Original Paper

Cells Tissues Organs

Cells Tissues Organs 2008;188:373–383
DOI: 10.1159/000140680

Accepted after revision: December 13, 2007
Published online: June 19, 2008

Mesencephalic Human Neural Progenitor Cells Transplanted into the Adult Hemiparkinsonian Rat Striatum Lack Dopaminergic Differentiation but Improve Motor Behavior

Marine Hovakimyan[a] Stefan Jean-Pierre Haas[a] Oliver Schmitt[a] Bernd Gerber[b]
Andreas Wree[a] Christian Andressen[a]

[a]Institute of Anatomy and [b]Clinic for Gynecology and Obstetrics, Medical Faculty, University of Rostock, Rostock, Germany

Key Words
Neurodegeneration · Neuroregeneration · Cell therapy · Growth factors · 6-Hydroxydopamine

Abstract
The clinical outcome of cell replacement therapies depends upon the successful survival and differentiation of transplanted cells. Here, we transplanted human neural progenitor cells derived from the ventral mesencephalon of an 8-week-old embryo into the ipsilateral (right) striatum of unilateral 6-hydroxydopamine-lesioned adult rats. To assess the therapeutic potency of grafted cells, 2 independent behavioral tests were conducted 12 weeks after transplantation: in the rotation test, a mild behavioral improvement was detected, and in the cylinder test, transplanted animals overcame the lesion-induced right forepaw preference. To address this behavioral improvement to a dopaminergic differentiation capacity of transplanted cells in vivo, immunohistochemistry for tyrosine hydroxylase was performed, showing a total lack of immunoreactivity. However, we found a considerable number of transplanted human nuclei-positive cells preferentially differentiated into neurons. In addition, glial fibrillary acidic protein-expressing cells were also detected. Our results show that behavioral improvement does not necessarily correlate with a differentiation of transplanted precursors into dopaminergic neurons, indicating other factors to be involved in a partial functional recovery.

Abbreviations used in this paper

6-OHDA	6-hydroxydopamine
BSA	bovine serum albumin
CNS	central nervous system
DA	dopamine
DAB	diaminobenzidine
DAergic	dopaminergic
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
FGF-2	fibroblast growth factor-2
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HN	human nuclei
hNPCs	human neural progenitor cells
ir	immunoreactive
NeuN	neuronal nuclei
NF 200	neurofilament 200 kDa
NPCs	neural progenitor cells
PBS	phosphate-buffered saline
PD	Parkinson's disease
SEM	standard error of the mean
TH	tyrosine hydroxylase

M.H. and S.J.-P.H. contributed equally to this work.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1422–6405/08/1884–0373$24.50/0

Accessible online at:
www.karger.com/cto

Dr. Christian Andressen
Institute of Anatomy, University of Rostock
Gertrudenstrasse 9, DE–18055 Rostock (Germany)
Tel. +49 381 494 8406, Fax +49 381 494 8402
E-Mail christian.andressen@med.uni-rostock.de

Nevertheless, for the development of a clinically useful cell therapy, it is important to overcome obstacles, namely the poor dopaminergic differentiation of human neural progenitor cells after grafting.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a relatively selective loss of midbrain dopaminergic (DAergic) neurons, with subsequent reductions in striatal dopamine (DA) levels [Hirsch et al., 1988]. The decline of DA in the striatum is associated clinically with progressive bradykinesia, tremor, rigidity and postural instability [Tedroff et al., 1999]. The primary PD treatment strategy involves a pharmacological approach to supply the missing neurotransmitter DA. The precursor of DA, L-3,4-dioxyphenylalanine, is currently the most effective therapeutic drug for alleviating PD symptoms. Unfortunately, L-3,4-dioxyphenylalanine slowly becomes less effective after long-term treatment and shows undesirable side effects [Fahn et al., 2004].

Clinical trials with neural transplantation have demonstrated the efficacy of primary human fetal neural tissue grafts in PD [Lindvall and Björklund, 2004]. However, there are several problems related to the use of this technique, including poor availability of sufficient quantities in combination with poor survival of transplanted cells limiting the widespread clinical application of neural transplants [Freed et al., 2003; Olanow et al., 2003].

An alternative strategy is the use of neural progenitor cells (NPCs), enabling the expansion in vitro to obtain large numbers of cells for transplantation. Additionally, these cells can be standardized, screened and manipulated prior to transplantation. Progenitor cells are functionally immature, self-renewing cells which, in contrast to the stem cells from which they are derived, are lineage restricted [McKay, 1997]. NPCs possessing the capacity to differentiate into all major cell types of the mature central nervous system (CNS) have been isolated from the developing or adult CNS [Weiss et al., 1996; Luskin et al., 1997; Temple and Alvarez-Buylla, 1999]. They can be expanded in vitro in the presence of mitogenic factors to provide much larger numbers of cells available for transplantation [Reynolds et al., 1992]. NPC proliferation is mediated and influenced by many growth factors, especially epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) [Kilpatrick and Bartlett, 1995; Tropepe et al., 1999]. Aiming at a significant restoration of striatal DA levels, the ventral mesencephalic area of the brain is suggested as an important source of NPCs suitable for the cell replacement therapy of PD [Lindvall and Björklund, 2004]. A therapeutic outcome, however, is thought to be dependent on the viability of NPCs that are destined to become DAergic neurons. Unfortunately, only a small fraction (about 5–10%, or even less of the progenitors), that are destined to become DAergic neurons, survives the grafting procedure [Brundin et al., 2000b].

For rat midbrain NPCs, it has been shown that they can proliferate and differentiate in vitro into DAergic neurons and, moreover, that the transplantation of these cells leads to a recovery in a rat model of PD [Studer et al., 1998]. Presently, there are only few reports about transplantation of human NPCs (hNPCs) derived either from embryonic stem cells [Ben Hur et al., 2004] or from fetal brain [Sanchez-Pernaute et al., 2001; Wang et al., 2004; Christophersen et al., 2006] into adult lesioned animals. Noteworthy, the number of surviving cells and their differentiation into tyrosine hydroxylase (TH)-immunoreactive (ir) cells varies widely, ranging from lack [Wang et al., 2004] to up to 1,000 cells per graft [Sanchez-Pernaute et al., 2001], depending on the expansion protocol and transplantation procedure. In addition, also the environment may play a crucial role in survival, migration and differentiation of transplanted cells. Here, we transplanted hNPCs derived from the ventral mesencephalic area of an 8-week-old human embryo into a DA-depleted striatum of adult rats. These cells have previously been shown to differentiate into TH-ir neurons accompanied by a behavioral benefit when transplanted into the lesioned striatum of juvenile animals [Hovakimyan et al., 2006]. In contrast to juvenile recipients, in the present parallel study, hNPC differentiation into DAergic neurons was not found, supporting the view of the environment as an instructive cue for neuronal specification and differentiation. Interestingly, we observed an almost complete functional recovery in the absence of DAergic innervation in the cylinder test. In the apomorphine-induced rotation test, again a mild improvement in the transplantation group was observed, whereas the sham control animals showed a progressive significant impairment. Our results suggest factors derived from transplanted hNPCs to be responsible for differences between the 2 groups.

Materials and Methods

In vitro Analysis

Isolation and in vitro Expansion of Mesencephalon-Derived hNPCs

Isolation and propagation of hNPCs were done in accordance with the guidelines and approved by the local ethics committee and that of the University of Rostock. NPCs isolated from the ventral mesencephalon of an elective aborted human fetus at 8 weeks gestational age were expanded in culture as described elsewhere [Hovakimyan et al., 2006]. If not specified otherwise, all material used for cell culturing was obtained from Invitrogen. In brief, hNPCs from ventral midbrain were cultured as monolayers on poly-L-lysine [10 μg/ml/cm/laminin-1 (2 μg/ml/cm^2); Sigma] coated dishes or glass coverslips with 12 mm diameter (for immunocytochemistry) and incubated in a 95% air and 5% CO_2 humidified atmosphere at 37°C in a serum-free cultivation medium composed of Dulbecco's modified Eagle's medium (DMEM)/F12 medium mixture (1:1, high glucose), supplemented with transferrin (100 mg/ml), insulin (25 mg/ml), progesterone (20 nM), putrescine (62 mM) and sodium selenite (30 nM). Mitogenic stimulation was achieved by adding EGF (20 ng/ml; Sigma) and FGF-2 (20 ng/ml; Sigma). Half the growth medium was replenished every second day. Passaging was undertaken every 8th to 10th day before reaching confluence, due to cell proliferation and doubling of cell numbers per passage. Proliferating hNPCs were expanded as adherent cells on monolayers, in order to prevent differentiation of neuroepithelial cells as observed in neurosphere preparations, at least for 6 months up to 19 passages.

In vitro Predifferentiation for Transplantation

To allow organotypic neuroepithelial in vitro differentiation, neurospheres were generated by cultivation of cells in serum-free proliferation medium, containing 20 ng/ml human EGF and FGF-2, in bacteriological (nonadherent) dishes for 6 days [Campos et al., 2004]. After 6 days, spheres were collected and triturated to generate single-cell suspensions (100,000 cells/μl DMEM) for transplantation purposes.

In vitro Differentiation

DAergic capacity of hNPC-derived neurospheres was performed by replating spheres on laminin/poly-L-lysine-coated tissue glass coverslips (12 mm diameter) placed in culture dishes and a further incubation in DMEM/F12 supplemented with N2 (Sigma) for 24 h.

In vitro Cell Characterization

For in vitro analysis of neural progenitor phenotype or DAergic capacity of the above-mentioned cells cultured on glass coverslips, immunoflurescent stainings against nestin (progenitor phenotype) or TH (pacemaker enzyme of DA synthesis as indicator for DAergic differentiation) were performed. Cells were fixed in 3.7% paraformaldehyde for 1 h at room temperature and then rinsed 3 times in 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by a preincubation in PBS containing 3% bovine serum albumin (BSA), 0.05% Triton X-100 and 5% normal goat serum for 1 h. Primary antibodies (anti-nestin, 1:500, mouse monoclonal, Chemicon, or anti-TH, 1:500, mouse monoclonal, Sigma) were solved in PBS containing 1% BSA and 0.025% Triton X-100 and incubated overnight at 4°C. After 3 rinses in PBS, secondary antibodies (goat anti-mouse, CY3 conjugated, 1:500; Dianova) were incubated for 3 h at room temperature followed by 3 rinses in PBS. Finally, sections were mounted on glass slides and embedded in anti-fading fluorescence mounting medium (Vector Laboratories) and covered by coverslips.

In vivo Experiments

Animals

A total of 15 male Wistar rats weighing between 280 and 320 g at the beginning of the experiment were housed at 22 ± 2°C under a 12-hour light/dark cycle with free access to food and water. All animal-related procedures were conducted in accordance with NIH and local ethical guidelines and approved by the animal experimentation committee of the University of Rostock.

Generation of Hemiparkinsonian Rats

Rats (n = 15) were deeply anesthetized with pentobarbital-Na^+ (45 mg/kg intraperitoneal) and unilaterally lesioned by an injection of 6-hydroxydopamine (6-OHDA-HCl; Sigma) into the right medial forebrain bundle [26 μg/4 μl, solved in 0.9% saline containing 0.8 mg ascorbic acid (Merck), coordinates referring to IA: AP +6.7, ML –1.5, V +1.5] [Paxinos and Watson, 1998].

Intrastriatal Cell Transplantation

Twelve weeks after the lesion, intrastriatal stereotaxic transplantation was conducted on the successfully lesioned animals (n = 15) by applying the microtransplantation technique [Nikkhah et al., 1994]. Rats were randomly classified into 2 groups, transplanted with hNPCs (transplantation group) or vehicle (DMEM, sham control group). The transplantation group (5 animals) received about 100,000 viable hNPCs (1 μl suspension) into the right striatum using the coordinates with reference to bregma: A = +0.5, L = –3.5, V = +5.5 [Paxinos and Watson, 1998]. At the same time point, the sham control group (10 rats) received an injection of vehicle alone (1 μl DMEM) using the same coordinates as for the transplantation group. Cells from the same passage and cell preparation were transplanted both in juvenile [Hovakimyan et al., 2006] and adult animals. To obtain similar conditions with our previous parallel transplantation study using these cells [Hovakimyan et al., 2006], no immunosuppression was performed.

Behavioral Tests

Apomorphine-Induced Rotations

Ten weeks after the lesion, apomorphine-induced rotations (0.25 mg/kg, subcutaneous; Teclapharm) were monitored for 40 min using an automated rotometer system according to Ungerstedt and Arbuthnott [1970]. The time lapse of 10 weeks between lesioning and testing is necessary because it is widely accepted that the increase in DA receptors, that is, in consistence with the number of rotations, reaches its final plateau during this time [Schwarting and Huston, 1996]. All animals (n = 15) displayed more than 4 contralateral rotations/min, indicating a unilateral death of about 97% of the nigrostriatal DAergic neurons [Ungerstedt and Arbuthnott, 1970] and, therefore, were used for the transplantation studies. Four, eight and twelve weeks following transplantation, the apomorphine-induced rotation test was again conducted for both groups.

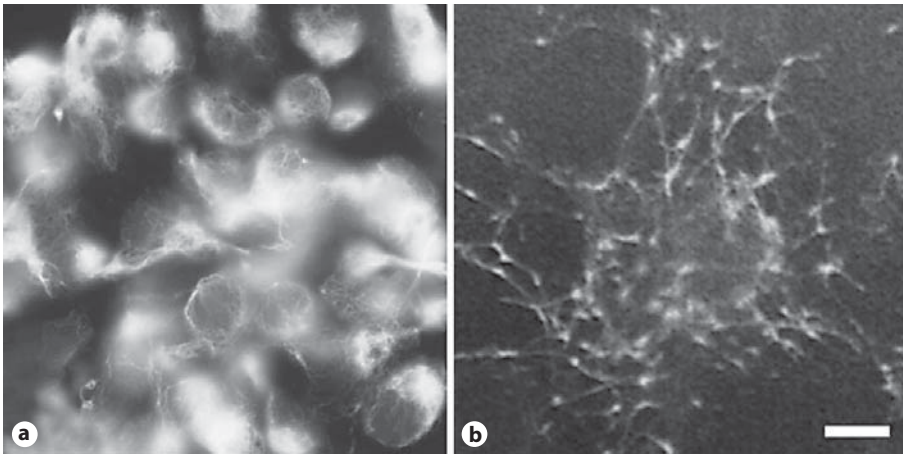

Fig. 1. In vitro morphology of hNPCs. Undifferentiated hNPCs possess a flat neuroepithelial morphology and are nestin-ir (**a**), whereas after differentiation as plated spheres (**b**) the cells show a neuronal morphology with elongated processes and a moderate number express TH. Scale bar: **a** 10 μm, **b** 100 μm.

Cylinder Test

This test was also conducted 10 weeks after the lesion and 12 weeks after transplantation for the same groups, as described previously [Kirik et al., 2000; Hovakimyan et al., 2006]. At these time points, each rat was individually placed in a transparent glass cylinder and videotaped. Twenty consequent wall contacts executed independently with the left or right forepaw were counted to determine the percentage of left forepaw use.

Tissue Processing and Analysis

Rats were injected with an overdose of pentobarbital (60 mg/kg) and transcardially perfused with ice-cold 0.9% sodium chloride (50 ml), followed by 400 ml of 3.7% paraformaldehyde. Brains were immediately removed from the skull, postfixed for 4 h, and transferred into PBS containing 20% sucrose (overnight, 4°C). The cryoprotected brains were frozen in isopentane (–50°C) and stored at –80°C until further processing.

Brains were cut with a cryostat at 30 μm and serial sections were collected free-floating in PBS. To identify and map the grafts' location, every seventh section was stained by the silver staining method as previously described by Gallyas et al. [1980]. For immunohistochemical analysis, sections were pretreated with 3% H_2O_2, blocked with 3% BSA, normal horse serum (1:67, polyclonal; Vector Laboratories) and 0.05% Triton X-100 for 1 h at room temperature, and then incubated with mouse anti-human nuclei (HN, 1:400, monoclonal; Chemicon), mouse anti-TH (1:1,000, monoclonal; Sigma) or mouse anti-OX-42 (1:1,000, monoclonal; Chemicon) primary antibodies overnight at 4°C. This was followed by incubation with biotinylated horse anti-mouse secondary antibody (1:200; Vector) overnight at 4°C. The secondary antibody step was followed with avidin-biotin peroxidase-conjugated complex (1:50; Vector) for 2 h at room temperature; 0.02% 3,3′-diaminobenzidine (DAB) was used as a chromogen for the visualization. Mounted sections were dehydrated in graded alcohols, followed by an incubation in xylol and finally embedded in DePeX mounting medium (Serva).

For immunofluorescent staining, blocking was performed with 3% BSA, 5% normal goat serum and 0.05% Triton X-100 (2 h). All sections were stained with HN antibody (anti-HN, 1:200) paired with rabbit anti-neurofilament 200 kDa (NF 200, 1:100; Sigma) or anti-glial fibrillary acidic protein (GFAP, 1:100; Sigma). For antigen visualization, secondary antibodies conjugated with anti-mouse CY3 (goat polyclonal, red fluorescence, 1:500; Dianova), anti-rabbit CY2 (donkey polyclonal, green fluorescence, 1:400; Dianova) or anti-rabbit AMCA (goat polyclonal, blue fluorescence, 1:100; Dianova) were used. The sections stained with anti-HN and anti-GFAP (detected with Cy3- and AMCA-conjugated antibodies) were further incubated with FITC-conjugated anti-NeuN (mouse-monoclonal, 1:200; Chemicon).

Statistical Analysis

Comparisons between groups were made using the nonparametric Mann-Whitney U test in SPSS 11.01 (SPSS Inc.). The minimum level of statistical significance was set at $p < 0.05$. All data are expressed as means ± SEM.

Results

Characteristics of hNPCs in vitro

After proliferation and expansion for 19 passages, hNPCs continuously contained the neural progenitor marker nestin (fig. 1a) and the intermediate filament vimentin as described elsewhere [Hovakimyan et al., 2006]. However, these cells were able to develop a considerable number of TH-ir neurons, when allowed to differentiate in vitro, indicating their DAergic differentiation capacity (fig. 1b), independently of the number of passages.

Behavioral Recovery of Hemiparkinsonian Rats

Apomorphine-induced rotations were counted as a marker for functional impairment (fig. 2a). In this test, all animals (n = 15) with a unilateral 6-OHDA lesion displayed a robust initial rotation response to apomorphine 10 weeks after lesioning, and there was no significant difference between the groups before grafting. The time lapse of 10 weeks between lesioning and testing was nec-

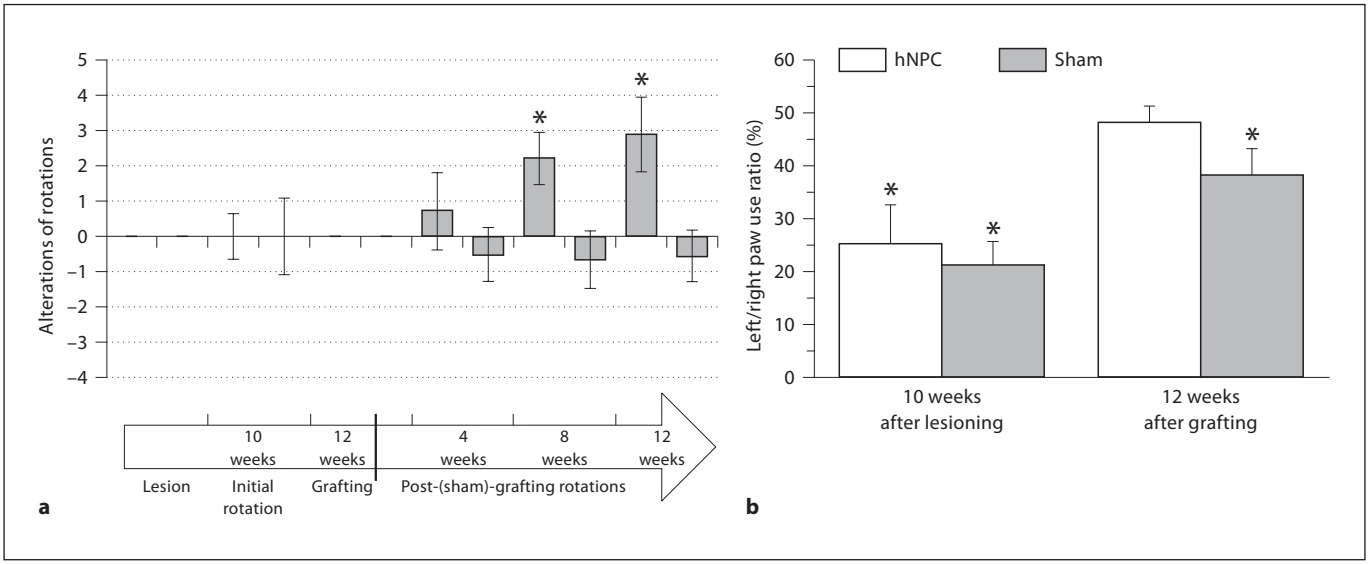

Fig. 2. a Motor asymmetry tests. Changes in apomorphine-induced rotation balanced to initial rotation values of the sham control (left) and the transplantation group (right) 10 weeks after lesioning. Sham control animals (n = 10) displayed a significant ($p \leq 0.05$) increase in rotations per minute (+2.88 ± 1.05). In the transplantation group, the rotation number decreased from 9.10 (after the lesion) to 8.55 rotations per minute (–0.55 ± 0.72). **b** The cylinder test showed a significant impairment in the left forepaw use 10 weeks after lesioning for the sham control group (21 ± 4.7, $p \leq 0.05$) and the transplantation group (25 ± 7.58, $p \leq 0.05$). Twelve weeks after grafting, the animals of the sham control group still revealed a significant left forepaw use preference (38% ± 5.28% left; $p \leq 0.05$), whereas those of the transplantation group showed a loss of forepaw preference (48% ± 3.39% left forepaw use without any significance; $p > 0.05$). *$p \leq 0.05$.

essary because it is widely accepted that the number of rotations reaches its final plateau by this time [Schwarting and Huston, 1996]. The rotation test was again performed 4, 8 and 12 weeks following transplantation. In the transplantation group, rats showed a mild behavioral recovery. At 12 weeks after transplantation, the average rotation score was decreased from 9.10 to 8.55. In contrast, in the sham control group, rotations in the direction contralateral to the lesion significantly increased from 7.72 to 10.6 ($p \leq 0.05$).

In the cylinder test, again a lesion became obvious by preferred right forepaw use (fig. 2b). For the sham control group 10 weeks after lesioning, the percentage of left forepaw use (left forepaw use ratio) was about 21%. Despite this ratio reaching about 38% at 12 weeks after transplantation, it remained statistically significant, indicating only an incomplete recovery. In the transplantation group, again a preferred left forepaw use by about 25% became obvious 10 weeks after lesioning ($p \leq 0.05$). However, 12 weeks after grafting, this group showed a forepaw use ratio of about 48% that is similar to our observations for healthy animals [Hovakimyan et al., 2006].

Assessment of DAergic Deafferentiation of the Striatum

The 6-OHDA lesions of the right nigrostriatal bundle produced, within 10 weeks, a nearly complete loss of DAergic neurons in the ipsilateral substantia nigra (fig. 3a). Accordingly, the lack of TH-ir in the ipsilateral right striatum was also obvious (fig. 3b), whereas in the contralateral (left) striatum, a rich innervation by afferent fibers from the contralateral intact substantia nigra remained visible in lesioned animals (fig. 3a, b). The TH-ir in the sham control group was similar as in those animals only lesioned (fig. 3c). This was also the case for the transplantation group (fig. 3d).

Cell Survival, Migration and Differentiation

The cell grafts could be easily identified in brain sections following HN immunostaining (fig. 4a, b). No marked differences were observed in graft morphology or size between animals. By HN-ir, most transplanted cells were confined to the striatum (fig. 4a, b), scattered around the needle track, whereas a minority showed migration into the surrounding parenchyma. Under higher magnification, cells with nuclei of different size and mor-

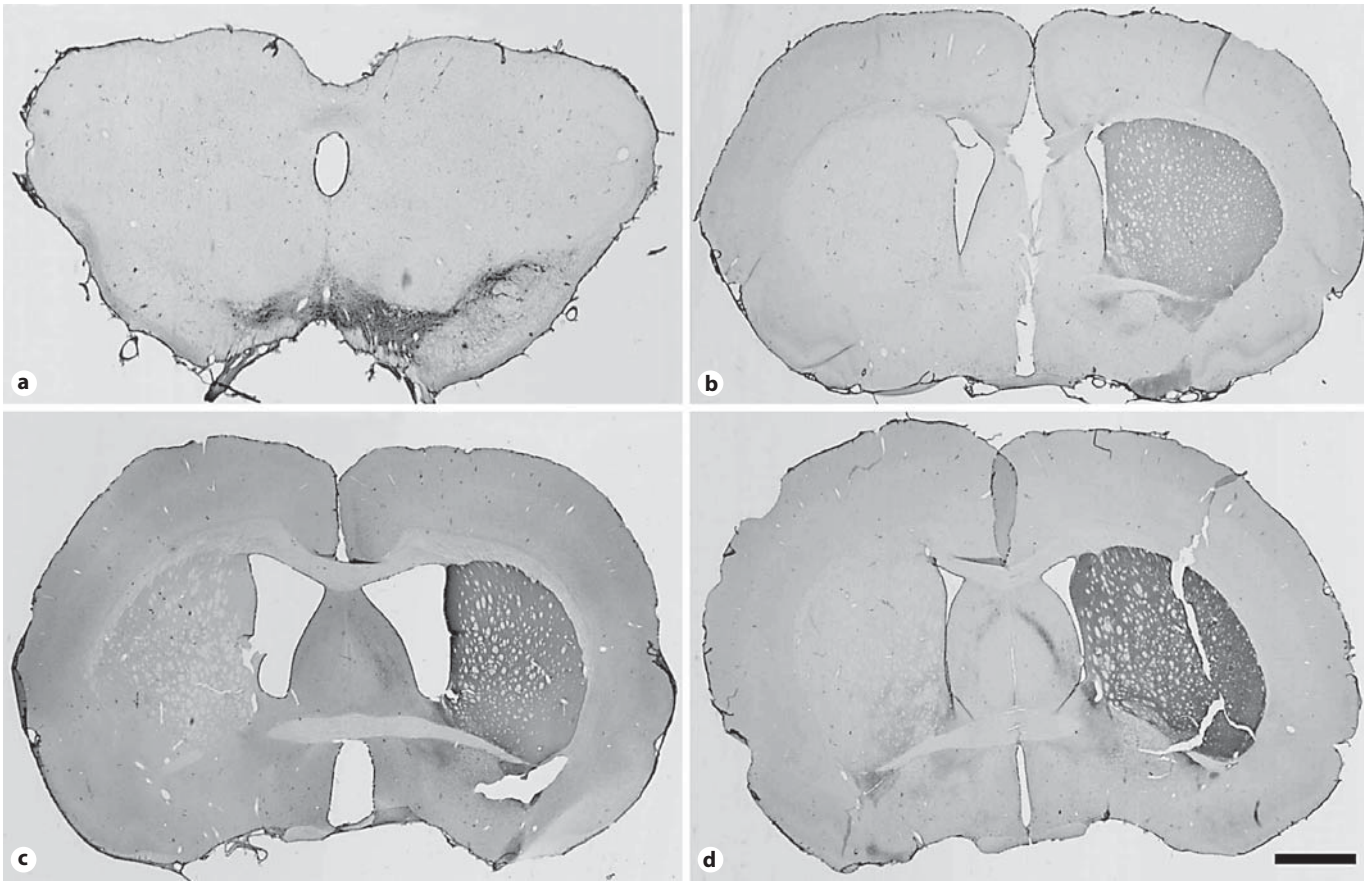

Fig. 3. TH-ir in frontal sections containing the region of the midbrain (**a**) or the striatum (**b–d**) of the various animal groups. Unilateral 6-OHDA lesioning leads to a nearly complete loss of DAergic neurons in the substantia nigra (**a**) and subsequently to a downregulation of TH-ir in the ipsilateral striatum (**b**) as shown 10 weeks after lesion. In contrast, the unaffected contralateral substantia nigra contains intensely stained DAergic neurons (**a**, right side) and a rich DAergic innervation of the striatum (**b**, right side). **c** Sham control animals show similar TH contents 12 weeks after sham transplantation, that is, 22 weeks after lesion. **d** Rats grafted with hNPCs also possess a comparably low TH-ir in the lesioned and subsequently transplanted hemisphere. Scale bar: **a** 4,500 μm, **b–d** 2,000 μm.

phology could be discerned (fig. 4b). In transplanted animals, we never observed tumor formation.

DAB-labeled HN-ir cells were counted in serial sections to obtain an estimation of cell survival. The mean number of absolute cell counts of transplanted cells per striatum was 4,376 ± 64.1, which was approximately 4.4% of the total number of initially transplanted viable cells.

Activation of microglia as a marker for inflammation was visualized by the morphology of OX-42-ir cells. Only moderate differences were found between the sham control and the transplantation group (fig. 4c, d). The majority of stained cells revealed a ramified morphology, typically for their resting state. Only around the stab wound, OX-42-ir was more pronounced, probably due to the mechanical injury.

Multipotent differentiation of grafted cells was confirmed by double immunofluorescent staining and confocal microscopy using HN (fig. 5a, d, g) paired with either NeuN (fig. 5b, e) or NF 200 (data not shown) or GFAP (fig. 5h). In accordance with the light microscopy observations, the immunofluorescence analysis again revealed a dense aggregate of HN-ir cells, located at the implantation site. Only a small number of cells migrated up to 600 μm into the host parenchyma (fig. 5b, c). Numerous neurons were identified by colabeling of HN (fig. 5d) with NeuN (fig. 5e, f). In addition to neuronal differentiation, a certain number of cells containing HN

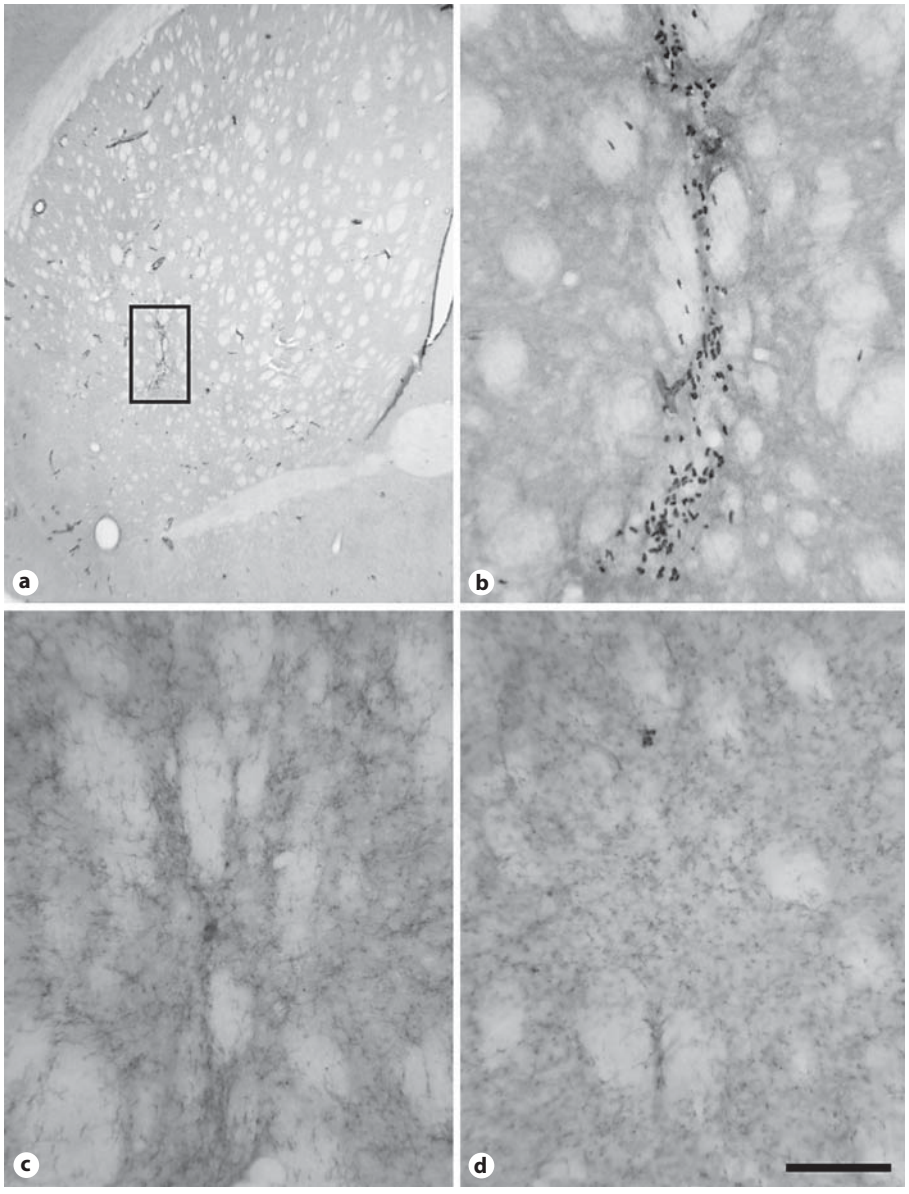

Fig. 4. Distribution of grafted cells stained for HN and host inflammation response revealed by OX-42-ir. **a** Overview of a frontal section through the striatum shows the position of HN-ir-grafted cells (inset) by DAB visualization. **b** By higher magnification of the inset, mainly 2 phenotypes of HN-ir cell nuclei can be observed, round or elongated. **c** Detection of OX-42-ir in transplanted animals reveals only a moderate amount of immunoreactivity in the region of the injection tract, containing only single activated microglial cells. **d** In sham-transplanted animals, a slight increase in OX-42-ir is also detectable in the region of the stab wound, compared to the surrounding areas. Scale bar: **a** 800 μm, **b–d** 100 μm.

(fig. 5g) was found to express the astroglial marker GFAP (fig. 5h, i). We observed that the number of grafted cells expressing neuronal markers (NeuN or NF 200) was comparable with those expressing the astroglial marker GFAP.

Discussion

It is generally accepted that the generation of large numbers of graftable neurons is an important prerequisite for a successful clinical treatment of PD. To fulfill this criterium, one possibility is the in vitro expansion of fetal hNPCs derived from the ventral midbrain. Recently, we have shown that such cells derived from human fetus can be expanded in culture for up to 6 months. When transplanted into the striatum of hemiparkinsonian juvenile rats, their differentiation into TH-ir neurons resulted in a significant motor behavior recovery [Hovakimyan et al., 2006]. Taking into account the view of the neonatal brain as a more permissive environment for neuronal differentiation [Schwarz and Freed, 1987], in this parallel experiment we were interested in the integration and differentiation capacity of these expanded hNPCs after

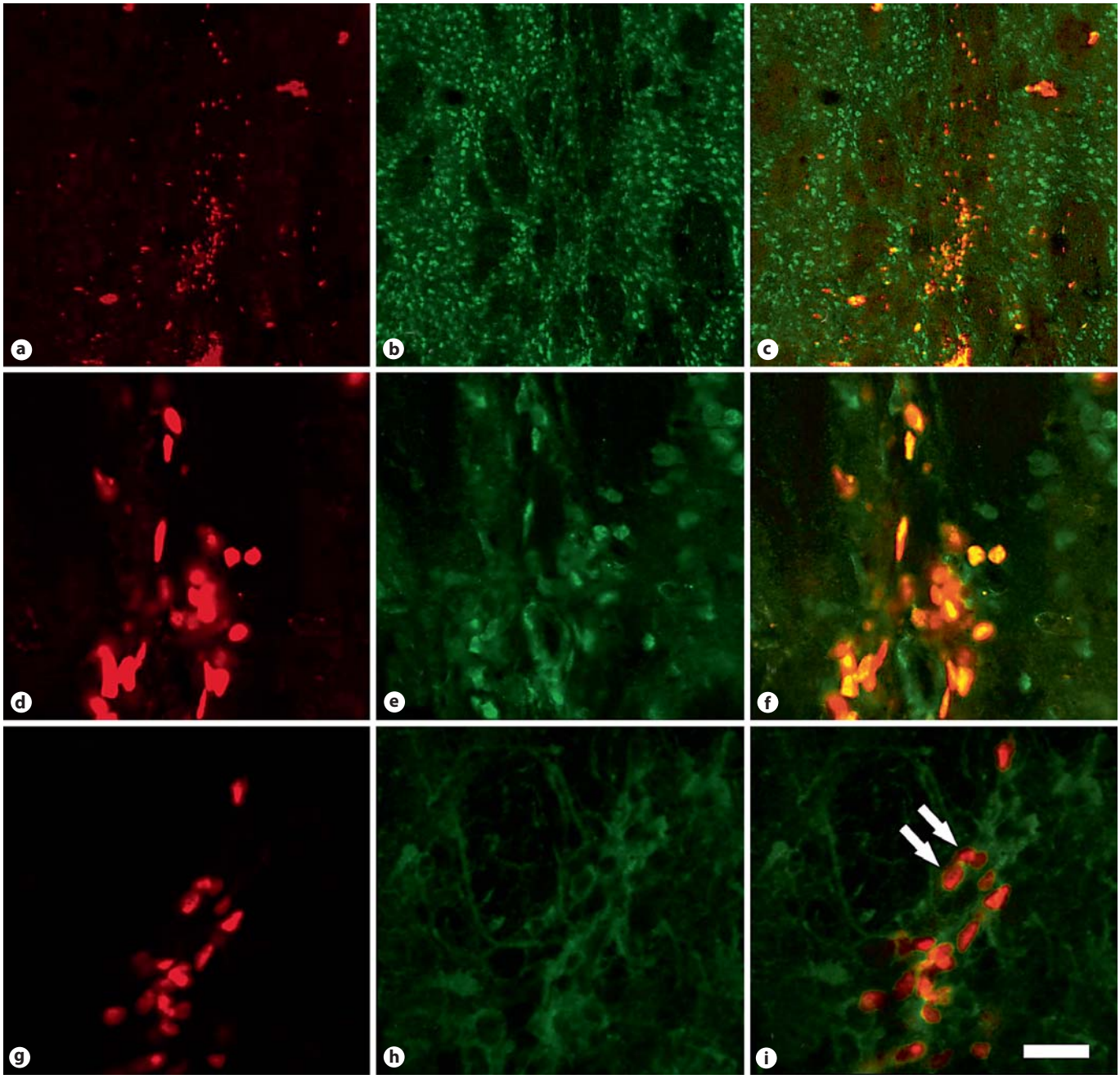

Fig. 5. Survival, migration and differentiation of transplanted hNPCs in the rat striatum. Multipotential differentation of transplanted hNPCs was evaluated by red fluorescent staining of HN (**a**, **d**, **g**) and green fluorescent labeling of NeuN (**b**, **e**) or GFAP (**h**), merged in **c**, **f** or **i** using confocal laser scanning microscopy. By high-power magnification, double-labeled neurons (**f**, yellow-orange fluorescence) or astrocytes (**i**, arrows) can be clearly discerned. Scale bar: **a–c** 125 μm, **d–i** 18 μm.

transplantation into the 6-OHDA-lesioned striatum of adult rats, focusing our attention on the DAergic differentiation and functional effects.

Fate of Transplanted Cells

Concerning differences between the characteristics of cells grafted into either neonatal or adult rat striatum, the most striking observation is the total absence of TH-ir cells in the striatum of adult animals, suggesting selective effects on the DAergic differentiation by the adult environment. This observation is in good agreement with a recent report on transplantation of expanded ventral mesencephalic hNPCs from a 12-week-old fetus into the hemiparkinsonian adult rat striatum [Wang et al., 2004]. In contrast, Svendsen et al. [1997] reported on the differentiation of hNPC-derived neurons that expressed TH at very low numbers and were sufficient to partially ameliorate lesion-induced behavioral deficits in only 2 animals. Interestingly, in this case the precursor cell population was from mixed brain regions. This might be of importance, since it is well known that DA-containing neurons are present in various areas of the CNS including the telencephalon [Bear et al., 2001]. Therefore, one can speculate whether hNPCs of nonmesencephalic, for example telencephalic, origin display a better DAergic differentiation capacity after intrastriatal transplantation.

The limited ability of prosencephalic or mesencephalic progenitors to differentiate into DAergic neurons in the mature host brain [Wang et al., 2004; Christophersen et al., 2006] highlights a general problem of transplanted NPCs to become committed to a certain neuronal lineage. Since our results show that like in juveniles, high numbers of neurons as well as GFAP-ir glial cells derived from transplanted cells were found in adult recipients, the knowledge about differences between the adult and juvenile striatum, selectively affecting certain lines of neuronal, especially DAergic differentiation, are truly of tremendous importance for the optimization of the cell replacement therapy for PD. However, unbiased stereology, concerning neuronal or glial differentiation, has not been performed due to technical limitations and, therefore, these observations have to be considered as preliminary.

Functional Effects

Despite the lack of TH-ir cells in striatum following transplantation, a therapeutic effect was observed in animals of the transplantation group when compared to the sham control group, indicating that additional factors have to be responsible for the observed functional recovery. This is in agreement with a recent study evaluating the effects of neurotrophic factors like glial cell line-derived neurotrophic factor (GDNF) both in humans [Gill et al., 2003] and nonhuman primates [Grondin et al., 2002]. In the latter study, a significant recovery in behavior is paralleled by only a small increase in mean striatal DA levels, suggesting a functional plasticity in the basal ganglia beyond a mere DA innervation [Brundin, 2002]. In this context, it is noteworthy that in our study, a considerable portion of cells underwent differentiation in the astroglial direction, as shown by double immunofluorescence for HN and astroglial marker GFAP. It seems likely that these cells might express GDNF or other neurotrophic factors that have been shown to be involved in a functional recovery in animal models of PD [Yoshimoto et al., 1995; Gash et al., 1996; Chaturvedi et al., 2006; McCoy et al., 2006]. This speculation is based on the evidence that NPCs can express GDNF and other neurotrophic factors not only in vitro, but also after grafting into adult CNS [Lu et al., 2003; Gao et al., 2006]. Alternatively, grafted cells could give rise to another type of neurons, e.g. γ-aminobutyric acid expressing, which could lead to perturbations in other neurotransmitter systems, contributing partially to the behavioral recovery [Winkler et al., 1999]. The fact remains that unknown factors are responsible for the improvement in motor behavior. Further investigations are required to determine the nature of these factors.

Regarding our results, it is notable that the release of neuroprotective factors by activated microglia [Nagatsu and Sawada, 2006] has to be ruled out because both in the transplanted as well as in the sham-operated animals the vast majority of OX-42-ir cells exhibited the typical ramified resting stage morphology [Kreutzberg, 1996]. Only a small number of partially activated cells with a rod-like shape were detected, preferentially located around the needle track, probably caused by mechanical injury. Consequently, observed behavioral differences between the 2 groups have to be referred to the grafted hNPCs.

Concluding Remarks

In conclusion, our results show that, despite immunosuppression, considerable numbers of neurons and glial cells derived from transplanted hNPCs were found for up to 12 weeks after transplantation. However, in contrast to juveniles, hNPCs grafted in adult animals did not give rise to TH-ir neurons. Despite their lack in the transplants, we observed a mild therapeutic effect in the apomorphine-induced rotation test. A more obvious effect

was detected in the cylinder test. Since both NeuN-ir neuronal and GFAP-ir glial differentiation of transplanted cells was detected in similar amounts in juvenile as well as adult recipients, we suggest local factors of the environment of adult rats, affecting selectively certain lines of neuronal, especially a DAergic differentiation. Therefore, in accordance with Brundin et al. [2000a] and Grondin et al. [2002], we propose that not only a reconstitution of a DAergic innervation, but additional yet unknown factors should be taken into account for the development of cell replacement therapies for PD.

Acknowledgements

We acknowledge Dr. Perrine Barraud and Dr. Deniz Kirik (Wallenberg Neuroscience Center, Lund, Sweden) for sharing their knowledge about microtransplantation surgery and animal behavioral testing. Marine Hovakimyan was financially supported by the German Academic Exchange Service (DAAD).

References

Bear, M., B. Connors, M. Paradiso (2001) Neuroscience: Exploring the Brain. Baltimore, Lipincott, Williams & Wilkins.

Ben Hur, T., M. Idelson, H. Khaner, M. Pera, E. Reinhartz, A. Itzik, B.E. Reubinoff (2004) Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. Stem Cells *22:* 1246–1255.

Brundin, P (2002) GDNF treatment in Parkinson's disease: time for controlled clinical trials? Brain *125:* 2149–2151.

Brundin, P., J. Karlsson, M. Emgard, G.S. Schierle, O. Hansson, A. Petersen, R.F. Castilho (2000a) Improving the survival of grafted dopaminergic neurons: a review over current approaches. Cell Transplant *9:* 179–195.

Brundin, P., O. Pogarell, P. Hagell, P. Piccini, H. Widner, A. Schrag, A. Kupsch, L. Crabb, P. Odin, B. Gustavii, A. Björklund, D.J. Brooks, C.D. Marsden, W.H. Oertel, N.P. Quinn, S. Rehncrona, O. Lindvall (2000b) Bilateral caudate and putamen grafts of embryonic mesencephalic tissue treated with lazaroids in Parkinson's disease. Brain *123:* 1380–1390.

Campos, L.S., D.P. Leone, J.B. Relvas, C. Brakebusch, R. Fassler, U. Suter, C. ffrench-Constant (2004) β1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance. Development *131:* 3433–3444.

Chaturvedi, R.K., S. Shukla, K. Seth, A.K. Agrawal (2006) Nerve growth factor increases survival of dopaminergic graft, rescue nigral dopaminergic neurons and restores functional deficits in rat model of Parkinson's disease. Neurosci Lett *398:* 44–49.

Christophersen, N., X. Meijer, J.R. Jorgensen, U. Englund, M. Gronborg, A. Seiger, P. Brundin, L.U. Wahlberg (2006) Induction of dopaminergic neurons from growth factor expanded neural stem/progenitor cell cultures derived from human first trimester forebrain. Brain Res Bull *70:* 457–466.

Fahn, S., D. Oakes, I. Shoulson, K. Kieburtz, A. Rudolph, A. Lang, C.W. Olanow, C. Tanner, K. Marek (2004) Levodopa and the progression of Parkinson's disease. N Engl J Med *351:* 2498–2508.

Freed, C.R., M.A. Leehey, M. Zawada, K. Bjugstad, L. Thompson, R.E. Breeze (2003) Do patients with Parkinson's disease benefit from embryonic dopamine cell transplantation? J Neurol *250:* 44–46.

Gallyas, F., J.R. Wolff, H. Bottcher, L. Zaborszky (1980) A reliable and sensitive method to localize terminal degeneration and lysosomes in the central nervous system. Stain Technol *55:* 299–306.

Gao, J., D.S. Prough, D.J. McAdoo, J.J. Grady, M.O. Parsley, L. Ma, Y.I. Tarensenko, P. Wu (2006) Transplantation of primed human fetal neural stem cells improves cognitive function in rats after traumatic brain injury. Exp Neurol *201:* 281–292.

Gash, D.M., Z. Zhang, A. Ovadia, W.A. Cass, A. Yi, L. Simmerman, D. Russell, D. Martin, P. A. Lapchak, F. Collins, B.J. Hoffer, G.A. Gerhardt (1996) Functional recovery in parkinsonian monkeys treated with GDNF. Nature *380:* 252–255.

Gill, S.S., N.K. Patel, G.R. Hotton, K. O'Sullivan, R. McCarter, M. Bunnage, D.J. Brooks, C.N. Svendsen, P. Heywood (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med *9:* 589–595.

Grondin, R., Z. Zhang, A. Yi, W.A. Cass, N. Maswood, A.H. Andersen, D.D. Elsberry, M.C. Klein, G.A. Gerhardt, D.M. Gash (2002) Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys. Brain *125:* 2191–2201.

Hirsch, E., A.M. Graybiel, Y.A. Agid (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. Nature *334:* 345–348.

Hovakimyan, M., S.J.P. Haas, O. Schmitt, B. Gerber, A. Wree, C. Andressen (2006) Mesencephalic human neural progenitor cells transplanted into the neonatal hemiparkinsonian rat striatum differentiate into neurons and improve motor behaviour. J Anat *209:* 721–732.

Kilpatrick, T.J., P.F. Bartlett (1995) Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. J Neurosci *15:* 3653–3661.

Kirik, D., C. Rosenblad, A. Björklund, R.J. Mandel (2000) Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J Neurosci *20:* 4686–4700.

Kreutzberg, G.W. (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci *19:* 312–318.

Lindvall, O., A. Björklund (2004) Cell therapy in Parkinson's disease. NeuroRx *1:* 382–393.

Lu, P., L.L. Jones , E.Y. Snyder, M.H. Tuszynski (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol *181:* 115–129.

Luskin, M.B., T. Zigova, B.J. Soteres, R.R. Stewart (1997) Neuronal progenitor cells derived from the anterior subventricular zone of the neonatal rat forebrain continue to proliferate in vitro and express a neuronal phenotype. Mol Cell Neurosci *8:* 351–366.

McCoy, M.K., T.N. Martinez, K.A. Ruhn, D.E. Szymkowski, C.G. Smith, B.R. Botterman, K.E. Tansey, M.G. Tansey (2006) Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. J Neurosci *26:* 9365–9375.

McKay, R. (1997) Stem cells in the central nervous system. Science *276:* 66–71.

Nagatsu, T., M. Sawada (2006) Cellular and molecular mechanisms of Parkinson's disease: neurotoxins, causative genes, and inflammatory cytokines. Cell Mol Neurobiol *26:* 781–802.

Nikkhah, G., M. Olsson, J. Eberhard, C. Bentlage, M.G. Cunningham, A. Björklund (1994) Microtransplantation approach for cell suspension grafting in the rat Parkinson model: a detailed account of the methodology. Neuroscience *63:* 57–72.

Olanow, C.W., C.G. Goetz, J.H. Kordower, A.J. Stoessl, V. Sossi, M.F. Brin, K.M. Shannon, G.M. Nauert, D.P. Perl, J. Godbold, T.B. Freeman (2003) A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann Neurol *54:* 403–414.

Paxinos, G., C. Watson (1998) The Rat Brain in Stereotaxic Coordinates. London, Academic Press.

Reynolds, B.A., W. Tetzlaff, S. Weiss (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci *12:* 4565–4574.

Sanchez-Pernaute, R.L., Studer, K.S. Bankiewicz, E.O. Major, R.D. McKay (2001) In vitro generation and transplantation of precursor-derived human dopamine neurons. J Neurosci Res *65:* 284–288.

Schwarting, R.K.W., J.P. Huston (1996) The unilateral 6-hydroxydopamine lesion model in behavioural brain research: analysis of functional deficits, recovery and treatments. Prog Neurobiol *50:* 275–331.

Schwarz, S.S., W.J. Freed (1987) Brain tissue transplantation in neonatal rats prevents a lesion-induced syndrome of adipsia, aphagia and akinesia. Exp Brain Res *65:* 449–454.

Studer, L., V. Tabar, R.D. McKay (1998) Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. Nat Neurosci *1:* 290–295.

Svendsen, C.N., M.A. Caldwell, J. Shen, M.G. ter Borg, A.E. Rosser, P. Tyers, S. Karmiol, S.B. Dunnett (1997) Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. Exp Neurol *148:* 135–146.

Tedroff, J., A. Ekesbo, E. Rydin, B. Langstrom, G. Hagberg (1999) Regulation of dopaminergic activity in early Parkinson's disease. Ann Neurol *46:* 359–365.

Temple, S., A. Alvarez-Buylla (1999) Stem cells in the adult mammalian central nervous system. Curr Opin Neurobiol *9:* 135–141.

Tropepe, V., M. Sibilia, B.G. Ciruna, J. Rossant, E.F. Wagner, D. van der Kooy (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol *208:* 166–188.

Ungerstedt, U., G.W. Arbuthnott (1970) Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. Brain Res *24:* 485–493.

Wang, X., Y. Lu, H. Zhang, K. Wang, Q. He, Y. Wang, X. Liu, L. Li, X. Wang (2004) Distinct efficacy of pre-differentiated versus intact fetal mesencephalon-derived human neural progenitor cells in alleviating rat model of Parkinson's disease. Int J Dev Neurosci *22:* 175–183.

Weiss, S., B.A. Reynolds, A.L. Vescovi, C. Morshead, C.G. Craig, D. van der Kooy (1996) Is there a neural stem cell in the mammalian forebrain?. Trends Neurosci *19:* 387–393.

Winkler, C., C. Bentlage, G. Nikkhah, M. Samii, A. Björklund (1999) Intranigral transplants of GABA-rich striatal tissue induce behavioral recovery in the rat Parkinson model and promote the effects obtained by intrastriatal dopaminergic transplants. Exp Neurol *155:* 165–186.

Yoshimoto, Y., Q. Lin, T.J. Collier, D.M. Frim, X.O. Breakefield, M.C. Bohn (1995) Astrocytes retrovirally transduced with BDNF elicit behavioral improvement in a rat model of Parkinson's disease. Brain Res *691:* 25–36.