Efferent Synaptic Connections of Grafted Dopaminergic Neurons Reinnervating the Host Neostriatum: A Tyrosine Hydroxylase Immunocytochemical Study¹

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Abstract

In adult rats with a unilateral 6-hydroxydopamine-induced destruction of the nigrostriatal dopamine (DA) pathway, grafts of embryonic substantia nigra can establish a new dopaminergic terminal fiber plexus in the previously denervated neostriatum and compensate for some of the behavioral deficits induced by the nigrostriatal lesion. In the present study the synaptic connections of the ingrowing DA fibers from the graft were analyzed ultrastructurally, using immunocytochemical localization of tyrosine hydroxylase (TH), in animals whose lesion-induced motor asymmetry had been completely compensated for by the nigral grafts. In two of the animals, horseradish peroxidase-wheatgerm agglutinin conjugate was injected into the graft in order to trace possible reciprocal afferent connections to the graft from the host striatum.

TH-immunoreactive axons from the graft were seen to make abundant symmetric synapses with neuronal elements in the host neostriatum. Between 85 and 90% of these synapses were on dendritic shafts and spines, and the rest were on neuronal perikarya. Two principal targets were identified: (i) dendrites of spiny neurons, the majority of which are likely to be striatal projection neurons; and (ii) the cell bodies of giant neurons, most (or perhaps all) of which are known to be cholinergic interneurons. The synapses made on dendritic spines, which constituted about 40% of all TH-positive synapses formed by the TH-positive neurons in the graft, resembled those seen in normal animals, both in that they made contacts with spine necks and in that they invariably were associated with an asymmetric TH-negative synapse contacting the spine head. The innervation of the giant cell perikarya, which constituted about 6% of all TH-positive synapses found, was strikingly abnormal in that the graft-derived TH-positive fibers formed dense pericellular "bas-

kets" selectively around the giant cell bodies. Such arrangements were never seen in the normal striatum, nor did they occur in the intact contralateral striatum in the grafted animals. It is proposed that this apparent dopaminergic hyperinnervation from the graft could provide a powerful inhibition of the cholinergic interneurons in the reinnervated host striatum, and that such an inhibitory mechanism could assist in the graft-induced functional recovery by potentiating the functional effects of DA synapses terminating on the spiny efferent neurons. This dual innervation may thus help to explain why restoration of only a small proportion of the striatal DA innervation by the graft is sufficient to induce complete compensation of, e.g., motor asymmetry in the lesioned rats. In the horseradish peroxidase-wheatgerm agglutinin-injected animals no labeled cells could be identified in the caudate-putamen, globus pallidus, or mesencephalic raphe of the host, which indicates that the grafted DA neurons function in the absence of several of the normal main inputs to nigral DA neurons.

Intracerebral grafts of dopamine (DA)-producing tissues are able to compensate for motor or sensorimotor deficits induced by neurotoxin lesions of the nigrostriatal DA pathway in adult rats (Björklund and Stenevi, 1979; Perlow et al., 1979; Björklund et al., 1980, 1981; Freed et al., 1980, 1981; Dunnett et al., 1981a, b, c). The operational mode of such grafts, however, is still poorly understood. It seems clear from experiments with grafts of adrenal medullary tissue (Freed et al., 1981; Freed, 1983) that grafted cells at least under certain conditions may exert their functional effects through a diffuse release of their active compounds into the cerebrospinal fluid or locally into the extracellular fluid. This is probably true also for grafts of hypothalamic hormone-producing neurons whose functional effects most likely are mediated via neurovascular links (Gash et al., 1980; Krieger et al., 1982). In the case of grafted embryonic DA neurons, there is, in contrast, good evidence that the behavioral effects of the grafts are directly dependent on the ability of the grafted nerve cells to establish new axonal networks in specific regions of the initially denervated host striatum, thus suggesting that grafted neurons function primarily through their efferent connections via a release of the DA transmitter onto receptive elements in the host striatum (Björklund et al., 1980; Dunnett et al., 1981c, 1983; Fray et al., 1983).

These observations on intrastriatal nigral grafts raise the important question whether their functional effects are due to a re-establishment of specific synaptic contacts with the initially denervated striatal neurons by the DA neurons of the graft, or whether the effects result

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from a more nonspecific neurohumoral type of action, i.e., a local diffuse release of the transmitter from nonsynaptic terminals in the host striatum. In the present study the ultrastructural features and postsynaptic targets of the ingrowing dopaminergic axons have been studied by means of tyrosine hydroxylase (TH) immunocytochemistry. In addition, horseradish peroxidase (HRP)-wheatgerm agglutinin (WGA) conjugate was injected into the grafts in order to clarify to what extent the behavioral function of the nigral grafts depends on the formation of reciprocal connections with the host striatum.

Materials and Methods

Experimental procedures

6-Hydroxydopamine lesion and transplantation surgery. Young, adult, female Sprague-Dawley rats (ALAB, Stockholm, Sweden; 180 to 200 gm) received unilateral 6-hydroxydopamine (6-OHDA) lesions of the right nigrostriatal DA pathway. Under methyl hexital anesthesia (Brietal, Eli Lilly & Co., Indianapolis, IN; 40 mg/kg), 8 μ g of 6-OHDA (6-OHDA-HCl, calculated as the free base; AB Hässle, Göteborg, Sweden) in 4 μ l of ascorbate-saline (0.2 mg of ascorbic acid/ml) were injected over 4 min, as detailed elsewhere (Björklund et al., 1980, 1983). The completeness of the striatal DA denervation was assessed by monitoring the amphetamine-induced turning response, as described below, 7 to 10 days after the 6-OHDA injection.

The rats received embryonic nigral transplants in a two-step grafting procedure, as described elsewhere (Björklund et al., 1980). In brief, the rats were prepared for transplantation by making a small suction cavity (about 2 $\times$ 3 mm) in the dorsal parietal cortex to expose the dorsal surface of the head of the denervated caudate-putamen, and the cavity was filled with Gelfoam. This operation was performed 2 weeks after the 6-OHDA lesion. Six weeks later, the cavity was re-exposed and the Gelfoam was removed. Graft tissue, consisting of the ventral mesencephalon dissected from a 16-day old rat fetus (of the same inbred strain), was finally inserted into the cavity in contact with the vessel-rich pia that had grown out over the walls of the cavity during the intervening period.

Rotation tests. The turning response to a single intraperitoneal injection of 5 mg/kg of metamphetamine was assessed in an automated "rotometer," according to the method of Ungerstedt and Arbuthnott (1970). The turning behavior was monitored continuously in hemispheric perspex bowls for 90 min after injection. The rats received one test prior to grafting and two further tests at 1½ and 3½ months after grafting.

Preparation of tissue sections. Four grafted animals, functionally completely compensated in the rotation test, were processed for light and electron microscopic immunocytochemistry at 3½ to 4½ months after transplantation. Two of these received a 20- μ l injection of an HRP-WGA conjugate (final concentration, approximately 3% HRP) into the nigral transplant under chloral hydrate anesthesia, 24 hr before perfusion. The animals were perfused through the heart, first with Tyrode's solution (1 to 2 min), followed by a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in sodium phosphate buffer for 30 min at room temperature (Somogyi and Takagi, 1982). The brain was postfixed for 1 to 2 hr by immersion in the same fixative.

Approximately 2 $\times$ 4 $\times$ 5 mm large blocks containing the striatum and nigral transplant were dissected and washed in several changes of 0.1 M phosphate buffer, pH 7.4, followed by 10% and 20% sucrose in the same buffer, until the blocks sank. They were then rapidly frozen in liquid N₂ and thawed in 0.1 M phosphate buffer (4°C). Frontal sections, 80 μ m thick, were cut on a Vibratome (Oxford Instruments, San Mateo, CA) and processed for immunocytochemistry. Sections from the two HRP-WGA-injected animals were reacted to reveal HRP activity prior to the immunostaining as follows.

Figure 1. Amphetamine-induced rotation in the four SN-grafted animals used in the electron microscopic immunocytochemical study. Prior to transplantation (Pre-Trpl.), the 6-OHDA-lesioned rats showed a strong turning response toward the lesioned side. After transplantation the turning response was abolished or even slightly reversed. The solid circles represent the two animals used in the quantitative study (Table I).

After washing in 0.05 M Tris-HCl buffer (pH 7.4) and preincubation in diaminobenzidine (DAB) (50 mg/100 ml of Tris buffer) for 20 min, the sections were incubated in the same solution with the addition of 0.01% hydrogen peroxide. Incubation was carried out for no longer than 5 to 8 min to minimize background. After washing with Tris buffer followed by phosphate buffer, these sections were also ready for immunostaining.

Immunocytochemical procedure

Antibody. The production and characterization of the anti-TH antibody has been published elsewhere (van den Pol et al., 1984). Briefly, TH was purified from bovine adrenal medullae, and a Dutch rabbit was injected with 75 μ g of TH in Freund's complete adjuvant; after 1 month, another injection was given in incomplete adjuvant. Two weeks later, the rabbit was bled, and serum was collected. The specificity of the antibody has been biochemically

Figure 2. A, Low power light micrograph of an 80- μ m-thick frontal section cut through the nigral transplant (NT) and rostral striatum (ST). The dorsal surface of the striatum bordering the transplant is indicated by dashed lines. The section has been incubated to reveal TH immunoreactivity and postfixed in OsO₄ for subsequent electron microscopy. The nigral transplant contains TH-immunoreactive cell bodies aggregated near the midline and at the border with the striatum. B, The framed area in A is shown at higher magnification. Three giant cells are labeled (G₁, G₃, and G₄), and a dense TH-immunoreactive fiber network is visible, covering approximately the dorsal third of the host striatum. Some of the medium-sized cell bodies are also indicated (small arrows); they are only lightly stained at background level. Only the perikarya of the three giant cells appear to be surrounded by TH-positive fibers, also shown at higher magnification in C to E, G, and H. C to H, High power light micrographs of giant cells (three of them also seen in A and B), located in the reinnervated dorsal third of the striatum and surrounded by TH-immunoreactive fibers (small arrows). Large arrows label medium-sized perikarya (less than half the size of giant cells), which were never seen to be surrounded by TH-positive fibers. One of the giant cells (G₁) is also shown at electron microscopic level in Figure 3 (use Fig. 2G for correlation). Scales: A, 200 μ m; B, 50 μ m; C to H, 10 μ m.

Figure 2

characterized by precipitation of enzyme activity and determination of components recognized by the antibody. Immunocytochemical tests have also been performed.

Incubations were carried out in the following order at room temperature, using phosphate-buffered saline (PBS) containing 1% normal goat serum (NGS; Miles Laboratories, Elkhart, IN) for all of the washes and antibody dilutions unless otherwise stated: 1 hr in 20% of NGS; 1-hr wash; overnight in rabbit anti-TH serum diluted 1:1000 at 4°C; three 30-min washes; 6 hr in goat anti-rabbit IgG (Miles) diluted 1:40; three 30-min washes; overnight in rabbit peroxidase-antiperoxidase complex (Miles) diluted 1:100 at 4°C; three 30-min washes (in PBS only). The vials containing the sections were subjected to agitation in all of the above steps.

After washing in 0.05 M Tris-HCl buffer (pH 7.4) and preincubation in DAB (50 mg/100 ml of Tris buffer) for 20 min, the sections were incubated in the same solution with the addition of 0.01% hydrogen peroxide for 5 to 8 min. After washing (Tris buffer followed by phosphate buffer), the sections were treated with 1% OsO₄ in 0.1 M phosphate buffer (pH 7.4) for 40 min. Following a few washes in the same buffer, the sections were dehydrated in ethanol (1% uranyl acetate was included in the 70% ethanol stage for 40 min), mounted on slides in Durcupan ACM resin (Fluka) under a coverslip, and cured for 2 days at 56°C. The slides could then be stored permanently.

Areas of interest were photographed and re-embedded in Durcupan in plastic capsules as described for immunocytochemical material (Somogyi and Takagi, 1982). Serial ultrathin sections were finally cut from the re-embedded thick sections; they were mounted on Formvar-coated single slot grids and stained with lead citrate (Reynolds, 1963). Electron micrographs were taken at 80 kV on Philips 201C and JEOL EM 100B electron microscopes.

Results

Turning behavior

Prior to grafting, the 6-OHDA-lesioned rats used in the present study exhibited a mean amphetamine-induced turning rate of >6.5 turns/min over 90 min after injection. This criterion selects rats with at least 98% depletion of striatal DA ipsilateral to the lesion (Schmidt et al., 1982, 1983). As shown in Figure 1, the four grafted animals selected for the ultrastructural study had all become completely compensated in their motor asymmetry by 3½ months after implantation of the graft, signifying a significant functional DA fiber ingrowth into the host striatum from the graft (Björklund et al., 1980). The two rats indicated by *solid circles* in Figure 1 are the ones used for synapse counting in Table I.

Light microscopic observations

The nigral transplant. The nigral transplant was situated on the dorsal surface of the head of the striatum, close to the rostral pole, in each of the four animals. The TH-immunoreactive perikarya formed groups in the center of the graft with an accumulation toward the striatal surface (Fig. 2A). The immunostaining had a different penetration from area to area inside the transplant, but generally it was much deeper than in the striatum. This could perhaps be explained by the better vascularization of the graft, which allows a better fixation, but in spite of that, also a deeper penetration of the antibodies. Usually only the proximal dendrites were stained besides the perikarya; otherwise, axonal processes could not be distinguished with certainty from dendritic ones in the graft. The immunoreactive processes formed a very dense network in parts of the

neuropil of the graft, and they could be followed across the graft-host border into the striatum. Many non-immunoreactive perikarya were also seen in the graft, surrounding or mixed with, groups of TH-positive cells.

The striatum. The dorsal one- to two-thirds of the striatum were reinnervated by TH-immunoreactive fibers from the graft forming a very dense network, which extended a few hundred micrometers in rostral and caudal directions from the TH-positive, cell-rich regions of the implant, and covered the entire mediolateral width of the striatum. Immunoreactive fibers could not be detected in any other parts of the caudate-putamen (i.e., ventral third; caudal part of the head, and tail) or in globus pallidus, and only occasional fibers occurred in nucleus accumbens. In the ipsilateral substantia nigra, which was carefully searched for surviving TH-positive neurons, only single stained cells were found. Consistent with the pretransplant rotation test, these observations provide sufficient evidence that the initial 6-OHDA lesion of the intrinsic nigrostriatal DA pathway was complete in the animals used in the present study.

The contralateral caudate-putamen was homogeneously covered by TH-positive fibers like that in normal animals. The immunoreactive elements of the contralateral globus pallidus, nucleus accumbens, and substantia nigra-ventral tegmental area (SN-VTA) were qualitatively identical to these areas of normal animals. Ipsilateral to the 6-OHDA lesion, only very few TH-immunoreactive cell bodies were detectable in the SN, whereas more such cells remained in the medial part of the VTA.

The density of TH-immunoreactive fibers in the reinnervated part of the ipsilateral striatum was similar to that of the normal striatum. The density was highest immediately below the graft and, going ventrally, the fibers became gradually more sparse. Sometimes individual fibers could be followed for a long distance as they ran parallel to the frontal plane of the section; they were frequently seen to give rise to thinner collaterals. Such fiber paths are not seen in frontal sections through the striatum of normal animals, since the normal TH-positive fibers coming from the SN-VTA are running in an oblique caudorostral direction. The axons were rarely varicose and had a diameter between 0.1 and 1.2 µm. As in normal animals, the TH-positive fibers had an even, continuous staining; thus, isolated individual TH-positive varicosities were never seen.

Associations with giant cells at the light microscopic level. All perikarya on the surface of the sections were stained very weakly at background level. After osmication, which also intensifies contours, each perikaryon became visible on the surface. This enabled us to identify all giant cell perikarya by their relative size (longest diameter, 25 to 35 µm), without using other procedures like Golgi impregnation, acetylcholinesterase histochemistry, or choline acetyltransferase immunocytochemistry (cf. "Discussion"). The most striking observation in this study was that, in all animals analyzed most, if not all, giant cell bodies of the reinnervated striatal area, were completely surrounded by TH-immunoreactive fibers (Fig. 2). When focusing from the top to the bottom of the giant perikarya, TH-positive fibers were seen to cover them at all focal planes, and they frequently followed the proximal dendrites as well. Such pericellular arrangements have never been seen in normal animals, and they did not occur in the contralateral intact striatum in the transplanted animals. None of the striatal cell bodies of the medium-sized

Figure 3. A, The upper right portion of the perikaryon of giant cell G₁, illustrated in Figure 2, G and H, is shown at the electron microscopic level. The angular shape of the soma and the edge of the perikaryon of a medium-sized cell on its right-hand side help its identification. Besides its size, the high density of rough endoplasmic reticulum in the cytoplasm and the deeply indented nucleus serve to characterize this cell as a giant cell. The arrows point to TH-immunoreactive fibers completely covering the cell body. The framed area is shown in B at higher power. B, the thin TH-immunoreactive fiber is in symmetric, en passant type synaptic contact (arrow) with the soma of the giant cell (G₁). C, The same giant cell (G₁) receives another symmetrical synaptic contact (large arrow) from a weakly immunostained fiber. This contact was found at a level deeper than 5 µm below the surface of the thick section, where all specific immunostaining is weaker because of the limited penetration of the antibodies. However, the fiber is still identifiable as TH positive on the basis of the immunoperoxidase reaction end product precipitated mainly around a mitochondrion (small arrows). Scales: A, 1 µm; B and C, 0.2 µm.

Figure 3

TABLE I
Postsynaptic target elements of dopaminergic fibers in transplanted and normal animals

Postsynaptic target elements of dopaminergic fibers were studied quantitatively in two transplanted animals (columns 2 to 7) and compared to similar data derived from normal animals (columns 8 and 9, taken from Freund et al., 1984). The data obtained from the two transplanted animals were similar, but they differed notably from those recorded in normal rats: the percentage of postsynaptic spines was lower and those of postsynaptic dendritic shafts and perikarya were higher in the grafted animals. The most striking difference from normal was not quantitative but qualitative: more than half of the postsynaptic perikarya were identified as giant cells in the grafted animals, whereas no such contacts were found in the normal striatum.

Postsynaptic Targets	6-OHDA-lesioned Animals with Nigral Transplants						Normal Animals (data from 6 animals)	
	Animal No. 1		Animal No. 2		Data Pooled		Number	Percentage
	Number	Percentage	Number	Percentage	Number	Percentage		
Spines	51	36.2	41	35.6	92	35.9	232	56.45
Dendritic shafts	76	53.9	59	51.3	135	52.7	149	36.25
Perikarya								
Total	14	9.9	14	12.2	28	11.0	25	6.1
Giant cells	9	6.4	7	6.1	16	6.3		
Axon initial segments			1	0.9	1	0.4	5	1.2
Total	141	100	115	100	256	100	411	100

category (10 to 15 μm), which are known to constitute about 95 to 97% of the total neuronal population, were surrounded by immunoreactive fibers in this way. Sometimes single axons could be seen to pass along one side of such perikarya, but the length of these immediate contacts was always very short and most frequently limited to only one point. Thus, the heavy innervation of the giant perikarya by TH-immunoreactive fibers from the grafts appeared to be highly selective.

Electron microscopic observations

Fiber morphology. The ultrastructural characteristics of TH-immunoreactive fibers were very similar to those in normal animals. None of them had myelin sheaths, and their diameter varied between 0.1 and 1.2 μm . The thicker main axons (0.6 to 1.2 μm) were never synaptic, and they contained large amounts of microtubules and mitochondria. The thinner fibers (0.1 to 0.4 μm) were usually presynaptic but only rarely formed bouton-like swellings (Figs. 3 to 6). They were full of large, clear vesicles, most of which were nearly round, but a few had pleomorphic shape. The immunoperoxidase reaction product had a fairly homogeneous distribution along the stained fibers, and it surrounded the outer membranes of all cytoplasmic organelles (vesicles, mitochondria, etc). The reaction product was typically precipitated on the presynaptic triangle-shaped "protein frames" (Figs. 3, B and C, 4B, and 5 B to D).

In Table I a total of 256 synaptic contacts established by TH-immunoreactive fibers have been identified in two of the transplanted animals, and all of them were classical symmetrical (Gray's type II) synapses. A slight accumulation of vesicles was seen at the synaptic active zones, but the fibers were usually not varicose at the junction. These *en passant* types of synaptic contacts are typical of TH-positive axons of intact, non-grafted animals as well (Freund et al., 1984).

Postsynaptic targets. Six light microscopically identified giant cells were sampled for correlated electron microscopy in order to establish whether the TH-immunoreactive "baskets" around their perikarya and proximal dendrites were synaptic. The giant cells could easily be identified at the ultrastructural level as well, since the outer rim of

their cytoplasm is full of granulated endoplasmic reticulum arranged in parallel laminae. This feature may explain why their cytoplasm appears to be darker than that of the medium-sized cells in the light microscope. The perinuclear region contained extensive Golgi cisternae and several deep indentations were found in the nucleus (Figs. 3A and 4A). A series of 40 to 60 ultrathin sections was studied in the electron microscope from each of the six giant perikarya. Altogether, 21 synapses established by immunoreactive fibers could be identified on them (Figs. 3, B and C, and 4B). In the case of one giant cell, where the serial sections were cut from the tip of the perikaryon that contained no nucleus, a tangle of TH-positive fibers was seen to be invaginated into the soma (Fig. 4). These fibers also established symmetric synaptic contacts with the perikaryon (Fig. 4B). The same giant cell bodies received a few symmetric synapses from immuno-negative terminals as well, which were densely packed with small round vesicles.

The distribution of postsynaptic target profiles of the TH-immunoreactive fibers was studied quantitatively in two animals, and the results are summarized in Table I. Only the randomly found synapses were counted; thus, the data of the correlated light and electron microscopy on giant cells, described above, are not included in Table I. The results are compared to a similar analysis done in normal animals earlier (Freund et al., 1984).

The percentage of synaptic contacts on dendritic spines was lower than in normal animals. The TH-positive fibers established symmetric synaptic contacts on the neck of the spines (Figs. 5, C to E, and 6A), whereas the head invariably received an asymmetrical synapse from an immuno-negative terminal containing small round vesicles (Fig. 5D). This synaptic arrangement on spines is typical for TH-positive fibers in normal animals as well. In the transplanted animals, however, an additional third synapse rarely appeared on the same spines; it was always symmetrical and established by TH-negative terminals containing small pleomorphic vesicles.

Synaptic contacts on dendritic shafts were more frequent than in normal animals. Most postsynaptic dendrites could be identified as densely spiny in serial reconstructions (Figs. 5, A, B, and E, and 6B), but occasionally smooth dendrites containing long mitochondria

Figure 4. A, Low power electron micrograph of a section cut from the tip of a giant cell's perikaryon (G_2). This neuron already was identified as a giant cell at the light microscopic level, but its ultrastructure is also typical (note the relatively large amount of rough endoplasmic reticulum). A network of TH-immunoreactive fibers (small arrows) is invaginated into the cell body, establishing several symmetric synaptic contacts with the perikaryal membrane. Two of these contacts (framed area) are shown at higher power in B. Open arrows label the points where TH-positive fibers enter the soma of the giant cell. B, The invaginated TH-positive fibers are in symmetric synaptic contact (large arrows) with the somatic membrane of giant cell G_2 . The synapse on the right is cut tangential in this micrograph, but during serial reconstruction it proved to be symmetrical also. The point where the TH-positive fiber on the left enters the soma is indicated by a small arrow. Scales: A, 0.5 μm ; B, 0.2 μm .

Figure 4

also received synaptic contacts from immunoreactive terminals (Fig. 6C).

The percentage of perikaryal contacts was also higher in the transplanted animals; however, in this case, the qualitative differences seem more important than the quantitative ones. More than half of the 11% perikaryal synapses were on giant cells in the transplanted animals, whereas the rest of the postsynaptic cell bodies were of medium size, containing round nonindented nuclei and limited amounts of cytoplasmic organelles (granulated endoplasmic reticulum, Golgi apparatus, etc). In normal animals (columns 8 and 9 in Table I), the 6.1% perikaryal synapses do not include any synapses on giant cells; they were all found on medium-sized cell bodies, some of which had been identified as striatonigral efferent neurons by means of retrograde HRP transport.

Only one axon initial segment was found postsynaptic to TH-immunoreactive fibers in the transplanted animals. It could not be followed back to the parent cell body; therefore, the type of cell to which this axon initial segment belonged could not be determined. In normal animals TH-immunoreactive contacts on axon initial segments were more frequent, but again, the origins of the postsynaptic axons could not be identified.

HRP-WGA injections into the graft

In the two injected animals the HRP-WGA injections were confined to the transplant, and no spread of HRP was found in the surrounding cortex or in the striatum below (Fig. 7). The injections were located in the central portions of the grafts (where many TH-positive neurons occurred) and covered approximately one-fourth of the entire graft volume. A peculiar finding was that, within the graft, but outside the area of passive diffusion of the injection, many of the non-TH-immunoreactive cells contained a large amount of the characteristic transported HRP granules, whereas TH-positive perikarya only very rarely showed this kind of labeling (Fig. 7B). The granules of transported HRP reaction end product could be recognized in the immunostained perikarya as well, since they were usually much darker than the diffuse immunostaining and had sharp contours. The entire ipsilateral striatum (Fig. 7C), globus pallidus, and the mesencephalic raphe nuclei—which are known to project to the SN normally—were carefully searched for labeled neurons, but no signs of retrograde HRP labeling could be found in these areas.

Discussion

Although the mode of termination of catecholamine axons in various parts of the CNS remains partly controversial, there is an emerging consensus that the DA axons of the nigrostriatal pathway establish morphologically recognizable synaptic contacts with neuronal elements in the caudate-putamen (Hattori et al., 1973; Arluison et al., 1978a, b; Descarries et al., 1980, 1981; Groves, 1980; Pickel et al., 1981; Freund et al., 1984). Thus, some 10 to 20% of all synapses in the neostriatum have been reported to be dopaminergic (Arluison et al., 1978a; Pickel et al., 1981). In normal intact rats, Freund et al. (1984) have shown that the TH-immunoreactive boutons make predominantly symmetric synaptic contacts with dendrites or dendritic spines of striatal neurons, consistent with the findings of Descarries et al. (1980, 1981) and Pickel et al. (1981). As shown in columns 8 and 9 in Table I, about 56% of all TH-positive synapses were on dendritic spines and about 36% were on dendritic shafts. Only a few percent appear to contact cell bodies directly (all of

which appear to be medium sized) or to contact their axon initial segments. Combined experiments with HRP labeling from the SN have, in addition, provided evidence that the striatonigral projection neurons constitute one of the primary targets for the nigrostriatal DA projection. Moreover, as summarized in Figure 8A, the close association between TH-positive symmetric synapses on spine necks and TH-negative asymmetric synapses on spine heads suggests that the DA terminals interact with the corticostriatal afferent fibers on the same spines.

To what extent the nigrostriatal DA terminals also normally make synaptic contacts with non-projecting striatal neurons is unclear. There is abundant biochemical and pharmacological data to indicate that the cholinergic interneurons, which are identical to the aspiny giant cells in the striatum (Henderson, 1981; Woolf and Butcher, 1981; Eckenstein and Sofroniew, 1983; Levey et al., 1983; Bolam et al., 1984a, b), receive an inhibitory dopaminergic input (see Bartholini et al., 1975; Guyenet et al., 1975b; Lehmann and Langer, 1983, for reviews). Morphologically defined dopaminergic synapses so far have not been demonstrated on dendrites or perikarya of identified giant neurons. This is also suggested by our own unpublished results in normal animals, in which Golgi-stained dendrites and perikarya of identified giant cells were serially reconstructed in the electron microscope. The same sections were also immunostained for TH prior to Golgi impregnation. Although close associations between TH-immunoreactive fibers and giant cell perikarya or dendrites rarely occurred, no unequivocal synapses could be identified. Thus, it seems possible that such synapses normally are sparse, or even entirely absent, and that the action of DA onto the striatal cholinergic neurons may be mediated, at least in part, in a more "neurohumoral" manner across nonjunctional contacts, whereas the direct dopaminergic effect on the striatal efferent neurons would chiefly be mediated by conventional synapses (see Lehmann and Langer, 1983, for discussion).

Afferent connections to the graft. In normal animals it is well known that the function of the nigrostriatal DA neurons is controlled by reciprocal striatonigral connections. Therefore, one may expect that at least some afferent connections from the host brain to the grafted nigral neurons should be re-established in order to obtain behavioral recovery. However, no such connections could be identified by means of HRP-WGA injections into the graft in the present study. Nevertheless, it should be pointed out that the labeling of neurons by retrograde axonal transport depends on the percentage of its total axonal network which is exposed to the injected tracer. Therefore, some neurons, for example, from the host striatum, may well project to the transplant while still having most of their axonal ramifications in the host SN and within the striatum (as local collaterals). Thus, with this kind of experiment, a weak projection from the host to the transplant cannot be excluded. Perhaps anterograde tracing procedures would be more conclusive in this regard. Inside the transplant (at any distance from the injection site), many of the TH-negative perikarya were strongly labeled, which suggests that they had extensive local axonal arborizations in the graft. By contrast, very few TH-positive cell bodies were labeled, and they were restricted to the area close to the injection site. This suggests that the DA neurons in the graft had most of their axons projecting outside the graft, above all in the host striatum.

Previous biochemical studies of DA turnover in nigra-transplanted animals (Schmidt et al., 1982, 1983) have suggested the presence of mechanisms regulating the activity of the grafted DA neurons.

Figure 5. Electron micrographs of sections from animal 1 in Table I, showing synaptic contacts of TH-immunoreactive fibers with different postsynaptic elements. A and B, TH-positive terminals are in symmetric synaptic contact (*large arrows*) with spiny dendritic shafts (*ds*). The origins of spines are labeled by *small arrows*. C and D, Two dendritic spines (*s*) are contacted by TH-positive terminals on the neck. The synapses (*large arrows*) are again of symmetrical *en passant* type. Both spines received an asymmetrical synapse as well from unstained boutons (*star* in D; not seen in C, but found during serial reconstruction). The origin of the postsynaptic spine neck (*s*) from a dendritic shaft (*ds*) is indicated by a *small arrow* in C. E, A TH-immunoreactive fiber is in symmetric synaptic contact (*arrows*) with a dendritic shaft (*ds*) and a spine (*s*), which also receives an asymmetrical synapse from an unstained bouton with small round vesicles (*star*). Scales, 0.2 μ m.

Figure 5

Figure 6. Electron micrographs of sections from animal 2 in Table I, showing the different types of postsynaptic elements of the ingrowing TH-positive fibers from the nigral grafts. A, Two spine necks receive symmetrical synaptic contacts (arrows) from a single large TH-immunoreactive varicosity. B and C, TH-positive terminals are in symmetrical synaptic contact (arrows) with dendritic shafts (ds), which have been identified as spiny (in the case of B) and spine free (in the case of C) during serial sectioning. D, The only axon initial segment (IS) which was found to be in synaptic contact (large arrow) with a TH-immunoreactive bouton in this study. Another symmetric synapse (small arrow) between an unstained terminal and a spine-like appendage of the axon initial segment is also indicated. Scales, 0.2 μ m.

Figure 7. *A*, Low power light micrograph illustrating the center of the HRP-WGA injection site (asterisk) in the nigral transplant (NT). The graft, which contains many TH-immunoreactive neurons (arrows), is situated on the dorsal surface of the striatum (ST); the border between them is marked by a dashed line. CC, corpus callosum. *B*, TH-immunoreactive cells (small arrows) and neurons retrogradely labeled by HRP but not immunoreactive for TH (large arrows) are shown within the graft at a distance from the HRP injection site. *C*, Light micrograph of the dorsal part of the reinnervated neostriatum, close to the graft. None of the striatal cells (large arrows) were found to be labeled by retrograde HRP transport at any distance from the graft. TH-immunoreactive fibers form a dense network in the dorsal third of the striatum, but only thicker fibers (small arrows) are clearly visible at this low magnification. Scales: *A*, 200 μ m; *B* and *C*, 20 μ m.

The apparent absence of any major reciprocal afferent connections to the nigral grafts indicates that such regulations should either take place locally within the reinnervated striatal circuitry, e.g., by presynaptic interactions, or through autoregulatory mechanisms within the graft.

Efferent synaptic connections of the grafted DA neurons. The present results show that the ingrowing TH-positive axons from the nigral grafts are capable of forming abundant mature and morphologically defined synapses with target neurons in the previously denervated host caudate-putamen. This lends further support to the view that the functional effects of intrastriatal nigral grafts are mediated via the connections established by their reinnervating efferents, and that the specificity of the behavioral recovery, previously seen with nigral grafts in different forebrain locations (Dunnett et al., 1981c,

1983; Björklund et al., 1983), may reflect the nature and function of those host target neurons which become innervated by the grafted DA neurons.

The principal features of the dopaminergic graft-host connections, as interpreted from the present observations, are summarized in Figure 8*B* and are compared with the main features of the normal nigrostriatal DA connections in the striatum in Figure 8*A*, as derived from the study of Freund et al. (1984). Two principal targets of the ingrowing TH-positive axons from the graft were identified: dendrites of spiny neurons and cell bodies of giant neurons. Most probably the neurons in the first category include the striatal projection neurons (which constitute most if not all of the spiny neurons in the striatum) (Somogyi and Smith 1979; Bolam et al., 1981; Somogyi et al. 1981), whereas most, or perhaps even all, giant neurons are

Figure 8. Summary diagram of the proposed synaptic arrangements in the striatum of normal and transplanted animals. **A**, Normal animals. The striatonigral neurons receive their excitatory input from the cortex and/or thalamus, the boutons of which terminate mainly on the head of the spines of the projection neurons. This excitatory input is inhibited by the dopaminergic projection coming from the SN-VTA. These boutons mainly contact the spine necks of the projection neurons. The cortical excitation is facilitated by the local striatal cholinergic interneurons (i.e., the giant cells), which terminate on dendritic shafts of the striatonigral neurons. Thus, dopaminergic and cholinergic inputs have opposing effects on the activity of striatonigral neurons. The nigrostriatal dopaminergic fibers may also contact the distal dendrites of giant cells (*dashed line*), but such connections have so far not been demonstrated. **B**, 6-OHDA-lesioned animals with nigral transplants. After 6-OHDA lesion, the striatonigral neurons lost their original dopaminergic inhibitory control, but their cortical thalamic and cholinergic inputs are assumed to be morphologically largely unchanged. The most striking feature of the ingrowing dopaminergic fibers of the nigral transplant is that they completely surround the perikarya of each giant cell of the reinnervated area. Besides this abnormal connection, they establish synaptic contacts on dendritic shafts and spines of the medium spiny neurons as in normal animals. The latter innervation is suggested to be inhibitory on the striatal projection neurons, and this effect is amplified by the inhibition mediated by the synapses acting on the facilitatory cholinergic interneurons. This amplification may well be present in normal striatum as well, but it is achieved via presynaptic inhibition of the cholinergic terminals, probably by DA released at nonsynaptic axoaxonal contacts. (not indicated in A).

known to be cholinergic interneurons (see Bolam et al., 1984a, b; Eckenstein and Sofroniew, 1983; Graybiel and Ragsdale, 1983; Groves, 1983; Levey et al., 1983).

The ingrowing TH-positive axons established symmetric synaptic contacts predominantly on dendritic spines and shafts, similar to the predominant types of TH-positive synaptic contacts in normal rats. The synapses formed on dendritic spines resembled those seen in normal animals both because they made contacts with the spine necks and because they invariably were associated with an asymmetric TH-negative synapse contacting the spine head (Freund et al., 1984). Although the overall abundance of TH-immunoreactive synapses in the reinnervated portion of the host striatum appeared to be fairly similar to normal, the relative proportion of synapses in different location on the host striatal target cells was abnormal, as summarized in Table I. Thus, the proportion of synapses on dendrites was lower in the reinnervated grafted specimens, about 85 to 90%, than in normal animals where they constituted greater than 90% of all TH-positive synapses. The contacts on spines were less frequent and contacts on shafts were more frequent than normal. Conversely, TH-positive synapses on perikarya were twice as frequent in the grafted animals as in normals. The most striking abnormality in the graft-derived TH-positive innervation was the dense pericellular fiber arrangements seen around the giant cell (i.e., cholinergic) perikarya. The vast majority of, perhaps all, giant neurons within the reinnervated region of the host striatum had such TH-positive fiber "baskets" around them. These fiber baskets were seen only in association with

the giant cells; they have never been seen in the normal striatum, nor did they occur in the intact contralateral sides in the grafted animals. Ultrastructurally, the pericellular fibers had formed frequent synaptic contacts, indicating that the cholinergic giant interneurons had become hyperinnervated by the ingrowing TH axons from the nigral grafts.

Interestingly, whereas the nigrostriatal axons in mature animals rarely make somatic contacts (Descarries et al., 1980, 1981; Groves 1980; Pickel et al., 1981; Freund et al., 1984), Specht et al. (1981) have reported that such contacts constitute a large percentage of the few synapses that have been formed in 14- to 15-day-old embryos. The relatively higher percentage of somatic synapses established by the ingrowing TH axons from the graft may thus be interpreted as a sign of immaturity of the newly formed striatal DA innervation. However, in embryos, Specht et al. (1981) found that the dopaminergic fibers formed junctions primarily with perikarya, whereas in the transplanted animals the perikaryal synapses still represented only 11% of the total. The 50% increase seen in perikaryal synapses in the grafted rats was the result of synapses on giant cell bodies which do not occur in normal adult animals and have not so far been reported during development either. The percentage of contacts on medium-sized perikarya was not significantly different from normal. Thus, the specific innervation of giant cell perikarya from the nigral grafts most probably represents an abnormal rather than an immature type of connection.

Functional implications. The apparent perikaryal hyperinnervation

of the cholinergic giant cells has interesting implications for the interpretation of the cellular mechanisms underlying the functional and behavioral effects of the grafts. There is considerable pharmacological data indicating that cholinergic and dopaminergic mechanisms act antagonistically on striatum-related behaviors. Thus, cholinergic antagonists or agonists can potentiate or depress, respectively, the functional effects induced by DA receptor activation in the striatum (e.g., see Fuxe et al., 1970; Scheel-Kruger, 1970; Klawans et al., 1972; Pycocock et al., 1978; Fung and Schwarz, 1983). This is readily explained if one assumes, as in Figure 8A, that cholinergic and dopaminergic synapses act antagonistically on the striatal efferent neurons. Single-unit recordings have shown that the predominant action of iontophoretically applied acetylcholine is excitatory in the striatum (Bloom et al., 1965; Bevan et al., 1975; Bernardi et al., 1976; Woodruff et al., 1976), whereas the predominant action of DA probably is inhibitory (see Siggins, 1978; Herrling and Hull, 1980). Since DA is also inhibitory on the striatal cholinergic neurons (Agid et al., 1975; Bartholini et al., 1975; Guyenet et al., 1975a; Trabucchi et al., 1975; for review, see Guyenet et al., 1975b; Lehmann and Langer, 1983), it seems likely that the dopaminergic hyperinnervation from the graft would provide a powerful inhibition of the cholinergic interneurons in the reinnervated host striatum. One may speculate that this direct synaptic connection of the reinnervating DA axons onto the perikarya of the cholinergic neurons could replace the inhibitory presynaptic action of DA onto the cholinergic terminals, which is likely to be the most important mode of dopaminergic regulation of cholinergic transmission in the striatum of normal animals (for review, see Lehmann and Langer, 1983). Therefore, a direct inhibitory DA input to the parent cell bodies of the extensive cholinergic terminal system would seem to offer a powerful mechanism whereby the grafted DA neurons with a minimum of divergence could regain normal control over striatal cholinergic transmission. It is conceivable that such an inhibitory action on the striatal cholinergic neurons could by itself produce some functional recovery in the 6-OHDA-lesioned animals, but, above all, it may assist in the behavioral compensation by potentiating the functional effects of DA synapses terminating on the spiny striatal efferent neurons as illustrated in Figure 8B. Perhaps hyperinnervation of the striatal cholinergic neurons is a mechanism whereby a subnormal density of DA afferents could restore relatively normal function. If so, this mechanism could help to explain why restoration of only a small proportion of striatal DA innervation from the nigral transplants is sufficient to induce complete compensation of, e.g., the motor asymmetry induced by unilateral destruction of the nigrostriatal DA pathways (Björklund et al., 1980; Schmidt et al., 1982).

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