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Mesencephalic human neural progenitor cells transplanted into the neonatal hemiparkinsonian rat striatum differentiate into neurons and improve motor behaviour

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Abstract

Neural stem cell transplantation is a promising strategy for the treatment of neurodegenerative diseases. To evaluate the differentiation potential of human neural progenitor cells (hNPCs) as a prerequisite for clinical trials, we intracerebrally transplanted *in vitro* expanded fetal mesencephalic hNPCs into hemiparkinsonian rats. On postnatal day one (P1), 17 animals underwent a unilateral intraventricular 6-hydroxydopamine injection into the right lateral ventricle. At P3, animals (n = 10) received about 100 000 hNPCs (1 μL) in the right striatum. Five weeks after birth, animals underwent behaviour tests prior to fixation, followed by immunohistochemistry on brain slices for human nuclei, glial fibrillary acidic protein, S100β, neuronal nuclei antigen, neuron-specific enolase and tyrosine hydroxylase. Compared with the apomorphine-induced rotations in the lesioned-only group (7.4 ± 0.5 min^{-1}), lesioned and successfully transplanted animals (0.3 ± 0.1 min^{-1}) showed a significant therapeutic improvement. Additionally, in the cylinder test, the lesioned-only animals preferred to use the ipsilateral forepaw. Conversely, the lesioned and transplanted animals showed no significant side bias similar to untreated control animals. Transplanted human nuclei-immunoreactive cells were found to survive and migrate up to 2000 μm into the host parenchyma, many containing the pan-neuronal markers neuronal nuclei antigen and neuron-specific enolase. In the striatum, tyrosine hydroxylase-immunoreactive somata were also found, indicating a dopaminergic differentiation capacity of transplanted hNPCs *in vivo*. However, the relative number of tyrosine hydroxylase-immunoreactive neurons *in vivo* seemed to be lower than in corresponding *in vitro* differentiation. To minimize donor tissue necessary for transplantation, further investigations will aim to enhance dopaminergic differentiation of transplanted cells *in vivo*.

Key words cell therapy; growth factors; neural stem cell transplantation; Parkinson's disease; substantia nigra.

Introduction

Parkinson's disease (PD) is a slowly progressive degenerative disorder of the central nervous system (CNS), caused by the chronic loss of dopaminergic (DAergic) neurons in the substantia nigra and subsequently depletion of dopamine in the striatum, the main projection area of the substantia nigra (Bernheimer et al. 1973; Hirsch et al. 1988). As the loss of striatal dopamine causes PD symptoms, most drugs used to treat PD are aimed at the replenishing of dopamine. DAergic drugs are generally effective at first in reducing many PD symptoms, but lose their effect over time and have severe side-effects (Clarke & Guttman, 2002).

Over the past two decades, cell replacement therapy has become a promising approach for the treatment of neurological diseases, including PD (Björklund & Lindvall, 2000). Clinical investigations and studies on animal models have shown that cell grafts can functionally integrate and restore dopamine release (Freed et al. 2001; Hagell & Brundin, 2001; Burnstein et al. 2004).

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The current primary treatment strategies focused on cells with potential to differentiate into DAergic neurons (Roybon et al. 2004).

Stem cells are immature, unspecialized cells that have the ability to renew themselves indefinitely, and, under appropriate conditions, can give rise to a wide range of mature cell types. Multipotent neural stem cells can be derived from the subventricular zone or germinal matrix of the developing fetal brain and develop into neurons, astrocytes and oligodendrocytes (Snyder et al. 1992). Neural progenitor cells (NPCs) have been demonstrated to be a population with self-renewal and multidifferentiation properties (McKay, 1997). Under certain conditions, these cells can be controlled to differentiate into specific neuronal phenotypes, such as DAergic neurons (Studer et al. 1998). Relatively little is known about human progenitor cells, because most studies have used progenitor cells obtained from rodents (Li et al. 2003; Sun et al. 2003). Human neural progenitor cells (hNPCs) can be expanded in large numbers for significant periods of time to provide a reliable source of neural cells for transplantation in neurodegenerative disorders such as PD.

A few studies have reported therapeutic effects of transplantation of NPCs from human sources in animal models (Ben-Hur et al. 2004; Yang et al. 2004). Mesencephalic NPCs are considered to be the most suitable candidates for cell replacement therapy for treating PD, because some researchers have shown that, compared with those from other brain areas, these NPCs differentiated with higher probability into DAergic neurons after transplantation (Svendsen et al. 1996). The main problems in progenitor grafting in PD are (1) the low rate of cell survival after transplantation and (2) the small percentage of those converting into the DAergic phenotype, suggesting that environmental factors or epigenetic stimulations are needed to support the survival, differentiation and integration processes. Strategies to improve the survival and differentiation of mesencephalic progenitors include (1) genetic modification, (2) pretreatment with growth factors, antioxidants and cytokines, and (3) modified implantation procedures (Brundin et al. 1986; Nakao et al. 1994; Yurek et al. 1996; Carvey et al. 2001; Kodama et al. 2004). Among those manipulations, growth factors in particular regulate neural stem cell behaviour. During development, growth factors provide important extracellular signals for regulating the proliferation and fate determination of stem and progenitor cells in the CNS (Calof, 1995).

In the present study, hNPCs were propagated *in vitro* in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2). *In vitro* studies have shown that both factors can maintain responsive neural progenitors in the cell cycle, thus expanding the progenitor population and delaying differentiation (Bouvier & Mytilineou, 1995).

After *in vitro* expansion we transplanted hNPCs derived from the ventral mesencephalon of an 8-week-old embryo into the DAergic deafferentiated striatum of neonatal rats. The aim of our study was to characterize (1) the differentiation of hNPCs after transplantation into the striatum of neonatal hemiparkinsonian rats, and (2) the possible therapeutic benefit.

Materials and methods

Isolation and *in vitro* expansion of mesencephalon-derived hNPCs

The isolation and propagation of hNPCs were in accordance with the Ethical Principles for Medical Research involving Human Subjects of the Declaration of Helsinki and were approved by the local ethics committee and that of the University of Rostock. Isolation and propagation of cells were essentially as described for retinal precursors (Andressen et al. 2003). In brief, the ventral midbrain (one individual from the 8th week of gestation) was dissected, including the subependymal regions. For the expansion of precursor cells, tissue samples were cut into small pieces, incubated in Hanks' buffer (HBSS) including 0.1% trypsin/EDTA (Invitrogene, Germany) for 30 min at room temperature. A single cell suspension was achieved by gentle trituration using a fire-polished Pasteur pipette. After sedimentation of the tissue debris by gravity, cells in the supernatant were collected, centrifuged, resuspended and transferred to culture dishes coated with poly-L-lysine (PLL, 10 μg mL^{-1} cm^{-2}) followed by laminin-1 (2 μg mL^{-1} cm^{-2}). Cells were placed in a humidified incubator at 37 °C, 5% CO_2 in air and cultivated in serum-free Dulbecco's modified Eagle's medium (DMEM) (high glucose)/F-12 medium mixture (1 : 1), supplemented with transferrin (100 mg mL^{-1}), insulin (25 mg mL^{-1}), progesterone (20 nM), putrescine (62 mM) and sodium selenite (30 nM). Additionally, 20 ng mL^{-1} of human recombinant EGF and FGF-2 were added to the medium (all supplements from Sigma, Germany). Cells were cultivated replenishing half of the medium every second day. Before reaching

confluence, cells were passaged by careful trituration in Ca^{2+}/Mg^{2+}-free, 0.1% EDTA containing HBSS using a fire-polished Pasteur pipette starting at a density of 5×10^4 cells mL^{-1}. Neural progenitor phenotype was assessed routinely by immunocytochemistry for Nestin and Vimentin.

Proliferating Nestin- and Vimentin-containing hNPCs were expanded for 6 months with passaging every 8th–10th day (19 passages). To allow mesencephalic hNPCs to predifferentiate, cells were cultivated in serum-free, 20 ng mL^{-1} human EGF and FGF-2 containing proliferation medium in bacteriological (non-adherent) dishes to generate neurospheres. This technique has recently been shown to mimic the three-dimensional situation in the developing CNS (Miyata et al. 2001; Campos et al. 2004). After 6 days, spheres were collected, and triturated to generate single cell suspensions for (1) transplantation or (2) further *in vitro* analysis. For the latter, suspended cells were again allowed to form spheres for a further 5 days (serum-free medium, without human EGF and FGF-2) which were then plated on PLL/laminin-1-coated glass cover slips to allow adherence for a further 24 h, followed by immunocytochemical examination with antibodies against the neuronal markers MAP5 and tyrosine hydroxylase (TH).

Animals

A total of 27 female Wistar rats were used in the experiment. Three groups were investigated: (1) intact controls (n = 10), (2) lesioned-only rats (n = 7), and (3) lesioned and transplanted rats (n = 10). Animals were housed at 22 ± 2 °C under a 12-h light/dark cycle with free access to food and water, first with their mothers, then in groups of 4–6 per cage. All animal-related procedures were conducted in accordance with local ethical guidelines and approved animal care protocols.

Neonatal 6-OHDA lesions

The hemiparkinsonian neonatal rats (n = 17) were generated by unilateral (right) intraventricular injection of 2 μL 6-OHDA-HCl (Sigma, 120 μg/10 μL 6-OHDA-HCl in 0.9% NaCl solution, containing 0.2 mg mL^{-1} ascorbic acid) on postnatal day one (P1), using the coordinates (relative to bregma): AP –0.6, L –0.8, V –2.1 (dura). Details of the handling and the operative procedures by using a neonatal stereotaxic device (Stoelting Co., Wood Dale, IL, USA) are described in detail elsewhere (Cunningham & McKay, 1993). The neurotoxin 6-OHDA induces rapid cell death of DAergic neurons within 1–3 days (Shimohama et al. 2003).

Cell transplantation

Intrastriatal stereotaxic transplantation was conducted on lesioned animals (n = 10) at P3. About 100 000 predifferentiated viable cells (1 μL suspension) from the 19th passage were transplanted into the right striatum by using a glass capillary with an outer diameter of 50–70 μm connected to a 5-μL Hamilton microsyringe. The coordinates from bregma were set as: AP +0.7, L –1.8, V –2.9 (dura), according to Nikkhah et al. (1995a,b).

Behavioural assessments

For the lesioned and transplanted group (n = 10), only those animals were included in the statistics for behaviour tests where the graft position was centred exactly in the striatum, as confirmed by immunohistochemistry (n = 5).

Apomorphine-induced rotations

Five weeks after transplantation lesioned-only animals (n = 7) and lesioned and transplanted animals (n = 5) were tested for apomorphine-induced contralateral rotations (0.25 mg kg^{-1}, s.c., Teclapharm, Germany). Their mean net contralateral rotations were collected for 40 min using a self-constructed automated rotometer system as described by Ungerstedt & Arbuthnott (1970).

Cylinder test

At the same time point, forelimb preference was evaluated with the cylinder test in intact control (n = 10), lesioned-only (n = 7), and lesioned and transplanted (n = 5) animals. The use of the forepaws during vertical exploration in a glass cylinder with a diameter of 20 cm was documented and analysed with a video camera system (Sony) as described by Kirik et al. (2000). Thirty consecutive forepaw contacts with the glass cylinder were counted per animal and differences between the right and left paw were evaluated for all three groups. To prevent subjective bias, contacts made by each forepaw with the cylinder wall were scored from the videotapes by an observer blinded to the animals' identities.

Immunohistochemistry

For *in vitro* immunohistochemistry cells were fixed for 20 min with 4% paraformaldehyde in 0.1 M PBS. After preincubation with 10% blocking serum for 1 h, the cultures were stained with antibodies to Nestin (1 : 500, Chemicon), Vimentin (1 : 200, Sigma) or double-stained with antibodies to MAP5 (1 : 500, Sigma) and TH (1 : 500, Sigma). CY2- (1 : 400) and CY3- (1 : 500) conjugated secondary antibodies (both Dianova) were used for visualization. Preparation of the animals for immunohistochemistry was performed 6 weeks after birth. Rats were injected with an overdose of pentobarbital (60 mg kg^{-1}) and transcardially perfused with ice-cold 0.9% sodium chloride (50 mL), followed by 300 mL of 3.7% paraformaldehyde (dissolved in 0.1 M PBS, pH 7.4). Brains were immediately removed from the skull, post-fixed for 4 h, and transferred into PBS (pH 7.4) 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 cryostate at 30 μm and serial sections were collected. For free-floating immunohistochemical stainings using 3,3′-diaminobenzidine (DAB), sections were pretreated with 3% H_2O_2 to quench endogenous peroxidases, then blocked with 3% bovine serum albumine (BSA), normal horse serum (1 : 67, polyclonal, Vector Laboratories) and 0.05% Triton-X100 for 1 h, and subsequently incubated with mouse anti-human nuclei (HN; 1 : 400, monoclonal, Chemicon) or mouse anti-TH (1 : 1000, monoclonal, Sigma) primary antibodies overnight at 4 °C. Then sections were washed three times in PBS and incubated with biotinylated horse anti-mouse secondary antibody (1 : 200, Vector) overnight at 4 °C. After three rinses in PBS, sections were incubated with peroxidase-conjugated avidin–biotin complex (1 : 50, Vector Laboratories) for 2 h at room temperature, washed three times in PBS, followed by incubation with 0.02% DAB for 8 min at room temperature. Mounted sections were dehydrated through a graded series of alcohol and embedded in DePeX mounting medium (Serva).

For immunofluorescence, after preincubation in 3% BSA, 5% normal goat serum and 0.05% Triton-X100 (2 h), all sections were stained with mouse anti-HN (1 : 200) together with the following polyclonal primary antibodies overnight at 4 °C: rabbit anti-neurofilament 200-kDa (NF 200, 1 : 100, Sigma), rabbit anti-neuron-specific enolase (NSE, 1 : 800, Chemicon), rabbit anti-S100β (1 : 100, Sigma) or rabbit anti-glial fibrillary acidic protein (GFAP, 1 : 100, Sigma). For antigen visualization, anti-mouse CY3- (goat, red fluorescence, 1 : 500, Dianova), anti-rabbit CY2- (donkey, green fluorescence, 1 : 400, Dianova) or anti-rabbit AMCA- (goat, blue fluorescence, 1 : 100, Dianova) conjugated secondary antibodies were applied. The sections stained with anti-HN and anti-GFAP (detected with CY3- and AMCA-conjugated antibodies) were finally incubated with FITC-conjugated anti-neuronal nuclei (NeuN) (mouse, 1 : 200, Chemicon) for neuronal phenotype detection of transplanted human cells. It was not possible to detect HN and TH simultaneously by immunoflourescent stainings, because both primary antibodies used in our laboratory were derived from the same host species (mouse).

Micrographs were taken via confocal laser scanning microscopy (CLSM), and bright-field microscopy. The 24-bit RGB micrographs derived from CLSM were segmented by applying the global intensity-based algorithm of Otsu (1979) to filter background staining.

Results

In vitro characterization of hNPCs

After dissection of the ventral mesencephalon followed by trituration, selection in proliferation medium containing the mitogens FGF-2 and EGF resulted in adherence of neuroepithelial cells to the coated culture dish. Neuroepithelial cells proliferated for 19 passages and expressed the intermediate filament proteins Nestin (not shown) and Vimentin (Fig. 1A). Under these conditions, a continuous proliferation was observed for at least 6 months, with no differences in proliferation and morphology of precursor cells. In the experiments parallel to the transplantation, immunohistochemical analysis of cells that were allowed to form spheres revealed that a high number of them expressed the neuronal marker MAP5 (Fig. 1B). Some of these MAP5-immunoreactive (ir) neurons were considered to be of DAergic phenotype, as they also synthesized TH (Fig. 1B).

Behavioural recovery of hemiparkinsonian rats

For apomorphine-induced rotations, two groups of rats were tested (lesioned-only, and lesioned and transplanted). The results are summarized in Fig. 2(A). In the lesioned-only group rats displayed rotations in

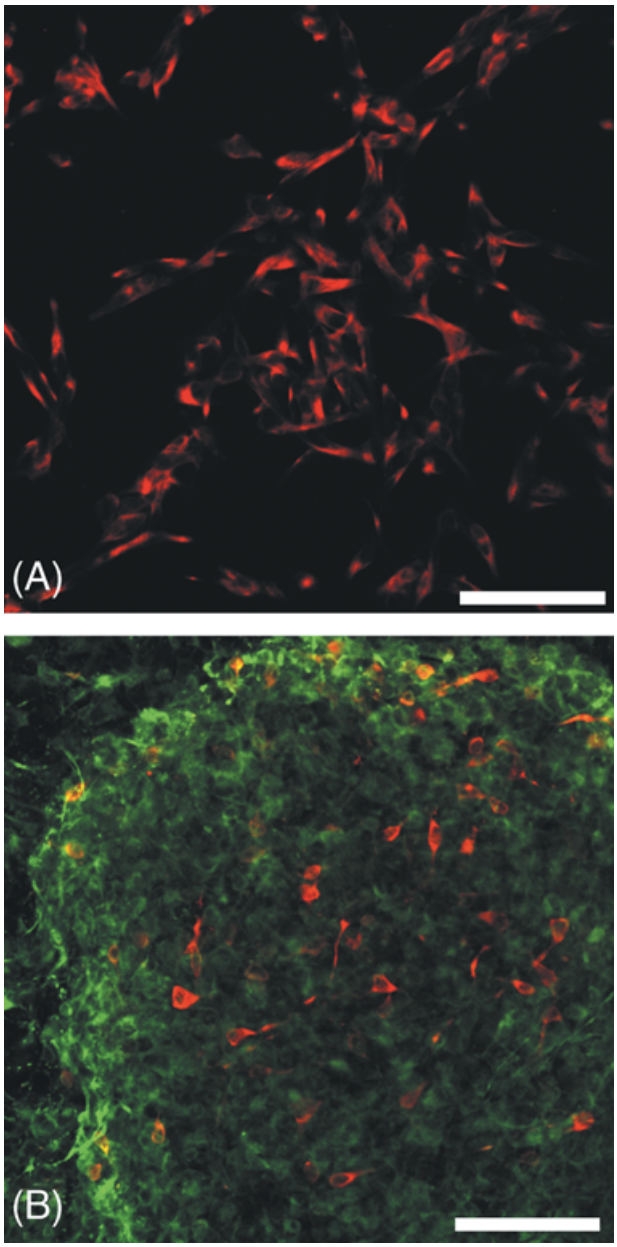

Fig. 1 Proliferation and differentiation of mesencephalic hNPCs *in vitro*. When cultured in serum-free medium supplemented with EGF and FGF-2 most cells expressed Vimentin (A). In plated spheres differentiated cells coexpressed MAP5 (green) and TH (red) as revealed by double immunocytochemistry (B). Note that the MAP5-staining intensity was electronically reduced in favour of TH-staining. Scale bars = 100 μm.

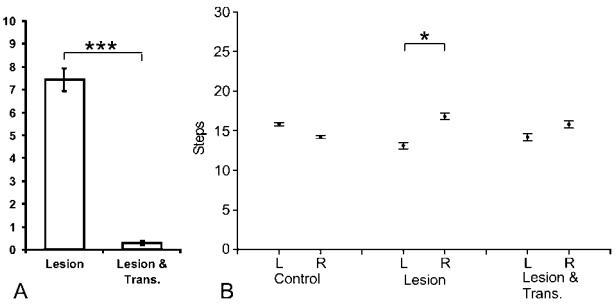

Fig. 2 Behavioral analysis. Apomorphine-induced rotations in lesioned-only animals (n = 7) were 7.4 ± 0.5 min^{-1}, whereas the rotation score in lesioned and transplanted animals (n = 5) was significantly lower (0.3 ± 0.1 min^{-1}; P = 0.001, U-test) (A). The forelimb preference tests in intact controls displayed no significant side bias (right to left ratio: 14.2 ± 0.2–15.8 ± 0.2), whereas lesioned-only animals displayed a significant (P = 0.05, Wilcoxon-test) right forepaw preference (right to left ratio: 16.9 ± 0.4–13.1 ± 0.4). However, lesioned and transplanted animals exhibited no significant differences in forepaw use (right to left ratio: 15.8 ± 0.5–14.2 ± 0.5) (B).

the direction contralateral to the lesion (7.4 ± 0.5 min^{-1}), consisting of a rapid and nearly complete depletion of DAergic neurons in the midbrain and deafferentiation of the striatum after 6-OHDA administration (Shimohama et al. 2003). By contrast, the lesioned and transplanted rats displayed significantly fewer rotations per minute, decreasing the number of rotations to 0.3 ± 0.1 min^{-1} (P = 0.001, U-test).

The cylinder test was performed on thr three groups of rats (intact control, lesioned-only, and lesioned and transplanted) (Fig. 2B). In the group of intact controls the number of touches performed by right and left forepaws was nearly identical (right to left ratio: 14.2 ± 0.2–15.8 ± 0.2). However, hemiparkinsonian rats showed a significant impairment in their left paw use (right to left ratio: 16.9 ± 0.4–13.1 ± 0.4). By contrast, the lesioned and transplanted animals again showed no significant side bias, the right to left ratio being 15.8 ± 0.5–14.2 ± 0.5 (P = 0.05, Wilcoxon-test), thus matching intact controls.

Survival, migration and differentiation of transplanted cells

Light microscopic analysis of TH-immunostained brain sections of 6-OHDA-lesioned animals revealed an almost complete depletion of TH-ir terminals in the right striatum (Fig. 3A) due to loss of DAergic neurons of the ipsilateral midbrain (Fig. 3B). For the lesioned and transplanted group five animals were excluded from the statistics due to ectopic localization (n = 2) or lack of HN-ir cells (n = 3), whereas successfully positioned hNPCs grafts centred in the striatum were found in five animals as assessed by HN immunohistochemistry (Fig. 4). Most of the transplanted hNPCs were confined

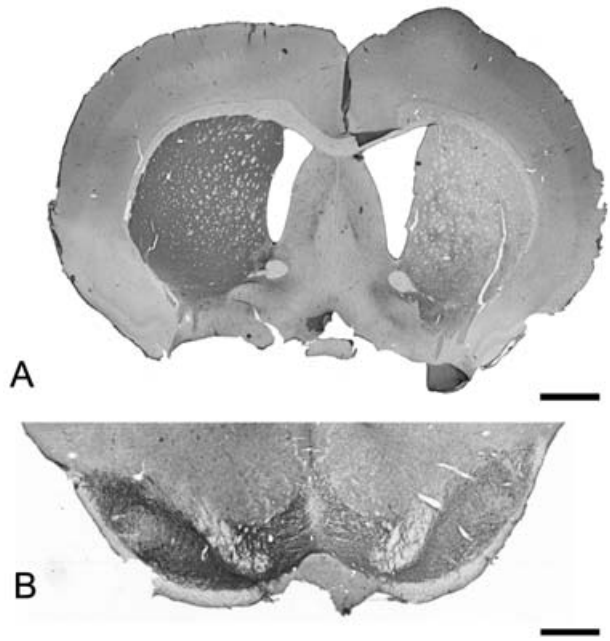

Fig. 3 Immunocytochemical TH-staining of the striatum and ventral mesencephalon including the substantia nigra of a hemiparkinsonian rat. The unilateral 6-OHDA lesion resulted in a depletion of the TH-positive fibres in the striatum (A) due to a profound DAergic cell loss in the substantia nigra (B). Scale bars = 1000 μm (A) and 500 μm (B).

to the striatum and loosely scattered around the injection site (Fig. 4A,B). However, some grafted cells migrated up to 2000 μm to the subependymal region of the lateral ventricle or along the corpus callosum (Fig. 4C). In the centre of the transplant (Fig. 4B) as well as in the surrounding area (Fig. 4D), in the corpus callosum (Fig. 4E) and the subependymal region (Fig. 4F) cells with different nuclear morphologies were detected. HN-ir cells in the white matter showed elongated nuclei parallel to the host fibre tracts, while cells concentrated within the grey matter exhibited mostly large round nuclei.

Multipotent differentiation of grafted cells was confirmed by double-immunofluorescent staining and confocal microscopy (Fig. 5A–F). A large number of HN-ir cells were detected in the striatum around the needle track (Fig. 5A). Double staining with HN paired with the mature neuronal marker NeuN revealed a considerable number of human cells with neuronal phenotype (Fig. 5B–D). The neuronal phenotype of grafted cells was also confirmed by the neuronal marker NSE (Fig. 5E) and NF 200 (not shown). Three-dimensional confocal analysis also revealed cells of human origin expressing astroglial markers GFAP (Fig. 5F) or S100β (not shown).

TH immunohistochemistry showed transplanted human progenitor cells converted into a DAergic phenotype *in vivo* located mostly in the periphery of a graft. TH-positive cells with clearly delineated processes, ramifying and extending in different directions, were only found in the striatum of lesioned and subsequently transplanted animals (Fig. 6A–C), but never in lesioned-only rats.

Discussion

Over recent decades, transplantation of fetal dopaminergic tissue into the denervated striatum has been considered as an approach for replenishing striatal dopamine levels and reforming the nigrostriatal pathway (Björklund & Lindvall, 2000). Having the capacity of both self-renewal and multiple differentiation, NPCs are an appropriate source of cells for clinical application and offer a promising future for cell replacement therapies (Gage, 2000). There have been many studies on the transplantation of animal-derived neural progenitors, whereas the therapeutic efficacy of NPCs of human origin has seldom been discussed. For clinical treatment of PD, however, an important goal is the generation of large numbers of graftable neurons from expanded human cultures.

In the experiment presented here, we transplanted hNPCs derived from the ventral mesencephalon of an 8-week-old embryo. These progenitors, with the capacity to proliferate and differentiate, have paramount potential for use in transplantation therapies for the treatment of neurodegenerative diseases. It has already been reported (Carpenter et al. 1999) that hNPCs exist in the first trimester and can be expanded *in vitro*, maintaining the capacity to differentiate into neurons, astrocytes and oligodendrocytes. This expansion depends on mitogens and does not require genetic modification of the cells. A number of growth factors support the proliferation of neural precursor cells and the differentiation of their progenitors. Growth factors not only carry out a traditional mitogenic function, but also enhance survival of dividing precursors (Kwon, 2002).

In particular, EGF and FGF-2 have been found to stimulate the division of embryonic or adult CNS precursors (Kilpatrick et al. 1995). To generate sufficient numbers of hNPCs from a limited amount of fetal mesencephalic tissue, we expanded human progenitors for 6 months in a medium containing FGF-2 and EGF (Chalmers-Redman et al. 1997; Svendsen et al. 1998). Removal of

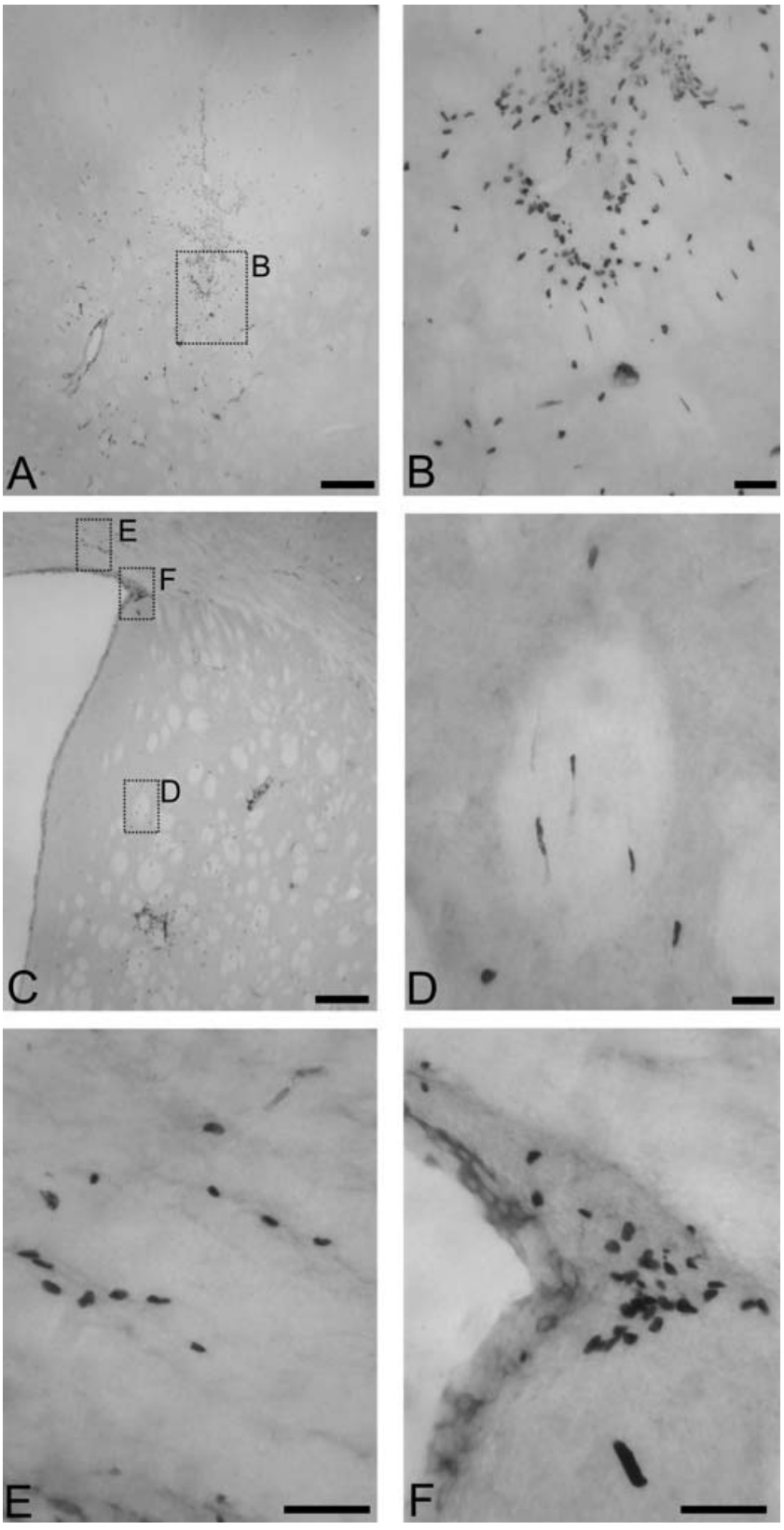

Fig. 4 Survival and migration of grafted hNPCs in hemiparkinsonian rats. Transplanted cells were detected by HN staining (A–F). Most cells were confined to the striatum (A–C), some cells migrated to the lateral ventricle and corpus callosum (C). D–F show enlargements of boxes outlined in C. Scale bars = 500 μm (A,C) and 50 μm (B,D–F).

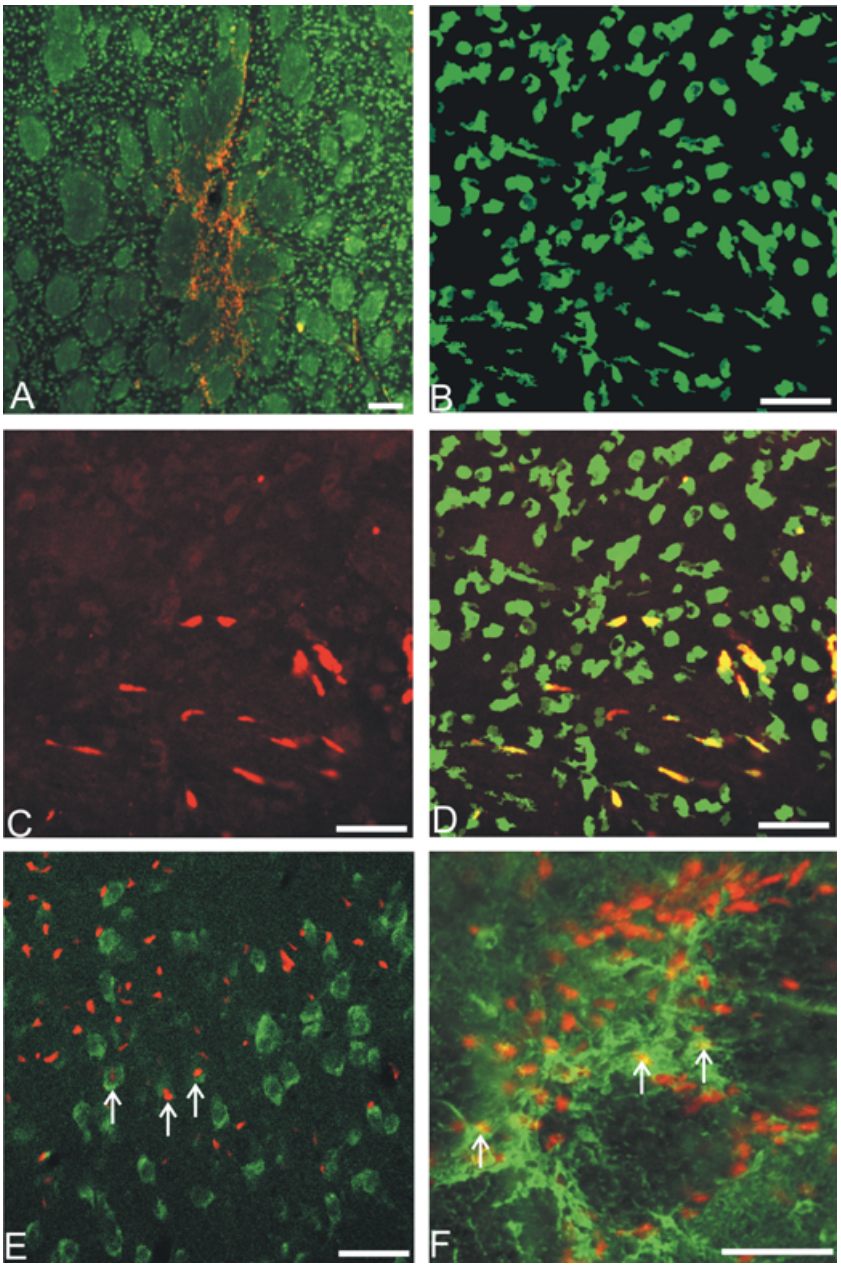

Fig. 5 Grafted hNPCs in hemiparkinsonian rats. HN-ir cells (red fluorescence) are confined to the striatum, counterstained with anti-NeuN (green fluorescence) (A). The differentiation of grafted hNPCs into neurons was revealed with NeuN (green) (B) and HN (red) (C). The co-localization (yellow) became obvious in the merged image (D). NSE (green) and HN (red) double labelling is shown in E. Astroglial differentiation of grafted cells was confirmed by staining with HN (red) paired with the astroglial marker GFAP (green) (F). Arrows in E and F indicate double labelled cells. Scale bars = 50 μm (A–F).

mitogens and addition of serum result in the differentiation of the progenitor cells into neurons and glia (Vescovi et al. 1999). The first steps of this differentiation were allowed to take place in spheres that are known to develop a three-dimensional structure with a cellular distribution and extracellular matrix composition similar to that of the developing neuroepithelium (Campos et al. 2004).

To determine whether transplanted cells led to a behavioural improvement, two behavioural assessment tests were performed: the apomorphine-induced rotation test and the cylinder test. The apomorphine-induced rotation scoring test relies on apomorphine stimulating supersensitive dopamine receptors in the dopamine-denervated striatum, which induces contralateral rotations in the rat. In this regard, the average rotation score in the lesioned-only rats (7.4 min^{-1}) is indicative of a unilateral loss of about 95% of the DAergic neurons in the substantia nigra (Ungerstedt & Arbuthnott, 1970), although structural perturbations in the striatum caused by cannulas or inflammation can make this test of apomorphine-induced behaviour unreliable (Dunnett et al. 1988; Jeyasingham et al. 2001). In accordance with other reports of microtransplantation

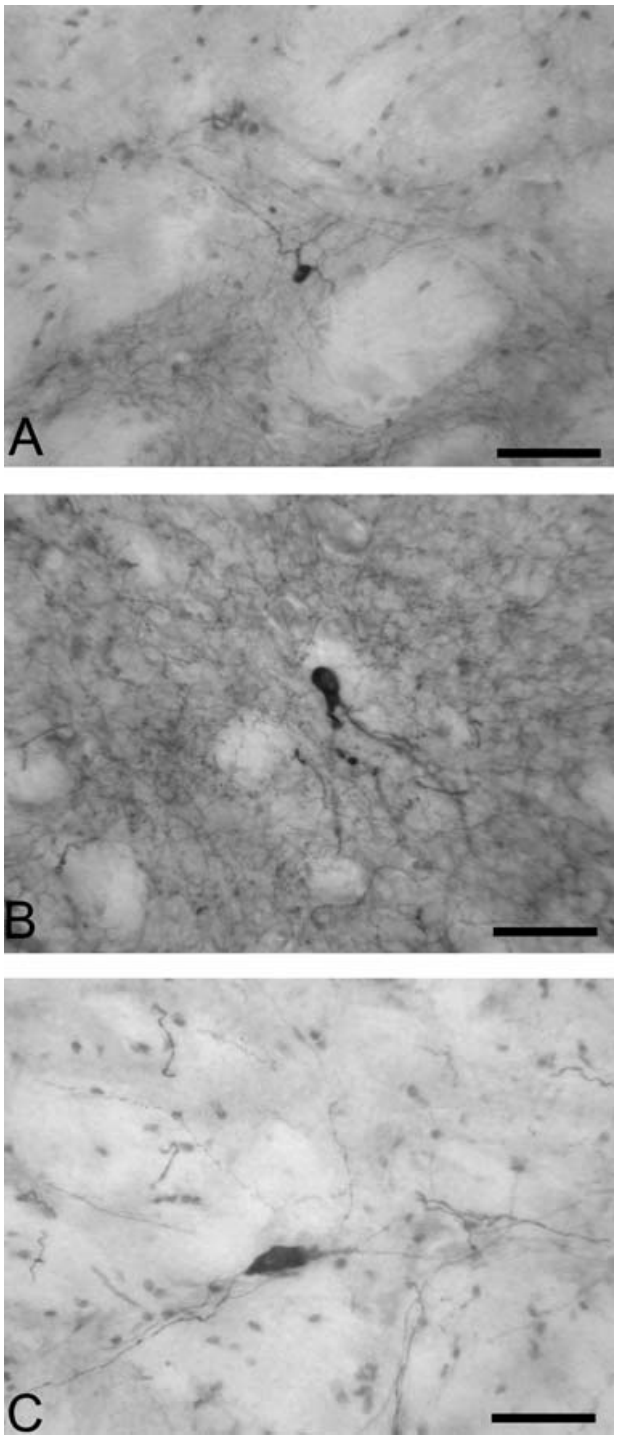

Fig. 6 Dopaminergic differentiation of grafted hNPCs. TH-ir cells with obvious processes were found in the right (lesioned and transplanted) striatum (A–C). Scale bars = 50 μm.

technique minimizing brain damage to overcome this limitation (Nikkhah et al. 1995a,b), we also performed microtransplantation using glass capillaries with an outer diameter of about 50–70 μm.

In addition, we used the cylinder test for the further evaluation of motor behaviour. Whereas intact control animals showed no preference in forepaw use, lesioned-only animals exhibited a significant impairment in the use of the left (contralateral) forepaw. It is well known for rats and mice lesioned as adults that a significant impairment in skilled paw use appears only after a cell loss in the range 60–80% of DAergic neurons in the substantia nigra (Espejo et al. 2001; Iancu et al. 2005), resulting in an 80–90% loss of DAergic fibres in the striatum (Lee et al. 1996). Compared with cylinder test experiments using adult hemiparkinsonian animals (Kirik et al. 2000), the observed side bias in the lesioned-only group was low, but significant. One possible explanation for this phenomenon could be a higher plasticity of the juvenile brain resulting in a compensatory effect of motor deficits in the extrapyramidal system.

Transplantation of hNPCs led to a significant improvement of motor behaviour in both tests. Mild improvements in motor behaviour were found after transplantation of non-neural cells (Shults et al. 2000), but in our study the improvement correlated with TH-ir cells with massive outgrowths detected in the right striatum of the lesioned and transplanted animals. Normally there are no DAergic neurons in the striatum of rats (Betarbet et al. 1997). Thus, the TH-ir cells seen in the striatum of transplanted animals must be derived from the grafted human cells. Moreover, we never observed TH-ir cells in the caudate putamen of lesioned-only animals.

In experimental animals there is a documented correlation between the numbers of surviving DAergic neurons in grafts and the degree of restoration of behavioural deficiencies due to lesions of the nigrostriatal pathway (Chaturvedi et al. 2003; Wang et al. 2004). In our experiment, the marked behavioural improvement (the decrease of rotational score from 7.4 ± 0.5 min^{-1} in lesioned-only rats to 0.3 ± 0.1 min^{-1} in lesioned and transplanted rats) was apparently caused by a relatively low number of TH-positive cells. It should be noted, however, that graft-derived TH-ir cells had clearly delineated outgrowths extending into the host striatum. Notably, Freed et al. (1981) showed that adrenal medulla tissue implanted in the lateral ventricle adjacent to the deafferentiated striatum had the capacity to reduce apomorphine-induced rotations even in the absence of any evidence of fibre re-innervation of the host brain.

Immunofluorescent analysis revealed convincingly that most of the transplanted cells became post-mitotic

neurons, as shown by NeuN, NSE or NF 200 immunoreactivity. A profound population of HN-ir cells was found to express the astrocyte-specific marker GFAP (or S100β). Notably, the vast majority of grafted cells positive for either neuronal or astroglial markers were found preferentially within the striatum. By contrast, cells which displayed extensive migration tendency, reaching the subependymal zone of the lateral ventricle and the corpus callosum, were negative for NeuN and GFAP (and S100β). Although it may be possible to detect oligodendroglial differentiation in the white matter, a similar study of the transplantation of human mesencephalic progenitors into the neonatal brain has shown that none of the migrated cells expressed appropriate markers (Englund et al. 2002). Our results of neuronal or astroglial differentiation detected preferentially in the striatum around the injection site correlate with those obtained after the grafting of human cells into the unlesioned developing rat striatum and hippocampus (Rosser et al. 2000). Accordingly, we also consider that cells that have migrated from the injection site are a population of as yet undifferentiated cells. It is noteworthy that in our study this population was relatively small. By contrast, for adult rats a larger number of undifferentiated cells have been described after transplantation (Fricker et al. 1999), focusing on the role of local environmental factors for neuronal differentiation. This view of the neonatal brain as a more permissive environment has recently been extended on dopaminergic differentiation of transplanted mesencephalic progenitors (Grothe et al. 2004). However, in our experiments, the rate of TH-ir neurons after transplantation into the striatum was found to be lower than expected from parallel *in vitro* differentiation, indicating that even the developing striatum is not an ideal environment for the generation of this neuronal subtype.

To overcome these limitations, cytokines and/or neurotrophic factors have been used to increase the number of pre-DAergic hNPCs *in vitro* before transplantation (Storch et al. 2001). Bearing in mind the poor differentiation of progenitors into DAergic cells *in vivo*, future studies will look at the role of local environmental cues for the induction and/or survival of certain types of neurons. The importance of the environment as a master regulator for DAergic differentiation will be assessed by orthotopic transplantation of mesencephalic hNPCs into the substantia nigra.

In summary, our results show that mesencephalic hNPCs can be isolated and subsequently propagated in culture using mitogens. These cells have the capacity to migrate and differentiate partially into neurons and glia when transplanted into the unilaterally lesioned neonatal rat brain. In spite of the marked neuronal differentiation and behavioural improvement observed, the number of surviving DAergic neurons in grafts was low. Taking the correlation between the number of DAergic neurons and behavioural outcome as a key hypothesis, strategies to improve th former will need to be developed to reach clinical applicability.

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