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RESEARCH ARTICLE

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Fetal ventral mesencephalon of human and rat origin maintained in vitro and transplanted to 6-hydroxydopamine-lesioned rats gives rise to grafts rich in dopaminergic neurons

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Abstract Free-floating roller tube cultures of human fetal (embryonic age 6–10 weeks post-conception) and rat fetal (embryonic day 13) ventral mesencephalon were prepared. After 7–15 days in vitro, the mesencephalic tissue cultures were transplanted into the striatum of adult rats that had received unilateral injections of 6-hydroxydopamine into the nigrostriatal bundle 3–5 weeks prior to transplantation. Graft survival was assessed in tyrosine hydroxylase (TH)-immunostained serial sections of the grafted brains up to post-transplantation week 4 for the human fetal xenografts and post-transplantation week 11 for the rat fetal allografts. D-amphetamine-induced rotation was monitored up to 10 weeks after transplantation in the allografted animals and compared with that of lesioned-only control animals. All transplanted animals showed large, viable grafts containing TH-immunoreactive (ir) neurons. The density of TH-ir neurons in the human fetal xenografts and in rat fetal allografts was similar. A significant amelioration of the amphetamine-induced rotation was observed in the animals that received cultured tissue allografts. These results promote the feasibility of in vitro maintenance of fetal human and rat nigral tissue prior to transplantation using the free-floating roller tube technique.

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Introduction

It has now been well established that embryonic neuronal grafts can exert a functional effect on the host animal (Perlow et al. 1979; Dunnett et al. 1983, 1988; Brundin et al. 1986; Strecker et al. 1989; for review, Dunnett 1991; Nikkhah et al. 1993; Strömberg et al. 1991, 1995; Cenci et al. 1993; Frodl et al. 1994). Evidence for afferent and efferent graft-host interconnections have been provided (Doucet et al. 1989; Freund et al. 1985a, b; Strömberg et al. 1989). Moreover, clinical trials in which human fetal cells were transplanted to the brain of patients with severe Parkinson's disease have shown improvement in clinical symptoms to various degrees (for review, see Lindvall 1994). In clinical trials, tissues are transplanted either as fresh cell suspensions (Lindvall et al. 1989, 1992; Peschanski et al. 1994), cryo-preserved cell blocks (Spencer et al. 1992), fresh solid implants (Zabek et al. 1994), hibernated solid implants (Kordower et al. 1995) or as solid strands of tissues maintained in vitro for several days prior to transplantation (Freed et al. 1992; Breeze et al. 1995). To improve functional outcome of patients, tissue pooled from several fetuses is transplanted at several sites in the caudate and putamen and bilateral grafting has been attempted (Widner et al. 1992).

The availability of sufficient viable tissue with dopamine-producing cells is a general problem. Several ventral mesencephalon (VM) explants need to be prepared within hours before the cell suspension transplants. This implicates that abortions must be planned on specific days, that ultrasound-guided, low-pressure abortion technique may be introduced to better preserve the central nervous system (CNS), and that the search for the VM in the aborted material is performed within the shortest possible time. Furthermore, the substantia nigra primordium represents only a relatively small band of dopami-

nergic cells within the VM, and, in order not to exclude any functional tissue, pieces slightly larger than the actual substantia nigra primordium have to be explanted such that varying amounts of serotonergic and GABAergic neurons are included (Spenger et al. 1995).

Although tissue preservation can elude some of these problems, it is usually combined with a loss of cells during storage (Fawcett et al. 1995). However, in vitro preservation of tissue allows for the collection of tissue over longer time periods and testing of the functional capacity of the explanted tissue by measuring dopamine or its metabolites released into the culture medium (Spenger et al. 1995; Studer et al. 1995, 1996).

Basically, storage of human fetal tissue is possible using hibernation, cryopreservation (Frodl et al. 1994) or tissue culture techniques (Spenger et al. 1994). Dopaminergic cells from dissociated cultures were reported to survive transplantation when kept for 2 days in vitro, whereas graft survival was poor after preculturing for 9 days (Brundin et al. 1988). Studies using reaggregate cultures of rat (Strecker et al. 1989) and porcine mesencephalon (Spector et al. 1993) have shown that the time in vitro can be extended when the spheres are not dissociated before transplantation.

In this paper, free-floating roller tube cultures of human fetal VM were transplanted after maintaining the tissue for 7–15 days in vitro. The grafts were placed into the striatum of unilaterally 6-hydroxydopamine (OHDA)-lesioned rats on the ipsilateral side. Graft survival was assessed by immunohistochemical investigation for tyrosine hydroxylase (TH) in the fixed brains collected between 1 and 4 weeks post-transplantation (p.t.). In addition, free-floating roller tube cultures of rat fetal VM were prepared and transplanted into the brain of unilaterally 6-OHDA-lesioned rats and the behavioural response to D-amphetamine was recorded.

Materials and methods

Experimental design

Experiment I: allografts

Five adult female Sprague-Dawely rats (BRL, Füllinsdorf, Switzerland), each with unilateral 6-OHDA lesions, received ipsilateral grafts of cultured rat fetal [embryonic day 13 (ED 13), CRL 9 mm] VM previously maintained in vitro for 8 days by the free-floating roller tube technique (Spenger et al. 1994). The rotational behaviour of the rats with transplants was assessed and compared with another five rats with 6-OHDA lesions that were only injected with medium at the same site (control rats). Another set of nine rats with 6-OHDA lesions and grafted with VM previously maintained in vitro for 7 days served to confirm the above results. Immunohistochemical examination of the grafts was performed to assess survival and TH-ir cell density in the grafts 11 weeks p.t.

Experiment II: xenografts

Twenty-nine adult male Wistar rats (animal care centre of the University of Bern) with unilateral 6-OHDA lesions received ipsilateral grafts of cultured human fetal VM (embryonic age of fetal tissue, 6–10 weeks post-conception, p.c.) maintained in vitro for 7–15 days by the free-floating roller tube technique (Spenger et al. 1994). Immunohistochemical investigation of the grafts was performed at different time points between 1 and 4 weeks p.t. Three of these rats were excluded from the analysis due to infection, graft rejection or penetration of the transplantation needle through the ventro-lateral surface of the brain. All the other 26 rats were used for analysis and the data refers to these animals. Xenografted animals were not behaviourally tested, as no effect was expected to be observed within the 1st month after grafting.

Animals and lesion surgery

Animals (200–220 g body weight) were given unilateral 6-OHDA (Sigma) lesions of the right ascending mesotelencephalic pathway, as described previously (Brundin et al. 1988). Briefly, 4 µl of 32 mM 6-OHDA were injected over 4 min using a 10-µl Hamilton microsyringe at the following coordinates in relation to bregma: A –3.3 mm, L 1.5 mm to the right, V 8.5 mm ventral to the dura, with the tooth bar (TB) set at zero.

Preparation of tissue cultures

Tissue cultures of 11 *human* fetal VM (experiment II) were prepared as described earlier (Spenger et al. 1994). Briefly, human embryonic mesencephalic tissue was obtained from routine suction abortions with the permission of the ethics committee of the Medical Faculty of the University of Bern. After dissection of the fetal brain, the ventral part of the mesencephalon was dissected out and chopped on a tissue chopper (McIllwain) in 250-µm steps. Tissue slices of appropriate size (0.25×1×1 mm) were transferred into 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 drum was tilted 5°. The composition of the culture medium was as follows: Dulbecco's modified Eagle medium (DMEM; Gibco No. 041–02320M) 55%; Hanks balanced salts solution (HBSS; Gibco No. 041–04020M) 33%; total glucose 1.18%; fetal calf serum (FCS; Gibco No. 013–06290) 10%; HEPES 0.01 M (Merck). Antibiotics (1%; Gibco, No. 061–05240) were added during the first 4 days in vitro. Medium change took place at 3- or 4-day intervals.

For cultures of *rat* fetal VM (experiment I) the same culture technique and medium was used as described above and the VM prepared as described by Dunnett and Björklund 1992. The dissected VM was further divided into four equally large pieces by a longitudinal and a transverse incision. Each piece was then placed into a separate culture tube containing 1 ml medium.

Transplantation surgery and immunosuppression

For transplantation, cultures were loaded into a 20-gauge lumbar puncture needle (Unisis, Tokyo) loaded from the tip. Six to ten cultures of human fetal VM (experiment II) or five cultures of rat fetal VM (experiment I) were loaded at a time. Under chloral hydrate anaesthesia (0.4 mg/kg i.p.), cultures were carefully injected into the right caudate putamen (within 20 s) at the following coordinates in relation to bregma: A +1.0 mm, L 3.0 mm to the right, V 5.0 mm ventral to the dura, with the tooth bar (TB) set at zero. The cannula was slowly retracted (1 mm/min) after an interval of 3 min. The xenografted animals were immunosuppressed with cyclosporin-A (CyA; 100 mg CyA/l drinking water; 50 mg Sandimmun/ml; Sandoz, Camberley, UK) combined with a daily administration of tetracycline hydrochloride (0.1 mg/l Achromycin; Lederle, Gosport, UK) through the drinking water. Immunosuppression was started 2 days before grafting.

Behavioral testing

D-Amphetamine-induced (2.5 mg/kg i.p.) rotational behaviour was monitored in automated rotometer cylinders (Columbus Instru-

ments) over a period of 90 min. The lesioned rats were rotated (between 4 and 5 weeks post-lesion) prior to transplantation in order to confirm the completeness of the lesion. Rats with a mean rotational score of at least 4 net turns per min ipsilateral to the lesion side were included. Postoperative rotation of allografted rats was assessed at 4, 6 and 10 weeks p.t. in a first experiment and at 3, 5 and 9 weeks p.t. in a second experiment.

Perfusion, fixation, sectioning

Under deep pentobarbital anaesthesia, rats were perfused through the ascending aorta with 200 ml 0.1 M phosphate buffer (PB) with Heparin [1000 immunizing units (IE)/100 ml; room temperature], followed by 300 ml ice-cold 4% paraformaldehyde in PB. Four hours after fixation, the brains were dehydrated in 20% sucrose/PB and cut at 30–50 µm thickness in coronal planes on a freezing microtome. Slices were mounted on gelatine/chrome-alum precoated glass carriers.

Tyrosine hydroxylase immunocytochemistry

Brain sections were washed three times in PB, incubated in 0.3% Triton-X-100/PB solution for 30 min, washed again and incubated with 1.5% normal goat serum (Vector, Burlingame, Calif.) for 20 min, then washed again and incubated with a polyclonal anti-TH-antibody 0.048 µg/ml (1:500; Pel-Freez Bio) in PB for 12 h at 4°C. Following washes to remove unbound primary antibody, sections were incubated with a biotinylated anti-rabbit antibody (Vector, Burlingame, Calif.) at a concentration of 1:200 in PB for 45 min. Specifically bound antibody was detected following incubation with an avidin-biotinylated horseradish peroxidase complex (Vector, Burlingame, CA) for 30 min and with a metal-enhanced 3,3′-diaminobenzidine (DAB) substrate kit (Pierce, No. 34065) for 10 min at room temperature. Endogenous peroxidase was inhibited by 3.3% H_2O_2/10% methanol in PB after adding the secondary antibody before adding avidin-peroxidase complex.

Morphological assessment

Counting of the TH-positive cells within the transplant was performed in 30-µm- and 50-µm-thick coronal sections of rat brains. Only cells containing a clearly visible nucleus were included in the counting. Every cell fulfilling the criteria was counted within a graft section. The assessed cell number was corrected for the slice thickness and nucleus size according to Abercrombie (Abercrombie 1946). The size of the transplant was assessed using a computer-assisted (Mac II, Image 1.2) device with a CCD camera attached to a Zeiss Axioplan microscope. Cell density was revealed as the number of TH-ir cells divided by the size of the graft section in cubic millimetres. Usually every third section was assessed and the absolute graft volume and TH-ir cell number projected. For the reconstruction of a brain section, a three-dimensional neuron tracing system was used (NTS; Eutectic; Capowski 1989).

Nestin immunocytochemistry

The anti-nestin antiserum 130 has been used previously to characterize the class VI intermediate filament nestin in human CNS tumours (Dahlstrand et al. 1992a). The coronal brain sections were washed three times in PB, incubated in 0.3% Triton X-100/PB solution for 30 min, washed again and incubated with 1.5% normal goat serum (Vector, Burlingame, Calif.) for 20 min, washed again and incubated with anti-nestin antiserum 130 (1:2000) in PB for 12 h at 4°C. Following washes to remove unbound primary antibody, sections were incubated with a biotinylated anti-rabbit antibody (Vector, Burlingame, Calif.) at a concentration of 1:200 in PB for 45 min. Specifically bound antibody was detected as described above.

Statistical analysis

For the statistical analysis, a commercially available software package was employed (Statistica 5.0; StatSoft, Tulsa, Okla.). The ANOVA for repeated measure followed by the Scheffe post hoc test was used to compare rotational scores (control versus grafted animals). All results in the text are expressed as the mean±SEM.

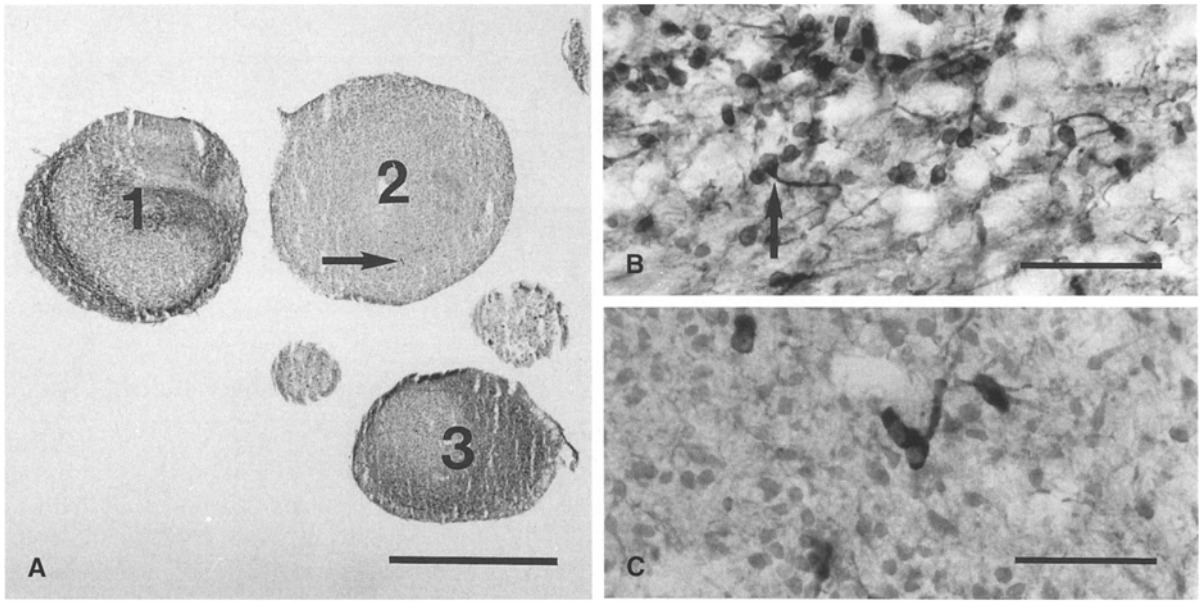


Fig. 1A–C Tyrosine hydroxylase (TH)-immunostained 30-µm-thick cross sections of free-floating roller tube cultures of human fetal ventral mesencephalon. **A** Overview. *Scale bar* 1 mm. Cultures *1* and *3* show areas of intense TH immunoreactivity and areas of poor TH immunoreactivity. Culture *2* shows no major immunostaining. However, some TH-immunoreactive (ir) neurons in culture *2* are present in the area indicated by the *arrow*. These neurons are shown in **C**. **B** Culture *1* at higher magnification. TH-ir neuron marked by the *arrow*. **C** Culture *2* at higher magnification. **B, C** Bright field. *Scale bar* 50 µm

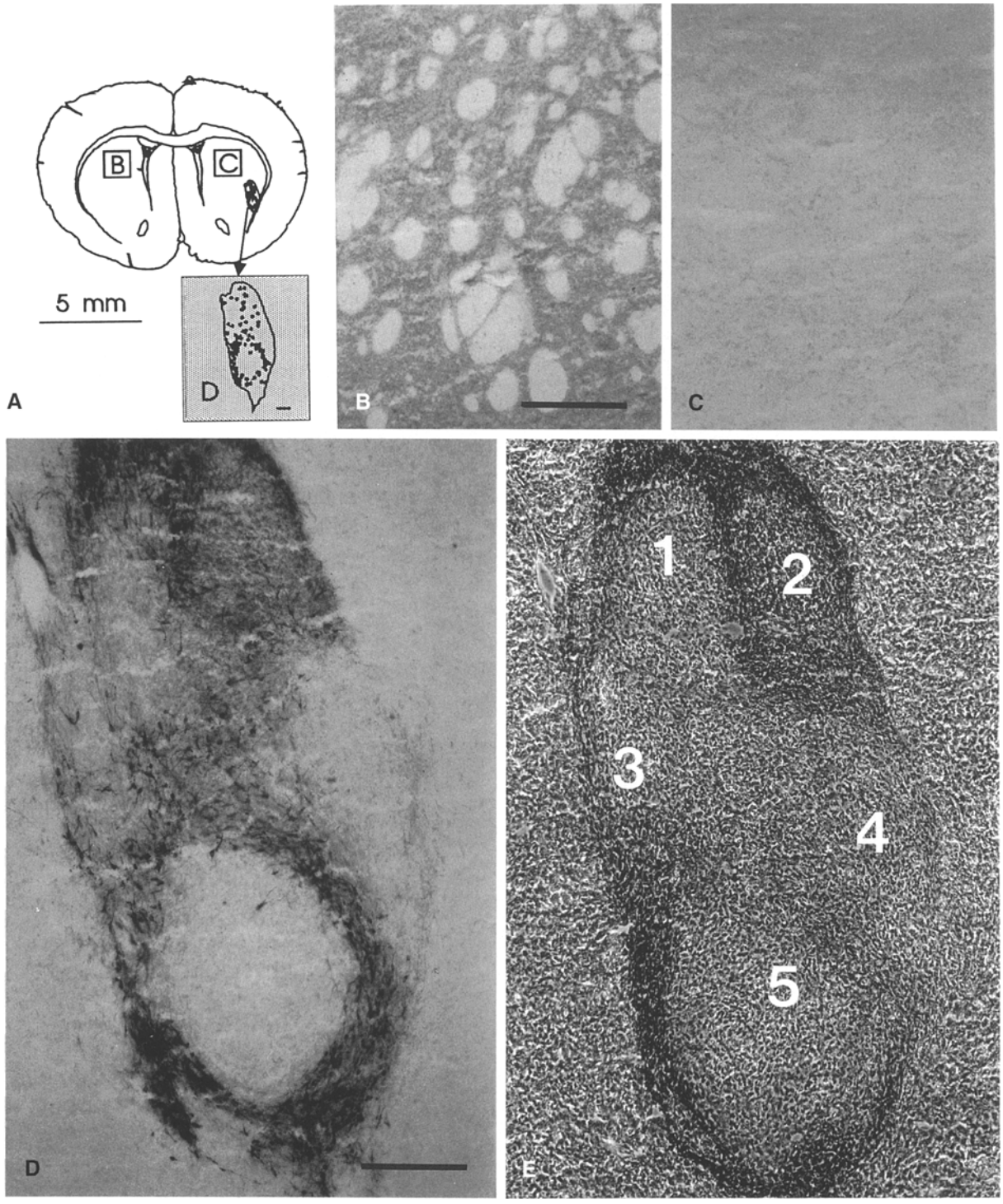


Fig. 2A–E Xenografted rat brain 16 days post-transplantation. **A** Schematic illustration of a coronal section. *Scale bar* 5 mm. *Rectangles B and C*, site of photographs **B** and **C**. **B, C** TH staining of intact and lesioned striatum, respectively. *Scale bar* 200 µm. **D, E** Photomicrograph of TH-stained transplant using bright field or phase contrast, respectively. *Scale bar* 200 µm. The transplant is composed of five original spherical cultures that have fused. The spheres are numbered *1–5* in **E**

Results

Overall assessment of the tissue cultures

The cultures formed small spheres of approximately 0.5–1.5 mm in diameter after 1 week in vitro. Immunostained cross sections of free-floating roller tube cultures of human and rat fetal VM gave rise to numerous TH-ir neurons. Dopaminergic cells were distributed throughout the culture with a tendency to form more dense groups in

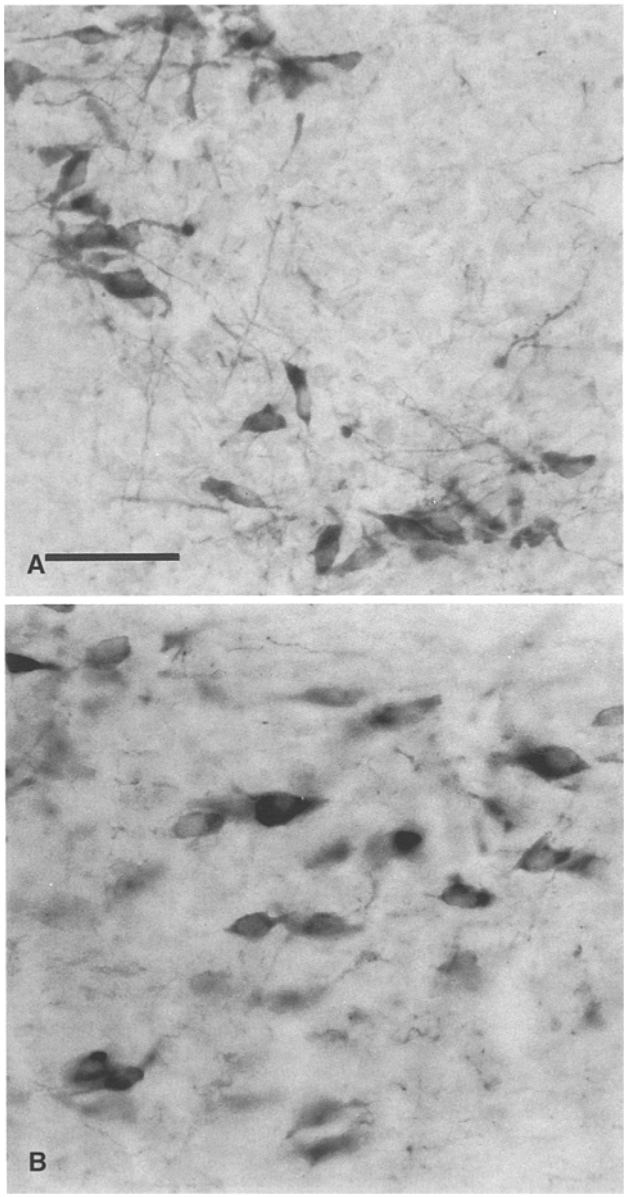


Fig. 3A, B TH-ir neurons in two different human fetal xenografts. **A** Fourteen days post-transplantation (p.t.) **B** Twenty-seven days p.t. (bright-field illumination). *Scale bar* 50 μm

some areas. For human fetal cultures, the number of TH-ir cells varied considerably among different individual cultures from the same fetus. This is well recognized in Fig. 1A, where two cross sections of two human fetal tissue cultures show areas of intense TH-ir, while a cross section of another culture does not. This difference is explained by the fact that each culture only represents a fraction of the explanted VM, and, while some cultures are derived from the centre of the developing substantia nigra, others are derived from more distal parts of the developing substantia nigra.

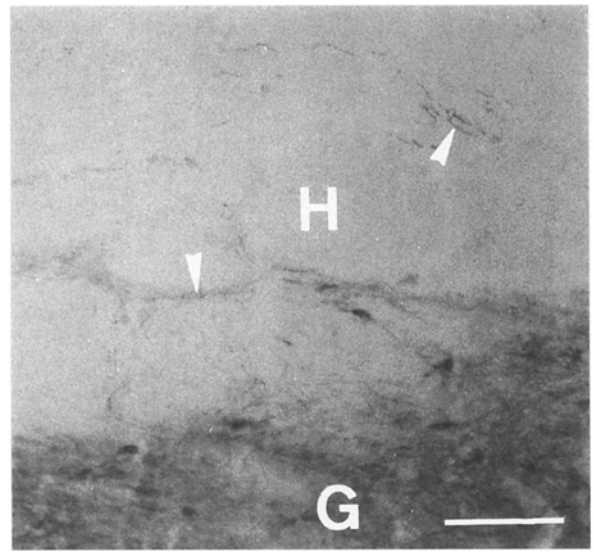


Fig. 4 TH-ir fibres (*white arrowheads*) growing from the xenograft (*G*) into the host brain (*H*). Human fetal xenograft 27 days p.t. Bright field. *Scale bar* 100 μm

Qualitative aspects of human fetal xenografts

The mean graft volume ±SEM was 0.380±0.04 mm^3 as assessed in xenografted animals 1–4 weeks p.t. As can be seen in Fig. 2, the grafts were well circumscribed and spindle shaped, and transplanted cultures fused at their borders, although the spherical shape of the individual cultures generally remained visible. All grafts contained TH-ir neurons, though at different cell densities. Necrotic areas were not observed in any of the 26 grafts. In cultures with lower densities of TH-ir cells, the TH-ir neurons were more frequently arranged at the outer zone of the transplanted cultures. Some of the grafted cultures were found to be densely populated with TH-ir cells, while within the same graft others are not, although all cultures are derived from the same fetus (Fig. 2D). The TH-ir neurons were generally spindle shaped or pyramidal and gave rise to multiple processes (Fig. 3).

TH-ir fibres formed a dense network within the grafts. Nerve fibres growing out of the transplant could already be recognized 2 weeks p.t. but were better seen in grafts 4 weeks p.t. (Fig. 4).

Quantification of human fetal xenografts

The number of TH-ir cells per graft, the graft volumes and the TH-ir cell densities of the grafts in the rats which received a xenograft of cultured human fetal VM are given in Table 1. The mean graft volume derived from six to ten transplanted cultures was 0.380±0.043 mm^3 (n=26), the mean TH-ir cell number per graft was 697±181 (n=26) and the mean TH-ir cell density of the grafts was 1949±404 cells/mm^3 (n=26). The mean TH-ir cell density of transplants derived from fetuses younger than 56 days p.c. was 2527±919 cells/mm^3 (n=10) and that of

Table 1 Quantitative assessment of human fetal xenografts. For each rat (experiment) the embryonic age (EA) of the donor tissue (days post conception (p.c.)), the time of preculturing the tissue (DIV = days *in vitro*) prior to transplantation, the time point at which histological examination was performed (days p.t.), the total number of TH-ir cells in the xenograft (TH cells), the graft volume (graft vol [mm^3]), the TH cell density (TH cells/[mm]^3) and the presence of rosettes in the grafts is given (Y = yes, present; N = no, not present). Average = mean ± SEM

Experiment	EA (days p.c.)	DIV	Days p.t.	TH cells	Graft vol (mm^3)	TH cell/mm^3	Rosettes
1	43	7	16	3801	0.520	7315	Y
2	43	7	16	582	0.457	1274	N
3	44	15	25	373	0.264	1416	N
4	46	10	26	50	0.244	205	N
5	46	10	26	192	0.378	508	N
6	46	10	26	292	0.088	3333	N
7	50	13	13	177	0.571	310	N
8	50	13	20	302	1.019	297	Y
9	53	12	14	922	0.359	2566	Y
10	53	12	7	3424	0.426	8043	N
11	62	10	15	861	0.254	3391	Y
12	62	10	15	256	0.374	684	Y
13	62	10	22	299	0.161	1863	N
14	62	10	22	17	0.269	63	Y
15	63	8	14	711	0.935	760	N
16	63	8	14	292	0.251	1164	Y
17	63	8	14	277	0.313	886	N
18	63	6	21	76	0.211	361	Y
19	63	11	28	71	0.249	285	Y
20	64	12	14	828	0.213	384	Y
21	64	12	21	647	0.310	2088	Y
22	68	15	21	814	0.330	2467	Y
23	68	15	21	1175	0.667	1763	Y
24	72	14	27	82	0.434	189	Y
25	72	14	27	511	0.306	1169	N
26	72	14	27	1099	0.283	3888	Y
Mean±SEM	58.3±1.9	11.0±0.5	19.7±1.1	697±181	0.380±0.043	1949±404	

fetuses older than 56 days p.c. was 1588±320 cells/mm^3 (n=16).

Rosette-like cell formations in human fetal xenografts

Using phase-contrast microscopy, the xenografts were screened for the presence of rosette-like structures. The presence of such cell arrangements has been described earlier in human fetal cultures maintained in vitro by the free-floating roller tube technique (Spenger et al. 1994). These structures were comprised of cylindrical cells arranged in a rosette-like manner, as can be seen in Fig. 5. These cells were always negative for TH and most often localized within TH-poor regions of the grafts. However, these cells stained strongly positive for nestin.

Morphological analysis of rat fetal allografts

All animals receiving grafts of cultures (8 days in vitro) of rat fetal (ED 13) mesencephalon showed large grafts, rich in TH-ir neurons, as can be seen in Fig. 6. Five cultures were transplanted per rat. The mean graft volume was 0.534±0.089 mm^3, the mean number of TH-positive neurons per graft was 986±195 TH-ir cells per graft and the mean TH-ir cell density in the rat fetal allografts was 1986±430 TH-ir cells/mm^3. Interestingly, this density was very similar to that observed in human fetal xenografts, which was 1949±404 TH-ir cells/mm^3 (Table 1).

Behavioural testing of animals receiving rat fetal allografts

The rats were tested for D-amphetamine-induced motor asymmetry both pre- and post-transplantation. The mean preoperative scores for the grafted and the sham-operated animals were 6.2±0.9 (n=5) and 9.3±2.0 (n=5) rotations per minute, respectively. This difference was due to one control animal with a very high preoperative score (15.6 rotations per minute). Therefore postoperative values were calculated as changes relative to the preoperative scores. As shown in Fig. 7, the motor asymmetry scores following transplantation decreased by 71±12%, 73±9% and 89±8% for the animals with transplants and increased by 65±33%, 79±52% and 127±68% for sham-operated animals after 4, 6 and 10 weeks postoperatively. The observation that tissue culture grafts can ameliorate D-amphetamine-induced rotation behaviour was confirmed in a second experiment, where rats with unilateral 6-OHDA lesions (preoperative rotation scores 8.5±0.8 rotations per minute, n=9) following grafting of cultured VM tissues showed a significant reduction (P<0.001) of D-amphetamine-induced rotation of 73%, 103% and 104% at 3, 5 and 9 weeks postgrafting, respectively (Meyer et al. 1996).

Discussion

The present report demonstrates that both human and rat fetal dopaminergic neurons precultured in vitro using the

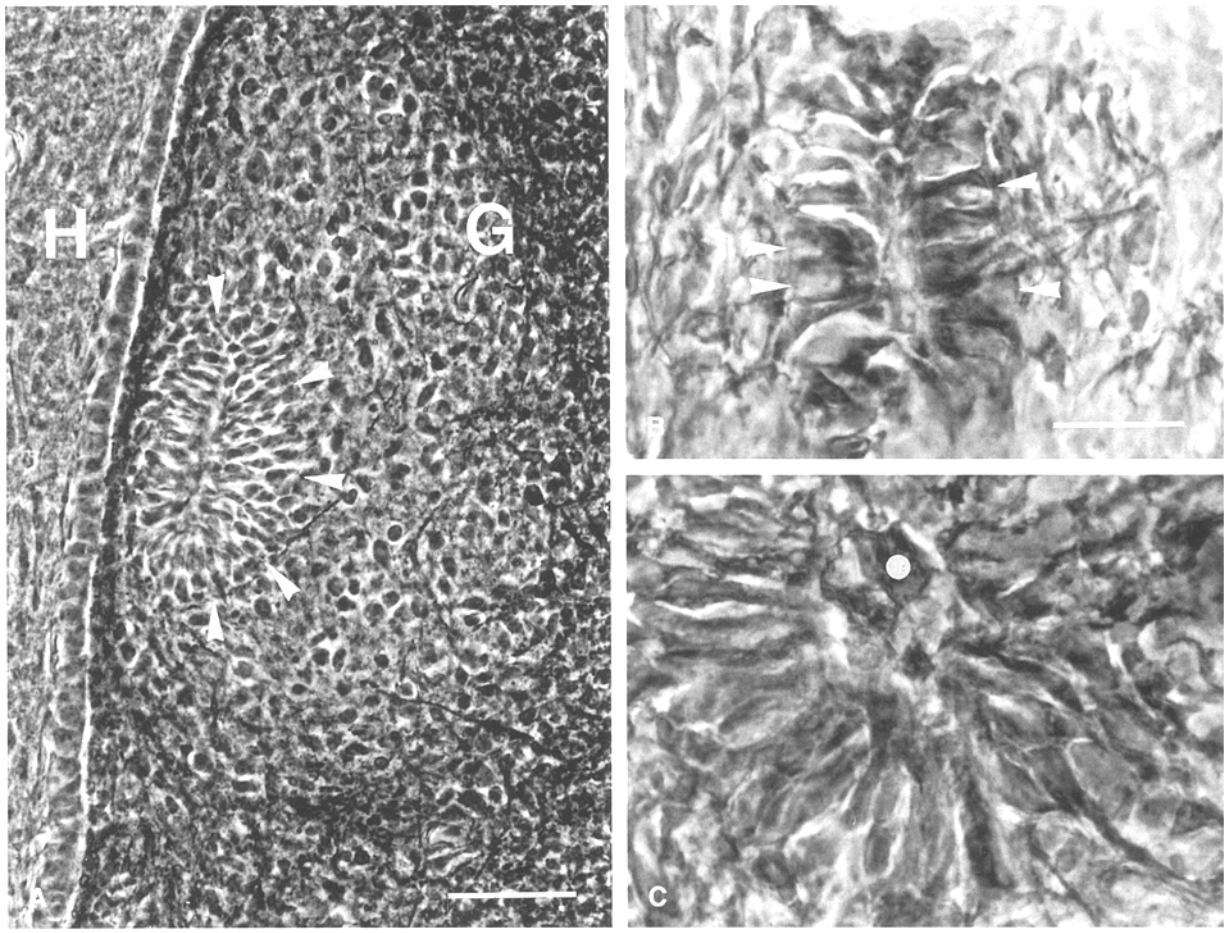


Fig. 5 Rosettes in human fetal xenografts (**A**) 21 days and (**B, C**) 27 days p.t. **A** Phase-contrast photomicrograph of TH-stained slide. Dark TH-ir fibres mark the outer zone of the graft. The graft (*G*) is placed at the ventricular border. *Scale bar* 50 μm (*H* right lateral septal nucleus of host brain). **B** Nestin immunostaining of a rosette-like formation of cells in a human fetal xenograft 27 days p.t. The apical part of the cytoplasm of the cells is densely filled with immunoreaction product. **C** Nestin-immunoreactive rosette-like formation of cells in another human fetal xenograft. Two nestin-immunoreactive cells, one of which is marked by a *white spot* in the centre of the rosette, are readily recognized. **B, C** Oil-immersed bright-field illumination. *Scale bar* 20 μm

free-floating roller tube system can be transplanted to brains of 6-OHDA-lesioned rats. Precultured human fetal xenografts survived for at least 4 weeks and gave rise to large, dopaminergic neuron-rich grafts. Similar results were obtained for precultured rat fetal allografts, which survived for at least 11 weeks, and moreover this treatment resulted in a reversed D-amphetamine-induced rotational behaviour, indicating a functional response of the system.

Brundin and colleagues (Brundin et al. 1988) have shown that dissociated rat dopaminergic neurons grafted after being cultured 2 days in vitro can survive transplantation and reverse amphetamine-induced rotational behaviour of 6-OHDA-lesioned rats. However, the survival of the tissue cultured for 7 days was poor and their function ineffective. It was reasoned that redissociation of the cultured cells prior to transplantation resulted in substantial cell damage in older, differentiated cultures (Brundin et al. 1988). Thus, several groups attempted to develop methods that allow the maintenance of tissues or cells in vitro such that the cells can be harvested and transplanted without the step of dissociation prior to transplantation. Three culture systems, namely the reaggregate culture system (Hemmendinger et al. 1981; Pulliam et al. 1988; Strecker et al. 1989), the organotypic roller tube culture system (Sørensen et al. 1994) and the free-floating roller tube culture system (Spenger et al. 1994) offer the possibility of long-term cultures of mesencephalic dopaminergic neurons that can easily be harvested for transplantation. It has been shown that rat fetal mesencephalic cells from reaggregated cultures kept for 9 days in vitro give rise to grafts rich in dopaminergic neurons and that motor asymmetry was influenced by these grafts (Strecker et al. 1989). A more recent study showed that reaggregate tissue cultures of porcine fetal mesencephalon after 15 days in vitro gave good graft survival and functional effects when transplanted to rats with 6-OHDA lesions (Spector et al. 1993). In the present study we show for the first time that human fetal mesencephalic cells can also be maintained in cultures for at least 15 days prior to transplantation when using the free-

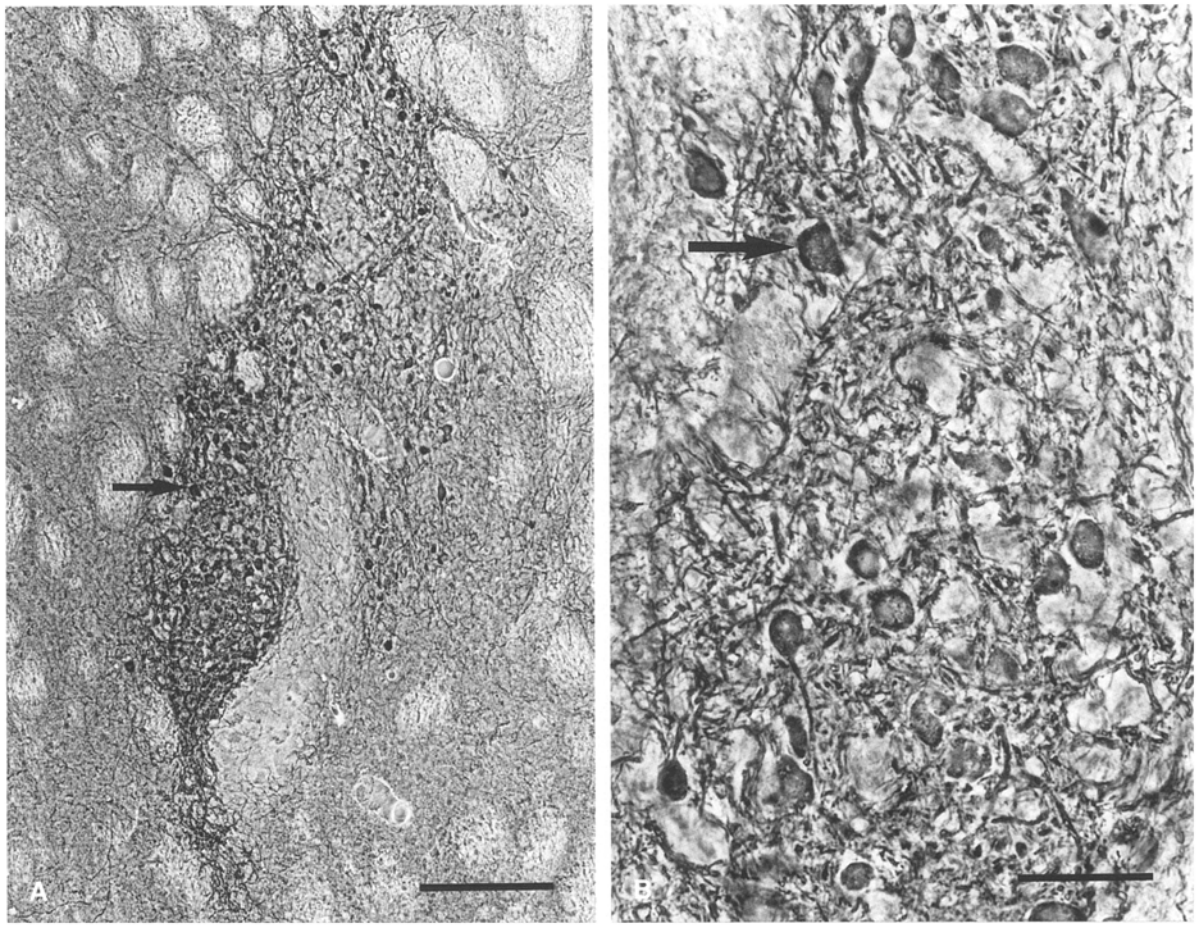
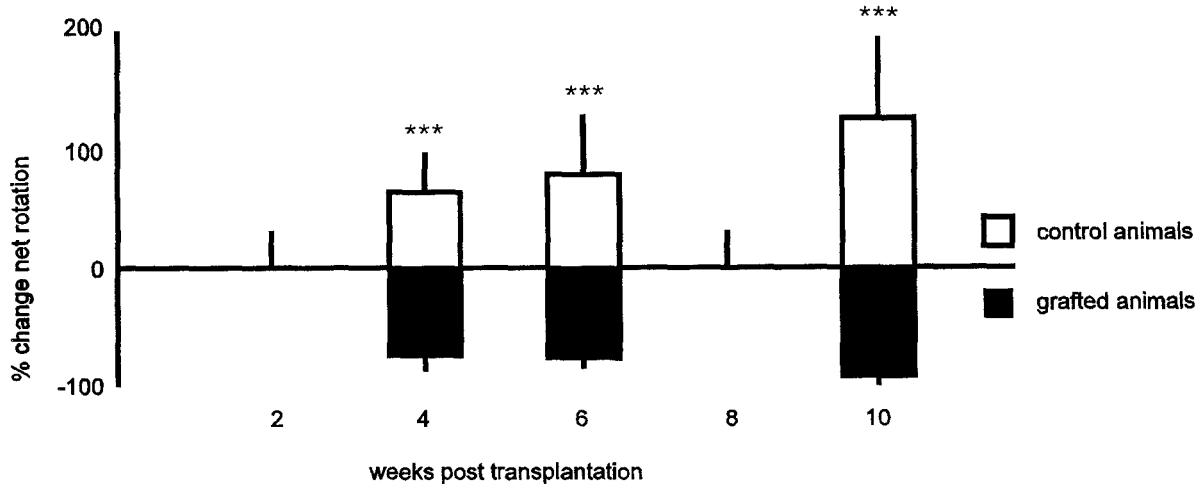


Fig. 6A, B Phase-contrast photomicrograph of a TH-immunostained rat fetal allograft 11 weeks p.t. **A** Overview. *Black arrows* mark same cell in **A** and **B**. *Scale bar* 200 μm. **B** Same transplant as in **A**. Numerous TH-ir neurons, some of which gave rise to TH-ir fibres, are recognized. *Scale bar* 50 μm

Fig. 7 Mean±SEM of percentage change in D-amphetamine-induced net rotation of animals grafted with cultured rat fetal ventral mesencephalon. *Black bars*, grafted animals; *open bars*, sham-operated animals. Statistical analysis was done using ANOVA for repeated measurements, followed by the Scheffe post hoc test (***$P<0.001$)

floating roller tube system and that the cultured tissues give rise to dopaminergic cell-rich transplants.

There is a critical time window in which human (Brundin et al. 1986, 1992), rat (Brundin et al. 1985, 1988) or non-human primate (Sladek et al. 1993) fetal neurons can be used for transplantation using the cell suspension technique with an upper age-limit of 8 weeks p.c. for human fetal suspension grafts (for references, see Brundin et al. 1988). However, solid pieces of human fetal mesencephalon 12 weeks p.c. were reported to survive transplantation (Strömberg et al. 1989). Sørensen



and colleagues reported that slices of ventral mesencephalon from neonatal rats grown in organotypic slice cultures for 1 week survived subsequent grafting (Sørensen et al. 1994). Here we have shown that when the free-floating roller tube technique is employed, tissues from fetuses 9 weeks and 10 weeks p.c. give rise to similar TH-ir cell-rich grafts as compared to tissues from fetuses 6–8 weeks p.c. Thus, employing the described technique allows the use of human fetal tissue throughout a wider range of embryonic age than for the suspension graft technique.

The quantitative histological examination of the allografts showed high TH-ir cell densities (1986 TH-ir cells/mm^3). These numbers were very similar to TH-ir cell densities in the human fetal xenografts (1949 TH-ir cells/mm^3). This compares well with TH-ir cell densities found in studies using the cell suspension technique (Nikkhah et al. 1994a, b), grafts of VM reaggregate cultures of rat (Strecker et al. 1989) and porcine origin (Spector et al. 1993). Of the 11 fetuses examined, 9 gave rise to transplants with mean group cell densities between 936 and 5304 cells/mm^3 and 2 fetuses gave rise to transplants with only 323 (EA 63 days p.c) and 303 cells/mm^3 (EA 50 days p.c). This may reflect in the two cases an inappropriate choice of the site of dissection of the fetal material. In fact the dissection of the VM can be very difficult, as the tissue is often fractured in suction abortions. Testing tissues for the presence of dopaminergic markers prior to transplantation may avoid such errors (Studer et al. 1995, 1996).

Rosette-like structures in free-floating roller tube cultures of human fetal VM (Spenger et al. 1994) and in reaggregate cultures of fetal porcine tissue (Spector et al. 1993) have been described. The nature of these cell arrangements is not clear, but it has been demonstrated that these cells are positive for vimentin, which suggests that these cells represent stem or precursor cells (Bignami et al. 1982; Stagaard and Mollgard 1989). In the present paper we describe the observation of rosette-like cell arrangements in the xenografts. It is assumed that the rosette-like cell arrangements in the grafts are associated with those observed in the cultures. These rosettes were predominantly seen in TH-ir-negative centre parts of the grafts. When the xenografts were immunostained for nestin, these cells were strongly immunoreactive. This provides further evidence that rosettes are comprised of immature cells, as nestin is an intermediate neurofilament protein expressed by immediate precursors to neurons and glia in rats and humans (Lendahl et al. 1990; Dahlstrand et al. 1992b, 1995; Tohyama et al. 1992; Clarke et al. 1994). Nestin expression, however, has also been demonstrated in endothelial cells and in radial glial cells (Clarke et al. 1994). In addition, it was shown that nestin is also temporarily expressed in reactive astrocytes after kainic acid-induced lesions (Clarke et al. 1994).

In the present study we have shown that the free-floating roller tube technique allows the maintenance of tissue in vitro for a considerable time period prior to transplantation. This was true for tissue of rat and human origin. As a consequence of the prolonged culturing period, exposure of these cells to a variety of treatments becomes available, e.g. neurotrophic factors that improve properties of mesencephalic neurons. Such an approach seems to be feasible given our recent results showing that BDNF enhances TH-ir cell number and functional capacity of human fetal ventral mesencephalic cultures in vitro using the free-floating roller tube technique (Spenger et al. 1995). In summary, this culture system provides a powerful tool for effective in vitro maintenance and manipulation of tissues prior to transplantation. The pre-treatment of human fetal neuron cultures with neurotrophic factors is an important topic for future investigations, with the potency of improving cures of neurodegenerative diseases.

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