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Recovery of Chronic Parkinsonian Monkeys by Autotransplants of Carotid Body Cell Aggregates into Putamen

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Summary

We have studied the effect of unilateral autografts of carotid body cell aggregates into the putamen of MPTP-treated monkeys with chronic parkinsonism. Two to four weeks after transplantation, the monkeys initiated a progressive recovery of mobility with reduction of tremor and bradykinesia and restoration of fine motor abilities on the contralateral side. Apomorphine injections induced rotations toward the side of the transplant. Functional recovery was accompanied by the survival of tyrosine hydroxylase–positive (TH-positive) grafted glomus cells. A high density of TH-immunoreactive fibers was seen reinnervating broad regions of the ipsilateral putamen and caudate nucleus. The nongrafted, contralateral striatum remained deafferented. Intrastriatal autografting of carotid body tissue is a feasible technique with beneficial effects on parkinsonian monkeys; thus, this therapeutic approach could also be applied to treat patients with Parkinson's disease.

Introduction

Parkinson's disease (PD) results from the progressive degeneration of dopaminergic neurons in the substantia nigra innervating the striatum; hence, one of the therapeutic approaches to this disease has been the intrastriatal grafting of dopamine-secreting cells (recently reviewed by Hagan et al., 1997; Lang and Lozano, 1998a, 1998b; Rosenthal, 1998). In the past 2 decades, numerous transplantation studies have been done in animal models of PD as well as in Parkinson's patients using dopamine-rich neural and paraneural tissues. Autografts of adrenal and other paraneural cells have been almost abandoned because they appear to induce only transient recovery; however, grafting of fetal mesencephalic neurons has been shown to produce sustained motor improvement in parkinsonian animals and PD patients (see Björklund and Stenevi, 1985; Björklund et al., 1987; Sladek and Gash, 1988; Yurek and Sladek, 1990; Zigmond et al., 1990; Freed et al., 1992; Kordower et al., 1995; Nakao et al., 1995; and Schwarting and Huston, 1996, for reviews and references). More recent grafting procedures have incorporated new technologies, such as the use of porcine dopaminergic neurons or engineered dopamine-secreting cells (Senut et al., 1996; Deacon et al., 1997; see also Rosenthal, 1998). The clinical use of transplantation is, however, restricted by several limiting factors, such as the difficulty in obtaining human embryonic mesencephalic tissue, the need for immunosuppression, the possibility of intraspecies viral infections, and the ethical and legal issues raised by the use of fetal allografts or xenographs.

We have recently shown that intrastriatal transplants of carotid body cell aggregates produce rapid and long-lasting cellular and functional recovery of parkinsonian rats (Espejo et al., 1998). The carotid bodies contain glomus or type I cells, which derive from the neural crest and are among the cells with the highest dopamine content in the organism, and glial-like type II cells (see Fidone and González, 1986). Grafted glomus cells showed excellent survival in the brain parenchyma, where they appeared organized in clusters emitting neurites and retaining the ability to secrete dopamine. After some months, the presence of carotid body cells induced striatal dopaminergic reinnervation to a degree similar to that existing before destruction of the substantia nigra (Espejo et al., 1998; our unpublished data). The special durability of transplanted glomus cells could possibly be related to their sensitivity to hypoxia, a condition probably favored inside intracerebral grafts, which may act as a "trophic" factor inducing cell growth and excitability and dopamine synthesis and release (Fishman et al., 1985; López-Barneo et al., 1988; Czyzyk-Krzeska et al., 1992; Stea et al., 1992, 1995; Ureña et al., 1994; López-Barneo, 1996). Due to the optimal results of our initial study in rats, we suggested that transplantation of carotid body cell aggregates might be used to treat PD (Espejo et al., 1998; see also Rosenthal, 1998). Intrastriatal autografting of carotid body tissue offers numerous potential clinical advantages because surgical resection of carotid bodies is technically simple and has no significant side effects in humans (reviewed by Honda, 1992).

This study was designed to evaluate the possible beneficial effects of intrastriatal autotransplants of carotid body cell aggregates in an animal, like the monkey, closer to the human in brain organization and function. We used two monkeys rendered parkinsonian by systemic administration of the neurotoxin MPTP for 10 months. Transplants were performed several months after the last MPTP injection, once the animals had reached a severe and stable parkinsonism. So, the monkeys presented a chronic motor syndrome resembling

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Table 1. Behavioral Recovery of Parkinsonian Monkeys after Carotid Body Grafts into the Left Putamen

Monkey	MPTP (mg/kg)	Number of Doses/Months after Last MPTP Dose	Disability Score[a]		Fine Motor Task After Graft (s)[b]		Circling Behavior	
			Before Graft	After Graft	Right Hand	Left Hand	Before Graft	After Graft
CYN-1	5.25	16/3 months	20.4 ± 1.1	10.8 ± 0.85	5.3 ± 0.1 >80%[c]	10.1 ± 0.5 <10%[c]	(−)	(+)
CYN-2	4.95	13/7 months	21.8 ± 0.83	12.2 ± 0.84	ND[d]	ND[d]	(−)	(+)

[a] Average (mean ± SEM) of five evaluation sessions.
[b] Time spent performing the task (mean ± SEM of 15 complete successful trials).
[c] Percentage of successful trials.
[d] Not done.

the situation in PD patients. We here show that unilateral carotid body autotransplants induced a clear amelioration of the global parkinsonism of MPTP monkeys with marked improvement of the motor abilities on the contralateral side of the graft. Functional recovery was paralleled by survival of glomus cells in the striatum and ipsilateral reinnervation of large regions of the putamen and the caudate nucleus.

Results

Behavioral Recovery of Parkinsonian Monkeys

After chronic administration of MPTP, the monkeys developed a severe bilateral parkinsonism, which consisted of loss of spontaneous activity, bradykinesia, impairment of balance, postural tremor, and freezing (see supplemental movies at http://www.neuron.org/cgi/content/full/22/4/743/DC1) (Langston et al., 1984; Pérez-Otaño et al., 1991). In monkey CYN-1, which had received carotid body grafts into the left putamen, a marked decrease of parkinsonian features was observed on the side contralateral to the implant 2 weeks after grafting. Tremor was the first sign to ameliorate followed by a slow but significant improvement of the bradykinesia in the contralateral limbs. In addition, the severity of parkinsonism clearly improved as evidenced by increased spontaneous activity and better maintenance of posture and execution of movements. Maximal recovery was observed 2 months after grafting and remained stable during the survival time (5 months). Disability score was reduced by 50% (Table 1). The most obvious consequences of carotid body implants into the left putamen were the appearance of behavioral signs indicative of asymmetric striatal dopamine function. Before becoming parkinsonian, monkey CYN-1 had been trained to perform a fine motor task (see Experimental Procedures), which was executed by each hand at approximately the same speed (2.5 s total time to perform the task) and with a similar percentage of successful trials (>90%). MPTP treatment resulted in a marked bilateral increase in the number of failures and in the time spent performing the task. After implantation of carotid body cells into the left putamen, the ability to perform the task with the contralateral (right) hand was partially recovered. This was manifested by faster movements and, more importantly, by an increase in the number of successful trials (see Table 1). Another sign of striatal function asymmetry was the appearance of circling behavior to the left side after administration of apomorphine, indicating a decreased dopamine receptor supersensitivity in the transplanted striatum. Apomorphine-induced rotations were already observed in monkey CYN-1 2 weeks after grafting and were maintained throughout the survival period. This behavioral pattern was so pronounced that the animal was not able to move around the cage in any direction except to the left side, so it often remained still in order to avoid turning.

Monkey CYN-2 received carotid body grafts into the left putamen and one injection of Tyrode solution into the right side to test for possible nonspecific effects induced by the surgical procedure. In this animal, the beneficial effects of the grafts, observed after the first month, started more slowly than in monkey CYN-1, probably because it had a more severe parkinsonian syndrome (Table 1). Amelioration of parkinsonism was characterized by enhanced spontaneous activity, improved balance, and reduction of contralateral bradykinesia, which was evidenced by more skillful movements of the right hand such as the preference of the animal to use it when reaching for food. At the end of the experimental period (3 months after transplantation), the disability score of monkey CYN-2 was reduced by 40% (Table 1). The evolution of fine motor tasks could not be studied in monkey CYN-2 because it had not been trained before becoming parkinsonian. However, like in monkey CYN-1, injections of apomorphine elicited systematically, beginning the first month after the transplant, a circling behavior to the left side suggesting an asymmetrical striatal sensitivity to dopamine. Apomorphine injections induced in both monkeys abnormal movements (dyskinesias) consisting of rapid and stereotyped flexo-extensions of the knee, which can be attributed to the persistence of dopamine receptor supersensitivity in the denervated, nongrafted striatum (Boyce et al., 1990; Luquin et al., 1992).

Survival of Grafted Glomus Cells and Striatal Reinnervation

Histological studies were done in brain sections stained either with thionine, for precise cytoarchitectonic localization of the needle tracts, or using an anti-TH antibody, to specifically identify dopaminergic cells and fibers. Figure 1A (left and right) shows photographs at low magnification of brain coronal sections at the caudal level of the putamen stained with thionine. The images illustrate the symmetrical location of the carotid body graft, near the center of the putamen (left), and the scar produced by the needle tract and Tyrode solution injection

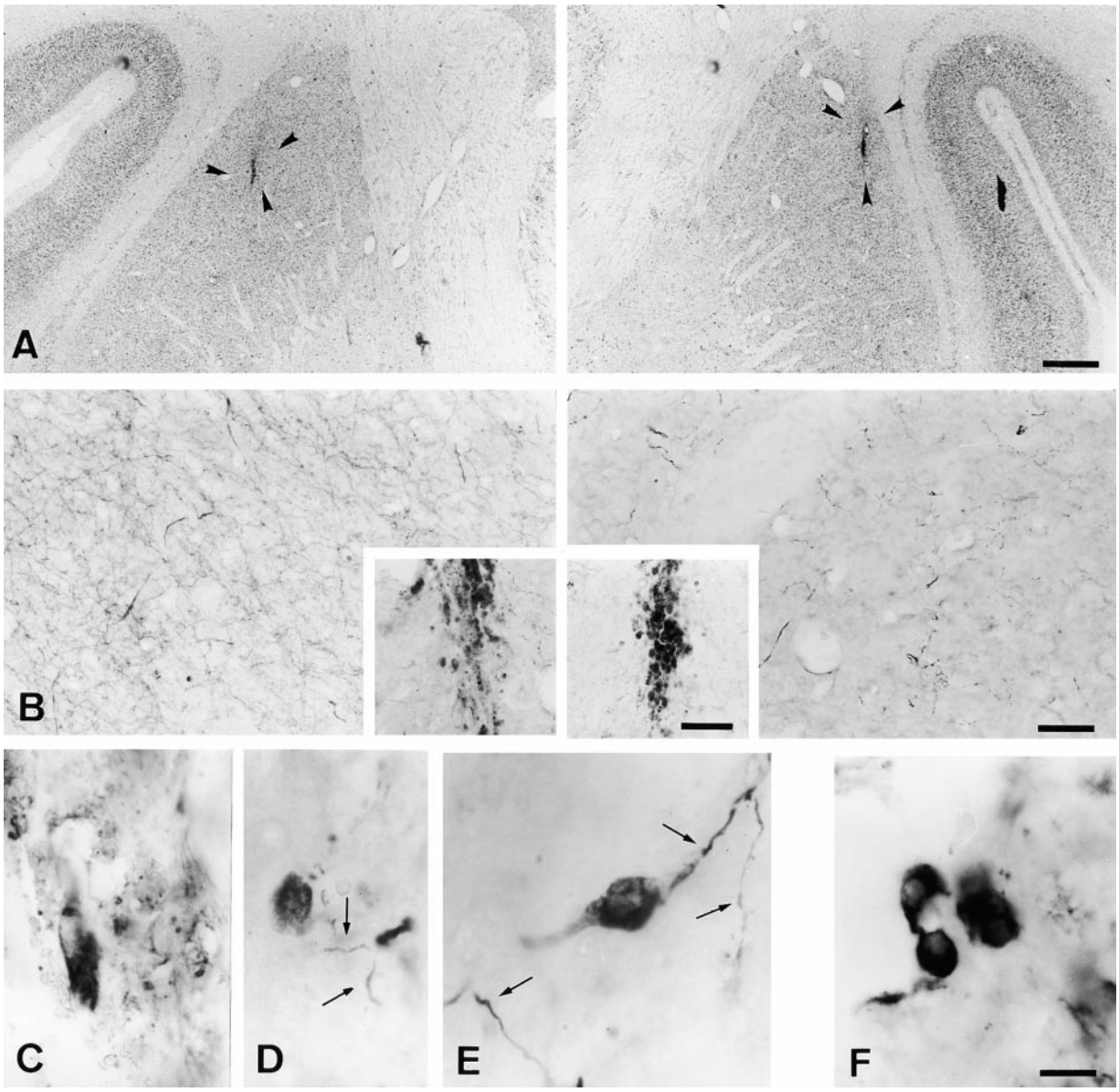

Figure 1. Carotid Body Grafts in Putamen and Morphological Features of TH-Positive Grafted Glomus Cells

(A) Brain coronal sections at the caudal level of the putamen of monkey CYN-2 stained with thionine. The carotid body graft in the left putamen (left) and the lesion produced by the needle and Tyrode solution injection into the right putamen (right) are indicated by arrowheads.

(B) Differential density of dopaminergic fibers on the left and right putamen in a region near the carotid body graft (left) or the Tyrode solution injection (right). The insets show enlargements of the areas of the carotid body graft (left) and needle scar (right) immunostained with an antiserum against TH.

(C–E) Representative isolated TH-positive glomus cells near a graft in the left putamen. Note the ovoid or bipolar shape of some cells and the emission of neurites (arrows) from the soma.

(F) Cluster of TH-positive glomus cells in a carotid body aggregate similar to those used for grafting.

Calibration bars: 1200 μm (A), 60 μm (B), 100 μm (insets), 12 μm (C–F).

in the right striatum (right). At the same level of the putamen, TH immunoreactivity was clearly apparent on the side of the graft, with the presence of numerous TH-positive fibers (Figure 1B, left), whereas, as is typical in MPTP-treated monkeys, the contralateral putamen was almost completely denervated (Figure 1B, right). The insets in Figure 1B show at higher magnification the appearance of the graft with a condensation of dark-colored TH-immunoreactive cells and fibers intermingled with macrophages (left) that were nonspecifically stained and appeared under the microscope with a characteristic lighter color. Only a macrophage infiltration was observed at the nongrafted putamen, which showed a complete absence of TH-positive fibers (right). Individual TH-positive glomus cells were difficult to visualize within the grafts, and the number was roughly estimated to be below 80–100 cells per injection. Due to the lack of a specific marker of type II cells, their number in the graft could not be estimated. This relatively small number of glomus cells observed was not unexpected,

since we detected in the carotid bodies resected from MPTP-treated monkeys an interstitial fibrosis and a low density of TH-immunoreactive glomus cells. Examples of individual TH-positive glomus cells located at the periphery of the graft with the characteristic ovoid or bipolar shape and emission of long neurites are shown in Figures 1C–1E. For a comparison, Figure 1F illustrates at the same magnification a group of glomus cells from a piece of the same carotid body used for the transplant.

Besides the long survival of carotid body cells and the appearance of TH-positive fibers near the grafts, a striking morphological feature of transplanted monkeys was the reinnervation of large regions of the ipsilateral striatum. Figure 2A shows at low magnification the marked contrast of TH-positive staining between the left and right putamen contained in the same coronal section of the brain. The areas included in the rectangles are shown enlarged in Figure 2B to illustrate the appearance of a dense striatal innervation by TH-positive fibers on the side ipsilateral to the grafts (left) and the sparse appearance of dopaminergic fibers on the right side of the brain. The difference in dopaminergic innervation between the left and right striata was apparent even at the level of the head of the caudate nucleus (Figures 2C and 2D), several millimeters rostral to the location of the carotid body implants.

Discussion

Our results indicate that unilateral autologous transplants of carotid body cell aggregates into the putamen ameliorate the severity of parkinsonism in chronically MPTP-treated monkeys. Carotid body resection and intrastriatal grafting of the tissue can be easily performed in the same operatory act and, as is already known of humans (see Honda, 1992), carotid body removal did not produce any appreciable alteration in the monkeys.

Amelioration of the parkinsonian symptoms started between 2 and 4 weeks after transplantation and progressed or was sustained throughout the 3–5 months of the study. Disability score was reduced in the two transplanted monkeys by 40%–50%; however, further amelioration of parkinsonism could have possibly been obtained with bilateral implants. Behavioral recovery was paralleled by the presence of TH-immunoreactive glomus cells in the graft and ipsilateral dopaminergic reinnervation of large regions of the putamen and the caudate nucleus. The behavioral and histological improvement of transplanted monkeys cannot be simply explained by recovery with time since in each monkey the nontransplanted side, which served as internal control, remained denervated. Tyrode injection into the right putamen of one of the monkeys also failed to induce reinnervation or recovery of function attributable to that hemisphere. Moreover, chronic MPTP monkeys with similar features as those used in this study are known to exhibit progressive and persistent loss of striatal dopamine and signs of neuronal degeneration several years after the MPTP treatment (for a recent work, see Brownell et al., 1998). The ipsilateral striatal reinnervation observed in transplanted monkeys rules out the remote possibility that what was seen in the rodent hemi-parkinsonian model (Espejo et al., 1998) was a carotid body transplant–mediated migration of dopaminergic neurons from the unlesioned hemisphere to the contralateral, 6-OHDA-lesioned side of the brain. As was described for carotid body implants in rats (Espejo et al., 1998), some glomus cells adopted ovoid or fusiform shape and grew long neurites. The number of TH-positive glomus cells that could be detected in the grafts was relatively small as compared with the number of glomus cells surviving in grafted rats (Espejo et al., 1998). In carotid bodies from MPTP monkeys, we observed a sparse distribution of TH-immunoreactive cells, so it is likely that a lower number of TH-positive cells was transplanted. Although the number of cases studied is obviously too small, it appears that chronic MPTP treatment damages a population of glomus cells or induces them to be TH negative. Nevertheless, glomus cells seem to be highly resistant to MPTP since, in spite of the high vascularization of the carotid body, a large proportion of cells maintained a TH-positive phenotype after chronic intravenous administration of the neurotoxin. In contrast, $<0.1\%$ of substantia nigra cells, probably receiving a smaller amount of MPTP, remained TH positive. Resistance to MPTP is possibly a characteristic of peripheral catecholaminergic neurons, since it is known that this neurotoxin does not significantly affect mouse adrenal catecholamine levels (Bohn et al., 1987). Increased resistance to MPTP could result from the expression of a lower density of dopamine transporter in carotid body cells as compared to substantia nigra neurons (our unpublished data).

Transplanted TH-positive glomus cells appeared intensely stained, indicating that they were metabolically active and capable of releasing dopamine in the graft and neighboring regions. In fact, dopamine release has been demonstrated to occur inside intrastriatal carotid body grafts in rats (Espejo et al., 1998). However, the broad reinnervation observed in distant regions of the putamen and caudate nucleus suggests that carotid body cell aggregates activated sprouting of spared dopaminergic cells or induced TH expression in other neurons (or differentiation of neural progenitors) after transplantation. Intrinsic reinnervation has been postulated to occur after adrenal (Freed et al., 1981; Bohn et al., 1987) and carotid body (Espejo et al., 1998) grafts in rats and is not related to brain injury or reactive gliosis since, as shown here, the level of TH-immunoreactive fibers was almost negligible on the sham-operated side of the brain. Thus, it seems that carotid body, and possibly other paraneural cells, may produce trophic factors that stimulate dopaminergic neurons to grow and sprout. The high concentration of neurotrophic substances in the carotid body (within either glomus cells, type II cells, or both; see below) and the long survival of grafted carotid body cell aggregates are factors that can explain why a few hundred or a thousand implanted cells are able to induce reinnervation of large regions of the striatum and significant functional recovery. As already suggested (Espejo et al., 1998), the durability of glomus cells in low oxygen tension, a condition likely strengthened within intracerebral grafts, may facilitate their adaptation and survival in the brain parenchyma. It has also been shown in embryonic and neonatal rats that the carotid body is one of the organs outside the CNS with the

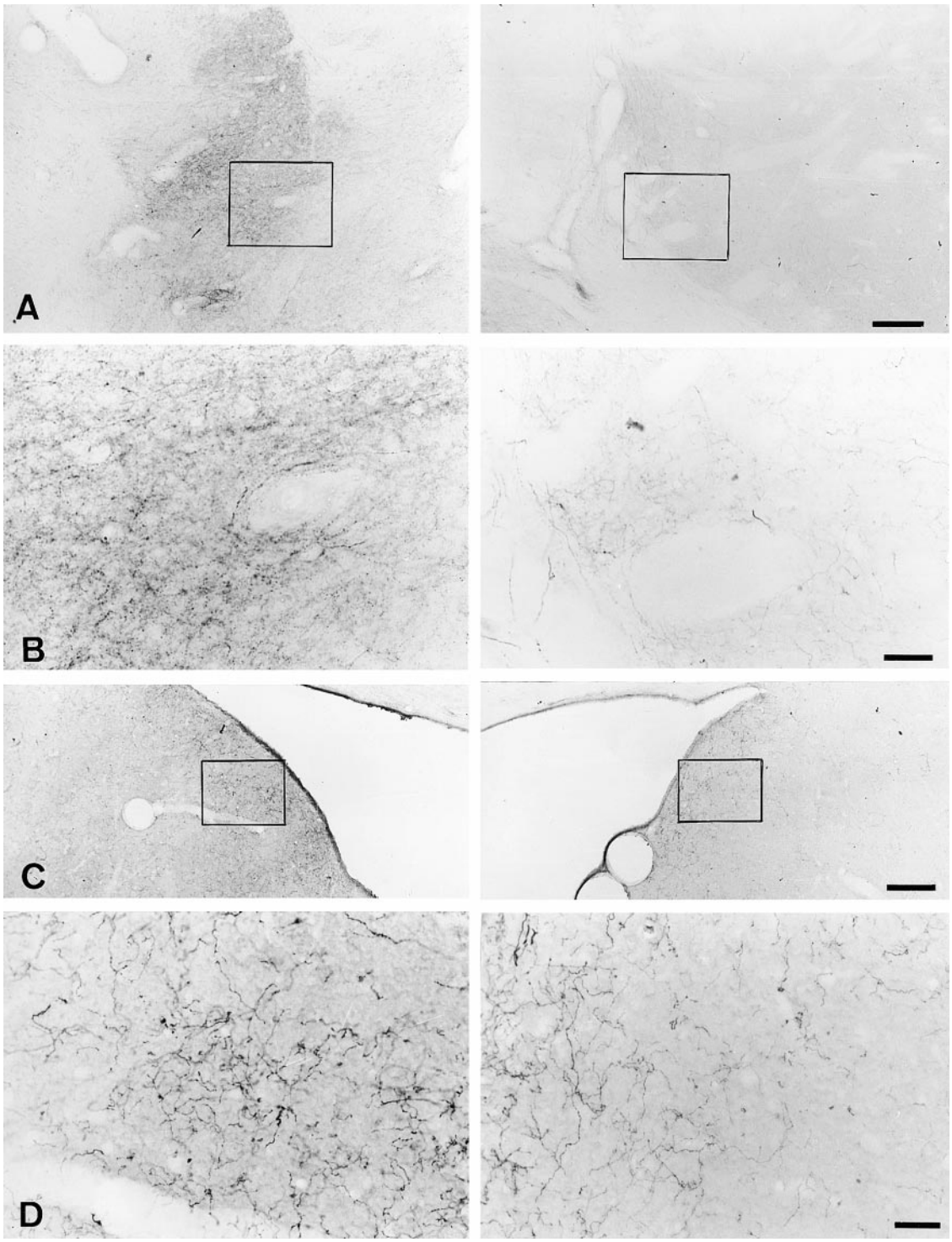

Figure 2. Ipsilateral Reinnervation of Putamen and Caudate Nucleus after Grafting Carotid Body Cell Aggregates into the Left Putamen

(A and B) Coronal sections at the level of the putamen processed for TH immunohistochemistry in monkey CYN-1 (A). Note the difference in staining between the left (grafted) and right sides of the brain. The areas in the rectangles are shown in (B) at higher magnification to illustrate the differential density of TH-positive fibers.

(C and D) TH immunohistochemistry of coronal sections at the level of the head of the caudate nucleus in monkey CYN-1 (C). The difference in fiber staining between the left (grafted) and right sides of the brain in the areas indicated by the rectangles can be appreciated at higher magnification in (D).

Calibration bars: 300 μm (A and C), 60 μm (B and D).

highest concentration of glial cell line–derived neurotrophic factor (GDNF) (Nosrat et al., 1996). GDNF and related molecules represent a family of growth factors belonging to the transforming growth factor β superfamily, which are extraordinarily potent (active at picomolar concentrations) and capable of promoting survival and differentiation of mesencephalic dopaminergic neurons in vivo and in vitro (Lin et al., 1993; Poulsen et al., 1994; Tomac et al., 1995; Gash et al., 1996; Horger et al., 1998). Interestingly, GDNF gene expression in the adult striatum is known to be upregulated by electrical excitation (Schmidt-Kastner et al., 1994), a condition favored by acute and chronic hypoxia in glomus cells (López-Barneo et al., 1988; Stea et al., 1992, 1995; Ureña et al., 1994). Intrastriatal injection of GDNF can elicit dopaminergic reinnervation in parkinsonian rats (Rosenblad et al., 1998), and a high single dose of GDNF in chronic parkinsonian monkeys also induces functional activation of nigral dopaminergic neurons and increased dopamine levels in the globus pallidus (Gash et al., 1996). GDNF injection alone, however, does not produce any significant increase of dopamine levels in the monkey caudate nucleus and putamen (Gash et al., 1996), which contrasts with the pattern of dopaminergic reinnervation observed in the present study after carotid body grafts. Therefore, it is possible that, besides GDNF, carotid body cells can also express other factors (see Kotzbauer et al., 1996; Baloh et al., 1998; Milbrandt et al., 1998) that promote dopaminergic neurons.

In conclusion, unilateral intrastriatal autotransplants of carotid body cell aggregates can induce amelioration of chronic parkinsonism in MPTP monkeys and marked recovery of the motor abilities on the contralateral side of the implant. Functional recovery is paralleled by survival of glomus cells and marked dopaminergic reinnervation of the ipsilateral striatum, which might be due to the release of growth factors from carotid body cells. Further studies to characterize the presence and regulation of dopaminergic neuron–promoting trophic factors in the carotid body are essential. Finally, the present paper should lead to clinical investigations aimed at assessing the efficacy of carotid body autotransplants in PD patients.

Experimental Procedures

Animals

Two Cynomolgus monkeys (*Macaca fascicularis*), CYN-1 and CYN-2, were used in these experiments. Animals were housed in a room under standard conditions of humidity (50%), air exchange (16 l/min), and dark/light cycles (8 a.m. to 8 p.m.). They were fed fresh fruit and commercial pellets and had free access to water. Both animals were rendered parkinsonian by weekly intravenous injections of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (0.5–1 mg/kg, total MPTP dose 16.6 and 14.3 mg) for 10 consecutive months. Marked reduction of spontaneous activity, bilateral action tremor, and posture and balance impairment were the most prominent features observed after concluding MPTP exposure. To avoid the possibility that spontaneous recovery, which usually occurs after MPTP administration, could resemble the behavioral effect of carotid body grafting, animals were allowed to recover from the last MPTP dose for at least 3 months prior to transplantation (see Table 1). All experiments were carried out according to the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Surgery and Intracerebral Grafting

Unilateral carotid body resection and stereotaxic surgery were performed under general anesthesia with endotracheal intubation and continuous blood pressure and EKG monitoring. Surgery started by exposing the superficial neck muscles in the vicinity of the lower maxilla, identifying the submandibular gland, and opening the carotid sheath, usually found under the digastric muscle, which had to be sectioned in order to expose the carotid bifurcation. Removal of the carotid body was performed under microscopy by dissecting out of the carotid wall all tissue from the last portion of the common carotid artery to several millimeters above the origin of both internal and external carotid arteries. The neck incision was sutured in layers. After removal, the carotid body was processed following the same basic methodology described by Espejo et al. (1998). Briefly, the carotid body was cleaned of surrounding adipose and connective tissue and trimmed into pieces of ~0.2–0.3 mm in diameter. A small piece was fixed and processed for histological examination (see below). The tissue was incubated for 20 min in Ca^{2+}- and Mg^{2+}-free Tyrode solution with collagenase (1 mg/ml), trypsin (1 mg/ml), and DNase (0.5 mg/ml). Cell aggregates were washed and resuspended in 5 ml of normal Tyrode solution to remove the enzyme. During carotid body processing, the animal was placed in the stereotaxic frame and deep anesthesia was maintained with a mixture of ketamine (10 mg/kg) and midazolam (1 mg/kg). A hole was drilled in the skull at the level of the right frontal ventricle according to the atlas of Szabo and Cowan (1984), and a ventriculography was performed by injecting 0.4 ml of Omnigrass into the right ventricle. The intercommissural line (AC–PC line) was measured and the coordinates for the putamen nucleus were adjusted according to the atlas. In both animals, carotid body cell aggregates (three to five per tract) were injected into the left putamen in two sites along the rostrocaudal axis using a Hamilton microsyringe. In CYN-2, one additional injection of Tyrode solution (2 ml) was delivered into the right putamen at the caudal level. The putamen coordinates used were as follows: rostral putamen, AP +3.4 mm from the midpoint of the AC–PC line, ML 12 mm from the longitudinal sinus, and VD 15 mm below dura mater; and caudal putamen, AP midpoint of the AC–PC line, ML 12.4 mm lateral from the longitudinal sinus, and VD 15 mm below dura mater. Animals received antibiotics (ampicillin 250 mg/day, i.m.) prophylactically for 2 weeks and analgesia with nonsteroidal anti-inflammatory drugs (flunixin, 2.5 mg/kg).

Behavioral and Pharmacological Assessment

Motor deficits induced by MPTP were assessed according to a nonhuman primate disability rating scale, which scores independently from 0 (normal) to 3 (maximal disability) parkinsonian features such as tremor (intensity and duration), bradykinesia, posture, balance, feeding, and freezing and from 0 (normal) to 5 (maximal disability) the reduction in spontaneous activity, thus giving a total maximal score of 25 (Herrero et al., 1993). Monkey CYN-1 had been trained before becoming parkinsonian to perform a fine motor task consisting in the capture of four peanuts with each of the two hands. Time spent in performing the motor task was measured every 2 weeks (see Table 1). In addition, apomorphine (0.1 mg/kg, i.m.) was also given every 2 weeks to test the appearance of circling behavior as a consequence of decreased dopamine receptor supersensitivity in the grafted striatum. After carotid body grafting, evaluations were undertaken every other week. The disability scores given in Table 1 are average values of five evaluations done during the last 2 weeks before transplantation (before graft) and sacrifice (after graft). All the evaluation sessions were recorded on standardized video tapes.

Histology

At the end of the experimental period (5 and 3 months after grafting for CYN-1 and CYN-2, respectively), animals were transcardially perfused with 4% paraformaldehyde in PB. The brains were postfixed overnight in the same fixative at 4°C and then immersed in 30% sucrose in PBS until brain dissection. Coronal sections, 30 μm thick, were cut on a freezing microtome and collected in PB. Tissue sections were incubated in PB with Triton X-100 (0.3%) and normal sheep serum for 30 min. Sections were incubated overnight in agitation with a monoclonal anti-TH antibody (1/1000, Boehringer-Mannheim). Subsequently, the sections were incubated in biotinylated anti-mouse antibody (1/200, Boehringer-Mannheim). The vector ABC procedure 1:100 (vectasin elite ABC kit, Vector Laboratories) was followed without blocking endogenous peroxidase. Staining for

peroxidase was performed in buffer acetate imidazole (0.125 M acetate and 0.010 imidazole [pH 7.2]) using 0.05% diaminobenzidine, 0.001% H_2O_2, and 0.1% $NiSO_4$ for 2–10 min. After washing in PB, sections were mounted in gelatin-coated slides, dehydrated, and coverslipped without counterstaining. Neural elements containing TH were visualized as blue-black. Nonspecifically stained macrophages typically appeared with a lighter color. A similar procedure as in the brain was applied to the piece of carotid body removed during animal surgery. Some sections of the brain were not used for TH immunocytochemistry but for staining with thionine to better localize the needle tract.

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