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Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: ultrastructural evidence for synapse formation using tyrosine hydroxylase immunocytochemistry*

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Summary. Human fetal mesencephalic dopamine (DA) neurons, obtained from 6.5–9 week old aborted fetuses, were grafted to the striatum of immunosuppressed rats with 6-hydroxydopamine lesions of the ascending mesostriatal DA pathway. The effects on amphetamine-induced motor asymmetry were studied at various timepoints after grafting. At eight weeks, functional graft effects were not evident but after 11 weeks small effects on motor asymmetry could be monitored and rats tested 19–21 weeks after grafting exhibited full reversal of the lesion-induced rotational behaviour. Four rats were sacrificed at different timepoints between 8 and 20 weeks and the grafted DA neurons were studied in tyrosine hydroxylase (TH) immunocytochemically stained sections at the light and electronmicroscopic level. The grafts contained a total of 500–700 TH-positive neurons in each rat. In one rat sacrificed 8 weeks after grafting the grafted neurons were TH-positive but exhibited virtually no fiber outgrowth. In another rat, sacrificed after 11 weeks, a sparse TH-positive fiber plexus was seen to extend into the adjacent host neostriatum. Two rats sacrificed after 20 weeks both contained TH-positive neurons that gave rise to a rich fiber network throughout the entire host neostriatum, and this fiber network was also seen to extend into the globus pallidus and nucleus accumbens. Very coarse TH-positive processes, identified as dendrites in the electron microscope, projected up to 1.5–2.0 mm from the graft into the host striatum. Ultrastructural analysis revealed that the grafted neurons had formed no TH-positive synaptic contacts with host striatal neurons after 8 weeks, and at 11 weeks some few TH-positive synapses were identified. Twenty weeks after transplantation, abundant TH-positive synaptic contacts with host neurons were seen throughout the neostriatum, and such contacts were identified in the globus pallidus as well. Thus, the present study provides tentative evidence for a time-link between the development of synaptic contacts and the appearance of functional graft effects. Similar to the normal mesostriatal DA pathway, ingrowing TH-positive axons formed symmetric synapses and were mainly seen to contact dendritic shafts and spines. However, in comparison to the normal rat striatum there was a higher incidence of TH-immunoreactive boutons forming synapses onto neuronal perikarya. The TH-positive dendrites that extended into the host striatum were seen to receive non-TH-immunoreactive synaptic contacts, presumably arising from the host neurons. These results suggest that human fetal DA neurons are able to develop a reciprocal synaptic connectivity with the host rat when grafted to the adult brain. Grafting of human fetal DA neurons may therefore be expected to provide a means of restoring regulated synaptic DA release in patients with Parkinson's disease.

Key words: Neural transplantation – Dopamine neurons – Human fetus – Tyrosine hydroxylase immunocytochemistry – Synaptic contacts – Parkinson's disease

Introduction

The success of studies utilizing grafted fetal dopamine (DA) neurons in experimental Parkinson's disease (PD) has stimulated research into developing a clinical application of the neural grafting technique in patients with PD.

Intrastriatal grafts of mesencephalic DA-containing neurons from rat fetuses are able to compensate

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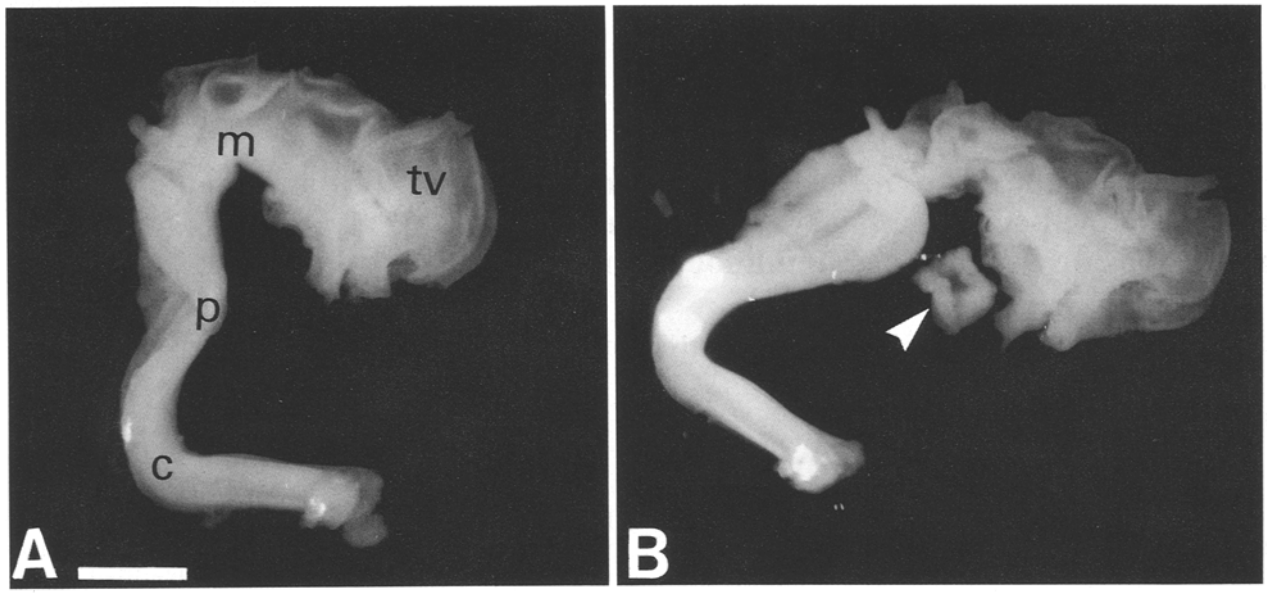

Fig. 1. **A** The dissected central nervous system obtained from the PC-7 fetus. In **B** the ventral mesencephalon (arrowhead) has been dissected out. Tv = telencephalic vesicle, m = mesencephalic flexure, p = pontine flexure, c = cervical flexure. Scale bar = 2 mm

for motor and sensorimotor deficits resulting from selective lesions of the mesotelencephalic DA system in adult rats (Björklund and Stenevi 1979; Perlow et al. 1979; for review see Brundin and Björklund 1987). More recent findings have shown that fetal DA neural grafts can reinnervate the striatum of monkeys with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced parkinsonism and produce some functional effects (Bakay et al. 1985; Redmond et al. 1986; Sladek et al. 1986). In the rat, at least, the functional recovery is clearly dependent on the DA fiber ingrowth into the denervated striatum (Björklund and Stenevi 1979; Björklund et al. 1980). The growth of the grafted DA neurons exhibits a high degree of specificity and the distributional pattern of the outgrowing fibers is reminiscent of that found in the normal brain (Björklund et al. 1983b). Electron microscopic immunocytochemical studies of rat-to-rat grafts have demonstrated abundant synaptic contacts between the ingrowing graft-derived DA fibers and the host striatal neurons (Freund et al. 1985; Mahalik et al. 1985; Triarhou et al. 1987). The grafts are metabolically (Schmidt et al. 1982, 1983) physiologically (Wuerthele et al. 1981) and biochemically (Strecker et al. 1987; Zetterström et al. 1986) active in that they exhibit transmitter synthesis, normal firing patterns, and spontaneous DA release. The grafted DA neurons also respond like normal DA neurons to the local application of DA receptor agonists and antagonists (Strecker et al. 1987; Wuerthele et al. 1981; Zetterström et al. 1986). There is evidence that the functional effects executed by these grafts may be due to a well-controlled synaptic release of DA and not to a simple diffusion of the transmitter over large distances from the terminals (Brundin and Björklund 1987; Brundin et al. 1987; Strecker et al. 1987; Strömberg et al. 1985).

In order to provide a further basis for future clinical applications of the neural grafting technique in PD, we have initiated a series of experiments in which human fetal mesencephalic DA neurons have been implanted into the DA denervated striatum of immunosuppressed rats. In two previous studies (Brundin et al. 1986b, 1988) we have reported that grafted human DA neurons reinnervating the host neostriatum are capable of restoring DA release to normal levels and of compensating for deficits in both spontaneous and drug-induced behaviours. The objective of the present study was to explore whether the grafted human DA neurons also establish synaptic connections with host striatal neurons.

Material and methods

Four 6-hydroxydopamine (6-OHDA)-lesioned rats with grafts of human fetal mesencephalic tissue were included in the present electron microscopic morphological study. An additional two 6-OHDA lesioned rats, also receiving human fetal grafts, were only followed behaviourally.

Animals and lesion surgery

Female Sprague-Dawley rats (ALAB, Stockholm, Sweden), weighing 180–200 g at the start of the experiment, were given two

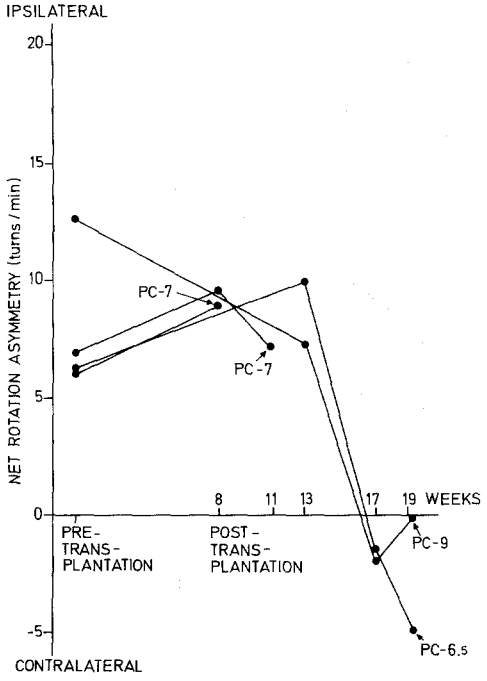

Fig. 2. Amphetamine-induced net rotation asymmetry score (turns contralateral to lesion subtracted from turns ipsilateral to lesion) before transplantation and at various timepoints, between 8 and 19.5 weeks, after transplantation, plotted for the four individual transplant recipients that were studied ultrastructurally

stereotaxic injections of 6-OHDA in the right ascending mesostriatal DA pathway under equithesin anesthesia (0.3 ml/100 g). First, 2.5 µl of 6-OHDA (3 µg/µl, free base, dissolved in 0.2 mg/ml ascorbate-saline) were injected at the following coordinates (in mm): A = –4.4; L = 1.2; V = 7.8; with reference to the bregma and dura respectively (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 (in mm): A = –4.0; L = 0.8; V = 8.0 with reference to the bregma and dura respectively (toothbar set at 3.4 mm above the interaural line). These two 6-OHDA injections produce an ipsilateral denervation not only of the caudate-putamen (Schmidt et al. 1982, 1983) but also of the nucleus accumbens and the olfactory tubercle (Dunnett et al. 1984).

Donor tissue and transplant groups

With the approval of the Research Ethical Committee at the University of Lund, 3 fragmented human fetuses were obtained at suction curretage abortions. Their post-conceptional ages (time from fertilization) were 6.5 weeks (PC-6.5), 7 weeks (PC-7) and 9 weeks (PC-9). The pregnancies were timed by measurements of the length of the fetus using ultrasound technique and by noting distinguishing developmental characteristics on the fetuses. Two to 5 h after the abortion, rats that had sustained complete 6-OHDA lesions of the right mesostriatal DA pathway 1–5 months earlier (see above) received neural implants. From the recovered central nervous tissue (Fig. 1), the region of the ventral mesencephalon, containing the developing DA cell groups, was dissected and prepared according to the cell suspension method (see e.g. Björklund et al. 1983a), with the trypsin step being omitted in the case of the PC-6.5 fetus. Using a 10 µl Hamilton microsyringe fitted with a 0.25 mm inner diameter cannula, two or three 2 µl deposits of cell suspension were implanted in the head of the right caudate-putamen using the following coordinates (in mm): two injections at: (1) A = +1.0; L = 3.0; V = 5.0; (2) A = +1.0; L = 3.0; V = 4.1; or alternatively three injections at: (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; (with reference to bregma and dura respectively, and with the tooth-bar set at zero). All transplantation surgery was conducted under equithesin anesthesia.

Immunosuppression

All rats were given daily injections of approximately 10 mg/kg, i.p., of Cyclosporin A (Sandimmune®, Sandoz, 50 mg/ml diluted to 10 mg/ml in sterile saline) starting on the day of graft surgery. To reduce the risk of opportunistic infections in the graft recipients, they were given tetracyclin (Terramycin, Pfizer, approx. 20–50 mg/kg daily) through the drinking water.

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). All rats used for grafting exhibited a mean of at least 6.1 full body turns per min ipsilateral to the lesion. The rotation test was repeated up to 3 times during the subsequent 8–21 weeks after transplantation (see Fig. 2). For the illustration of graft effects on motor asymmetry, a net rotation asymmetry score was calculated by subtracting turns contralateral to the lesion from ipsilateral turns.

Tyrosine hydroxylase immunocytochemistry

At timepoints varying between 8 and 20 weeks after transplantation 4 of the rats were perfused for tyrosine hydroxylase (TH) immunocytochemistry, essentially according to the procedure of Freund et al. (1985). The rats were anaesthetized with chloral hydrate (3.5 mg/kg). After a preperfusion through the ascending aorta (descending aorta clamped) with 100 ml of 0.9% saline at room-temperature, each rat was perfused for 15 min with 500 ml of ice-cold 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 m phosphate buffer (PB, pH 7.4). The brain was then either stored in PB for 3 days prior to being cut on a vibrating microtome (Bio-rad, USA) or cut immediately. Seventy micron thick coronal sections, were obtained from 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 for 30 min. The sections were then incubated for 48 h at 4° C in anti-TH serum (kindly given by Dr. J. F. Powell, Oxford, UK) diluted to 1 : 1600 in PBS. The production and characterization of the TH antibody has been described previously (van den Pol et al. 1984). After rinsing, the sections were incubated in goat-anti rabbit IgG (1 : 40, Miles Laboratories, U. K.) 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, according to the peroxidase-anti peroxidase method (Sternberger et al. 1970). 3,4-diaminobenzidine (0.05%; Sigma, USA) was employed as the chromogen in the visualization reaction. Finally, the sections were treated with buffered 1% OsO_4 (pH 7.4) which served as a final fixative of the tissue for electron microscopy, as well as to enhance the

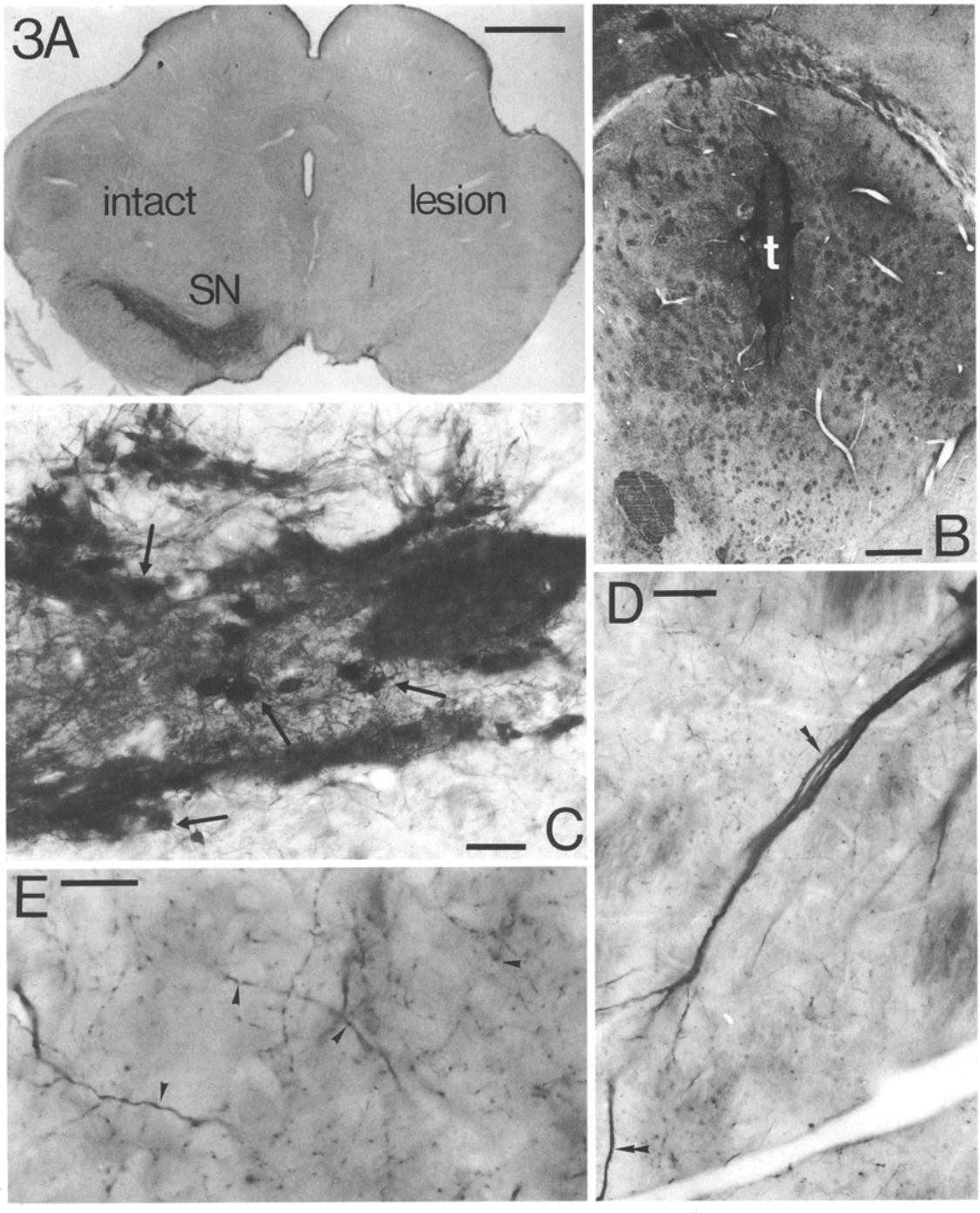

Fig. 3A–E. Light microscopic pictures of TH-stained 70 μm vibratome sections from a rat receiving human fetal mesencephalic grafts (20 weeks survival). **A** Light microscopic picture of a section through the mesencephalon stained for TH-immunocytochemistry, to show the effects of the 6-OHDA lesion. On the intact side, numerous TH-immunoreactive neurons are seen in the substantia nigra (SN) and ventral tegmental area. On the lesion side, however, virtually no TH-immunoreactivity is visible. **B** Low power light photomicrograph of the grafted neostriatum, illustrating the size and position of a long-term survival TH-stained transplant (t) (PC-9 donor; 20 weeks post-grafting). Coarse fibers, thought to be dendrites (see **D**), can be seen emanating from the graft into the surrounding host brain. **C** Higher magnification of a group of TH-immunoreactive neurons (arrows) situated within the human mesencephalic graft illustrated in **B**. The graft is also seen to have a very dense TH-positive neuropil, comprised of fine immunoreactive fibers. Note that the photograph is oriented so that dorsal is to the right and medial is upward. **D** Higher magnification of a bundle of the coarse processes (double arrowheads) which extend into the host brain from the graft tissue. At the EM-level, these processes could be identified as TH-immunoreactive dendrites. The dendrites are surrounded by punctate structures and fine TH-positive fibers which are identifiable as TH-immunoreactive axons and boutons. **E** High power light micrograph of graft-derived fine-calibre TH-positive fibers (arrowheads) present in the host neostriatum. Due to the limited penetration of the antibody only structures at the surface of the sections are stained. Scale bars: **A** 1 mm; **B** 0.5 mm; **C** 75 μm; **D** 50 μm; **E** 15 μm

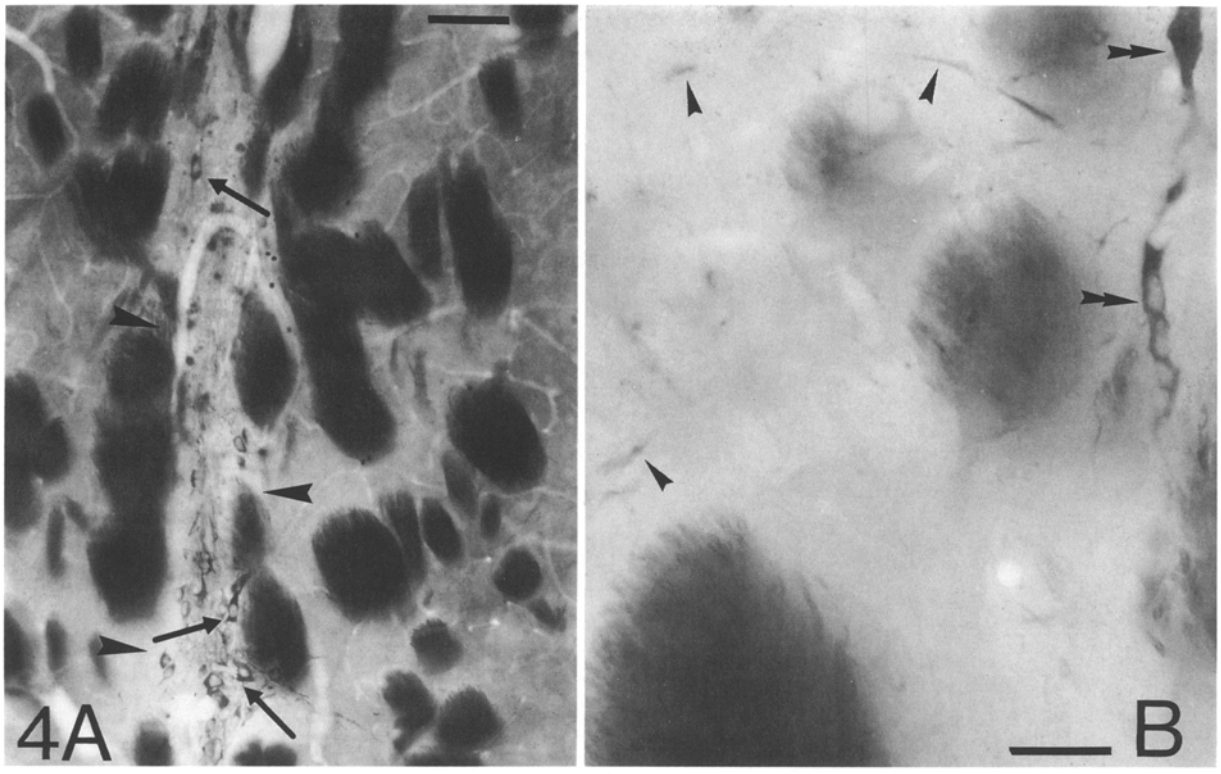

Fig. 4. A Light microscopic picture of the graft (arrowheads) from the rat perfused after 11 weeks (PC-7 donor). Note the numerous TH-immunoreactive neurons (arrows) within the graft but the paucity of fiber outgrowth into surrounding host neostriatum (compared to the 20-week specimen in Fig. 3B, C). **B** High power light photomicrograph showing fiber outgrowth from the same specimen as in **A**. A segment of a swollen dendrite (double arrowheads) extends from the graft and a few scattered TH-immunoreactive fibers and varicosities (arrowheads) are visible (compare with Fig. 3D, E). Scales: **A** 100 μm; **B** 20 μm

immunoreaction product such that fine immunoreactive fibers could be more easily discerned in the light microscope. The sections were routinely dehydrated with the inclusion of 1% uranyl acetate at the 70% ethanol stage and embedded in Durcupan ACM resin (Fluka, Switzerland). Following careful examination in the light microscope, areas of the striatum containing graft-derived processes were re-embedded for electron microscopy and ultrathin sections were cut on a Reichert OMU3 ultramicrotome. They were collected on single slot Formvar-coated copper grids, counter-stained with lead citrate (Reynolds 1963) and examined in a Philips 201 electron microscope.

Results

In order to study the relationship between the formation of synapses by human fetal grafts and functional graft effects, rats were studied morphologically at time points before (8 weeks) and just around (11 weeks) the time when functional effects usually appear, and finally after long-term survival (20 weeks) when the functional effects are fully manifested in the amphetamine-induced rotation test (Brundin et al. 1986b). The rat receiving tissue from a PC-6.5 fetus and studied after 20 weeks graft survival, was included as part of a previously reported behavioural study (Brundin et al. 1988).

Motor asymmetry testing

The results of the amphetamine-induced motor asymmetry tests are summarized in Fig. 2. Briefly, the 2 long-term surviving rats (receiving mesencephalic tissue from the PC-6.5 and PC-9 donors) both exhibited complete graft-induced reversal of the lesion-induced rotational asymmetry with more turns in the direction contralateral than ipsilateral to the lesion, as assessed 19.5 weeks after grafting. In contrast, the two short-term surviving rats (receiving grafts from the PC-7 donor) did not show a marked reduction in net rotation asymmetry at either the 8 or 11 week post-grafting timepoints. However, the rat perfused after 11 weeks exhibited marked (total 86 full turns over 90 min) turning in the direction contralateral to the lesion at the 11-week timepoint, probably representing early signs of a functional graft effect (Brundin et al. 1986a, b). Two additional rats from the same PC-7 group were followed for an additional 10 weeks. At 21 weeks after grafting, both animals showed complete reversal of the lesion-induced turning, with more turns contralateral to the lesion than ipsilateral.

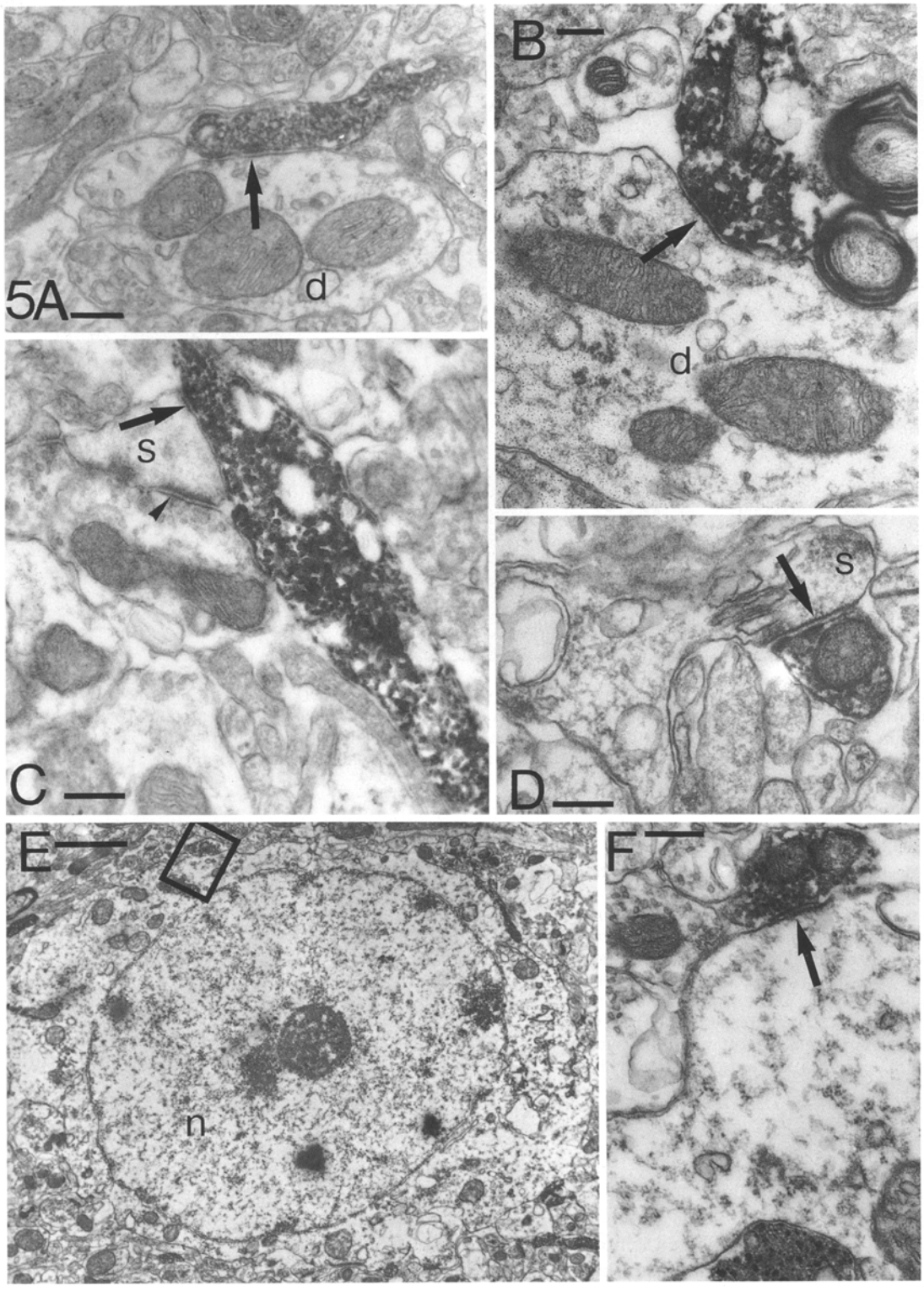

Fig. 5A–F. Examples of synaptic specializations made between TH-immunoreactive boutons of graft origin and neuronal elements within the host neostriatum (20 weeks post-grafting). **A, B** TH-positive boutons forming symmetrical synapses (arrows) onto dendritic shafts (d). **C** A large TH-immunoreactive fiber forms a symmetrical synaptic contact (arrow) with a dendritic spine (s). This spine also receives an asymmetrical synapse (arrowhead) from a non-immunoreactive bouton. **D** A TH-positive bouton forms a symmetrical synaptic specialization (arrow) with a dendritic spine (s). This spine can be clearly identified by the presence of the spine apparatus. **E** Low power electron micrograph of a neuron (n), with typical ultrastructural features of a medium-sized spiny neuron, which is surrounded by several TH-immunoreactive elements. The area contained within the box is shown, several sections serially, at higher magnification in **F**. **F** The boxed area in **E** 5 serial sections away, at higher magnification, showing a TH-immunoreactive bouton forming a symmetrical synapse (arrow) with the neuronal perikaryon of the neuron in **E**. Scales: **A–D, F** 0.25 μm; **E** 1.9 μm

Table 1. Relative distribution of postsynaptic targets of TH-positive boutons making symmetric synaptic contacts in the host striatum in rats receiving grafts of human fetal DA neurons, compared to previous observations in normal rat striatum and in the striatum reinnervated by rat-to-rat grafts

Post-synaptic target	Human to rat grafts		Normal striatum[a]	Rat-to-rat grafts[b]
	Number	Percentage	Percentage	Percentage
Dendritic shafts	39	50.6	36.25	52.7
Dendritic spines	25	32.5	56.45	35.9
Cell bodies	13	16.9	6.1	11.0
Axon initial segment	0	0	1.2	0.4

[a] Data from Freund et al. 1984
[b] Data from Freund et al. 1985

Light microscopy

Virtually no TH-immunoreactive neurons were visible in either the substantia nigra or the ventral tegmental area ipsilateral to the 6-OHDA lesion (Fig. 3A). In contrast a normal density of intensely immunoreactive neurons was present on the contralateral intact side. Surviving grafts were readily located in the neostriatum of all animals (Figs. 3B and 4A). The grafts contained between 500 and 700 TH-immunoreactive neurons in total in each rat. The TH-positive neurons were multipolar and of irregular shape (Figs. 3C and 4A). The smallest TH-positive neurons typically measured 12 × 16 μm whereas another, less frequent, group was much larger (up to 50 μm along their major axis). In the rats with 8–11 week graft survival times, the dendrite-like processes emanating from the perikarya were coarse but short, and in the 8-week specimen they did not even extend into the host brain. However, in the rats with 20 week surviving grafts, the coarse TH-positive dendrite-like processes extended up to 1.5–2 mm into the surrounding caudate-putamen (Fig. 3D), coursing predominantly in a rostromedial direction from the graft. In these two rats, the grafts were surrounded by a dense TH-positive fiber network (Fig. 3E) and some thicker fibers, which were identified as axons in the electron microscope, could be traced across the graft-host border into the host caudate-putamen. The rat perfused after 8 weeks showed no fiber outgrowth (Fig. 4B). In contrast, the axonal fiber outgrowth in the 20-week specimens reached almost the entire caudate-putamen, and to some extent also the nucleus accumbens and globus pallidus. Outside the graft placement site, the outgrowth appeared as a network of very fine calibre TH-positive axons (Fig. 3D, E). The density of this network approached that of the contralateral non-denervated caudate-putamen. Thus there seemed to be a progressive increase in the fiber outgrowth into the host striatum from grafted human fetal DA neurons, such that the outgrowth started between 8 and 11 weeks after grafting, and expanded greatly between 11 and 20 weeks.

Electron microscopic observations

Blocks of the material from the 4 rats were randomly coded so that it was not known from which animal sections were being examined at the time of the electron microscopy. In the 8-week specimen, no TH-positive synaptic boutons were identified within the host caudate-putamen despite careful scrutiny of many ultrathin sections. In the 11-week specimen, there were 12 examples of TH-immunoreactive boutons of presumed graft origin, which were observed to make synaptic contacts with host neuronal elements in the caudate-putamen in regions clearly separated from the body of the graft. In the 20-week specimens, where extensive fiber outgrowth was seen in the light microscope, many TH-immunoreactive boutons were identified in virtually all areas of the neostriatum and examples of synaptic boutons were also found within the nucleus accumbens and globus pallidus.

The TH-immunoreactive boutons were easily identified by their characteristic electron dense appearance due to the deposition of the immunoreaction product around synaptic vesicles (Fig. 5). These boutons tended to be larger (0.6–1.2 μm in diameter) than similarly stained boutons in the contralateral, non-grafted striatum (0.4–0.8 μm in diameter). All the synaptic contacts identified were of the classical symmetric type, similar to the previously described dopaminergic synaptic specializations in the rat neostriatum (Bouyer et al. 1984b; Descarries et al. 1980; Freund et al. 1984; Pickel et al. 1981).

The percentage distribution of post-synaptic targets of the graft-derived TH-positive boutons is reported in Table 1. The predominant post-synaptic target was dendritic elements, either shafts (Fig. 5A, B) or spines (Fig. 5C, D). There was, however, an increased incidence of TH-immunoreactive boutons forming synapses onto neuronal perikarya (Fig. 5E, F). These post-synaptic elements were of various neuronal types, conforming to both projection neurons and intrinsic interneurons, according to their ultrastructural appearance (Bolam 1984). There was no obvious hyperinnervation of neostriatal giant cell bodies as has previously been described for syngeneic rat-to-rat solid grafts placed in a cortical cavity overlying the striatum (Freund et al. 1985). In the

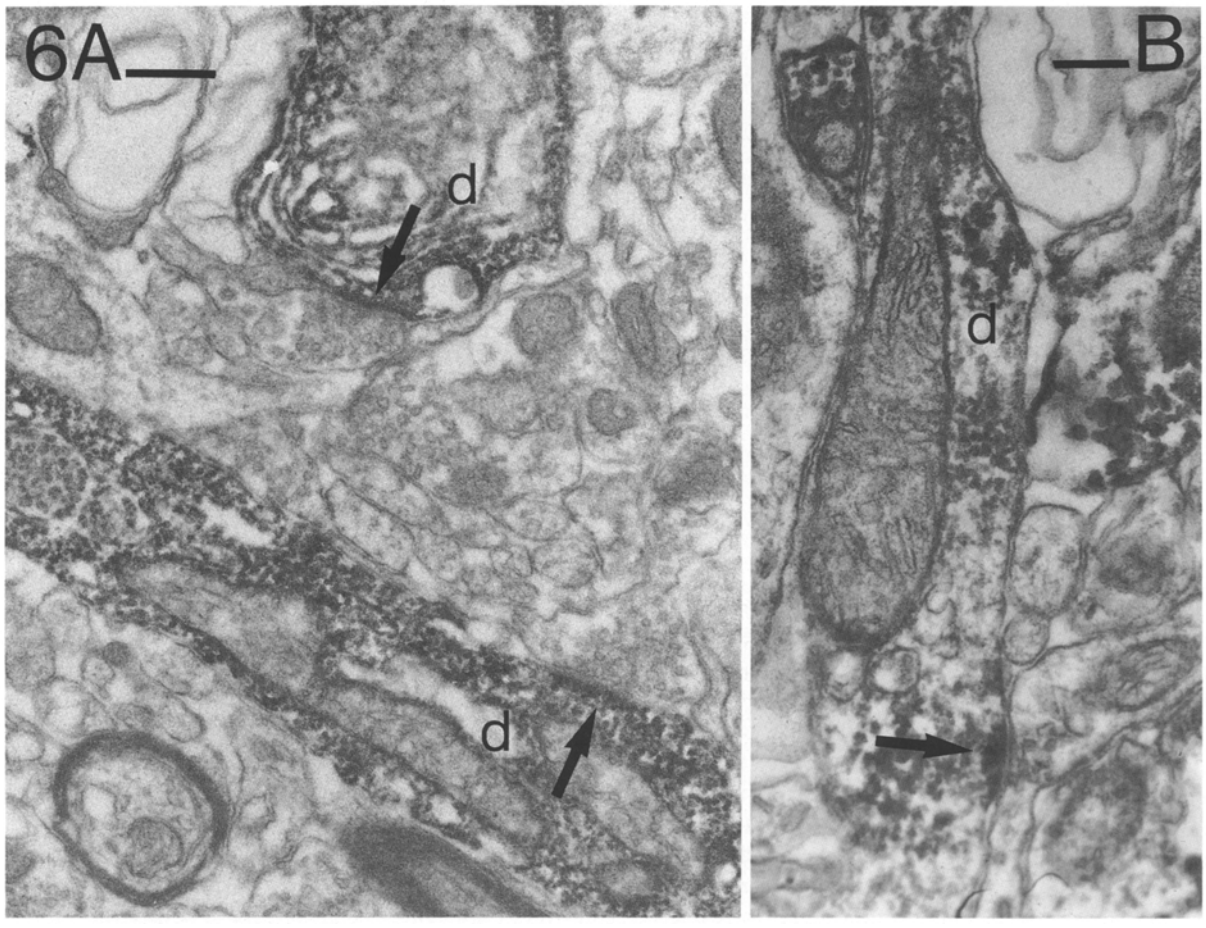

Fig. 6A, B. Examples of TH-immunoreactive dendrites (d) of graft origin which receive synaptic contacts (arrows) from unlabeled boutons of presumed host origin within the host neostriatum in a long-term survival graft (20 weeks post-transplantation). The example in **A** was found 1.0 mm away from the graft placement site and the example in **B** was 1.5 mm distant from the graft. Scales: **A** 0.35 μm; **B** 0.25 μm

nucleus accumbens, the few synaptic boutons examined formed symmetrical synapses with dendritic shafts and spines, similar to what has been reported in intact rats (Bouyer et al. 1984a; Voorn et al. 1986), and in the globus pallidus the 6 identified TH-immunoreactive boutons all contacted dendritic shafts.

In addition to the network of fine calibre TH-positive axons, a population of coarse processes extending up to 2 mm from the grafted TH-immunoreactive cell bodies into the host caudate-putamen could be identified as dendrites in the electron microscope. Their diameter measured up to 1 μm, and they were seen to receive synaptic contacts from non-immunoreactive boutons. These afferent synaptic contacts were seen also along the portions of the dendritic processes extending deep into the host neostriatal tissue. Because of the deposition of the peroxidase reaction product within the dendrites these synapses could not clearly be classified as symmetric or asymmetric.

Discussion

This study provides evidence that grafted human fetal DA neurons can extensively reinnervate the host brain and form synaptic contacts with rat striatal neurons. The neurons retain many of the morphological characteristics that they would have acquired if allowed to develop in situ. Moreover, the type of synaptic contacts formed by the grafted human fetal DA neurons largely resemble those seen with normal or grafted rat DA neurons. The observation of non-immunolabelled boutons synapsing onto TH-immunoreactive dendrites that extend from the graft into the surrounding host neostriatum provides tentative evidence for the existence of host afferents to the grafted DA neurons.

The symptoms of PD have been estimated to appear when the patients have lost about 70–80% of their nigrostriatal DA neurons (cf. Agid et al. 1987; Bernheimer et al. 1973). Until this stage, the progressive loss of DA neurons is most probably compen-

sated for by an increased metabolic activity and DA turnover in remaining neurons and by the development of supersensitive postsynaptic DA receptors in the striatum (Agid et al. 1987). When levodopa therapy is given in the early phase of PD, most patients show considerable improvement of motor function without any significant fluctuations. However, after some years of levodopa treatment 50% or more of all PD patients exhibit a loss of efficacy of levodopa associated with diurnal oscillations in motor performance ("on-off" phenomena) and dyskinesias (Marsden et al. 1980). The precise cause of the decline in the therapeutic response to levodopa is unclear, but one major hypothesis suggests that it is due to the degeneration of nigrostriatal DA neurons that continues during the course of the levodopa therapy. With the progressive loss of storage capacity for DA in the striatum, variations in dopa-plasma levels can no longer be buffered by the brain, which results in severe fluctuations in symptomatology (Marsden et al. 1980; Nutt 1987). Experiments in rats have shown that even without any remaining DA neurons, levodopa can be converted to DA in the striatum through decarboxylation in sites such as serotonergic terminals, non-aminergic neurons and non-neuronal cells (Melamed et al. 1980). However, the storage and controlled physiological transmitter release at synaptic sites is likely to be lost in parallel with the degeneration of the nigrostriatal DA neurons. Therefore it seems reasonable to assume that the ability to form appropriate synaptic contacts with the host striatum and the restoration of a well-controlled DA neurotransmission at these sites is a critical factor determining the usefulness of DA-rich neuronal implants in patients with PD.

Freund et al. (1985) and Mahalik et al. (1985) have previously reported that the ingrowing dopaminergic axons from solid rat-to-rat grafts, placed in a cortical cavity, form synaptic contacts onto dendrites and cell bodies in the host striatum. Similar to the DA input in normal animals, the principal target of the DA boutons from these rat-to-rat grafts was the dendrites of medium-sized spiny neurons, which is the major class of striatal projection neurons (Bolam 1984). The density of DA synapses in the reinnervated portion of the striatum seemed to be close to normal, whereas the relative proportion of synapses on different sites of the host striatal neurons was abnormal. In the study by Freund et al. (1985) DA synapses were found to be less frequent on dendrites and more abundant on neuronal perikarya in the graft innervated striatum compared to normal. The proportion of contacts on spines was lower, and contacts on shafts higher, than normal in the grafted animals. Most conspicuously, these neurons formed dense pericellular DA fiber arrangements with frequent synaptic contacts around giant cholinergic interneurons (Freund et al. 1985).

The present results show that TH-positive axons from human mesencephalic grafts are able to form synapses of the normal symmetric type with target neurons in the previously denervated rat striatum. As with rat-to-rat grafts the relative proportion of TH-positive synapses on dendrites was lower in the reinnervated striatum (about 83%) than in the normal brain (about 93%) whereas TH-positive synapses were more frequent on perikarya in the grafted animals (Table 1). In contrast to grafted rat DA neurons, the human DA neurons formed no hyperinnervation of giant neurons. Freund et al. (1985) suggested that the DA hyperinnervation of the cell bodies of giant cholinergic neurons might play a special role in the graft-induced functional recovery. However, this seems less likely since even in the absence of this hyperinnervation, as with the present human mesencephalic implants, equivalent functional effects occurred in both spontaneous and drug-induced behaviours (Brundin et al. 1986b, 1988).

The graft-derived DA reinnervation of the denervated striatum is but one example that fibers from grafted neurons can make functional connections with host elements. For the DA grafts the fiber ingrowth is characterized by a high degree of specificity. The DA fibers grow into the host only if the grafted DA neurons are located in a DA terminal area, such as the striatum, and in such regions the axons tend to establish normal terminal patterns (Björklund et al. 1983b; Herman et al. 1986). The present and previous studies, moreover, have demonstrated that the ultrastructural features of the DA innervation in the striatum is fairly similar in grafted and normal animals. Although the factors involved in the reformation of specific neuronal circuitry in the brain are virtually unknown, the results obtained with xenografts of mouse (Brundin et al. 1985) and human (Brundin et al. 1986b; Nilsson et al. 1988; Strömberg et al. 1986) neurons in the rat brain indicate that these factors are similar across different mammalian species. Therefore it seems probable that the specificity of the connections between the grafted DA neurons and the host brain depends on complex interactions between host and graft elements that can operate between phylogenetically widely disparate species, such as rat and man.

Human mesencephalic DA neurons grafted in the rat model of PD have previously been shown to produce an extensive new DA-containing terminal network in the previously denervated striatum, and to reduce or even normalize amphetamine-induced, apomorphine-induced and spontaneous motor

abnormalities in rats with unilateral lesions of the mesostriatal DA system (Brundin et al. 1986b, 1988; Strömberg et al. 1986). The present results indicate that the grafted human DA neurons begin to extend a DA fiber network into the host between 8 and 11 weeks after grafting. At this time the grafted fetal cells are approximately 15–18 weeks old. This fetal age coincides with, or somewhat exceeds, the approximate time when human fetal DA neurons are extending their axons and forming terminal varicosities in their target areas during normal ontogenetic development in situ (Nobin and Björklund 1973; Olson et al. 1973; Pickel et al. 1980). The rate of development of cell suspension grafts of human DA neurons is markedly slower than that of rat DA neurons, which are known to develop an extensive DA fiber network in the host striatum within 4–6 weeks (Björklund et al. 1983b). These observations are consistent with the observation that the functional effects of grafted human DA neurons appear much later than those resulting from grafted rat DA neurons (Brundin et al. 1986b). Thus, both with rat and human donors the functional effects seem to appear around the time when the DA fibers develop from the graft and extend into the host striatum. This time-course is compatible with the idea that grafted human fetal neurons retain an "internal clock" which governs their rate of development even when they are removed from their normal developmental context and placed into the fully developed target area (Lindvall et al. 1987) or into the anterior chamber of the eye (Olson et al. 1987). These observations are consistent, e.g., with those of Sotelo and Alvardo-Mallart (1987) who have observed that the time-course of development and migration of mouse fetal Purkinje cells grafted to the cerebellum of adult mice follows that of Purkinje cells developing in the normal fetal environment. In the present study, the larger TH-positive cells and boutons seen in the human fetal grafts, as compared to what is normally found in the rat, provide another example of the grafted DA neurons retaining species-specific characteristics.

There is evidence from electrophysiological experiments that intrastriatal grafts of rat DA neurons can receive some afferent input from the host (Arbuthnott et al. 1985). Morphological evidence for host afferent input to mesencephalic grafts, however, is poor (Freund et al. 1985; Mahalik et al. 1985). Bolam et al. (1987) have shown that identified DA neurons in solid rat-to-rat grafts receive abundant synaptic inputs of several different types, but the synaptic contacts were all located within the graft boundaries and may thus have been derived from intrinsic local connections. The present data demonstrate a synaptic input, presumably from the host, onto those portions of the dendrites of the grafted TH-positive neurons which extended into the depth of the host caudate-putamen. In studies of fetal striatal tissue grafted to the adult striatum Pritzel et al. (1986) and Clarke et al. (1988) have demonstrated extensive host DA afferents to the striatal grafts. This illustrates the potential functional importance of host-to-graft afferent connections, as the striatal grafts have been shown to respond with an increase in GABA release when the host DA system is activated with amphetamine (Sirinathsinghji et al. 1988). The possibility that host neurons can form afferents to intrastriatally implanted human fetal DA grafts is of particular interest when extended to the clinical setting, as it suggests a possible means of extrinsic regulation of the activity of the grafted DA neurons.

With the intracerebral dialysis technique, the grafted human DA neurons have been found to restore spontaneous DA release to normal levels in the reinnervated host striatum, and there is evidence indicating that the DA reuptake system which normally operates in the mesostriatal system is functioning also in the grafted human DA neurons (Brundin et al. 1988). The present data indicate that the DA release may take place at normal mature synaptic contacts with previously denervated neuronal elements of the host striatum. Although the relative importance of synaptic versus non-synaptic transmitter release in the mediation of graft-induced functional recovery in 6-OHDA treated rats is poorly known, circumstantial evidence indicates that DA synapses may be of functional significance. First, there is a direct relationship between the extent of DA fiber outgrowth from mesencephalic grafts at the light microscopic level and the degree of functional recovery in the amphetamime-induced rotation test (Björklund and Stenevi 1979; Björklund et al. 1980). Second, although adrenal medullary grafts, that do *not* form synapses with the host striatum, can contain at least as high amounts of DA as mesencephalic neural grafts (Freed et al. 1984), experiments in rats indicate that they do not possess the same functional potential as fetal mesencephalic grafts (see Lindvall et al. 1987, for discussion). Finally, studies employing the in vivo voltammetry technique to monitor DA release within the normal intact striatum indicate that the concentration of DA in the synaptic cleft is one or two orders of magnitude higher than it is extrajunctionally, suggesting that DA released from mesostriatal axon terminals will reach more efficient concentrations within the synaptic cleft and that the diffusion of DA in extrajunctional spaces is very limited (Gonon and Buda 1984). Indeed, in the

present experiment, the rats sacrificed after 8–11 weeks, which exhibited no or only few TH-positive synapses in the host striatum, showed no or only minor functional effects in the amphetamine-induced rotation test although these grafts were as rich in TH-positive neurons as were the functional grafts.

In conclusion, the morphological and functional properties of human fetal DA neurons, as revealed by grafting experiments in rats, indicate that these neurons would be able to restore physiologically regulated and synaptically localized DA neurotransmission in the denervated host striatum. We feel that in future human trials this may be of decisive importance for the capacity of the grafts to induce long-term and stable improvements of motor performance in patients with PD, without causing adverse effects, such as dyskinesias, due to uncontrolled transmitter release.

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