In vitro and in vivo characterization of neural progenitor cells as putative candidates for experimental studies and clinical trials in cell replacement therapy for neurodegenerative diseases

Dissertation

zur

Erlangung des akademischen Grades

Doctor rerum naturalium (Dr. rer. nat.)

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Rostock

vorgelegt von

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geboren am 28.10.1977 in Armenien

Rostock, September 2008

urn:nbn:de:gbv:28-diss2009-0119-0

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Das wissenschaftliche Kolloquium fand am 08.06.2009 statt.

Content

Abbreviations

BDNF	brain-derived neurotrophic factor
BMP	bone morhpogenic protein
CAG	cytosine-adenine-guanine
c-myc	myelocystomatosis cellular oncogene
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSF	colony stimulating factor
Cy A	cyclosporin A
DA	dopamine
DAergic	dopaminergic
EGF	epidermal growth factor
EPO	erythropoetin
ES cell	embryonic stem cell
FGF-2	fibroblast growth factor 2
GABAergic	gamma aminobutyric acid-ergic
GDNF	glial cell-line derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HD	Huntington's disease
hNPCs	human neural progenitor cells
IFN	interferon
IL	interleukin
ir	immunoreactive
L-DOPA	L-3,4-dioxyphenylalanine
LIF	leukaemia inhibitory factor
MAP2	microtubule-associated protein 2
MAP5	microtubule-associated protein 5
MFB	medial forebrain bundle
NeuN	neuronal nuclei
NF160	neurofilament 160 kDa
NF200	neurofilament 200 kDa
NGF	nerve growth factor

NSE	neuron specific enolase
NPCs	neural progenitor cells
NSCs	neural stem cells
NT3	neurotrophin 3
6-OHDA	6-hydroxydopamine
PD	Parkinson's disease
PDGF	platelet-derived growth factor
p75NGFR	nerve growth factor receptor p75
RNA	ribonucleic acid
RT-PCR	real time polymerase chain reaction
SN	substantia nigra
SV40	Simian-Virus-40
SVZ	subventricular zone
TGFα	and transforming growth factor alpha
TH	tyrosine hydroxylase
TNF	tumour necrosis factor
TrkA	tropomyosin-related kinase A
TrkB	tropomyosin-related kinase B
TrkC	tropomyosin-related kinase C
VM	ventral mesencephalon
v-myc	myelocytomatosis viral oncogene
VZ	ventricular zone

Abstract

There is increasing interest towards in vitro expanded neural progenitor cells (NPCs) as promising candidates for cell replacement therapy in neurodegenerative diseases. In vitro properties of rat fetal conditionally immortalized striatal cells ST14A and human NPCs (hNPCs) derived from the mesencephalic area of an 8-week-old embryo have been investigated in this study. ST14A cells showed a high proliferative ability and expression of neural progenitor cell (NPCs) markers nestin and vimentin at permissive temperature of 33° C. In contrast, at the nonpermissive temperature of 39° C, cell proliferation was ceased, nestin and vimentin expression was decreased, and cells underwent neuronal differentiation. The cells were, however, immunoreactive (ir) only for neuronal markers typical for immature neurons, lacking expression of mature neuronal markers. It has been concluded, that for further application for transplantation purposes into animals, these cells have to be induced to produce a mature neuronal phenotype. In contrast, hNPCs showed in vitro proliferation only in presence of growth factors when expanded as free-floating neurospheres. They stopped to proliferate after removal of growth factors, and differentiated in tyrosine hydroxylase (TH)-expressing neurons. When transplanted into hemiparkinsonian neonatal and adult rat striatum, hNPCs survive even in the absence of immunosupression, and ameliorate motor deficits caused by lesion, as shown by two behavioural tests: apomorphine-induced rotation and the cylinder test. However, surivival, and migration of grafted hNPCs differed in neonatal and adult rats: cells survived much better in neonate animals, and migration distance was also longer in neonates. In neonatal rats hNPCs underwent differentiation into neurons, among these TH-expressing, and astroglia. In contrast, in adult ones no TH differentiation was observed, although most of the cells differentiatied into neurons and astrocytes.

Both tests showed a behavioural recovery in transplanted animals (neonates as well as adults) in contrast to sham-operated ones. The presence of significant behavioural recovery even in absence of dopaminergic differentiation in adult animals indicates that not only TH-expressing, but other types of neurons, and astrocytes also might contribute to amelioration of motor deficits.

General outline of the thesis

This thesis consists of three chapters, with a general introduction at the beginning (Chapter 1). Results and their discussion are presented in the chapter 2. The reprints of all three publications are presented in the chapter 3. The cells derived from embryonic rat striatum (Publication 1) and human embryonic VM (Publications 2 and 3) were expanded in vitro and their proliferation and differentiation capacities have been evaluated. Cells derived from these two brain areas were chosen as they are of special interest as putative candidates for preclinical experiments and clinical trials for the treatment of two common, wide-spread neurodegenerative disorders, Parkinson's and Huntington's diseases. The in vitro observations concerning hNPCs were followed by the transplantation studies (Publications 2 and 3). The survival, migration and differentiation of hNPCs were evaluated after grafting into neonatal (Publication 2) and adult (Publication 3) striatum of hemiparkinsonian rats.

Chapter 1: General introduction

1.1. Neurodegenerative disorders and fetal transplantation

Neurodegenerative diseases are an assortment of central nervous system (CNS) disorders, characterized by neuronal loss in the different brain areas.

Although neurodegenerative diseases have different causes, the dysfunction and loss of certain groups of neurons is common to all these disorders and allows the development of similar therapeutic strategies for their treatment.

Parkinson's disease (PD) is the second most-common neurodegenerative disease of genetic, toxic as well as slow progressing idiopathic ethiology affecting around 2% of the population over 65 years of age (Roybon et al., 2004). The disorder was originally described by James Parkinson in 1817 (Parkinson 1817). The pathological hallmarks include progressive loss of dopaminergic (DAergic) projection neurons in the substantia nigra pars compacta (SN) and cytoplasmatic inclusions called Lewy bodies (Dawson and Dawson, 2003). The following depletion of dopamine (DA) transmitter in the striatum is clinically associated most often with tremor, rigidity, progressive bradykinesia and postural instability (Tedroff, 1999). Idiopathic PD symptoms become apparent when about 50% of nigral DAergic neurons and 70-80% of striatal dopamine are lost (Dunnett and Björklund, 1999). Pharmacological treatment with L-DOPA (L-3,4-dioxyphenylalanine) works initially, but is often related to undesirable side effects, including motor complications.

Likewise, currently no pharmacological treatment exists for another neurological disorder, Huntington's disease (HD). First described by George Huntington in 1872, HD is a relatively common hereditary neurological disease, caused by the expansion of a CAG (cytosine-adenine-guanine) triplet repeat in the huntington gene, resulting in the selective dysfunction and loss of gamma aminobutyric acid-ergic (GABAergic) neurons in the striatum which project to the globus pallidus and SN. Clinically, the disease presents with progressive motor, emotional and cognitive disturbances until death within 15 to 20 years (Ona et al., 1999). No specific treatment is known to slow, stop or reverse the progressive nature of the disease.

Functional replacement of specific neuronal populations through transplantation of neural tissue represents an attractive therapeutic strategy for treating neurodegenerative disorders such as HD and PD. Given that most neurodegenerative diseases affect the neuronal populations of specific neurochemical phenotypes, an ideal source material for transplantation would be a cell capable to limited self reproducing and assumption of desired neuronal phenotype upon differentiation

(Isacson, 2003). The successful transplantation requires selective replacement of lost phenotypes and the re-establishment of the original connection patterns with local and distant host partners (Rossi and Cattaneo, 2002).

Experimental transplantation studies for PD have been carried out during the last 30 years on rodents and non-human primates using a wide spectrum of cells including chromaffin cells (Freed et al., 1990; Unsicker, 1993), human neuroblastoma cells (Manaster et al., 1992), human amnion epithel cells (Kakishita et al., 2000). The best results, however, have been obtained only after transplantation of DAergic tissue derived from the ventral mesencephalon (VM) of different species into the striatum (for a review, Herman and Abrous, 1994). Studies have shown that grafted cells survived (Björklund et al., 1983), form synapses with host striatum (Björklund et al., 1983; Mahalik et al., 1985; Doucet et al., 1989), are able to produce dopamine (Schmidt et al., 1983; Triarhou et al., 1994,) leading to functional improvements in lesioned animals (Dunnett et al., 1983; Fisher and Gage, 1993).

Since 1987, when the first clinical neural transplantation trials were initiated, some 350 Parkinson's disease patients have received intrastriatal grafts of human fetal mesencephalic tissue (Lindvall and Björklund, 2004; Winkler et al., 2005). The outcomes of these studies demonstrate that transplanted human DAergic neurons survive and ameliorate many of the motor symptoms of advanced PD (Freeman et al., 1995a; Kordower et al., 1995; Defer et al., 1996; Hauser et al., 1999; Piccini et al., 2000; Mendez et al., 2005).

In animal models of HD transplanted fetal rat striatal cells have been demonstrated to survive, grow and reverse spontaneous motor abnormalities and at least partly normalize the metabolic hyperactivity in the extrapyramidal neural system (Deckel et al., 1983; Isacson et al., 1984; Campbell et al., 1993). Normal development of striatal grafts, connections with host brain and behavioural improvement in rodent models of HD have been described using human donor striatal grafts (Freeman et al., 1995b; Sanberg et al., 1997). In non-human primate models of HD, bradykinesia and dyskinesias induced by unilateral excitotoxic lesions of the striatum can be reversed by intrastriatal allografts of fetal striatal tissue (Kendall et al., 1998). Clinical trials have been performed on the basis of these experimental data. The transplantation of human fetal striatal tissue for the treatment of HD (Freeman et al., 2000) has shown that grafts can survive, develop and remain unaffected by the underlying disease process, at least for 18 months after transplantation in patients with HD, indicating no histological evidence of immune rejection.

Despite these promising results, substitutive therapy in the treatment of neurodegenerative disorders like PD and HD, based on the intracerebral implantation of human fetal tissue appears,

at this time, to be hampered because of ethical and technical difficulties.

Besides the ethical concerns related to the use of aborted human material, the viability and purity can also not be controlled. An additional obstacle for the use of fresh human tissue for transplantation is the poor survival of grafts. Only approximately 1 to 20% from grafted cells survives the transplantation procedure (Dunnett and Björklund, 1999). That means that 4-8 fetal brains are required for a significant reduction of symptoms in only one patient (Brundin et al., 2000). Concerning both disorders, there are many technical and conceptual problems which are to be resolved before treatment approaches tested in the laboratory can be used in clinical trials.

1.2 Stem and progenitor cells

Stem cells biology has its own lexicon, often confused by uncertainty in definitions "stem" and "progenitor" and by establishing degree of "stemness". Numerous reviews on stem cell biology have addressed the issue of a continuum of cell fate or differentiation from the most primitive precursor cells to the most differentiated adult somatic cell (reviewed by Steindler, 2007) for a complete list of definitions of stem and progenitor cells. To avoid misunderstandings while reading the thesis, here, the definitions and features of stem and progenitor cells will be given in brief.

A stem cell is a cell from the embryo, fetus or adult, which has the ability for reproducing itself for long periods, or in the case of adult stem cells throughout the life of the organism. The potency of a stem cell represents a range of cell types it can generate. Stem cells are the basic cell type from which all others emanate through restriction of potency.

Totipotent is called the cell able to generate the entire organism. The fertilized egg is considered to be totipotent as it has the potential to give rise to virtually all cells.

Embryonic stem (ES) cells are derived from the inner cell mass of the embryo in the blastocyst, one of the earliest stages (4-5 day) of development. They can in vitro replicate indefinitely. ES cells are pluripotent and have the potential to differentiate into all three germ layers of the mammalian body-the mesoderm, endoderm and ectoderm.

Adult stem cells are undifferentiated cells that occur in a certain tissue. They are self-renewing and become specialized to yield all of the specialized cell types of the tissue from which they originated.

Progenitor or precursor cells occur in fetal or adult tissues and are partially specialized. They are defined on the basis of two functional properties: limited capacity for self renewal and the ability to generate multiple mature neural cells. Stem and progenitor cells are distinguished in the

following way: a stem cell has the unlimited capacity to self renewal via symmetric or asymmetric division, whereas a progenitor cell has the ability to self-renew only for a limited period of time, before terminally differentiate into cells that are committed to a particular lineage.

Tab 1: Basic features of stem and progenitor cells.

Feature	Stem cell	Progenitor cell
Self-renewal	Unlimited	Limited
Plasticity	Pluripotent	Unipotent or multipotent

Neural stem cells (NSCs) are cells of the nervous system (central as well as peripheral) that are self-renewing and multipotent. As the mammalian CNS develops, that is presumed that a gradual restriction occurs in the differentiation potential of NSCs.

Neural progenitor cells (NPCs) present a population of cells with limited capacity for self renewal. They are a step further along than NSCs in the differentiation process; NPCs have committed to a particular lineage (neuronal or glial) and have begun to express lineage-specific markers. As the mammalian CNS develops, that is presumed that a gradual restriction occurs in the differentiation potential of neural stem and progenitor cells.

Hence, NPCs has been defined as a relative undifferentiated population of cells that (i) are capable to generate the broad array of specialized neurons and glial cells in CNS, (ii) have limited capacity for self renewal, and (iii) can give rise to cells other than themselves through asymmetric cell division (Gage, 2000).

1.3 In vitro expansion of neural progenitor cells

ES cells clearly represent an interesting option for cell-based transplantation studies. While these cells can efficiently generate neurons “of interest” in vitro, after grafting they are affected with problems, including propensity of teratoma formation. The potential of mouse ES cells to generate functional DAergic neurons and to ameliorate behavioural deficits after grafting into parkinsonian rats has been demonstrated (Björklund et al., 2002). When low numbers of undifferentiated mouse ES cells were transplanted into the rat DA-depleted striatum, the cells differentiated into functional DAergic neurons, reducing parkinsonian symptoms. However, teratoma-like tumours were formed in 20% of animals at the implantation site. In an alternative approach, highly enriched populations of midbrain NPCs were developed in vitro from mouse ES cells and transplanted into parkinsonian rats (Kim et al., 2002). These cells survived

transplantation procedure, led to the recovery from Parkinsonism, and did not form teratoma tumors. The possibility to grow NPCs in laboratory unlimitedly and to induce their differentiation by providing them the factors required for full maturation make them an attractive cell source for cell-based therapies.

It was previously suggested that in mammalian CNS progenitor cells were present only during development. However, neural progenitor cells have been isolated from various regions of the adult brain, including hypothalamus, dentate gyrus of the hippocampus and the forebrain structures subventricular zone (SVZ) and olfactory bulb (Temple and Alvarez-Buylla, 1999). In recent years, neurogenesis has been reported to occur in other regions of the adult brain such as the neocortex (Gould et al., 1999) and the SN (Zhao et al., 2003). More recently clonogenic NPCs have been isolated from mouse tegmentum and shown to express nestin and other NPCs markers. They formed spheres, differentiate in vitro in neurons, astrocytes and oligodendrocytes, had significant telomerase activity, displaying morphological and functional properties of mature neuronal subpopulations (Hermann et al., 2006).

NPCs can be either directly extracted from fetal or adult nervous tissue and proliferate in culture (Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Ling et al., 1998; Svendsen et al., 1999; Vescovi et al., 1999; Akiyama et al., 2001; Arsenijevic et al., 2001), or embryonic stem cells can be extracted, proliferated and differentiated into neural precursor cells (Li et al., 1998; Brüstle et al., 1999; Lee et al., 2000; Schuldiner et al., 2001; Kim et al., 2007). NPCs can be isolated and propagated in vitro either by exposure of mitogens, or by producing immortalized cell lines.

Epidermal growth factor (EGF) and fibroblast growth factor (FGF)-2 are factors most commonly used for in vitro propagation of NPCs. These factors have been found to stimulate the devision of embryonic and adult CNS progenitors (Kilpatrick and Bartlett, 1995). Cells isolated from the rat hippocampus in the presence of FGF-2 have been shown to express region-specific migration and neuronal differentiation after transplantation into the adult rat brain (Gage et al., 1995a; Suhonen et al., 1996). Embryonic mouse or rat forebrain progenitors expanded in the presence of EGF, by contrast, develop into predominantly glial phenotypes in vivo, as observed after transplantation into adult rat spinal cord (Hammang et al., 1997) or the developing rat forebrain (Winkler et al., 1998). Cells, obtained from the embryonic (6,5-9 week of gestation) human forebrain and expanded in culture in the presence of EGF, FGF-2 and leukaemia inhibitory factor (LIF), have been shown to survive well after transplantation to both neurogenic and non-neurogenic sites, showing migration, integration and site-specific differentiation into both neurons and glia

(Fricker et al., 1999). NPCs obtained from a mixed brain fragment of a 22-week-old fetus and expanded in vitro in presence of EGF and FGF-2 have also demonstrated good survival and differentiation into neurons and astrocytes, responded appropriately to regional determinants after grafting in neonatal brain (Rosser et al., 2000).

In the CNS, blockade of cell proliferation constitutes one of the events that are necessary for cells to undergo terminal differentiation. Likewise, they can be manipulated in vitro to stop proliferation to undergo terminal differentiation. This can be achieved by withdraw of mitogens (mitogen-expanded cells) or elevation of temperature (temperature-sensitive immortalized cell lines). These in vitro expanded cells can be used not only in transplantation experiments for the repair of CNS injuries and diseases, but also allow detailed study of the mechanism of neural proliferation, differentiation, and the role of different factors on cells phenotypic specialization (Schwartz et al., 2003).

In vitro CNS-derived neural progenitors exhibit regionalization. Neurospheres generated from different CNS regions express region-appropriate markers and generate region-appropriate progeny: spinal cord-derived cells generate spinal cord progenitors (Mayer-Proschel et al. 1997), whereas forebrain-derived cells generated GABA-ergic neurons (Potter et al., 1999). Consistently, striatal progenitors derived into GABAergic neurons (Kallur et al., 2006), mesencephalic progenitors give rise into DAergic neurons, (Storch et al., 2001; Storch et al., 2004; Miljan et al., 2008). The differentiation program can be influenced by exposure to various factors such as cytokines.

Cytokines, a diverse group of polypeptides that are generally associated with inflammation, immune activation and cell differentiation or death, include interleukins (IL), interferons (IFN), tumour necrosis factors (TNF), chemokines and growth factors. Hematopoietic cytokines are a large group of proteins normally found in blood that are involved with clonal expansion and lineage restriction of stem and progenitor cells within the hematopoethic system. Many of these cytokines, including erithropoetin (EPO), colony stimulating factor (CSF), and several members of the IL-family, are found within the brain and have been shown to influence the development of neurons (Mehler et al., 1993). Another subclass of cytokines, growth factors, also plays an important role in regulating of NPCs proliferation and differentiation. While EGF, FGF-2 and transforming growth factor alpha (TGFα) are mitogens for neural stem cells both in vivo and in vitro, other growth factors including bone morphogenic proteins (BMPs), platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) can regulate stem cell differentiation. Chemically defined media, including cytokines, growth

factors and other substances supporting for cell differentiation, have been developed for obtaining desired cell phenotypes (Ling et al., 1998; Carvey et al., 2001; Yan et al., 2001; Le Belle et al., 2004; Wang et al., 2004; Christophersen et al., 2006; Molina-Hernandez and Velasco, 2008).

Another possibility for in vitro expansion offers the immortalization of cells. Immortalized cell lines are established by transduction of neural progenitors with a vector encoding an immortalizing oncogene. It has been shown that the immortalization process holds cells at particular stages of development and prevents their terminal differentiation (Ryder et al., 1990). Owing to the fact of immortalization cells remain in continuous cell cycle, multiply limitlessly providing large numbers for transplantation purposes. In addition they offer a possibility for genetic manipulations, which enables the production of growth factors and enzymes.

Many immortalized cell lines seem to share in vitro properties with primary progenitors, for example, they express nestin and can express cell adhesion molecules (reviewed by Snyder, 1994).

For the production of immortalized cell lines, cells are transfected with oncogenes of different isoforms of myc, neu, p3, adenoviral E1A and SV large T antigen. V-myc propagated NPC lines include C17.2, derived from mouse cerebellum (Ryder et al., 1990), H6, derived from human 15-week-old telencephalon (Flax et al., 1998), HNSC.100, derived from human 10-week-old forebrain (Villa et al., 2002), Mes2, derived from human 8-week-old VM (Lotharius et al., 2002). Such cells response to EGF and FGF-2: they proliferate in response to EGF and FGF-2 and after removal of growth factors undergo terminal differentiation. A clonal NPC line from human fetal cortical tissue was established via immortalization with v-myc (Cacci et al., 2007). The cells showed a high proliferative ability in the presence of EGF and FGF-2. When growth factors were withdrawn, proliferation and expression of v-myc and telomerase were dramatically reduced, and the NPCs differentiated into glia and neurons (mostly glutamatergic and GABAergic as well as DAergic). NPCs derived from VM of 10-week old embryos and immortalized with the c-mycER^{TAM} transgene have shown after removal of growth factors EGF and FGF-2 differentiation into neurons, astrocytes and oligodendrocytes; the cells were able to generate TH-positive neurons demonstrating that even though they are multipotent, they still displayed some brain region restricted differentiation (Miljan et al., 2008).

Other authors immortalized neural cells with the temperature-sensitive mutant of the SV40 Large T antigen establishing stable homogenous cell populations in vitro: cells, derived from developing rat cerebellum (Frederiksen et al., 1988), CSM14.1 cells derived from VM region of E14 rat (Zhong et al., 1993), ST14A cells derived from striatum of E14 rat (Cattaneo et al.,

1994). The cell lines immortalized with SV40 are useful for preclinical transplantation studies, because they proliferate in culture at a permissive temperature of 33° C, and begin the differentiation after elevation of the temperature to the nonpermissive 39° C, which is in correspondence with the temperature of the rodent brain, allowing them to stop proliferation and differentiate after transplantation.

Immortalized cell lines can be induced in vitro to produce certain neuronal phenotypes. By an effective in vitro protocol a homogenous population of functional GABAergic neurons were obtained from rat striatal line ST14A (Bosch et al., 2004). These cells survived transplantation into the adult rat brain and maintained their acquired fate in vivo.

The propensity of immortal non-transformed rat mesencephalic cell line AF5 to differentiate in response to high confluence culture condition and serum starvation to exhibit GABAergic lineage-specific properties has been also demonstrated (Sanchez et al., 2006).

Immortalized cell lines have shown capacity to differentiate into several neuronal cell types when transplanted (Martinez-Serrano and Björklund, 1997). The transplantation of immortalized cells into the neonatal brain resulted in a differentiation into neurons and glia with apparent region-specific morphology (Renfranz et al., 1991; Snyder et al., 1992; Cattaneo et al., 1994). When transplanted into the adult rat striatum, immortalized cells generated from embryonic hippocampus or striatum, usually form glia (Lundberg et al., 1997). Cells derived from embryonic rat VM and immortalized with SV-40 have been detected to form tyrosine hydroxylase (TH)-ir neurons after grafting, improving motor behaviour in parkinsonian rats (Haas et al., 2007).

Chapter 2: General results and discussion

2.1 In vitro characterization of the striatal neuronal precursor cell line ST14A

The use of neural stem cells for transplantation is a rapidly expanding field.

An important source for cell transplantation purposes present immortalized cell lines (Frederiksen et al., 1988; Whittemore and Onifer, 2000). Immortalized cells can undergo limitless numbers of divisions and are often resistant to apoptosis (Hahn and Meyerson, 2001). Thus, such cells provide a supply for unlimited numbers of homogenous and stable cells for research, allowed controlled genetic modifications and are highly suited for the extensive characterization and validation of data needed for their clinical use.

The most commonly used oncogens for immortalizing cells are members of the myc oncogene family or a temperature sensitive mutant of SV40 large T antigen (Gage et al., 1995b). In neural cell lines developed by constitutively expressing oncogenes such as myc, the mitotic activity of oncogene is always present, and cells proliferate continuously in culture. Differentiation of the immortalized progenitor cells into neurons may require sufficient down-regulation of the oncogene, but attempts to induce terminal differentiation often induce apoptosis (Rubin et al., 1993). Additional factors are required to achieve terminal differentiation of neural cell lines immortalized with myc. To obtain a regulatable expression of the immortalizing oncogene, a temperature sensitive mutant of SV40 large T antigen (tsA58) has been used for the conditional immortalization (Mehler et al., 1993).

In the Publication 1 in vitro properties of immortalized cell line ST14A cells have been investigated to evaluate their potential for experimental studies. Embryonic striatal conditionally immortalized ST14A cell line was established via retroviral transduction of the temperature-sensitive mutant of the SV40 Large T antigen (ts A58/U19 allele) into primary cells derived from embryonic day 14 primordial rat striatum. Like their immature progenitors from which they where derived, ST14A cells maintained the expression of nestin at a permissive temperature of 33° C and exhibited a response to FGF-2 stimulation (Cattaneo and Conti, 1998). Upon shifting to the nonpermissive temperature of 39° C, the expression of the Large T antigen was down regulated, as has also been shown by experiments conducted in other immortalized cells (Renfranz et al., 1991). In the same study ST14A cells have been shown to express microtubule associated protein 2 (MAP2), messenger RNAs of various neutrophins (NGF, NT3, BDNF, bFGF, CNTF; shown by RNase protection assay) and produce neutrophin receptors (TrkA, TrkB,

TrkC, p75NGFR shown by Western blotting or RT-PCR).

Here, we investigated in vitro properties of ST14A cells for up to 14 days. As expected from previous studies, cells proliferated extensively producing neural progenitor cell markers nestin and vimentin at the permissive temperature in serum containing medium. The cells also continued to multiply their number at nonpermissive temperature, but the proliferation was drastically slowed down. In the serum-free medium at 39° C almost the same number of cells was found at days 0 and 14, indicating the entire stop of proliferation. It was concluded, that only the elevation of the temperature is not enough for stop of proliferation.

The expression of NPCs markers nestin and vimentin was drastically decreased after shifting the temperature to 39° C. Low levels of both markers, however, were weakly detectable even after 14 days of culture at the nonpermissive temperature, indicating that cells partially (or at least some of them) maintained the expression of progenitor markers.

Concerning differentiation, neuronal markers appearing earlier in the neural development such as (neuronal specific enolase) NSE, NF160 (neurofilament 160), NF200 (neurofilament 200) and microtubule associated protein 5 (MAP5) were weakly detectable already at permissive temperature. Similar observations have been made by Ehrlich et al. (2001), suggesting that even in the immortalized state, ST14A cells are expressing antigens characteristic of neurons which have progressed beyond the progenitor state. The expression of these markers increased after shifting of the temperature to 39° C. Even at 39° C neuronal markers NeuN (neuronal nuclei) and MAP2, typical for mature neurons have been never observed. In another study MAP2 (Cattaneo and Conti, 1998) has been detected by Western-blot analysis. Here, it can be speculated, that mature neuronal markers are expressing yet only at low levels, making them undetectable for immunocytochemistry.

The phenotypic range of a precursor that is immortalized depends on the potential of the precursors normally prevalent in a given region at the given developmental stage. By an effective culture condition including factors supporting for neural differentiation ST14A cells have been shown to differentiate into GABAergic neurons in vitro (Bosch et al., 2004).

The temperature-sensitive variant of SV40 large T antigen has been the main choice for the conditional immortalization. However, many studies failed to demonstrate full differentiation of the immortalized cells by only switching the temperature from the permissive to the nonpermissive one (Frederiksen et al., 1988; Mehler et al., 1993). It can be concluded, that additionally factors like growth factors and cytokines and appropriate substrates are needed in addition to elevation the temperature to provoke complete differentiation of immortalized

precursor cells.

2.2. In vitro characterization of mesencephalic hNPCs

Neural transplantation using tissue from the fetal human CNS offers a possible therapeutic approach for treatment of PD (Lindvall, 1994). However, a wide application of this therapy is hindered. One factor limiting the widespread application of neural cell transplantation as a treatment for neurodegenerative diseases is the lack of availability of suitable donor tissue for transplantation. In vitro propagation of NPCs enables to study their intrinsic properties more detailed and to obtain large numbers of cells for transplantation studies.

The success in treating neurodegenerative animal models of PD (Nishino et al., 2000) with rodent NPCs, has prompted efforts to develop and produce human NPCs (hNPCs) with regard to their DAergic differentiation. Midbrain DAergic neurons are generated from the precursor cells in the ventricular zone (VZ) of the developing VM. After mitosis in the VZ, the precursor cells migrate, start expressing specific markers including TH, and differentiate into mature DAergic neurons (Kawano et al., 1995). Many laboratories have investigated the potential of growth factors expanded neural progenitors to integrate and restore function in animal models of neurodegenerative diseases. Under optimal conditions mesencephalic progenitors have been reported to yield neurons in which 24% are DAergic (Studer et al., 1998). Attempts have been made to enhance DAergic differentiation: Ling et al. (1998), reported, that mesencephalic progenitor cells derived from fetal rat brain could give rise to DAergic neurons using a combination of interleukin-1 (IL-1), IL-11, LIF and GDNF. In the culture of rat mesencephalic progenitor cells the DAergic differentiation was increased after treatment with ascorbic acid (Yan et al., 2001).

For clinical treatment of different types of neurodegenerative diseases, however, an important but yet unfulfilled goal is the generation of large numbers of neurons from expanded human cultures, which possesses more advantages than those from animals. The successful conversion of rodent NPCs to DAergic neurons has prompted intensive efforts to develop hNPCs with the view to their differentiation into DAergic neurons.

NPCs obtained from the human first semester forebrain were expanded in vitro as free-floating neurospheres, and their TH-differentiation was induced by factors including acidic FGF, combined with factors that activate protein kinase C (Christophersen et al., 2006).

In the present study (Publication 2 and Publication 3) cells from the VM of a human embryo of 8 weeks of gestation have been used. Mesencephalic precursor cells derived from the VM of

developing brain appear to be a good candidate cell source for DAergic precursors. These cells have transcription factors characteristic of their midbrain origin, and can respond to environmental signals by rapidly differentiation into DAergic neurons. The DA character of VM-derived cells has made them an attractive candidate source for donor cells that can be used in cell-based therapy for PD. The twofold purpose of this study was the (i) characterization of mesencephalic hNPCs in vitro and (ii) obtaining sufficient numbers of cells for transplantation experiments.

We expanded the cells in the medium containing EGF and FGF-2 for up to 6 months.

Long term neurosphere cultures have been generated using this combination (Vescovi et al., 1993). Nestin and vimentin are markers which are predominantly expressed in proliferating NPCs. A high percentage of NPCs isolated from human embryonic VM (7-8 weeks) has shown immunoreactvity for vimentin (Riaz et al., 2002). Here, we show that hNPCs from VM of 8-weeks old embryo proliferate continuously in the presence of EGF and FGF-2, expressing nestin and vimentin. The expression of nestin and vimentin in undifferentiated hNPCs is consistent with the presence of these intermediate filament proteins in stem and progenitor cells in the mammalian CNS (Lendahl et al., 1990).

To induce cell differentiation they were allowed to form spheres, in serum-free medium without human EGF and FGF-2, which were then plated on poly-L-lysin/laminin-1-coated glass cover slips for adherence. In principle, the in vitro differentiation of neurosphere-forming cells includes five steps: stop of proliferation, attachment of the neuroshpere to the surface of cell culture dishes, detachment of cells from the neurosphere, migration of these cells away from the sphere and their differentiation into different cell types in response to environment.

24 hours after plating the immunocytochemical analysis was performed. We have shown here, that these cells, expanded as neurospheres, are positive for microtubule associated protein 5 (MAP5). MAP5 is essential for the growth and elongation of neuronal processes and is expressed earlier than all other MAPs. It is suggested that phosphorylated MAP5 is expressed in developing axons, decreases with maturation and remains as its non- phosphorylated form in cell bodies and dendrites at adulthood (Ohyu et al., 1997).

Some of these MAP5 positive cells were also expressing TH. As TH is the rate limiting enzyme involved in the synthesis of catecholaminergic neurotransmitters, these neurons co-expressing MAP5 and TH and exhibiting traditional DA neuron morphology with long processes, were considered to be of DAergic phenotype.

2.3. In vivo characterization of in vitro expanded hNPCs, transplanted into the lesioned rat brain

In vitro culture systems permit testing the cellular properties of isolated cell lines and their behaviour under different conditions and factors affecting the fate of expanded cells. In the next step, however, it is very important to test these cells in vivo. After intracerebral stereotactic transplantation the differentiation of cells can differ noticeably compared to the in vitro conditions.

Here, we generate an animal model of PD to investigate the behaviour and functional effects of in vitro expanded hNPCs after grafting into lesioned neonatal and adult striatum. To investigate the characteristics of hNPCs after grafting and to address how factors in their environment might influence their survival, differentiation and migration, we transplanted in vitro expanded hNPCs both into developing and adult rat brain

Animal models of PD allow researchers to study the pathology of PD while mimicking physical and behavioural changes during the entire disease course. Animal models of PD are a useful tool not only for understanding the pathology of this disorder, but also for the preclinical trials, including cell replacement therapy. In the present study (Publication 2 and Publication 3) animals were unilaterally lesioned with 6-hydroxydopamine (6-OHDA).

The 6-OHDA rat lesion model is the most extensively used animal model for PD, because it reflects many symptoms of the disorder and give rise to a reliable and typical lesion pattern (Cenci, 2002). 6-OHDA is a neurotoxin that selectively destroys catecholaminergic neurons and it is typically injected unilaterally, since bilateral injections cause high mortality in adult animals (Iancu et al., 2005). Cytotoxity of 6-OHDA is due to its pro-oxidant activity: the toxin undergoes rapid auto-oxidation in the extracellular space promoting a high rate of reactive oxygen species formation which is associated with activation of the apoptotic cascade (Blandini et al., 2007). Intracerebral injection of 6-OHDA into the rat nigrostriatal pathway has been shown to permanently degenerate virtually all DAergic neurons in the SN pars compacta (Javoy et al., 1976; Jeon et al., 1995) leading to stable motor deficits over the time. The toxin can also be injected intrastriatally or directly into the SN. The advantage using unilateral 6-OHDA lesioned model is to afford a more easy assessment of motor impairments by utilizing tests that examine for a side bias- and drug-induced rotation (Ungerstedt and Arbuthnott, 1970).

In an animal model of PD, bilateral nigrostriatal lesions in rats (typically made by central injection of the toxin 6-OHDA into the medial forebrain bundle (MFB)) induce akinesia, rigidity, and catalepsy, whereas unilateral lesions produce deficits in tests of lateralised function,

including rotation in response to DAergic drugs, impairments in use of contralateral limb and increased grip strength with the contralateral paw (Jeyasingham et al., 2001). In the present study (Publications 2 and Publication 3) we have explicitly compared lesioned only rats with those transplanted with hNPCs after lesion and sham transplanted ones using two tests of locomotor lateralisation of motor functions i.e. apomorphine-induced rotation test and the cylinder test.

Both tests allow not only to characterize the extent of lesion but also to detect therapeutical effects of mesencephalic grafts.

It is generally accepted that xenografts (between animals of different species) and allografts (between outbred individuals of the same species) typically show rapid rejection occurring in a matter of days in non immunoprivileged organs like the liver or the kidney.

Cyclosporin A (Cy A) is a potent immunosuppressive drug widely used in clinical transplantation to prevent graft rejection. Cy A has been found in prolonging survival of a variety of xenografts transplanted to rat brain (Lund et al., 1987). However Cy A has been reported to enhance DAergic function (Borlongan et al., 1995) and, therefore, could theoretically contribute to the clinical benefit by xenotransplantation. The use of Cy A is complicated because the long-term costs and task of justifying the symptomatic relief.

In our study neither neonatal nor adult animals have been immunosuppressed. The brain is considered to be an immune-privileged site, which permits xenotransplantaton of the graft without the requirement for immunosuppression (Carson et al., 2006; Galea et al., 2007). Many explanations of immunologic privilege of CNS have been offered, including blood-brain barrier that prevents the access by the immune system to the site of injection. We have not found any evidence of immune response, using immunohistochemistry against OX-42. All cells positive for OX-42 exhibited morphology typical for the resting stage, concluding that microglia was not activated. In a nonimmunosuppressed adult rat the host immune response to a xenotransplant into the brain is typically initiated within 2 days following grafting and the complete destruction of the xenotransplant occurs within 30 days of transplantation. Human retinal pigment epithelial cells, transplanted into lesioned adult rat striatum survived 18 weeks without immunosuppression and showed no detectable evidence of a graft versus host immune reaction (Subramanian et al., 2002).

In the present work human cells have been shown to survive in the rodent environment without immunosuppression. It has been already shown that EGF-responsible human progenitor cells grown for 3 weeks in vitro can survive transplantation procedure into the lesioned rat striatum, but after 4 weeks postgrafting the transplants appeared very diffuse with only few scattered

DAergic neurons (Svendsen et al., 1996). Likewise, FGF-2-responsive human progenitors survive transplantation into adult rat striatum when grafted at very high density, although, again, the survival time has been assessed only 2-3 weeks (Sabate et al., 1995). Here, we show, that when expanded in vitro in presence of both factors, hNPCs survive at least 12 weeks after transplantation into adult hemiparkinsonian rat striatum.

Nevertheless, it should be pointed out that the survival was much better in the neonatal striatum than in the adult. The reasons for the poor survival of transplanted cells are yet to be clarified. It seems likely that poor cell survival owes to many factors such as mismatch conditions, lack of supporting trophic factors, free radical-mediated toxity and apoptosis. Not only survival, but also migration and differentiation were delayed in the adult hemiparkinsonian animals, confirming the view that specific, time-dependent patterns of promoting and inhibiting factors of the extracellular matrix and neural receptors of neonatal and adult striatum differ. In neonates some of the grafted hNPCs migrated up to 2000 μm to the subependymal region of lateral ventricle or along the corpus callosum, while in the adult environment only a small minority of cells exhibited migration to the surrounding parenchyma (600 μm).

Here, we performed heterotopic transplantation, grafting hNPCs derived from mesencephalon into the striatum. Although the reinnervation of other DA-denervated basal ganglia such as the SN and subthalamic nucleus may also be important, optimal placement of the graft within the striatum may be necessary to maximize the functional recovery (Baker et al., 2002).

Concerning TH-expression, and therefore a putative DAergic differentiation, the neonatal and adult environments also differed strongly. In neonates a small number of TH-ir cells were detected. Nevertheless, adult ones lacked DA-ergic differentiation, suggesting one more time that the adult striatum is not able to support DAergic differentiation.

Other studies speak in support of this proposal: neural mesencephalic progenitor cells of the immortalized cell line CSM 14.1 did not differentiate in TH positive neurons after transplantation into the adult striatum (Anton et al., 1995), whereas the transplantation of the same cells into the neonatal dopamine-depleted striatum resulted in a DAergic differentiation, followed by motor behaviour improvement (Haas et al., 2007).

A recent study on rodent neural progenitor cells (13.5 day of gestation) has shown that grafted VM cells developed to TH-expressing mature neurons strongly at 3 weeks, peaked at 4 weeks, following by the decrease of TH-expression at 5 and 6 weeks postgrafting (Park et al., 2007). In neonate animals 5 weeks after transplantation TH-ir cells were detected. Immunohistochemical analysis in adults was only performed once (12 weeks postgrafting), and it can be also speculated

that by this time TH differentiation has been gradually reduced.

Although non-expanded precursors transplanted into developing brain have shown extraordinary plasticity in neuronal differentiation (Vicario-Abejon, 1995), in vitro expanded cells have much lower success in generation of neurons especially when grafted into adult environment. The transplantation of in vitro expanded human mesencephalic progenitors did not result in sufficient differentiation of DAergic neurons to completely resolve the behavioural defects in an animal model of PD (Svendsen et al., 1996; Svendsen et al., 1997). Rat mesencephalic NPCs expanded in the presence of FGF-2 have been also clearly shown to lose the efficiency of DAergic differentiation after long-term expansion and passaging (Yan et al., 2001).

It is notable that in our studies the vast majority of survived cells underwent neuronal differentiation and was immunopositive for the neuronal marker NeuN in both neonates and adults, suggesting that hNPCs can undergo neurogenesis even in the lesioned non-neurogenic environment of the adult striatum and assume a neuronal phenotype. This is in agreement with a work showing marked neural differentiation following grafting of cortical NPCs into DA-depleted striatum but no TH expression, whereas the same cells were able to express TH in vitro (Yang et al., 2002). It is unknown why in vitro expansion of cells might result in a loss of their potential to differentiate into DAergic neurons after transplantation. It can be proposed that under conditions used for cell expansion in vitro cells altered and lost their identity as DAergic precursors. Endogenous precursor cells have access to spatiotemporal information and environmental molecular signalling that is important for adopting a specific fate. Long-term culture under stimulation with growth factors causes the loss of such information for unknown reasons, which are to be clarified (Santa-Olalla et al., 2000).

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 (Wang et al., 2004). In motor behaviour tests demonstrated neonatal animals a marked improvement of impaired motor functions. It should been pointed, that only a sparse TH-differentiation of grafted cells was observed. It seems unlikely, that a robust behavioural effect is caused by only rare TH-ir cells. Moreover, no TH-differentiation was observed in cells, grafted into the adult striatum. A behavioural improvement, however, has been also observed. Similar results have been recently obtained by Miljan and colleagues (Miljan et al., 2008). Implantation of the undifferentiated human mesencephalic immortalized NPCs into the striatum and SN of 6-OHDA unilaterally lesioned rats, displayed sustained improvements in a number of behavioural tests, whereas histological analysis shown no evidence of differentiation

into TH-positive neurons.

It is obvious to speculate, that not only DA produced by grafted cells is contributed to motor behavioural improvement of lesioned animals, but also further factors are responsible for these effects. hNPCs, transplanted both in neonatal as well as in adult hemiparkinsonian rats, differentiated not only in neurons but also in astrocytes, as shown by immunohistochemistry for the astroglial marker glial fibrilary acidic protein (GFAP). This may indicate some important roles played by these exogenous astrocytes in improving local striatal environments and thus the overall behavioural improvement after transplantation. It has been shown that intrastriatal administration of GDNF in 6-OHDA lesioned rats exhibits functional recovery as well as supports the regeneration of the nigrostriatal dopaminergic circuitry (Gash et al., 1996). It can be supposed, that GDNF, expressed by astrocytes is at least partially contributed to the alleviation of PD symptoms. Alternatively, grafted cells could give rise to another type of neurons, for example GABAergic, which could lead to perturbations in other neurotransmitter systems, contributing partially to the behavioural recovery (Winkler et al., 1999). The fact remains that unknown factors are responsible for the improvement in motor behaviour. Further investigations are required to determine the nature of these factors.

The usefulness of NPCs transplantation in PD treatment is now well-accepted. However, results from either rodent or primate animal models remain inconsistent. Before human progenitor cells transplantation becomes a routine method for the treatment of patients with PD, many related problems are to be resolved. On the present basis of results obtained till now in animals and humans, it is possible to identify a set a requirements that need to be fulfilled by neural progenitor cells to induce marked clinical improvement: firstly, the cells should release DA in a regulated manner and show the molecular, morphological and electrophysiological properties characteristic for midbrain neurons. Secondly, the cells must be able to reverse in animals those motor deficits that resemble the symptoms in persons with PD. Finally, the grafts must become functionally integrated into host neural circuitries.

The results presented here are promising in the line of survival, differentiation and migration of hNPCs following grafting into the striatum of lesioned animals. We show, that even in the absence of immunosuppression, human cells survive, and moreover, are able to marked differentiation into neurons and glia. In the future, attempts will be made to improve their survival and enhance the fate of TH-differentiation.

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Chapter 3: Publications (Reprints):

Publication 1: Hovakimyan M, Weinreich K, Haas SJ, Cattaneo E, Rolfs A, Wree A (2008) In vitro characterization of embryonic ST14A cells. International Journal of Neuroscience 118: 1489-1501

Publication 2: Hovakimyan M, Haas SJ, Schmitt O, Gerber B, Wree A, Andressen C (2006) Mesencephalic human neural progenitor cells transplanted into the neonatal hemiparkinsonian rat striatum differentiate into neurons and improve motor behaviour. Journal of Anatomy 209: 721-732

Publication 3: Hovakimyan M, Haas SJ, Schmitt O, Gerber B, Wree A, Andressen C (2008) Mesencephalic human neural progenitor cells transplanted into the adult hemiparkinsonian rat striatum lack dopaminergic differentiation but improve motor behaviour. Cells Tissues Organs 188: 373-383

Title: IN VITRO CHARACTERIZATION OF EMBRYONIC ST14A-CELLS

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Key words: differentiation, Huntington's disease, immortalization, neural progenitors, cell transplantation, striatum

ABSTRACT

The embryonic striatal temperature sensitive immortalized ST14A-cell line was characterized *in vitro* by immunocytochemistry when cultured at 33°C and at nonpermissive temperature of 39°C for up to 14 days. At 33°C in DMEM/10% FCS, cells proliferated, were extensively expressing the neural progenitor cell markers nestin and vimentin contrary to neuronal markers. However, when cultured at 39°C the proliferation was delayed and cells began to increase the expression of neuronal markers, followed by a decrease of nestin and vimentin. In serum-free medium the process of neuronal differentiation became more obvious, indicating the potential to use these cells for experimental restorative therapies.

Abbreviations

BSA, bovine serum albumin

CNS, central nervous system

DMEM, Dulbecco's modified Eagle's medium

FCS, fetal calf serum

MAP2, microtubule associated protein 2

MAP5, microtubule associated protein 5

NF160, neurofilament 160

NF200, neurofilament 200

NeuN, neuron-specific nuclear protein

INTRODUCTION

Cell replacement therapy for the treatment of central nervous system (CNS) disorders has recently become widespread (Lindvall & Kokaia, 2006). The cells for transplantation purposes should fulfil a number of requirements including unlimited availability, long-term stability *in vitro* as well as *in vivo* and suitability for genetic manipulations.

The immortalization of cell lines allows yielding a large number of appropriate cells in homogeneity (Cattaneo et al., 1994; Englund et al., 2002; Thompson, 2005; Xu et al., 2004).

Conditionally immortalized neuronal progenitor cells are interesting candidates for cell transplantation in experimental neurodegenerative processes, as the nonpermissive temperature for the immortalizing oncoprotein corresponds to the brain temperature of rodents (Björklund, 1993; Gage et al., 1991; Martinez-Serrano & Björklund, 1997; McKay, 1992). The most commonly used oncogens for immortalizing cells are members of the myc oncogene family or a temperature sensitive mutant of SV40 large T antigen (Gage et al., 1995).

The embryonic striatal conditionally immortalized ST14A-cell line, established via retroviral transduction of the temperature-sensitive mutant of the SV40 Large T antigen (ts A58/U19 allele) into primary cells derived from embryonic day 14 rat striatum primordia, has the ability of self renew und to give rise to various terminally differentiated cell types (Cattaneo & Conti 1998). At the permissive temperature of 33°C ST14A-cells proliferate over extended periods of time and express the immortalizing oncoprotein and the intermediate filament protein nestin, whereas at the nonpermissive temperature of 39°C the immortalizing oncoprotein is down regulated, the proliferation is reduced followed by a decrease of expression of nestin and the differentiation into neuronal cells. This cell line was chosen from among many clones because of high proliferative ability (Cattaneo & Conti, 1998).

ST14A-cells present a homogenous, stable and highly transfectable cell line, providing an opportune source for gene manipulations to express particular genes of interest (Böttcher et al., 2003; Corti et al., 1996; Pahnke et al., 2004; Weinelt et al., 2003).

Most recently ST14A-cells and their transfectants were used as a cellular model for the investigation of the role of different factors in cell development (Bajorat et al., 2005), proliferation and motility (Cacci et al., 2003), differentiation (Peters et al., 2004), migration (Gambarotta et al., 2004) and death (Wang et al., 2001).

ST14A-cells, transduced retrovirally to express a factor for regression of glioma tumors (Barresi et al., 2003; Benedetti et al., 2000), had a strong reducing effect on glioma cells.

Moreover, native ST-14A cells were shown to have the ability to inhibit tumour outgrowth when implanted into rats, those indicating the usefulness for the treatment of intracranial tumours (Staflin et al., 2004).

Under certain culture conditions the ST14A-cells display several characteristics specific to striatal medium-size spiny neurons, albeit immature ones (Ehrlich et al., 2001).

When transplanted into embryonic rat brain, ST14A cells aggregate into clusters and migrate while changing both their antigenic properties and ability to proliferate (Cattaneo et al., 1994).

Following intracerebral grafting procedure into the adult striatum, ST14A cells survive without forming tumors, differentiate preferentially into mature glial cells, including astrocytes, oligodendrocytes and pericytes, fully integrated with the resident glial population (Lundberg et al., 1996).

In the light of increasing interest towards ST14A cells as putative candidates for preclinical approaches in animal models, here, to define the potential and limits of these cells, we investigated their proliferation and differentiation capacities under different culture conditions for up to 14 days in vitro.

MATHERIAL AND METHODS

Cell culture

ST14A-cells were routinely grown in petri-dishes in 5% CO_2/95% air in a humidified incubator at 33°C and were fed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco). After expansion the cells were resuspended by trypsinization and collected by centrifugation (10 min, 600 rpm). Single cell suspensions were prepared (DMEM+10% FCS), the final cell density being about $2x10^5$ cells/ml. For further analysis of cell differentiation under various culture conditions (temperature, medium, time) cell suspensions were transferred to poly-D-lysine coated culture-slides (8 chamber slides, Becton Dickinson), 5 µl of cell suspension to each chamber, i.e. about 1000 to 1500 cells per 0,69 cm^2. Cells were allowed to adhere for 12 h (33°C, DMEM+10% FCS), thereafter changes of temperature and media were initiated. Cells were further cultured (i) in DMEM+10% FCS at 33°C for 3 days, (ii) in DMEM+10% FCS at 39°C for 3, 7 and 14 days, (iii) in serum free medium (Start V-medium, Biochrom) at 33°C for 3 days and (iv) at 39°C for 3, 7 and 14 days. Media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) and changed every third day.

Immunocytochemistry

Prior to immunofluorescence analysis cells were washed with 0.9% sodium chloride, fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C for 15 min, rinsed in 0.1 M Tris buffer (pH 7.4; 3 x 10 min) and preincubated in 0.1 M Tris buffer (pH 7.4) containing 3% bovine serum albumin (BSA) (Sigma), 3% normal goat serum (Gibco) and 0.05% Triton for 1h. Thereafter, cells were incubated with the primary antibodies, diluted in 0.1 M Tris buffer (pH 7.4) containing 1% BSA and 0.025% Triton over night at 4°C, against nestin (1:4, supernatant, diluted in Dulbecco´s phosphate buffered saline, monoclonal antibody rat 401 anti nestin, kind gift from R. McKay), vimentin (1:200, mouse monoclonal, Sigma), neuron specific enolase (NSE, 1:1000, rabbit polyclonal, Chemicon), microtubule associated protein 2 and 5 (MAP2, 1:500, mouse monoclonal Sigma; MAP5, 1:500, mouse monoclonal, Sigma), neurofilament 200 (NF200, 1:100, rabbit polyclonal, Sigma), neurofilament 160 (NF160, 1:50, mouse monoclonal, Sigma) and neuron-specific nuclear protein (NeuN, 1:1000, mouse monoclonal, Chemicon). Then the cell preparations were washed 3 x 10 min in 0.1 M Tris buffer and further incubated overnight at 4°C with polyclonal secondary anti-mouse (FITC-conjugated, 1:100, Chemicon) or anti-rabbit (Cy3-conjugated, 1:500, Dianova) antibodies. Finally, specimen were again washed for 10 min in 0.1 M Tris buffer, and cell nuclei were stained for 5 min with 0.03% (w/v) with 4´,6-diamidino-2-phenylindole (DAPI) in 0.1 M Tris buffer at room temperature, again washed 2 x 10 min in 0.1 M Tris buffer followed by destilled water, and mounted in anti-fading fluorescence mounting medium. Controls consisted of omission of primary antisera. They exhibited non-specific background fluorescence.

Analysis

Cell cultures were analyzed for the intensity of labelling with the various antibodies with a fluorescence microscope (Leitz Aristoplan, Wetzlar, Germany) using the respective filter units. Relative intensity of immunostaining was judged by the observer and scaled as - (not detectable), or if positive as + (sparse or weak), ++ (moderate) or +++ (extensive or strong). Cell growth was monitored at different time points under different culture conditions by counting the cell nuclei after nuclear staining with DAPI, the numbers of at least twelve chambers being expressed as mean ± SD per well.

RESULTS

In serum containing medium at 33°C cells rapidly proliferated increasing the number per chamber from 1206 ± 500 to 6514 ± 2734 in 3 days, whereas at 39°C respective numbers (9013 ± 3016) were reached after cultering for 14 days (Fig. 1a). However, culture in serum free medium at 39°C stopped cell devision so that comparable cell counts were found at day 0 (1464 ± 546) and at day 14 (1421 ± 455) (Fig. 1b).

Cultured at the permissive temperature in DMEM+10% FCS medium ST14A-cells grow in a monolayer and retained a flat, polygonal fibroblast-like morphology with stunted processes. Cells were extensively immunostained against nestin (Fig. 2a) and vimentin (Fig. 2f), moderate against NSE, NF200 and NF160, sparse against MAP5 (Fig. 2c), but were immunocytochemically negative for MAP2 and NeuN. In the DAPI staining mitotic figures were often seen (not shown). When cultured at the nonpermissive temperature of 39°C in DMEM+10% FCS medium ST14A-cell growth slowly proceeded (Cattaneo & Conti, 1998) up to 14 days, the cells retained their flat morphology developing short processes only. Depending on culture time at the nonpermissive temperature (3 to 14 days), immunostaining changed speaking in favour of a neuronal differentiation. Immunostaining against both nestin (Fig. 2b) and vimentin (Fig. 2g) drastically decreased but was still sparsely detectable after 14 days, however, that against the neuronal markers NSE, NF200, NF160 and MAP5 increased over time, most obviously seen for MAP5 (Fig. 2d). Even at 14 days at the nonpermissive temperature, ST14A-cells were immunocytochemically negative for MAP2 and NeuN. In the DAPI staining a decreasing number of mitotic figures were seen with increasing culture time. After 3 days of culture in serum free medium (Start-V-medium) at 33°C ST14A-cells were moderately immunostained against nestin and only sparsely against vimentin, extensively against NF200 and NF160, and sparsely against MAP5, NSE. In the DAPI staining mitotic figures were also seen. Depending on culture time at the nonpermissive temperature (3 to 14 days) in serum free medium, immunostaining drastically changed towards neuronal differentiation. Immunostaining against nestin was only sparsely detectable after 14 days and vimentin was immunocytochemically undetectable from day 7 onwards. After 14 days in serum free medium immunostaining was undectable against NeuN, sparse against MAP5 and NF160, moderate against NSE, but extensive against NF200.

DISCUSSION

Concerning transplantation of immortalized neural progenitors some studies provide very strong evidence that the final pathway of their *in vivo* differentiation is determined by local cues provided by the host tissue environment (Lundberg et al., 2002; Macklis, 1993; Renfranz et al., 1991; Shihabuddin et al., 1995; Snyder et al., 1997). For clinical application in the future great efforts should been made for the development and optimization of appropriate protocols for derivation and propagation of the transplanted cells.

In the present study we investigated further properties of the ST14A-cell line proliferation and differentiation in culture up to 14 days with respect to the expression of different specific markers for neural progenitor cells or neurons under various culture conditions. Here, we show that at the permissive temperature (33°C) ST14A-cells exhibit a high proliferative ability. The cells also continue to multiply their number at nonpermissive temperature, but the proliferation is drastically slowed down. In the serum-free medium at 39°C almost the same number of cells was found at days 0 and 14. This contrasts with data (Cattaneo & Conti, 1998), demonstrating that the removal of serum associated with the shift in temperature induces cell death within 2-3 days of incubation. However, Cattaneo and Conti investigated the cell proliferation for only up to 6 days. Here, we show, that despite a light increase in the cell count after 3 days culturing in serum-free medium at 39°C, the comparable cell counts are to find at 0 and 14 days of culturing, indicating that proliferation is stopped. At 33°C cells expressed the neural progenitor cell markers nestin and vimentin. The elevation of temperature to 39°C led to a massive decrease of the nestin and vimentin immunoreactivity, whereas these markers are still detectable after 14 days, only much less intensively. Some studies have shown that immortalized cells, obtained from rodent primary CNS cells retain some of the properties of the immature progenitors from which they were derived (Frederiksen & McKay, 1988; Mehler et al., 1993). In consistence with this fact, ST14A cells partially maintained the expression of nestin and vimentin. Other studies have also provided evidences that at the permissivie temperature these cells express nestin and vimentin, whereas at the nonpermissive temperature (39°C) *in vitro* or after transplantation into rodent brain the immortalizing oncoprotein is down regulated, the proliferation is reduced followed by a decrease of expression of these markers (Cattaneo et al., 1994; Cattaneo & Conti, 1998; Lundberg et al., 1997).

When cultured at 33°C in serum-containing medium ST14A-cells were moderate immunostained against NSE, NF200, NF160, and sparsely against MAP5. The data from

Ehrlich et al. (2001) has also shown that even in the mitotic state these cells expressed NSE and ß3 tubulin when grown in FCS-containing medium, indicating that even in the immortalized state, ST14A cells are expressing antigens typically characteristic of neurons which have progressed beyond the progenitor state.

In concordance with Cattaneo and Conti (1998) the shift of the temperature to 39°C induced cell differentiation, seen in an increasing expression of NSE, NF200, NF160 and MAP5. As could be expected, culturing in serum-free medium at the permissive temperature tentatively had a comparable effect on ST14A cells differentation: nestin and vimentin expression is reduced and neuronal differentiation promoted seen in an increasing expression of NSE, NF200, NF160 and MAP5.

In our study ST14A-cells were never found to be immunopositive for NeuN or MAP2. It is known that NeuN does not stain the nuclei of immature nerve cells until they achieve a stage of development that at least approaches mature function (Sarnat et al., 1998). Likewise, NeuN was not detectable in immortalized CSM14.1-cells by immunohistochemistry, whereas cells expressed NeuN as shown by Western-blotting (Haas & Wree, 2002). ST14A-cells were found to express MAP2 as shown by Western-blot analysis by Cattaneo and Conti (1998). It is known that MAP2 antigen concentration varies with the degree of neuronal maturation and dendritic differentiation, and that its expression is proportional to the state of differentiation (Sims et al., 1988). In our study cells lack MAP2 and NeuN as shown by immunocytochemisty, but express MAP5, a marker which is generally expressed in early developmental stages (Bates et al., 1993). We conclude that MAP2 and NeuN are present only in low concentrations, indicating that ST14A-cells have not reached late stages of differentiation.

Here, we show that additional factors, supporting for the cell differentiation, ST14A cells are required for a fully matured neuronal phenotype.

Following intrastriatal grafting only a low number of ST 14A cells (up to 3%) expressed MAP2 and expressed neuron-like morphology (Lundberg et al., 1997).

Concerning ST14A-cells the lack of neuronal differentiation of cells in *vivo* is a major limitation of this cell model for transplantation purposes. Nevertheless, by an efficient culture procedure ST14A-cells were induced to a stable GABAergic phenotype *in vitro* and showed a long-term survival and phenotype maintenance following transplantation into the adult brain in a model of Huntington's disease (Bosch et al., 2004).

The transfection with genes coding for functionally relevant factors to repair the diseased brains also makes this cell line a potential candidate for the implementation of cell replacement therapies (Hoffrogge et al., 2007; Pahnke et al., 2004; Weinelt et al., 2003).
If validated for preclinical use, in animal models of neurodegenerative disorders, conditionally immortalized ST14A cells could be of great value as they combine proliferative properties for the generation of enough cell material with the ability to stop their division at the fitting time.

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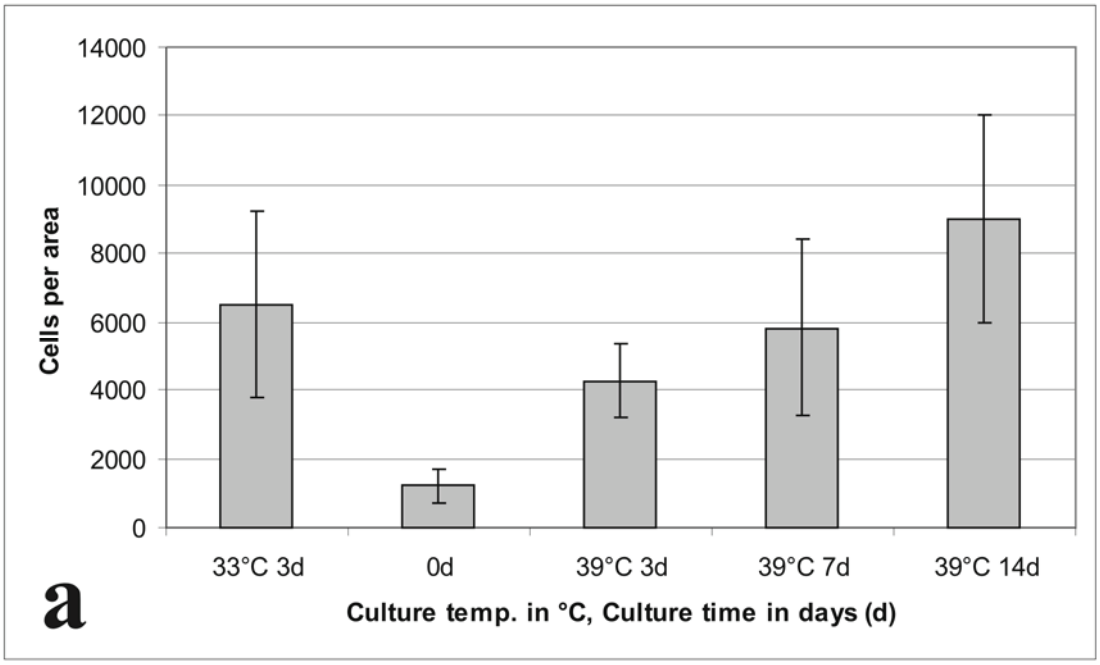

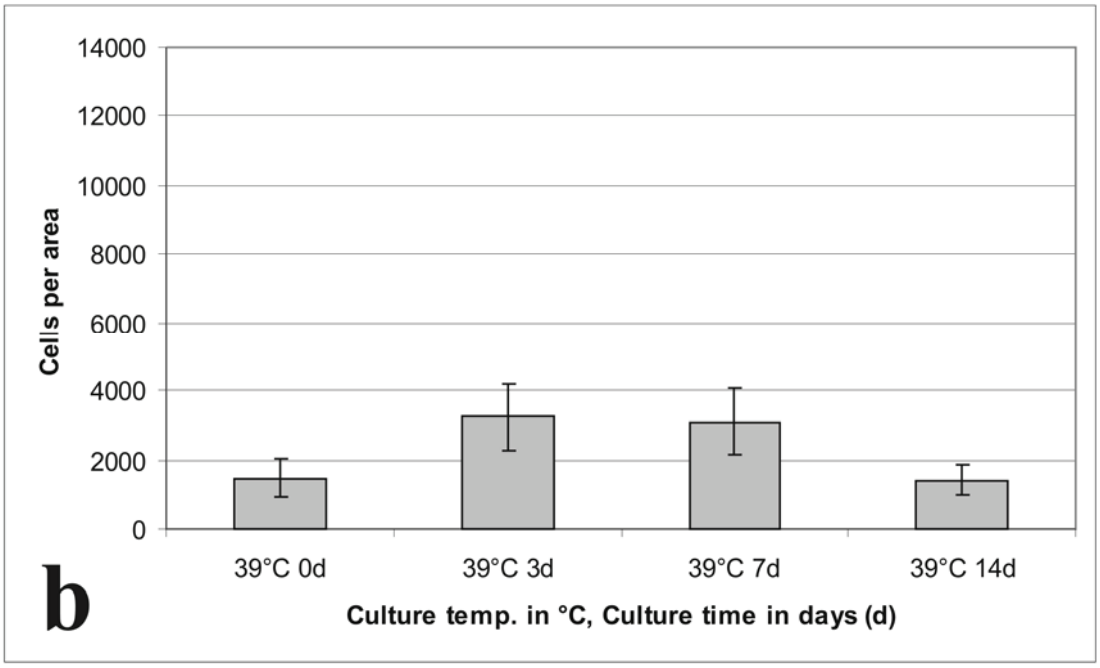

Fig 1. Proliferation of ST14A cells. A - ST14A cells proliferate extensively at 33°C, increasing the cell number from 1206 ± 500 to 6514 ± 2734 in 3 days. At nonpermissive temperature (39° C) the proliferation is delayed: the cell number after 14 days is only 9013 ± 3016.

B - cell division ceases after serum removal, and comparable counts of cells are found at days 0 (1464 ± 546) and 14 (1421 ± 455).

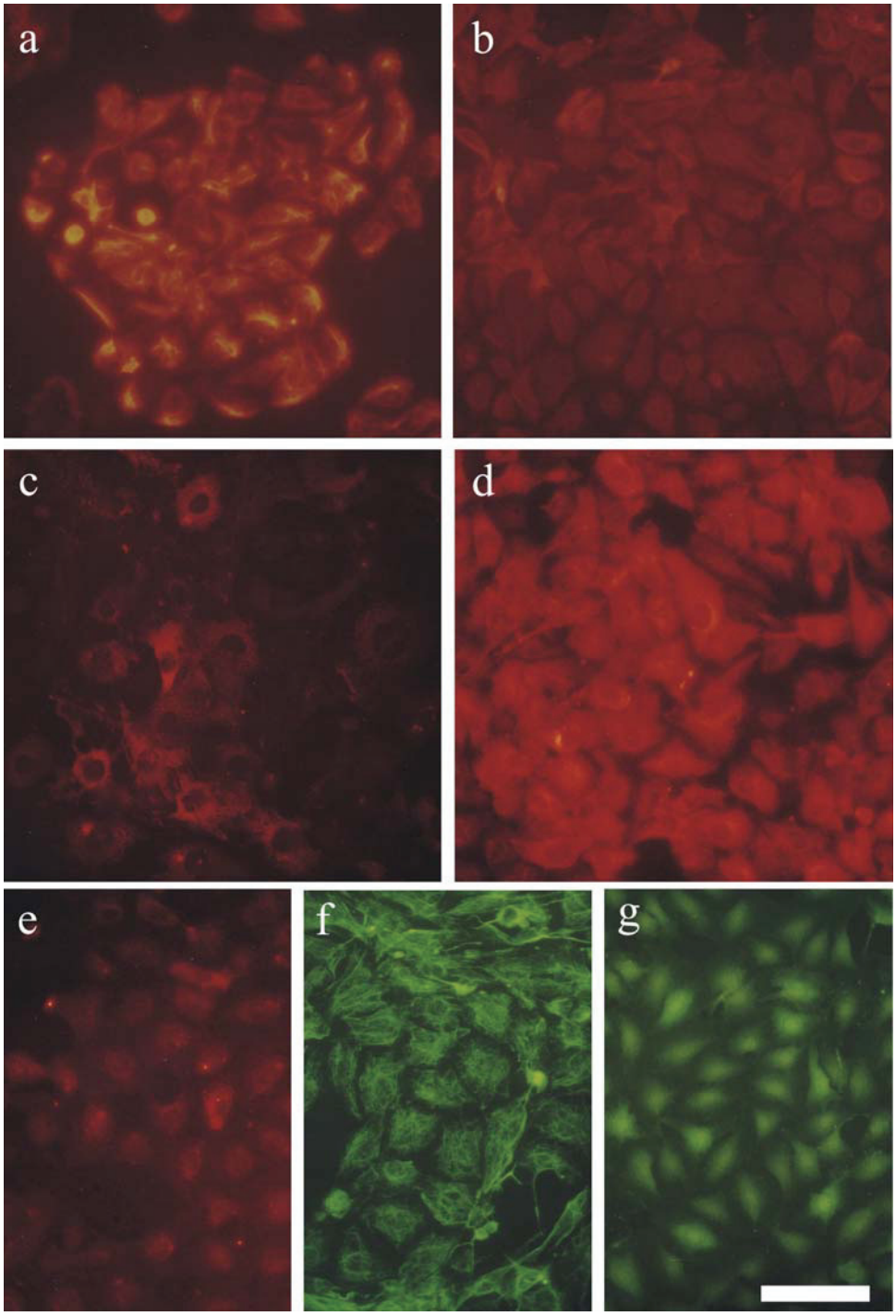

Fig 2. At 33°C in serum containing medium cells are extensively stained against the progenitor cell markers nestin (a) and vimentin (f). Even in these cell culture conditions some cells are sparsely stained against neuronal cell marker MAP5 (c). At nonpermissive temperature of 39° C cells continue to express MAP5, but much more extensively (d), accompanied by a decreasing intensity of the nestin (b) and vimentin (g) stainings. e - negative control. Scale bar = 50 μm

J. Anat. (2006) **209**, pp721–732 doi: 10.1111/j.1469-7580.2006.00654.x

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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Accepted for publication *24 July 2006*

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.

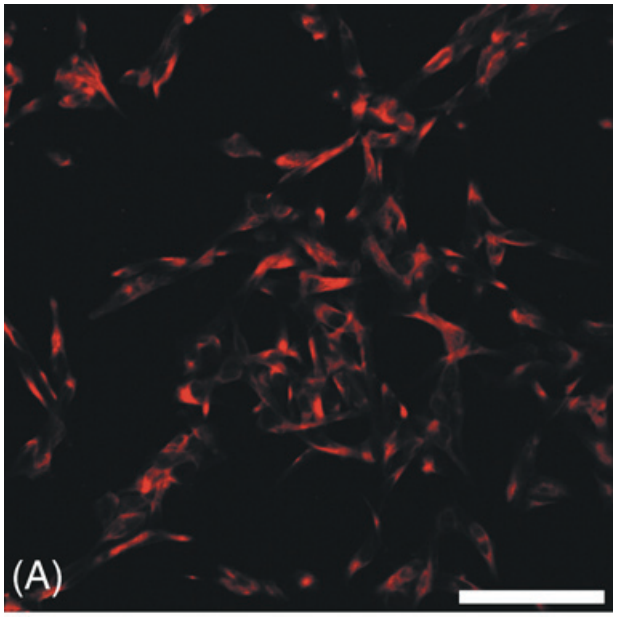
Results

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



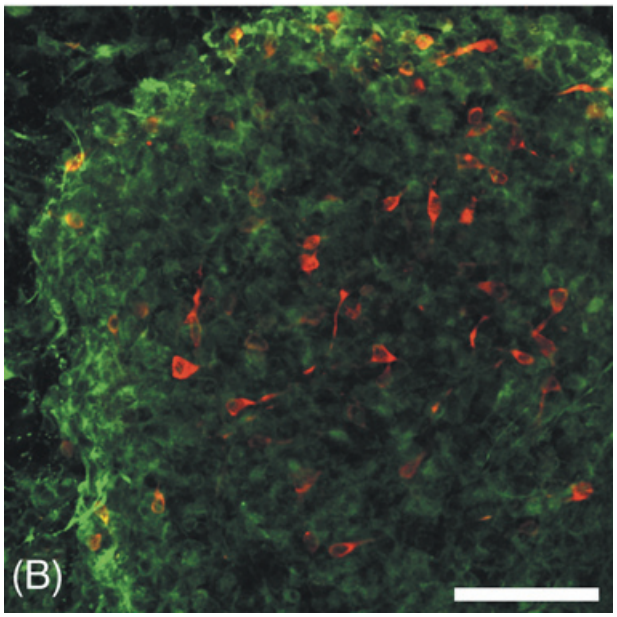


Fig. 1 Proliferation and differentiation of mesencephalic hNPCs *in vitro*. When cultured in serum-free medium supplemented with EGF and FGF-2 most cells expressed Vimentin (A). In plated spheres differentiated cells coexpressed MAP5 (green) and TH (red) as revealed by double immunocytochemistry (B).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

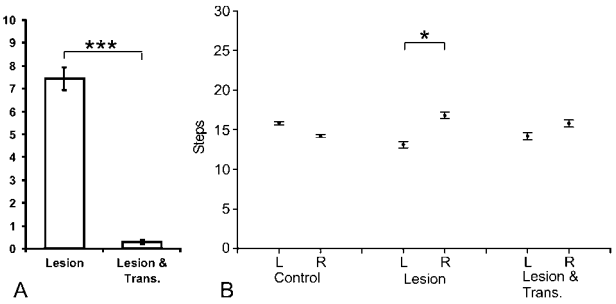


Fig. 2 Behavioral analysis. Apomorphine-induced rotations in lesioned-only animals (n = 7) were 7.4 ± 0.5 min^{-1}, whereas the rotation score in lesioned and transplanted animals (n = 5) was significantly lower (0.3 ± 0.1 min^{-1}; P = 0.001, U-test) (A). The forelimb preference tests in intact controls 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

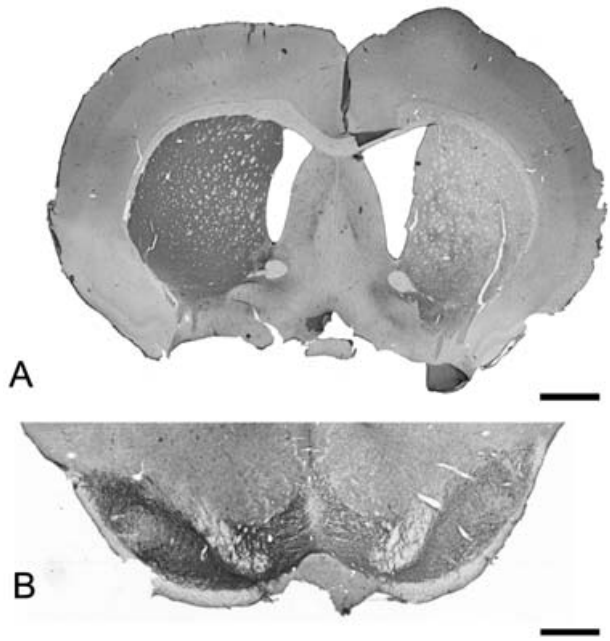


Fig. 3 Immunocytochemical TH-staining of the striatum and ventral mesencephalon including the substantia nigra of a hemiparkinsonian rat. The unilateral 6-OHDA lesion resulted in a depletion of the TH-positive fibres in the striatum (A) due to a profound DAergic cell loss in the substantia nigra (B).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

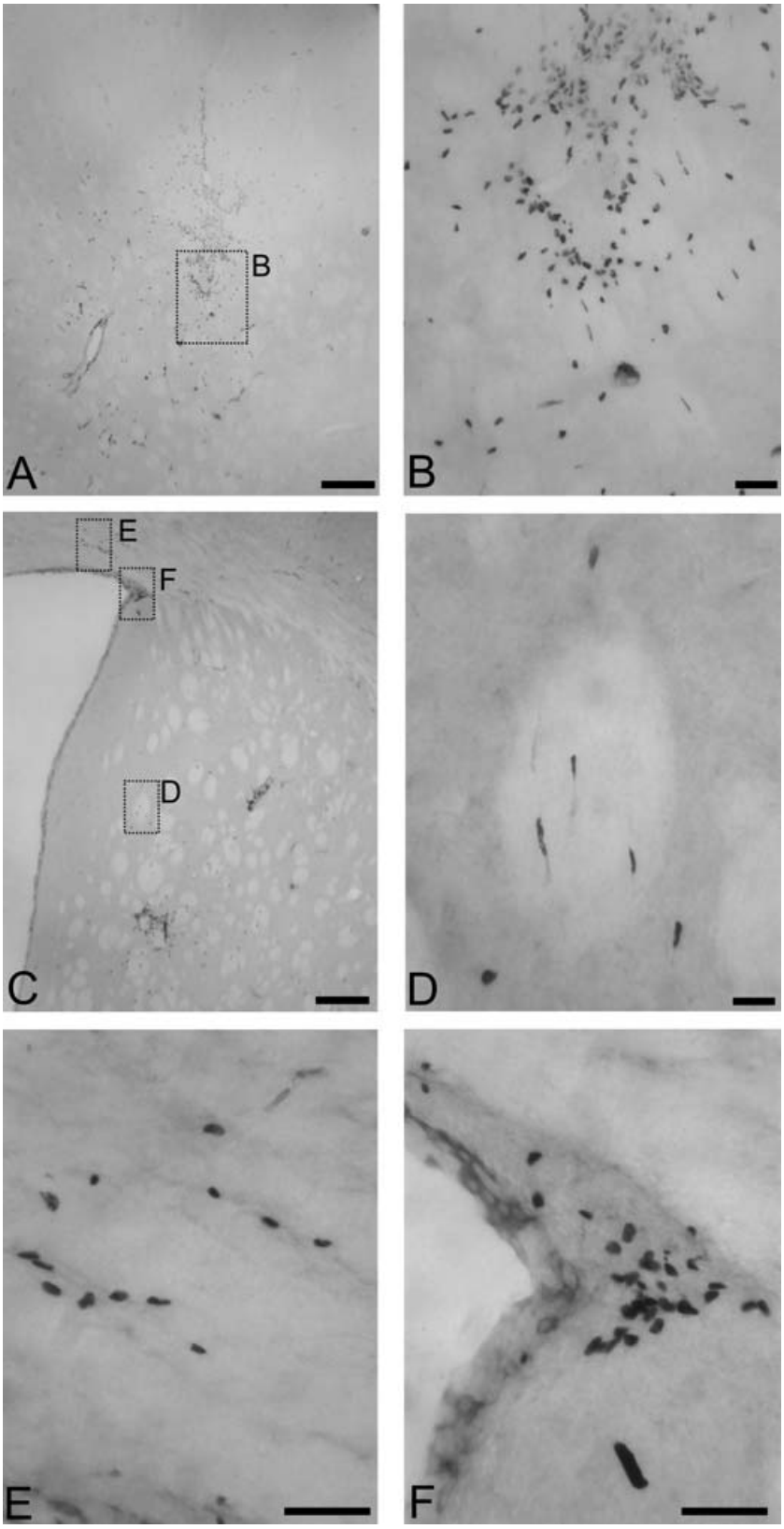


Fig. 4 Survival and migration of grafted hNPCs in hemiparkinsonian rats. Transplanted cells were detected by HN staining (A–F). Most cells were confined to the striatum (A–C), some cells migrated to the lateral ventricle and corpus callosum (C). D–F show enlargements of boxes outlined in C. Scale bars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

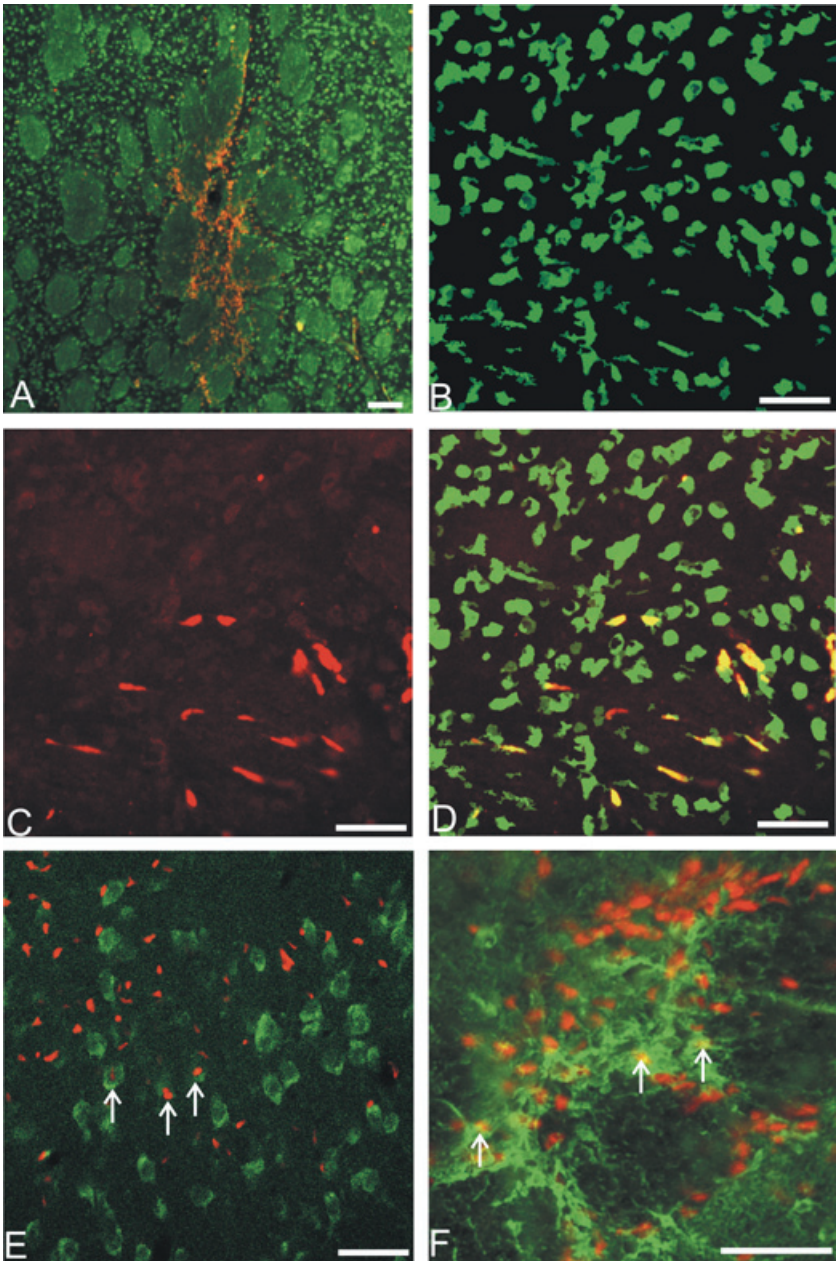


Fig. 5 Grafted hNPCs in hemiparkinsonian rats. HN-ir cells (red fluorescence) are confined to the striatum, counterstained with anti-NeuN (green fluorescence) (A). The differentiation of grafted hNPCs into neurons was revealed with NeuN (green) (B) and HN (red) (C). The co-localization (yellow) became 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

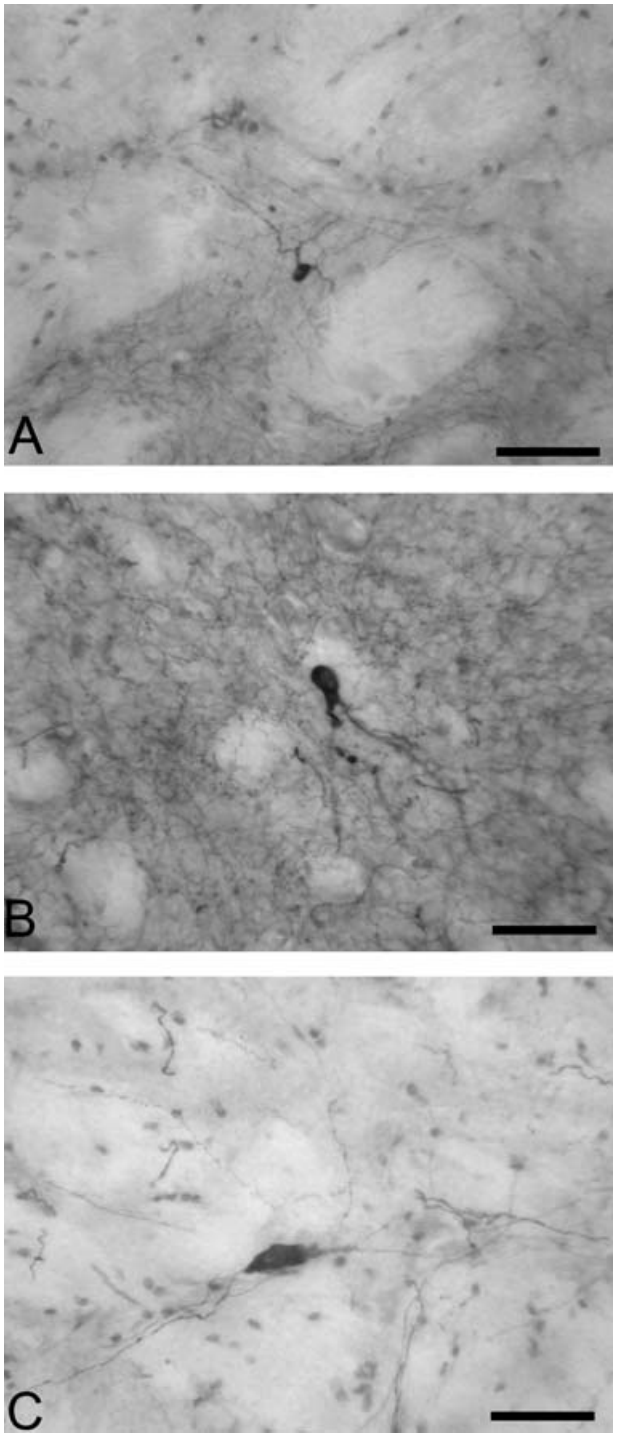


Fig. 6 Dopaminergic differentiation of grafted hNPCs. TH-ir cells with obvious processes were found in the right (lesioned and transplanted) striatum (A–C). Scale bars = 50 μm.

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.

Acknowledgements

We acknowledge Dr Perrine Barraud and Dr Deniz Kirik (Wallenberg Neuroscience Center, Lund, Sweden) for sharing their knowledge of neonatal rat surgery, and also Anke Sund (Institute of Anatomy, University of Rostock) for skilful technical assistance. M.H. was financially supported by the German Academic Exchange Service (DAAD).

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Original Paper

Cells Tissues Organs

Cells Tissues Organs
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

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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.

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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.

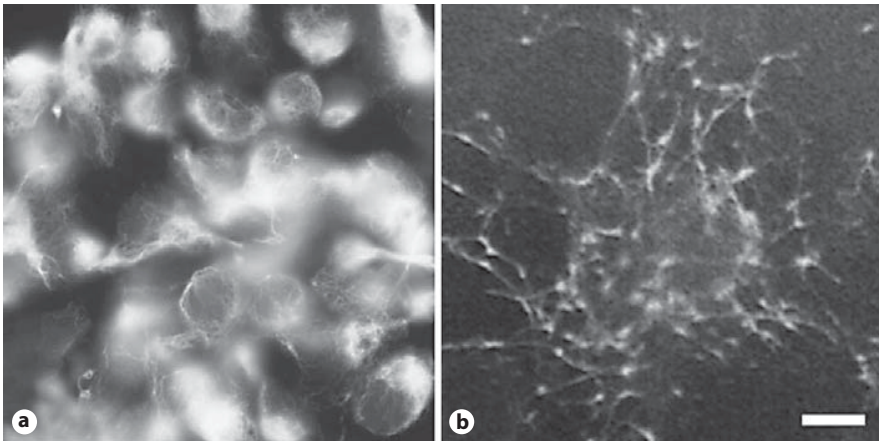

Fig. 1. In vitro morphology of hNPCs. Undifferentiated hNPCs possess a flat neuroepithelial morphology and are nestin-ir (**a**), whereas after differentiation as plated spheres (**b**) the cells show a neuronal morphology with elongated processes and a moderate number express TH. Scale bar: **a** 10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

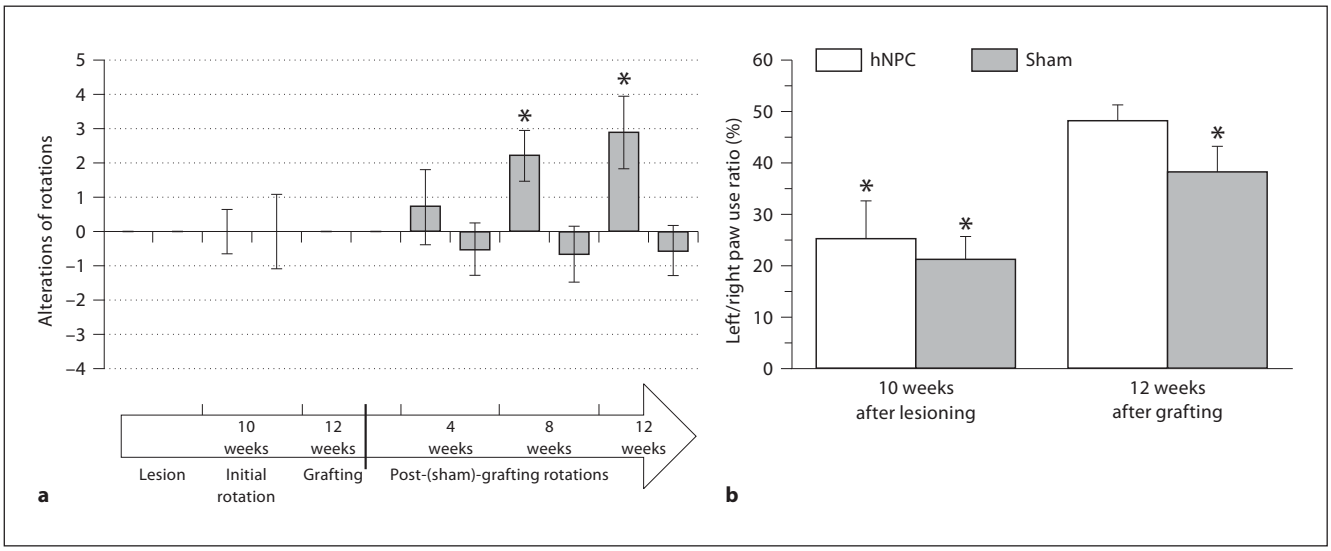

Fig. 2. a Motor asymmetry tests. Changes in apomorphine-induced rotation balanced to initial rotation values of the sham control (left) and the transplantation group (right) 10 weeks after lesioning. Sham control animals (n = 10) displayed a significant ($p \leq 0.05$) increase in rotations per minute (+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-

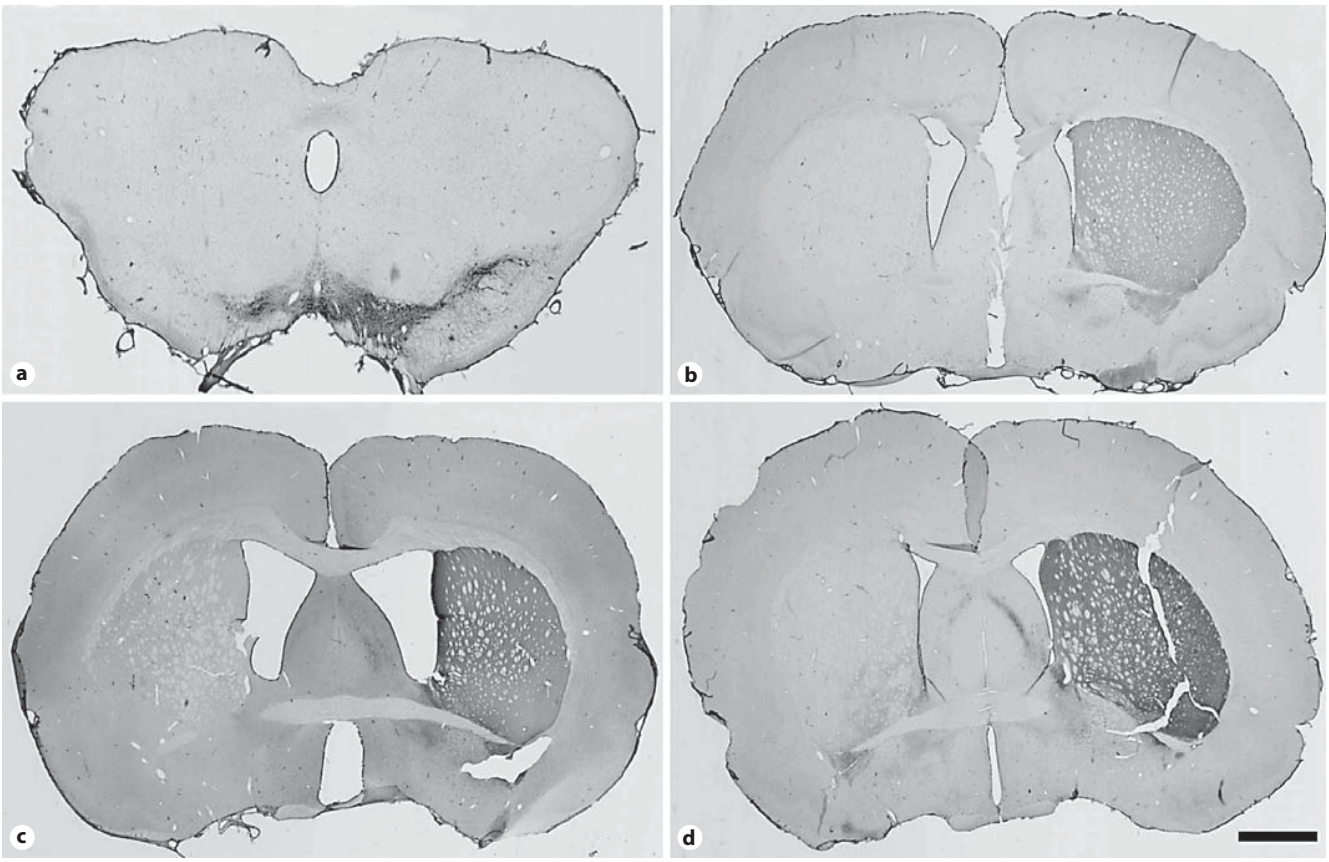

Fig. 3. TH-ir in frontal sections containing the region of the midbrain (**a**) or the striatum (**b–d**) of the various animal groups. Unilateral 6-OHDA lesioning leads to a nearly complete loss of DAergic neurons in the substantia nigra (**a**) and subsequently to a downregulation of TH-ir in the ipsilateral 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

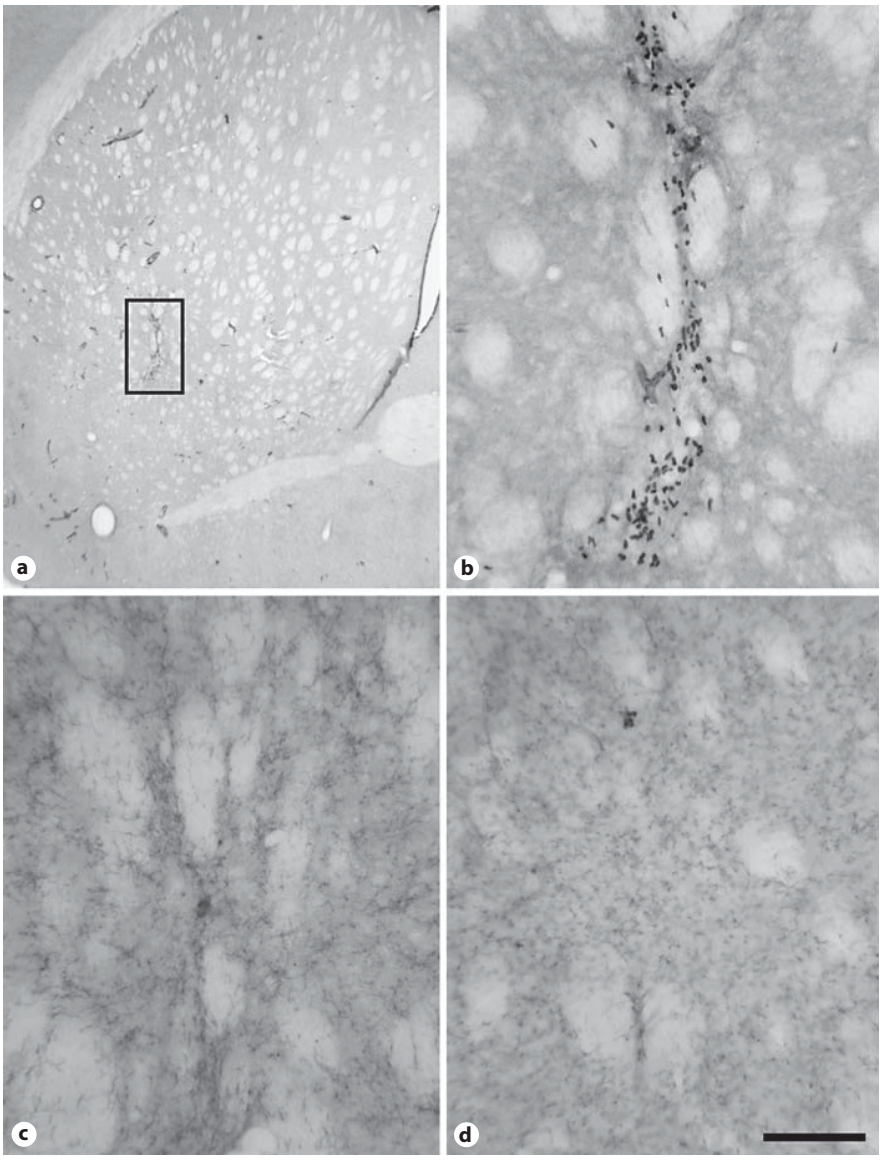

Fig. 4. Distribution of grafted cells stained for HN and host inflammation response revealed by OX-42-ir. **a** Overview of a frontal section through the striatum shows the position of HN-ir-grafted cells (inset) by DAB visualization. **b** By higher magnification of the inset, mainly 2 phenotypes of 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

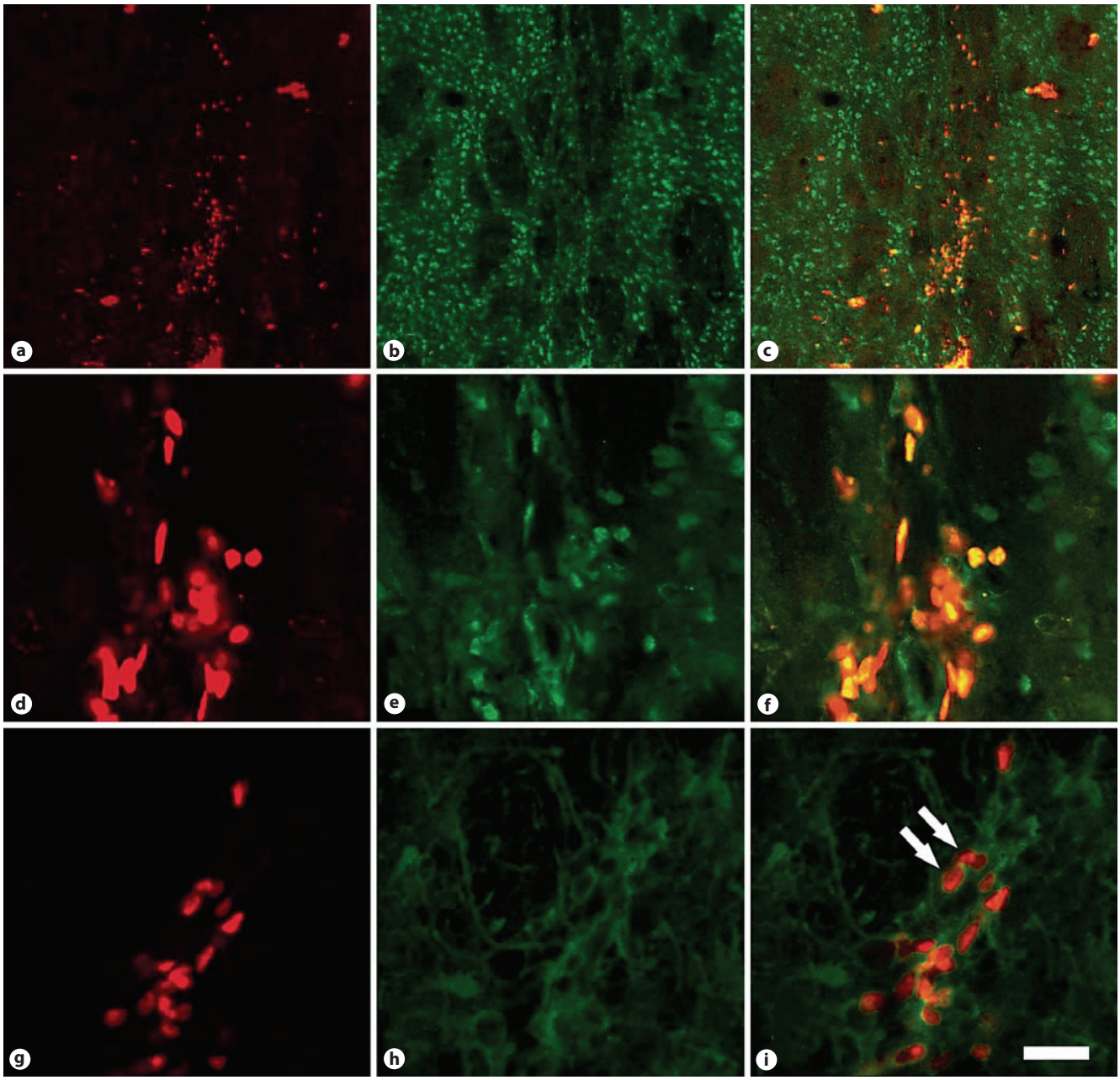

Fig. 5. Survival, migration and differentiation of transplanted hNPCs in the rat striatum. Multipotential differentation of transplanted hNPCs was evaluated by red fluorescent staining of HN (**a**, **d**, **g**) and green fluorescent labeling of NeuN (**b**, **e**) or GFAP (**h**), merged in **c**, **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).

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Declaration on contribution to publications

Publication 1: Hovakimyan M, Weinreich K, Haas SJ, Cattaneo E, Rolfs A, Wree A (2008) In vitro characterization of embryonic ST14A cells. International Journal of Neuroscience 118: 1489-1501

- Culturing of ST14A cells under various culture conditions
- In vitro immunocytochemistry
- Fluorescence microscopy (in collaboration with SJP Haas)
- Statistical analysis

Publication 2: Hovakimyan M, Haas SJ, Schmitt O, Gerber B, Wree A, Andressen C (2006) Mesencephalic human neural progenitor cells transplanted into the neonatal hemiparkinsonian rat striatum differentiate into neurons and improve motor behaviour. Journal of Anatomy 209: 721-732

- In vitro immunocytochemistry
- Contribution to the behavioural tests and evaluation of results
- Immunohistochemistry on brain slices
- Light and fluorescence microscopy

Publication 3: Hovakimyan M, Haas SJ, Schmitt O, Gerber B, Wree A, Andressen C (2008) Mesencephalic human neural progenitor cells transplanted into the adult hemiparkinsonian rat striatum lack dopaminergic differentiation but improve motor behaviour. Cells Tissues Organs 188: 373-383

- In vitro immunocytochemistry
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ACKNOWLEGEMENTS

Firstly, I would like to thank Prof. Dr. Andreas Wree for giving me an opportunity to conduct this study in the Department of Anatomy of the University of Rostock.

I would like to express sincere gratitude to my research adviser Prof. Dr. Oliver Schmitt for his support during my research in Germany and for the fruitful suggestions and constant encouragement.

Thanks to Dr.rer.nat. Stefan Jean-Pierre Haas for introducing me to laboratory work and special techniques like animal behaviour testing, immunohistochemistry, immunofluorescence, light and fluorescence microscopy. The daily work, inspirations and the scientific expertise of Stefan were extremely helpful.

I am also grateful to PD Dr.rer.nat. Christian Andressen for sharing his knowledge in cell culturing, immunocytochemistry as well as many discussions about our publications.

I appreciate the efforts of all colleagues of the "Journal club" for being a part of the vital scientific discussions. Their critical suggestions were highly influential on my research work and their help admirable.

I thank Barbara Kuhnke, Frauke Winzer and Antje Schümann for being very friendly co-workers and for their efficient technical assistance.

A lot of thanks go to Gabriele Janik for her help in the official matters and for being always kind and friendly.

My particular thanks also go to Prof. Dr. Dieter Weiss for his support, fruitful discussions and help.

I also thank Prof. Dr. Stefan Porembski for every advice, support and for always having time for me.

I am grateful to Prof. Dr. Rudolf Guthoff for his continuous motivation and for believing in me.

I thank Prof. Dr. Armen Zakarian for lending helping hands to me whenever required and the sympathy he showed to me, in addition to scientific suggestions.

The study was made possible by the financial support of DAAD (German Academic Exchange Service) and actually of the SFB Transregio 37.

Finally, I am deeply indebted to my family, especially my husband Vahan, my daughter Anna-Maria and my mother Anahit for their sincere care and continuous support without which it would not have been possible to accomplish the targets.

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EDUCATION

October, 2004 – PHD student at the University of Rostock

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Yerevan State University, Yerevan, Armenia

Faculty of Biology

Concentrations: Biochemistry and Biophysics

Diploma thesis: The venom influence of Transcaucasian vipera lebetina obtusa on free radical oxidation of membranous lipids of tissue of some vertebrates

Master of Biology

September, 1996 – June, 2000

Yerevan State University, Yerevan, Armenia

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Concentrations: Biochemistry

Diploma thesis: The influence of gibberellic acid on amylase and dehydrogenate activeness of the wheat

Bachelor in Biochemistry

ADDITIONAL EDUCATION

August – September, 2004

Herder Institut, Leipzig, Germany

Intensive course German as a foreign language (diploma)

SCHOLARSHIPS AND AWARDS

September, 2005

Awarded to participate in Spetses (Greece) Summer School, course "Structural Biology"

June, 2005

Participation in the Interdisciplinary Lindau Meeting of Nobel Laureates 2005 (recommended by DAAD)

May, 2004

German Academic Exchange Service (DAAD)

Awarded the DAAD-scholarship to undertake the 10 month postgraduate research at the University of Rostock (Germany) starting in October, 2004.

May, 1994

Ministry of Education of the Republic Armenia

Awarded Gold medal 'For outstanding results in studies' completing the secondary school.

PROFESSIONAL EXPERIENCE

August, 2007-present

Department of Ophthalmology, Medical Faculty of University of Rostock

September, 2005 – August 2007

Institute of Anatomy, Medical Faculty of University of Rostock

Work on the project: "Transplantation of expanded human neural progenitor cells into unilaterally 6-OHDA depleted striatum of neonatal and adult rats"

January, 2003 – August, 2004

Scientific-Research Institute of Spa treatment and Physical medicine, Ministry of Health, Armenia

PUBLICATIONS:

1. Ayvazyan N, Zakharyan A, Hovakimyan M (2003) "New approaches in studies of Vipera lebetina obtusa venom action on lipids of nervous tissue of vertebrates". 12th Ordinary General Meeting of societas Europaea Herpetologica, 12-16 August, St. Petersburg, Russia

2. Ayvazyan N, Zakharyan A, Hovakimyan M (2003) "Influence of snake venom upon lipid peroxidation of brain". Materials of the 1st International Conference "Modern aspects of rehabilitation in medicine", 23-25 September Yerevan, Armenia

3. Hovakimyan M, Haas SJP, Briese V, Ulfig N, Wree A, Andressen C (2005) Human neural stem cells differentiate into neurons and astrocytes after transplantation into the adult rat brain. Ann Anat: 187 (Suppl) ip. 113

4. Hovakimyan M, Haas SJP, Schmitt O, Gerber B, Wree A, Andressen C (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

5. Haas SJP, Hovakimyan M, Schmitt O, Gerber B, Wree A, Andressen C (2007) Transplantation of mesencephalic human neural progenitor cells into the caudate putamen of Hemiparkinsonian rats. Proceedings of seventh Göttingen meeting of the German Neuroscience Society, T3-3C

6. Hovakimyan M, Weinreich K, Haas SJP, Cattaneo E, Rolfs A, Wree A (2008) In vitro characterization of embryonic ST14A-cells. Int J Neurosci 118: 1489-1501

7. Hovakimyan M, Haas SJP, Schmitt O, Gerber B, Wree A, Andressen C (2008) Mesencephalic human neural progenitor cells transplanted into the adult hemiparkinsonian rat striatum lack dopaminergic differentiation but improve motor behaviour. Cells Tissues Organs 188: 373-383

8. Hovakimyan M, Spasova M, Wree A, Schmitt O (2008) In vitro differentiation of the immortalized mesencephalic progenitor cell line CSM 14.1 occurs independently from hematopoietic cytokines. Submitted to In Vitro Cell Dev Biol Anim.

9. Stachs O, Zhivov A, Kraak R, Hovakimyan M, Wree A, Guthoff R (2009) Mapping of subbasal nerve fibers in the normal cornea, after refractive surgery and penetrating keratoplasty by in vivo laser scanning confocal microscopy. Submitted to J Refract Surg.

ADDITIONAL SKILLS

Languages: Armenian - native, Russian - fluent, German – fluent, English-very good, French-basic knowledge

Computers: MS Access, MS Excel, MS Word, MS PowerPoint, Corel Draw, Photoshop

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ERKLÄRUNG

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den ....

Marine Hovakimyan