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Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour, and dopamine release

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Summary. We have used a rat model of Parkinson's disease (PD) to address issues of importance for a future clinical application of dopamine (DA) neuron grafting in patients with PD. Human mesencephalic DA neurons, obtained from 6.5–8 week old fetuses, were found to survive intracerebral cell suspension xenografting to the striatum of rats immunosuppressed with Cyclosporin A. The grafts produced an extensive new DA-containing terminal network in the previously denervated caudate-putamen, and they normalized amphetamine-induced, apomorphine-induced and spontaneous motor asymmetry in rats with unilateral lesions of the mesostriatal DA pathway. Grafts from an 11.5-week old donor exhibited a lower survival rate and smaller functional effects. As assessed with the intracerebral dialysis technique the grafted DA neurons were found to restore spontaneous DA release in the reinnervated host striatum to normal levels. The neurons responded with large increases in extracellular striatal DA levels after the intrastriatal administration of the DA-releasing agent d-amphetamine and the DA-reuptake blocker nomifensine, although not to the same extent as seen in striata with an intact mesostriatal DA system. DA fiber outgrowth from the grafts was dependent on the localization of the graft tissue. Thus, grafts located within the striatum gave rise to an extensive axonal network throughout the whole host striatum, whereas grafted DA neurons localized in the neocortex had their outgrowing fibers confined within the grafts themselves. In contrast to the good graft survival and behavioural effects obtained in immunosuppressed rats, there was no survival, or behavioural effects, of human DA neurons implanted in rats that did not receive immunosuppression. In addition, we found that all the graft recipients were immunized, having formed antibodies against antigens present on human T-cells. This supports the notion that the human neurons grafted to the non-immunosuppressed rats underwent immunological rejection. Based on an estimation of the survival rate and extent of fiber outgrowth from the grafted human fetal DA neurons, we suggest that DA neurons that can be obtained from one fetus may be sufficient to restore significant DA neurotransmission unilaterally, in one putamen, in an immunosuppressed PD patient.

Key words: Neural transplantation – Spontaneous behaviour – Human fetus – Dopamine release – Intracerebral dialysis – Immunization – Cyclosporin A – Parkinson's disease

Introduction

Over the last decade several experimental studies have demonstrated that grafted neural tissue can survive and function in experimental animals (Björklund et al. 1987). The possibility to use intrastriatal implants of fetal dopamine (DA) neurons as an experimental therapy in patients with Parkinson's disease (PD) has been discussed repeatedly over the last few years. The finding that human fetal mesencephalic DA neurons can survive grafting to immunosuppressed rats and reduce amphetamine- and apomorphine-induced motor asymmetry in animals with unilateral lesions of the mesostriatal DA pathway has raised hopes that they could be used for clinical grafting (Brundin et al. 1986; Strömberg et al. 1986). Studies using rat donor tissue have shown that grafted fetal DA neurons also can affect spontaneous behavioural deficits both in rats with 6-hydroxydopamine (6-OHDA) induced lesions of the meso-

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striatal system (Dunnett et al. 1981, 1983, 1986, 1987, for review see Brundin and Björklund 1987) and in rats with age-related motor impairments (Gage et al. 1983). Moreover, rat DA neurons grafted intrastriatally have been found to spontaneously release DA (Zetterström et al. 1986; Strecker et al. 1987), and to establish normal mature synaptic connections with the previously denervated neuronal elements of the host (Freund et al. 1985; Mahalik et al. 1985).

These data, obtained using rat donor tissue, support the idea that grafted DA neurons may be employed as a therapy to ameliorate neurological deficits also in PD patients. However, it has so far been unclear to what extent grafted human fetal DA neurons are able to affect spontaneous behaviour or to restore spontaneous DA release in the DA-denervated striatum. Another critical issue, which is important to clarify before undertaking any clinical trials, is to what extent intracerebral grafts of human fetal CNS tissue from the first trimester can give rise to an immunological reaction in the host. Moreover, it is important to know whether immunosuppression is necessary in order to obtain good graft survival when grafting human fetal CNS tissue across a histocompatibility barrier. The present study was undertaken to clarify these points. The ability of grafted human fetal DA neurons to establish synaptic connections in the DA denervated striatum is reported in a parallel communication (Clarke et al. 1987).

Material and methods

Subjects and lesion surgery

In order to unilaterally deplete the right striatum of DA, female Sprague-Dawley rats (ALAB, Stockholm, Sweden), weighing 180–200 g at the time of lesion surgery, were given unilateral stereotaxic injections of 6-OHDA in the right ascending mesostriatal DA pathway. Briefly, 2.5 µl of 6-OHDA (3 µg/µl in 0.2 mg/ml ascorbate-saline) were injected under equithesin anesthesia (0.3 ml/100 g) at the following coordinates: 4.4 mm caudal to bregma; 1.2 mm lateral to midline; 7.8 mm ventral to dural surface; with the toothbar set at 2.4 mm below the interaural line. A second injection of 2 µl of 6-OHDA (same concentration as above) was performed at the following coordinates: 4.0 mm caudal to bregma; 0.8 mm lateral to midline; 8.0 mm ventral to dural surface; with the toothbar set at 3.4 mm above the interaural line.

Donor tissue and transplant groups

At 1.5–6 months after the 6-OHDA lesion, 3 groups of rats with complete 6-OHDA lesions of the right mesostriatal DA pathway (see below) received implants of mesencephalic tissue obtained from aborted human fetuses of different post-conceptional ages (time from fertilization). Lesioned rats without grafts served as controls in the dialysis experiment. With the approval of the Research Ethical Committee at the University of Lund, 3 fragmented fetuses were obtained at suction curretage abortions. Their post-conceptional ages were 6.5 weeks (PC-6.5), 8 weeks (PC-8) and 11.5 weeks (PC-11.5). The pregnancies were timed by measurements of the length of the fetus using ultrasound technique and by securing distinguishing developmental characteristics on the fetus. The neural grafting was performed within 2 to 5 h after the abortion.

The region of the ventral mesencephalon containing DA neurons was dissected and prepared according to the cell suspension method (see Brundin et al. 1985a) for the PC-8 fetus (in 50–60 µl glucose-saline) and the PC-11.5 fetus (in 60–70 µl glucose-saline). In the case of the PC-6.5 fetus (in 30–40 µl glucose-saline) the trypsin step was omitted. Cell viability was assessed soon after dissociation, using acridine orange and ethidium bromide as a vital stain (Brundin et al. 1985a), and was as follows: PC-6.5 = 68%; PC-8 = 86%; PC-11.5 = 65%. The cell suspension prepared from the PC-6.5 and PC-8 fetuses were grafted into 3 and 9 recipient rats, respectively. Two × 3 µl (PC-6.5) and 2 × 2 µl (PC-8) aliquots of the cell suspensions were implanted into the head of the caudate-putamen of each host (coordinates: A = +1.0; L = 3.0; V = 5.0 and 4.1; in mm, with reference to bregma and dura respectively, and with the toothbar set at zero) using a 10 µl Hamilton microsyringe fitted with a 0.25 mm inner diameter cannula. In the PC-11.5 group, three 2 µl deposits were implanted in 6 recipient rats (coordinates: (1) A = +1.8; L = 2.5; V = 4.5; (2) A = +0.6; L = 2.0; V = 4.5; (3) A = +0.6; L = 3.2; V = 4.5; in mm; with reference to bregma and dura respectively, and with the toothbar set at zero). All transplantation surgery was conducted under equithesin anaesthesia.

Immunosuppression

All rats in the PC-6.5 and PC-11.5 groups, and 5 of the 9 PC-8 rats, were given daily injections of approximately 10 mg/kg, i.p., of Cyclosporin A (CyA) (Sandimmune®, Sandoz, 50 mg/ml diluted to 10 mg/ml in sterile saline) starting on the day of transplantation surgery. To reduce the risk of opportunistic infections in the graft recipients, all animals (also the non-immunosuppressed rats) were administered tetracyclin (Terramycin, Pfizer, approx. 20–50 mg/kg daily) through the drinking water.

Motor asymmetry tests

Amphetamine-induced rotation. Three to 4 weeks after the 6-OHDA lesion, the rats were given 5 mg/kg of d-amphetamine, i.p., and their rotational behaviour was monitored in automated "rotometer bowls" for 90 min (Ungerstedt and Arbuthnott 1970). Eighteen rats that exhibited a mean of at least 6.2 full body turns per min ipsilateral to the lesion were selected for transplantation. This test was repeated 3 to 4 times during the subsequent 12–19.5 weeks after transplantation (see Fig. 1A–C). For the analysis of graft effects on motor asymmetry, a net rotation asymmetry score was calculated by subtracting turns contralateral to the lesion from ipsilateral turns. An additional 4 rats, which reached the same rotation criterion, were used as 6-OHDA lesion controls in the intracerebral dialysis experiment.

Apomorphine-induced rotation. Prior to grafting and at 18 weeks after grafting the rats in the PC-8 group were tested for apomorphine-induced rotation (0.05 mg/kg, s.c., in the neck) during 40 min. One of the 4 rats in the non-immunosuppressed PC-8 group which reached the pre-transplant criterion on amphetamine-induced rotation (11.6 turns/min), but which did not show a marked apomorphine-induced rotation after the 6-OHDA lesion in the pre-transplant test, was not included in the post-transplantation analysis of apomorphine-induced rotation.

Spontaneous rotation. At 19 weeks after grafting, 8 rats in the PC-8 group were tested for their spontaneous (i.e. non-drug-induced) motor asymmetry in the rotometers for a 10 h period during their dark period (approx. 20.00 h–06.00 h). The rats were allowed to habituate to the rotation bowls for approximately 10 min before the start of the test and the total number of left or right half turns was counted.

Intracerebral dialysis

Spontaneous and drug-induced DA release in the graft-innervated striatum was measured with the intracerebral dialysis technique (Ungerstedt 1984). The measurements of DA release were performed 20–21 weeks post-grafting in 8 CyA-treated rats which were later shown to have surviving grafts (one rat in the PC-6.5 group, 4 in the PC-8 group, and 3 rats in the PC-11.5 group), and which showed less than 6 turns/min amphetamine-induced net rotation asymmetry in the post-transplantation test. Four 6-OHDA lesioned rats without grafts, were used as controls. The day before the collection of dialysis samples, rats were anesthetized with chloral hydrate (0.35 g/kg, i.p.) and dialysis loops were surgically implanted bilaterally into the striatum in a position about 0.6 mm from the presumed graft sites (A = +1.2 mm from bregma, L = 2.5 mm, V = 5.3 mm from dura with the tooth bar set at zero). These dialysis loops were made from hollow, flexible, saponified cellulose ester dialysis fiber (CD Medical International, Ltd) with an outer diameter (wet) of 0.27 mm and a molecular weight cut-off of 10,000 Daltons. Both ends of the tubing were glued inside stainless steel tubing (inner diameter 0.26 mm), leaving a 4 mm length to be exposed to the brain. Prior to implantation this tubing was moistened and folded in half, making the dorsal-ventral length of the exposed probe equal to 2 mm (see Sharp et al. 1986 for further details). Throughout the dialysis sample collection the rats were maintained under halothane anesthesia (1.2% halothane-air mixture), and body temperature was kept at 37° C using an incandescent light. The dialysis loops were continually perfused at a rate of 2 µl/min with Ringer solution. After discarding the first 30 min of striatal perfusate, the perfusates were collected during 15 min sampling periods into plastic Eppendorf microtubes, containing 2 µl of an additives solution which contained 28.5 mg EGTA and 18 mg reduced glutathione per ml at a pH of 6–7 (pH adjusted with 6N NaOH). At least four baseline release samples were collected, followed by two 15 min samples in which the DA releasing agent d-amphetamine sulphate (10^{-5} M) was added to the Ringer perfusion medium. After returning to the Ringer only perfusion medium and allowing enough time for DA levels to approach baseline values again, the DA reuptake blocker nomifensine hydrogen maleate (0.67×10^{-5} M; Hoechst) was also added to the Ringer for 2 samples. Immediately after collection, perfusates were frozen in liquid nitrogen and stored at –80° C for up to 1 week prior to assay. At the conclusion of the experiment, the grafted rats were perfused on the same day for catecholamine histofluorescence (see below), while the 6-OHDA lesion-only rats were decapitated and their brains were dissected on ice, allowing the identification of the dialysis loop tracts in the striatum, as well as the collection of bilateral striatum tissue samples (in the vicinity of the dialysis loop) for later assay of tissue DA content.

The dialysis samples were assayed by a radioenzymatic method that combined incubation and extraction steps modified from the procedure of Peuler and Johnson (1977), and separation and counting steps modified from Schmidt et al. (1982). Twenty-five µl of each perfusate sample were added to incubates containing (expressed as final concentration) 100 mM Tris, 30 mM MgCl, 10 mM EGTA, 4 µl of a previously prepared catechol-O-methyltransferase (COMT) enzyme solution (Nikodejevec et al. 1970), 1.4 mM dithiothreitol and 1.5 µl S-adenosyl-L-methionine-(^{3}H-methyl) (Amersham, specific activity 90 Ci/mmol). The total incubation volume was 50 µl and the pH was 8.1–8.3. DA was added as an internal standard to additional samples of incubation mixture plus 25 µl of perfusate. Blank tubes containing the complete incubation mixture, without the addition of perfusate or standards, and internal standards ranging from 0.01 to 0.2 ng of DA were always run with the pooled residual dialysis samples.

The samples were incubated at 37° C for 40 min. The reaction was stopped by the addition of 25 µl of a solution consisting of 800 mM boric acid, 80 mM EDTA-Na_2 and 4 mM of 3-methoxytyramine in 1 N NaOH. The resulting solution (pH 10.0 ± 0,2) was vigorously mixed and the ^{3}H-3-methoxytyramine derivative was extracted into 1 ml of toluene/isoamylalcohol (3 : 2). Aqueous and organic phases were separated by centrifugation, and 0.95 ml of the organic layer was decanted into a second tube containing 50 µl of 0.1 N acetic acid and the ^{3}H-3-methoxytyramine derivative was extracted into the aqueous layer with vigorous shaking. The samples were then placed on dry ice, and the organic layer was aspirated and discarded. The aqueous layer was then washed with 0.5 ml of toluene/isoamylalcohol (3 : 2) and after centrifugation and freezing, the organic layer was discarded again. Finally, 35 µl of the acid, together with 30 µg of carrier amine, was chromatographed with chloroform/methanol/70% ethylamine (16 : 3 : 2) onto silica gel plates (Whatman LK5DF). The spot corresponding to DA was scraped into scintillation vials, extracted with 0.3 ml ethanol-ammonium hydroxide (100 : 20), and counted with 0.3 ml ethoxyethanol and 10 ml scintillation mixture (Instafluor, Packard). This assay had a twice blank sensitivity of 5–10 pg for dopamine. Tissue samples from the lesion-only rats were sonicated in ice-cold 0.1 M perchloric acid at a dilution of 1 : 25, after which 25 µl of the supernatant was assayed by the procedure of Schmidt et al. (1982).

Antibody detection

In order to identify an immunological response towards the grafted tissue, sera from grafted and control animals were analysed for the presence of antibodies directed against human cells. Purified human T-cells were assayed for direct binding of sera detected by the indirect fluorescent antibody technique in a quantitative fluorocytometer according to a modification of the method of Möller (1961). Approximately 2 ml of blood was taken from each rat immediately prior to perfusion. After centrifugation, serum was collected from the blood sample and then kept frozen at –20° C until the antibody assay was performed. Samples were taken from all grafted rats that had survived throughout the 19–21 weeks post-transplantation period and from 3 unoperated control rats housed under the same conditions.

T-lymphocytes were chosen as the human target cells, since these cells express high levels of major histocompatibility complex (MHC) antigens and several other antigens that display polymorphism. For the antibody assay, human lymphocytes were prepared from buffy-coats (leukocyte concentrates) from 2 different healthy blood donors. The blood was diluted in equal parts of saline and Earles balanced salt solution (BSS, Gibco) and separated by a gradient centrifugation of ficoll-isopaque (Lymphoprep®, Nygaard A/S), 1500 rot./min for 15 min at 4° C. The lymphocyte cellband was harvested, and washed × 3 in BSS. The cells were suspended in complete medium to a concentration of 1×10^7/ml in complete RPMI 40 medium (Gibco), supplemented with 5% fetal calf serum and kept in culture over night. T-lymphocytes were separated from B-lymphocytes by sheep erythrocyte rosetting. On the day of assay, human T-lymphocytes at a concentration of 1×10^6/ml in BSS were mixed with an equal volume of 1% AET-(2-amino-ethyl-isothioromiun-bromide;

Sigma)-labelled sheep red blood cells (Statens Veterinärmedicinska Laboratorium) suspended in 40% fetal calf serum and BSS. The mixture was centrifuged to obtain a pellet and then incubated for 15 min on ice. The pellet was gently resuspended in the supernatant and the T-cell-erythrocyte rosettes was separated by centrifugation on ficoll-isopaque as described above. The harvested T-cells were washed twice. Thereafter, the bound erythrocytes were lysed by an osmotic shock of distilled water for 15 s. The purified T-cells were subsequently counted and resuspended to a concentration of 1×10^7/ml in BSS. The viability was more than 90% as determined by trypan blue exclusion. One ml of the suspension was transferred to hemolysis tubes and centrifuged to a pellet. The supernatant was discarded and the cells resuspended in 50 µl of sera from either normal control rats or operated animals. The cells were then incubated for 15 min at room temperature under constant agitation to allow binding of rat immunoglobulins to the surface of the human cells and were rinsed twice in 2 ml of BSS in order to wash off unbound immunoglobulins. Fifty µl of Fluoroscein isothiocyanate-(FITC) labelled goat anti-rat-immunoglobulin (1 : 10 in BSS, Southern Biotechnologies Inc.) was added and incubated for 15 min at room temperature under agitation. The fluorochrome labelled antiserum was ultracentrifuged for 5 min at 100 000 g to eliminate complexes. Afterwards, the cells were washed three times in BSS, resuspended in 1 ml BSS and kept on ice. The cell suspension was run in a quantitative fluorocytometer (Ortho Diagnostics, Spectrum III). The lymphocyte population (as determined by the cell size and granulation) was analysed for fluorescence intensity. A cut-off level was arbitrarily defined as the level of fluorescence intensity which was surpassed by less than 2% of the lymphocytes in the 3 control sera (Fig. 4A). For each serum the percentage of lymphocytes that displayed a fluorescence intensity above the arbitrary cut-off level was determined. Increased binding of rat immunoglobulins on human target cells was considered to occur when this percentage exceeded the mean percentage + 2 standard deviations for the 3 control sera.

Catecholamine histofluorescence

Twenty–21 weeks after transplantation, the brains were processed for catecholamine histofluorescence according to the ALFA method (Lorén et al. 1980; procedure I). Briefly, the rats were perfused through the ascending aorta with an ice-cold aluminum-formaldehyde solution under high pressure, and the forebrains were dissected and rapidly frozen in a mixture of propane-propylene cooled by liquid nitrogen. After freeze-drying, the specimens were reacted with formaldehyde vapour for 1 h at 80° C. After embedding in paraffin, sections were cut at 15 µm thickness. Catecholamine cell body counts in the grafts were performed in every third section on blind coded slides and the cell number estimated according to the formula of Abercrombie (1946).

Tyrosine hydroxylase immunohistochemistry

One rat from the PC-6.5 group, which showed functional recovery in the amphetamine-induced rotation test (marked *c* in Fig. 1B), was perfused for tyrosine hydroxylase (TH) immunohistochemistry 20 weeks after transplantation, essentially according to the procedure of Freund et al. (1985). After a preperfusion through the ascending aorta (descending aorta clamped) with 100 ml of 0.9% saline at room-temperature, the rat was perfused during 15 min with 500 ml of ice-cold 2% paraformaldehyde + 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was stored in PB for 3 days prior to being cut on a vibrating microtome (Bio-rad, USA). Sections, 70 µm thick, were cut through the caudate-putamen and the mesencephalon at the level of the substantia nigra. The sections were rinsed 3 times in PB-saline (PBS) and then preincubated in normal goat serum +0.05% Triton X100 for 30 min. The sections were then incubated for 48 h at 4° C in anti-TH serum (kindly donated by Dr. J.F. Powell, Oxford, UK) diluted to 1 : 1600 in PBS containing 0.05% Triton X100. The production and characterization of the TH antibody has been described elsewhere (van den Pol et al. 1984). After rinsing the sections were incubated in goat-anti-rabbit IgG (1 : 40, Miles) for 12 h at 4° C, and then incubated with rabbit peroxidase-anti-peroxidase complex (1 : 100, Miles) for 3 h at room temperature under constant agitation. In the peroxidase reaction diaminobenzidine (0.05%) was used as chromogen and the sections were treated with OsO_4 to intensify the reaction product. The sections were dehydrated and embedded in Durcupan ACM resin (Fluka) and examined in the light microscope. The number of graft TH-immunoreactive cell bodies were counted on every section and a representative sample were measured. This material was subsequently processed for electron microscopy as part of a parallel immunocytochemical study (Clarke et al. 1987).

Results

One rat in the PC-6.5 group and one rat in the PC-11.5 group died during the course of the experiment and are therefore not included in the behavioural and morphological analysis. The brain of the PC-11.5 rat was immersion fixed after death (16 weeks post-grafting), cryostat sectioned and cresyl violet stained. The right cerebral hemisphere was found to be heavily infiltrated throughout with small round cells, indicating the presence of an ongoing inflammatory response.

Motor asymmetry

Amphetamine-induced rotation. As summarized in Fig. 1, the CyA-treated rats receiving grafts from young donors (PC-6.5 and PC-8) showed marked reductions in rotation asymmetry (Fig. 1B), whereas rats in the non-immunosuppressed PC-8 group did not show any permanent effects of the grafts (Fig. 1A) and rats in the CyA-treated PC-11.5 group exhibited variable and only minor graft effects (Fig. 1C).

As the rats in the CyA-treated PC-8 and PC-6.5 groups were found to have comparable graft survival in the microscopic analysis (see below) they were analysed together for the amphetamine-induced rotation tests. Of the 7 CyA-treated rats in the PC-6.5 and PC-8 groups, 6 rats showed a marked (> 94%) reduction in amphetamine-induced net rotation asymmetry in at least one of the post-grafting rotation tests (Fig. 1B). The seventh rat from the PC-8 group (labelled *a* in Fig. 1B), which only attained a 15% reduction in net rotation asymmetry did, how-

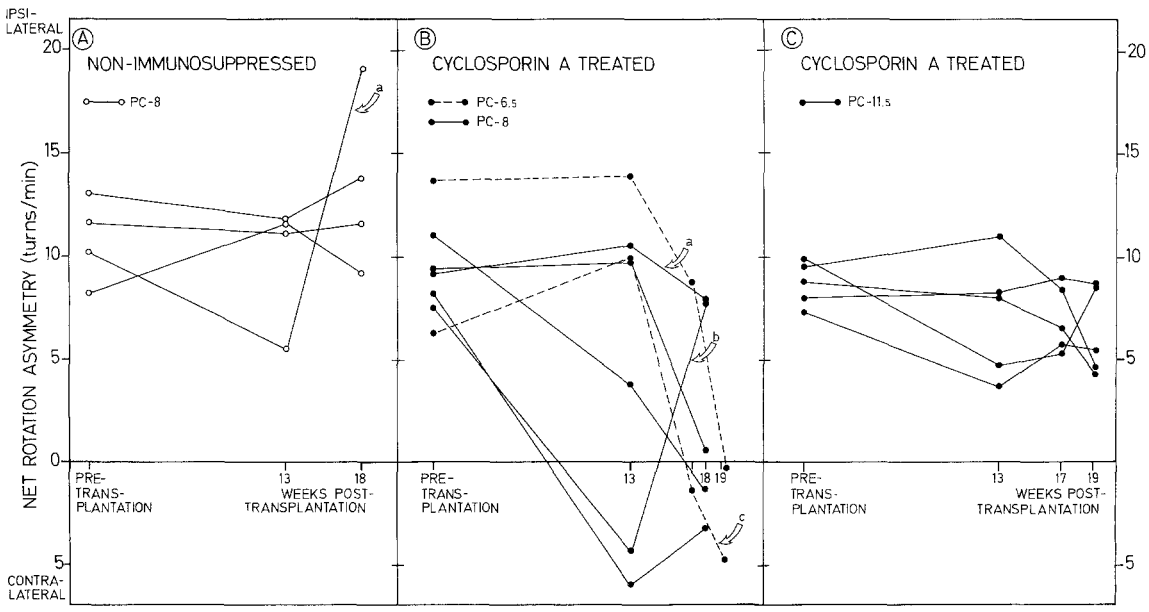

Fig. 1A–C. The amphetamine-induced net rotation asymmetry score (turns contralateral to lesion subtracted from turns ipsilateral to lesion) plotted for each individual transplant recipient before transplantation and at various timepoints, between 13 and 19.5 weeks, after transplantation. **A** Non-immunosuppressed rats (n = 4) receiving transplants from the PC-8 fetus (rat marked a is discussed in the text). **B** CyA-treated rats (n = 7) receiving transplants from the PC-6.5 and PC-8 fetuses (rats marked *a, b* and *c* are discussed in the text). **C** CyA-treated rats (n = 5) receiving transplants from the PC-11.5 fetus

ever, exhibit 102 full contralateral left body turns over 90 min at the 18 week test compared to 0 left turns in the pre-graft test. This ability to turn in the direction contralateral to the 6-OHDA lesion has previously been found to be a sensitive indicator of DA graft function (Herman et al. 1985; Brundin et al. 1986). Of the 5 rats that exhibited a complete reversal of net rotation asymmetry after 18–19.5 weeks, 3 rats showed no reduction in net rotation asymmetry at 13 weeks. However, all the CyA-treated PC-6.5 and PC-8 rats performed between 5 and 567 (mean = 172) full contralateral turns in the 13 weeks test, compared to 0 for all these rats at the pre-graft test, which indicates that the grafts were somewhat functional already at 13 weeks.

One rat in the PC-8 group which showed a complete reversal of net rotation asymmetry after 13 weeks (labelled *b* in Fig. 1B) returned to its pre-transplantation value after 18 weeks. Such a pattern of behavioural changes is suggestive of an existing graft ceasing to function between 13 and 18 weeks after transplantation. This rat exhibited marked weight loss and signs of malaise over the last few weeks and, at subsequent histological analysis (see below), extensive pathological changes were observed in the transplant-containing hemisphere. Therefore this rat was excluded from the statistical analysis of amphetamine-induced rotation (see below).

Three of the 4 rats in the non-immunosuppressed PC-8 group did not exhibit any changes in amphetamine-induced rotation after grafting (Fig. 1A). The fourth rat showed a 48% reduction in net amphetamine-induced rotation 13 weeks after grafting but, presumably as the result of graft rejection, the rotation asymmetry increased to above pre-transplantation levels at 18 weeks. This rat exhibited 255 full contralateral body turns over the 90 min test period 13 weeks after grafting, whereas prior to grafting and also 18 weeks after grafting it did not perform any full contralateral body turns.

In the CyA-treated PC-11.5 group functional graft effects were less marked than in the immunosuppressed PC-6,5 and PC-8 rats (Fig. 1C). One rat which died after the 13 week timepoint (not included in Fig. 1C) exhibited a 84% reduction in amphetamine-induced rotation asymmetry. Of the remaining 5 rats, one showed an increase in net rotation asymmetry and the other 4 rats exhibited decreases of between 13% and 52% of their respective pre-transplantation scores. The rat with the 52% decrease in rotation asymmetry performed 200 full body turns (over 90 min) contralateral to the lesion, at 19.5 weeks after grafting, which indicates the presence of a functional graft. When the 19.5 week rotation asymmetry values were compared, using a paired Student's t-test, to the respective pre-transplantation values, there was no change in the PC-11.5

group (mean ± S.E.M.; pre-grafting = 8.7 ± 0.45; 19.5 weeks post-grafting = 6.3 ± 0.97; t(4) = 2.25, $p > 0.05$).

When the scores for the pre-graft and final rotation test were compared for the 3 groups (the immunosuppressed PC-6.5 + PC-8 group, the immunosuppressed PC-11.5 group, and the non-immunosuppressed PC-8 group) there was a significant group x time interaction (2-factor repeated measures ANOVA, G × T, F(1,22) = 3.66, $p < 0.02$, the mean score for the 17 and 19.5 week timepoints for the PC-6.5 and PC-11.5 rats was treated as one timepoint). There was no difference between the 3 groups before grafting or at 13 weeks after grafting (one factor ANOVA; $p > 0.05$) whereas at the 18 week timepoint the CyA-treated PC-6.5 + PC-8 rats showed significantly less rotation when compared to the non-immunosuppressed PC-8 rats (one factor ANOVA; F(2,13) = 12.26 with post-hoc Scheffé's test, $p < 0.05$). When the 18 week timepoint rotation asymmetry values were compared to the respective pre-transplantation values, there was a significant reduction in the CyA-treated PC-6.5 + PC-8 rats (t(5) = 3.36, $p < 0.03$; paired Student's t-test) whereas the non-immunosuppressed PC-8 rats displayed no change (t(3) = 1.30, $p > 0.05$).

Apomorphine-induced rotation. Eighteen weeks after grafting the CyA-treated PC-8 rats showed a 58–79% reduction in apomorphine-induced rotation compared to their pre-transplantation score (paired Student's t-test: t(3) = 6.24, $p < 0.01$) whereas the non-immunosuppressed rats showed unchanged or increased values (t(2) = 1.03, $p > 0.05$) (Fig. 2A) (one rat in the non-immunosuppressed group was excluded from the analysis, see Material and methods). The difference between the CyA-treated and the non-immunosuppressed rats was statistically significant (Student's t-test: t(5) = 4.84, $p < 0.01$) (Fig. 2A).

Spontaneous rotation. In the test for spontaneous motor asymmetry, which was performed in the 8 healthy rats in the PC-8 group, the non-immunosuppressed rats all exhibited a marked asymmetry towards the lesioned side, whereas the immunosuppressed rats were either fully symmetric or biased in the opposite direction. This difference in side bias was statistically significant (Student's t-test: t(6) = 2.65, $p < 0.05$) (Fig. 2B).

Intracerebral dialysis

In the intracerebral dialysis experiment the grafts restored basal extracellular DA levels to normal or

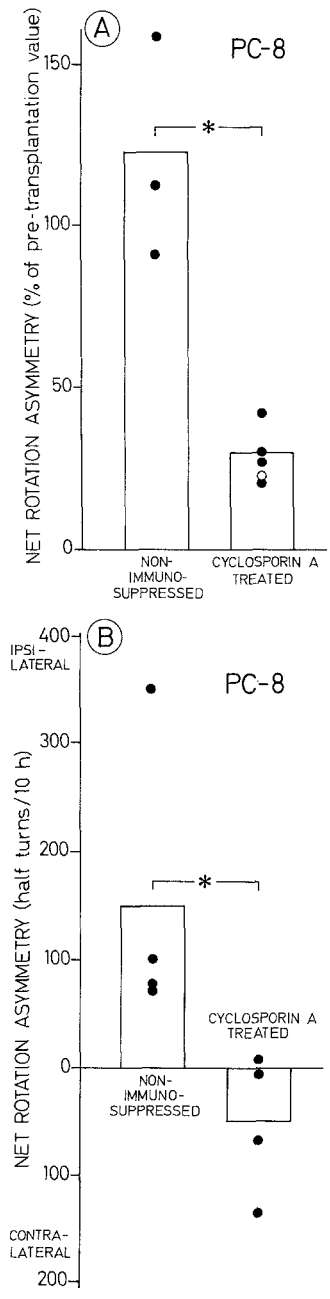

Fig. 2. A Apomorphine-induced net rotation asymmetry (turns ipsilateral to lesion subtracted from turns contralateral to lesion; expressed as a percentage of pre-transplantation score) 18 weeks after grafting for the non-immunosuppressed (n = 3) and CyA-treated (n = 5) rats receiving transplants from the PC-8 fetus. The open circle represents the result for the rat which exhibited cerebral pathology (see Results). This rat was excluded from the calculation of the mean and from the statistical analysis. **B** Spontaneous rotation asymmetry monitored overnight for 10 h during the rats' dark period 19 weeks after transplantation in the non-immunosuppressed (n = 4) and CyA-treated (n = 4) rats receiving transplants from the PC-8 fetus. "Ipsilateral" and "contralateral" with reference to the side of the lesion. Dots represent individual rats and bars show group means. * indicates $p < 0.02$ in **A** and $p < 0.05$ in **B** (Student's t-test)

supranormal values, and administration of amphetamine or nomifensine produced large increases in the amount of DA recovered from the grafted animals, although these drug-induced increases were smaller than those seen in the contralateral intact striatum (Fig. 3A, B). For statistical analysis the dialysis data were subjected to logarithmic transformation in order to reduce the heterogeneity of the variance between different groups. Baseline DA release data was statistically analyzed using both one and 2-factor ANOVA, with the one factor ANOVA being coupled with post-hoc Scheffé's tests, while drug responses were analyzed with one factor ANOVA coupled with post-hoc Scheffé's tests (between groups) and one-tailed paired Student's t-tests (within groups).

The pre-drug mean baseline level of DA seen in the intact striata was 0.19 ± 0.06 (± S.E.M.) pmol per 25 µl of sample perfusate. All 8 intact striata showed a very large increase in extracellular DA when d-amphetamine (10^{-5} M) was added to the perfusion medium (15.1 fold increase over the mean group baseline; $t(7) = 15.12$, $p < 0.0001$) (solid line in Fig. 3A). Similarly, nomifensine (0.67×10^{-5} M) produced a dramatic increase in all the intact striata tested ($t(6) = 5.79$, $p < 0.001$) (hatched bars in Fig. 3B). In contrast, the striata in rats with unilateral 6-OHDA lesions of the mesostriatal pathway had barely detectable levels of extracellular DA in either baseline perfusate samples (mean ± S.E.M. = 0.022 ± 0.002 pmol/25 µl), or samples collected during amphetamine administration (mean ± S.E.M. = 0.066 ± 0.034 pmol/25 µl; see Fig. 3A), which did not produce a significant DA level increase in the 6-OHDA lesion-only rats ($t(3) = 1.63$, $p > 0.05$). The analysis of tissue DA content in the striatum of lesion-only rats also revealed an almost complete depletion of DA. The striatal DA depletion in these 4 rats ranged from 95.1 to 98.6% of the DA content in their respective normal intact sides (mean = 97.4% depletion).

The mesencephalic grafts were able to restore extracellular DA levels in the 6-OHDA denervated

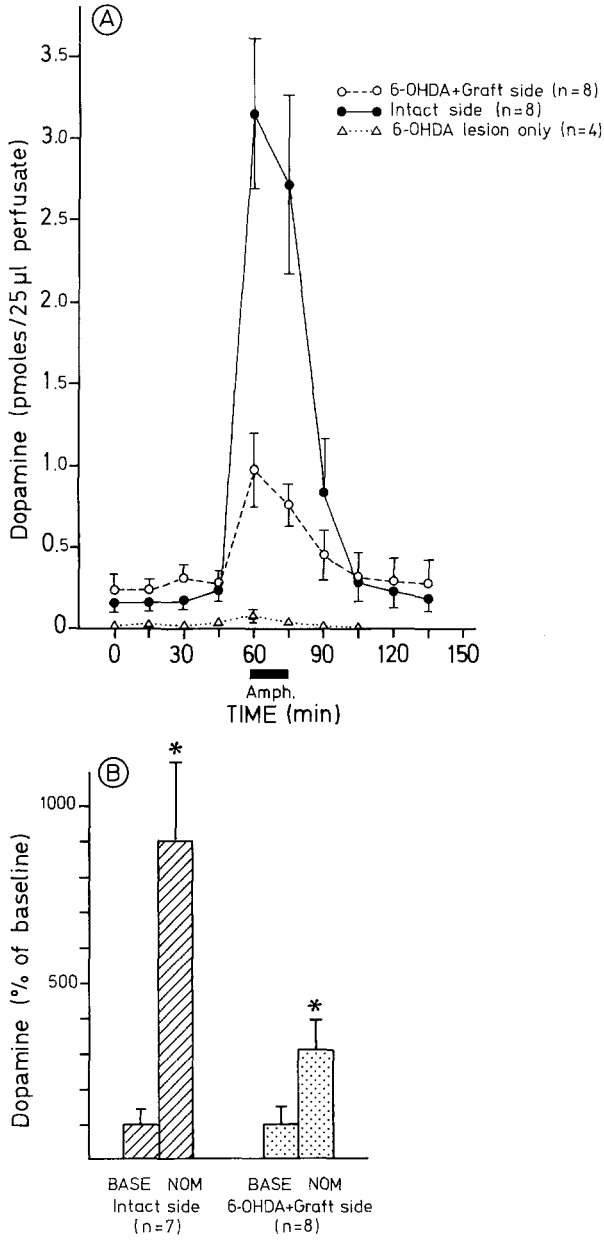

Fig. 3. **A** Mean ± S.E.M. (bars) DA levels measured in striatal perfusates collected by intracerebral dialysis from 6-OHDA-lesioned plus grafted striata (dashed line, n = 8), the contralateral normal intact striata (solid line, n = 8) and from a group of rats with only a unilateral 6-OHDA lesion but no graft (dotted line, n = 4). Each 15 min sample provided 30 µl of perfusate (2 µl/min perfusion rate) of which 25 µl was assayed. Each data point represents the total amount of DA measured in the 25 µl of perfusate. Amphetamine (10^{-5} M), which was added to the Ringer perfusion medium for two 15 min samples and then removed, produced a large increase in the extracellular DA levels measured in both the intact and grafted groups, but produced no effect in the lesion-only group. Baseline DA levels for the intact and grafted groups were not significantly different from each other, although baseline DA levels in both groups were significantly greater than in the lesion-only group (ANOVA followed by post-hoc Scheffé's test, $p < 0.05$, for details see text). **B** Effect of nomifensine (0.67×10^{-5} M added to the perfusion medium) on extracellular DA levels measured in striatal perfusates from the normal intact group (n = 7) and the grafted group (n = 8). The mean baseline (BASE) DA level for each striatum was calculated from the 3 samples preceding nomifensine (NOM) treatment, and this value was used to calculate the percentage increase during nomifensine administration. Note that since the DA levels did not differ between the two nomifensine samples their averaged value is shown here. Although only 5 of the 8 grafted striata produced clear increases in measurable DA levels during the nomifensine treatment, the increase in the graft group was significant (* indicates $p < 0.05$; Student's t-test)

striata (Fig. 3A). The mean baseline DA level was 0.26 ± 0.08 (± S.E.M.) pmol per 25 µl of sample perfusate (range 0.11 to 0.75 pmol/25 µl), an amount that was not significantly different from the amount of DA seen on the intact contralateral sides at any pre-drug baseline time point (post-hoc Scheffé's test; $p > 0.05$). The one grafted rat which had usually high basal DA levels (0.75 pmol/25 µl) was later found to have had the dialysis loop directly in contact with grafted tissue, rather than adjacent to it as intended. Amphetamine produced large increases in extracellular DA in 7 out of the 8 grafted striata (3.7 fold mean increase; t = 5.23, $p < 0.002$; dashed line in Fig. 3A). This increase was significantly less than that seen on the intact side (post-hoc Scheffé's test; $p < 0.05$). Nomifensine, produced on average a 3-fold increase in extracellular DA levels. However, the response was quite variable, with only 5 out of the 8 grafted striata showing a clear nomifensine-induced DA increase. The group effect, was nevertheless clearly seen when the data were plotted as the percentage increase over baseline (Fig. 3B; mean percentage of baseline ± S.E.M. = 310 ± 82%; t = 1.90, $p < 0.05$).

Antibody detection

Increased binding of rat immunoglobulin purified human T-lymphocytes was detected in the sera of all rats when compared to normal control sera, indicating that all the grafted rats were immunized. There was no difference in immunoglobulin binding to human lymphocytes between immunosuppressed and non-immunosuppressed rats. The median for the percentage of lymphocytes that displayed a fluorescence intensity above the cut-off level was, for immunosuppressed animals, 23.0% (range 5.7–58.7%) and for the non-immunosuppressed group 21.1% (range 6.5–51.4%). The control sera values were 1.4%, 1.5% and 1.9% (for examples see Fig. 4A, B).

Graft survival and morphology

Non-immunosuppressed rats. In the 4 non-immunosuppressed PC-8 rats no surviving DA neurons or axons were detected. The implantation site was discernable as an area surrounded by orange fluorescent presumed macrophages. The greatest tissue necrosis was observed in the rat that exhibited a transient graft effect on amphetamine-induced rotation 13 weeks post-transplantation. In this rat, the area with abundant orange autofluorescent cells, indicative of a rejection process, encompassed approximately 1.2 mm at its widest point mediolaterally in the coronal plane of the striatum.

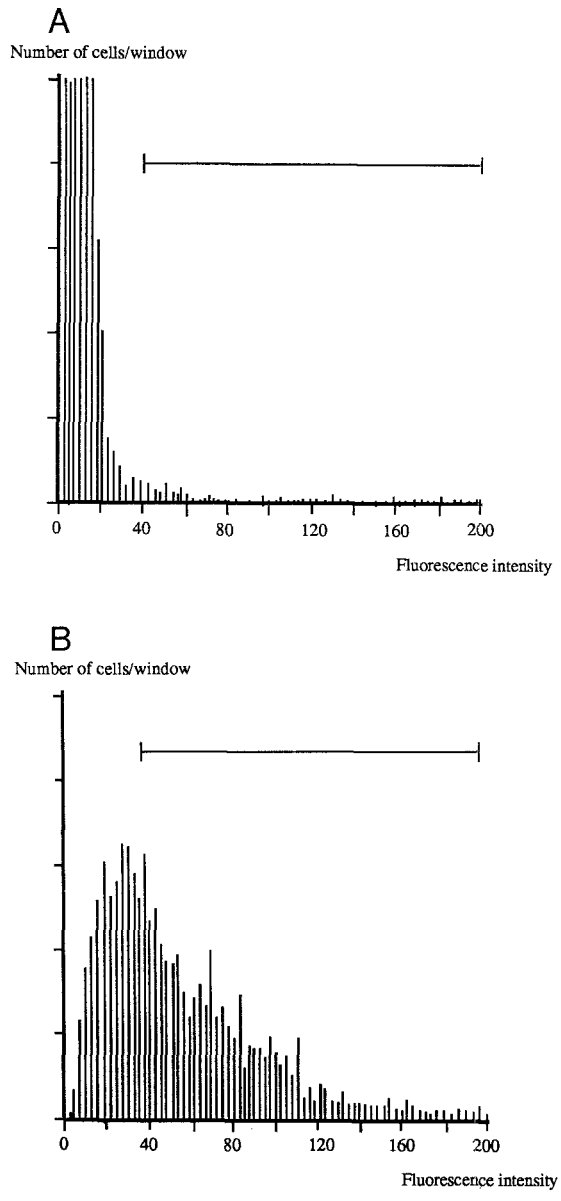

Fig. 4A, B. Rat immunoglobulin binding to human T-cells in vitro measured by quantitative fluorocytometry. Shown is the intensity of fluorescence in arbitrary units (x-axis) and the number of cells (arbitrary scale) counted at a given window of fluorescence intensity. The horizontal bar indicates the interval of fluorescence intensities which exceeded the cut-off level (see text). The proportion of cells (in % of total number of cells counted) that exhibited a fluorescence intensity above the cut-off level was calculated for each analysed serum. **A** Illustrates the result for a control serum in which 1.4% of the cells exhibited fluorescence intensities above the cut-off level. **B** Illustrates the result for a serum from the grafted rat in the non-immunosuppressed PC-8 group which is marked *a* in Fig. 1A. When exposed to this rat's serum, 51.4% of the T-cells were labelled at fluorescence intensities above the cut-off level

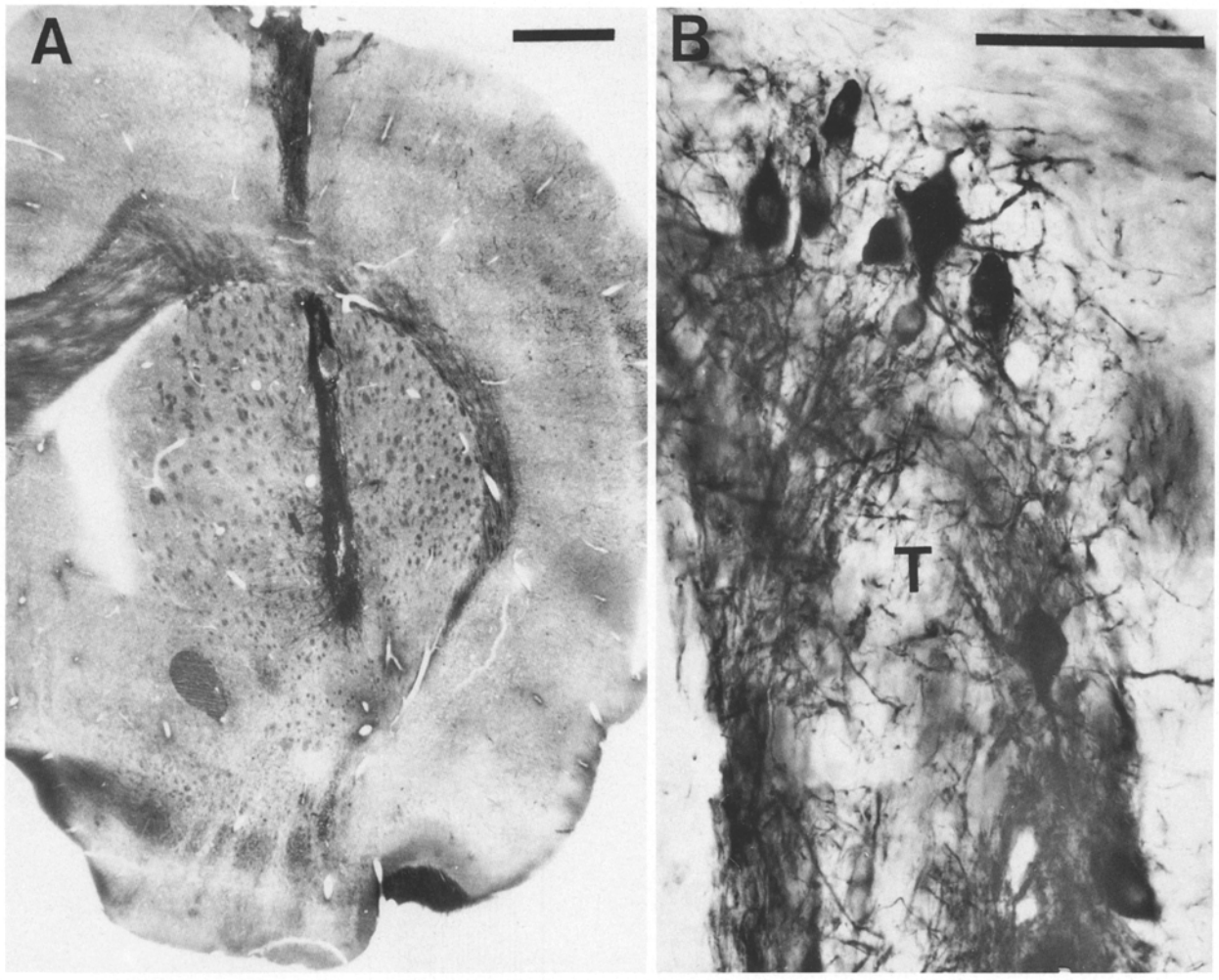

Fig. 5. A Photomicrograph of a TH-immunostained coronal section through the head of the caudate-putamen of rat receiving a PC-6.5 graft (marked *c* in Fig. 1B). The graft is seen as darkly stained tissue extending through the neocortex into the caudate-putamen: At this magnification coarse fiber outgrowth is just visible close to the graft in the caudate-putamen. Scale bar = 1 mm. **B** A higher magnification of a portion of the transplant (T) situated just ventral to the corpus callosum (light region in upper right of photograph) from the same section as illustrated in (**A**). Several darkly stained TH-immunoreactive perikarya which extend fibers both within and outside the graft are visible. Scale bar = 100 μm

CyA-treated rats. The immunosuppressed recipients bearing tissue from the PC-11.5 donor generally showed poor or no graft survival, whereas immunosuppressed recipients bearing grafts from the PC-8 and PC-6.5 donors consistently exhibited good graft survival.

In the 5 rats from the PC-11.5 group that survived until perfusion there was evidence of graft derived DA innervation in 3 rats. In 2 of these rats, although there was clear graft derived DA innervation radiating from the injection tract, it was not possible to discern actual DA graft neurons. These rats exhibited 24% and 51% reductions in net rotation asymmetry, respectively, 19.5 weeks after grafting. In the third rat, 194 surviving grafted DA neurons were found. This particular rat had shown a 52% decrease in net rotation asymmetry.

The rats from the PC-8 and PC-6.5 groups all exhibited surviving grafts containing between 402 and 4249 DA fluorescent neurons. The rat in the PC-8 group which showed clear evidence of graft function in amphetamine-induced rotation after 13 weeks, but a return of the lesion-induced rotation asymmetry after 18 weeks (labelled *b* in Fig. 1B), contained an intrastriatal graft with approximately 800 DA neurons. However, the right hemisphere of this rat's brain was pathologically affected and displayed widespread infiltration of orange autofluorescent cells. Although the possibility of a rejection process cannot be entirely excluded, it seems unlikely

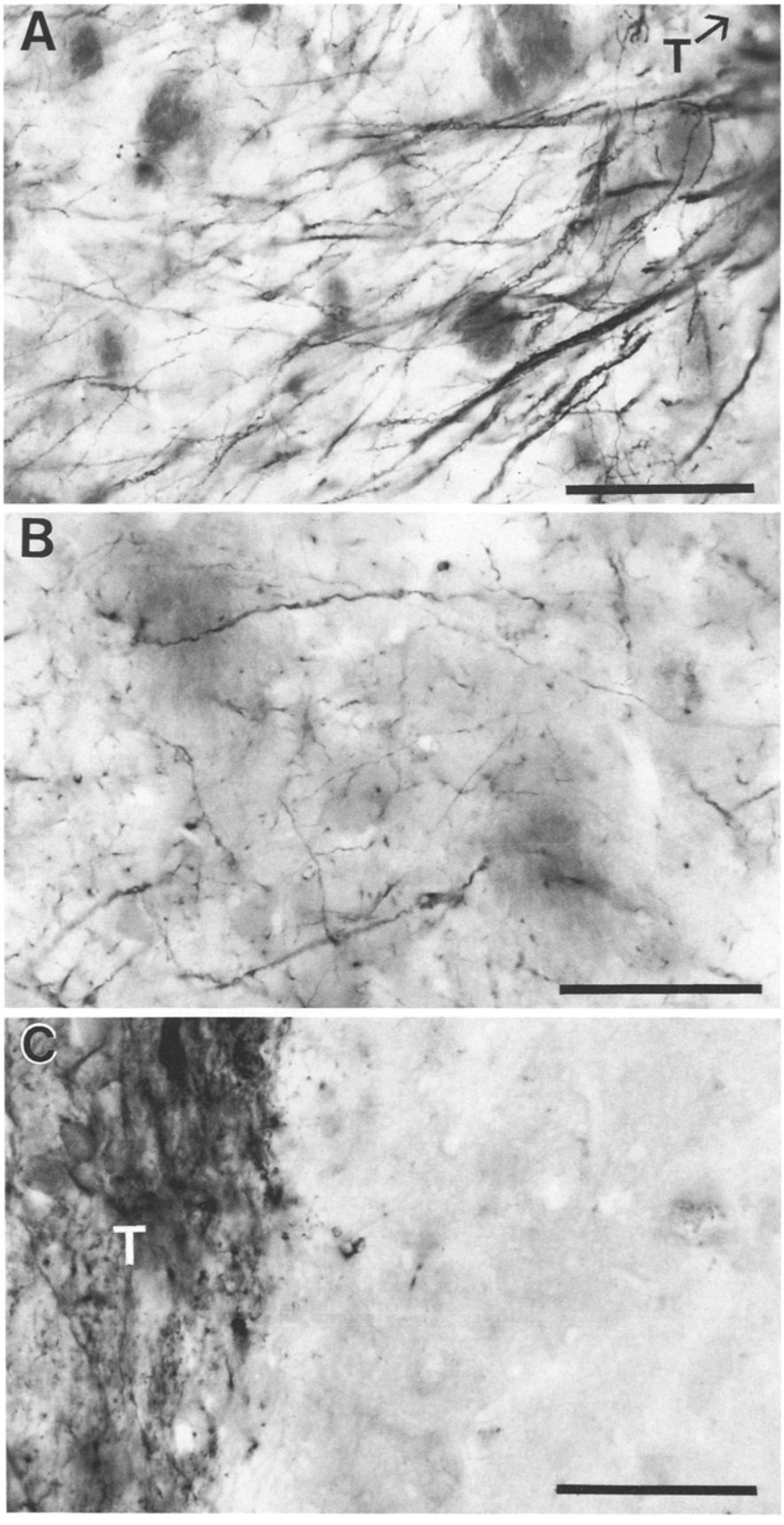

Fig. 6. A High magnification of fine and coarse TH-immunoreactive process extending from the transplant (illustrated in Fig. 5A, B) into the host caudate-putamen. Large rounded dark area are fiber bundles of the internal capsule. The transplant border is located just out of view in the direction of the arrow from T. Scale bar = 100 μm. **B** Graft-derived TH-immunoreactive fibers innervating the host caudate-putamen. Scale bar = 50 μm. **C** A portion of the transplant (T) located in the neocortex overlying the caudate-putamen (see Fig. 5A). Although there were several TH-immunostained cell bodies in this part of the graft, there was almost a total absence of fiber outgrowth from the graft tissue into the adjacent neocortex (right), in contrast to the dense fiber network within the graft itself. Scale bar = 50 μm

since the cellular infiltrate was distributed throughout the right hemisphere and *not* focused on the graft, and the graft tissue itself was still present and contained DA fluorescent neurons. The other rat which exhibited contralateral turning as an indication of graft survival 18 weeks after grafting, but did not show a reduction in amphetamine-induced net rotation asymmetry (labelled *a* in Fig. 1B), was found to have the smallest graft in the CyA-treated group with 402 DA neurons. The mean number of surviving DA neurons in the CyA-treated PC-8 hosts was 1626 (if the pathologically affected rat is excluded).

In rats that had undergone intracerebral dialysis a distinct probe tract was evident in the center of the striatum as described previously (Zetterström et al. 1986; Strecker et al. 1987). In the rat that showed the highest basal DA release in the dialysis experiment, the tract from the dialysis probe was found to partly encroach upon the graft tissue itself.

In the TH-immunostained specimen from the PC-6.5 group, there was a complete absence of TH-positive neurons in the ventral tegmental area and substantia nigra on the side ipsilateral to the 6-OHDA injections. Surviving graft tissue was observed extending through the right striatum up into the overlying neocortex (Fig. 5A). The graft contained approx. 700 intensely TH-immunoreactive neurons of characteristic morphology (Fig. 5B). On average, the TH-positive perikarya measured 32 × 20 μm along their major and minor axes, respectively. There seemed to be 2 main populations of TH immunoreactive neurons, one large and less frequent type, which in extreme cases was up to 50 μm long, and one smaller type (mean 25 × 19 μm). Most of the neurons were multipolar with long coarse processes which extended into the host striatum (Fig. 6A). In the parallel ultrastructural study (Clarke et al. 1987) some of these processes have been identified as dendrites. Within the host neostriatum a dense plexus of TH-immunoreactive fibers of presumed graft origin was present throughout the whole rostrocaudal extent of the head of the caudate-putamen (Fig. 5A, 6B) and, also, to some extent in the globus pallidus and nucleus accumbens. Single TH-immunoreactive cells had sometimes presumably migrated to areas of the striatum that were up to 1–1.5 mm from the actual bulk of the graft tissue. Very little TH-positive fiber outgrowth into the neocortex was seen from the part of the graft which extended into the overlying parietal cortex (Fig. 6C).

Discussion

There are at present two main strategies for the development of a transplantation therapy in PD, namely the transplantation of adrenal medullary cells or fetal DA neurons (cf. Lindvall et al. 1987b). In the clinical trials reported so far (Backlund et al. 1985; Lindvall et al. 1987a; Madrazo et al. 1987) adrenal medulla autotransplantation has been performed to the striatum in patients with severe PD. The effects have varied from partial and transient recovery (Backlund et al. 1985; Lindvall et al. 1987a) to very marked improvement up to at least a year postoperatively (Madrazo et al. 1987). Although the mechanisms underlying the improvement in the latter patients remain unclear, it seems possible to conclude from these clinical trials that implantation of catecholamine producing cells could be a valuable therapeutic approach in PD patients. Considerably more animal experimental work has so far been performed with grafts of fetal mesencephalic tissue, and available experimental data seem to favour the strategy of using fetal DA neurons. Of particular importance is that, in contrast to what has been shown after grafting of fetal DA neurons, no long-lasting influence on spontaneous behaviour or striatal DA release has been found with adult adrenal medulla implants in experimental animals (see Lindvall et al. 1987b for discussion). Furthermore, Strömberg et al. (1985) have reported that the effect of adrenal medulla grafts on drug-induced rotational asymmetry in rats with unilateral mesostriatal lesions is not permanent, and the grafted adrenal cells will undergo involution and atrophy unless nerve growth factor (NGF) is infused into the brain.

According to the provisional ethical guidelines adopted by the Swedish Society for Medicine in 1985, it is considered acceptable from the ethical point of view to use DA neurons obtained from human fetuses from induced abortions in Sweden for the development of a transplantation therapy in patients with severe PD. In the present study we have used the unilateral 6-OHDA model of PD to address questions of particular importance as a basis for future clinical applications of DA neuron grafting. These issues include optimal donor age, yield of DA neurons from each human fetus, and their growth potential, transmitter metabolism and functional capacity in drug-induced and spontaneous behaviours. Furthermore, we have dealt with some of the immunological problems related to fetal neuron grafting in patients, particularly the need for immunosuppression.

Optimal donor age

In our previous study (Brundin et al. 1986) we examined the survival of human DA neurons obtained from fetuses of ages varying between 9 and

19 weeks. In that study, although some DA neuron survival was obtained with 11-week donor tissue, the optimal donor age for human mesencephalic DA neuron grafts was found to be less than 11 weeks, with good results attained with 9-week old donor tissue. This is corroborated by the present findings which show that only in some cases was there survival of grafted DA neurons in rats receiving 11.5-week old donor tissue and, furthermore, these PC-11.5 grafts were small in contrast to the relatively large grafts obtained when using 6.5 to 8-week old donors. Using the current trypsin-dissociated tissue procedure we have previously found that good survival of DA neurons obtained from fetal rat mesencephalon of different fetal ages is linked to a high initial in vitro viability of the cell suspension (Brundin et al. 1985a). The current results with human donor tissue parallel those obtained with rat donors as the in vitro viability with the trypsin dissociated PC-8 cell suspension was 86% compared to only 65% in the PC-11.5 cell suspension, which subsequently yielded relatively poor graft survival (note that the PC-6.5 tissue was mechanically dissociated without prior trypsin incubation and therefore the in vitro viability score for this cell suspension is not comparable).

Yield of DA neurons

On the average 1626 DA neurons survived in each of the CyA-treated PC-8 recipients. These neurons were derived from 4 µl injections of dissociated fetal tissue, and since the whole donor ventral mesencephalon was dissociated in a volume of 50–60 µl one can estimate that a total number of 20000–25000 mesencephalic DA neurons would survive xenografting from one PC-8 human fetus to an immunosuppressed host. As the normal number of DA neurons in a human mesencephalon have been estimated at around 450000 (German et al. 1983), this represents a DA neuron survival rate after grafting of about 5%. This figure is only slightly lower than the 10% survival rate that we have previously estimated to hold for syngeneic cell suspension grafts of rat fetal DA neurons (Brundin and Björklund 1987). Grafting to immunosuppressed PD patients in an allogeneic setting may, of course, result in a slightly different DA neuron yield compared to the present xenogeneic grafting to immunosuppressed hosts. Notably, certain neural allografts clearly survive longer than xenografts, and in some cases possibly permanently, in non-immunosupressed mice (Mason et al. 1986; Widner et al. 1988). Nevertheless, based on the above data it is of interest to consider to what extent the number of DA neurons that can be obtained from one human fetus, when xenografted to the rat, could be sufficient to replace the DA neurons that normally innervate one putamen. The putamen is of particular interest as a target region as it exhibits a greater DA depletion than the caudate nucleus in PD (Nyberg et al. 1983), and is implicated in the motor functions that are disturbed in PD (Lindvall et al. 1987b). Assuming that the proportion of mesencephalic DA neurons which innervate different forebrain regions is similar in rat and man, one can estimate that in the human approximately 60000 DA neurons give rise to the innervation of one putamen. Thus, using the present technique, the grafting of tissue from one human fetal mesencephalon should be sufficient to replace about 30–40% of the DA neurons that normally innervate one human putamen.

Morphological characteristics

The morphology of the DA neurons in the human grafts (Fig. 5B) was similar to that seen in the normal human brain (Bazelon et al. 1967; Pearson et al. 1983) and conformed to that seen in our previous study (Brundin et al. 1986). The grafted human DA neuron perikarya were on average larger than those of grafted rat neurons (Jaeger 1985; Bolam et al. 1987) and seemed to fall into two size categories. These presumably represent the 2 different populations of DA neurons normally found in the mesencephalon: the smaller DA neurons of the paranigral nucleus and the larger representing a subpopulation of substantia nigra neurons (Bazelon et al. 1967). The grafted DA neurons gave rise to a TH immunoreactive fiber network (Fig. 6B) that essentially reached the whole host neostriatum, nucleus accumbens and globus pallidus. This is in agreement with previous studies on human DA neurons (Brundin et al. 1986; Strömberg et al. 1986) but in marked contrast to what is observed with rat-to-rat DA grafts. Grafted rat DA neurons usually extend a DA fiber network only approximately 1.5–2 mm from the graft borders (Björklund et al. 1983). In a parallel study (Clarke et al. 1987) we have seen that fibers emanating from the human fetal mesencephalic grafts form TH-immunoreactive synaptic contacts with rat host striatal neurons. In agreement with previous work (Brundin et al. 1986; Strömberg et al. 1986), the present study demonstrated coarse TH-immunoreactive processes extending up to at least 600 µm into the surrounding host striatum. In the parallel ultrastructural study some of these coarse processes have been identified as dendrites (Clarke et al. 1987). Such dendritic processes may provide a

site for host input to the graft, as proposed by Mahalik et al. (1985). Indeed, in the parallel electron microscopic study we have observed graft-derived TH-labelled dendrites receiving non-labelled synaptic contacts in the host striatal neuropil (Clarke et al. 1987).

The host tissue volume that can be reached by the outgrowing axons from fetal DA neurons implanted in the brain of a PD patient will depend not only on the number of surviving DA neurons, but also on the number and location of the implanted cell deposits and the growth capacity of each DA neuron. The present data indicate that grafted human fetal DA cells have a greater growth potential than corresponding rat neurons. Thus, the grafted human DA neurons gave rise to a TH-immunoreactive fiber network that reached the whole rat caudate-putamen, nucleus accumbens and globus pallidus, which represents a growth distance of at least 3 mm (see also Brundin et al. 1986).

The volume of the human putamen is about 200 times greater than the putamen in the rat whereas the number of DA cells innervating the putamen is only 10 times higher in man than in rat (German et al. 1983; Björklund and Lindvall 1984). Therefore, it seems that each human DA neuron innervates a 20 times greater striatal tissue volume than is covered by a rat DA cell. A single deposit of a rat DA cell suspension has been shown to reinnervate a more or less spherical portion of the striatum with a radius of about 1.5–2 mm (Björklund et al. 1983). Assuming that human DA neurons reinnervate a 20 times greater volume than rat cells also when grafted, their maximal extent of growth when placed in a human striatum would be 4.1 to 5.4 mm away from the graft. If in a future clincial trial, fetal DA cells were deposited at two sites along 20 mm long injection tracts (as in the adrenal medulla autograft experiments of Lindvall et al. 1987a) this would lead to a maximum innervation of between 50% and 80% of the total putaminal volume. From the calculations of graft DA cell yield and growth capacity it seems reasonable, therefore, to assume that due to compensatory mechanisms, e.g. high activity in grafted DA neurons in combination with supersensitive postsynaptic DA receptors (cf. Brundin and Björklund 1987), the innervation provided by neurons that can be obtained from one fetus could lead to a significant recovery in unilateral striatal DA neurotransmission, and in putamen-related function in a PD patient. However, if multiple grafts in several locations (e.g. in the putamen, caudate nucleus and nucleus accumbens, on both sides) are necessary in order to obtain significant amelioration of the motor symptoms in a PD patient, it seems with the current grafting technique, as if mesencephalic tissue from several fetuses would have to be implanted. Alternatively, the current technique would have to be improved in order to obtain higher yields of surviving DA neurons from each donor fetus.

As with DA grafts from rats (Björklund et al. 1983; Herman et al. 1986) and monkeys (Sladek et al. 1986), the reinnervation from the human grafts displayed some target specificity. Whereas the graft displayed extensive TH-immunoreactive fiber outgrowth when placed in the striatum (Fig. 6A, B), graft portions located in the overlying neocortex displayed little or no TH-positive fiber outgrowth (Fig. 6C). In the TH-immunostained specimen, single TH-immunoreactive perikarya were found within to the host striatum up to 1–1.5 mm from the graft borders. Although some caution should be expressed as TH-immunoreactive perikarya have been reported in the striatum of monkeys with lesions of the intrinsic DA pathway (Sladek et al. 1986), our finding resembles the evidence for migration of xenografted mouse neurons previously seen in the rat hippocampus and striatum (Björklund et al. 1982; Low et al. 1983; Wells et al. 1985). Furthermore, in 2 rats from the PC-11.5 group, a graft-derived fiber network was detected around the implant without the concomitant demonstration of the parental graft perikarya. The DA innervation was most probably not of host origin as the fibers radiated from the area of the injection tract and there was no innervation in the other parts of the striatum. This also resembles results obtained in other xeno- or allogeneic graft settings in both the rat hippocampus and striatum (Low et al. 1983; Daniloff et al. 1985; Brundin et al. 1988, submitted). This suggests that some of the fetal DA neurons had migrated out of the central graft tissue mass and were difficult to visualize histochemically, perhaps due to reduced levels of the histochemical marker (in this case DA) in the cell bodies.

Functional characteristics

Previous studies with rodent donor tissue have already shown that mesencephalic DA neurons can reverse spontaneous, amphetamine- and apomorphine-induced rotation (Dunnett et al. 1981, 1983, 1986, 1987) and that they release DA spontaneously (Zetterström et al. 1986; Strecker et al. 1987). In agreement with our previous study (Brundin et al. 1986) the onset of functional graft effects on amphetamine-induced motor asymmetry occurred in the PC-6.5 and PC-8 rats between 13 and 18 weeks after transplantation. Moreover, some PC-6.5 and

PC-8 rats performed more turns contralateral to the lesion than in the ipsilateral direction. In a test of apomorphine-induced rotation the rats with surviving PC-8 grafts were significantly less asymmetric when compared either to their pre-transplantation values or to the non-immunosuppressed PC-8 rats that subsequently were found to lack surviving transplants. This suggests that the grafted DA neurons, by spontaneously releasing DA, had caused a reduction in DA receptor supersensitivity in the initially denervated host striatum and thereby a reduction in apomorphine-induced rotation asymmetry. Also spontaneous motor asymmetry was compensated in the CyA-treated PC-8 rats, which had surviving grafts, when compared to the non-immunosuppressed PC-8 rats, where the grafts had been rejected. In summary, grafted human fetal DA neurons thus seem to possess the same functional capacity as their rodent counterparts in tests of motor asymmetry.

The intracerebral microdialysis experiments support these behavioural data and provide evidence that the grafted human DA neurons are spontaneously active and that they respond with an increased DA release to amphetamine administration. Indeed within the area reinnervated by the grafts, the implanted human neurons restored spontaneous DA release to normal striatal DA release levels. Comparable results have been obtained with rat grafts which contain between 3000 and 5000 surviving DA neurons (Strecker et al. 1987). Similar release levels were obtained in the current study with a lower number of surviving DA neurons, which indicates that the DA release capacity of the human neurons is at least as good as that of the grafted rat neurons. The human DA grafts showed a significant increase in DA release in response to amphetamine. The amphetamine-induced increase was, however, significantly smaller than seen in intact rat mesostriatal DA neurons, which suggests that the DA neurons in the grafts may be releasing DA at maximal capacity during basal conditions and that their reserve capacity for drug-induced release is somewhat limited. Alternatively, this could be due to an inherent property of human DA neurons or, possibly, related to the fact that these neurons were not fully mature at the time of the dialysis experiment. The amphetamine effect on striatal DA release and DA related behaviour has been shown to develop slowly in rats during the postnatal period (Gazzara et al. 1986), which suggests that the development of amphetamine-induced physiological responses may be more protracted than the development of the ability to synthesize and release DA. Also the response to the DA reuptake blocker nomifensine was smaller and more variable in the graft-reinnervated striata than in the intact control striata, possibly because the expression of DA reuptake sites was not complete in the outgrowing human fetal DA neurons at the time of the dialysis experiment. However, the distinct nomifensine response in some of the rats supports the idea that nomifensine could be used as a marker for graft derived DA terminals when monitoring graft survival in PD patients. Indeed, radioactively labelled nomifensine has recently been introduced as a ligand for non-invasive positron emission tomography in order to monitor striatal DA terminals in primates (Leenders et al. 1988).

Immunological aspects

This study confirms that there is little or no survival of xenografted DA neurons in the rat striatum unless the host is immunosuppressed with CyA (Brundin et al. 1985b). As the use of immunosuppression inevitably will represent a certain health risk in patients (cf. Krupp et al. 1986; Maiorca et al. 1985) we felt it important to establish whether or not human fetal neural tissue can give rise to an immunological response in the host when grafted into the brain. The demonstration of donor specific antibodies in the present graft recipients is a definite indicator of the graft tissue being immunogenic. The anti-human immunoglobulins were detected by their ability to bind to antigens on human T-cells, but the exact nature of the antigenic structures was not determined in the present study. This particular method of determining immunization was chosen as other methods of assaying immunization, such as the primed lymphocyte test and a secondary mixed lymphocyte culture would not have been possible to perform in the animals that were on continuous immunosuppression.

Although these results show that the grafted rats were immunized by the grafts, it should be pointed out that immunoglobulins are not thought to play an important role in late rejection of a grafted tissue (Mason et al. 1986b). This is consistent with the observation in the present study that serum from animals with a rejected graft did not bind to a higher percentage of the human T-cells in vitro.

Although there are reports of survival of human tissue grafts into the adult rat brain without immunosuppression (Kamo et al. 1985, 1986, 1987) the present data show that intracerebral neural xenografts can set off immune reactions in rats and that this may compromise long-term survival in case immunosuppression is not used. Interestingly, one rat (marked *a* in Fig. 1A) in the non-immunosup-

pressed PC-8 group had evidence of a functioning graft as late as 13 weeks after grafting which then disappeared by 18 weeks, suggesting that there was a late rejection of the xenograft tissue. This rat demonstrated a larger zone of tissue necrosis (about 1.2 mm in width at its greatest point) than the other rats in the same group. If this is representative of the size of tissue damage that might occur after rejection of a DA allograft placed in the human striatum it would most likely have insignificant functional consequences. Allografting may, however, represent an immunologically different situation. Thus, in studies with intraparenchymal DA neuron allografts in primates (Bakay et al. 1985; Sladek et al. 1986) and mice (Widner et al. 1988) longterm graft survival has been obtained without immunosuppression.

The rationale for choosing daily CyA treatment (10 mg/kg) as immunosuppressive treatment was based on earlier experiences with xenografted neural tissue in the rat (e.g. Brundin et al. 1985b). This single drug therapy has been found to be sufficient to support xenograft survival in the rat. However, with prolonged treatment a small subgroup of the CyA treated rats will exhibit side effects such as opportunistic infections and gingival changes during prolonged treatment (unpublished observations; Ryffel 1982). The gingival pathology can lead to excess and misdirected growth of the incisors, causing feeding problems and eventual weight loss. In addition, two animals in the present series showed diffuse cerebral pathology possibly related to intracerebral infection, although the graft remained in at least one of the cases. This may be taken as an indication of the risks of long-term immunosuppressive treatment in intracerebrally grafted experimental animals.

Conclusions

The present study provides experimental data documenting that human fetal DA neurons, obtained through routine abortions, have a morphological and functional potential consistent with the requirements for grafting in PD patients. Several findings are of direct relevance for future clinical trials: First the optimal donor age with the present cell suspension technique has been confirmed to be less than 11 weeks of gestation. Second, the yield of DA neurons using the present grafting procedure and their estimated growth capacity makes it likely that the number of surviving DA neurons that could be obtained from a single fetus would be sufficient to produce a significant recovery of DA neurotransmission and function of one human putamen. For multiple bilateral implants in a PD patient, the yield of DA neurons (which we estimate to be around 5% in the present xenograft setting) would have to be improved, or DA neurons from several fetuses would have to be used. Third, the findings that human fetal neural tissue from the first trimester can evoke an immune reaction when implanted in a rat brain would make it advisable to use immunosuppressive treatment also in the clinical setting.

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