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Cellular and Functional Recovery of Parkinsonian Rats after Intrastriatal Transplantation of Carotid Body Cell Aggregates

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Summary

We have tested the suitability of chromaffin-like carotid body glomus cells for dopamine cell replacement in Parkinsonian rats. Intrastriatal grafting of cell aggregates resulted in almost optimal abolishment of motor asymmetries and deficits of sensorimotor orientation. Recovery of transplanted animals was apparent 10 days after surgery and progressed throughout the 3 months of the study. The behavioral effects were correlated with the long survival of glomus cells in the host brain. In host tissue, glomus cells were organized into glomerulus-like structures and retained the ability to secrete dopamine. Several weeks after transplantation, dopaminergic fibers emerged from the graft, reinnervating the striatal gray matter. The special durability of grafted glomus cells in the conditions of brain parenchyma could be related to their sensitivity to hypoxia, which is known to induce cell growth, excitability, and dopamine synthesis. This work should stimulate research on the clinical applicability of carotid body autotransplants in Parkinson's disease.

Introduction

Parkinson's disease (PD) is characterized by selective degeneration of dopaminergic neurons in the substantia nigra projecting to the striatum. Intrastriatal grafting of dopamine-secreting cells from neural and paraneural tissues, such as fetal mesencephalon or adrenal medulla, has been reported to result in amelioration of the motor syndrome in Parkinson's patients and in animal models of PD (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; Schwarting and Huston, 1996). Although transplantation is considered a promising treatment for PD, its clinical use is still restricted to few cases. The major limiting factors of this therapy are difficulty in obtaining sufficient viable embryonic mesencephalic tissue and the controversial ethical and/or legal issues raised by the use of human fetal allografts. In addition, the use of adrenal autografts has been abandoned because it induces only transient recovery in both Parkinson's patients and animals. These limitations have hastened the development of new technologies, with their own advantages and restrictions, for intrastriatal dopamine delivery, such as the use of polymer-encapsulated cells or xenografts with localized immunoprotection (Sanberg et al., 1996; Senut et al., 1996).

In search of an appropriate source for dopamine cell replacement in PD, we undertook the present study, which was stimulated by recent work in our laboratory showing that carotid body glomus, or type I, cells behave as oxygen-sensitive presynaptic-like elements capable of exocytotic dopamine release upon acute exposure to low oxygen tension (Ureña et al., 1994; Montoro et al., 1996; see also Fishman et al., 1985). These cells, which derive from the neural crest (Pearse et al., 1973), are among those with the highest dopamine content in the body (Fidone et al., 1982; Fidone and González, 1986) and offer potential clinical advantages because surgical resection of carotid bodies has no significant side effects (Winter, 1970; reviewed by Honda, 1992) and therefore can be used for autografts. Moreover, we presumed glomus cells to be particularly well-suited for neuronal transplantation due to their durability in low oxygen tension, a normal environmental condition in some regions of the brain parenchyma (Baumgärtl and Lübbers, 1983) that is likely to be accentuated inside intracerebral grafts. Hypoxia can have deleterious effects on neurons but seems to exert a "trophic" action on glomus cells. Chronic hypoxia is known to stimulate glomus cell growth and excitability (Edwards et al., 1971; Stea et al., 1992, 1995), as well as the synthesis of dopamine, due to induction of the tyrosine hydroxylase gene (Czyzyk-Krzeska et al., 1992; Czyzyk-Krzeska and Beresh, 1996).

We show here that intrastriatal grafting of cell aggregates of carotid body results in recovery of hemi-Parkinsonian rats, with complete disappearance of motor asymmetries and deficits of sensorimotor orientation analyzed. Transplanted animals ameliorated within a few days after surgery and improved progressively during the 3 months of the study. The behavioral effects were correlated with long survival of glomus cells in the host tissue, where they were organized in clusters, with fibers extending outside the graft, and retained their ability to secrete dopamine.

Results

Loss of Striatal Dopamine Content in Parkinsonian Rats

Rats were rendered hemi-Parkinsonian by injecting 6-hydroxydopamine (6-OHDA) into the left substantia nigra. Seven days after nigra lesions, animals exhibited the characteristic amphetamine-induced strong rotational behavior directed toward the side of the lesion (see below). Tyrosine hydroxylase (TH) immunohistochemistry revealed that intranigral 6-OHDA injection destroyed more than 90% of the TH-containing cell bodies in the unilateral site, leaving intact the cells of the contralateral nucleus (Figure 1A). No clear signs of regenera-

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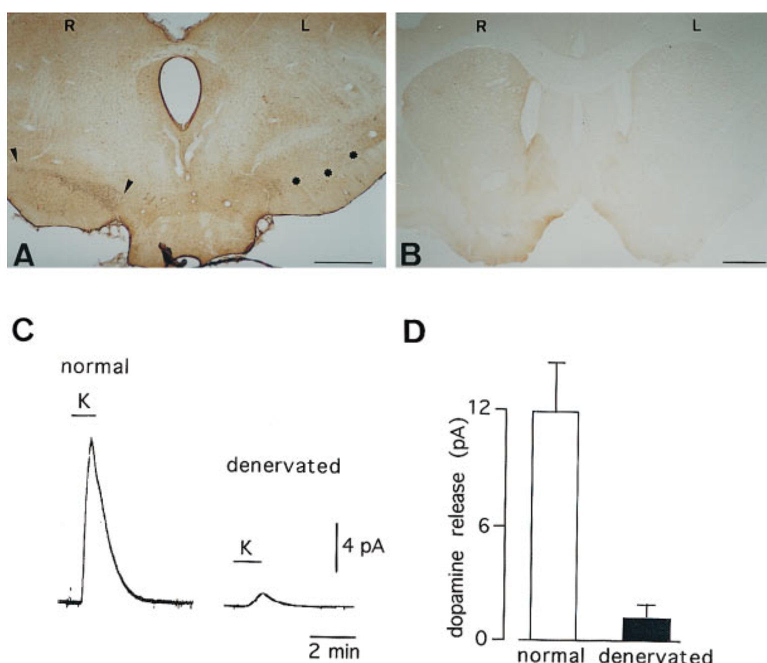

Figure 1. Depletion of Striatal Dopamine in 6-OHDA-Lesioned Rats

(A–B) Coronal sections through the brain stem (A) and striatum (B) of a rat 10 days after 6-OHDA treatment, processed for tyrosine hydroxylase (TH) immunohistochemistry. Injection of 6-OHDA induced a cell loss in the left substantia nigra ([A], asterisks), while the right substantia nigra remained intact ([A], arrowheads). Small clusters of surviving dopaminergic neurons were usually seen at the medial and lateral edges of the spreading area of the drug. Degeneration of dopaminergic cells results in dramatic decrease of immunoreactivity in the ipsilateral striatum ([B], L). TH-positive staining of the contralateral striatum ([B], R) is clearly appreciated. Scale bars, 1 mm.

(C) Amperometric signals due to oxidation of dopamine released from normal or denervated striatal slices upon exposure to an external solution with 66 mM K^+.

(D) Mean dopamine released in normal and denervated striata of an animal using the same electrode and experimental conditions. Values are: normal (11.8 $\pm$ 2.8 pA, mean $\pm$ SD, n = 6 measurements in 3 different slices) and denervated (1.2 $\pm$ 0.7 pA, mean $\pm$ SD, n = 7 measurements in 3 different slices). L, left; R, right.

tion of TH-positive cells were observed up to 3 months after lesion. In accordance with the extension of nigra lesions, the dopaminergic innervation in the ipsilateral striatum virtually disappeared (Figure 1B). The degree of functional striatal denervation in Parkinsonian animals was estimated by measuring depolarization-induced dopamine secretion with amperometric carbon-fiber electrodes (Ureña et al., 1994) placed in the gray matter of striatal thin slices. This methodology was also used to examine the status of striatal reinnervation in transplanted animals (see below). Figure 1C shows representative recordings of focal dopamine release in the normal and denervated striata of a lesioned animal. These measurements were repeated in several slices to obtain mean estimates of the amount of dopamine releasable over the entire region (Figure 1D). In excellent agreement with the histological observations, these data show marked and quite uniform reduction of functionally active dopaminergic terminals in the ipsilateral striatum of hemi-Parkinsonian animals (Zetterström et al., 1986; Fornaguera et al., 1994). The amount of depolarization-evoked dopamine secretion in the denervated striatum was well-correlated with the severity of the behavioral syndrome. In nigra-lesioned animals rotating more than 360 turns/60 min during the amphetamine test, the amplitude of the amperometric signal in the lesioned side was 11.8% $\pm$ 0.05% (mean $\pm$ SD, n = 3) of the mean value in the normal striatum (Marshall and Ungerstedt, 1977; Björklund et al., 1980, 1987). This percentage of ipsilateral versus contralateral dopamine sets an upper limit to the actual value, since it is likely that a small fraction of the amperometric signals was due to detection of oxidizable substances (e.g., serotonin) unaffected by nigra lesion.

Long-Term Survival of Grafted Glomus Cells and Striatal Dopaminergic Reinnervation

The morphological features of intrastriatal grafts were studied 10 days, 1 month, and 3 months after transplantation. Glomus cells were easily distinguished from the surrounding tissue by their strong TH immunoreactivity (Figures 2 and 3). The grafts had an oval shape of $\sim$0.5 mm $\times$ 0.3 mm, oriented in the dorsoventral direction (Figures 2A and 3A). In most cases, they were located either near the center of the striatum or displaced in the mediodorsal direction. Striatal host-graft interface showed scarce astroglial reaction, with some macrophages of characteristic bright yellowish color (Figures 2C and 4B). In 1-month-old transplants (Figure 2), glomus cells were distributed over the entire graft and were grouped in clusters of 5–20 (Figures 2B–2D), resembling the glomerulus-like organization of the carotid body. Some isolated cells were observed at the host-graft interface and, very rarely, outside the area of the transplant. The numbers of TH-positive cells counted in two 1-month-old transplants were 391 and 443, respectively. Glomus cells in the clusters had an oval or round shape of $\sim$10–12 μm in diameter with short neurite-like processes arising from the cell body (Figures 2C and 2D). Isolated cells displayed more variable fusiform or pear-like shapes with processes at their poles. Only a few TH-positive fibers were present in 1-month-old grafts (Figure 2D) and were almost undetectable in younger transplants.

Three months after transplantation, the grafts remained unaltered (Figures 3A and 3B) with numerous clusters of intensely stained cells maintaining the glomerulus-like organization (Figures 3C and 3D). Occasionally, individual glomus cells with neuron-like morphology were observed (e.g., Figure 3D). TH-positive

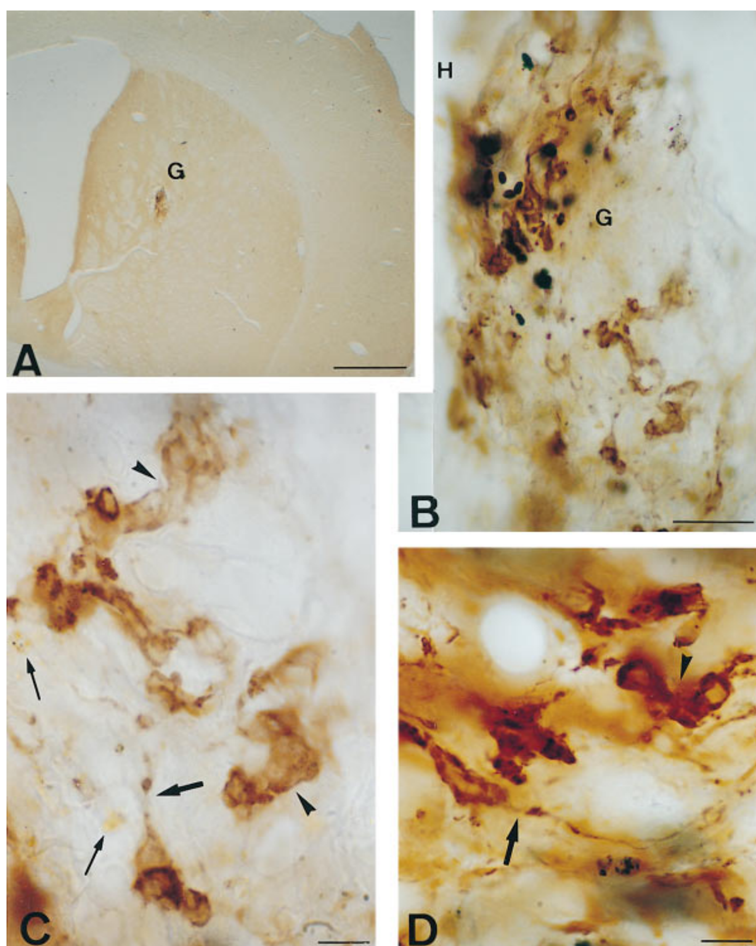

Figure 2. Morphological Features of 1-Month-Old Carotid Body Transplants

Coronal sections through the striatum show the shape ([A], "G") and cellular organization of the graft (B–D). Grafted cells remained at the site of injection ([B], "G") and organized in cell clusters ([C]–[D], arrowheads). Some cells adopted a fusiform morphology with processes arising from their poles ([C]–[D], thick arrows). Note the lack of strong glial reaction at the graft- ("G") host ("H") interface (B). Macrophages located within the graft are observed ([C], thin arrows). Scale bar, 1 mm in (A); 50 μm in (B); and 15 μm in (C) and (D).

cells counted in two 3-month-old transplants numbered 359 and 254, respectively. At this stage, long fibers were seen emerging from cell bodies (Figures 3C and 3D). Figure 4 illustrates the pattern of striatal reinnervation after carotid body transplantation ("G" in Figures 4A and 4B). TH-positive fibers were seen within the graft and running through the grey matter of the host striatum. These fibers were thin and followed a sinuous course with varicosities at regular distances along their length (Figures 4C and 4D), mimicking normal dopaminergic fibers (Pickel, 1982). In Parkinsonian animals transplanted with fragments of the carotid artery, the striatum remained denervated for up to 3 months after surgery (data not shown).

Striatal reinnervation after transplantation was also manifested by an increase in dopamine concentration. Figure 5A shows recordings of focal dopamine release in response to high external K^+ in slices of normal and reinnervated striata from an animal with behavioral recovery 3 months after transplantation. The mean amplitude of amperometric signals recorded within each striatum is represented by the bar diagram. The amount of dopamine released in the reinnervated region was about 65% of that observed in the contralateral side, indicating a clear recovery of dopamine content compared with the denervated striatum before grafting ($\sim$11%; Figure 1). Similar increases of striatal dopamine concentration were observed after grafting in two other animals studied. As in other Ca^{2+}-dependent secretory systems (Ureña et al., 1994), transmitter release in the normal and reinnervated striata was almost abolished by blockade of Ca^{2+} channels with 0.3 mM of Cd^{2+} added to the external solution (Figures 5B and 5C). Ca^{2+}-dependent dopamine release was also clearly detected inside the grafts by placing the carbon-fiber electrode near the clusters of glomus cells. However, these values were not as large as those measured just outside the transplant in the reinnervated gray matter. This could indicate that as glomus cells grow and send prolongations to the host brain parenchyma, secretory vesicles are preferentially accumulated at release sites away from the cell body.

Behavioral Recovery of Transplanted Animals

Substantia nigra destruction resulted in a Parkinson's syndrome with characteristic alterations of several motor and sensorimotor parameters (see Schwarting and Huston, 1996, for a detailed description of the syndrome). Animals exhibited akinesia, revealed by $\sim$50% reduction in distance traveled in open-field testing and by strong net spontaneous turning toward the ipsilateral side (Figure 6A). Amphetamine treatment induced rotational behavior (over 360 turns/60 min) toward the side of the lesion (Figure 6B). Sensorimotor orientation deficit was revealed by enhanced latency to sniff the probe

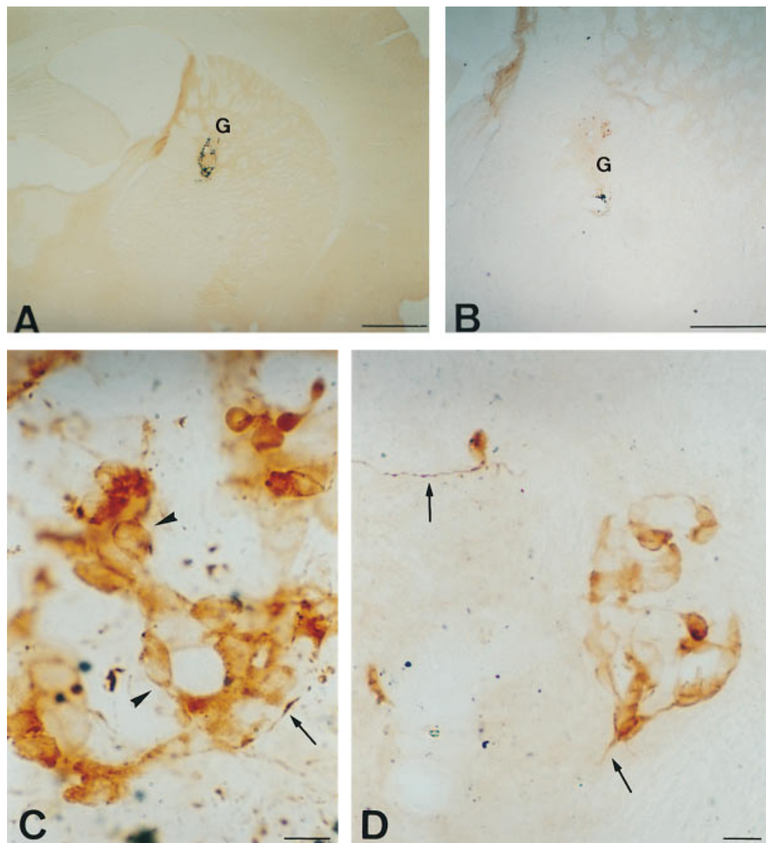

Figure 3. Morphological Features of 3-Month-Old Carotid Body Transplants

Coronal sections through the striatum show the shape ([A]–[B], "G") and cellular organization (C–D) of the grafts. Isolated and clustered glomus cells (arrowheads) developed neuron-like morphology with evident neuritic processes (arrows). Scale bar, 1 mm in (A); 500 μm in (B); and 15 μm in (C) and (D).

in the whisker-touch test (Figure 6C), along with net ipsiversive asymmetry in thigmotactic scanning in the open field (Figure 6D). The beneficial effects of carotid body transplants were already manifest 10 days after grafting. At this stage, spontaneous and amphetamine-induced ipsilateral rotation were nearly abolished (Figures 6A and 6B, closed squares). The net ipsiversive turning mean value of 55.1%, measured after nigra lesion, was reduced to 6.1% after grafting, a value quite similar to that observed in controls (3%). Similarly, four rats out of seven did not rotate after amphetamine administration and the other three (rotating more than 480 turns/60 min before grafting) exhibited a low number of rotations (mean value of 48.6 turns/60 min, $n = 3$).

Most behavioral parameters recovered completely within the first month after grafting and remained stable, or even improved, throughout the study. Spontaneous and amphetamine-induced turning behavior totally vanished within the first month after surgery (Figures 6A and 6B, closed squares). In these animals, amphetamine did not induce rebound circling movements toward the right (contralateral) side, indicating that left striatal supersensitivity to dopamine after denervation did not develop. Sensorimotor behavior tested here, particularly the thigmotactic scanning, recovered with a time course slower than motor asymmetries (Figures 6C and 6D, closed squares). This was probably related to the delayed development of dopaminergic striatal reinnervation (Björklund et al., 1987; Yurek and Sladek, 1990). Interestingly, akinesia was the only abnormality that did not ameliorate after transplantation. Sham-operated Parkinsonian animals implanted with fragments of carotid artery did not show signs of recovery in any of the functional tests used (Figures 6A–6D, open triangles). For convenience, the present study extended for only 3 months post-transplant, but it is likely that the salutary effect of the grafts in Parkinsonian animals can last much longer since at the moment of sacrifice the grafts were intact and showed no evidence of deterioration.

Discussion

Our findings indicate that intrastriatal grafting of aggregates of carotid body cells can revert most of the behavioral alterations studied in a rat model of PD. These beneficial effects are related to the presence in the implants of chromaffin-like glomus cells, which appear to adapt extraordinarily well to the conditions in the host brain parenchyma. Presumably, it is because of this adaptability that the glomus cells can survive and grow for long periods while maintaining their ability to secrete dopamine.

Functional Recovery after Carotid Body Transplants in Comparison with Other Grafting Procedures

Over the last decades, research in dopamine cell replacement in PD has concentrated on the use of two major sources of dopamine: chromaffin cells of the adrenal medulla, and fetal mesencephalic neurons (reviewed by Björklund et al., 1987; Yurek and Sladek, 1990). Adrenal implants can attenuate the motor disturbances in PD, but they often have only transient effects and do not effectively ameliorate the deficits of sensorimotor orientation (Freed et al., 1981; Bing et al., 1988; Abrous et al., 1993). For these reasons, their clinical use has

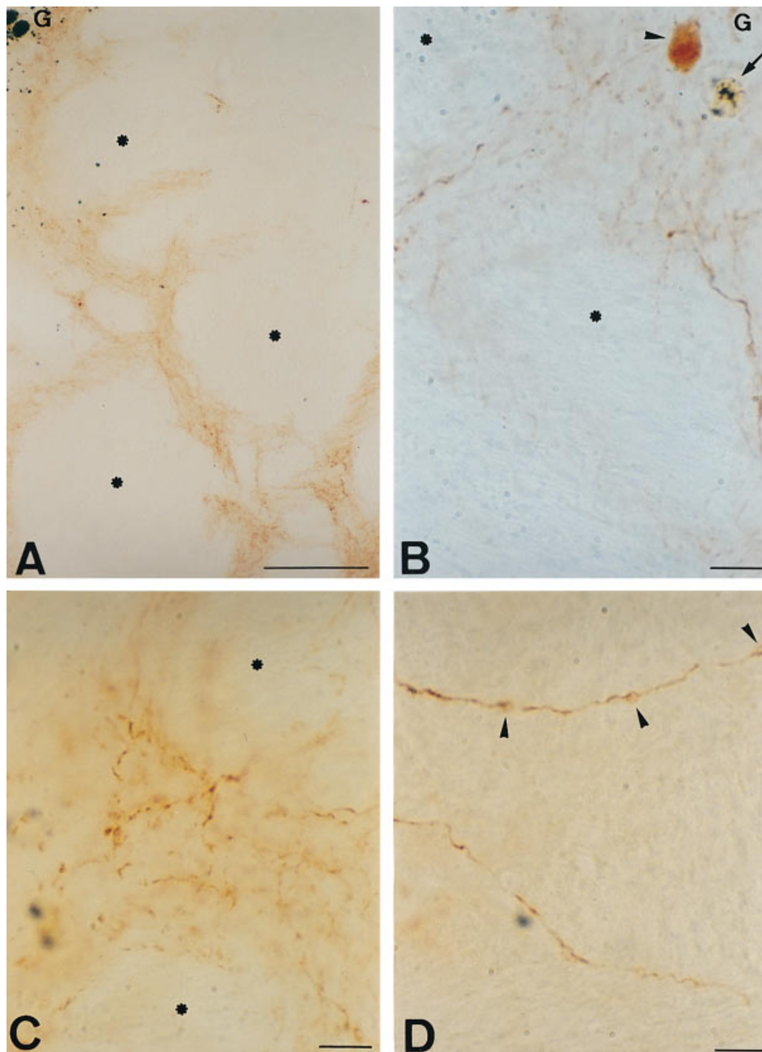

Figure 4. Dopaminergic Reinnervation of Striatum 3 Months after Grafting

(A) and (B) are microphotographs illustrating the course of dopaminergic fibers arising from the transplant ("G") and running through the gray matter. Fibers are thin and varicose (C–D), resembling normal dopaminergic axons. Asterisks in (A) through (C) indicate the striatal white matter bundles. Axonal varicosities are stressed by arrowheads in (D). In (B), a yellowish macrophage at the graft-host interface (arrow) contrasts with the dark brown-stained glomus cell (arrowheads). Scale bars, 100 μm in (A) and 15 μm in (B) through (D).

been practically abandoned. Intrastriatal allografts of fetal mesencephalic cells yield much better results in terms of compensation for spontaneous and drug-induced motor asymmetries and sensorimotor deficits; however, their clinical applicability has numerous limitations (Björklund et al., 1980; Bolam et al., 1987; Brundin et al., 1987; Freed et al., 1992). Apart from the ethical issues, there is the difficulty of obtaining human embryonic tissue and the need to administer cyclosporine A to depress the host immune system. In addition, long-term survival of fetal allografts depends on donor age restrictions. For example, in transplants of dissociated mesencephalic cells from young fetuses, less than 1% of the grafted cells remain alive after a few weeks, and cell death increases critically with donor age (Björklund et al., 1980; Brundin et al., 1985, 1987).

Most of the disadvantages summarized above appear, in principle, to be circumvented with carotid body transplants. Intrastriatal grafts of carotid body cell aggregates are more efficient than other paraneural grafts for the short- and long-term recovery of behavioral abnormalities in Parkinsonian rats, and the tissue can be easily used for autografts without side effects or need of postoperative immunosuppression. Like the most efficient mesencephalic neuronal grafts, carotid body transplants were able to compensate for spontaneous and amphetamine-induced directional bias as well as the deficits of sensorimotor orientation. Noteworthy motor asymmetries were rapidly abolished within the first month after carotid body transplant, whereas fetal mesencephalic grafts do not fully compensate for the motor asymmetries until 6–8 weeks post-grafting (Abrous et al., 1993; Isacson and Deacon, 1997). Moreover, the lack of rebound circling after amphetamine in our transplanted rats could be explained by the fact that grafted glomus cells induced a rapid recovery of striatal dopaminergic tonus, preventing the development of ipsilateral dopaminergic supersensitivity (Schwarting and Huston, 1996). Taken together, these findings suggest that grafted glomus cells rapidly released sustained amounts of dopamine, which, as discussed below, is possibly due to an increase of excitability and rate of dopamine synthesis after implantation in the host parenchyma. Amelioration of sensorimotor deficits with carotid body grafts was comparable to that observed with mesencephalic cells (Björklund et al., 1987; Bolam et al., 1987; Brundin et al., 1987). Akinesia did not improve after carotid body grafting in any of the animals studied. This finding is not surprising, since recovery of akinesia appears to require simultaneous dopamine replacement

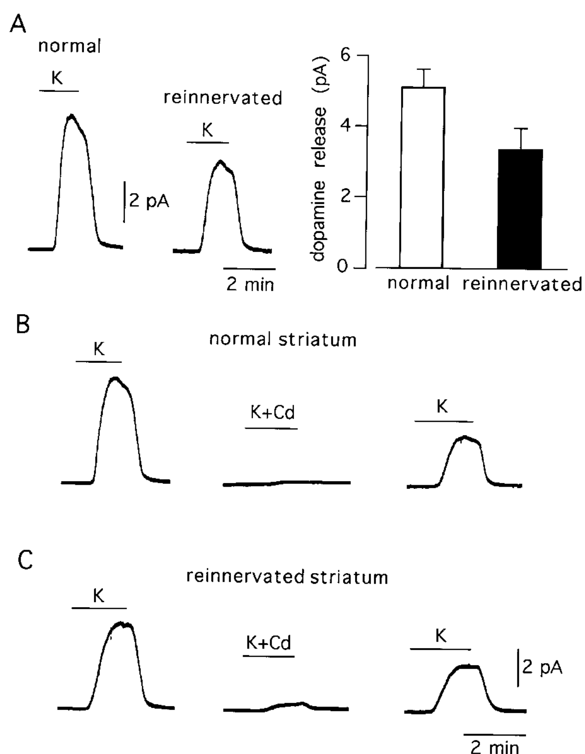

Figure 5. Dopamine Release in Normal and Reinnervated Striata 3 Months after Transplantation

([A], Left) Amperometric signals due to dopamine released from striatal slices upon exposure to high external K^+ solution (66 mM).
([A], Right) Comparison of mean dopamine released in normal and reinnervated striata of an animal using the same electrode and experimental conditions. Values are: normal (5.06 ± 0.46 pA, mean ± SD, n = 5 measurements in 5 different slices) and reinnervated (3.28 ± 0.66 pA, n = 5 measurements in 5 different slices).
(B) Recordings in a normal striatum showing that focal dopamine release evoked by high external K^+ is reversibly abolished by blockade of Ca^{2+} entry with 0.3 mM Cd^{2+}.
(C) Similar experiment as in (B) in the reinnervated striatum.

in nucleus accumbens as well as in striatum (Herman et al., 1986, 1988; Björklund et al., 1987; Brundin et al., 1987).

The good recovery of our transplanted animals is likely related, at least in part, to the procedure followed to prepare the carotid body tissue for transplantation. We tested striatal implantation of the whole carotid body in a Parkinsonian animal with negative results (data not shown). After sacrifice, the histology showed that connective tissue surrounding the carotid body isolated the graft from the host tissue. It is also known that intracerebral grafting of dispersed dopaminergic neural or paraneural cells results in high cellular mortality during the first weeks after transplantation (Yurek and Sladek, 1990), and that viability of grafted fetal dopaminergic neurons improves with the use of solid pieces of mesencephalon (Björklund et al., 1980; Brundin et al., 1987). To our knowledge, there is only one previous report describing the use of carotid body glomus cells for transplantation studies that were performed with enzymatically dispersed cells (Bing et al., 1988). This work showed that intrastriatal grafted glomus cells were even less efficient than adrenal cells to recover Parkinsonian rats. Compensation of motor asymmetries in these grafted animals was only ~50%, and most of the implanted cells died within 4 weeks after grafting. It is known that excitability and responsiveness to hypoxia in glomus cells is altered by cell-dispersion procedures (López-Barneo et al., 1998); hence, our satisfactory results possibly depend on the use of fresh cell aggregates, maintaining the tissular integrity, and the use of only mild enzymatic treatment.

Cellular Properties of Transplants

We have shown that restoration of the behavioral disturbances in transplanted animals was correlated with survival of glomus cells in the host brain, where they often appeared to be organized in glomerulus-like clusters resembling the structure of the native carotid body tissue (Fidone and González, 1986). Considering the small size of cell aggregates used for grafting, the number of cells in the transplants was relatively high and did not change appreciably with time, indicating that those cells overcoming the transplantation trauma were capable of long-term survival. Grafted glomus cells were able to grow processes and fibers extending outside the graft, with typical varicosities of catecholaminergic synapses, while retaining their ability to release dopamine. Thus, intrastriatal grafted glomus cells seem to have a higher capacity to survive in the brain parenchyma than adrenal or other paraneural cells, which might be related to their special molecular properties. Unlike other sympatoadrenal cells, glomus cells do not require nerve growth factor to survive and grow (Stea et al., 1992; Zhong and Nurse, 1995). In addition, as specialized arterial chemoreceptors, glomus cells are strongly stimulated by environmental oxygen tensions below 30 or 40 mm Hg (reviewed by López-Barneo, 1996). These values are normally observed in the brain parenchyma (Baumgärtl and Lübbers, 1983) and are likely accentuated within the graft. Chronic hypoxia, which can damage other cell types, has effects resembling those of growth factor on glomus cells. Low oxygen tension produces enhancement of cellular excitability due to sodium channel induction as well as Ca^{2+} mobilization, cell growth, and the appearance of neurite processes (Stea et al., 1992, 1995; Jackson and Nurse, 1995). Prolonged hypoxia also increases the rate of dopamine synthesis due to TH mRNA stabilization and gene induction (Czyzyk-Krzeska et al., 1992; Czyzyk-Krzeska and Beresh, 1996).

A constant feature in transplanted animals was the occurrence of striatal reinnervation by TH-positive fibers able to secrete dopamine in a Ca^{2+}-dependent manner. Reinnervation and recovery of striatal dopamine concentration have also been observed after grafting mesencephalic tissue (Björklund et al., 1980; Bolam et al., 1987; Brundin et al., 1987; Freed et al., 1992) and adrenal cells (Freed et al., 1981; Bohn et al., 1987; Bing et al., 1988). In 3-month-old carotid body transplants, many of the fibers running by the striatal gray matter arose from TH-positive cells inside the grafts. However, it is likely that, as previously suggested (Bohn et al., 1987; Bing et al., 1988), sprouting of intrinsic ipsilateral or contralateral fibers also occurs. Intrinsic reinnervation

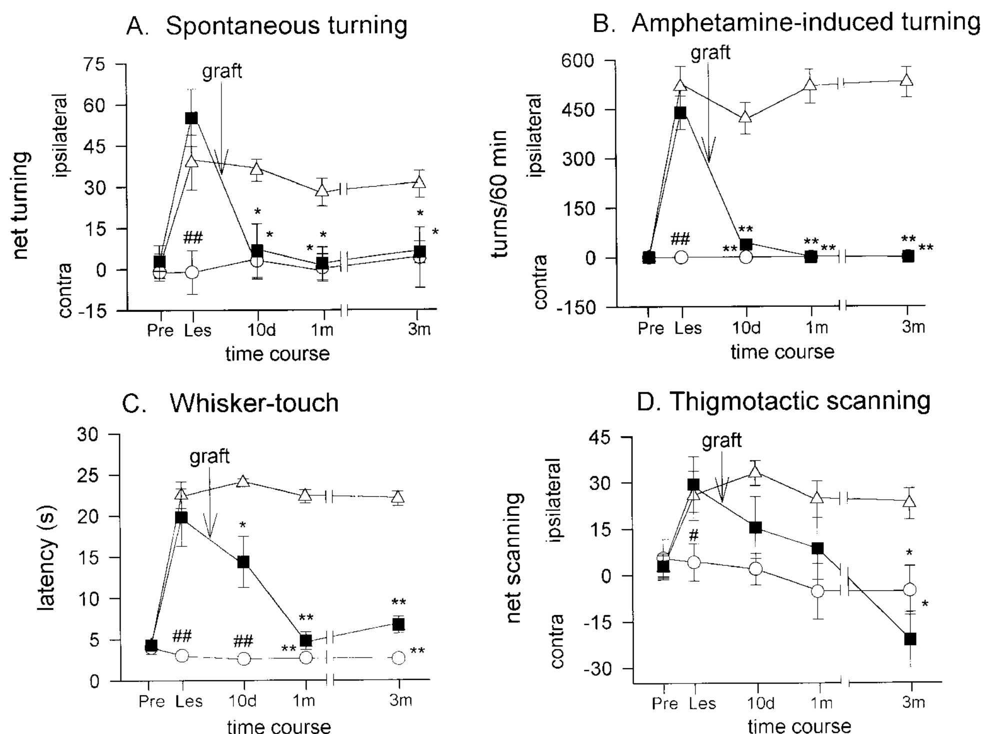

Figure 6. Time Course of Turning Behavior and Sensorimotor Orientation in Control, Lesioned, and Grafted Rats

Turning behavior was measured in control (open circles, n = 5 and 4 at 3 months), 6-OHDA-lesioned rats with carotid body grafts (closed squares, n = 7, 6, and 4 at 10 days, 1 month, and 3 months, respectively), and 6-OHDA-lesioned rats with sham grafts (open triangles, n = 6) through net spontaneous turning in the open field (A) and amphetamine-induced turning (B). Sensorimotor orientation was evaluated through the whisker-touch test (C) and net thigmotactic scanning in the open field (D). Two-way ANOVA for repeated measures (df 8, 69) indicated significant interaction effects for spontaneous turning ($F = 2.6$, $p < 0.01$), amphetamine-induced turning ($F = 58.2$, $p < 0.0001$), whisker-touch latency ($F = 11.1$, $p < 0.0001$), and thigmotactic scanning ($F = 2.3$, $p < 0.05$). Values are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ versus 6-OHDA-lesioned rats with sham grafts; # $p < 0.05$, ## $p < 0.01$ versus 6-OHDA-lesioned rats with either carotid body or sham grafts (Newman-Keuls test). Abbreviations: Pre, 7 days before lesion; Les, 7 days after 6-OHDA or sham lesion; 10d, 1m, and 3m are 10 days, 1 month, and 3 months after grafting, repectively. Grafts were implanted 10 days after nigra lesion (arrow).

cannot be explained simply by nonspecific injury- or tissue-induced neurotrophism (Yurek and Sladek, 1990), since no signs of striatal reinnervation, elevation of Ca^{2+}-dependent dopamine release, or behavioral recovery were observed in sham-operated animals. Therefore, the possible "trophic" factor stimulating striatal sprouting must be in the carotid body cell aggregates. In recent years, a glial-derived neurotrophic factor (GDNF) that promotes survival and differentiation of mesencephalic cells in vitro and in vivo has been identified (Lin et al., 1993; Tomac et al., 1995; Gash et al., 1996). It is therefore presumable that glomus cells or the glia-like type II cells existing in the carotid body contribute with trophic factors to stimulate cell growth within the graft or in the brain parenchyma.

In conclusion, we show here that intrastriatal grafting of cell aggregates of carotid body has excellent results with respect to the amelioration of some motor and sensorimotor anomalies in Parkinsonian rats. This work should stimulate future research aimed at the assessment of the possible clinical applicability of this therapy, as well as toward the characterization of the molecular properties of carotid body tissue that are relevant to their interaction with brain parenchyma (e.g., oxygen sensitivity or release of putative neurotrophic factors).

Experimental Procedures

Unilateral 6-OHDA-Induced Nigra Lesion

We used male Wistar rats (275–325 g) housed at a regulated temperature (22°C $\pm$ 1°C) in a 12 hr light-dark cycle (lights on at 08:00 hours). Food and water were available ad libitum. Thirty minutes before 6-hydroxydopamine (6-OHDA, RBI) lesion, rats were injected with desipramine (15 mg/kg i.p.) to protect noradrenergic terminals from 6-OHDA toxicity. Rats were anesthetized with chloral hydrate (450 mg/kg i.p.) and placed in a Kopf stereotaxic apparatus with the incisor bar set 3.3 mm below the interaural line. Saline solution (1 μl) containing 6-OHDA (4 μg/μl) and 0.2% ascorbic acid was injected over 15 min with a blunted 30-gauge cannula at the following coordinates with respect to bregma: AP = -5.3, L = -2.3, and V = -8.2. The cannula was left in place for 1 min after injection. After surgery, rats were injected with penicillin (100,000 I.U. i.m.). Control rats followed the same protocol except that a 6-OHDA-free solution (0.9% NaCl with 0.2% ascorbic acid) was injected.

Grafting Technique

Carotid body cell aggregates were obtained from isogenic male rats (275–325 g) under anesthesia. Carotid bifurcations were removed after neck incision, and carotid bodies were isolated, cleaned of surrounding adipose tissue, and trimmed into pieces ~1/4–1/5 the size of the whole carotid body. The estimated number of transplanted glomus cells varied between 400 and 600. The tissue was incubated for 20 min in a 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 centrifuged at 800 g for 5 min and resuspended

in 5 ml of normal Tyrode solution to remove the enzymes. The fragments of carotid artery used for sham grafts were prepared following the same procedure. Ten days after nigra lesion, Parkinsonian animals were anesthetized and placed into a Kopf stereotaxic apparatus. A burr hole was drilled over the denervated striatum, and a blunted 23-gauge cannula, connected to a 2 ml Hamilton syringe, was lowered to the injection site (coordinates: AP = +0.2, L = −3, and V = −5.5). Tyrode's solution (2 ml) containing a cell aggregate of carotid body or carotid artery was injected over 5 min. After surgery, rats were injected with penicillin (100,000 I.U. i.m.).

Immunohistochemistry

Rats were transcardially perfused with 150 ml of phosphate buffered saline (PBS) followed by 500 ml of ice-cold 4% paraformaldehyde in a phosphate buffer (PB) of 0.1 M (pH 7.2–7.4). After dissection, the brains were postfixed overnight in the same fixative at 4°C and immersed in 30% sucrose in PB until they sank. Coronal sections 30 μm thick were cut on a freezing microtome and collected in PB. Sections were incubated in PBS, 0.2% gelatin, and 0.1% Triton X-100 (PBS-G-T) with 3% normal goat serum (Vector) for 1 hr to block nonspecific sites. Sections were then incubated overnight with a polyclonal anti-TH antibody (1:3000, Chemicon) in PBS-G-T. After washing in PBS-T, sections were incubated with biotinylated anti-rabbit antibody (1:200, Vector). Thereafter, sections were incubated with the ABC kit (1:100, Vector) for 1 hr, and specifically bound antibody was revealed by using 3.3′-diaminobencidine (Sigma) as chromogen. After washing in PB, sections were mounted on gelatin-subbed slides, dehydrated, and coverslipped without counterstaining. Estimation of the number of cells was done with the Abercrombie correction (Abercrombie, 1946) in four grafts, counting individual cells in the complete series of histological sections.

Amperometry

Animals were decapitated under anesthesia, and then the brains were rapidly removed and placed in a cold (~5°C) Krebs-Ringer bicarbonate buffer containing (in mM): 126 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.5 $MgSO_4$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, and 10 glucose, bubbled with 95% O_2 and 5% CO_2. Coronal slices of the striatum (150–200 μm thick) were done with a Lancer vibratome. Slices were incubated at room temperature in the same Krebs-Ringer solution for 60 min. For amperometric measurements, a slice was transferred to the recording chamber, placed on the stage of a microscope, and continuously superfused with a control solution of the following composition (in mM): 150 NaCl, 2.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH = 7.4). To activate dopamine release, this solution was replaced by another of the same composition but with 66 mM KCl and 84 mM NaCl. In some experiments, 0.3 mM $CdCl_2$ was added to the high K^+ solution. Dopamine release was monitored in amperometric mode with a carbon-fiber electrode (12 μm in diameter) covered with polyethylene (Ureña et al., 1994). The electrode was connected to a current-to-voltage converter and polarized to a constant voltage of 650 mV, which in our experimental conditions is near the oxidation peak of dopamine. To measure dopamine release, electrodes were gently placed on top of the striatal gray matter with a micromanipulator. Amperometric signals were low-pass filtered at 50–100 Hz and stored on tape. Experiments were performed at room temperature (22°C–25°C).

Behavioral Studies

Rats were randomly allocated into three groups: 6-OHDA-lesioned with carotid body graft (n = 7), 6-OHDA-lesioned with sham graft (n = 6), and sham nigra-lesioned with sham graft (controls, n = 5). Motor and sensorimotor responses were evaluated 7 days before and after unilateral nigra lesion or sham operation, and at 10 days, 1 month, and 3 months after grafting. For these studies, we followed a methodology previously described (Ungerstedt and Arbuthnott, 1970; Marshall, 1979; Björklund et al., 1980, 1987; Schwarting and Huston, 1996). Akinesia was evaluated by measuring the distance traveled in the open-field test. Spontaneous directional bias was estimated through net turning (percent ipsilateral minus contralateral turning). Turning is defined as tight turns within a diameter of 30 cm, ipsilateral or contralateral to the side of the lesion. Each open-field test lasted 10 min. Drug-induced rotation was evaluated in the home cage by injecting D-amphetamine sulfate (5 mg/kg i.p., RBI), which induces dopamine release from dopaminergic neurons (Schwarting and Huston, 1996) and glomus cells (Docherty and McQueen, 1978). The number of ipsiversive or contraversive rotations were quantified from 30–90 min after injection. We used in this study only those animals observed to make more than 6 turns/min. Sensorimotor orientation was evaluated using the whisker-touch and open-field tests. The rat was approached from the right side (contralateral to the lesion) with a thin probe, and after touching the vibrissae, the latency in seconds to sniff the probe was quantified. A maximal latency of 25 s was scored if the animal failed to display a response within this period. In the open field test (1 × 1 m, quadrants 20 × 20 cm each), sensorimotor neglect was evaluated through net thigmotactic scanning (percent ipsilateral minus contralateral scanning). Thigmotactic scanning is defined as the time spent locomoting along the wall of the open field with the snout within a 5 cm range of the wall. Animal behavior in the open field and during the drug-induced rotation tests was videotaped. The software for the analysis was developed in our laboratory (Espejo et al., 1994). All tests were carried out during the last phase of the light period (17:00 to 20:00 hours). Behavioral data were analyzed by using two-way ANOVA (group as between factor, time point as within variable), followed by one-way ANOVA and Newman-Keuls tests for comparison between the three groups at the same time point. When variance was not homogeneous, the data were logarithmically (log[x]) transformed. Experiments were performed according the animal care guidelines of the European Communities Council (86/609/EEC).

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