

Transplantation of Immortalized Mesencephalic Progenitors (CSM14.1 Cells) Into the Neonatal Parkinsonian Rat Caudate Putamen

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The present study analyzed whether grafts of the mesencephalic progenitor cell line CSM14.1 into the neonatal rat caudate putamen (CPu) differentiate into neurons and whether this is accompanied by a functional improvement in 6-hydroxydopamine (6-OHDA)-lesioned animals. As in previous studies, a neuronal differentiation of CSM14.1 cells transplanted into the CPu of adult animals could not be observed, so we here used neonatal rats, because graft location and host age seemingly are crucial parameters for neural transplant differentiation and integration. Rats bilaterally lesioned at postnatal day 1 by intraventricular 6-OHDA-injections 2 days later received 100,000 CSM14.1 cells prelabelled with the fluorescent dye PKH26 into the right CPu. Five weeks after grafting, the cylinder test was performed, and the data compared with data from age-matched intact controls and bilaterally lesioned-only animals. Brain slices immunostained for tyrosine hydroxylase (TH) were quantified by optical densitometry. We observed a significant preference of left forelimb use exclusively in transplanted animals. In these rats, TH-containing perikarya were found in the grafted CPu, presumably leading to the significant increase of TH-immunoreactive fibers in this region. Moreover, confocal laser microscopy revealed a differentiation of transplanted PKH26-labelled CSM14.1 cells into neuronal nuclei antigen or TH-immunoreactive cells. Thus, CSM14.1 cells differentiate into TH-containing neurons, which most probably contribute to the preferred forelimb use, indicating a functional integration of CSM14.1 cells into the host basal ganglia loops during early postnatal development. These findings that are in contrast to observations in adult rats suggest instructive cues for neuronal differentiation and integration given by the neonatal microenvironment. © 2007 Wiley-Liss, Inc.

Key words: Parkinson's disease; 6-OHDA; cell therapy; neural progenitor cell; PKH26

In clinical transplantation studies, patients suffering from Parkinson's disease (PD) received autografts of adrenal cells (Backlund et al., 1985; Goetz et al., 1989, 1991; Apuzzo et al., 1990; Kordower et al., 1991; Olson et al.,

1991; Dunnett and Björklund, 1999), fetal mesencephalic xenografts from pig (Deacon et al., 1997; Dunnett and Björklund, 1999), or human mesencephalic neurons from aborted fetuses (Herman and Abrous, 1994; Olanow et al., 1996, 2003; Dunnett and Björklund, 1999; Piccini et al., 1999; Freed et al., 2001). The rationale for these clinical trials was to reconstitute the dopaminergic innervation. This approach has been pursued for 30 years and has proved to be successful in animal models of PD (Björklund, 1992; Brundin and Wictorin, 1993; Herman and Abrous, 1994). Moreover, since 1987, over 300 patients have received human fetal mesencephalic dopaminergic cells (Björklund and Lindvall, 2000; Lindvall and Hagell, 2002a). In some of these patients, L-Dopa could be withdrawn (Lindvall, 1997; Olanow et al., 1997), and, in post-mortem studies (Kordower et al., 1995), an extensive reinnervation of the striatum for up to 10 years was reported. However, in humans, success is still limited (Freed et al., 2001; Olanow et al., 2003), mainly because of the variety of cell-source material and posttransplantation treatment. Grafting of primary fetal cells as a routine therapeutic option (Piccini et al., 1999) is not available because of technical and ethical limitations (Martinez-Serrano and Björklund, 1997; Björklund and Lindvall, 2000).

In a search for alternatives for restorative therapies, other cell types have been investigated. For example, autologous cell grafts derived from the carotid body, containing catecholaminergic neurons, were applied for the treatment of experimental Parkinsonism in rodents and primates, which exhibited improvement in motoric functions (Espejo et al., 1998; Luquin et al., 1999). Neural progenitor cells obtained from the adult primate brain

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have also been autotransplanted with success (Brunet et al., 2005), indicating that neural progenitor cells are a promising source.

In the past, several conditionally immortalized cell lines of neural progenitor or neural stem cells were established from brain tissue of rodent embryos (Frederiksen et al., 1988; Redies et al., 1991; Snyder, 1994; Cattaneo and Conti, 1998). Such cells are promising candidates for restorative therapies of neurodegenerative disorders such as PD, because of their highly reproducible differentiation characteristics and ease in handling.

Our group demonstrated that the nestin-containing, temperature-sensitive, immortalized mesencephalic progenitor cell line CSM14.1 (Zhong et al., 1993; Anton et al., 1994, 1995), derived from a 14-day-old rat embryo, differentiates *in vitro* into tyrosine hydroxylase (TH)- and aldehyde-dehydrogenase 2-containing neurons (Haas and Wree, 2002). However, it has been reported that transplantation into the adult rat caudate putamen (CPu) did not result in a dopaminergic differentiation (Anton et al., 1994, 1995; our unpublished data). One possible reason for this observation could be the lack of instructive clues resulting from the nonpermissive ectopic microenvironment. Therefore, in the present study, we performed transplantation into the neonatal rat CPu. The hypothesis underlying these experiments is that a specific niche facilitates the differentiation and integration of CSM14.1 cells by morphogenic signals (Panchision and McKay, 2002; Doetsch, 2003), namely, the developing brain being a more permissive environment (Costantini and Isacson, 1999).

In addition to exploring the morphological changes of CSM14.1 cells transplanted into the CPu of neonatal rats that were dopamine depleted, the scope of this contribution is to investigate behavioral tests as a functionally and therapeutically relevant readout. Especially, we studied the survival, migration, integration, and differentiation of transplanted cells. Additionally, the motor behavior in the lesioned and subsequently unilaterally transplanted group was compared with an intact control group and bilaterally lesioned-only animals.

MATERIALS AND METHODS

Animals

In total, 33 male Wistar rats (Charles River, Sulzfeld, Germany) were used in the experiments. Litters were reared by the mothers until weaning at 25 days of age. They were housed at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under a 12-hr light/dark cycle with free access to food and water, first with their mothers, then in groups of four to six. All animal care and handling were conducted in compliance with the regulations of and under license from the local authorities. Animals were divided into three groups: intact control ($n = 10$), bilaterally lesioned only ($n = 17$), or bilaterally lesioned and subsequently unilaterally grafted ($n = 6$). At postnatal day 40 (P40), the cylinder test was performed, and, at P42, the animals were fixed by perfusion and the brains taken for histologic stains and immunohistochemistry.

6-OHDA Lesion

On the day after birth (P1), 23 animals received bilateral intraventricular injections of $2 \mu\text{l}$ 6-OHDA-HCl (Sigma, St. Louis, MO; $120 \mu\text{g}/10 \mu\text{l}$ 6-OHDA-HCl in 0.2 mg/ml ascorbic acid/saline) using the coordinates (according to bregma): AP -0.6 , L ± 0.8 ; V -2.1 (dura). Details of the handling and the operative procedures using a neonatal stereotaxic device (Stoelting) have been described by Cunningham and McKay (1993).

Cultivation and PKH 26 Labelling of CSM14.1 Cells

Conditionally immortalized CSM14.1 cells (Zhong et al., 1993; Haas and Wree, 2002) were cultured and expanded in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (all reagents from Gibco, Grant Island, NY) in a humidified incubator (95% air/5% CO_2 , at 33°C). The cells were passaged every third day before reaching confluence. PKH26 labelling (Sigma) prior to transplantation was conducted as described elsewhere (Haas et al., 2000). Viability, after labelling, was over 95% as determined by trypan blue. The final cell concentration was equilibrated to about 100,000 viable cells/ μl , and the cell suspensions were stored on ice until grafting.

Cell Transplantation

Unilateral grafting of PKH26-labelled CSM14.1 cell suspensions in the developing forebrain was conducted according to a microtransplantation approach (Nikkhah et al., 1995a,b). The transplantation surgery was performed at P3 in lesioned rats ($n = 6$), the time point when nearly all endogenous dopaminergic neurons are dead (Shimohama et al., 2003). Briefly, the hypothermic anesthetized pups were fixed in the neonatal stereotaxic device (Stoelting). The micrografts were implanted by using a glass capillary with an outer diameter of 50–70 μm connected to a 5- μl Hamilton microsyringe. A 1- μl deposit of the cell suspension (about 10^5 viable cells) was implanted into the right developing CPu at the following coordinates (according to bregma): AP $+0.7$, L -1.8 , V -2.9 (dura).

Behavioral Assessment

Five weeks after transplantation, forelimb preference was evaluated by using the cylinder test (Schallert et al., 2000) in age-matched, intact controls ($n = 10$), bilaterally 6-OHDA-lesioned rats ($n = 17$), and unilaterally transplanted animals that were also bilaterally 6-OHDA lesioned at P1 ($n = 6$). The use of forepaws during vertical exploration in a glass cylinder with a diameter of 20 cm was documented and analyzed with a Sony videocamera system as described by Kirik et al. (2000). Thirty consecutive forepaw contacts with the glass cylinder were counted per animal, and differences between the right (ipsilateral to the graft) and the left (contralateral to the graft) paw were evaluated for the different groups. The required time to perform these contacts was not monitored because it is of no relevance for this behavioral test.

Immunohistochemical Procedures

Preparation of the animals for immunohistochemistry and histology were performed 6 weeks after birth. After anesthesia with pentobarbital- Na^+ (60 mg/kg), the rats were perfused

transcardially with ice-cold 0.9% sodium chloride (50 ml), followed by 400 ml of 3.7% paraformaldehyde (PFA). Brains were postfixed for 4 hr, cryoprotected, and frozen in isopentane (-50°C).

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 CPU were stored freely floating overnight in phosphate-buffered saline (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. Free-floating sections were washed in 0.1 M PBS (pH 7.4) and preincubated 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). Antibodies directed against glial fibrillary acidic protein (GFAP; mouse monoclonal, 1:400 or rabbit polyclonal, 1:100; both from Sigma), anti-TH (mouse monoclonal, clone TH-2, 1:500; Sigma), or neuronal nuclei antigen (NeuN; mouse monoclonal, 1:1,000; Chemicon, Temecula, CA) were used. After washing, an incubation with Cy2-conjugated anti-mouse IgG (goat polyclonal, 1:400; Dianova, Hamburg, Germany), and AMCA-conjugated anti-rabbit IgG (goat polyclonal, 1:100; Dianova) followed. After rinsing in PBS, specimens were mounted on glass slides.

For light microscopy of TH immunohistochemistry—in the substantia nigra and the basal ganglia—coronal sections were visualized by the ABC method (Vector Laboratories, Burlingame, CA). The same staining procedure for all sections was performed for all animals to guarantee a standardized staining protocol. Thirty-micrometer-thick sections were incubated for 20 min in 3% H_2O_2 , then rinsed and preincubated in PBS containing 0.05% Triton X-100 (Sigma), 3% BSA, and 3% normal horse serum (Vector Laboratories), followed by an incubation with anti-TH (mouse monoclonal, clone TH-2, 1:1,000; Sigma). After rinsing, sections were incubated with biotinylated secondary antibodies (horse polyclonal, 1:200; Vector Laboratories), followed by washes and incubation in the ABC complex (1:50; Vector Laboratories). Final visualization was performed in 0.02% diaminobenzidine (DAB) for 8 min at room temperature (RT).

Densitometry and Statistical Analysis

Densitometry was performed on TH-DAB-stained sections. Sections were scanned at a resolution of $5.04\ \mu\text{m}$ using a dynamic range of 256 gray levels (Nexscan 4100, Heidelberg, Germany). Optical densities (Oberholzer and Östreicher, 1996)

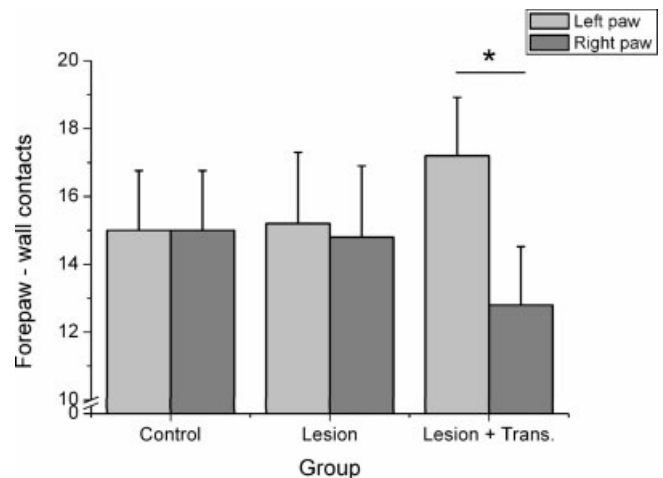


Fig. 1. Results of the cylinder test are shown. Intact controls used left and right forepaws equally (left paw: 15 ± 1.76 , right paw 15 ± 1.76). Lesioned animals also used both paws equally (left paw: 15.2 ± 2.1 , right paw 14.8 ± 2.1). Lesioned and subsequently transplanted animals preferentially used their left forepaws (left paw: 17.2 ± 2.1 , right paw 12.8 ± 1.72), with a statistical significance ($*P = 0.042$, U-test). Error bars show SEM.

of images were calculated by the formula $\text{OD} = \Sigma - \log[(I - B)/(W - B)]/n$, where I = intensity image, B = background image, W = white image, and n = number of pixels. Images were corrected with regard to nonspecific background staining to allow comparisons. The background of the corpus callosum of all slices was always used to obtain a correct background for subtraction. The right CPU was outlined interactively and OD calculated in terms of percentage of theoretical maximal immunoreactivity (black). In six different sections of the same animal, ODs were analyzed and averaged. The densitometric data were analyzed statistically by applying the U-test of Mann-Whitney for nonparametric data because of small sample sizes. Results were expressed as means \pm SEM of the different treatment groups. For statistical evaluation, data from the cylinder test were subjected to the Wilcoxon test. For all statistical analyses, $P < 0.05$ was considered significant. Tests were performed with SPSS 11.01 (SPSS Inc.).

RESULTS

Behavioral Assessment

The ability for preferential forepaw use was evaluated by the cylinder test, in a nondrug-induced voluntary exploration task (Fig. 1). In the intact control group, the animals used left and right forepaws equally, without significant differences (left paw: 15 ± 1.76 , right paw 15 ± 1.76). In the

Fig. 2. Tyrosine hydroxylase distribution in the brains of various groups. **a–a'**: Frontal sections containing the SNc of an intact control animal (a), a bilaterally lesioned rat (a'), and a bilaterally lesioned and transplanted animal (a'). **b–b'**: Frontal sections through the region of the CPU. A section of an intact control animal displaying a strong TH immunoreactivity (b). A section of a bilaterally lesioned animal is shown in b' and a section of a lesioned and transplanted (right hemi-

sphere) animal in b''. **c–c''**: Magnifications of the CPUs. The control CPU exhibits a strong TH-ir (b,c), whereby TH-ir in the deafferented CPU is weak (b',c'). Within sections of transplanted animals, we found several TH-ir neurons that also developed outgrowing fiber processes (b'',c''). These TH-ir neurons are magnified and shown in d–g, corresponding to the letters at arrowheads in c''.

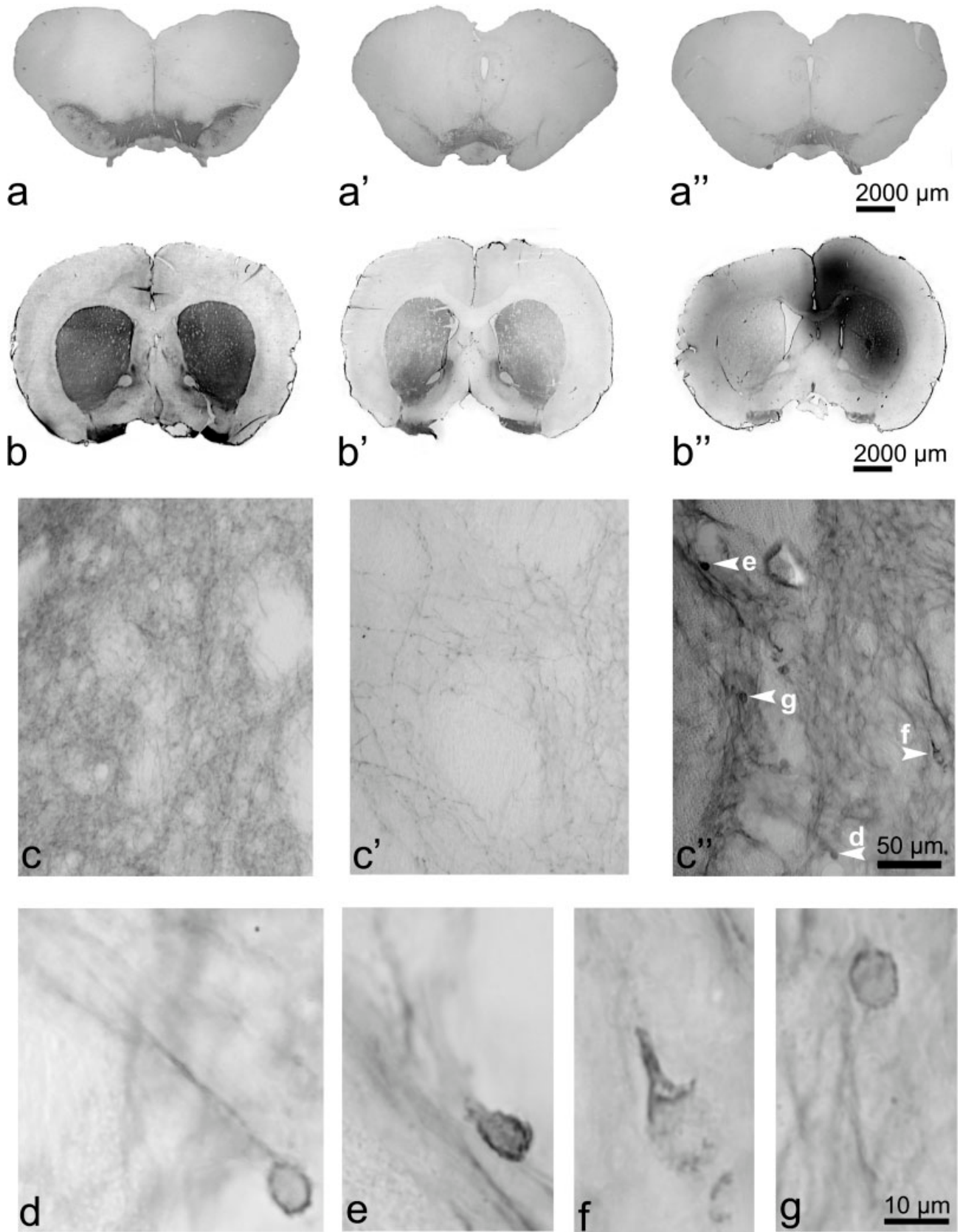


Figure 2

bilaterally lesioned-only group, a similar ratio was observed that was also not significantly different (left paw: 15.2 ± 2.1 , right paw 14.8 ± 2.1), indicating a comparable dopaminergic deafferentiation in both hemispheres. In contrast to these two groups, animals that were lesioned and transplanted with CSM14.1 cells into the right CPu preferentially used their left forepaws (left paw: 17.2 ± 2.1 , right paw 12.8 ± 1.72) with a statistical significance ($P = 0.042$, U-test).

Morphology and Densitometry

Injections of 6-OHDA into both lateral ventricles of neonatal rats result in a nearly complete dopaminergic (DA) neuron cell loss in the left and right substantia nigra (SN; Fig. 2a,a'), leading to a bilateral DA deafferentiation of the CPu (Fig. 2b,b') as demonstrated by TH immunohistochemistry. Grafting of CSM14.1 cells into the right CPu of bilaterally lesioned pups is followed by an increase in TH immunoreactivity (ir) compared with 1) the contralateral lesioned and nontransplanted CPu of the same animals (Fig. 2b'') and 2) the CPu of bilaterally lesioned-only rats (Fig. 2b'). Along the injection path of the glass capillary, TH-ir in the cerebral cortex was also observed. However, in other parts of the cortex, TH-ir zones were not observed (Fig. 2b''). Inside the needle track in dorsal parts of the cortex, PKH26-containing cells were found that were also TH-ir, whereas transplanted cells were never found in the lateral parts of the cortex (not shown). Moreover, TH-ir in the lateral part of the transplanted CPu showed a clear delineation to the cerebral cortex and corpus callosum (Fig. 2b''). At higher magnification, the CPu of intact controls exhibited a dense network of TH-ir fibers (Fig. 2c), which is drastically reduced in bilaterally lesioned-only animals (Fig. 2c'). In the transplanted CPu, a graft-derived high density of TH-ir fibers is reestablished, and TH-containing perikarya can be found (Fig. 2c'',d-g). In these transplanted animals, the SN is still devoid of TH-ir neurons, resembling the SN of lesioned-only rats (Fig. 2a',a'').

Densitometry of TH content in the right CPu of the three groups revealed (Fig. 3) that the CPu of intact controls had a mean OD of 11.11% ($\pm 2.73\%$) and that of lesioned-only animals possessed a significantly lower OD of 2.08% (± 0.39 ; $P = 0.02$). The CPu transplanted with CSM14.1 cells had an OD of 14.45% ($\pm 1.84\%$), which was significantly increased compared with lesioned-only animals ($P = 0.03$), but not significantly different from intact controls ($P = 0.116$).

Cell Migration and Differentiation

Most of the PKH26-labelled transplanted CSM14.1 cells were localized around the injection site within the CPu, but considerable amounts migrated over a distance of about 800–1100 μm into the host parenchyma (Figs. 2b'', 4a,b). Using CLSM, we identified transplanted CSM14.1 cells by the red fluorescence of PKH26 dye. The position of the grafted cells around the injection site is shown in Figure 4a,b, and we found NeuN-ir and PKH26-labelled

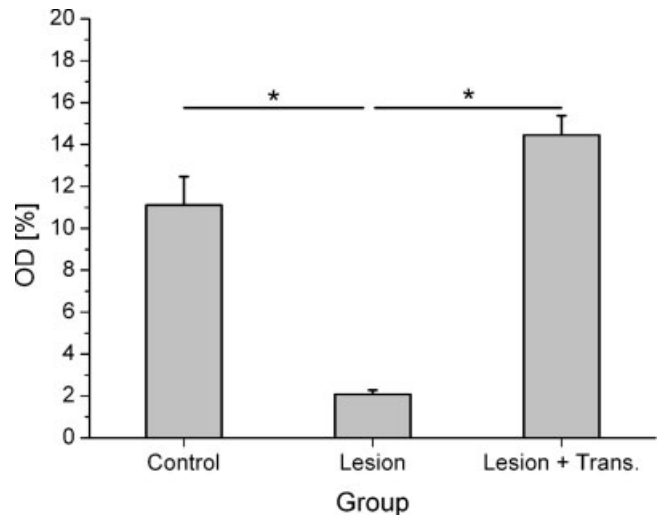


Fig. 3. Mean optical densities of the TH-ir of CPus of the three experimental groups. By the nonparametric U-test, significant TH-ir differences exist between the intact control group and the lesion group as well as between the lesion and the transplant group. However, no significant changes between the intact control group and the lesioned and subsequently transplanted group were found. Error bars show SEM. *Significant difference from the respective group; $P < 0.05$.

cells (Fig. 4c–e) in the proximity of the graft host-border, demonstrating that CSM14.1 cells differentiated to neurons. Colabelling of TH and PKH26 indicated the differentiation of transplanted cells into dopaminergic nerve cells (Fig. 4f–h). However, for the core of the transplants, we never observed PKH26-labelled and NeuN-containing CSM14.1 cells, whereas in this region PKH26-labelled cells were mainly colocalized with GFAP (not shown).

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 CPu of postnatal day 3 Parkinsonian Wistar neonates (ectopic allotransplantation in a nonneurogenic region). The transplantation was performed on the right 6-OHDA-lesioned CPu.

In a prior study, Haas and Wree (2002) described the neurogenic potential of CSM14.1 cells and the absence of immunoreactivity for glial proteins at nonpermissive temperature in vitro. However, it seems that these cells possess a wider phenotypic differentiation potential in vivo after transplantation than in vitro, as has been shown for other neural progenitor cells (Whittemore and Onifer, 2000). The CSM14.1 cells have been transplanted by Anton et al. (1994, 1995); however, so far there exist no published reports on their in vivo differentiation or phenotypic characterization in the CPu. Hence, a key point of this contribution is the investigation of the posttransplantational differentiation of CSM14.1 cells.

In our own experiments, we never observed a neuronal differentiation of CSM14.1 cells after ectopic trans-

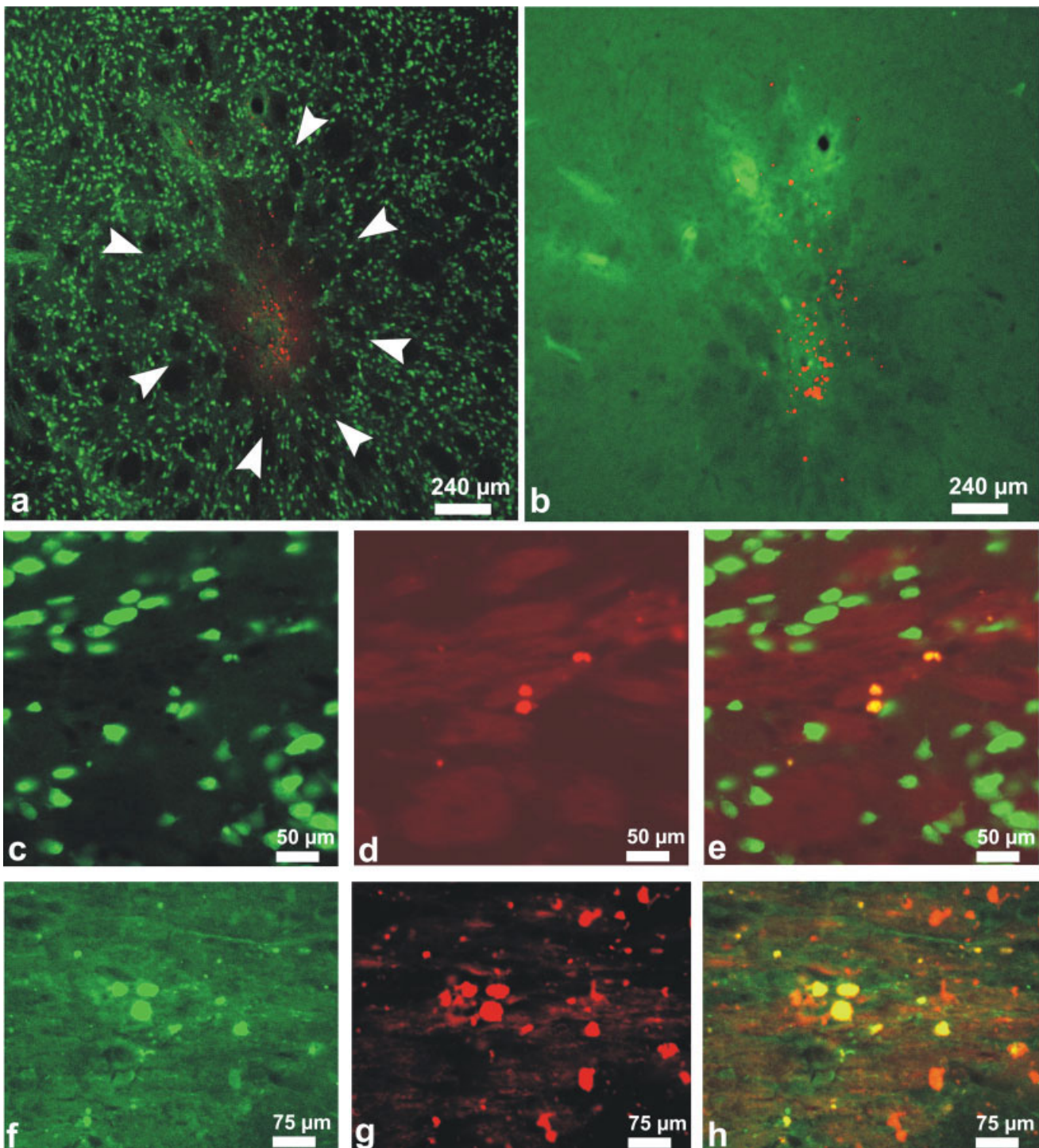


Fig. 4. Differentiation of transplanted cells (a–h). In the 0.2- μm -thick confocal planes. PKH26 labelling is red, and NeuN-ir or TH-ir is green. In **a**, the graft localization within the CPU is shown in a NeuN-stained brain slice, and, in **b**, a parallel section stained for TH also demonstrates graft localization in the CPU. At the border of the grafts (arrowheads in **a**), NeuN- and PKH26-labelled cells can be observed at higher magnifications (**c–e**). Single-channel CLSM images

of NeuN-ir neurons (**c**) and the PKH26 signal (**d**) are merged in **e**. Note that PKH26-labelled cells in the core of the graft are not doubly labelled (**a**). Dopaminergic differentiation was demonstrated by colabelling of TH-ir with PKH26 dye (**f–h**). Single-channel CLSM images of TH-ir cells (**f**) and the PKH26 signal (**g**) are merged in **h** and show that considerable amounts of transplanted cells contained TH.

plantation into the CPu of adult rats, whereas now they expressed mainly the glial marker GFAP. However, evidence was provided that neural progenitor cells showed a better survival, integration, and differentiation in neonate hosts (Herman et al., 1991; Bentlage et al., 1999; Sortwell et al., 2001). Most probably, specific, time-dependent patterns of promoting and inhibiting factors of the extracellular matrix and neural receptors of the neonate and adult CPu differ (Mendez et al., 1996; Krobot et al., 1997; Saunders et al., 1999; Mohapel et al., 2005). These patterns should contribute primarily to the survival, differentiation, and functional integration of grafted neural progenitors (Lindvall and Hagell, 2002b).

Indeed, we detected a dense TH-ir network of fibers around the transplant in the CPu and colocalization of the lipophilic fluorescent marker PKH26 with TH. For the first time, TH-ir perikarya of transplanted and differentiated CSM14.1 cells were found after transplantation into the neonatal brain. These morphologic findings coincide with behavioral improvement measured by the cylinder test and a significant densitometric increase of TH-ir of regions containing the transplants.

After bilateral 6-OHDA lesion in neonatal rats, no obvious motor deficits were observed. We never found severe deficits such as adipsy or aphagy as are observed sometimes after lesioning of adult animals (Snyder-Keller et al., 1989; Luthman et al., 1994; Neal-Beliveau and Joyce, 1999). The motoric behavior followed after lesioning and prior transplantation could not be monitored, because in neonates the visual and the motoric systems are not fully developed (Joyce et al., 1996), and the cylinder test was performed only 5 weeks after birth. The growth of axons of transplanted cells mainly occurs 2 weeks after transplantation, as shown by Snyder-Keller et al. (1989). At a later time point, the axonal pattern stays unaltered. The strong TH-ir that we have observed could be induced by a known normal up-regulation of TH resulting from physiologic development within P1–P14 (Hedner and Lundborg, 1981). We believe that the observed improvement of motoric impairment of forelimb use is due to this process of de novo synthesis of TH by transplanted cells, as shown here by colabelling for PKH26 and TH; moreover, we never found TH-ir cell somata in lesioned-only animals. This is corroborated by observations of Kirik et al. (1998) and Reader and Dewar (1999), who found that only 5–10% of dopaminergic neurons have to be substituted or 10% of TH synthesis has to be reached in order to yield significant motor improvements. Insofar as densitometric measurements (Oberholzer and Östreicher, 1996; Ermert et al., 2001; Xavier et al., 2005) gave rise to significant increases of TH-ir, the correlation of motoric improvement and TH up-regulation seems to be plausible.

We assume that the adult CPu synthesizes much more inhibiting factors, which may suppress differentiation and integration of neural progenitors and are not found in the neonate brain, as described elsewhere (Mendez et al., 1996; Olsson et al., 1997; Sortwell et al., 2001). Furthermore, adult astroglia may not support as strongly as in neonates a differentiation and synaptic inte-

gration of neural progenitors (Krobot et al., 1997; Joosten et al., 2004).

Taken together, these data might suggest that neonatal striatal transplantation of neural progenitor cells may provide a platform for investigating inhibiting and promoting factors relevant to neural progenitor differentiation and integration by compartmental (Schmitt et al., 2007) and developmental proteomic strategies, as proposed by several authors (Fountoulakis et al., 1999; Ballif et al., 2004; Yang et al., 2005). The understanding and experimental control of the above-mentioned factors in accordance with conditionally immortalized progenitor cells established from the human fetal brain (Martinez-Serrano and Björklund, 1997; Björklund and Lindvall, 2000) are auspicious for solving technical and obvious ethical problems regarding prospective clinical application.

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