was chosen, 15 slices were collected. Additionally, three-dimensional (3D) gradient echo (GE) images were collected (FLASH, TR/TE = 150/14 ms, flip angle = 15°) with a FOV of 2.56 × 2.56 × 0.48 and a matrix size of 128 × 96 × 24 (zero-filled to 128 × 128 × 32), resulting in a spatial resolution of 200 × 200 × 150 $(\mu m)^3$. The images are presented as raw data without any image processing.

Immunohistochemistry

At the end of the experiment (7 or 50 days posttreatment), animals were transcardially perfused with ice cold PBS following a solution of 4% paraformaldehyde and 4% sucrose in phosphate buffer (PB) according to a known protocol (NeuroScience Labs, NSA, Knoxville, TN). Brains were immersed in the perfusion solution for 24 h in 4°C following cryoprotection in 30% sucrose for additional 48 h before freezing. Twelve samples were processed by NSA (4 of the PBS group, 4 of the high MSC group, 4 of the high NTF-SC group, and 4 animals treated with high dose of NTF-SC cells that were sacrificed after a week). These samples were serially sectioned into 40 μm coronal sections and every eighth section throughout the striatum was dyed for human nuclei antigen and the adjacent section for tyrosine hydroxylase (TH).

The brains of animals treated with SPIOs-labeled cells were sectioned axially (8 μm) and dyed with Prussian blue stain (Sigma-Aldrich, according to manufacturer's instructions) for the detection of Fe particles. Adjacent sections were immunostained with anti-human nuclear antibody. Briefly, sections were microwave-boiled in a citrate buffer for antigen unmasking, and then immersed in a blocking and permeability solution (10% FCS, 2% bovine serum albumin, 1% glycine, and 0.05% Triton). Post-blocking, the samples were incubated overnight with anti-human nuclear antibody (1:200) in 4°C. Sections were dyed with anti-mouse IgG conjugated to Alexa Fluor 568 (Invitrogen; 1:500). Nuclei were counterstained with DAPI (1:500; Sigma-Aldrich). CD68 staining (1:500; Serotec, Oxford, UK) was conducted in a similar manner except the antigen unmasking process and the use of biotinylated anti-mouse IgG (ready to use; Zymed-Invitrogen) followed by streptavidin Alexa Fluor 568 (1:500; Invitrogen).

Stereological study

All sections stained for TH (n = 4 animals from each group) were quantified for the area with a positive stain in the striatum. By using an Olympus DP71 camera (Japan) at a 40× magnification, 2–3 images were photographed to cover all or almost all the striatum of each side and each animal. The images were then quantified by the ImagePro 5.1 software that measured the total area of positive staining, according to a unified cutoff. The operator of the software was blind to the origin of the images. The damage was calculated as the percent of the TH-positive area in the lesioned striatum divided by the TH-positive area of the untreated contralateral side.

Dopamine measurements by HPLC

Animals (n = 5 each of the following groups: PBS, high-dose MSC, and high-dose NTF-SC) were sacrificed by CO_2 and their brains were quickly removed and placed on ice. The striatae were dissected out and weighted. Each sample was sonicated in ice cold 1 mL of 0.1 M perchloric acid until homogeneity was achieved. The samples were centrifuged for 15 min (12,000 rpm, 4°C), and the supernatants were collected and transferred onto a 0.2 μm nylon filter tubes (Corning). The samples were centrifuged again (6,000 rpm, 5 min, 4°C) and the filtrates were stored in −80°C until analyzed. An aliquot of the filtrate was injected into the HPLC system (Waters, Milford, MA, USA) equipped with a C18 reverse phase, 3 μm LUNA column (100 mm × 2 mm; Phenomenex, Torrance, CA, USA). The sample was eluted by a mobile phase made of 25 mM NaH_2PO_4, 50 mM Na-citrate, 0.03 mM EDTA, 10 mM diethylamine HCl, and 2.2 mm sodium octyl sulfate (pH 3.2), 30 mL/L methanol and 22 mL/L dimethylacetamide at a flow rate of 0.4 mL/min. The dopamine peak was determined by electrochemical detection at a potential of 0.6 V. The dopamine content in the sample was calculated by extrapolating the peak area from a standard curve (range 1–200 pg of dopamine) constructed under the same conditions during each run by the Maxima Workstation (Waters). The results were normalized to the sample weight [44].

Statistical analysis

The results are expressed as means ± standard error. Student's t-test was used to compare means of 2 groups. Comparisons between several groups were done by ANOVA with Scheffe's post-hoc analysis. Repeated tests (amphetamine-induced rotations) were also analyzed by repeated ANOVA test. Statistical calculations were performed using SPSS v. 13.

Results

Induced human NTF-SC express neurotrophic factors

In vitro analysis of the NTF-SC revealed their NTF expression profile. They expressed astrocyte markers such as GFAP and GS. Moreover, The NTF-SC highly expressed the GDNF, BDNF, and IGF-1 proteins as indicated by immunocytochemistry (Fig. 1A–J). ELISA analysis showed that the differentiated NTF-SC secrete the neurotrophic factors into the culture supernatant. While untreated MSC secreted low levels of BDNF and GDNF (1,216 ± 725 pg/10^6 cells and 337 ± 27 pg/10^6 cells, respectively), after 6 days of induction the NTF-SC secreted over five times more BDNF (7,117 ± 1,335 pg/10^6 cells), and over twice the amount of GDNF (787 ± 206 pg/10^6 cells) (Fig. 1K, L).

Conditioned media from human NTF-SC protect neuroblastoma cell line against 6-OHDA toxicity

The protective effect of the induced MSC in vitro was examined using a neuroblastoma cell line, SH-SY5Y, exposed to increasing doses of 6-OHDA (32–160 µM). For the neuroprotection assay, we induced human MSC, removed the induction medium, and incubated it with fresh serum-free medium for additional 24 h to allow secretion of NTFs. For controls, we used supernatants of untreated MSC incubated with serum-free medium for 24 h and serum-free medium alone. The SH-SY5Y cells were incubated with the supernatants and 1 h later, 6-OHDA neurotoxin was added to the cultures. Cell viability was measured 24 h later using MTT. We observed a consistent reduction in viability of the SH-SY5Y cells incubated with DMEM and 32, 48, and 72 µM of 6-OHDA (31.7 ± 6.9%, 10.1 ± 0.2%, and 12.3 ± 0.4%, respectively, Fig. 2). In contrast, SH-SY5Y cells that were incubated with supernatants, collected from untreated MSC or NTF-SC media, demonstrated a statistically significant higher percentage of viability in the presence of 32, 48, and 72 µM 6-OHDA. The NTF-SC media demonstrated an added protective value upon the survival of the SH-SY5Y cells, although no statistically significant difference was demonstrated between the induced NTF-SC media treatment and the untreated MSC media treatment (90.2 ± 7.4%, 73.2 ± 9.6%, and 55.4 ± 15.9%, respectively, for the MSC group, $P < 0.05$, and 92.4 ± 5.0%, 93.4 ± 12.9%, and 80.3 ± 12% for the NTF-SC group, $P < 0.005$, Fig. 2). At 160 µM 6-OHDA, cell viability dropped to 15.0 ± 0.6% in the control group and similarly in the MSC group (11.7 ± 0.2%), while the NTF-SC media remained slightly beneficial (17.9 ± 0.6%, $P < 0.05$ compared to both groups).

Transplanted human NTF-SC attenuate 6-OHDA-induced rotational behavior in rats

Overall, the treated animals of all groups tolerated well the various therapies including immunosuppression. Of 56 animals, 2 died within the experimental follow-up, 1 of peritonitis and the other of an unknown cause. The animals failed to gain weight for 14 days posttreatment regardless of the group tested (excluding healthy controls for the open field test), and from that point on almost all animals gained weight in a similar manner.

The control (PBS) group demonstrated an statistically significant increase of amphetamine-induced ipsilateral rotations on the 14th day post-lesion (2.71 ± 0.79 net ipsilateral rotations per min) and the 28th day (4.11 ± 0.86), reaching a plateau on the 42nd day measurement (4.74 ± 1.07) suggesting that the pathological process of death of nigrostriatal dopaminergic nerve terminals was progressive in nature for at least 28 days.

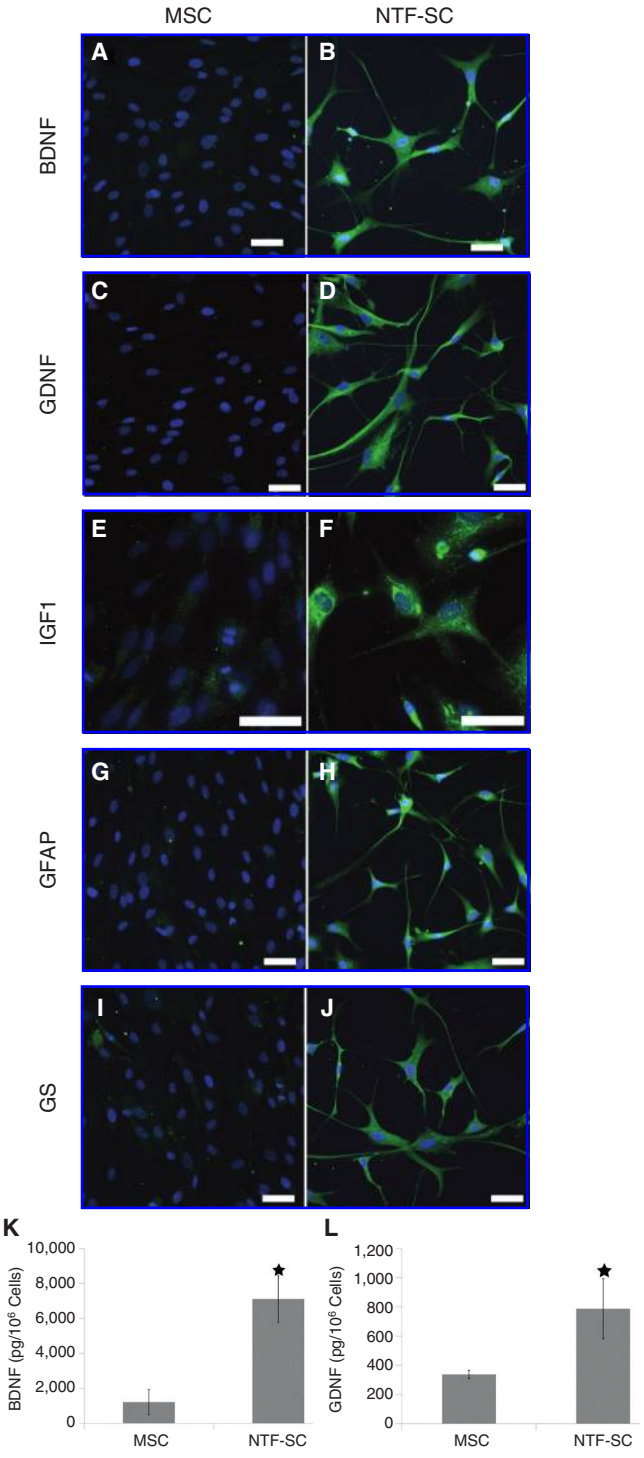

FIG. 1. Human bone marrow-derived mesenchymal stem cells (MSC) express and secrete neurotrophic factors. MSC and neurotrophic factors secreting cells (NTF-SC) were stained with antibodies against neurotrophic factors including brain-derived neurotrophic factor (BNDF) (**A**–**B**), Glial-derived neurotrophic factor (GDNF) (**C**–**D**) and insulin-like growth factor 1 (IGF-1) (**E**–**F**), and with antibodies against the astrocytic markers glial fibrillary acidic protein (GFAP) (**G**–**H**) and glutamine synthetase (GS) (**I**–**J**). All secondary antibodies used were conjugated to Alexa-488 (green). Nuclei were visualized with DAPI (blue). Scale bar = 50 µm. Neurotrophic factors secretion from human MSC before and after induction into NTF-SC, from 3 different donors analyzed by ELISA assay. *$P < 0.01$ for the MSC versus the NTF-SC for BDNF secretion (**K**) and $P < 0.05$ for GDNF secretion (**L**).

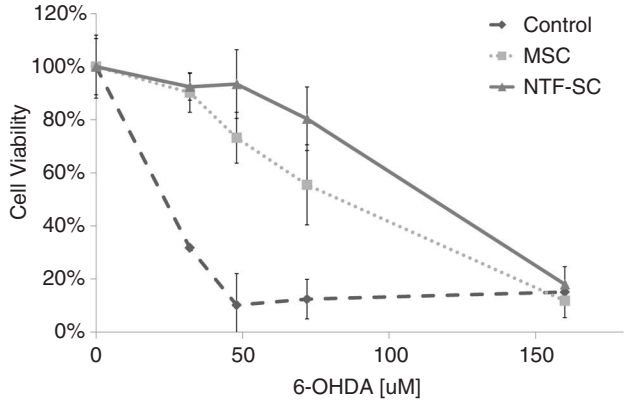

FIG. 2. Human mesenchymal stem cells (MSC) and neurotrophic factors secreting cells (NTF-SC) media attenuated 6-OHDA-induced neuroblastoma cells death. In the range of 32–160 μM, 6-OHDA treatment resulted in a survival rate of <35% compared to untreated SH-SY5Y neuroblastoma cells. Media from both cell types of cells media attenuated the 6-OHDA-induced cellular death in a statistically significant manner in comparison to neuroblastoma cells treated with 6-OHDA only ($P < 0.05$ for the range of 32–72 μM). A treatment with 160 μM of 6-OHDA resulted in a smaller but statistically significant increase in cellular viability by the NTF-SC media only and not by MSC media.

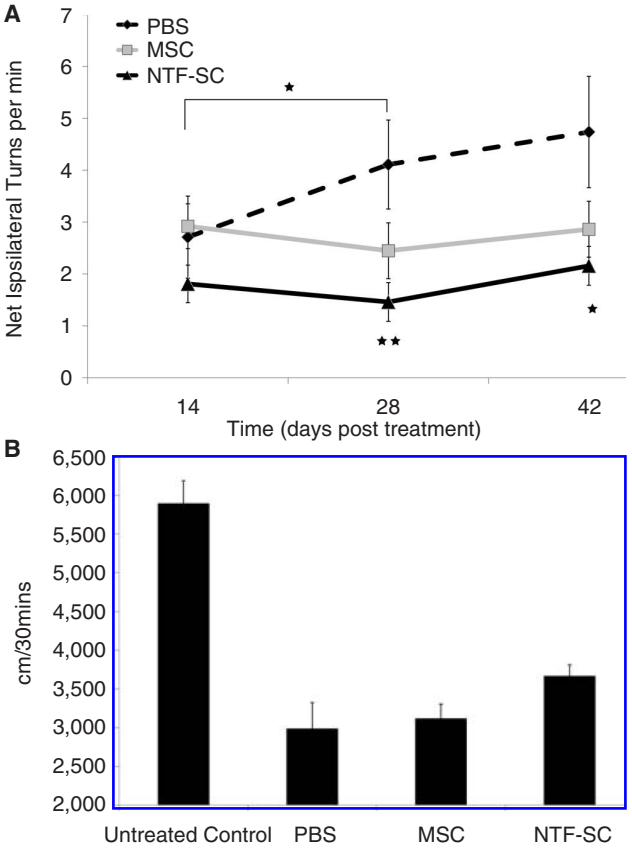

FIG. 3. Striatal transplantation of human neurotrophic factors secreting cells (NTF-SC) attenuated 6-OHDA-induced behavioral changes. (**A**) In the amphetamine-induced rotations test only NTF-SC ($n = 20$) treatment was beneficial compared to control ($n = 10$) at two time points (day 28 and day 42). No statistically significant difference was shown for the mesenchymal stem cells (MSC)-treated group ($n = 20$) as compared to the control group or the NTF-SC-treated group. The number of net ipsilateral turns was significantly increased over time only in the phosphate-buffered saline (PBS)-treated group (*$P < 0.05$, **$P < 0.01$ in a post hoc analysis after ANOVA, and * over bar indicates $P < 0.05$ in a paired *t*-test). (**B**) 6-OHDA induced a hypoactive motor behavior pattern in an open field test at 7 days posttreatment. NTF-SC treatment showed a positive effect in the voluntary mobility as compared to MSC- and PBS-treated groups (ANOVA, $P = 0.054$).

There were no statistically significant differences between the different cell doses, regardless of the type of treatment and the day of measurement. In the untreated MSC low-dose group, we found that the animals rotated at a rate of 3.54 ± 0.63, 2.61 ± 0.55, and 3.25 ± 0.75 net ipsilateral turns per min on days 14, 28, and 42 posttreatment, respectively, while the MSC high-dose-treated group had shown lower but not statistically significant measures: 2.05 ± 0.41, 2.22 ± 0.52, and 2.30 ± 0.78 at the same time intervals. The NTF-SC-treated group had shown the same trait between the doses: 1.19 ± 0.6, 1.29 ± 0.44, and 2.07 ± 0.81 in the low-dose-treated group compared to 2.12 ± 0.42, 1.49 ± 0.32, and 1.67 ± 0.52 in the high-dose-treated group. Hence, we combined the high- and low-dose groups for another statistical analysis. We found that both cellular treatment types produced a nonprogressing effect on the rotational behavior. The NTF-SC group demonstrated a lower result in the first measurement 14 days posttreatment, although it is not statistically different from the other groups. It had a statistically significant better result than the control group on the 2 later measurements (2.45 ± 0.54 and 2.86 ± 0.54 for the combined MSC-treated group on days 28 and 42 posttreatment, respectively, compared to 1.46 ± 0.37 and 2.16 ± 0.37 for the combined NTF-SC-treated group at the same time intervals, $P < 0.05$ compared to the PBS group). In summary, the MSC-treated group did not demonstrate a statistically significant improvement as compared to the control (PBS) group. In contrast, our novel induction-based treatment, the NTF-SC groups, demonstrated a marked decrease of 25% and 45% after 14 and 28 days post-transplantation (Fig. 3A).

All 6-OHDA-treated animals demonstrated overall motor hypoactivity in the open field test on the seventh day posttreatment. The control group activity indices were similar to those of the MSC-treated group. The total distance walked by the combined low- and high-dose NTF-SC-treated animals was 3,667 cm on average, which was 22% higher compared to the control group (2,989 cm) and 17% higher than the combined low- and high-dose MSC-treated group (3,119 cm) on average. However, there was no statistically significant difference between the groups ($P = 0.054$) (Fig. 3B).

Transplantation of human NTF-SC resulted in a greater preservation of TH-positive area in the 6-OHDA-lesioned striatum

The methodology of the stereological study is drafted on Supplementary Figure 3 (supplementary materials are available online at http://www.liebertpub.com). We found

that 6-OHDA-induced lesions with this specific protocol decreased the striatal TH-positive area by more than 5-fold in comparison to the control hemisphere (15.26 ± 2.95% of the contralateral striatum). Both types of cellular treatment, MSC and NTF-SC, demonstrated a protective effect. However, only the NTF-SC yielded in a statistically significant higher TH-positive area as a percent of the untreated side (31.15 ± 6.27%), by increasing it more than twice in comparison to the control group, while MSC treatment failed to induce a statistically significant difference (23.11 ± 3.5%, Fig. 4A–D).

Interestingly, when subdividing the different areas of the striatum into sections of ~1 mm thick, we found the NTF-SC to be beneficial in the anterior, rather than the posterior area of the striatum, the region of transplantation (Fig. 4E).

Cellular transplantation inhibited 6-OHDA-induced dopamine depletion in the lesioned striatae

We found that both types of cellular treatments, that is, MSC and NTF-SC, prevented the falls of striatal dopamine levels induced by neurotoxin injection by >2.5-fold compared with the control group (HPLC measurement of the levels of dopamine in the whole lesioned striatum was defined as a percentage of the untreated side). While the control group (n = 5) lesioned striatae contained only 21.3 ± 3.8% dopamine measured in comparison to the untreated side, MSC-treated animals (n = 5) had on average 68.7 ± 8.6% and the NTF-SC-treated group (n = 5) had 72.4 ± 16.4% striatal dopamine levels compared to the intact contralateral striatum. Only measurements from the NTF-SC-treated group reached a statistically significant difference compared to the control group ($P < 0.05$).

Tracking of transplanted cells by histology and in vivo MRI

Cell tracking was done at 3 different time points, by applying in vivo MRI and 3 different histological assessments. In vivo MRI was conducted on selected animals treated with iron particles-labeled cells (or SPIOs only as controls, n = 3 each) on day 35 posttreatment. The 3D T_2*-weighted images demonstrated 2 distinct hypointense regions for the PBS-treated group: the PBS/SPIO injection sites and the 6-OHDA injection sites (Fig. 5A). The latter probably resulted from bleeding in the site of 6-OHDA injection. No other hypointensities could be seen in the area between the 2 injections. In contrast, the 3D T_2*-weighted images of the cell-treated group demonstrated a migration pathway from the site of NTF-SC injection to the striatum (Fig. 5B, C). The 2D T_2-weighted image is shown in Figure 5G for anatomical reference. Histological staining with Prussian

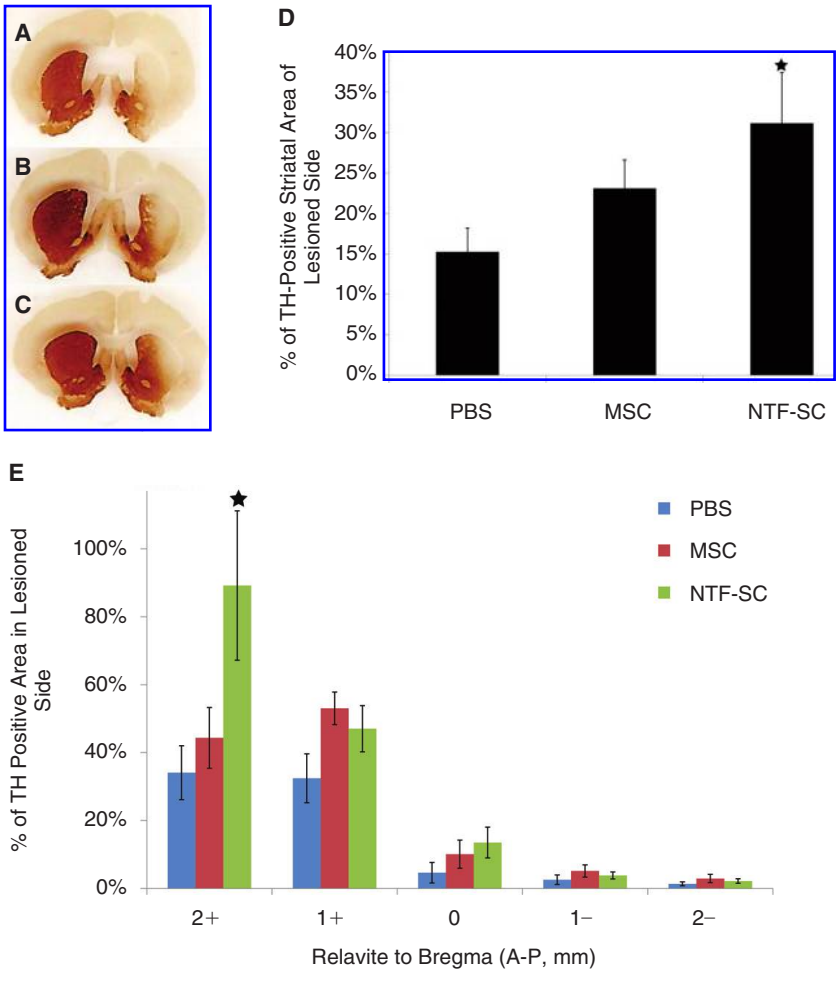

FIG. 4. Treatment with human neurotrophic factors secreting cells (NTF-SC) salvaged TH-positive striatal terminals damaged by 6-OHDA. (**A-C**) A macroview of TH staining in striatum with a representative slice for each group of rats (A-phosphate-buffered saline (PBS), B-mesenchymal stem cells (MSC), C-NTF-SC). (**D**) Digital quantification of the TH-positive area in the whole lesioned striatum as a percent of the untreated striatum, demonstrating the beneficial protective effect of NTF-SC treatment (n = 4 from each group, *$P < 0.05$ compared to PBS). (**E**) Quantification of TH-positive area of the lesioned side compared to the untreated side—a segmental comparison. The whole striatum was divided into five segments from anterior to posterior (each group represents ~1 mm thickness of the striatum, *$P < 0.05$ compared to PBS).

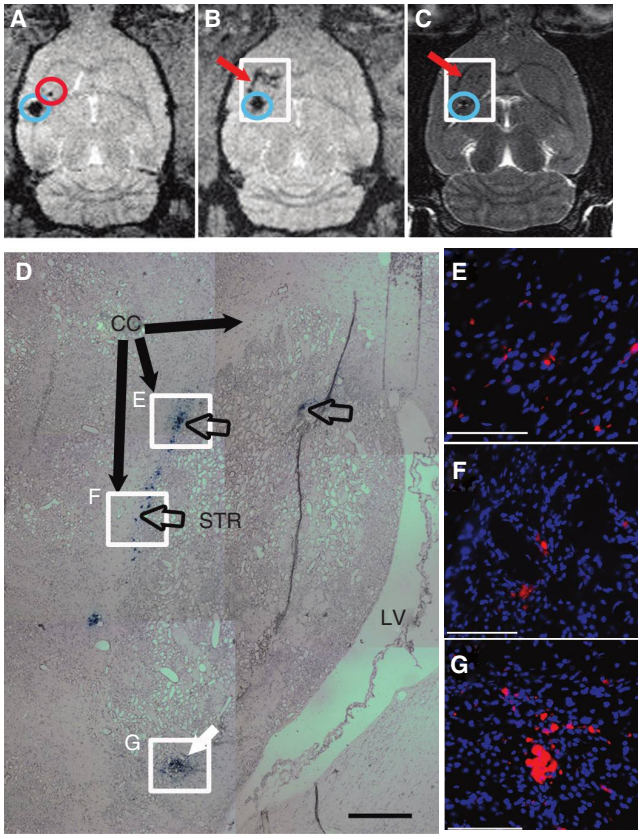

FIG. 5. Tracking of human neurotrophic factors secreting cells (NTF-SC) by in vivo MRI and corresponding histology. (**A**) T_2* MRI scan conducted at 35 days posttreatment of a control SPIOs-treated animal (without cells) demonstrating the SPIO injection site (blue circle) and the 6-OHDA injection site in the striatum (red circle). No other hypointense signals could be detected in the striatum. (**B**) The migratory pathway as demonstrated by an axial T_2*-weighted image. A marked hypointense signal is visible from the cell transplantation site (blue circle) to the 6-OHDA lesion site (red arrow). A good correlation to the Prussian blue staining was found (**D**) along the CC into the anterior striatum. The same trail (red arrow) can even be seen also in the T_2-weighted image (**C**), which is less sensitive to the inhomogeniety induced by the SPIOs. (**D**) Prussian blue stain to detect SPIOs-labeled cells in an axial section (50 days after treatment in the animal that underwent MRI scan displayed in **B-C**) demonstrating a migratory path from the cell transplantation site (white arrow), along the corpus callosum (CC, hollow black arrows), and into the anterior striatum (STR, LV—lateral ventricle, scale bar—500 μm). (**E-G**) immunostaining with anti-human nuclei antibody of the marked white boxes in (**D**) in adjacent sections (scale bar 100 μm).

blue for the iron particles was in excellent agreement with hypointensities in the MR images (Fig. 5D).

Further histological studies aimed at the search for human nuclear antigen. Four randomly selected animals treated with the higher dose of human NTF-SC were sacrificed for histological studies at the first time point, that is, 7 days after they were treated with 6-OHDA. The brains of the animals were serially sectioned as described. We found, using the NSA methods, a large cluster of cells around the injection site in each of these animals. In 3 out of the 4 specimens we found cells present along 620–1,920 μm from the site of transplantation in adjacent sections, indicating the beginning of cellular migration. In order to quantify the survival rate of the cells, we manually counted all the cells with normal morphology; hence smooth looking not segmented nuclei with a positive dye. This measurement revealed that only 0.34 ± 0.1% of the cells survived 1 week post-transplantation.

On the last day of the experiment, that is, 50 days post-treatment, we employed the NSA methods on 4 randomly selected animals from the PBS control group, the high MSC-treated group, and the NTF-SC-treated group. This examination revealed almost no cells. In fact, around the transplantation site we found only remnants of cells and signs of old bleeding. After sectioning other brains into 8 μm sections and using a more sensitive fluorescent-based stain, a small minority of our transplanted human cells after 50 days were observed. The staining was conducted on axial sections adjacent to those in which a positive Prussian blue dye was found, and all cells positive for human nuclear antigen were demonstrated along the migration path. However, using the fluorescence method, we could not find cells along the entire migration pathway indicated by the Prussian blue stain, indicating that iron deposits did not necessarily indicate living cells by the last day of the experiment (Fig. 5E–G). CD68 staining for the identification of macrophages in the route of the cells was negative (data not shown). In summary, migration of human cells within the lesioned rat striatum was noted by in vivo MRI and by histological analysis; a small minority of the cells was found to survive for at least 50 days.

Discussion

In this report, a robust induction protocol of adult human bone marrow-derived MSC into NTF-SC is described. Such cells produce and release a several NTFs including BDNF and GDNF. These cells conditioned media rescued 6-OHDA-treated neuroblastoma cells. In cyclosporinized rats with unilateral striatal 6-OHDA lesions, ipsilateral transplantation of human NTF-SC was beneficial and partially attenuated amphetamine-induced rotations and other abnormal behavior, striatal dopamine levels reduction, as well as the loss of TH-immunoreactive nerve terminal network. We also found that the NTF-SC migrated along the corpus callosum (CC) around the striatal 6-OHDA lesion into the anterior striatum, instead of migrating directly to the lesion area.

Stem cell-based therapy is a promising treatment for PD and other neurodegenerative diseases. A commonly investigated cell type in the context of neurodegenerative disorders is the adult-derived MSC. Such cells have demonstrated a beneficial effect in a variety of experimental animal models such as for PD [9] and stroke [45]. The positive effect of the cellular treatment was attributed to one of several possible mechanisms, for example, neural predisposition [31], trophic factors secretion [46], or immunomodulation [47]. Other works have shown the therapeutic potential of NTF in animal models of Parkinson's disease; however, NTF-based therapy is currently limited due to difficulties in their delivery [28,48].

In this article, we aimed to give MSCs an added value to the basal beneficial effect these cells posses. We intended to induce astrocytic differentiation (due to their NTF secretion property) in order to create a safe and efficient method to deliver NTF into the CNS. Indeed, several groups reported that MSC express partial astrocytic (or Schwann cells) phenotype [33,34,49]. Here we report that our novel protocol probably did not create a mature astrocyte, although it induced the expression of astrocytic markers, but importantly it met its primary goal by increasing the NTFs secretion. We found that the NTF-SC secrete significantly higher levels of NTFs compared to untreated MSCs. This protocol increased the levels of BDNF and GDNF 2.3- and 5.8-fold, respectively, as compared to control human MSC. This specific induction protocol resulted in significantly higher levels of NTFs secretion when compared to previously published protocols [50]. Immunocytochemical studies revealed that the induction process is robust, since almost all cells are positive for the tested NTF.

Cell-based delivery of NTFs may be potentially superior to direct infusion of NTFs since it does not require instruments of transplantation such as permanent catheters, rather than a single surgical procedure. Our proposed medium-based induction may also be superior to viral vector delivery for achieving genetic overexpression since it circumvents safety problems [51]. Moreover, we used xeno-free media for both the production of the MSC culture and for their induction procedure until transplantation, rendering our method more practical and acceptable for clinical use. The platelet-based growth medium did not alter the basic mesenchymal characteristics of the MSC in terms of CD markers and mesenchymal lineage differentiation (for more detail, see Supplementary Figs. 1 and 2; Supplementary materials are available online at http://www.liebertonline.com). We propose, therefore, that we have successfully produced a potentially safe and efficient cell base vehicle for delivering NTFs to the CNS.

Human neuroblastoma cells exposed to 6-OHDA are used as an in vitro model for PD due to similar cellular processes that occur in the degenerating dopaminergic neurons, such as oxidative stress and apoptosis [52,53]. The conditioned media of our MSC and NTF-SC protected the neuroblastoma cell line from a 6-OHDA-induced cell death. Although both MSC and NTF-SC demonstrated beneficial effects, NTF-SC-based treatment was more potent in protection from the 6-OHDA, specifically in higher insult concentrations. It is possible that in this in vitro model the basal levels of NTF secretions by untreated MSC are also protective, or that there are other protective soluble substances that we did not measure. The conditioned media used in this experiment consisted of a serum-free media placed on the cells 24 h postinduction. Therefore, it consisted of only the factors secreted by the cells, and not induction media. This strongly implies that the mechanism underlying the observed protection is the presence of secreted NTFs.

In order to perform in vivo testing for our assumption that NTF-SC-based treatment is beneficial in a PD animal model, we first calibrated the well-established 6-OHDA-induced hemiparkinsonian rat model by injecting the specific dose of 6-OHDA into 2 striatal locations. As opposed to injections into the medial forebrain bundle, we used a relatively mild and progressive model of PD, probably representing an early phase of PD in human subjects. The cellular transplantation treatment was given on the day of 6-OHDA injection, therefore aiming at neuroprotection rather than at regeneration of already-lost dopaminergic terminals. The animals were examined by 2 well-documented behavioral tests: amphetamine-induced rotations and spontaneous motor activity in an open field [54,55].

The effect of NTF-SC treatment in terms of amphetamine-induced rotations was comparable to that found by some researchers who utilized dopamine-producing cells in the same animal model, for example, Wernig et al. who transplanted differentiated iPS [6], and Cho et al. who used human-derived embryonic stem cells (ESCs) differentiated into dopaminergic neurons [56]. Our finding that NTF-SC transplantation reduces the amphetamine-induced rotations by 40% as compared to the control group indicates that our therapeutic strategy, of employing stem cells as inducers of neuroprotection, is efficient and comparable to dopaminergic cell replacement strategies. The NTF-SC treated group also demonstrated a positive trend in the open field test, whereas the MSC did not alter the hypoactive behavior compared to the PBS-treated group.

Following the 6-OHDA lesion, the striatal TH-immunoreactive fiber area of the MSC-treated group was larger than in the PBS-treated animals. However, only the NTF-SC-treated group demonstrated a statistically significant lesser destruction of DA nerve terminals attested by a markedly larger TH-positive area compared to the PBS-treated group. As stated, only the most anterior part of the striatum benefited from the NTF-SC treatment, at a distance from the transplantation site. It is interesting to note that in that area of the striatum, the damage was not as severe as in the posterior section. Hence, NTF-SC may prove more beneficial in a moderately injured tissue but not in a state of complete or near-complete loss of dopaminergic nerve terminals. We observed that the migratory path of the transplanted cells was through the CC to the anterior parts of the striatum and not directly from the transplantation site into the lesion. This might be an indication that the surviving cells affected the anterior, rather than the posterior striatum.

It is more likely that our cellular treatment protected the 6-OHDA-stressed dopaminergic terminals but we cannot yet totally rule out some reinnervating effect. On the one hand, the mechanism of the neuroprotective effect of NTFs seems straightforward, since the cells were transplanted on the day of the lesion. On the other hand, the best protective effect was found furthest from the transplantation site, where the cells probably arrived days after the damage was caused and terminals had begun to degenerate, therefore suggesting that there was a reinnervation process involved.

Notably, the spared dopaminergic fibers were associated with diminished depletion of dopamine levels in the striatae that indicate the functional recovery, and explaining the clinical improvement. Although MSC partially rescued dopamine levels, only the NTF-SC-treated group demonstrated a statistically significant lesser decrease of striatal dopamine levels that makes this treatment superior to the conventional noninduced MSC-based therapy.

An important issue in stem cell research is the survival of the transplanted cells in vivo. In this work we employed different methods over several time points in order to address this subject. Using in vivo MRI we were able to demonstrate the migration capacity of our cells, which was highly correlated to Fe histological staining. Our results correlate to a previous work conducted in our laboratory, indicating that rat-derived NTF-SC migrated toward a quinolinic acid-induced

striatal lesion [38]. In that study, the cells migrated along the internal capsule into the striatum. In the current study we observed a migratory route that bypassed the lesion site along the CC and led into the anterior striatum, a phenomenon that we believe was not previously observed. Such cellular migration proves 2 major points: first that the migrated cells survived in the CNS; and second that the cells moved toward a specific (yet unknown) signal traveling along a specific route.

In an attempt to quantify the number of surviving cells, we used a stereological method at 7 and 50 days after treatment. When applying this relatively insensitive but reproducible method, we found that the vast majority of the transplanted cells were rejected within a week post-transplantation, and that no cells were found in the treated striatum after 50 days, even though immunosuppressive therapy was given. A reduction in the number of cells to just 1% has been previously demonstrated [14]. This low surviving rate is probably due to immune rejection, and to some extent immediate cellular death due to shear forces when passing though a thin syringe into the tissue. When we used a more sensitive method, with a higher signal to noise ratio, the surviving cells were highlighted, as the tissue was sliced into thin 8 μm sections and a fluorescent dye was used. Moreover, the survival of the cells in the specific location in which they were found was supported by its high correlation to the MR images and the Prussian blue stain for iron particles in the specific specimens in which we used SPIOs-labeled cells. The absence of macrophages (CD68-expressing cells) along the migration route is additional evidence of the survival of the migrating transplanted cells. It is therefore implied that although only a minority of the cells survived throughout the experiment, they were sufficient to induce the described beneficial effect in the lesioned striatum. Another strong possibility is that the transplanted cells exerted their full beneficial efficiency soon after their placement into striatum and that there was no further need for their entire presence later on.

Further studies should aim at resolving 2 important issues. The first is whether autotransplantation would give rise to the superior survival of the transplanted cells, possibly achieved by transplanting rat-derived cells into the same animal model. The second issue is whether by examining the effect of the cells when transplanted later or further away from the lesion, NTF-SC treatment induces a reinnervating or only a neuroprotective effect on living stressed neurons.

Conclusions

In the current study, we described a novel protocol that induced human-derived MSC into NTF-SC, with a xeno-free production method. This induction gave rise to a pronounced increase in the production and secretion of various NTFs, mainly BDNF and GNDF, both known to be relevant to therapy in a variety of neurodegenerative diseases, such as PD. In a rat model for PD, we found that the efficacy of NTF-SC was superior to that of MSC in terms of behavioral, biochemical, and histological indices. We demonstrated that the surviving cells migrated toward the lesion, and had the most significant effect at the end of the migration trail. Since our novel technology is clinically compatible and safe, we suggest that in the future, cell therapy based on the transplantation of NTF-SC derived from autologous human MSC should become an important option in PD treatment.

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