

Trophic Restoration of the Nigrostriatal Dopaminergic Pathway in Long-Term Carotid Body-Grafted Parkinsonian Rats

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We studied the mechanisms underlying long-term functional recovery of hemiparkinsonian rats grafted intrastrially with carotid body (CB) cell aggregates. Amelioration of their motor syndrome is a result of the trophic actions of these grafts on the remaining ipsilateral substantia nigra neurons rather than of the release of dopamine from the CB grafts. The grafts maintain a stable morphological appearance and differentiated cell phenotype for the duration of the life of the host. Adult CB expresses high levels of glial cell line-derived neurotrophic factor (GDNF) and the multicomponent GDNF receptor complex. These properties may contribute to the trophic actions of the CB transplants on nigrostriatal neurons and to their extraordinary longevity. We show that CB glomus cells, although highly dopaminergic, are protected from dopamine-mediated oxidative damage because of the absence of the high-affinity dopamine transporter. Thus, intrastriatal CB grafts are uniquely suited for long-term delivery of trophic factors capable of promoting restoration of the nigrostriatal pathway.

Key words: nigrostriatal dopaminergic neurons; carotid body grafts; long-term recovery; glial cell line-derived neurotrophic factor; dopamine transporter; Parkinson's disease

Introduction

Because Parkinson's disease (PD) results primarily from degeneration of nigrostriatal neurons, intrastriatal transplantation of dopamine-secreting cells has been used as a therapeutic approach for this disorder (Lang and Lozano, 1998a,b; Rosenthal, 1998; Dunnett and Bjorklund, 1999). Among the various dopaminergic tissues tested in animal models of PD and in Parkinson's patients, allografts (and occasionally xenografts) of fetal mesencephalic neurons are an efficacious therapy in humans (for review and references, see Bjorklund et al., 1987; Sladek and Gash, 1988; Freed et al., 1992, 2001; Kordower et al., 1995; Deacon et al., 1997). These pioneer transplantation studies have, however, set the limitations of cell replacement therapies in PD. Intrastriatal grafts of mesencephalic neurons increase dopamine concentration locally but have little restorative action on the nigrostriatal pathway. Moreover, the use of human fetal cells raises numerous ethical, legal, and practical issues (e.g., scarcity of human embryonic tissue), whereas the use of xenografts is hampered by the need for sustained immunosuppression. Intracerebral adminis-

tration of trophic factors for dopaminergic neurons (Gash et al., 1996; Choi-Lundberg et al., 1997; Rosenthal, 1999; Kordower et al., 2000; Akerud et al., 2001) and autotransplantation of dopaminergic carotid body (CB) cell aggregates (Espejo et al., 1998; Luquin et al., 1999; Mínguez et al., 2001) are two approaches that have been developed recently in animal models to complement fetal cell grafts.

The CBs contain neural crest-derived, highly dopaminergic glomus cells, which function as arterial oxygen sensors releasing dopamine in response to hypoxia (for review, see López-Barneo et al., 2001). Unlike many other organs, CBs were presumed to be particularly well suited for intracerebral transplantation because hypoxia, an environmental condition that is likely to be present inside the grafts, makes the CB grow (McGregor et al., 1984). Moreover, CBs offer potential clinical advantages because their unilateral surgical resection has no significant side effects (Honda, 1992) and therefore can be used for autografts. Intrastriatal transplants of CB cell aggregates are able to effect notable histological and functional recovery of parkinsonian rats (Espejo et al., 1998) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys (Luquin et al., 1999). In addition, a pilot study on PD patients subjected to unilateral resection of the CB and bilateral transplantation of the tissue into the putamen has proven the feasibility and safety of this therapy in humans (Mínguez et al., 2001).

Although results obtained so far with CB grafts are promising, the mechanisms of action and long-term viability of the implants remain unknown. Here, we show that amelioration of parkinsonian rats with CB transplants is a result of their trophic effect on the ipsilateral nigrostriatal neurons rather than the local release of dopamine. CB grafts have an extraordinary durability and can

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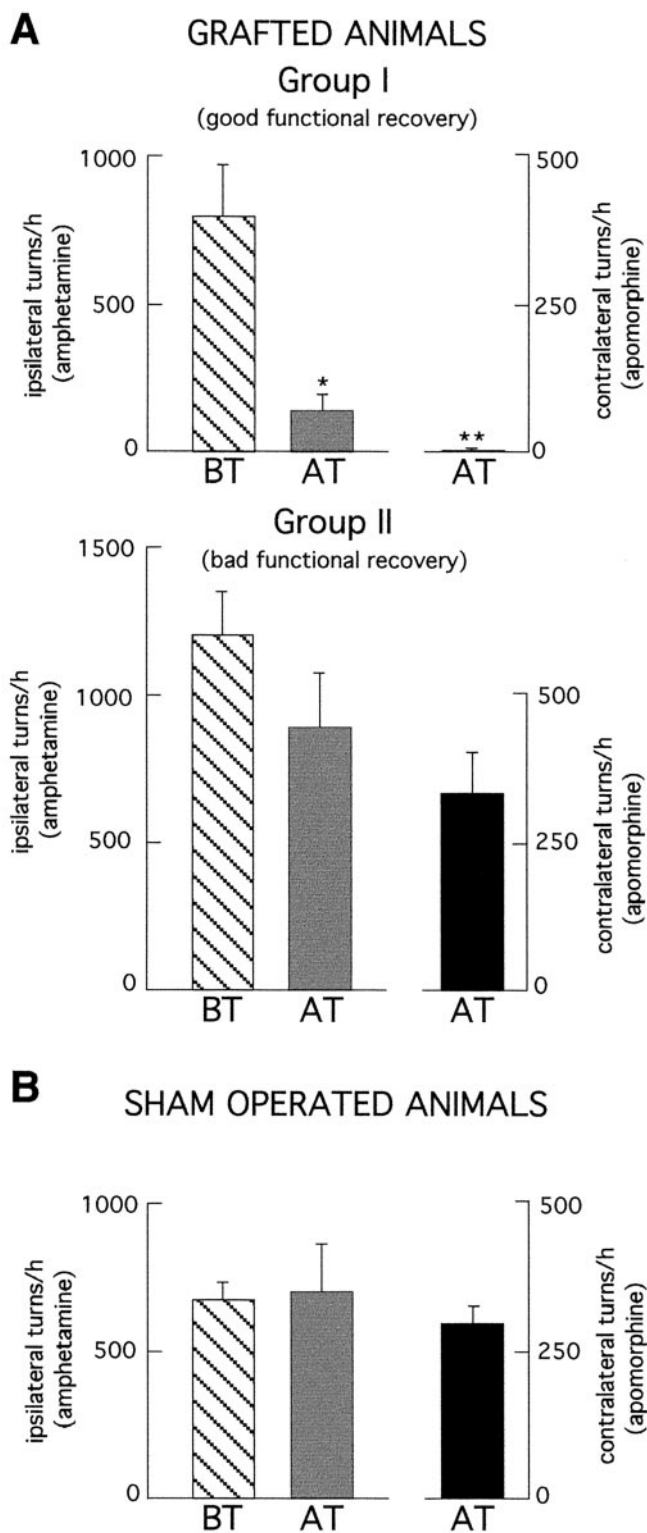


Figure 1. Long-term functional recovery of CB-grafted hemiparkinsonian rats. *A*, Evolution of the rotational behavior in CB-grafted animals measured before the transplantation (BT) or at the end of the period they were allowed to survive (between 5 and 15 months) (AT). Animals' behavior was evaluated by the amphetamine (left ordinate) and apomorphine (right ordinate) tests. Animals were classified in groups I and II depending on the behavioral and histological characteristics (see Results). Apomorphine tests were done only on animals that had some functional recovery in the amphetamine test to detect upregulation of dopamine receptors. Behavioral status is as follows (mean \pm SEM in turns per hour): group I, amphetamine test, BT = 787 ± 171 ($n = 6$) and AT = 138 ± 53 ($n = 6$); apomorphine test, AT = 3 ± 5 ($n = 5$); group II, amphetamine test, BT = 1206 ± 145 ($n = 6$) and AT = 872 ± 179 ($n = 6$); apomorphine test, AT = 328 ± 133 ($n = 3$). *B*, Same analysis as in *A* performed in the group of

last almost the entire life of the animal. We demonstrate that the adult CB expresses high levels of glial cell line-derived neurotrophic factor (GDNF), which may contribute to the longevity of the grafts and to their trophic effect on nigrostriatal neurons. We also show that CB glomus cells lack expression of the dopamine transporter (DAT) and are resistant to dopamine-mediated oxidative damage.

Materials and Methods

Unilateral 6-hydroxydopamine-induced substantia nigra lesion, CB grafting, and chronic MPTP-treated mice. Female Wistar rats (250–300 gm) were housed at regulated temperature ($22 \pm 1^\circ\text{C}$) in a 12 hr light/dark cycle, with access *ad libitum* to food and water. General procedures to produce unilateral 6-hydroxydopamine (6-OHDA) (Sigma, St. Louis, MO) substantia nigra (SN) lesions and transplantation surgery in rats were as described previously (Espejo et al., 1998; Toledo-Aral et al., 2002). Parkinsonian animals received transplants 10–15 d after SN lesion. For transplantation, carotid bodies were trimmed into pieces approximately one-third the size of the whole organ. A carotid body piece was placed on $1 \mu\text{l}$ of Tyrode's solution and injected into the striatum after stereotaxic coordinates in reference to bregma (in mm): anteroposterior (AP), +0.2; lateral (L), +3; ventral (V), -6. Sham-operated animals received only $1 \mu\text{l}$ of Tyrode's solution. Mice of the Swiss and C57BL/6 strains received subcutaneous MPTP injections either daily (5–30 mg/kg) or weekly (20–40 mg/kg) during 1 week to 3 months. All experiments were performed according to the animal care guidelines of the European Communities Council (86/609/EEC).

Histological analyses. Rat and mouse brains and carotid bifurcations were removed and fixed overnight at 4°C with 4% paraformaldehyde in PBS. Slices 30–100 μm thick were cut with a Vibratome (Vibratome, St. Louis, MO). Histological analysis was as previously described (Nieto et al., 1996; Pardo et al., 2000; Echevarría et al., 2001). For immunohistochemical studies, we used a polyclonal anti-tyrosine hydroxylase (TH) antibody [1:1000; for rat, Chemicon (Temecula, CA); for mice, Pel-Freez (Rogers, AR)] and a secondary anti-rabbit antibody (1:200; Pierce, Rockport, IL). The level of reinnervation by TH-positive fibers in the CB-grafted striatum was estimated by measuring the percentage of reinnervated area compared with the intact contralateral side. To make this calculation, all of the coronal sections of the striatum stained with the TH antibody (10–14 sections per animal) were photographed and digitized. The areas of TH immunoreactivity in both the normal and the grafted striatum of each slice were calculated by computer using the appropriate tools of Canvas 6.0 (Deneba Systems, Miami, FL).

In situ hybridization against TH and DAT was done using digoxigenin UTP-labeled antisense riboprobes synthesized from two plasmids (pCR2.1-TH and pRc/CMV-DAT) (Martres et al., 1998) containing partial cDNA sequences for TH and DAT, respectively. After hybridization, the sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000; Roche Diagnostics, Mannheim, Germany).

Under chloral hydrate anesthesia (350 mg/kg, i.p.), some rats received Fluorogold (FG) (Fluorochrome, Denver, CO) injections 24 hr before the brain was removed. Two stereotaxic deposits of 0.1 μl of a 4% Fluorogold solution in saline were done (AP +1.3, L +2.5, V -5.5; and AP -0.9, L +3.5, V -5.5). Injections were made with a Stoelting (Wood Dale, IL) infusion pump at a rate of 0.05 $\mu\text{l}/\text{min}$. The cannula was left in place for 10 min after each injection before being slowly retracted. Heterozygous GDNF/lacZ mice 4 months old were used for the 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining (Sánchez et al.,

sham-operated animals that rotated <900 turns per hour. Behavioral status is as follows (mean \pm SEM in turns per hour): amphetamine test, BT = 671 ± 54 ($n = 8$) and AT = 702 ± 159 ($n = 8$); apomorphine test, AT = 298 ± 27 ($n = 3$). * $p < 0.02$, statistically significant with respect to BT in the same group (*t* test); ** $p < 0.02$, statistically significant with respect to the same test in group II and in *B* (*t* test).

1996). For colocalization studies, TH immunohistochemistry was performed after the X-gal staining.

Behavior and pharmacological assessment. Hemiparkinsonian rats were randomly allocated into two groups: grafted ($n = 16$) and sham operated ($n = 16$). Within the first group, four animals were discarded later in our analysis because the graft was either dead or located outside the striatum. Amphetamine-induced (5 mg/kg, i.p.; Sigma) rotation was evaluated 8 d after unilateral nigra lesion and 10 d after transplantation. Apomorphine (0.5 mg/kg, s.c.; Sigma) tests were done only on animals that had some functional recovery in the amphetamine test to detect upregulation of dopamine receptors. Rats were tested monthly for rotational behavior induced by amphetamine and when necessary by apomorphine (Schwartz and Huston, 1996). Amphetamine and apomorphine tests were alternated every 2 weeks. Rotations were measured in an automated rotometer (Letica, Barcelona, Spain) after the injection. The parameter used for analysis was the number of rotations during the hour of maximum effect of the drug. Values are given as mean \pm SEM. The statistical significance of differences among parameters was considered at a value of $p \leq 0.05$ (Student's *t* test).

Molecular biology. To perform reverse transcription (RT)-PCR, poly(A⁺) RNA was extracted from either slices or intact carotid bodies using the Dynabeads mRNA Direct micro kit (Dyna, Oslo, Norway) as indicated by the manufacturer. Between 10 and 20 whole carotid bodies or the equivalent amount of tissue from the superior cervical ganglion (SCG) or the SN were used (Pardal et al., 2000; Echevarria et al., 2001). The reverse transcription reaction was performed immediately after the mRNA isolation using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA). Standard PCR was done with 1–5 μ l of first-strand cDNA. The primers used to amplify the different genes were as follows: for TH, sense, GGACATTGGACTTGCATCTCTGGG and antisense, TGAGAAGCAGTGTGGGAGGATGG; for GDNF, sense, TGAAGTTATGGGATGTCGTGGCTG, antisense A, AAGTGTATTGCAGTTAAGACGC (band of 372 bp), and antisense B, ATCCACACCGTTTAGCGGAATGC (band of 627 bp); for GDNF family receptor $\alpha 1$ (GFR $\alpha 1$), sense, ATGTTCCTAGCCACTCTGTACTTCG and antisense, GGTTGCAGACCTCGTTGGACATGC; for c-ret, sense, GCGCCCCGAGTGTGAGGAATGTGG, and antisense, GCTGATGCAATGGGCGGCTGTGC (as by Moreau et al., 1998); and for DAT, sense, TATAGAGACGCAATCATCACCAC and antisense, AGCAGGAAAAGTAGCCAGGACAAT. In every case, the band of the expected size was gel purified and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), and its identity was finally confirmed by sequencing using the dideoxynucleotide chain termination method with the Sequenase 2.0 kit (United States Biochemicals, Cleveland, OH). For TH, the partial sequence of 599 bp amplified by PCR and cloned into the pCR2.1 vector was the one used as a template to produce the antisense riboprobe after linearization of the pCR2.1-TH.

Results

Functional recovery of CB-grafted animals depends on nigrostriatal reinnervation

To perform this study, we prepared hemiparkinsonian rats with extensive lesions of the left SN (991 ± 64 ipsilateral turns per hour in amphetamine test; $n = 32$). Animals were left to recover between 5 and 15 months after either CB transplantation ($n = 16$) or sham surgery ($n = 16$) to study the long-term evolution of the grafts. We found healthy grafts in >80% of the animals that received transplants, even if they were left to recover for >7 ($n = 4$) or 11 ($n = 2$) months. The percentage of graft survival is similar to that observed in animals killed within 3 months after the surgery (our unpublished data), suggesting that the grafts of CB tissue that overcome the transplantation trauma can maintain a stable phenotype for almost the entire life of the animal.

Functional recovery of the whole group of CB-transplanted animals, estimated with the amphetamine test, was statistically significant (in turns per hour, 1000 ± 124 before transplantation and 505 ± 142 after transplantation; $n = 12$; $p < 0.02$). In contrast, the surgery had no significant behavioral effect on sham-

operated animals (in turns per hour, 999 ± 76 before transplantation and 737 ± 119 after transplantation; $n = 16$; $p = 0.2$). On the basis of the behavioral (apomorphine test) and histological characteristics of the CB-grafted animals (see below), we classified them in two clearly distinct groups. Animals in group I had an average $\sim 85\%$ stable recovery in the amphetamine test and did not rotate after administration of apomorphine ($n = 6$) (Fig. 1A), indicating a truly good functional recovery after transplantation with lack of dopamine supersensitivity. The analysis of the time course of recovery indicated significant functional improvement at 1 month (55%), which increased at 3 months (72%) and was almost complete (81%) at 5 months. Later, functional recovery remained stable or with slight additional improvement. Animals in group II manifested an asymmetrical sensitivity to apo-

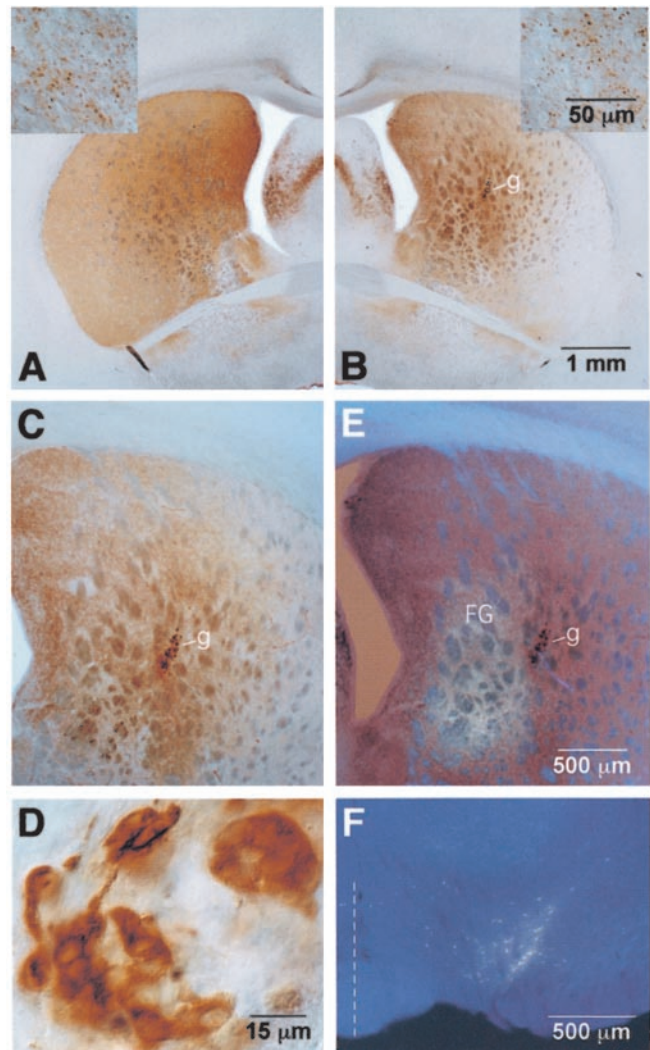


Figure 2. Long-term striatal reinnervation and survival of substantia nigra neurons in carotid body-grafted rats. *A–D*, Striatal dopaminergic reinnervation in a representative animal of group I killed 5.5 months after carotid body transplantation (*AT*). *BT*, Before transplantation. Normal (*A*) and reinnervated (*B*) striata are shown for comparison. *Insets* at high magnification show the appearance of TH-positive striatal fibers. The region with the carotid body graft (*g*) is shown in *C* and *D* at higher magnifications. Note the numerous typical glomus cells in the graft arranged in glomeruli. *E*, Localization of FG injection in the striatum near the graft. *F*, Retrograde labeling of numerous cells in the ipsilateral substantia nigra. Midline is indicated by the dotted line. *A–E*, Transmitted light and immunostaining with antibody against TH. *E, F*, UV illumination. Behavioral parameters of this animal (turns per hour) were as follows: amphetamine test, BT = 1512 and AT = 111; apomorphine test, AT = -9. Striatal reinnervation, 70.7%.

morphine and had a poor recovery in the amphetamine test ($n = 6$) (Fig. 1A), indicating a striatal denervation with a compensatory upregulation of dopamine receptors. Differences in the number of turns per hour before transplantation between animals distributed later on in groups I (787 ± 171) and II (1206 ± 145) were not statistically significant. We followed the parallel evolution of a group of sham-operated hemiparkinsonian animals with a mean value of turns (671 ± 54 turns per hour) comparable with that of group I animals. On average, these sham-operated animals did not recover in the amphetamine test, and those with some apparent amelioration manifested a clear motor asymmetry in response to apomorphine (Fig. 1B). Thus, these data indicated that long-term functional recovery of hemiparkinsonian rats required the presence of CB transplants in the striatum.

Differences in the level of functional recovery described above were correlated with distinct degrees of histological restoration (striatal reinnervation, $69.5 \pm 3.7\%$, $n = 6$ for group I; $4.9 \pm 2.2\%$, $n = 6$ for group 2). Figure 2A–D illustrates the appearance of the striata of a rat representative of group I after TH immunostaining. Comparison of the unlesioned (A) and transplanted (B) sides shows an important reinnervation that affects primarily the central and medial regions of the striatum. TH-positive fibers were particularly dense in the region surrounding the graft, which appeared as a dense mass of intensely stained TH-positive glomus cells (Fig. 2C) organized in clusters similar to the glomeruli of the carotid body (Fig. 2D). The origin of the dopaminergic fibers was studied by injection of FG, a fluorescent retrograde tracer, in the striatum near the center of the reinnervated area (Fig. 2E). Fluorescent cells were observed in the ipsilateral SN, primarily in the medial region of the pars compacta, and in the lateral part of the ventral tegmental area (VTA) (Fig. 2F). With the exception of a few cells encountered in the contralateral SN, TH-positive cells filled with FG were not appreciable in the CB graft or anywhere else in the brain. This distribution of FG-positive cells was repeated in the four group I animals studied. Intra-striatal FG injection in four sham-operated animals showed almost complete absence of fluorescent cells in the SN (data not shown). These results show that functional recovery of CB-grafted animals is correlated with striatal reinnervation by fibers originating in ipsilateral nigrostriatal neurons.

The histological appearance of the nigrostriatal system in animals with poor functional recovery (group II) is illustrated in Figure 3. The striatum ipsilateral to the lesion was almost completely denervated (Fig. 3, compare A, B) despite the presence of a large graft, well placed in the center of the striatum (Fig. 3B, C) with numerous intensely stained glomus cells (Fig. 3D, E). Striatal denervation was paralleled by an extensive destruction of ipsilateral mesencephalic dopaminergic neurons, with an almost absolute absence of TH-positive neurons in the SN and a marked reduction of staining in the adjacent VTA (Fig. 3F–I). These

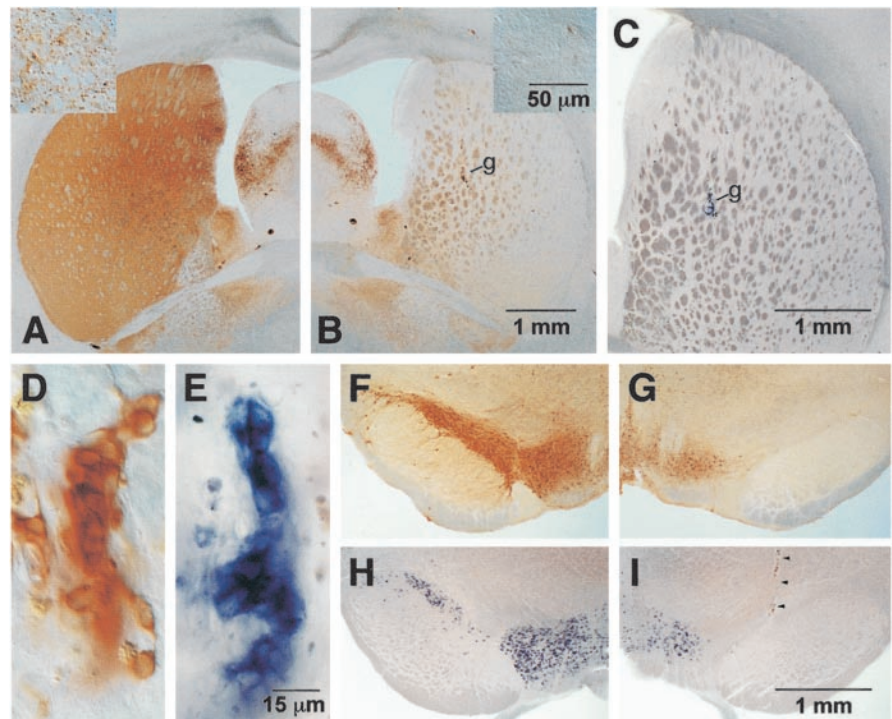


Figure 3. Lack of striatal reinnervation in carotid body-grafted animals with complete destruction of substantia nigra neurons. Comparison of the normal (A) and denervated (B) striata in a representative animal of group II killed 5 months after carotid body transplantation (AT). Insets at high magnification show the appearance of TH-positive striatal fibers. Localization of the graft (g) is shown in C and in D and E at higher magnification. Note the large size of the graft with abundant glomus cells. Appearance of the normal (F, H) and lesioned (G, I) substantia nigra in the same animal. Note the complete absence of dopaminergic cells in the lesioned substantia nigra and the marked reduction of stained cells in the ventral tegmental area. Arrowheads in I indicate the track of the needle used for 6-OHDA injection. Immunostaining with antibody against TH (A, B, D, F, G) and TH mRNA *in situ* hybridization (C, E, H, I). Behavioral parameters of this animal is as follows (turns per hour): amphetamine test, BT = 1293 and AT = 1473. Striatal reinnervation, 2.1%.

observations indicate that CB grafts by themselves are not sufficient to reverse parkinsonian symptoms in animals with complete nigra lesions. Together, the behavioral and histological analyses indicate that the beneficial effect of CB grafts on parkinsonian animals is predominantly a result of their trophic effects on the remaining SN neurons, which are induced to innervate the striatum.

GDNF is highly expressed in adult CB cells

We studied whether CB cells are the source of the potent dopaminotrophic factor GDNF and other members of the same family (Rosenthal, 1999) that are retrogradely transported to stimulate SN neurons. Using two different sets of primers derived from rat GDNF sequences, we amplified, by RT-PCR, cDNA fragments (of 372 and 627 bp) from carotid bodies and by sequencing confirmed them to be derived from rat GDNF (Lin et al., 1993). The oligonucleotides flanking the 627 bp fragment were used repeatedly to amplify GDNF in all of the different CB preparations tested ($n = 9$) (Fig. 4A). Several parallel attempts ($n = 5$) to detect GDNF from the neighboring large SCG have failed (Fig. 4A). The other GDNF-related factors studied (neurturin, artemin, and persephin) were not amplified by RT-PCR of CB tissue. However, the CB ($n = 4$) and the SCG ($n = 3$) expressed consistently the multicomponent receptor complex for GDNF (GFR α 1 and c-ret) (Rosenthal, 1999).

GDNF is expressed in limited amounts during specific developmental stages by target tissues, and its presence in embryonic rodent CB has been reported previously (Nosrat et al., 1996;

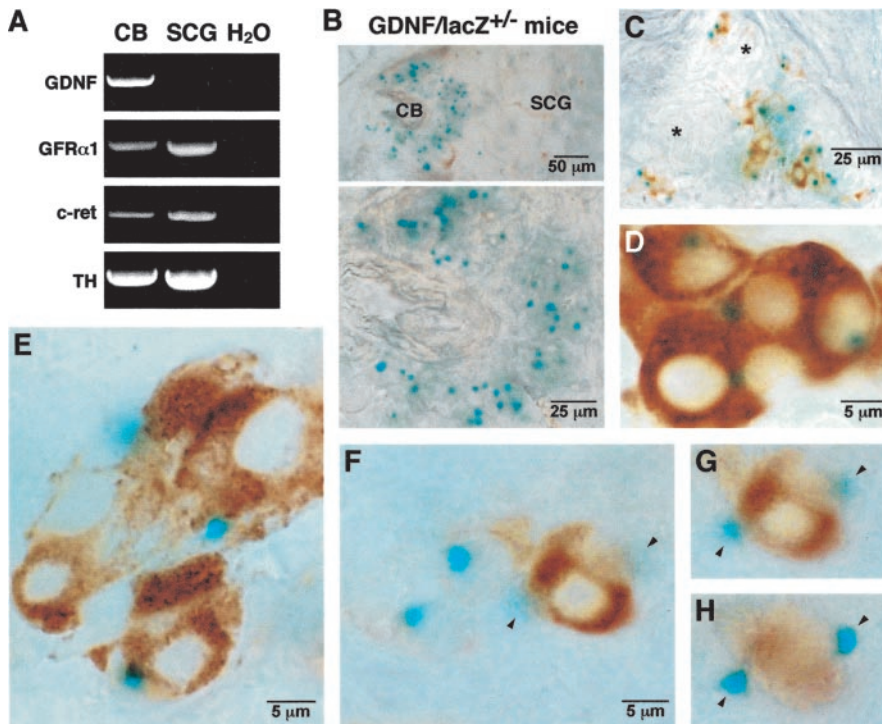


Figure 4. Identification and localization of GDNF and the GDNF multicomponent receptor complex in the carotid body. *A*, RT-PCR showing the existence of GDNF and the high-affinity GDNF receptor complex (GFR α 1 and c-ret) in the CB. Note the parallel experiment illustrating the absence of GDNF message in the SCG. TH is also amplified to test for mRNA stability. *B*, X-gal staining (blue) of histological sections at the level of the carotid artery bifurcation from heterozygous GDNF/lacZ knock-out mice at two magnifications illustrating the dense appearance of GDNF-positive cells in the CB and the complete absence of GDNF expression in the SCG. *C*, Colocalization of GDNF (blue)- and TH (brown)-positive cells in the same glomeruli. Note that TH immunostaining is absent from glomeruli without GDNF-positive cells (asterisks). *D*, Example at higher magnification of colocalization of TH and GDNF expression. *E–H*, Localization of GDNF-positive cells in close apposition with TH-positive cells. *F–H*, The focus was changed in steps of 4 μ m to illustrate how some GDNF-positive cells (indicated by arrowheads) are arranged juxtaposed on a glomus cell.

Erickson et al., 2001). The functional role of GDNF in the adult, however, is less well studied, because its normal low level of expression, a characteristic of trophic factors, hampers its unequivocal identification by standard histological and/or immunological techniques (*in situ* hybridization, immunostaining, or ELISA). To study precisely the cell localization and the pattern of GDNF expression in CB of adult animals, we used heterozygous knock-out GDNF/lacZ mice, in which the cells expressing GDNF can be labeled with the characteristic blue X-gal staining (Sánchez et al., 1996). These experiments revealed that GDNF is highly expressed in the adult CB but is totally absent in the SCG and other neighboring tissues (Fig. 4*B*). Simultaneous X-gal and TH immunostaining showed that GDNF-producing cells are colocalized with clusters of TH-positive glomus cells (Fig. 4*C,D*). The deposits of X-gal appear to be preferentially located in the periphery of TH-positive cells (Fig. 4*E,F*) and occasionally surrounding glomus cells (Fig. 4*F–H*). These data demonstrate that GDNF is highly and selectively expressed in CBs of adult rodents. The pattern of CB GDNF expression is consistent with its presence in glial-like subtentacular cells located in close apposition with glomus cells.

Differential expression of DAT and susceptibility to neurotoxins of SN neurons and CB glomus cells

The long-term functional recovery of transplanted rats was correlated with the survival of the CB tissue. Therefore, we studied whether, in addition to the local effects of trophic factors, such as

GDNF, the survival of CB grafts could also be facilitated by special features of dopamine metabolism in glomus cells that protect them from oxidative stress.

The high-affinity presynaptic dopamine reuptake system (DAT) plays a critical role in the susceptibility of SN neurons to degeneration (for review, see Jenner, 1998; Offen et al., 1999). Reuptake of dopamine results in the production of reactive oxygen species, which can induce neuronal death (Spina and Cohen 1989; Jenner, 1998; Offen et al., 1999). In addition, DAT is used for presynaptic transport of some molecules [e.g., MPP⁺ (methyl phenylpyridinium)] with toxic effects (Burns et al., 1983; Gainetdinov et al., 1997). Although glomus cells contain and release large amounts of dopamine (Pardal et al., 2000; López-Barneo et al., 2001), we hypothesized that they should have reduced DAT activity on the basis of our previous observations on parkinsonian monkeys, which had relatively well preserved carotid bodies despite their exposure to large doses of systemically administered MPTP (Luquin et al., 1999). Differential expression of DAT on SN and CB was initially studied by *in situ* hybridization of mesencephalic and CB slices. Figure 5*A* illustrates that, although DAT was abundantly expressed in SN neurons, it was not detected in the CB. Consecutive slices incubated with a TH probe show the high levels of expression of the enzyme in both SN and CB, demonstrating the stability of

the mRNA in the tissue. This experiment was repeated in SN and CB preparations from 12 different animals with identical results, suggesting that DAT is not present in CB cells. Additional support for this observation was obtained by high-sensitivity RT-PCR analysis performed on carotid body and mesencephalic slices, showing the complete absence of the DAT message in CB cells ($n = 3$ experiments) (Fig. 5*B*). To determine whether the absence of DAT expression has a functional consequence, we investigated whether glomus cells are protected from MPTP damage. We compared the effects of the neurotoxin on TH-positive mesencephalic and CB cells in two different MPTP-sensitive mouse strains. Whereas chronic systemic administration of MPTP resulted consistently in a marked destruction of substantia nigra and, to a lesser extent, VTA neurons, the CB glomus cells remained intact in all cases ($n = 7$) (Fig. 5*C*). These data show that CB cells, although highly dopaminergic, are protected from toxicity associated with the dopamine uptake system.

Discussion

In this article, we show that the beneficial effects of intrastriatal CB transplants in hemiparkinsonian rats are predominantly a result of their trophic action on intrinsic nigrostriatal neurons rather than their delivery of dopamine. Functional recovery, which was maintained for the entire life of the CB-grafted rats, is paralleled by the long-term survival of these CB grafts. We demonstrated that GDNF is highly expressed in the adult CB. In con-

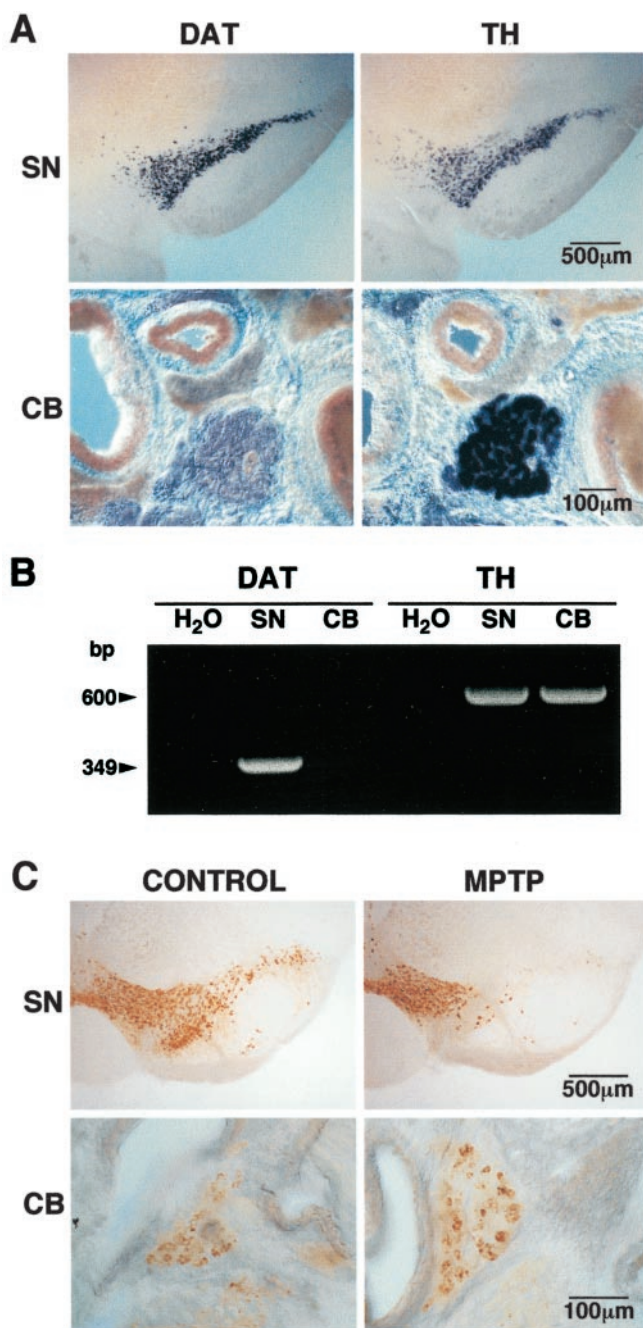


Figure 5. Differential expression of dopamine transporter in substantia nigra and carotid body cells. *A*, *In situ* hybridization in consecutive mesencephalic (SN) and CB slices with probes against DAT and TH mRNAs. DAT message was undetectable in CB slices. *B*, RT-PCR showing the lack of expression of DAT in the carotid body and comparison with SN. TH mRNA was amplified in parallel to demonstrate mRNA stability. *C*, TH immunohistochemistry of mesencephalic and carotid body slices from control and MPTP-treated (30 mg/kg daily for 55 d) C57BL/6 mice. Note that systemic administration of MPTP produced a marked destruction of dopaminergic SN neurons but left intact CB glomus cells.

trast, DAT, abundantly expressed in dopaminergic mesencephalic neurons, is absent from CB glomus cells, thus making them resistant to dopamine reuptake-derived toxicity. These properties of the glomus cells may contribute to the remarkable longevity of the CB grafts and to their trophic actions on nigrostriatal neurons.

For these experiments, we used hemiparkinsonian animals with more extensive SN lesions (average number of amphet-

mine-induced turns, ~990 turns per hour) and studied them for a longer time period (up to 15 months) than in our previous work (Espejo et al., 1998; Toledo-Aral et al., 2002). One-half of the CB-transplanted animals achieved an almost complete and stable recovery of their motor asymmetries. Postmortem histological analysis of these animals demonstrated that they had intrastriatal CB grafts with numerous TH-positive cells and a marked striatal dopaminergic reinnervation. In the remaining 50% of the animals with transplants, functional recovery was very poor. Although this last group of animals had well placed, large CB grafts with abundant metabolically active glomus cells, they had almost complete striatal denervation in parallel with extensive dopaminergic neuronal loss in the ipsilateral SN and adjacent VTA. These observations suggest that, to obtain a good functional recovery, CB-transplanted animals must retain a small percentage of dopaminergic SN neurons after the lesion such that the grafted CB cells are able to induce the remaining SN neurons to reinnervate the striatum. Intrinsic reinnervation has been postulated to occur after adrenal grafts (Bohn et al., 1987; Hansen et al., 1995; Gainetdinov et al., 1997) or in GDNF-injected rats (Rosenthal et al., 1998). The present study demonstrates directly that the striatal reinnervation induced by CB grafts is produced by fibers that originate almost exclusively in the ipsilateral SN and adjacent VTA. The lack of long-term recovery of any of our sham-operated animals indicates that striatal reinnervation does not result from a nonspecific reaction to brain damage or gliosis (but see Song and Haber, 2000).

The restorative activity of the CB grafts on nigrostriatal neurons could be attributable to the graft-mediated delivery of GDNF, which we showed to be highly expressed in adult CB tissue, and possibly other trophic factors. At picomolar concentrations, GDNF is capable of promoting survival and differentiation of mesencephalic dopaminergic neurons *in vivo* and *in vitro* (Lin et al., 1993; Tomac et al., 1995a; Gash et al., 1996). Moreover, GDNF delivered intrastrially is taken up and transported retrogradely to the soma of SN neurons (Tomac et al., 1995b). Interestingly, neurotrophin-3, another well known trophic factor (Fariñas et al., 1994), is also expressed in the CB (I. Fariñas, personal communication). Thus, CB grafts could offer stable and regulated pumps ideal for the safe delivery of the proper mixture of growth factors capable of promoting nigrostriatal sprout and reinnervation. We did not see obvious changes in the morphology of SN neurons in the side ipsilateral to the CB grafts compared with neurons in the contralateral intact side. However, elucidation of the biochemical and morphological modifications induced by the trophic action of CB grafts on SN neurons requires additional studies in a feasible and reproducible rodent model of chronic and diffuse bilateral parkinsonism, which needs to be developed.

A remarkable observation of our study is the extraordinary longevity of CB grafts, which appear to be active for almost the entire life of the animal. Glomus cells in the graft maintain a stable phenotype and the typical organization in glomeruli characteristic of the native CB. These properties may be attributable to the fact that we implanted CB cell aggregates rather than dispersed cells, suggesting that, after transplantation, the CB tissue has specific molecular and topological features that facilitate its adaptation to the environment in the brain parenchyma. We demonstrated that adult CB tissue expresses the GDNF multi-component receptor complex (GFR α 1 and c-ret) (Rosenthal, 1999). Therefore, it is plausible that GDNF released from subnucleolar cells has a trophic activity on glomus cells. In addition to the bases for the paracrine effects of GDNF, we show that CB

glomus cells, although highly dopaminergic, do not express DAT and are therefore not subjected to the oxidative stress inherent to dopamine metabolism, which is an important factor in the pathophysiology of PD (Spina and Cohen, 1989; Jenner, 1998; Offen et al., 1999; Conway et al., 2001). Systemic administration of MPTP in mice destroys most SN neurons but does not alter CB cells. These observations explain why glomus cells are less affected by MPTP than adrenal medulla or superior cervical ganglion cells (Ambrosio et al., 1990) and the relative preservation of carotid bodies in chronic MPTP monkeys (Luquin et al., 1999). The longevity of glomus cells could also be facilitated by their physiological role as oxygen sensors, because it is known that hypoxia, a deleterious condition in most tissues, makes the carotid body grow (McGregor et al., 1984; López-Barneo et al., 2001). Interestingly, hypoxia has also been shown to stimulate proliferation, survival, and differentiation of dopaminergic neural precursors (Morrison et al., 2000; Studer et al., 2000).

In conclusion, we show that intrastriatal CB transplants induce long-term cellular and functional recovery of hemiparkinsonian rats because of trophic restoration of the intrinsic nigrostriatal pathway. CB cells in adult rodents express high levels of GDNF but, although highly dopaminergic, are protected from dopamine-mediated oxidative damage because of the absence of the high-affinity dopamine transporter. These results should encourage studies designed to test the neuroprotective and restorative effects of CB autotransplants in PD patients.

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