Exp Brain Res (1998) 119:345–355

RESEARCH ARTICLE

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Comparison of mesencephalic free-floating tissue culture grafts and cell suspension grafts in the 6-hydroxydopamine-lesioned rat

Received: 11 March 1997 / Accepted: 19 August 1997

Abstract Ventral mesencephalon (VM) of fetal rat and human origin grown as free-floating roller-tube (FFRT) cultures can survive subsequent grafting to the adult rat striatum. To further explore the functional efficacy of such grafts, embryonic day 13 ventral mesencephalic tissue was grafted either after 7 days in culture or directly as dissociated cell suspensions, and compared with regard to neuronal survival and ability to normalize rotational behavior in adult rats with unilateral 6-hydroxydopamine (6-OHDA) lesions. Other lesioned rats received injections of cell-free medium and served as controls. The amphetamine-induced rotational behavior of all 6-OHDA-lesioned animals was monitored at various time points from 18 days before transplantation and up to 80 days after transplantation. Tyrosine hydroxylase (TH) immunostaining of the histologically processed brains served to assess the long-term survival of grafted dopaminergic neurons and to correlate that with the behavioral effects. Additional cultures and acutely prepared explants were also fixed and stored for histological investigation in order to estimate the loss of dopaminergic neurons in culture and after transplantation. Similar behavioral improvements in terms of significant reductions in amphetamine-induced rotations were observed in rats grafted with FFRT cultures (127%) and rats grafted with cell suspensions (122%), while control animals showed no normalization of rotational behavior. At 84 days after transplantation, there were similar numbers of TH-immunoreactive (TH-ir) neurons in grafts of cultured tissue (775 ± 98, mean ± SEM) and grafts of fresh, dissociated cell suspension (806 ± 105, mean ± SEM). Cell counts in fresh explants, 7-day-old cultures, and grafted cultures revealed a 68.2% loss of TH-ir cells 7 days after explantation, with an additional 23.1% loss after grafting, leaving 8.7% of the original number of TH-ir cells in the intracerebral grafts. This is to be compared with a survival rate of 9.1% for the TH-ir cells in the cell-suspension grafts. Immunostaining for the calcium-binding proteins calretinin, calbindin, and parvalbumin showed no differences in the neuronal expression of these proteins between the two graft types. In conclusion, we found comparable dopaminergic cell survival and functional effects of tissue-culture grafts and cell-suspension grafts, which currently is the type of graft most commonly used for experimental and clinical grafting. In this sense the result is promising for the development of an effective in vitro storage of fetal nigral tissue, which at the same time would allow neuroprotective and neurotrophic treatment prior to intracerebral transplantation.

Key words Neural transplantation · Tyrosine hydroxylase · Calcium-binding proteins · Parkinson's disease · Rat

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Introduction

The results of neural transplantation in patients with severe Parkinson's disease are promising, but the symptomatic relief is in general not yet of a magnitude that would justify treatment on a large scale (Freed et al. 1992; Freeman et al. 1995a; Lindvall 1994; Lindvall et al. 1990, 1992; Widner et al. 1992). One limitation is the low number of surviving graft dopaminergic neurons, combined with the limited accessibility and concern about the use of human fetal donor tissue.

Many clinical projects use cell suspensions of freshly dissociated cells (Defer et al. 1996; Lindvall et al. 1989, 1992; Peschanski et al. 1994; Widner et al. 1992), but transplantation of fresh solid tissue (Zabek et al. 1994), cryopreserved tissue blocks (Spencer et al. 1992), hibernated tissue blocks (Kordower et al. 1995), and solid

strands of tissue maintained in vitro for several days (Breeze et al. 1995; Freed et al. 1992) have also been reported. An increased donor age window for successful grafting has been demonstrated in solid grafts of human and rat origin as compared to cell-suspension grafts (Freeman et al. 1995b; Simonds and Freed 1990). In addition, transplantation of solid tissue may be less traumatic to the donor cells, with less mechanical injury and disruption of cell contacts.

The storage of dopaminergic neurons in tissue culture prior to transplantation offers the possibility of pregrafting treatment of the cells with growth factors, as well as pooling of tissue from several donors in order to obtain larger and more functional grafts. Provisional storage in culture would also facilitate planning and timing of the actual transplantation and allow testing for infectious agents and removal of undesirable cell types.

Different methods are available for culturing of brain tissue prior to intracerebral transplantation. Organotypic slice cultures of mesencephalic tissue from neonatal rats maintained in culture for 1 week (Sørensen et al. 1994) and primary cultures derived from fetal rat mesencephalon (embryonic day, E, 14, E15) or human fetal mesencephalon (7–11 weeks gestation) have been shown to survive implantation into the 6-hydroxydopamine (6-OHDA)-lesioned striatum of adult rats (Walters et al. 1992). Fetal (E14) rat dopaminergic neurons survive transplantation after 2.5 days in dissociated cultures, but survival is very poor when maintained for 7 days in culture (Brundin et al. 1988).

We have previously reported that mesencephalic tissue of human or rat origin kept as free-floating roller-tube (FFRT) cultures can survive transplantation to the striatum of 6-OHDA-lesioned adult rats and establish dopaminergic nerve connections with the host brain (Spenger et al. 1992, 1994, 1995, 1996). In the present study we compared the long-term survival and behavioral effects of 7-day-old FFRT cultures derived from E13 donors and cell suspensions of E13 rat ventral mesencephalon (VM) after grafting to the striatum of 6-OHDA-lesioned rats. For estimation of the survival of dopaminergic neurons in culture and after transplantation, cell counts were performed in freshly dissected fetal tissue, 7-day-old cultures, and grafted brains. In order to compare the survival of different subpopulations of ventral mesencephalic neurons, the content of neurons expressing the calcium-binding proteins (CBPs) calretinin (CR), calbindin (CB), and parvalbumin (PV) was also estimated in the two graft types.

The finding that FFRT cultures were equally effective as cell suspensions after transplantation to the lesioned rat brain is discussed in light of the need for further improval of graft survival and efficacy, which may be obtained by pregrafting treatment of the cultured donor tissue by, for example, neurotrophins. Some of the work has been published earlier in abstract form (Meyer et al. 1996).

Materials and methods

Preparation of cultures and cell suspensions

Under deep pentobarbital anesthesia, E13 fetuses were removed by Caesarian section from pregnant Sprague-Dawley rats (BRL, Füllinsdorf, Switzerland) and killed by decapitation. The brains were carefully removed, the VM isolated, and tissue cultures prepared as earlier described (Spenger et al. 1994). Briefly, the mesencephalic explants (2 mm × 1.5 mm × 1 mm in size) were divided into four equally large quadrants and transferred into labeled conical plastic tubes (Falcon No. 2095) containing 1 ml of medium. The tubes were placed in a roller drum (60 rev/h) in an incubator (37°C) with 5% CO_2. The culture medium was composed of 55% Dulbecco's modified Eagle medium (DMEM, Gibco No. 041-02320 M), 32.5% Hank's balanced salt solution (HBSS, Gibco No. 041-04020 M), 1.5% glucose, 10% fetal calf serum (FCS, Gibco No. 013-06290), and 1% 0.01 M Hepes (Merck). Antibiotics (Gibco No. 061-05240 D) were added during the first 4 days in vitro. The culture medium was changed twice a week.

The dissociation procedure for cell suspensions was performed as described by Dunnett and Björklund in 1992, with few modifications. Briefly, tissue fragments were incubated for 20 min at 37°C in an incubation medium containing 0.04% DNase (Sigma D-5025), 0.1% trypsin (Sigma T-8128), 0.9% NaCl, and 0.6% glucose. The tissue pieces were washed four times with a dissociation solution (0.04% DNase, 0.9% NaCl, and 0.6% glucose) and then gently triturated through the fire-polished tips of Pasteur pipettes of progressively smaller internal diameter until a turbid suspension, free of visible tissue fragments, was obtained.

Cell counts and viability measurements were done using a 0.4% trypan blue solution (Sigma T-8154) diluted 1:10 in 0.1 M phosphate buffer (PB). The cell counts at the time of surgery indicated that the cell suspension comprised approximately 100 000 cells/µl, with a viability of no less than 90%.

Striatal dopamine depletion and transplantation surgery

Forty female Sprague-Dawley rats (BRL, Füllinsdorf, Switzerland), weighing 180–220 g, were anesthetized (chloral hydrate 0.4 mg/kg i.p.) and placed in a stereotaxic device (Kopf Instruments, USA). Striatal dopamine depletion was made by injection of 4 µl 6-OHDA (Sigma) solution (32 mM 6-OHDA, 1% ascorbic acid, 0.9% NaCl) into the right ascending mesostriatal pathway through a small burr hole in the skull. The injection was performed over 6 min using a 10-µl Hamilton microsyringe, and the following coordinates in relation to bregma: posterior 2.8 mm, lateral 2.0 mm and 8.4 mm ventral to the dura, and the tooth-bar set at 3.9 mm below the interaural line. Eight weeks later, all rotating rats were randomly distributed into four groups for receiving transplants and as transplantation controls.

In one group (n = 9), each animal was grafted with four mesencephalic FFRT cultures, derived from one donor (E13) and maintained in vitro for 7 days. For transplantation the cultures were pooled in a petri dish, the medium was carefully removed, and all four tissue cultures were loaded from the tip into a 20-gauge spinal needle (Unisis, Tokyo). The cultures were carefully injected over 1 min. Three minutes after injection, the needle was slowly retracted.

In another group (n = 9), each animal received a deposit of 5 µl freshly dissociated fetal (E13) rat mesencephalic cells, derived from one donor. For injection of the cells we used a 10-µl Hamilton microsyringe fitted with a steel cannula with an inner diameter of 0.25 mm. In each case the rate of injection was 1 µl/min and the cannula was left in the right striatum for an additional 3 min before retraction.

A third group of animals (n = 7) was used as culture transplant controls. These rats each received a volume of medium estimated to correspond to four FFRT cultures using the 20-gauge spinal needle for injection.

The fourth group served as cell-suspension transplant controls (n = 7), as these rats were injected with 5 µl of medium using a

10-µl Hamilton syringe, i.e., the same volume and procedure used for cell-suspension grafting.

All transplantations and medium injections were performed under deep chloral hydrate anesthesia (0.4 mg/kg i.p.), using the following coordinates in relation to bregma: anterior 1.0 mm, lateral 2.5 mm and 4.5 mm ventral to the dura, with the tooth-bar positioned 2.5 mm below the interaural line.

Behavioral testing

The amphetamine-induced rotational behavior (Dunnett et al. 1981; Perlow et al. 1979; Ungerstedt and Arbuthnott 1970) was tested for all rats at the same time points before and after transplantation. Immediately after injection of D-amphetamine sulfate (2.5 mg/kg i.p.; Sigma A-5880) the animals were placed in automated rotometer cylinders (Columbus Instruments, USA) and monitored for 90 min. Lesioned rats were rotated 18 days and 10 days before transplantation in order to evaluate the completeness of the lesions. Rats with a mean rotational score of more than five net turns per min ipsilateral to the lesion ($n = 32$) were selected and distributed into the four experimental groups. After transplantation, the amphetamine-induced rotational behavior was tested on days 22, 38, 48, 66, and 80.

Histological processing

At 84 days posttransplantation, the rats were perfused through the ascending aorta under deep pentobarbital anesthesia, using 200 ml 0.1 M phosphate buffer (PB) with heparin (1000 IU/100 ml; Novo Nordisk), followed by 250 ml of cold 4% paraformaldehyde in PB with 0.16% picric acid. The brains were immediately removed, postfixed for 2.5 h in the same solution, cryoprotected by immersion in 15% sucrose in 0.1 M PB for no less than 48 h, and thereafter frozen. The frozen brains were cut at 30 µm in the frontal plane on a freezing microtome (2800 Frigocut N; Reichert-Jung) and the sections mounted onto gelatine chrome-alum-coated microscope slides.

Additional FFRT cultures, maintained for 7 days in vitro, and freshly prepared ventral mesencephalic explants were fixed by immersion (1.5 h), cryoprotected in sucrose, frozen, and thereafter sectioned at 20 µm and 12 µm, respectively. The resulting sections were collected systematically and mounted directly onto gelatine chrome-alum-coated microscope slides.

Tyrosine hydroxylase immunohistochemistry

Every third brain and culture section and every fifth section of the fresh explants were stained immunocytochemically for tyrosine hydroxylase (TH) using the biotin-avidin peroxidase method. In brief, the sections were washed three times in 0.1 M PB, incubated in a 0.3% Triton X-100 /PB solution for 30 min, washed in buffer, and incubated with 1.5% normal goat serum (Vector Laboratories, Burlingame, USA) in buffer for 20 min, washed thoroughly, and incubated with a polyclonal rabbit anti-TH antibody (1:500; Pel-Freez Bio) in 0.1 M PB for 12 h at 4°C. Unbound primary antibody was then washed off and the sections incubated for 30 min with biotinylated anti-rabbit antibody (Vector, Burlingame, USA), diluted 1:200. After rinsing in buffer, the sections were treated with a solution for blocking endogenous peroxidase activity (3.3% H_2O_2/10% methanol in 0.1 M PB) for 10 min. Visualization of bound antibody was performed using the avidin peroxidase-complex method (Vector, Burlingame, USA) for 45 min and the metal-enhanced 3,3-diaminobenzidine (DAB) substrate kit (Pierce No. 34065). Sections were dehydrated in alcohol, cleared in xylene, and mounted in Eukitt.

Calretinin, calbindin, and parvalbumin immunohistochemistry

Sections adjacent to TH-immunostained sections from the center of the grafts ($n = 5$), from the center of the FFRT cultures ($n = 8$), and from fresh explants ($n = 3$) were immunostained for CR, CB, and PV. In brief, the sections were washed in 0.1 M PB for 20 min, and thereafter preincubated for 60 min in PB with 0.4% Triton X-100 and 10% horse serum (HS). After washing, sections were incubated for 48 h at 4°C with the following primary antibodies diluted 1:5000 in PB with 0.1% Triton X-100 and 2.5% HS: rabbit polyclonal anti-calretinin, mouse monoclonal anti-calbindin-D_{28k} and mouse monoclonal anti-parvalbumin (primary antibodies were kindly provided by Dr. M. R. Celio, University of Friburg, Switzerland). Bound antibody was detected using biotinylated anti rabbit and anti mouse IgG, a preformed avidin-peroxidase conjugate, and DAB, as described for the TH staining.

Cell counts and morphometric analysis

TH-immunoreactive (ir) neurons containing a visible nucleus were counted in every third section from the grafts and cultures (×10 objective) and in every fifth section of the fresh explants using a ×40 objective. Cell numbers were corrected for double counting according to Abercrombie's formula (Abercrombie 1946), using the mean diameter of the nuclei of the TH-ir neurons (grafts, 6.4 ± 0.1 µm, $n = 92$; cultures, 6.7 ± 0.2 µm, $n = 150$; fresh explants, 5.3 ± 0.1 µm, $n = 128$). The size of the nuclei in the different tissues was estimated using the neuron tracing system from Eutectic Electronics, USA.

The volumes of the transplants were assessed by computer-assisted image analysis (NIH Image 1.49 for Macintosh). Briefly, microscope images were projected on the computer screen and the graft boundaries traced in a calibrated image-analysis window superimposed on to the video-microscope image. After automated computation of the area enclosed by each tracing, the cross-sectional areas were integrated across the rostrocaudal extent of the graft to yield the graft volume.

Fiber outgrowth from the grafts was estimated using NIH Image 1.49 for Macintosh. Briefly, microscope images were projected on the computer screen, the graft-host boundary was identified, and the length of the most conspicuous fibers was estimated. Data for TH-ir fiber outgrowth were evaluated in a semiquantitative manner.

Statistical analysis

All results are expressed as means ± SEM. A commercially available statistical software package (Systat 5.0; Systat, Evanston, I11., USA) was used for statistical analysis. Data were subjected to linear regression analysis or analysis of variance (ANOVA)/Student's *t*-test. Statistical significance level was set at $P < 0.05$.

Results

Assessment of the 6-OHDA lesions

In accordance with the pregrafting results of the amphetamine-induced rotational tests, and verified by immunohistochemical staining, 32 lesioned rats showed a nearly complete loss of TH-ir neurons in the right substantia nigra (SN; Fig. 1). Eight 6-OHDA-injected animals displayed only partial denervation (≤ 5 rotations/min) and were excluded from the experiment.

Behavioral testing of animals

The mean amphetamine-induced net rotation scores of 6-OHDA-lesioned rats with and without grafts are

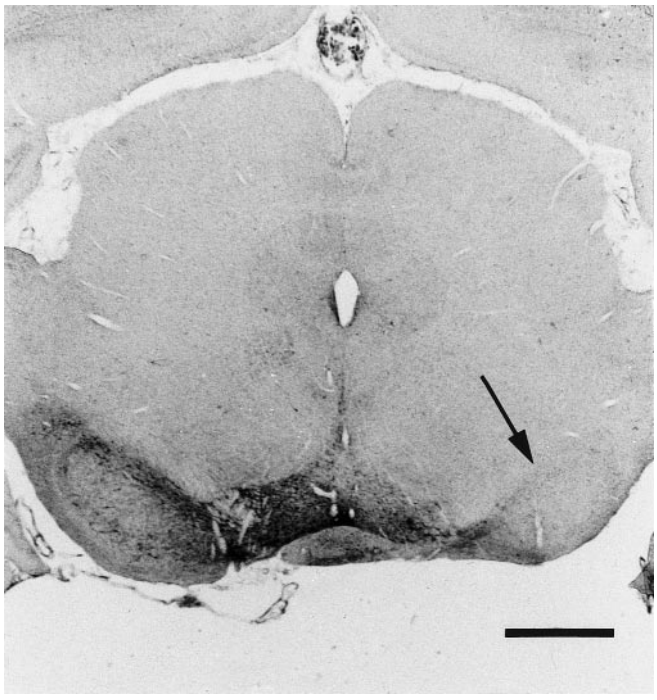

Fig. 1 Immunocytochemically stained section of the ventral mesencephalon from a 6-hydroxydopamine (6-OHDA)-lesioned rat showing loss of tyrosine hydroxylase (TH)-immunoreactive neurons (*arrow*) ipsilateral to the 6-OHDA injection. *Scale bar* 1 mm

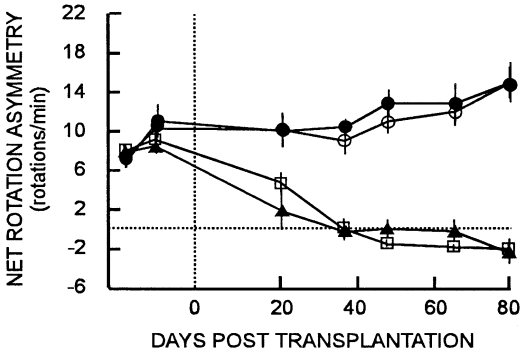

Fig. 2 The mean asymmetric net rotation scores (amphetamine-induced rotations contralateral to the lesion subtracted from rotations ipsilateral to the lesion) recorded in a 90-min test session and plotted for each group before transplantation/sham operation and at various time points between 22 and 80 days postgrafting. Rats grafted with free-floating roller-tube (FFRT) cultures (*filled triangles*), fresh cell suspensions (*squares*), sham-operated with the FFRT culture cannula (*filled circles*), and sham-operated with the cell-suspension cannula (*empty circles*). *Bars* indicate SEM

shown in Fig. 2. Lesioned rats, sham-grafted with the cell-suspension cannula or the tissue-culture cannula, displayed a mean increase in amphetamine-induced rotational behavior of 45% and 36%, respectively (group means, last rotation presham operation compared with last rotation postsham operation). The rotation asymmetry after the sham graftings did not differ significantly from pregrafting levels, and there was no significant difference between the two sham-grafted groups.

At 22 days posttransplantation, rats grafted with FFRT cultures displayed a significant, 77% reduction in the net ipsilateral turning response ($P < 0.01$), whereas rats grafted with dissociated cell suspension showed a nonsignificant reduction of 49%.

At 80 days posttransplantation, all grafted rats displayed a marked reduction in amphetamine-induced rotation asymmetry. For rats grafted with cultured tissue, the mean reduction (group means, last rotation pretransplantation compared with last rotation posttransplantation) was 127% ($P < 0.001$), compared with 122% ($P < 0.001$) for rats grafted with fresh cell suspensions.

Table 1 Quantitative data from rat allografts (mean±SEM) (*TH* tyrosine hydroxylase, *FFRT* free-floating roller-tube)

Graft type	TH-ir neurons	Graft volume (mm^3)	Neuron density (cells/mm^3)
FFRT-culture grafts	775±98	0.69±0.08	1201±179
Cell-suspension grafts	806±105	1.20±0.14	670±38

Cell counts and morphometric analysis of grafts

All recipient rats, killed for histological analysis 84 days posttransplantation, contained viable grafts correctly placed within the host striatum, with less than 5% of the graft volumes extending into the overlying neocortex. The four simultaneously injected FFRT cultures had in most recipients fused to form a single elongated piece of graft tissue located in the implantation tract. The TH-ir neurons in these grafts displayed a relatively uniform distribution, whereas the TH-ir neurons in the cell suspension grafts tended to be located in greater numbers near the graft-host interface (Fig. 3). In both graft types, the TH-ir neurons were typically bipolar or multipolar in shape, with extensive TH-ir fiber outgrowth into a 1- to 1.5-mm-wide zone of the surrounding host striatum. TH-ir neurons were found in almost identical numbers in the FFRT culture grafts (775 ± 98) and the cell suspension grafts (806 ± 105). Given that the tissue culture grafts were significantly smaller than the cell suspension grafts (0.69 ± 0.08 mm^3 compared with 1.20 ± 0.14 mm^3; $P < 0.01$), they accordingly had a significantly higher density of TH-ir neurons (1201 ± 179 cells/mm^3 compared with 670 ± 38 cells/mm^3; $P < 0.01$). The results in terms of TH-ir cell number, graft volume, and cell density for FFRT and cell suspension grafts are summarized in Table 1.

Correlation between cell number, graft volume, and behavioral recovery

In Fig. 4 the number of intrastriatal TH-ir graft-cells is plotted against the reduction in motor asymmetry as recorded at

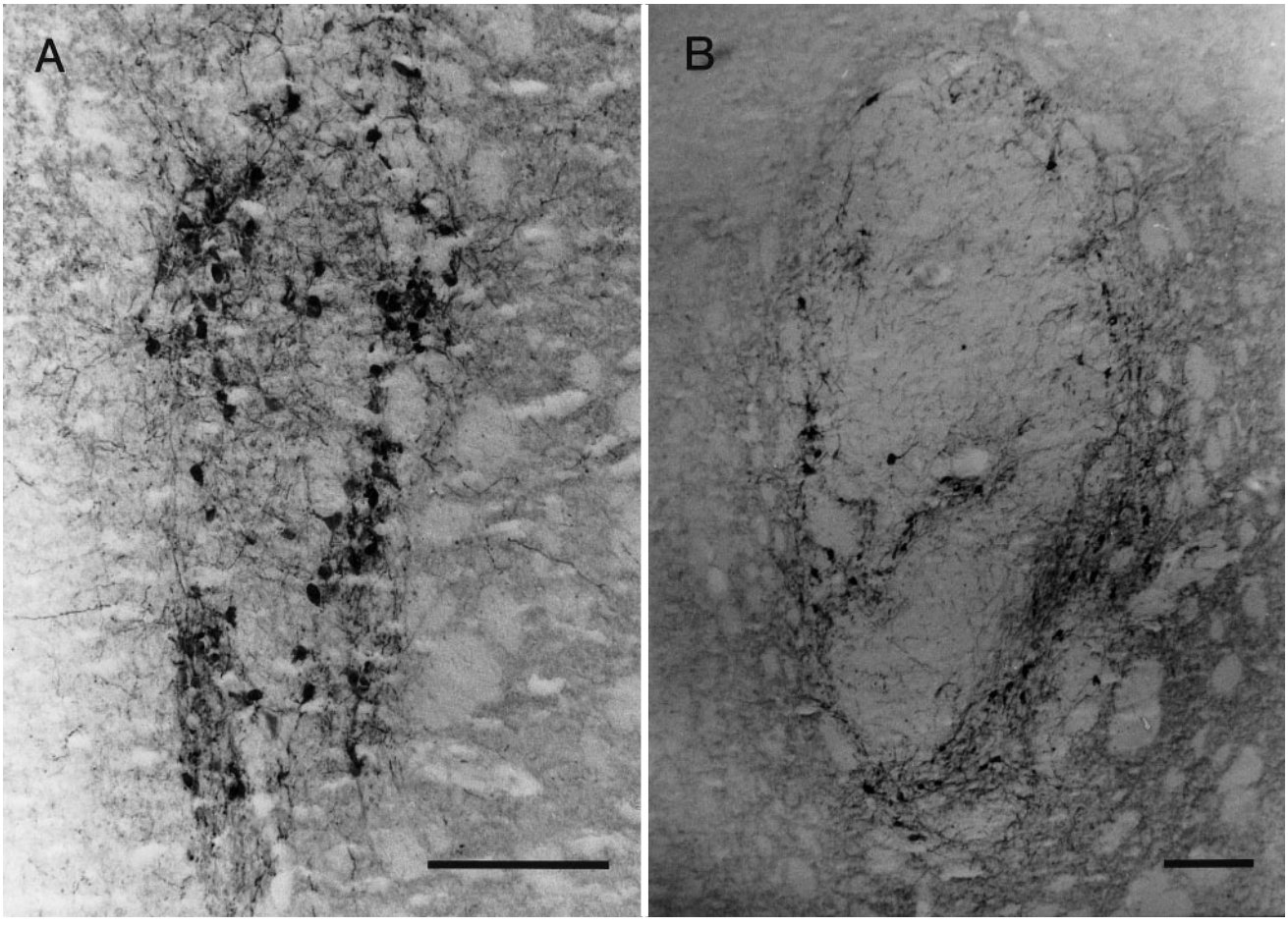

Fig. 3A,B Graft TH-ir neurons and fibers in the dopamine-depleted host striatum 12 weeks after grafting of FFRT cultures (**A**) and cell suspensions (**B**). *Scale bars* 200 μm

Table 2 Proportions of graft neurons expressing calcium binding proteins relative to TH-ir neurons (mean±SEM)

Graft type	Calretinin/ TH (%)	Calbindin/ TH (%)	Parvalbumin/ TH (%)
FFRT-culture grafts	158±30	30±5	18±4
Cell suspension grafts	174±32	26±4	14±2

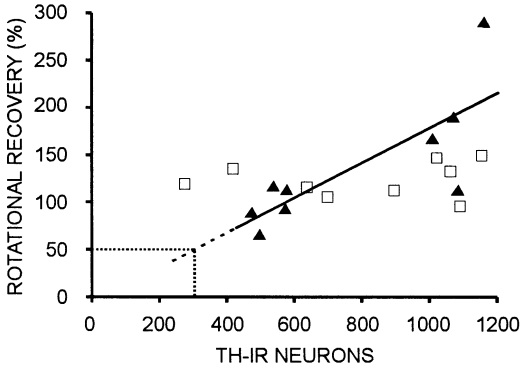

Fig. 4 Correlation of behavioral recovery with number of surviving TH-ir neurons in tissue-culture grafts (*filled triangles*) and cell-suspension grafts (*empty squares*). A significant correlation was only found for rats grafted with cultured tissue (linear regression analysis, $R = 0.78$, $P = 0.01$), whereas no significant correlation was detected for rats grafted with fresh cell suspensions ($R = 0.18$, $P = 0.64$). The *dotted lines* indicate the number of TH-ir graft cells needed for a 50% rotational recovery [Behavioral recovery = 100% × (rotation rate pregrafting–rotation rate postgrafting)/rotation rate pregrafting]

80 days posttransplantation. A significant correlation (linear regression analysis, $R = 0.78$, $P = 0.01$) between behavioral recovery and number of surviving TH-ir neurons was found for rats grafted with cultured tissue, but was not present for rats grafted with dissociated cell suspensions ($R = 0.18$, $P = 0.64$). For rats with culture grafts, but not for rats with cell-suspension grafts, there was also a significant correlation between behavioral recovery and graft volume (linear regression analysis, $R = 0.76$, $P = 0.02$; curve not shown).

For cell-suspension grafts, but not for culture grafts, there was a highly significant correlation between TH-ir cell number and graft volume (linear regression, $R = 0.93$, $P = 0.0003$; curve not shown).

Graft neurons expressing CBPs

Immunostains for the CBPs CR, CB, and PV in the grafts are shown in Fig. 5. Table 2 shows the proportions between the CBP-ir cells and the TH-ir cells as counted in

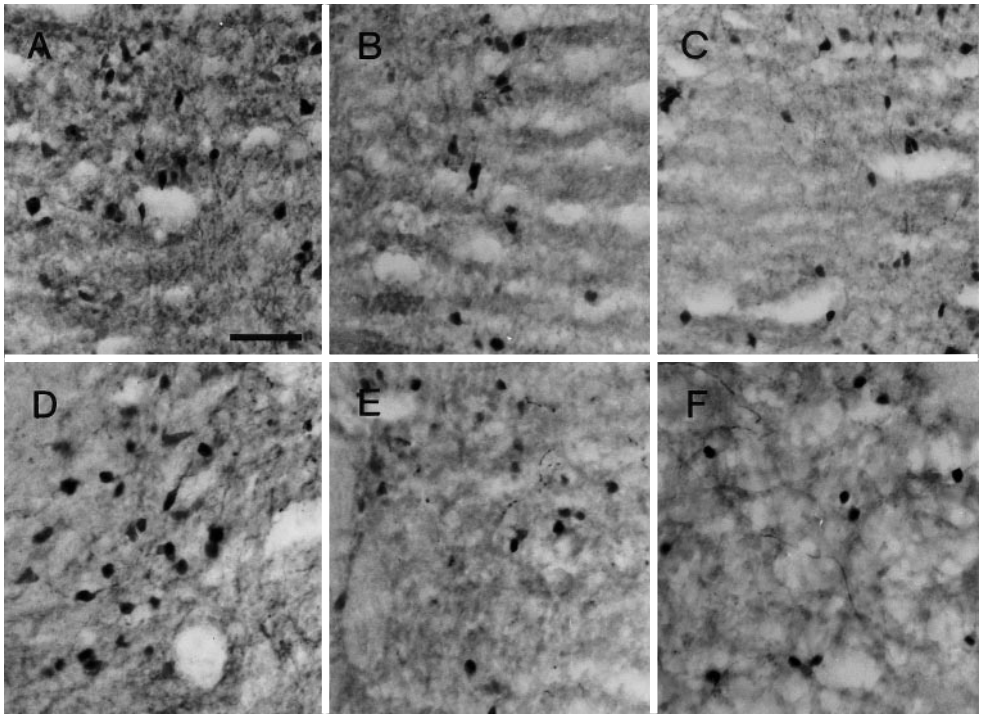

Fig. 5A–F Immunocytochemical staining for the calcium-binding proteins calretinin, calbindin, and parvalbumin in tissue culture grafts (**A–C**) and in cell suspension grafts (**D–F**). *Scale bar* 50 μm

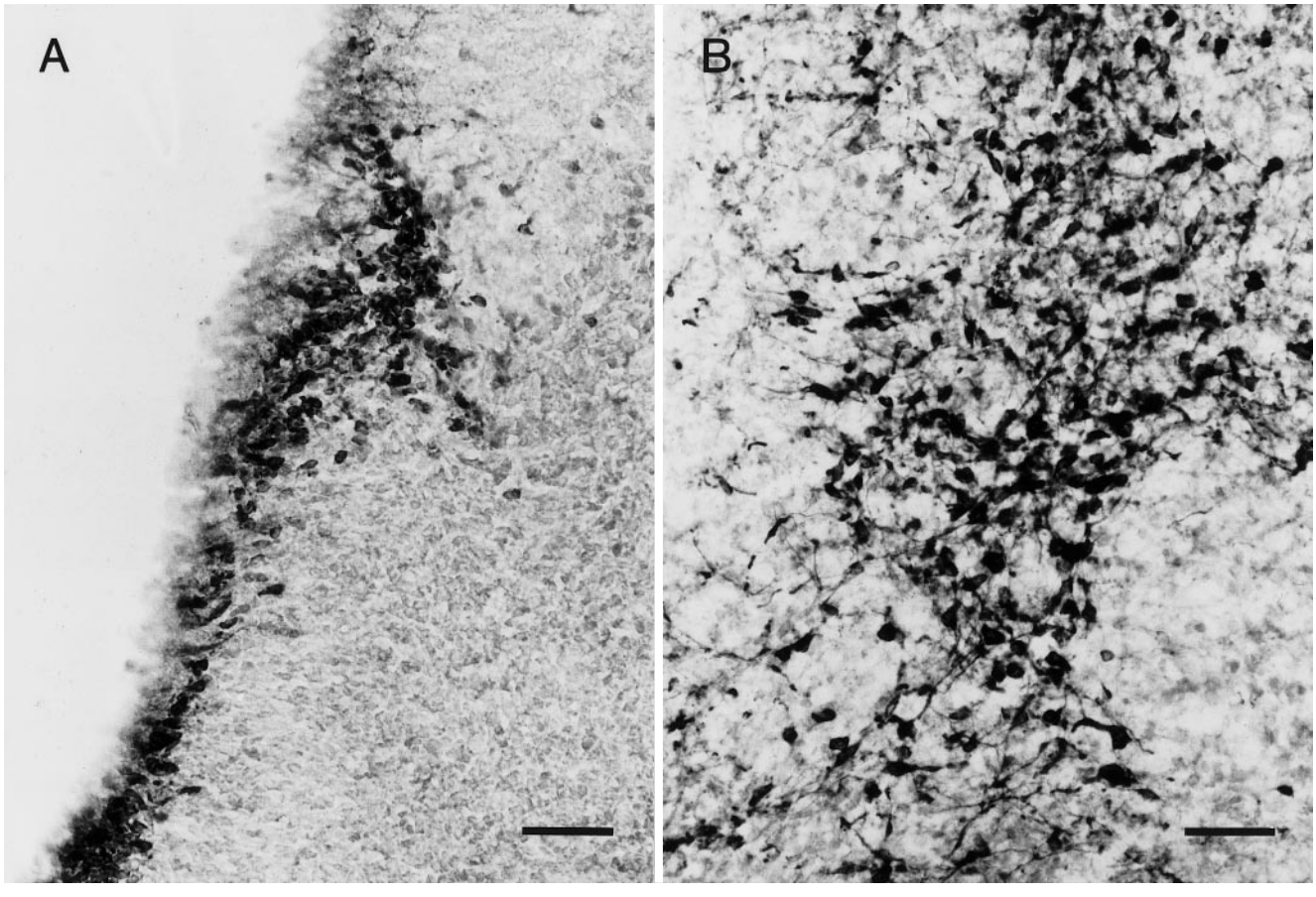

Fig. 6A, B Section from newly prepared ventral mesencephalic explant showing an intense TH immunoreactivity in area corresponding to the dopaminergic nuclei (**A**), and section from a 7-day-old FFRT culture with intensely stained TH-ir neurons (**B**). *Scale bars* 50 μm

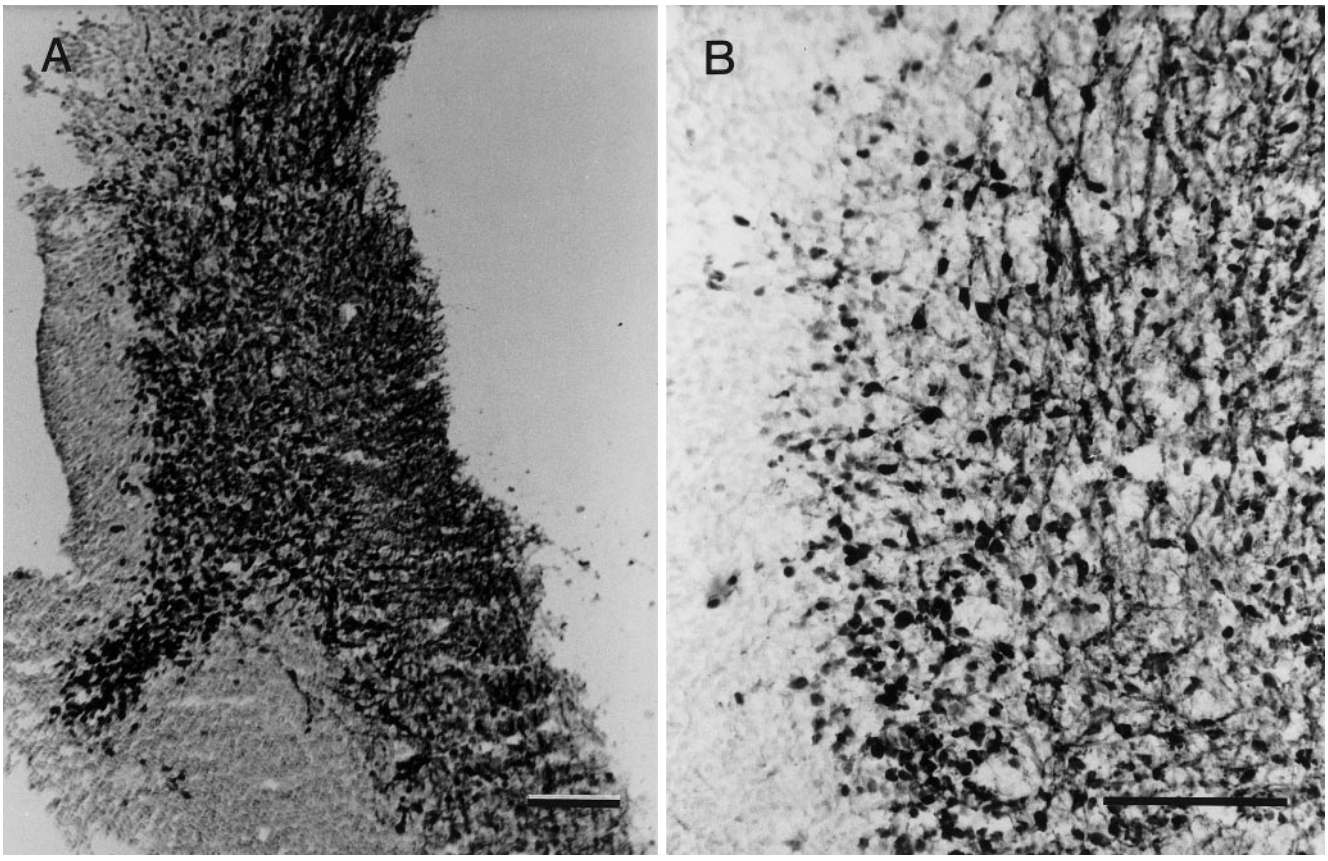

Fig. 7A, B Immunocytochemical staining for calretinin in a fresh mesencephalic explant (**A**), and for calretinin in a mesencephalic culture maintained 7 days in vitro (**B**). *Scale bars* 100 μm

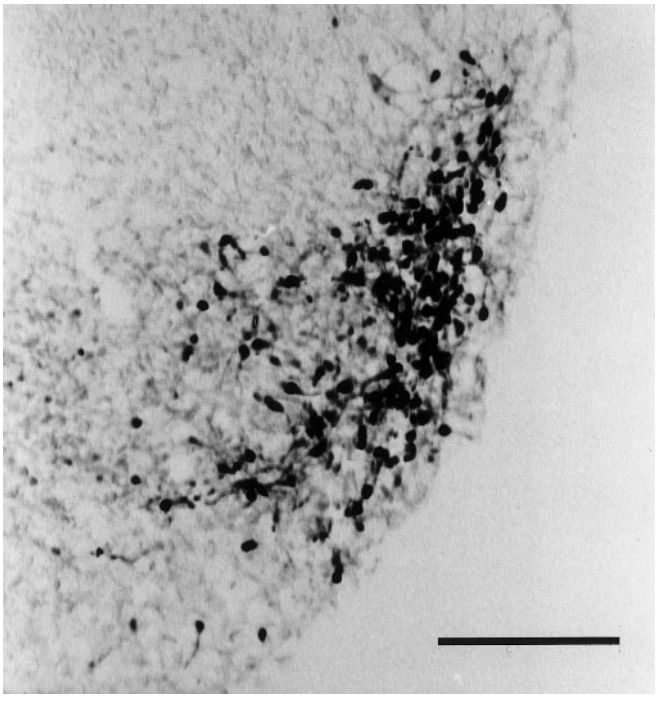

Fig. 8 Immunocytochemical staining for calbindin in a mesencephalic culture maintained 7 days in vitro. *Scale bar* 100 μm

adjacent sections. Both graft types had a high CR to TH ratio, a moderate CB to TH ratio, and a low PV to TH ratio, with no significant differences between the two graft types.

Comparison of 7-day-old cultures and fresh explants

After 7 days in vitro, the cultured VM tissue formed small spheres of approximately 0.5–1.5 mm in diameter. TH staining of the sectioned cultures revealed numerous TH-ir neurons of mainly bipolar or multipolar shape (Fig. 6B). Four cultures, derived from one mesencephalic explant and corresponding to a culture sample used for transplantation, contained a total of 2826 ± 261 (n = 11) TH-ir neurons.

Sections of fresh VM explants contained an area with intense TH-ir, corresponding to the dopaminergic areas of the VM (Fig. 6A). Counts of neurons with distinct TH staining gave matching numbers (8890 ± 504, range 7433–10373) for all five analyzed explants, confirming the accuracy and reproducibility of the dissection procedure.

Immunostaining for CR (Fig. 7) revealed that this protein was highly expressed, and to the same extent, in fresh explants and 7-day-old cultures (Table 3). CB was barely expressed in the immature fresh explants (not shown), but very distinct in 7-day-old cultures (Fig. 8), whereas PV was not detectable in fresh explants and only barely expressed in the cultures (Table 3).

Table 3 Counts of TH-ir and calcium-binding protein-ir neurons in newly prepared mesencephalic explants and in 7-day-old FFRT cultures (– not expressed, (–) barely expressed)

Tissue	TH-ir neurons	Calretinin/ TH (%)	Calbindin/ TH (%)	Parvalbumin/ TH (%)
Fresh explants	8890±504	259±32	(–)	–
FFRT cultures	2826±261	292±21	53±7	(–)

Discussion

The purpose of this study was to examine and compare the survival and behavioral efficacy of rat VM cells grafted to the dopamine-depleted striatum of adult rats, as either tissue block explants grown for 7 days as FFRT cultures or freshly prepared, dissociated cell suspensions. The results showed that FFRT culture grafts from E13 donor rats and corresponding fresh E13 cell-suspension grafts had the same functional effects on hemiparkinsonian rats, and that the "in vitro storage" of the tissue prior to transplantation had no negative effects on dopaminergic cell survival. With an earlier onset of the functional effects in the culture-grafted rats, the findings extend previous attempts to develop a protocol for storage – and pregrafting treatment – of fetal mesencephalic donor tissue.

Amphetamine-induced rotation

At 80 days posttransplantation, amphetamine-induced rotation scores revealed similar functional effects in rats grafted with FFRT cultures and in rats grafted with freshly prepared cell suspensions. The compensation of motor asymmetry was, however, faster in onset in the rats receiving culture grafts, as shown in the rotational test performed 22 days after transplantation. One explanation for this might be that the cultured dopaminergic neurons are more mature at the time of grafting, corresponding to the extra 7 days in culture. It is not likely that the behavioral difference between the two graft groups could be attributed to the use of transplantation needles of different size.

The fact that the sham-grafted rats displayed increasing or unchanged ipsilateral rotational behavior after sham-grafting surgery indicates that the sham-grafting by itself did not promote recovery by, for example, sprouting of remaining dopaminergic nerve fibers, in response to induced production or release of trophic factors. In this connection it is also worth noting that the post-sham-grafting rotation scores were very similar in the two sham groups although the transplantation needle used for grafting of cultures (and the corresponding sham-grafting) was larger than the one used for cell suspension grafts and sham-grafting. The increased turning rate for many of the sham-operated rats during the 80-day-long postoperative period is a common observation, which has been attributed to the combined effects of sensitization and conditioning (Annett et al. 1993).

TH-ir neuron survival

Both the FFRT culture grafts and the cell suspension grafts were rich in dopaminergic neurons and very well integrated in the host brains. A dense TH-ir reinnervation of the host striatum was present in a 1- to 1.5-mm-wide zone around both types of grafts. Owing to the larger volume of the cell suspension grafts in conjunction with a process extension that was similar to that seen for culture grafts, it is likely that a larger area of the host striatum is covered. Interestingly, this did not result in a better functional effect. Since we found similar numbers of surviving TH-ir neurons in both graft types, the cell-suspension grafts showed a markedly lower TH-ir cell density. It is proposed that this results in a different graft-host interaction and suggests that a smaller graft volume with higher cell density functionally can compensate for the smaller area covered. The reason for the larger volume of the cell-suspension grafts is not clear but might be explained by diffusion of the injected cell suspension, which is less likely to be expected for the solid culture implants.

In accordance with the similarity in functional effect, cell counts for TH-ir neurons were also similar in the two graft types. Cell counts of fresh (acutely made) explants, 7-day-old FFRT cultures, and grafted cultures showed a 68.2% loss of cells 7 days after explantation and an additional 23.1% loss after grafting, with 8.7% of the TH-ir cells found in the acute explants remaining. This is to be compared with a survival rate of 9.1% of the TH-ir cells in the cell-suspension grafts, which is in agreement with observations of other groups (Björklund 1993; Brundin et al. 1988; Brundin and Björklund 1987).

Relationship between graft survival and functional effects

In the rats grafted with FFRT cultures, there was a linear correlation between the number of TH-ir graft cells and the reduction in rotational behavior. Rats grafted with dissociated cell suspension did not display such significant linear correlation. The lack of correlation could indicate that the reliance on the amphetamine-induced rotation test as a measure of graft efficacy is problematic for large grafts that result in more than 100% recovery of the lesion-induced rotation asymmetry. Moreover, it is suggested that the missing correlation between TH-ir cell numbers and functional recovery for the larger cell suspension transplants may be the result of a different pattern of graft-host interaction.

For cell-suspension grafts of VM, it has previously been shown by logarithmic regression analysis that 100–600 surviving dopaminergic neurons are sufficient to elicit a 50% reduction in amphetamine-induced rotational asymmetry in 6-OHDA-lesioned rats (Brundin et al. 1988; Sauer and Brundin 1991; Sauer et al. 1992). Using linear regression analysis on data from culture grafts, we could confirm that approximately 300 dopaminergic neurons were needed to exert this effect. The variation in TH-

ir cell number (100–600 cells) reported to be needed for a 50% reduction in rotation score might be explained by the use of slightly variable coordinates for graft injection in the different studies, assuming that variable numbers of grafted neurons actually had innervated the dorsal and central subregions of the striatum of importance for rotational behavior.

CBPs in normal VM and in the grafts

It has been reported previously that CR-ir neurons in the rat substantia nigra (SN) are found in relatively high numbers in the SN pars compacta (SNc) and usually not found in SN pars reticulata (SNr). Many CR-ir neurons have also been found in the ventral tegmental area (VTA; Résibois and Rogers 1992; Rogers 1992). CB-ir neurons have been found in relatively small numbers in the rat SNc and are absent in the rest of the SN, but the protein is abundantly present in the VTA in conjunction with light-to-moderate fiber staining (McRitchie et al. 1996). PV-ir neurons in the rat SN are virtually restricted to the SNr, where the majority of the neurons express the protein, while only infrequently a few PV-ir neurons are found within SNc. In the VTA, PV-ir neurons are found in small numbers (Gerfen et al. 1985; McRitchie et al. 1996).

Between the two graft types of the present study, there were no differences in the numbers of CR-, CB-, and PV-positive neurons relative to TH-ir cells (Table 2).

The presence of many CR-ir neurons and moderate numbers of CB-ir neurons compared with the number of TH-ir neurons suggests survival of, in particular, SNc neurons and, to some extent, also VTA neurons (Rogers 1992). The very high CR to TH ratio found in both graft types, relative to the ratio found in the normal VM, might be explained by a preferential survival of CR-ir neurons in the grafts or by an increased expression of the CBP after grafting. The few PV-ir graft neurons found probably represent SNr neurons. For more precise estimates of the different neuronal subpopulations, however, double or triple immunocytochemical stainings for TH and the CBPs would be required.

Potential use of pregrafting storage

Provided that the neuronal survival is not compromised, pregrafting storage of donor tissue in culture has, in particular in clinical settings, several advantages compared with the use of fresh cell suspensions (Björklund et al. 1980) and fresh solid tissue (Freeman et al. 1995a). Even short-term storage of donor tissue will greatly facilitate the planning of surgery, just as more time will be available for screening of the donor tissue for potential infectious agents. Also the limited availability of donor tissue, relative to the need of up to ten fetuses for bilateral graftings, may be overcome, as pooling of tissue from several donors perhaps from different centers will be possible. Live storage of the donor tissue as FFRT cultures will also allow stimulation of the cells by relevant growth factors in order to support graft survival and differentiation, and high-pressure liquid chromatography (HPLC) determination of dopamine release into the medium will make it possible to select explants rich in dopaminergic cells for grafting (Studer et al. 1996).

It is expected that pretreatment of the VM cultures with neurotrophic factors should increase the yield of dopaminergic neurons. Treatment with brain-derived neurotrophic factor (BDNF) (10 ng/ml) has thus been shown to increase the density of TH-ir cells by 2.5-fold and the fiber density by 3.5-fold in FFRT cultures of human VM (Spenger et al. 1995). Qur Preliminary data also indicate that treatment of E14 mesencephalic FFRT cultures with neurotrophin (NT)-4/5 (10 ng/ml) and glial cell line-derived neurotrophic factor (GDNF; 10 ng/ml) increases spontanous dopamine release and survival of the TH-ir neurons.

Also postoperative intraparenchymal infusions of NT-4/5 next to nigral cell suspension grafts have been shown to stimulate fiber growth and behavioral recovery in 6-OHDA-lesioned rats (Haque et al. 1996). Alternative approaches include genetic engineering of cells for more effective intracerebral delivery of trophic factors (Takayama et al. 1995) and pregrafting mixing of dopaminergic neurons with naturally occuring or genetically engineered neurotrophin-producing cells. Another interesting approach is to increase the survival of the grafted dopaminergic neurons by the use of inhibitors of free radical formation or free radical scavengers such as lazaroids (Frodl et al. 1994; Nakao et al. 1994). Also in most of these experimental situations there is a great potential for the use of pregrafting culturing of donor tissue.

Conclusions

The recovery in amphetamine-induced rotational behavior of adult 6-OHDA-lesioned rats grafted with 7-day-old mesencephalic FFRT cultures had an earlier onset, but was otherwise similar to the recovery observed for rats grafted with freshly prepared cell suspensions. At 84 days posttransplantation, the survival rate of TH-ir neurons was the same in the two types of grafts. The two graft types also expressed the CBPs CR, CB, and PV in similar proportions to TH-ir neurons. The proportions moreover indicated that the surviving neurons mainly came from SNc and, to a certain extent, the VTA, with a few neurons originating from SNr.

The demonstration that FFRT culture grafts survived and functioned just as well as cell-suspension grafts, which currently is the type of graft most commonly used for experimental and clinical grafting, is promising for future developments of in vitro storage and neuroprotective and neurotrophic treatment of fetal nigral tissue prior to intracerebral transplantation.

Acknowledgements The skillfull technical assistance of Beatrice Bühler and Elvira Ciessynna is gratefully acknowledged. Thanks are also due to Prof. Jens Zimmer for his continuous support and ex-

tensive help with the manuscript. This study was supported by the Swiss National Science Foundation (grant 31-45558.95), the Swiss Federal Office of Education and Science (grant BBW 93.0349), the Swiss Parkinson Association, the Danish MRC, and the Danish Parkinson Association.

References

Abercrombie M (1946) Estimation of nuclear population from microtome sections. Anat Rec 94:239–247

Annett LE, Reading PJ, Tharumaratnam D, Abrous DN, Torres EM, Dunnett SB (1993) Conditioning versus priming of dopaminergic grafts by amphetamine. Exp Brain Res 93:46–54

Björklund A (1993) Better cells for brain repair. Nature 362:414–415

Björklund A, Schmidt RH, Stenevi U (1980) Functional reinnervation of the neostriatum in the adult rat by use of intraparenchymal grafting of dissociated cell suspensions from the substantia nigra. Cell Tissue Res 212:39–45

Breeze RE, Wells TH, Freed CR (1995) Implantation of fetal tissue for the management of Parkinson's disease: a technical note. Neurosurgery 36:1044–1047

Brundin P, Björklund A (1987) Survival, growth and function of dopaminergic neurons grafted to the brain. Prog Brain Res 71: pp 293–308

Brundin P, Barbin G, Strecker RE, Isacson O, Prochiantz A, Björklund A (1988) Survival and function of dissociated dopamine neurons grafted at different developmental stages or after being cultured in vitro. Brain Res Dev Brain Res 39:233–243

Defer GL, Geny C, Ricolfi F, Fenelon G, Monfort JC, Remy P, Villafane G, Jeny R, Samson Y, Karavel Y, Gaston A, Degos JD, Peschanski M, Cesaro P, Nguyen JP (1996) Long-term outcome of unilaterally transplanted parkinsonian patients. I. Clinical approach. Brain 119:41–50

Dunnett SB, Björklund A (1992) Staging and dissection of rat embryos. In: Dunnett SB, Björklund A (eds) Neural transplantation: a practical approach. IRL, Oxford, pp 1–19

Dunnett SB, Björklund A, Stenevi U, Iversen SD (1981) Behavioural recovery following transplantation of substantia nigra in rats subjected to 6-OHDA lesions of the nigrostriatal pathway. I. Unilateral lesions. Brain Res 215:147–161

Freed CR, Breeze RE, Rosenberg NL, Schneck SA, Kriek E, Qi J, Lone T, Zhang Y, Snyder JA, Wells TH, Olson Ramig L, Thompson L, Mazziotta JC, Huang SC, Grafton ST, Brooks D, Sawle GV, Schroter G, Ansari AA (1992) Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. N Engl J Med 327:1549–1555

Freeman TB, Olanow CW, Hauser RA, Nauert GM, Smith DA, Borlongan CV, Sanberg PR, Holt DA, Kordower JH, Vingerhoets FJG, Snow BJ, Calne D, Gauger LL (1995a) Bilateral fetal nigral transplantation into the postcommisural putamen in Parkinson's disease. Ann Neurol 38:379–388

Freeman T, Sanberg PR, Nauert GM, Boss BD, Spector DH, Olanow CW, Kordower JH (1995b) The influence of donor age on the survival of solid and suspension intraparenchymal human embryonic nigral grafts. Cell Transplant 4:141–154

Frodl EM, Nakao N, Brundin P (1994) Lazaroids improve the survival of cultured rat embryonic mesencephalic neurons. Neuroreport 5:2393–2396

Gerfen CR, Baimbridge KG, Miller JJ (1985) The neostriatal mosaic: compartmental distribution of calcium-binding protein and parvalbumin in the basal ganglia of the rat and monkey. Proc Natl Acad Sci USA 82:8780–8784

Haque NSK, Hlavin ML, Fawcett JW, Dunnett SB (1996) The neurotrophin NT-4/5, but not NT-3, enhances the efficacy of nigral grafts in a rat model of Parkinson's disease. Brain Res 712:45–52

Kordower JH, Freeman TB, Snow BJ, Vingerhoets FJG, Mufson EJ, Sanberg PR, Hauser RA, Smith DA, Nauert GM, Perl DP, Olanow CW (1995) Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. N Engl J Med 332:1118–1124

Lindvall O (1994) Neural transplantation in Parkinson's disease. In: Dunnett SB, Björklund A (eds) Functional neural transplantation. Raven Press, New York, pp 103–137

Lindvall O, Rehncrona S, Brundin P, Gustavii B, Åstedt B, Widner H, Lindholm T, Björklund A, Leenders KL, Rothwell JC, Frackowiak R, Marsden CD, Johnels B, Steg G, Freedman R, Hoffer BJ, Seiger Å, Bygdeman M, Strömberg I, Olson L (1989) Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. a detailed account of methodology and a 6-month follow-up. Arch Neurol 46:615–631

Lindvall O, Brundin P, Widner H, Rehncrona S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD (1990) Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science 247:574–577

Lindvall O, Widner H, Rehncrona S, Brundin P, Odin P, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Björklund A, Marsden CD (1992) Transplantation of fetal dopamine nerons in Parkinson's disease: One year clinical and neurophysiological observations in two patients with putaminal implants. Ann Neurol 31:155–165

McRitchie DA, Hardman CD, Halliday GM (1996) Cytoarchitectural distribution of calcium binding proteins in midbrain dopaminergic regions of rats and humans. J Comp Neurol 364:121–150

Meyer M, Widmer HR, Evtouchenko L, Guzman R, Seiler RW, Zimmer J, Spenger C (1996) Transplantation of fresh cell suspensions versus cultured tissue of fetal rat mesencephalon to 6-hydroxydopamine lesioned rats. Soc Neurosci Abstr 22:1492

Nakao N, Frodl EM, Duan WM, Widner H, Brundin P (1994) Lazaroids improve the survival of grafted rat embryonic dopamine neurons. Proc Natl Acad Sci USA 91:12408–12412

Perlow MJ, Freed WJ, Hoffer BJ, Seiger Å, Olson L, Wyatt RJ (1979) Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. Science 204:643–646

Peschanski M, Defer G, N Guyen JP, Ricolfi F, Monfort JC, Remy P, Geny C, Samson Y, Hantraye P, Jeny R, Gaston A, Keravel Y, Degos JD, Cesaro P (1994) Bilateral motor improvement and alteration of *l*-dopa effect in two patients with parkinson's disease following intrastriatal transplantation of foetal ventral mesencephalon. Brain 117:487–499

Résibois A, Rogers JH (1992) Calretinin in rat brain: an immunohistochemical study. Neuroscience 46:101–134

Rogers JH (1992) Immunohistochemical markers in rat brain: colocalisation of calretinin and calbindin-D28k with tyrosine hydroxylase. Brain Res 587:203–210

Sauer H, Brundin P (1991) Effects of cool storage on survival and function of intrastriatal ventral mesencephalic grafts. Restor Neurol Neurosci 2:123–135

Sauer H, Frodl EM, Kupsch A, Bruggencate, Oertel WH (1992) Cryopreservation, survival and function of intrastriatal fetal mesencephalic grafts in a rat model of Parkinson's disease. Exp Brain Res 90:54–62

Simonds GR, Freed WJ (1990) Effects of intraventricular substantia nigra allografts as a function of donor age. Brain Res 530:12–19

Sørensen JC, Østergaard K, Zimmer J (1994) Grafting of dopaminergic ventral mesencephalic slice cultures to the striatum of adult rats. Exp Neurol 127:199–206

Spencer DD, Robbins RJ, Naftolin F, Marek KL, Vollmer T, Leranth C, Roth RH, Price LH, Gjedde A, Bunney BS, Sass KJ, Elsworth JD, Kier EL, Makuch R, Hoffer PB, Redmond DE (1992) Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of patients with Parkinson's disease. N Engl J Med 327:1541–1548

Spenger C, Hyman C, Studer L, Hotayt L, Seiler RW (1992) Free floating microcultures of human fetal ventral mesencephalon (abstract). J Neural Transplant Plast 3:327

Spenger C, Studer L, Evtouchenko L, Egli M, Burgunder JM, Markwalder R, Seiler RW (1994) Long-term survival of dopaminergic neurones in free-floating roller tube cultures of human fetal ventral mesencephalon. J Neurosci Methods 54: 63–73

Spenger C, Hyman C, Studer L, Egli M, Evtouchenko L, Jackson C, Dahl-Jørgensen A, Lindsay RM, Seiler RW (1995) Effects of BDNF on dopaminergic, serotonergic, and GABAergic neurons in cultures of human fetal ventral mesencephalon. Exp Neurol 133:50–63

Spenger C, Haque NSK, Studer L, Evtouchenko L, Bühler B, Lendahl U, Dunnett SB, Seiler RW (1996) Fetal ventral mesencephalon of human and rat origin maintained in vitro and transplantated to 6-OHDA lesioned rats gives rise to grafts rich in dopaminergic neurons. Exp Brain Res 112:47–57

Studer L, Psylla M, Bühler B, Evtouchenko L, Vouga CM, Leenders KL, Seiler RW, Spenger C (1996) Non- invasive dopamine determination by reversed phase HPLC in the medium of free-floating roller tube cultures of rat fetal ventral mesencephalon. Brain Res Bull 41:143–150

Takayama H, Ray J, Raymon HK, Baird A, Hogg J, Fischer LJ, Gage FH (1995) Basic fibroblast growth factor increases dopaminergic graft survival and function in a rat model of Parkinson's disease. Nature Med 1:53–58

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

Walters AM, Clarke DJ, Bradford HF, Stern GM (1992) The properties of cultured fetal human and rat brain tissue and its use as grafts for the relief of the Parkinsonian syndrome. Neurochem Res 17:893–900

Widner H, Tetrud J, Rehncrona S, Snow B, Brundin P, Gustavii B, Björklund A, Lindvall O, Langston JW (1992) Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). N Engl J Med 327:1556–1563

Zabek M, Mazurowski W, Dymecki J, Stelmachów J, Zawada E (1994) A long term follow-up of fetal dopaminergic neurons transplantation into the brain of three parkinsonian patients. Restor Neurol Neurosci 6:97–106