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Orthotopic transplantation of immortalized mesencephalic progenitors (CSM14.1 cells) into the substantia nigra of hemiparkinsonian rats induces neuronal differentiation and motoric improvement

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Abstract

Neural progenitor cell grafting is a promising therapeutic option in the treatment of Parkinson's disease. In previous experiments we grafted temperature-sensitive immortalized CSM14.1 cells, derived from the ventral mesencephalon of E14-rats, bilaterally in the caudate putamen of adult hemiparkinsonian rats. In these studies we were not able to demonstrate either a therapeutic improvement or neuronal differentiation of transplanted cells. Here we examined whether CSM14.1 cells grafted bilaterally orthotopically in the substantia nigra of hemiparkinsonian rats have the potential to differentiate into dopaminergic neurons. Adult male rats received 6-hydroxydopamine into the right medial forebrain bundle, and successful lesions were evaluated with apomorphine-induced rotations 12 days after surgery. Two weeks after a successful lesion the animals received bilateral intranigral grafts consisting of either about 50 000 PKH26-labelled undifferentiated CSM14.1 cells (n = 16) or a sham-graft (n = 9). Rotations were evaluated 3, 6, 9 and 12 weeks post-grafting. Animals were finally perfused with 4% paraformaldehyde. Cryoprotected brain slices were prepared for immunohistochemistry using the freeze-thaw technique to preserve PKH26-labelling. Slices were immunostained against neuronal epitopes (NeuN, tyrosine hydroxylase) or glial fibrillary acidic protein. The CSM14.1-cell grafts significantly reduced the apomorphine-induced rotations 12 weeks post-grafting compared to the sham-grafts ($P < 0.05$). There was an extensive mediolateral migration (400–700 μm) of the PKH26-labelled cells within the host substantia nigra. Colocalization with NeuN or glial fibrillary acidic protein in transplanted cells was confirmed with confocal microscopy. No tyrosine hydroxylase-immunoreactive grafted cells were detectable. The therapeutic effect of the CSM14.1 cells could be explained either by their glial cell-derived neurotrophic factor-expression or their neural differentiation with positive effects on the basal ganglia neuronal networks.

Key words cell therapy; glial cell-derived neurotrophic factor; neural transplantation; Parkinson's disease.

Introduction

Parkinson syndrome comprises different severe and progressive neurodegenerative disturbances primarily affecting the extrapyramidal system. After several years of treatment with levodopa, motoric fluctuations develop in many patients with Parkinson's disease. The symptoms of Parkinson syndrome follow if 80–85% of dopaminergic neurons of the substantia nigra (SN) undergo cell death accompanied by a related 60–80% dopaminergic deafferentiation of the striatum (Marsden, 1982).

The reconstitution of dopaminergic innervation by cell grafting to a deafferentiated striatum has been conducted over the past 30 years and has proved to be successful in animal models of Parkinson's disease (Björklund, 1992; Brundin & Wictorin, 1993; Herman & Abrous, 1994). Since 1987 over 300 patients have received human fetal mesencephalic progenitor cells (Björklund & Lindvall, 2000; Lindvall & Hagell, 2002). In some of these patients levodopa could be deposed (Lindvall, 1997; Olanow et al. 1997), and in *post mortem* studies (Kordower et al. 1995) a reinnervation of the striatum of up to 10 years was reported. However, success is still limited in humans (Freed

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et al. 2001; Olanow et al. 2003). Moreover, grafting human dopaminergic neural progenitors as a routine therapeutic option (Piccini et al. 1999) has not been accomplished due to ethical limitations (Martinez-Serrano & Björklund, 1997; Björklund & Lindvall, 2000).

Advances in neural stem cell biology have demonstrated how multipotent neural progenitors can be isolated, expanded, and used for brain transplants. However, the location of transplantation, the type of multipotent neural progenitors, the graft and host species have been the subject of many studies. Several conditionally immortalized cell lines of multipotent neural progenitors were established from brains of rodent embryos (Frederiksen et al. 1988; Redies et al. 1991; Snyder, 1994; Cattaneo & Conti, 1998). Haas & Wree (2002) demonstrated that nestin-expressing CSM14.1 cells (Zhong et al. 1993; Anton et al. 1994, 1995) differentiate *in vitro* to tyrosine hydroxylase and aldehyde dehydrogenase (ALDH2)-containing neurons. CSM14.1 cells were derived from the mesencephalon of a 14-day-old rat embryo, retrovirally immortalized with the temperature-sensitive SV40 Large T-antigen. In CSM14.1 cells a post-mitotic dopaminergic phenotype is induced by temperature shift. Dopaminergic predifferentiated multipotent neural progenitors give rise to a motoric improvement after transplantation in animal models (Studer et al. 1998; Kawasaki et al. 2000; Sawamoto et al. 2001; Storch et al. 2001; Kim et al. 2002). Hence CSM14.1 cells seem to be promising candidates for transplantation into hemiparkinsonian rats. Transplanting CSM14.1 cells to the orthotopic SN may regulate differentiated graft-neurons by striatonigral projections. The release of dopamine in the SN affects the motoric behavior (Kozlowski et al. 1980; Jackson & Kelly, 1983a,b; Robertson & Robertson, 1989). Nikkhah et al. (1994) demonstrated that microtransplantation of fetal dopaminergic cells into the SN pars reticulata (SNr) yields an excellent survival of the grafted cells and a dense reinnervation of the SNr. These effects following multipotent neural progenitor-substantia nigra transplantation have been confirmed by other investigators (Olsson et al. 1995; Mendez et al. 1996; Yurek, 1997).

The scope of this contribution is to investigate behavioral and morphological changes followed by orthotopic transplantation of CSM14.1 cells into the SN of adult rats and to compare these results with previous findings concerning heterotopic striatal grafting.

Materials and methods

Animals

We used 25 adult male Wistar rats (Charles River, Sulzfeld, Germany), weighing 280–310 g at the beginning of the experiments. They were housed at 22 ± 2 °C under a 12-h light/dark cycle with free access to food and water. All animal care and handling was conducted in compliance with the regulations and licensing of the local authorities.

6-Hydroxydopamine (6-OHDA) lesion surgery

The substantia nigra of these rats – deeply anesthetized with pentobarbital-Na^+ (45 mg kg^{-1} i.p.) – were unilaterally lesioned by an injection of 6-OHDA-HCl (Sigma, Taufkirchen, Germany) into the right medial forebrain bundle (4 μL per 26 μg, coordinates referring to IA: AP + 6.7, ML – 1.5, V + 1.5; Paxinos & Watson, 1998). Prior to injection of 6-OHDA, animals received desipramine (Sigma, 2.5 mg kg^{-1} i.p.) to protect peripheral and central catecholaminergic non-dopaminergic cells.

Cultivation of CSM14.1 cells

Conditionally immortalized CSM14.1 cells (kindly provided by Prof. Dale Bredesen, Buck Institute for Age Research, Novato California) were cultured and expanded as described previously (Zhong et al. 1993; Haas & Wree, 2002) in Petri dishes (Nunc, Wiesbaden, Germany) containing DMEM supplemented with 10% fetal calf serum (FCS), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (all reagents from Gibco, Karlsruhe, Germany) in a humidified incubator (95% air, 5% CO_2, at 33 °C). The cells were passaged every 3rd day before reaching confluence. For Western blotting, cells were washed with sterile phosphate-buffered saline (0.1 M PBS, pH 7.4), scraped from the Petri dishes and solved in PBS, followed immediately by three freezing and thawing cycles. The whole protein content of these lysates was determined by a spectrophotometer (Beckman, Model DU640, Fullerton, USA) and a bicinchoninic assay (Pierce Chemical Co., Rockford, USA). For immunocytochemistry cells were cultured on poly-L-lysine coated glass slides until reaching confluence, then washed with isotonic sodium chloride solution and finally fixed with 3.7% paraformaldehyde dissolved in PBS (pH 7.4).

Intranigral cell transplantation

Fourteen days after successful unilateral lesion of the substantia nigra pars compacta (determined by apomorphine-induced rotations) 16 animals received bilateral intranigral cell grafts, each containing about 50 000 viable CSM14.1 cells, solved in 0.5 μL DMEM (coordinates referring to IA: AP + 3.8, ML ± 2, V + 2; Paxinos & Watson, 1998) injected using a 5-μL syringe (Hamilton, Bonaduz, Switzerland). Prior to transplantation the cells were labelled with the lipophilic fluorescent dye PKH26 (Sigma) according to Haas et al. (2000). In brief, CSM14.1 cells were trypsinized, collected by centrifugation, washed with DMEM and pellets containing approximately 2×10^7 cells were solved in 1 mL of diluent C (PKH26 Fluorescent Cell Linker Kit, Sigma). Cells were then suspended, immediately mixed with an equal volume of a 2× PKH26 stock solution in diluent C

and further suspended at room temperature. After 5 min, an equal volume of FCS was added to stop the labelling reaction. The cells were again pelleted, transferred to a fresh centrifuge tube and washed in DMEM/10% FCS. Following labelling, cells were washed three times in DMEM and finally resuspended in DMEM. Viable cell number was estimated using Trypan blue staining (Sigma) and viability, after labelling, was over 95%. Final cell concentration was about 10^5 viable cells μL^{-1}. Nine animals received sham-grafts into the same region consisting of 0.5 μL of DMEM. Surgeries were performed under pentobarbital-Na^+-anesthesia (45 mg kg^{-1} i.p.).

Apomorphine-induced rotations and statistics

We used the D2-receptor agonist apomorphine, which causes contralateral rotations, as a way to determine completeness of the 6-OHDA-lesions in living animals, as it is difficult to determine the completeness of a striatal dopaminergic deafferentiation by amphetamine (Hefti et al. 1980; Heikkila et al. 1981; Casas et al. 1988; Carman et al. 1991; Hudson et al. 1993; Moore et al. 2001). Apomorphine-induced rotations (Teclapharm, 0.25 mg kg^{-1} s.c.) were monitored over 40 min, 12 days after lesion and regularly for 12 weeks after transplantation using an automated rotometer system according to Ungerstedt & Arbuthnott (1970). Only animals which rotated more than 4 contralateral rotations min^{-1} 12 days after lesion, indicating a unilateral death of about 97% of the nigrostriatal dopaminergic neurons (Ungerstedt & Arbuthnott, 1970), were used for transplantation or sham-transplantation studies. As an internal control the rotations of unlesioned animals (n = 8) were also tested (0.15 rotations min^{-1} ± 0.32). Thereafter, rotations were measured 3, 6, 9 and 12 weeks after CSM14.1 cell-transplantation or DMEM injection. Finally, the mean net-rotations of the sham group (n = 9) and the transplanted group (n = 16) were compared. Because the sample sizes are relatively small the distributions of the absolute rotations were tested by means of the Kolmogorov–Smirnov test. As this test produced a significant result for the existence of normal distributed data, the apomorphine-induced rotational behavior of the animals was analyzed statistically by the t-test. Differences of $P \leq 0.05$ are considered to be significant. Tests were performed with SPSS 11.01 (SPSS Inc., Chicago, USA).

Fast Blue injections

To investigate the possible outgrowth of intranigrally transplanted CSM14.1 cells into the lesioned caudate putamen we used the retrograde neuronal tracer Fast Blue (Sigma). Transplanted (n = 4) and sham-transplanted animals (n = 3) – deeply anesthetized with pentobarbital-Na^+ (45 mg kg^{-1} i.p.) – received 4 × 0.5 μL injections of a solution containing 2% of 'Fast Blue' at different sites into the dopaminergic deafferentiated caudate putamen 5 days before being euthanized. The coordinates referring to bregma according to Paxinos & Watson (1998) were: (1) AP –1, ML –4, V (dura) –3, (2) AP –1, ML –4, V (dura) –5, (3) AP + 1, ML –2.5, V (dura) –3, (4) AP –1, ML –2.5, V (dura) –5.

Immunohistochemical procedures

Preparation of the animals for immunohistochemistry and histology was done 12 weeks after transplantation. After anesthesia with ether, the rats were perfused transcardially with ice cold 0.9% sodium chloride (50 mL), followed by 400 mL of 3.7% paraformaldehyde (dissolved in 0.1 M PBS, pH 7.4). Brains were immediately removed from the skull, postfixed for 4 h, and transferred into PBS (pH 7.4) containing 20% sucrose overnight at 4 °C. The cryoprotected brains were frozen in isopentane (–50 °C) and stored at –80 °C until further processing.

For immunofluorescent stainings of brain sections, containing the PKH26-labelled grafted cells, immunohistochemistry was performed as described previously for the staining of brain tissue sections without the use of detergents (Haas et al. 2000). In brief, 30-μm-thick coronal cryostat sections containing the mesencephalic region were stored free floating overnight in PBS containing 30% sucrose at 4 °C. Cryoprotected brain sections were pretreated for immunohistochemical staining by successive freeze/thaw cycles (five times) in hyperosmotic medium and liquid nitrogen, to make intracellular epitopes accessible for antibodies. The free-floating sections were then washed twice in 0.1 M PBS (pH 7.4) and incubated for 2 h at room temperature in PBS containing 3% bovine serum albumin (BSA, Sigma) and 5% normal goat serum (Gibco), followed by an incubation overnight at 4 °C with primary antibodies (solved in PBS, 1% BSA) against tyrosine hydroxylase (TH, mouse monoclonal, clone TH-2, 1 : 500, Sigma), glial fibrillary acidic protein (GFAP, mouse monoclonal, 1 : 400 or rabbit polyclonal, 1 : 100, both from Sigma) or neuronal nuclei antigen (NeuN, mouse monoclonal, 1 : 1000, Chemicon). Tissue sections were then washed three times in 0.1 M PBS and incubated overnight at 4 °C with Cy2-conjugated anti-mouse IgG (goat polyclonal, 1 : 400, Dianova, Hamburg, Germany) and AMCA-conjugated anti-rabbit IgG (goat polyclonal, 1 : 100, Dianova). After three rinses in 0.1 M PBS, specimens were mounted on gelatine-coated glass slides and embedded in anti-fading fluorescence mounting medium.

For light microscopy of TH-immunohistochemistry – in the substantia nigra and the basal ganglia – coronal or sagittal sections were visualized by the ABC-method (Vector Laboratories, Burlingame, USA) with the chromophore DAB. The same staining procedure for all sections was performed for all animals to guarantee a standardized staining protocol. After two rinses in 0.1 M PBS, 30-μm sections were incubated for 20 min in 3% H_2O_2 to quench endogenous

peroxidases. Sections were then rinsed three times in 0.1 M PBS and incubated for 1 h in 0.1 M PBS containing 0.025% Triton X-100 (Sigma), 3% BSA and 3% normal horse serum (Vector Laboratories), followed by an incubation over 48 h at 4 °C with the primary antibodies against TH (mouse monoclonal, clone TH-2, 1 : 1000, Sigma), solved in 0.1 M PBS containing 0.05% Triton X-100 and 1% BSA. After three washes in 0.1 M PBS, sections were incubated overnight at 4 °C with biotinylated secondary antibodies directed against mouse IgG (horse polyclonal, 1 : 200, Vector Laboratories) followed by three washes in 0.1 M PBS, then for 2 h in the ABC-complex (1 : 50 for solutions A and B, Vector Laboratories), and after three rinses in 0.1 M PBS the final detection with 0.02% DAB (Sigma) for 8 min at room temperature was performed. Sections were mounted onto gelatine-coated glass slides, dehydrated in graded alcohol concentrations and coverslipped with DePeX mounting medium (Serva, Heidelberg, Germany).

Nissl- and immunocytochemical stainings of cultured cells

Fixed CSM14.1 cells were stained for 3 min at room temperature with 0.1% cresyl violet acetate (Sigma), washed in water, dehydrated in graded alcohol concentrations and also coverslipped with DePeX mounting medium (Serva). Immunocytochemistry of fixed CSM14.1 cells was performed as described previously (Haas & Wree, 2002). Anti-glial cell line-derived neurotrophic factor (GDNF, goat polyclonal, 1 : 100) and anti-ciliary neurotophic factor (CNTF, goat polyclonal, 1 : 100) were obtained from R&D Systems (Wiesbaden, Germany). Immunoreactive structures were also detected by the ABC-Method (Vector).

Stereology

An estimation of the numerical density of TH-immunoreactive (TH-ir) neurons of the whole substantia nigra pars compacta was performed by means of unbiased stereological counting using the optical fractionator method (West et al. 1991). The total number of TH-ir neurons could not be determined because different stainings were applied to serial sections. Evaluation was realized with the STEREOINVESTIGATOR program (Microbrightfield Inc., Williston, VT) and a BX-51 microscope (Olympus, Hamburg, Germany) equipped with a three-axis motor system and a microcator. On each section the substantia nigra pars compacta was outlined. Counting frames (70 × 70 μm) were placed at the intersection of randomly placed grids. The coefficient of error (West & Gundersen, 1990) was smaller than 0.1.

Western blotting

Detection of GDNF and CNTF contents in CSM14.1-protein lysates was done using Western blotting as previously described (Haas & Wree, 2002). Cell-lysates containing 50 μg of whole protein were loaded per lane. Anti-GDNF (goat polyclonal, 1 : 1000) and CNTF (goat polyclonal, 1 : 1000) were also obtained from R&D Systems. Recombinant rat GDNF and rat CNTF (both from R&D Systems) were used as positive controls. The housekeeping protein β-actin was detected as well on these Western blots to verify loading of cell lysates (anti-β-actin, 1 : 3000, Sigma). Immunoreactive bands were detected by the enhanced chemoluminescence method (ECL-Kit, Amersham, Buckinghamshire, UK) on X-ray films (Agfa, Mortsel, Belgium).

Documentation of various stainings

For light microscopy, specimens were observed and documented with a Leitz Aristoplan microscope (Wetzlar, Germany) equipped with a digital camera (Coolpix 4500, Nikon, Tokyo, Japan). Fluorescent stainings and colocalizations were also observed and documented with a Leitz Aristoplan microscope or with a confocal laser scanning microscope (Model TCS SP2, Leica, Wetzlar, Germany).

Results

Morphologic control of lesioning

Rat brains were stereotactically lesioned by a 6-OHDA injection into the right medial forebrain bundle leading to a nearly complete dopaminergic neuron cell loss in the injected hemisphere. This is demonstrated by immunohistochemical visualization of TH in Fig. 1a, showing the deafferentiation of the Cpu, and in Fig. 1b, which shows the less intensive immunoreactive ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) in coronal and sagittal sections (Fig. 1c,d). Prior to transplantation, we ensured successful lesioning by performing the apomorphine-induced rotation test. For further experiments only those animals were used which exhibited a minimum of 4 contralateral rotations min^{-1}.

Neuronal cell counts

Unbiased stereology revealed no obvious differences in the number of TH-ir neurons in the substantia nigra pars compacta of CSM14.1- or sham-transplanted rats, either between the intact hemispheres or in the lesioned contralateral hemispheres. However, in both animal groups the dopaminergic cell loss in the lesioned substantia nigra pars compacta was over 97% compared to that in the intact contralateral side. The numerical density of TH-ir cells in the different experimental groups is summarized in Table 1.

Neurotrophin contents in CSM14.1 cells

Using Western blotting we documented GDNF expression in lysates of undifferentiated CSM14.1 cells, cultured

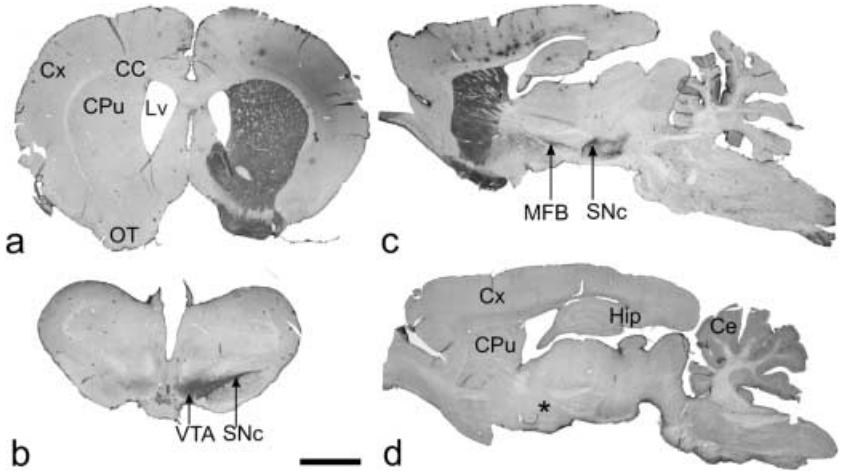

Fig. 1 Tyrosine hydroxylase distribution in the Hemiparkinsonian rat model. (a) Frontal section through the region of the CPu. The right hemisphere, i.e. the intact side, displays a strong TH-ir. The contralateral lesioned hemisphere contains no more obvious TH-ir after dopaminergic deafferentiation. (b) Frontal section through the mesencephalon containing the SNc. Immunoreactivity was found on the intact side at the right. On the lesioned side only a limited TH-ir was observed in the SNc and the VTA. (c) Sagittal section of an intact control hemisphere demonstrating TH-ir of the CPu, SNc, VTA and medial forebrain bundle (MFB). The arrows points to the intact MFB and SNc. (d) Sagittal section of a 6-OHDA lesioned hemisphere demonstrating abundance of TH-ir. The asterisk is placed over the location of 6-OHDA injection. Scale bar: 160 μm (a), 80 μm (b), 250 μm (c,d).

Table 1 Mean numerical density of TH-ir neurons [number mm^{-3}] in the intact and lesioned substantia nigra pars compacta of various groups

Group	Mean	SEM	*n*
Sham-transplanted intact side	3591	263	4
Sham-transplanted lesioned side	55	9	4
CSM14.1-transplanted intact side	3810	270	11
CSM14.1-transplanted lesioned side	45	6	11

under the permissive temperature of 33 °C (Fig. 2a). This was affirmed by immunocytochemistry as shown in Fig. 2b. An expression of CNTF was not found by Western blotting (Fig. 2d), which was also in agreement with a negative immunocytochemistry for CNTF (Fig. 2e).

Behavior

The differences of initial contralateral rotations (12 days after lesion) and 3, 6, 9 and 12 weeks after transplantation are shown in Fig. 3. The CSM14.1 transplantation group (transplanted group) rotated on average 9.59 times min^{-1} ± 2.49) and the sham-transplanted group (sham group) 7.2 times min^{-1} (± 2.3). The sham group exhibited an increase of rotations 3 weeks afterwards, to 7.74 rotations min^{-1} (± 2.58). Twelve weeks after sham-transplantation the sham group showed an increase of rotations of 9.39 min^{-1} ± 2.47). This amounts to a total increase of 3.19 rotations min^{-1} ± 2.47), or 35%, indicating that the rotational behavior deteriorated continuously. Conversely, the number of rotations of the transplanted group decreased in all by 2.93 rotations min^{-1} down to 6.66 min^{-1} (± 1.91). The differences of rotations min^{-1} between the transplanted group and the sham group were statistically significant at 9 weeks ($P = 0.053$) and 12 weeks ($P = 0.008$).

Migration and differentiation

Transplanted CSM14.1 cells were, due to the red fluorescent PKH26-labelling, localized around the injection site within the mesencephalon. Considerable amounts migrated over a distance of 700–1400 μm in the rostrocaudal or up to 400 μm in the mediolateral direction into the host tissue. Most transplanted CSM14.1 cells were localized in the substantia nigra (Fig. 4a–f). There were no obvious differences between the lesioned and the contralateral control side concerning migration. A tumor formation of transplanted cells or host tissue adjacent to the injection side was never observed.

The differentiation of a portion of transplanted cells into astrocytes, mainly of cells located directly in the injection tract, was revealed by GFAP-immunohistochemistry (Fig. 4e,f). We never found TH-ir of transplanted CSM14.1 cells. Hence, we exhibit confocal laser scanning microscopy to visualize colocalizations of PKH26 and TH at a suitable resolution. TH-ir neurons of the substantia nigra, which were not affected by the 6-OHDA lesion, are shown in Fig. 4(g,i). Cells in Fig. 4(j) that are exhibiting a yellow fluorescence, pretending a PKH26/TH colocalization, stem from different cells that spatially overlap. Moreover, we never detected transplanted PKH26-labelled cells that contained the retrograde tracer Fast Blue, indicating that transplanted cells did not project to the denervated

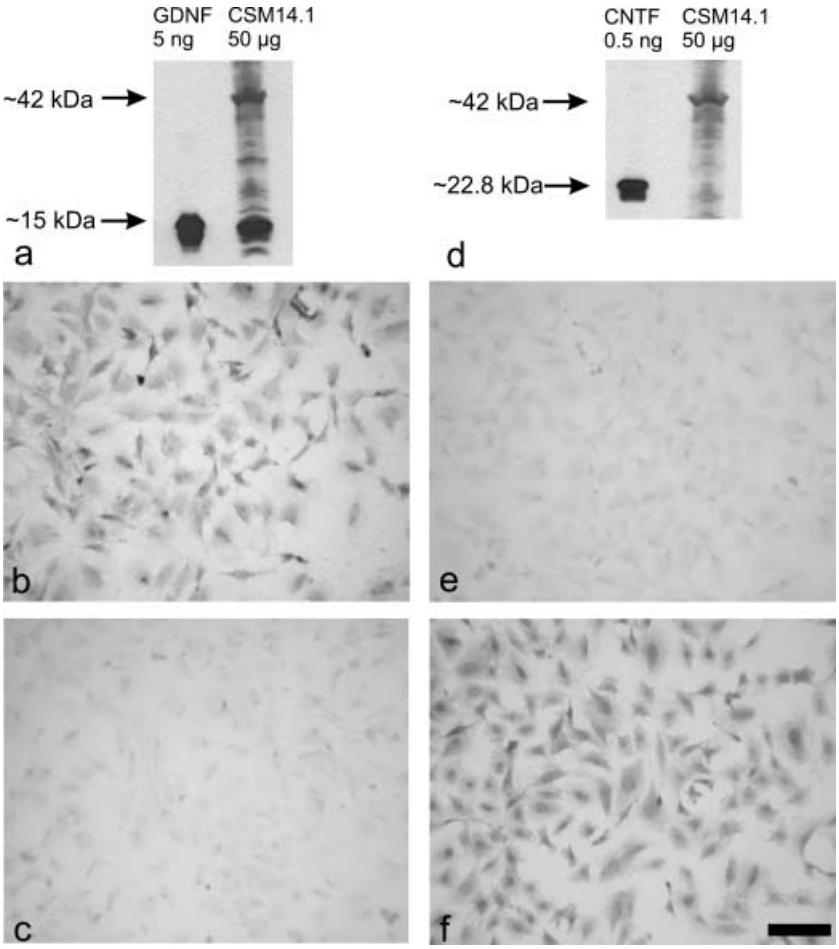

Fig. 2 Neurotrophine contents of CSM14.1 cells *in vitro*. (a) 50 µg protein lysate of CSM14.1 cells and the control probe of 5 ng recombinant rat GDNF were analyzed by Western blotting for GDNF. Specific bands in both probes around 15 kDa can be detected. A second specific band in protein lysates of CSM14.1 cells around 42 kDa belongs to the protein product of the housekeeping-gene β-actin, demonstrating the correct loading. Moreover, a distinct positive immunocytochemical reaction for GDNF in undifferentiated CSM14.1 cells was found (b), whereas the negative control (c) shows clear immunonegativity. (d) In a Western blot of another probe of CSM14.1 cells no immunoreactivity for CNTF can be detected, whereas 0.5 ng of recombinant rat CNTF shows a specific band around 22.8 kDa. Again in the CSM14.1-cell lysates the band around 42 kDa belongs to β-actin, demonstrating clearly the correct loading of the gel. The immunocytochemical staining for CNTF also does not show any immunopositive reaction (e), similar to the immunonegative control in (c). The Nissl-stain (f) demonstrates the morphology of adherent undifferentiated CSM14.1 cells. Scale bar: 100 µm (b,c,e,f).

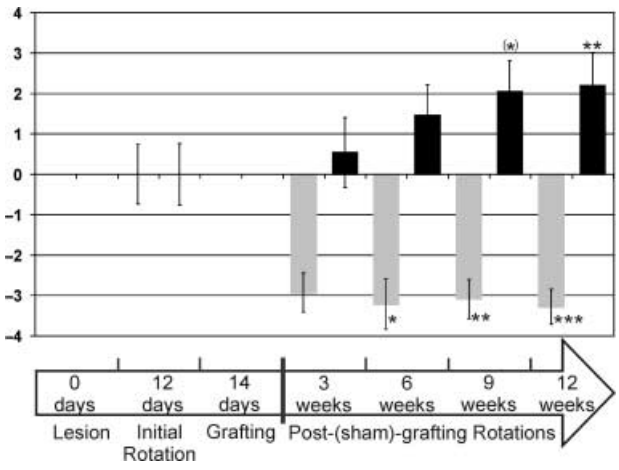

Fig. 3 Time scale of experiments and development of apomorphine-induced rotation behavior. The diagram shows the mean net-rotations/min of the experimental groups. Gray bars represent CSM14.1-transplanted animals (n = 16) and black bars the results of the sham-transplanted group (n = 9). The arrow below illustrates the time scale of experiments and behavioral measurements. It starts with the event of lesioning (0 days), the 6-OHDA injection effect was evaluated 12 days later by an initial rotation test and 2 days after these initial rotation measurements, (sham-)transplantations were performed. Three weeks, 6 weeks, 9 weeks and 12 weeks after (sham-)transplantation the rotations were further registered. Subtracting the number of contralateral rotations after the (sham-)transplantation from the number of initial rotations showed a trend (P = 0.053) towards a significant decrease of rotations after 9 weeks in the CSM14.1-transplanted group. This significant decrease declined further at 12 weeks (P = 0.008) after transplantation. Error bars show SEM.

striatum (not shown). Contrary to this observation, the several remaining endogenous nigrostriatal TH-ir neurons, lacking PKH26-labelling, in many cases contained Fast Blue (not shown).

The neuronal differentiation of intranigrally transplanted CSM14.1 cells was proved by colocalization of NeuN and PKH26 (Fig. 5). This type of colocalization was visible on the lesioned (Fig. 5c) as well as on the control side (Fig. 5d). This result was confirmed by confocal laser scanning microscopy (CLSM) (Fig. 5e). In the sham-transplanted substantia nigra, NeuN-ir but no PKH26-dye was detectable (Fig. 5f). The red immunofluorescence arose partly from autofluorescence of macrophages; however, that autofluorescence was clearly distinguishable from the brighter PKH26-labelling.

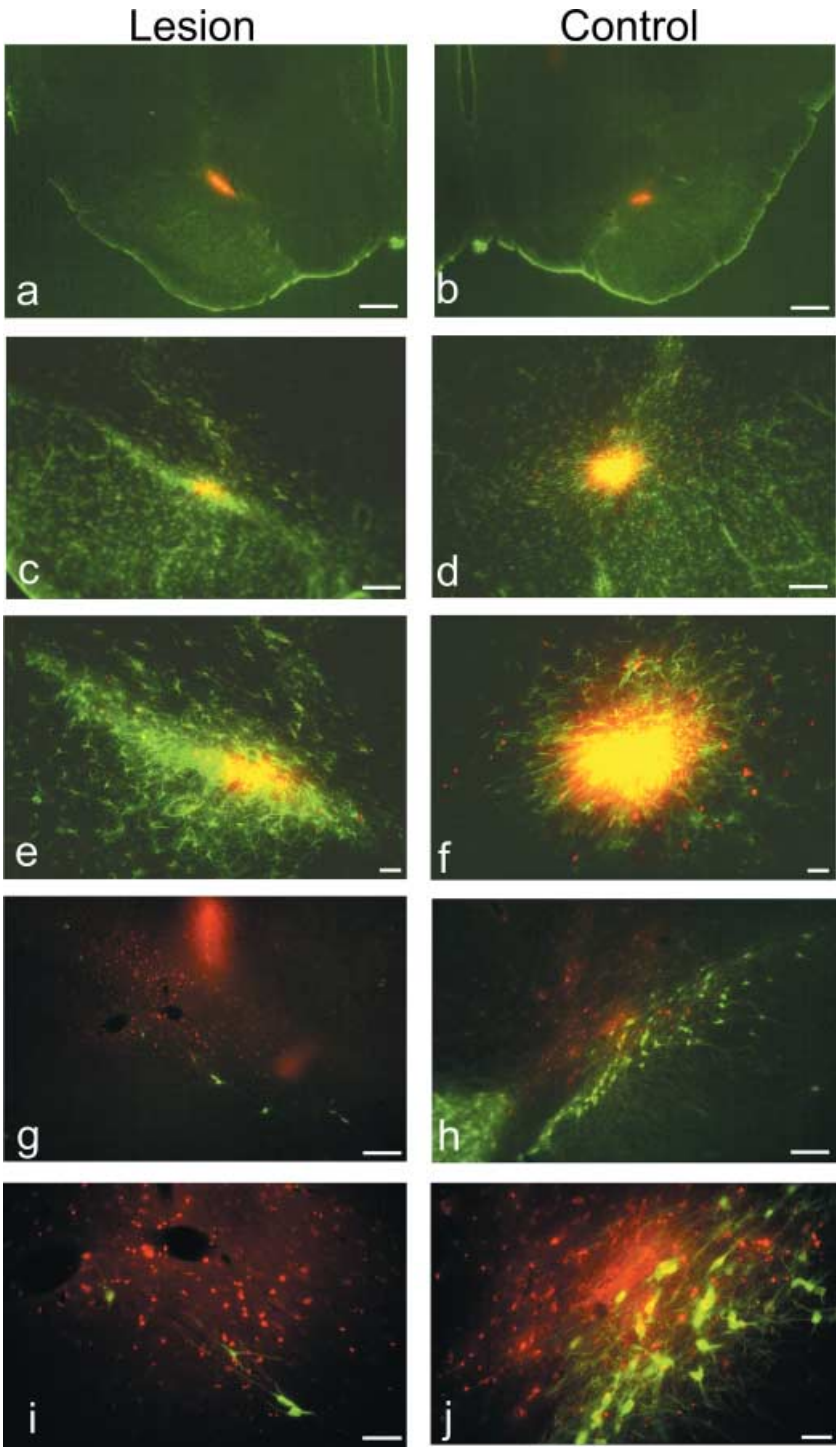

Fig. 4 Graft localisation and morphology in the SNc. Immunofluorescence micrographs of the lesioned hemisphere (left) and the contralateral intact control side (right). Immunofluorescence of GFAP (green) and PKH26 (red) marked CSM14.1 cells in the substantia nigra are shown for the lesioned side (a) and the contralateral intact side (b) as an overview at low magnification. PKH26-labelled transplanted cells migrated over a distance of about 400 mm and no obvious differences between the lesioned (c) or the control side (d) were observed. (e,f) Images represent a magnification of the specimen of the top. Parallel sections stained for TH (green fluorescence), demonstrate the correct placement of the PKH26-labelled grafts (red fluorescence) in the region of the lesioned SNc (g) or the intact contralateral control hemisphere (h). The distinct vertical transplantation needle tract can be detected in the upper part (g). Single dopaminergic TH-immunoreactive neurons, lacking PKH26-labelling survived the 6-OHDA lesion as demonstrated by green immunofluorescence (i), whereas the intact control side shows many TH-ir neurons (green) and also the survival of transplanted PKH26-labelled CSM14.1 cells (j). As well as in the lesioned SNc (i) and the intact control SNc (j) no colocalizations of PKH26 and TH of transplanted CSM14.1 cells within the VTA were observed (g,h). The yellow fluorescence signal (j) mimicking a PKH26/TH colocalization stems from different cells lying on top of another. Scale bars: 1000 μm (a,b), 200 μm (c,d, g,h), 70 μm (e,f,i,j).

Discussion

In this study we transplanted cell suspensions of the temperature-sensitive large-T-antigen immortalized neural progenitor cell line CSM14.1 derived from the embryonic (E14) ventral mesencephalon into the substantia nigra of adult hemiparkinsonian rats. The transplantation was exhibited on the 6-OHDA lesioned right SN as well as on the unlesioned left control SN of the same animal.

In a prior study, Haas & Wree (2002) described the neurogenic potential of CSM14.1 cells and the absence of immunoreactivity for glial proteins at non-permissive temperatures *in vitro*. These cells possessed a wider phenotypic differentiation potential after transplantation

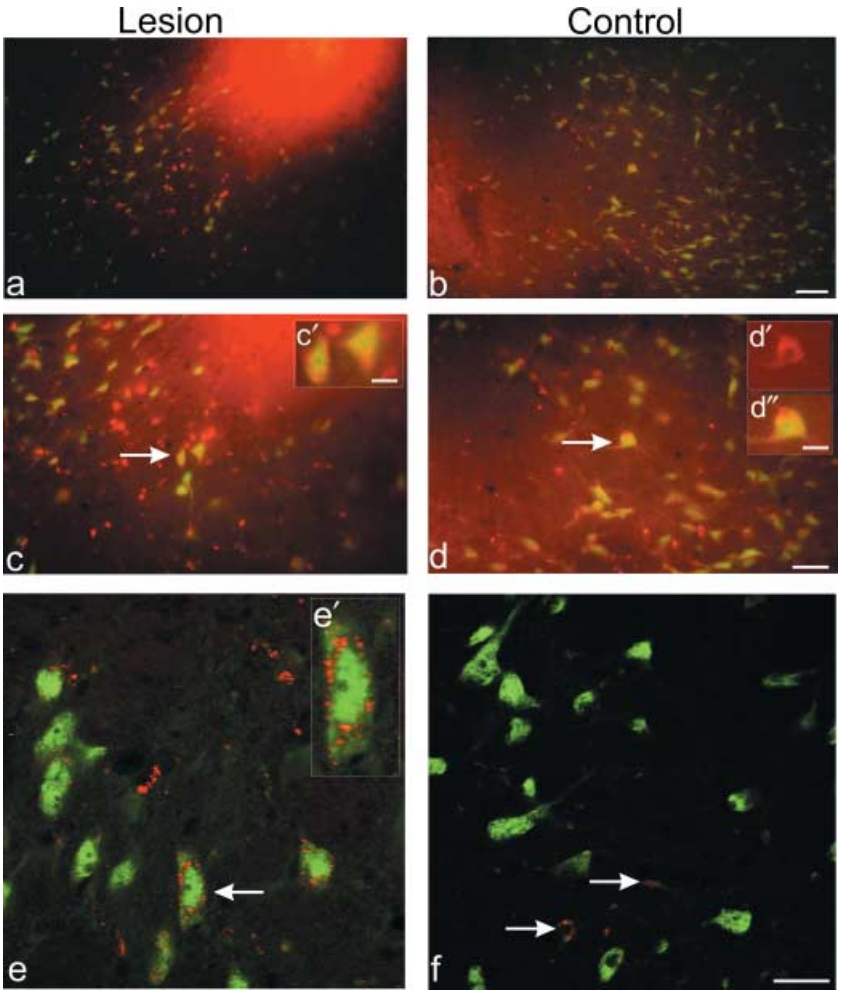

Fig. 5 Neuronal differentiation of CSM14.1 cells. NeuN-stained sections (green fluorescence) from the region of the SNc in the lesioned (left) and intact control side (right) are illustrated. Immunofluorescent micrographs of PKH26-labelled cells show a distinct NeuN-immunoreactivity, indicating an obvious neural phenotype of transplanted CSM14.1 cells in the lesioned (a) or intact control side (b). The core of the graft in the lesioned hemisphere appears with an intense red fluorescence in the upper right corner (a). Further evidence at higher magnification is displayed in (c) and (d) as c′, d′ and d″. CSM14.1 cells in the lesioned side are labelled by PKH26 and contain NeuN (c and c′). PKH26 (d′) and NeuN-colocalization (d″) of a transplanted CSM14.1 cell in the intact control side is also shown. CLSM-imaging (2-μm-thick optical sections) supplies further evidence for CSM14.1-cell survival and differentiation into NeuN-immunoreactive neurons in the lesioned side (e). The arrow points to a cell which is also documented at higher magnification (e′). Due to the lack of PKH26-labelled cells sham-transplanted animals never showed a PKH26/NeuN colocalization (f). In these animals only red autofluorescent host-derived macrophages, mainly lying around blood vessels (arrows), were detectable. These cells never contained NeuN. Moreover, due to their location and morphology they were clearly distinguishable from PKH26-labelled and transplanted NeuN-immunoreactive cells from transplanted animals. Scale bars: 70 μm (a,b), 50 μm (c,d), 20 μm (c′,d′,d″), 30 μm (e,f).

in vivo than *in vitro*. CSM14.1 cells have also been transplanted by Anton et al. (1994, 1995); however, so far there are no reports on their *in vivo* differentiation and phenotypic characterization due to the lack of appropriate cell labelling. Hence, a key point of this contribution is the investigation of the post-transplantational differentiation of CSM14.1 cells.

To compare *in vitro* and *in vivo* behavior of CSM14.1 cells we also investigated relevant neurotrophic factors that might be synthetized. By Western blotting we detected a robust GDNF synthesis at the permissive temperature; however, CSM14.1 cell lysates did not contain CNTF. In contrast, we observed a strong endogenous CNTF immunoreactivity around the stab wound of transplanted and sham-transplanted brains (unpublished observations).

For cell labelling and tracking we used the lipophilic fluorescent dye PKH26, which is non-toxic and also remains for a long period in the labelled cells (Haas et al. 2000; Modo et al. 2002). PKH26 does not label endogenous neuronal cells or cells exhibiting phagocytic activity, i.e. microglial cells or macrophages in the host tissue, when PKH26-labelled and lysed cells are injected into the host parenchyma, as has been shown by Weiss et al. (2003). We observed that the PKH26-labelled CSM14.1 cells survive at least 3 months after transplantation and migrate within this time over a rostrocaudal distance of approximately 700–1400 μm. This is interesting when compared with the adult striatum, where the migration of transplanted CSM14.1 cells is weak (own unpublished observations). Importantly, a tumor-like proliferation was never observed.

Altogether, three subpopulations of transplanted cells can be distinguished. The first exhibits typical features of a differentiation into a morphological neuronal phenotype. Additionally, the morphological neural morphology was correlated with NeuN-ir. The immunohistochemical detection of NeuN was possible at least at 3 weeks after transplantation (unpublished observations). Rubio et al. (2000) reported a NeuN-ir still present 1 week afterwards, by using transplanted human neural precursor cells (HNSP.100) developed by Villa et al. (2000). Because similar amounts of NeuN-ir and PKH26-labelled transplanted cells were found on the lesioned as well as on the non-lesioned

side it seems that the lesion does not influence the neural differentiation of transplanted cells. So far, we have not observed TH-ir cells colocalized with PKH26. Other transmitter phenotypes were not investigated whereby γ-aminobutyric (GABA) and cholinergic types come into question.

The second subpopulation synthetized glial proteins of which GFAP was shown here. The third group was labelled with PKH26; however, a classification to one of the later groups was not possible because these cells do not exhibit immunoreactivity with the applied markers. These cells had a small and round morphological appearance.

Typically, cells of the glial group expressing GFAP were located most frequently close to or around the stab of injection. We observed immunoreactivity in these zones for CNTF as well (not shown here). The *in situ* CNTF synthesis of the host tissue may be a major driving force for the differentiation of transplanted CSM14.1 cells to a astroglial phenotype (Levison et al. 1996; Lisovoski et al. 1997; Lee et al. 2000; Haas et al. 2004). In combination with the fact that the SN represents a non-neurogenic zone where it is more probable that transplanted cells give rise to glial phenotypes (Svendsen et al. 1996, 1997; Fricker et al. 1999; Rubio et al. 2000; Lie et al. 2002) the findings presented here agree with observations made by other groups. Unknown restrictive factors and signals seem to be responsible for a suppression of neural predifferentiation of transplanted neural progenitor cells (Keirstead, 2001; Cao et al. 2001).

The location of the transplantation target turns out to be a critical component of the transplantation experiment with regard to cell integration and functional differentiation. Different regions of the rat brain characterized either as neurogenic or non-neurogenic zones may lead to different graft reactions. A second aspect is the conformance of the region from which the cells to be transplanted originate, as well as the transplantation target, could be orthotopic or ectopic (Wichterle et al. 1999). In this context, spatial and temporal transplantation patterns are variable and give rise to modified structural and functional integration of transplants. Finally, the stage of development – prenatal (Campbell et al. 1995; Olsson et al. 1997, 1998), neonatal, juvenile or adult (Wictorin et al. 1990; Labandeira-Garcia et al. 1991) – constitutes an important parameter for the strategy of transplantation. Intranigral transplantation of tissue derived from the ventral mesencephalon or GABAergic striatal tissue improves motoric behavior in parkinsonian rat models. Dopamine agonist-induced rotations as well as complex motoric behavior like stepping postural balance and disengage behavior are improved, whereas amphetamine-induced rotations and the so-called skilful forelimb use are not (Nikkhah et al. 1994; Olsson et al. 1995; Yurek, 1997; Winkler et al. 1999; Mukhida et al. 2001; Palmer et al. 2001). The motoric behavior demonstrated by the rotational test correlates with these results. The fact that the sham-transplanted group exhibited an increase of rotations emphasizes the relationship between the transplantation of CSM14.1 cells and the improvement of motoric behavior. However, these findings should be considered carefully, as the observations are based on apomorphine-induced rotations only and not on further behavioral tests, i.e. amphetamine-induced rotations, cylinder test, staircase test, stepping test, stride-length analysis and locomotor activity measurements (Schallert et al. 2000; Kirik et al. 2001; Metz et al. 2005). The intrastriatal stereotactic injection of Fast Blue did not label any of the transplanted CSM14.1 cells either on the lesioned or on the unlesioned side. This finding correlates with the lack of TH-ir and dopaminergic reinnervation of the striatum on the lesioned side. In agreement with other publications (Nikkhah et al. 1994; Olsson et al. 1995; Yurek, 1997; Starr et al. 1999; Winkler et al. 1999; Palmer et al. 2001; Collier et al. 2002) no reinnervation of the CPu was found, which may be ascribed to inhibiting factors released along myelinated fiber tracts (Schwab et al. 1993; Fawcett & Asher, 1999). Therefore, other mechanisms for the significant changes found must contribute to the motoric behavior (Nikkhah et al. 1994; Winkler et al. 1999); in particular, the neuroprotective and neuroregenerative roles of GDNF that reduce apomorphine-induced rotations (Hoffer et al. 1994; Bowenkamp et al. 1995; Kearns & Gash, 1995; Lapchak et al. 1997; Tseng et al. 1997). Due to the intense background in the anti-GDNF labelling in the 30-μm-thick tissue slices we were not able to document a robust GDNF content in transplanted cells. This could also be due to the down-regulation of GDNF contents in CSM14.1 cells 3 months after transplantation. However, counting of TH-ir cells in the lesioned SNc revealed no obvious differences in the survival or regeneration of dopaminergic neurons between the CSM14.1- and the sham-transplanted animals, whereas in the intact contralateral side we found similar cell numbers as found by other groups for the intact SNc (Wright et al. 2004; Yazdani et al. 2006).

Therefore, further mechanisms have to be attributed to the *de novo* neurons developed from CSM14.1 cells. Evidence was provided that these new neurons are immunonegative for TH; however, we can not exclude a GABAergic phenotype. Such GABAergic differentiated CSM14.1 cells may inhibit the original nigral GABAergic output (Nikkhah et al. 1994; Winkler et al. 1999). This mechanism seems more plausible as the apomorphine-induced rotations decrease abundantly 3 weeks post-transplantation, a time span which appears to be necessary for differentiation. Conversely, other studies reported that intranigrally transplanted differentiated dopaminergic or GABAergic neurons cause a decrease of rotation immediately after 1 week (Palmer et al. 2001).

Taken together, these data might suggest that intranigral transplantation of neural progenitor cells may provide a

platform for restorative therapies of parkinsonian movement disorders. Still unsolved are the problems concerning the influence of inhibitory factors that prevent axonal sprouting from the intranigral transplant to the striatum. A further essential point is that modes of physiological TH expression of transplanted cells need to be explored to facilitate the inauguration of a balanced striatal control.

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