Signaling of Glial Cell Line–Derived Neurotrophic Factor and Its Receptor GFRα1 Induce Nurr1 and Pitx3 to Promote Survival of Grafted Midbrain-Derived Neural Stem Cells in a Rat Model of Parkinson Disease

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INTRODUCTION

Parkinson disease (PD) is a neurodegenerative disorder characterized by a progressive degeneration and loss of the midbrain dopaminergic (mDA) neurons. Signs of PD typically occur when approximately 80% of the dopamine-producing cells are damaged (1). Replacing lost cells by grafting dopamine-expressing neurons has the potential to alleviate some of the deficits in affected patients. One promising source of renewable cells for transplantation in PD is neuronal progenitor cells. Ventral mesencephalic (VM) progenitors can be stimulated to proliferate, differentiate toward the DA phenotype, integrate into the host tissue after transplantation, and mediate behavioral improvements in a rat model of PD (2–4). The success of this treatment, however, greatly depends on the rapid and reliable production of desired quantities of mDA neurons with long-term survival. Thus, it is very important to elucidate the molecular mechanisms of neuronal progenitor cell differentiation into DA neurons. To date, the controlling mechanisms of this differentiation are incompletely understood, but several key molecular events have recently been reported to activate cascades of other signaling molecules and transcription factors that lead to the final differentiation of DA neurons (5–8). Moreover, recent studies also indicate that major transcriptional determinants regulating the development of mDA neurons have led to new hypotheses about the etiopathogenesis of PD (9).

A critical transcription factor involved in the induction and maintenance of midbrain DA neurons is the orphan nuclear receptor Nurr1. Nurr1 is crucial for expression of a set of genes involved in DA metabolism that includes tyrosine hydroxylase (Th), vesicular monoamine transporter (Vmat2), dopamine transporter (Dat), and aromatic L-amino acid decarboxylase (Aadc) (10, 11). In addition, the level of Nurr1 mRNA in peripheral blood lymphocytes of PD patients is significantly decreased (12). Thus, the expression of Nurr1 may be a reliable biomarker for PD and its progression. Moreover, recent evidence suggests that there may be cross-talk between Nurr1 and several other transcriptional factors in different development and maturation stages of mDA neurons (13). The bicoid-related homeodomain-containing transcription factor Pitx3 is expressed exclusively in mDA neurons of both the substantia nigra compacta (SNc) and the
ventral tegmental area (VTA) and is involved in development and/or maintenance of these neurons (8, 14, 15). Although the expression of Pitx3 is independent from the Nurr1-mediated transcriptional cascade, Nurr1 may be critical for the maintenance of Pitx3 in the late stages of embryonic mDA neuron development. Moreover, there is growing evidence suggesting that Nurr1 and Pitx3 are interconnected at a functional level. Pitx3 regulates the Nurr1 transcriptional complex by inducing the release of the SMRT/HDAC–mediated repression from Nurr1 (10).

Glia1 cell–line–derived neurotrophic factor (GDNF), a member of the transforming growth factor β family, was originally discovered because of its ability to promote the survival of ventral midbrain DA neurons (16). Glial cell line–derived neurotrophic factor is expressed throughout the central nervous system during development (17) and is an essential factor for the maintenance and survival of adult DA neurons (18). A major site of action of GDNF is the midbrain in which the growth factor is expressed at low levels under basal conditions (19). The major source of GDNF to the midbrain is the striatum, where GDNF is transported retrograde by DA neurons of the SNc and the VTA (20). This suggests that GDNF is a target-derived factor for dopamine neurons in the adult brain (21). Glial cell line–derived neurotrophic factor signals by specific binding to glycosphosphatidylinositol-anchored receptors termed GFRα1, in collaboration with signaling receptor subunits such as the RET tyrosine kinase or neural cell adhesion molecule transmembrane receptors. Glial cell line–derived neurotrophic factor is efficacious in the remediation of, or protection against, degeneration of the nigrostriatal dopamine pathway in models of PD (22). Furthermore, GDNF promotes differentiation and development of DA neurons in rodent animals and primates (5, 23). This effect might be associated with Nurr1, which involves in the expression of RET in midbrain DA neurons and in the brainstem (24), and with Pitx3 (23). Neurons isolated from the SNc and VTA of Nurr1−/− mice fail to express TH after treatment with GDNF, suggesting that these cells lack a functional GDNF receptor. Hence, the interaction between Nurr1 and GDNF may play an important role in the development of DA neurons (25). The exact mechanism of interaction between Nurr1 and GDNF and what role each plays in the differentiation of transplanted mNSCs into DA neurons and maintenance have remained unclear.

We have investigated the effect of GDNF on grafted mNSCs in a PD rat model. Results from the analysis of in vitro and in vivo experiments show that GDNF promotes the survival and differentiation of mNSCs grafts in the host striatum by enhancing the expression of Nurr1 and Pitx3 and restores the motor function in an animal model of PD.

**MATERIALS AND METHODS**

**Animals and Tissue Collection**

Male Sprague-Dawley rats (300–350 g) were housed 4 to 5 per cage with ad libitum access to food and water in a room 25°C under 12-hour light/dark conditions. Animal care and handling were carried out according to the guidelines of the Animal Care and Use Committee at Southeast University. All efforts were made to minimize animal suffering and reduce the number of animals used. Ventral mesencephalon was obtained from fetuses of the same inbred strain.

**Preparation of mNSCs Cultures**

Sprague-Dawley rat ED12 VM neural stem cells were prepared as previously described (26–28). In brief, VM tissue was isolated and mechanically triturated through a Nitex filter to a quasi-single-cell suspension. Viable cells were counted using the Trypan Blue exclusion method. Cell suspensions were plated in 24-well tissue culture plates at a density of $1.25 \times 10^5$ cells/cm² in 0.4 ml of expansion medium. The expansion medium consisted of Dulbecco Modified Eagle Medium/F12 (GIBCO, Beijing, China) containing 1% N2 (Invitrogen, Beijing, China), 1% B27 (Invitrogen), 20 ng/mL epidermal growth factor (R&D Systems, Shanghai, China), and 20 ng/mL basic fibroblast growth factor (R&D Systems). Cells were cultured in an incubator with 3% ± 2% CO₂ at 37°C. These culturing methods resulted in the formation of mNSC neurospheres.

After 3 days, the cultures were supplemented with fresh medium plus 20 ng/mL GDNF (R&D Systems) or with a combination of GDNF and GFRα1 small interfering RNA (siRNA; 40 nmol/L, Santa Cruz Biotechnology, Santa Cruz, CA) cells were treated with Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s guidelines. Untreated cultures served as controls. At day 7, the cell spheres were passaged using 700 μg/mL collagenase/dispace (Roche Applied Science, Indianapolis, IN) with agitation on an orbital mixer incubator (80 rpm, 20 minutes). Cells were again plated at a density of $1.25 \times 10^5$ cells/cm². These cultures were referred to as passage 1. For the differentiation assay, spheres were collected, resuspended in differentiation medium, and added 0.5% (vol/vol) fetal bovine serum (GIBCO), then plated onto poly-D-lysine and laminin-coated plates (10 μg/mL each) and left to differentiate for 6 days. All differentiation experiments were performed in quadruplicate. For passage 2 cultures, passage 1 spheres were split again after an additional 7 days and treated as described previously mentioned. After 7 or 13 days in vitro, differentiation and partial expansion cell cultures were fixed for 30 minutes in 4% paraformaldehyde (PFA) in 0.15 mol/L phosphate buffer, pH 7.4, and rinsed in phosphate-buffered saline (PBS) for immunohistochemistry (IHC).

To label dividing cells, selected cultures, expanded for 7 days in vitro in the presence of GDNF (20 ng/mL) or not (control), were exposed to 10 μmol/L bromodeoxyuridine (BrdU; Sigma, St Louis, MO) for 20 hours before fixation or transplantation.

**6-Hydroxydopamine Lesion**

Male Sprague-Dawley rats received a 4-μL injection of 6-hydroxydopamine (6-OHDA; 6 mg of free base/mL of 0.1% ascorbate in saline) into the left medial forebrain bundle, as previously described (29). At 3 weeks after lesion, apomorphine (0.25 mg/kg)-induced rotation was measured, and animals making greater than 7 rotations per minute during a 30-minute period (lesion >90%) were selected for grafting.
(28). Rotational scores were expressed as net contralateral round per minute.

Cell Transplantation and Behavioral Evaluation
At 5 weeks after the 6-OHDA lesion, the animals were randomly stratified into groups based on behavioral testing. Passage 1 spheres were expanded for 1 week and treated as described previously mentioned. Midbrain-derived neural stem cells spheres were collected, mechanically triturated into cell suspensions by gentle pipetting, and used for transplantation. Quantities of 5 μL of the suspensions (cell density, about 5 × 10^5 cells/mL) were stereotaxically injected into the left side of the striatum (A, 0.5 mm anterior to the bregma; L, 2.5 mm; V, 5 mm below the dura) of the hemiparkinsonian model rats. The injection rate was 1 μL/min, and the syringe was left in place for an additional 5 minutes. A total of 23 rats received sham grafts (5 μL of Dulbecco modified Eagle medium/F12), 23 rats received GDNF-treated mNSCs, 23 rats received GDNF plus GFRα1 siRNA-treated mNSCs, and an additional 23 rats received GDNF plus GFRα1 control siRNA-treated mNSCs. Behavioral testing was carried out 2 weeks before grafting and at 2, 4, 6, and 8 weeks after transplantation.

The animals were divided into 3 independent experiments. For IHC analysis (n = 8 per group), animals were perfused through the ascending aorta first with 150 to 200 mL of saline and then with 300 mL of 4% PFA in 0.1 mol/L PBS, pH 7.4. After overnight postfixation in PFA at 4°C, brains were transferred to PBS and stored at 4°C. For reverse transcription–polymerase chain reaction (RT-PCR; n = 3 per group) and Western blotting (n = 4 per group), the animals were decapitated, the brains removed, and striata were dissected out and weighed; they were then frozen in dry ice and stored at −86°C.

Reverse Transcription–Polymerase Chain Reaction
Total RNA was extracted and quantified as previously described (30). In brief, RNA was extracted from tissues (n = 3 per group) using Tri-reagent (Invitrogen) treated with RNase-free DNase and used for complementary DNA (cDNA) synthesis. Pitx3, Nurr1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control) cDNA fragments were amplified, using the following primers: Pitx3 (forward 5'-CCCCGTTCGCTTTCAACTCG-3', reverse 5'-CGAGGCGTAAGGGCAAGGACAC-3'), Nurr1 (forward 5'-CTGGCGTTTCCAAGAGTGC-3', reverse 5'-TGGACCTGTATGCTAAGCGTA-3'), TH (forward 5'-TGTCACGTCC-3', reverse 5'-GGGCGAGGGCGGTCTC TAAGT-3'), and GAPDH (forward 5'-ACCACAGTCCA TGCCATAC-3', reverse 5'-TCCACCACCTGGTCT GTA-3'). DNA was amplified by 1 cycle of denaturing at 94°C (5 minutes), 25 to 30 cycles of denaturing at 94°C (45 seconds), annealing at 60°C (45 seconds), elongation at 72°C (45 seconds), and 1 cycle of final elongation at 72°C (5 minutes). The amplified DNA fragments were separated on agarose gel and visualized with 0.5 μg/mL ethidium bromide, using FluorChem 8900 Imager (Alpha Innotech, Cell Biosciences, Santa Clara, CA).

Western Blot
Cells or striatal tissue (n = 4 per group) were extracted in ice-cold RIPA lysis buffer. Lysates were then centrifuged for 10 minutes at 14,000 × g at 4°C. Samples containing 80 μg of protein from each supernatant were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride, blocked for 2 hours in 5% nonfat milk, and incubated with polyclonal anti-Pitx3 (1:500; Chemicon, Billerica, MA), anti-Nurr1 (1:500; Santa Cruz Biotechnology), anti-TH (1:500; ABR, Novus Biologicals, Littleton, CO), anti-GFRα1 (1:500; Abcam, Cambridge, MA), or anti–β-actin (1:10000; BioVision, Mountain View, CA). Incubations took place overnight at 4°C. After washing 3 × 15 minutes in Tris-buffered saline-Triton 2% (TBST), samples were incubated with peroxidase-conjugated secondary antibody for 1 hour, then washed in TBST, and exposed to Kodak film using ECL chemiluminescence (Pierce, Thermo Fisher, Rockford, IL). The identified proteins were quantified by measuring optical densities of the bands. Data from each experimental band were normalized to β-actin before statistical analysis of variance (Student t-test).

IHC and Cell Counting
To characterize the stem cells during proliferation and their development and differentiation after grafting, IHC was performed on cultured cells and on brain sections containing the grafts. In brief, for cultured cells, the fixed cell cultures were blocked in 3% goat serum with 0.3% Triton X-100 and incubated with primary antibodies. Appropriately coupled secondary antibodies were used for single or double labeling. The extent of the 6-OHDA lesion in the substantia nigra was verified in midbrain sections by IHC. For the brain sections, at 4 or 8 weeks after transplantation, the animals (n = 8 per group) were deeply anesthetized and transcardially perfused with 4% PFA. Brains were removed, postfixed overnight, and dehydrated in 30% sucrose in 0.1 mol/L phosphate buffer. For fluorescent IHC, 30-μm free-floating sections were washed 3× in TBS. Sections were blocked with 3% normal goat serum (NGS) in TBST for 1 hour. Sections were incubated in primary antibodies made up in 3% NGS/TBST overnight, followed by 2-hour incubation in appropriate secondary antibodies, coupled to fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate, made up in 1% NGS/TBST (all from Santa Cruz Biotechnology). Specimens were examined with a confocal laser scanning microscope (LSM510; Carl Zeiss Microimaging, Thornwood, NY). The following antibodies from Santa Cruz Biotechnology and dilutions were used: polyclonal rabbit anti-rat caspase 3 (1:300), polyclonal rabbit anti-rat Bcl-2 (1:300), polyclonal rabbit anti-rat nestin (1:200), polyclonal rabbit anti-rat glial fibrillary acidic protein (GFAP; 1:500), polyclonal rabbit anti-rat microtubule-associated protein 2 (1:200), polyclonal rabbit anti-rat Nurr1 antibody (1:200), and polyclonal rabbit anti-rat tyrosine hydroxylase (TH) antibody (1:200). Monoclonal mouse anti-GFAP (1:500; Sigma-Aldrich), polyclonal rabbit anti-rat Pitx3 antibody (1:200; Chemicon), monoclonal mouse anti-rat TH (1:500; ABR), and monoclonal mouse anti-rat BrdU antibody (1:200; Calbiochem, San Diego, CA) were also used.
FIGURE 1. Proliferation of midbrain-derived neural stem cells (mNSCs) induced by glial cell line–derived neurotrophic factor (GDNF) in serum-free medium with epidermal growth factor and basic fibroblast growth factor 2. (A–C) Immunoassayed image of BrdU-positive, Nestin-positive mesencephalic cell spheres at 7 days in vitro (DIV). (D, E) Phase-contrast photomicrographs of representative spheres of mNSCs during GDNF expansion at 7 DIV. (F) GDNF increases cell sphere diameter. Data are reported as mean ± SEM (n = 5 experiments; 10–12 microscopic fields were randomly selected for counting in each experiment). *, p < 0.05 versus control group by unpaired 2-tailed Student t-test. Scale bars = (A–C) 100 μm; (D, E) 30 μm.
FIGURE 2. Effects of glial cell line–derived neurotrophic factor (GDNF) on apoptosis of midbrain-derived neural stem cells (mNSCs). (A–P) GDNF decreases the fluorescence intensity of caspase 3–positive cell spheres (A–F, O) and simultaneously enhances the fluorescence intensity of Bcl-2–positive cell spheres (G–L, P) at 7 days in vitro (DIV, n = 12 per group). Immunoblots for Bcl-2 and activated caspase 3 in mNSCs spheres treated or not treated with GDNF at 7 DIV (M). The corresponding bar graph represents the Bcl-2 and activated caspase 3 protein levels in 4 independent experiments, normalized against β-actin (N). Data are reported as mean ± SEM. *, p < 0.05; **, p < 0.01 versus control group by unpaired 2-tailed Student t-test. Scale bar = 100 μm.
used. No immunoreactivity and cross-reactivity was generated using different combinations of secondary antibodies, as determined by control experiments in which the primary antibody was omitted from the IHC procedure. For identification of signal colocalization within a cell, optical thickness was kept to a minimum, and orthogonal reconstructions were obtained. To access the number of positive cells in or around the graft, 1 series of the sections from each graft (n = 8) labeled by IHC for TH and Nurr1, Pitx3, or BrdU were sampled using confocal microscopy. Quantification of coexpressed positive cells was performed in every section in which a graft was identifiable as described (31). To determine the percentage of TH-positive/Pitx3-positive cells or Nurr1-positive cells within the culture slide, a minimum of 10 random selected fields in each slide were counted for positive cells, using a 20× objective lens and 3 to 4 independent experiments. Quantification was performed by an independent observer in a blinded manner.

To quantify fluorescence intensity, digital images were taken using fluorescence microscopy (×20 magnification) under identical camera exposure and compensation settings for control and experimental conditions, as previously described with modifications (32). Each image was analyzed using Adobe Photoshop CS software. Background fluorescence in the cell-free regions for each image was determined. The fluorescence intensity in each neurosphere was then measured and corrected for the background. A total of at least 30 individual randomly selected neurospheres were analyzed for each experimental condition. The overall level of fluorescence in the cell-free regions for each image was determined. The mean fluorescence intensity per neurosphere was calculated.

Statistical Analysis

All statistical tests were performed with SPSS statistical software (version 15). One- or two-way analysis of variance with Student-Newman-Keuls post hoc test or 2-tailed Student t-tests were used; p < 0.05 was considered significant, unless stated otherwise.

RESULTS

GDNF-Induced Neurogenesis From mNSCs In Vitro

After culture in serum-free medium supplemented with growth factors, stem cells from VM proliferated well, making floating cell spheres that were BrdU-Nestin-positive (Figs. 1A–C). The rate of proliferation was faster in GDNF-treated mNSCs, resulting in larger cell spheres versus those of untreated mNSCs (Figs. 1D–F; p < 0.05). To investigate whether the prosurvival effects were due to a decrease in cell death, the expression of activated caspase 3 and Bcl-2 was assayed. When compared with control, GDNF-treated mNSCs displayed a significant reduction in caspase 3-positive cell spheres (Figs. 2A–F; O, p < 0.05) and caspase 3 protein expression (Figs. 2M, N; p < 0.01). In addition, there was a substantial increase of anti-apoptosis Bcl-2-positive cell spheres (Figs. 2G–L, P; p < 0.05) and Bcl-2 protein expression level (Figs. 2M, N; p < 0.01).

Every 7 days, the cell spheres were mechanically dissociated almost to the single-cell level and replated with fresh medium, and cell proliferation was maintained for at least 4 months. Once the spheres stuck to the bottom of the culture plate, the cells started to differentiate (Figs. 3A–D). Exposure to GDNF was sufficient to induce expression of Pitx3, Nurr1, and GFRα1 in mNSCs (Figs. 3E, F). Accordingly, exposure to GDNF was used to screen clones for percentage of conversion to the TH phenotype. Glial cell line–derived neurotrophic factor treatment induced a significant increase in spheres immunoreactive to TH relative to total spheres versus control treatment (Fig. 3I). The ratio of TH-positive/Pitx3-positive and TH-positive/Nurr1-positive cells in cultures was highly increased by GDNF (Figs. 3J, K). Many of the cells in these cultures did not exhibit typical neuron morphology. Numerous clusters of GFAP-positive cells were seen (Fig. 3A), indicating that clonally expanded individual progenitor cells from the VM were capable of differentiation into more than 1 cell type. Expanded mNSCs without treatment with GDNF did not exhibit significant TH-positive cell conversion (Figs. 3I–K).

GFRα1 siRNA Impairs the Effect of GDNF on mNSCs In Vitro

Because GDNF upregulated the expression of Nurr1, Pitx3, and GFRα1 in the cultured mNSCs, we further investigated whether knocking down GFRα1 expression by specific siRNA treatment affected the expression of Nurr1 and Pitx3 in the cultured mNSCs after GDNF treatment. The results clearly showed that GFRα1 siRNA significantly reduced the expression level of Nurr1, Pitx3, and GFRα1 (Figs. 3G, H). More importantly, GFRα1 siRNA treatment could prevent GDNF-induced upregulation of the ratios of TH-positive/Pitx3-positive and TH-positive/Nurr1-positive cells (Figs. 3J, K). These results demonstrate that GDNF could improve the differentiation of incubated mNSCs into DA neuron by increasing expression of Nurr1 and Pitx3 via a GFRα1 signaling pathway.

GDNF Has Neurorestorative Effects in an Experimental PD Model

Next, we probed the neurorestorative effect of GDNF on grafted mNSCs. 6-Hydroxydopamine (24 μg in 4 μL) induced substantial degeneration of TH-positive cells in the SNC (Figs. 4B, C). Striatal injection of GDNF-treated mNSCs or GDNF plus GFRα1 control siRNA-treated mNSCs significantly reduced apomorphine-induced contralateral rotational behavior compared with the mock group with maximum effect measured at 8 weeks after lesion (Fig. 4A). However, the difference between GDNF-treated mNSCs and GDNF plus GFRα1 control siRNA-treated mNSCs groups was not statistically significant. Nevertheless, when the GFRα1 siRNA was delivered into mNSCs, there were differences between the GDNF- and GFRα1 siRNA-treated groups (++, p < 0.01) and between GFRα1 control siRNA- and GFRα1 siRNA-treated group (+, p < 0.05; ++, p < 0.01) (Fig. 4A).
FIGURE 3. Differentiation of rat midbrain-derived neural stem cells (mNSCs) in vitro. (A–D) Confocal micrograph at 6 days after whole sphere differentiation in vitro showing the emergence of glial fibrillary acidic protein (GFAP)-positive (green, A), Map-2-positive (green, B), Pitx3-positive/tyrosine hydroxylase (TH)-positive (C), and Nurr1-positive/TH-positive (D) cells. (E) Cells from rat mesencephalic progenitors were converted on poly-L-lysine for 6 days using glial cell line–derived neurotrophic factor (GDNF; 20 ng/mL), and whole-cell lysates were prepared and subjected to immunoblotting with anti-Pitx3, anti-Nurr1, anti-GFRα1, or anti–β-actin antibodies. (F) The corresponding bar graph represents the Nurr1, Pitx3, and GFRα1 protein levels in 3 or 4 independent experiments, normalized against β-actin. (G) mNSCs were treated with GFRα1 siRNA, GDNF, GDNF plus GFRα1 control siRNA, or GDNF plus GFRα1 siRNA. At 72 hours after GDNF (20 ng/mL) treatment, cells were incubated with GFRα1 siRNA for an additional 72 hours. Whole-cell lysates were prepared and subjected to immunoblotting with anti-Pitx3, anti-Nurr1, anti-GFRα1, or anti–β-actin antibodies. (H) The corresponding bar graph represents the Nurr1, Pitx3, and GFRα1 protein levels in 3 or 4 independent experiments, normalized against β-actin. (I) GDNF significantly augmented the proportion of TH-positive spheres out of total spheres versus control. (J, K) Ratio of TH-positive to Pitx3-positive or Nurr1-positive cells, respectively. Data are shown as mean ± SEM. *, p < 0.05; **, p < 0.01, one-way analysis of variance with Tukey post hoc test followed by unpaired 2-tailed Student t-test. Scale bar = 100 μm.
FIGURE 4. In vivo behavioral and histologic analyses after transplantation (n = 8). (A) Time course of apomorphine-induced rotational behavior in mock-, glial cell line–derived neurotrophic factor (GDNF) + midbrain-derived neural stem cells– (mNSCs), GDNF + mNSCs + GFRα1 control siRNA-, and GDNF + mNSCs + GFRα1 siRNA-grafted animals. Glial cell line–derived neurotrophic factor + mNSCs and GDNF + mNSCs + GFRα1 control siRNA transplants showed significant behavioral improvement; GDNF + mNSCs + GFRα1 siRNA transplants resulted in restoration of behavior. *Significant difference from baseline. +Significant difference from both GDNF and GFRα1 control siRNA groups. Transplantation was made at time 0. Abscissas, weeks; ordinates, turns per minute. Data are shown as mean ± SEM. * and +, p < 0.05; ++ and **, p < 0.01, analysis of variance with Tukey post hoc test. (B–L) B, normal group; C, 6-hydroxydopamine (6-OHDA) lesion (PD) group. Low-power photomicrographs of tyrosine hydroxylase (TH) immunostaining show degeneration of TH-positive cells in the substantia nigra and ventral tegmental area after GDNF-expanded mNSCs were injected into striatum. At 8 weeks after transplantation, BrdU-positive/Pitx3-positive (D–F), BrdU-positive/Nurr1-positive (G–I), and BrdU-positive/TH-positive (J–L), colabeled cells are still apparent in or around the needle tract. Scale bar = (B–L) 200 μm.
FIGURE 5. Development of rat midbrain-derived neural stem cells (mNSCs) grafts for 8 weeks after mNSCs transplantation into the 6-hydroxydopamine (6-OHDA)-lesioned striatum. (A) RT-PCR analyses show Nurr1, Pitx3, and tyrosine hydroxylase (TH) mRNA levels in the injury ipsilateral striatum of 6-OHDA rat at 8 weeks after transplantation. Lanes 1 to 4 represent treatment with mock, glial cell line–derived neurotrophic factor (GDNF) + mNSCs, GDNF + GFRα1 control siRNA plus mNSCs, and GDNF + GFRα1 siRNA + mNSCs, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to ensure equal complementary DNA loading. (B) The corresponding bar graph represents the Nurr1, Pitx3, and TH mRNA levels in 3 independent experiments, normalized to that of GAPDH and expressed relative to the respective control value. Data are shown as mean ± SEM. *, p < 0.05; **, p < 0.01. (C) Western blot shows Nurr1, Pitx3, and TH protein levels in the injury ipsilateral striatum of 6-OHDA rat at 8 weeks after transplantation. Lanes 1 to 4 represent treatment with mock, GDNF + mNSCs, GDNF + GFRα1 control siRNA + mNSCs, and GDNF + GFRα1 siRNA + mNSCs, respectively. The β-actin blot confirms equal loading of protein samples. (D) The corresponding bar graph represents the Nurr1, Pitx3, and TH protein levels in 3 or 4 independent experiments, normalized against β-actin. Data are shown as mean ± SEM. *, p < 0.05; **, p < 0.01. (E–J) Photomicrographs are representative of grafts at 8 weeks (E, G, I). Immunofluorescent staining, combined with confocal laser scanning, was used to determine colocalization of Nurr1 (red) and TH (green), Pitx3 (red) and TH (green), and BrdU (red) and TH (green), respectively. Quantification (F, H, J) revealed that the increased number of Nurr1-positive/TH-positive, Pitx3-positive/TH-positive, and BrdU-positive/TH-positive cells in the ipsilateral striatum at 4 and 8 weeks after GDNF plus mNSCs transplantation. This effect was significantly abolished by GFRα1 siRNA administration at 4 and 8 weeks after transplantation. (K) Double labeling for glial fibrillary acidic protein (GFAP) with Pitx3. (L) Double labeling for BrdU with microtubule-associated protein 2. Data in B, D, F, H, and J are shown as mean ± SEM (n = 8 at each time point in each group). *, p < 0.05; **, p < 0.01, 1-way analysis of variance followed by unpaired 2-tailed Student t-test. Scale bars = (E, G, I) 100 μm; (K, L) 20 μm.
GDNF Promotes the Expression of Nurr1, Pitx3, and TH in the Grafts and Ameliorates mNSC Survival and Differentiation After Transplantation

At 8 weeks after transplantation, Nurr1-positive/TH-positive, Pitx3-positive/TH-positive, and BrdU-positive/TH-positive grafts were visible as large clusters of cells along or in the area around the needle tract (Figs. 4D–L). Moreover, the apparent upregulation of the expression of Nurr1, Pitx3, and TH in the mNSCs grafts by GDNF treatment was verified using RT-PCR and Western blot (Figs. 5A, C). The mRNA or protein from cell lysates of mNSCs grafts exposed to medium-only, GDNF-only, and GDNF and GFRα1 siRNA or GFRα1 control siRNA mixture (Figs. 5A, C) were assessed for Nurr1, Pitx3, and TH. Both mRNA and protein levels of Nurr1, Pitx3, and TH were detectable in the cultures exposed to medium, increased in cultures incubated with GDNF or GDNF and GFRα1 control siRNA mixture. GFRα1 siRNA abolished the effect of GDNF on the expression of Nurr1, Pitx3, and TH in the mNSCs grafts (Figs. 5B, D; *, p < 0.05; **, p < 0.01).

Midbrain-derived neural stem cells were evaluated by histology at 4 or 8 weeks after transplantation. Immunofluorescence staining revealed that the grafted mNSCs cells survived in the DA-depleted striatum and were distributed diffusely in the area surrounding the site of transplantation (Figs. 4D–L, 5E, 6A, B, G, and I). The numbers of Nurr1-positive/TH-positive, Pitx3-positive/TH-positive, and BrdU-positive/TH-positive neurons were enhanced by GDNF and GDNF plus GFRα1 control siRNA-treated grafts in DA-depleted striatum (Figs. 5E–J). This effect was markedly impaired by GFRα1 siRNA treatment (Figs. 5F, H, J; *, p < 0.05; **, p < 0.01). Moreover, GFAP was not colocalized with Pitx3-positive cells in the grafts in DA-depleted striatum (Fig. 5K); thus, GDNF delivery might induce the differentiation of grafted newborn cells into mature neurons (Fig. 5L).

DISCUSSION

We have investigated the effect of GDNF on the expression of dopamine-related transcription factors Nurr1 and Pitx3 in mNSCs grafts. Our results show that GDNF improves the survival and differentiation of mNSCs into DA phenotype neurons in vitro and in vivo. The underlying mechanism might involve in increasing the expression of Nurr1 and Pitx3 in the mNSCs grafts. Animals that received GDNF- or GDNF plus GFRα1 control siRNA-treated mNSCs showed a significant reduction of apomorphine-induced rotational asymmetry beginning at 2 weeks after transplantation compared with pregrafting values. This observation is consistent with previous reports assessing the potential of transplantation of cultured VM donor tissue (33).

Our data showed that GDNF promotes the expression of Nurr1 and Pitx3 in both cultured and grafted mNSCs. In development, Nurr1 expression begins in midbrain DA neuron at approximately E10.5 and its expression subsequently extends to other CNS regions, including the cortex and hippocampus (6). Because Nurr1 can regulate several proteins that are required for dopamine synthesis and regulation, it is considered to be a key intrinsic factor involved in the specification and maturation of DA neurons. Our results suggest that both Nurr1 and the Nurr1-regulated factor TH are expressed in mNSCs grafts and that Nurr1 expression may be directly involved in GDNF-induced TH expression.

Induction of midbrain DA neuron maturation by Nurr1 is complemented by other transcription factors, including Pitx3. Pitx3-deficient mice display a progressive loss of DA neurons in the substantia nigra as early as E12 (34). Furthermore, Pitx3 expression begins concurrently with TH expression (35) and is specifically involved in the terminal differentiation and/or early maintenance of SNc (8, 36). Interestingly, VTA DA neurons are maintained in Pitx3-deficient mice until adulthood, suggesting that none of these transcription factors are individually sufficient to instruct the midbrain DA neuron phenotype. Moreover, the functional cooperative actions of Pitx3 and Nurr1 in vivo promote Nurr1-mediated transcription in midbrain DA neurons (10). Our results indicate that GDNF simultaneously increased the expression of Pitx3 and Nurr1 in both cultured and transplanted mNSCs, which indicates that Pitx3, by acting in concert with Nurr1, is also a crucial factor for the specification of the DA phenotype in the grafted mNSCs.

GFRα1 is the only receptor known to be absolutely required for the effects of GDNF in all systems studied to date; it is the only membrane-associated molecule known that is capable of binding GDNF with high affinity (16, 37). Thus, GFRα1 is likely to function as the indispensable GDNF-sensing component of several different receptor complexes. In most cases studied so far, transmembrane partner molecules have been shown to associate with glycoporphosphatidylinositol-anchored receptors for signal transduction. In our in vitro model, the enhanced expression levels of Nurr1, Pitx3, and GFRα1 and the increased ratio of TH-positive/Pitx3-positive and TH-positive/Nurr1-positive cells (Fig. 3) were abolished in cultured mNSCs by GFRα1 siRNA treatment. Glial cell line–derived neurotrophic factor promotes neuronal maturity and specifically increases the yield of cell type–specific functions of DA neurons when it was coexpressed with Nurr1 or Pitx3 from cultured mNSCs (Fig. 3). These findings suggest a requirement for GDNF signaling via GFRα1 for neuronal differentiation and DA development of mNSCs neurospheres. In addition, GDNF-induced survival effect may also be due to induction of Nurr1 and Pitx3 in the grafted mNSCs. It is well established that deletions of Nurr1 and Pitx3 negatively affect mDA neuron survival and maintenance (38, 39).

Poor survival of grafted DA neurons is one of the major obstacles to the success of clinical trials in PD (40). The effects of GDNF on cell survival that we have identified are potentially important to the field of neural transplantation in PD. Previous studies of DA neuron transplants in animal models of PD had been focused on the use of cells derived from the developing VM at a stage when most of the developing DA neurons are postmitotic (E12–13 in the mouse or E14–15 in the rat) (41). In fact, at this stage of development, DA neurons that survive after transplantation to the DA-depleted striatum originate from cells that have exited, or are about to exit, the cell cycle. A recent study indicated that the yield of DA neurons in grafts of E12 rat VM tissue was
5-fold larger than in standard E14 transplants (42). Thus, in our study, the cells obtained from E12 VM tissue may provide an even more efficient source of mDA neurons on grafting. Furthermore, consistent with our in vitro findings, 4 and 8 weeks after transplantation, we found that mNSCs treated with GDNF or in combination with GFRα1 control siRNA yielded more Nurr1-positive/TH-positive, Pitx3-positive/TH-positive, and BrdU-positive/TH-positive neurons than those treated with GDNF plus GFRα1 siRNA. In agreement with these results, we found that rotational behavior was ameliorated in 6-OHDA–lesioned rats grafted with mNSCs treated with GDNF or in combination with GFRα1 control siRNA and that this effect was impaired in rats grafted with mNSCs treated with GDNF plus GFRα1. These observations further enhance the likelihood that GDNF signaling can induce a DA phenotype, possibly through regulation of Nurr1 and Pitx3 signaling pathways in vivo. Our signaling can induce a DA phenotype, possibly through regulation of Nurr1 and Pitx3 signaling pathways in vivo. Our results also showed that drug-induced rotation behaviors had reached a maximum improvement at 8 weeks after grafting, whereas the survived cells in the graft were already decreasing. One possible explanation is that various growth factors secreted by astrocyte-derived from grafted-mNSCs could further improve drug-induced rotation behaviors.

In conclusion, we show that GDNF promotes the generation of midbrain DA neurons and improves the survival and differentiation of mNSCs grafts and functional recovery in the 6-OHDA–lesioned rat brain by simultaneously increasing the expression of Nurr1 and Pitx3. The synergistic action between Nurr1 and Pitx3 may enhance the efficacy of cell replacement therapies for PD.

REFERENCES
36. Maxwell SL, Ho HY, Kuehner E, et al. Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of...


Lee HS, Bae EJ, Yi SH, et al. Foxa2 and Nurr1 synergistically yield A9 nigral dopamine neurons exhibiting improved differentiation, function, and cell survival. Stem Cells 2010;28:501–12

