Functional Regeneration in a Rat Parkinson's Model after Intrastratial Grafts of Glial Cell Line-Derived Neurotrophic Factor and Transforming Growth Factor β_1 -Expressing Extra-Adrenal Chromaffin Cells of the Zuckerkandl's Organ

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Intrabrain transplantation of chromaffin cell aggregates of the Zuckerkandl's organ, an extra-adrenal paraganglion that has never been tested for antiparkinsonian treatment, induced gradual improvement of functional deficits in parkinsonian rats. These beneficial effects were related to long survival of grafted cells, striatal reinnervation, and enhancement of dopamine levels in grafted striatum. Grafted cells were not dopaminergic, but they expressed glial cell line-derived neurotrophic factor (GDNF) and transforming growth factor- β_1 . These factors were detected in the host striatal tissue, indicating that chromaffin cells secreted them after grafting. Because glial cell line-derived neurotrophic factor possesses neurorestorative prop-

erties over dopaminergic neurons, and transforming growth factor- β_1 is a cofactor that potentiates the neurotrophic actions of GDNF, functional regeneration was likely caused by the chronic trophic action of neurotrophic factors delivered by long-surviving grafted cells. This work should stimulate research on the clinical applicability of transplants of the Zuckerkandl's organ in Parkinson's disease.

Key words: Parkinson's disease; graft; Zuckerkandl's organ; extra-adrenal cell; adrenal cell; glial cell line-derived neurotrophic factor; transforming growth factor- β_1 ; stereology; 6-hydroxydopamine

Parkinson's disease is caused by the loss of dopaminergic neurons of substantia nigra projecting to striatum. The most prevalent therapy is levodopa administration, but it is not efficacious after several years of treatment. In search of an alternative therapy, intrastratial grafts of dopamine-secreting cells from neural or chromaffin tissues, such as fetal mesencephalon, adrenal medulla, and carotid body, has been reported to ameliorate functional deficits in animal models of Parkinson's disease (Bolam et al., 1987; Bohn et al., 1987; Goetz et al., 1991; Espejo et al., 1998; Luquin et al., 1999). Transplantation is considered a promising treatment for human Parkinson's disease, but its clinical use is still restricted to few cases. The major limiting factors regarding fetal mesencephalic cells are the ethical, practical, and safety issues associated with tissue derived from aborted human fetuses and the difficulty in obtaining sufficient viable embryonic mesencephalic tissue (Dunnett and Björklund, 1999). Adrenal cells are no longer used because their long-term survival is very poor in the brain, and beneficial effects are transient either in Parkinson's

patients or in animals (Yurek and Sladek, 1990). The clinical effects of glomus cell transplants still have to be investigated.

Another grafting strategy is to introduce potentially neuroprotective molecules that stimulate regeneration in the damaged nigrostriatal system. Among these molecules, glial cell line-derived neurotrophic factor (GDNF) has potent *in vivo* effects (Lin et al., 1993; Tomac et al., 1995; Beck et al., 1995; Gash et al., 1998), and promising results have been obtained with fibroblasts engineered to secrete GDNF (Tseng et al., 1997) or viral vectors expressing GDNF (Mandel et al., 1997; Kirik et al., 2000). In this context, the antiparkinsonian effects of cell grafts of the chromaffin lineage are attributed not only to dopamine release from grafted cells but also to dopaminergic reinnervation of the denervated striatum (Freed et al., 1981; Bohn et al., 1987; Espejo et al., 1998; Dunnett and Björklund, 1999; Luquin et al., 1999), and chromaffin cells are known to express and release GDNF (Unsicker and Kriegstein, 1996; O'Connor, 1999). However, as explained, adrenal chromaffin cells induce a transient amelioration, a fact that perhaps has precluded the use of extra-adrenal cells, of similar embryological origin.

Nevertheless, the extra-adrenal tissue is not homogeneous, and different extra-adrenal paraganglia can be found. Among them, the Zuckerkandl's organ is the biggest extra-adrenal paraganglion (although its size is reduced over life), representing an important source of circulant catecholamines (Ahonen et al., 1987). It is located adjacent to the lower abdominal aorta and can be easily removed (Testut and Latarjet, 1978; Bohn et al., 1982). In the present study, the antiparkinsonian efficacy of the Zuckerkandl's organ grafts was tested in rats in search for an alternative source of cells for cell replacement in Parkinson's disease. Here we show

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that intrastriatal transplantation of extra-adrenal chromaffin cells of the Zuckerkandl's organ induce progressive improvement of functional deficits in parkinsonian rats and that these chromaffin cells present a long survival after grafting, unlike adrenal chromaffin cells that only induce a transiently beneficial effect.

MATERIALS AND METHODS

Unilateral 6-hydroxydopamine-induced nigra lesion. Wistar rats were housed at a regulated temperature ($22 \pm 1^\circ\text{C}$) in a 12 hr light/dark cycle (lights on at 8:00 A.M.). Food and water were available *ad libitum*. Thirty minutes before 6-hydroxydopamine (6-OHDA; Research Biochemicals, Natick, MA) lesion, rats were injected with the antibiotic ceftriaxone (10 mg/0.3 ml, i.m.), and desipramine (15 mg/kg, i.p.) to protect noradrenergic terminals from 6-OHDA toxicity. Rats were anesthetized with chloral hydrate (425 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 (1.2 μl /site) containing 6-OHDA (5 $\mu\text{g}/\mu\text{l}$) and 0.2% ascorbic acid was injected over 5 min with a blunted 30 gauge cannula at the following coordinates with respect to bregma: anteroposterior (AP) -5.2 , -5.4 , lateral (L) -2.2 , and ventral (V) -8.2 (Paxinos and Watson, 1997). The cannula was left in place for 1 min after injection. Control rats followed the same protocol except that a 6-OHDA-free solution (0.9% NaCl and 0.2% ascorbic acid) was injected.

Grafting procedure. Extra-adrenal paraganglia and adrenal glands were obtained from young rats (200–250 gm) under anesthesia (chloral hydrate, 425 mg/kg, i.p.). Abdominal skin and muscles were incised, and intestinal asae were displaced to expose the abdominal aorta. After carefully dissecting retroperitoneum, the Zuckerkandl's organ was located lying on the abdominal aorta between the emergence of inferior mesenteric and iliac arteries, and the adrenal gland was located on the superior pole of the left kidney. Then, the paraganglion and the adrenal gland were gently removed and cleaned of surrounding adipose tissue, and the adrenal medulla was isolated from the surrounding cortical tissue. They were then coronally sectioned, and each section was trimmed into pieces of $\sim 0.75\text{ mm}^3$ in volume. The tissue for grafting was incubated for 20 min in a Ca^{2+} - and Mg^{2+} -free Tyrode's solution with collagenase (1 mg/ml), trypsin (1 mg/ml), and DNase (0.5 mg/ml) (Espejo et al., 1998). After enzymatic treatment, cell aggregates were resuspended in 5 ml of normal Tyrode's solution to remove the enzymes. Afterward, an anesthetized parkinsonian or control animal was 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 μl Hamilton syringe, was lowered to the injection site (coordinates: AP $+1.5$, L -3 , and V -5.5) (Paxinos and Watson, 1997). Tyrode's solution (2 μl) containing a cell aggregate of extra-adrenal paraganglia, adrenal medulla or without it (control and sham-grafted rats) was injected. Pieces used for transplantation were randomly chosen. All the experiments were performed in accordance with the European Communities Council Directive for the employment of laboratory animals (86/609/EEC).

Behavioral study. Rats were randomly assigned to four groups: 6-OHDA-lesioned with organ of Zuckerkandl's graft (referred to as "Zuckerkandl group"; $n = 12$), 6-OHDA-lesioned with adrenal graft (referred to as "adrenal group"; $n = 10$), 6-OHDA-lesioned with sham graft (referred to as "sham-grafted group"; $n = 10$), and sham nigra lesion with sham graft (referred to as "control group"; $n = 10$). Motor and sensorimotor deficits were evaluated 15 d before and after unilateral nigra lesion or sham operation, and at 1, 2, 3, and 5 months after grafting. For behavioral study, we followed a methodology previously described (Ungerstedt and Arbuthnott, 1970; Marshall, 1979; Schwarting and Huston, 1996; Espejo et al., 1998). In summary, locomotor directional bias was evaluated by quantifying ipsilateral rotations induced by amphetamine (5 mg/kg, i.p.). The number of ipsiversive rotations were quantified from 30–90 min after injection, and only those animals observed to make >420 turns per hour were used for grafting. Forelimb asymmetry was evaluated by the cylinder test (Kirik et al., 2000), in which the animal is allowed to move freely in a transparent cylinder ($50 \times 30\text{ cm}$) until it has displayed 20 rearing postures, during which it leans at least one paw against the cylinder wall. The number of left and right forepaw contacts are counted, and the data are presented as percentage of right forepaw contacts (right paw use ratio). Hemiparkinsonian rats with lesion in the left substantia nigra present a significant impairment in the contralateral (right) paw use. Sensorimotor orientation was evaluated through the whisker-touch and odor tests. These tests are based on the impaired orientation of hemiparkinsonian rats to stimuli presented contralaterally

(Stricker and Zigmond, 1986). In the whisker touch test, the orientation latency toward a tactile stimulus is measured. Thus, a thin probe is approached from the right side, and after gently touching the vibrissae, the latency for moving the head toward the probe is quantified (whisker-touch). In the odor test, head orientation latency trying to avoid an aversive stimulus is measured. Thus, a probe with the tip impregnated in ammonia is approached from the right side near the nose, and the latency for shaking the head off the probe is quantified. Behavioral data in the cylinder test was analyzed by using Student's *t* test (independent groups) at 1 and 5 months after grafting. Two-way ANOVA was used for analyzing the other tests (group, between variable; time point, within variable) followed by one-way ANOVA and Newman–Keuls test for comparisons among groups at the same time point. If distribution was found not to be normal and variance was not homogeneous, as revealed by the *F* test, the data were logarithmically ($\log[x]$) transformed before ANOVA analysis. Sphericity of repeated measures was always assessed before ANOVA treatment by using the Mauchly's *W* test, to reveal that sphericity was not violated.

Immunohistochemistry and immunofluorescence. Rats were transcardially perfused with 150 ml of PBS followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2–7.4. After dissection the brains were post-fixed overnight in the same fixative at 4°C and immersed in 25% sucrose in PBS for cryoprotection before being stored. Pieces of the Zuckerkandl's organ or the adrenal medulla not used for transplantation were immersed in 4% paraformaldehyde in 0.1 M PB overnight. Coronal sections of brains, paraganglia, or adrenal medulla (50- μm -thick) were cut on a vibratome and collected in PBS. The sections taken from the pieces of paraganglia and adrenal medulla were used for tyrosine hydroxylase (TH) and dopamine- β -hydroxylase (DBH) immunofluorescence. The sections taken from five grafted brains with Zuckerkandl's organ were mounted onto glass slides in sequence and subsequently used for thionin staining (Nissl method), TH and DBH immunohistochemistry, and TH–DBH immunofluorescence. Regarding the remainder grafted brains with extra-adrenal cells ($n = 7$), four brains were used for HPLC and ELISA analyses (see corresponding section), and sections taken from three brains were subsequently stained with GDNF, transforming growth factor (TGF)- β_1 , and acid fibroblast growth factor (aFGF) antisera. Finally, 15 brains of controls ($n = 5$), sham-grafted rats ($n = 5$), and rats with adrenal transplants ($n = 5$) were also sectioned and used for TH immunohistochemistry, and 12 brains from controls ($n = 4$), sham-grafted rats ($n = 4$), and rats with adrenal transplants ($n = 4$) were used for HPLC and ELISA analyses.

For immunohistochemistry, endogenous peroxidase activity was quenched by placing sections into 3% H_2O_2 in 0.05 Tris buffer, pH 7.6, for 10 min. Then sections were incubated in PBS and 0.25% Triton X-100 (PBS-T) with 3% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 hr to block nonspecific sites. Sections were incubated overnight with monoclonal mouse anti-tyrosine hydroxylase (anti-TH) (1:1000; Diasorin), rabbit anti-dopamine- β -hydroxylase (DBH) antibodies (1:1000; Diasorin), rabbit anti-TGF- β_1 (1:1000; Chemicon, Temecula, CA), chicken anti-GDNF (1:2000; Chemicon), or rabbit anti-aFGF (1:1000; Chemicon) in PBS-T. After washing in PBS-T, sections for DBH, TGF- β_1 , and aFGF immunohistochemistry were incubated for 1 hr with biotinylated anti-rabbit antibody (1:200; Chemicon), those for GDNF immunohistochemistry were incubated with biotinylated anti-chicken antibody (1:200; Chemicon), and those sections for TH immunohistochemistry were incubated with biotinylated anti-mouse antibody (1:200; Diasorin). Then, all sections were incubated with the ABC kit (1:100; Vector Laboratories) for 1 hr, and specifically bound antibody were revealed by using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) as chromogen and 0.01% hydrogen peroxide. Negative control sections were incubated in the same solutions for the same incubation times as the other brain sections, with the exception that the primary antibody solution was replaced by a PBS-T solution containing 1% goat serum without the primary antibody. Sections were washed in PBS and mounted on glass slides and coverslipped with DPX.

For immunofluorescence, after quenching and blocking, sections were incubated overnight with monoclonal mouse anti-TH (1:1000; Diasorin) and rabbit anti-DBH antibodies (1:1000; Diasorin) in PBS-T. After washing in PBS-T, sections were incubated for 1 hr with biotinylated anti-rabbit antibody (1:200; Chemicon), and then they were incubated for 2 hr with goat cyanine-2-streptavidin (green color; 1:1000) and goat cyanine-5-anti-mouse antibody (red color; 1:200). Finally, sections were mounted on glass slides and coverslipped with glycerol and PB (1:1).

Stereological methods and density of striatal TH+ innervation. Volumet-

ric measurements were done by applying the principle of Cavalieri, using photo sections (Lagares and Avendaño, 1999). Every five coronal sections ($50\ \mu\text{m}$ width, $\tau = 200\ \mu\text{m}$) of the Zuckerkandl's organ, adrenal medulla, or the striatal tissue were randomly sampled by using a reticle of hitting points where the area associated with each sampling point was $0.11\ \text{mm}^3$ ($a(p)$). The estimate of the volume was calculated as $\sum V = \tau \cdot a(p)$. $\sum Pi$, where $\sum Pi$ = total number of hitting points. The coefficient of error (CE) was calculated according to a formula that takes into account the shape of the organ (e.g., 4.1 for a fusiform organ such as the Zuckerkandl's paraganglion; Gundersen and Jensen, 1987). The number of chromaffin cells was calculated applying the physical disector method (Sterio, 1984). For this procedure, six systematically chosen pairs of adjacent sections ($\tau = 50\ \mu\text{m}$) were used. Four disector frames were used for every section, and the area of the disector frame covered $16,104\ \mu\text{m}^2$ ($a(\text{ret})$). The estimated disector volume or $\sum V(\text{dis})$ was calculated as $\sum V(\text{dis}) = \text{number of disectors} \cdot \tau \cdot a(\text{ret})$. The estimate of the number of cells (N) was calculated as $N = (\sum Q^- \cdot \sum V(\text{ref}))/\sum V(\text{dis})$, where $\sum Q^-$ was the total number of cells counted in all the disectors, and $V(\text{ref})$ was the estimate of the volume of the chromaffin tissue (in the paraganglion, adrenal medulla, or grafted striatum), as measured by the principle of Cavalieri.

The optical density of striatal TH+ innervation was measured at five different rostrocaudal levels corresponding to approximately +1.5, +1, +0.5, -0.5, and -1 mm relative to bregma (Paxinos and Watson, 1997). Images from coronal sections of ipsilateral and contralateral striata were taken with a high-resolution digital camera from a microscope equipped with a natural density filter to give constant illumination throughout the specimen. The digitalized images were analyzed using Scion Corp. (Frederick, MD) Image for the personal computer, four subdivisions per section being assessed (dorsal, medial, lateral, and ventral regions of dorsal striata), avoiding TH+-transplanted cells in grafted striata. Optical density readings were corrected for background staining (the corpus callosum, a nondopaminergic region of the tissue). To avoid differences in staining intensity between individual animals, all values are expressed as a percentage of the intact contralateral striatum.

HPLC. Four rats per group were killed by decapitation at 5 months after sham (sham-grafted and control groups) or chromaffin cell grafting. The brains were quickly removed and placed on ice, and the left striata were immediately dissected and weighed. The dissected tissue was then chopped into pieces, mixed, and divided equally into two halves (with similar weight) and frozen at -80°C separately (one part for HPLC measurements, the other for GDNF and TGF- β_1 ELISA). Later, tissue samples were homogenized in 0.5 ml of an ice-cold solution containing (in M): 0.4 HClO_4 , 0.5 sodium acetate, and 0.5 acetic acid, and centrifuged at 27,000 rpm for 60 min at 4°C . The supernatants were decanted and filtered through a $0.45\ \mu\text{m}$ filter (Sartorius), and frozen at -80°C until HPLC assay. The electrochemical performance was based essentially on the method described by Saito et al. (1992). Aliquots ($10\ \mu\text{l}$) of each sample were injected directly into the HPLC system (System Gold; Beckman Instruments, Fullerton, CA), consisting of a solvent delivery pump with a pulse-dampener, an automatic sample injector (Carnegie Medicine), and an analytical C18 reverse-phase column (Ultrasphere 3 μm particle size; $75 \times 4.6\ \text{mm}$ inner diameter; Beckman). The ESA model 5100 A Coulchem electrochemical detection system consisted of a model 5021 conditioning cell (detector setting, +400 mV) followed in sequence by a model 5011 dual electrode analytical cell (cell 1, +100 mV; cell 2, -260 mV). The output signal from the final electrode was amplified by a 5100 A controller and relayed to an integrator (model 106; Beckman). The detection limit of the system was $0.1\ \text{pg}/\mu\text{l}$. The mobile phase for the separation of catecholamines and their metabolites was a mixture of 0.075 M Na_2HPO_4 , 1.2 mM sodium heptanosulfonate, 0.097 mM EDTA, and 8% methanol (v/v) adjusted to pH 3.6. The buffer solution was filtered through a $0.45\ \mu\text{m}$ membrane filter and degassed. The flow rate was set to 1.7 ml/min, and pressure was $\sim 2000\ \text{psi}$. The mobile phase was recycled for 2 weeks of continuous use before being replaced with fresh solution. The entire chromatographic system was run at ambient temperature. Peaks of biogenic amines and metabolites were identified by comparing the retention time of each peak in the sample solution with that in the standard solution. Dihydroxybenzylamine (DHBA) was used as internal standard for extraction variability, being added to the HPLC samples just before homogenization. The program System Gold 2.01 (Beckman) was used to calculate monoamines levels in each sample. The intrastriatal contents of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were quantified (Espejo and Miñano, 2001). Dopamine turnover was estimated by the

DOPAC–DA ratio. Neurochemical data were compared by using nonparametric Kruskal–Wallis tests followed by *post hoc* Mann–Whitney *U* tests.

Determination of neurotrophic factors by ELISA. For determination of tissue GDNF and TGF- β_1 proteins, tissue from left striata were sonicated in a homogenization buffer containing 150 mM NaCl, 50 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, $0.6\ \mu\text{M}$ leupeptin, and 1% Triton X-100, pH 7.4, at a tissue concentration of 50 mg/ml (wet weight per volume). Tissue levels of GDNF and TGF- β_1 were determined from tissue homogenates by ELISA using commercial kits, according to supplier's recommendations (G1230, G3520; Promega, Madison, WI). The results were compared by using nonparametric Kruskal–Wallis test followed by *post hoc* Mann–Whitney *U* tests.

RESULTS

Induction of parkinsonism

Rats were rendered hemiparkinsonian by injecting 6-hydroxydopamine, a toxin that destroys dopaminergic neurons, into the left substantia nigra. Those animals that showed a strong ipsilateral rotational behavior after the administration of amphetamine (>420 turns per hour), indicative of the destruction of $>85\%$ of the dopaminergic neurons in the substantia nigra (Fornaguera et al., 1994), were selected for grafting. These animals presented an overt hemiparkinsonian syndrome characterized by drug-induced turning, forepaw use asymmetry, and sensorimotor neglect.

Structure of the Zuckerkandl's organ, adrenal medulla, and grafts

Hemiparkinsonian rats were grafted with aggregates of extra-adrenal or adrenal cells 7 d after the amphetamine test. Cell aggregates instead of dispersed cells were used because it is known that the viability of grafted cells improves with the use of pieces of tissue (Björklund et al., 1980; Espejo et al., 1998; Luquin et al., 1999). Grafts were aimed at the dorsal striatum. To discern the number of grafted chromaffin cells per rat, stereological measurements were made. Thus, the estimate of the volume of the Zuckerkandl's organ ($n = 5$) was $9.5 \pm 1.1\ \text{mm}^3$ (mean $\pm$ SEM; mean CE = 0.17), and the estimate of the volume of chromaffin tissue inside the organ was $2.1 \pm 0.3\ \text{mm}^3$ (22% of the organ; mean CE = 0.19). The estimate of the number of chromaffin cells, as measured by the physical disector method, was $45,500 \pm 512$ cells (mean CE = 0.15). Because the mean volume of the pieces used for transplantation was $0.75 \pm 0.1\ \text{mm}^3$, and assuming the same percentage volume of chromaffin tissue in the randomly chosen pieces as that in the organ (22%, $0.16\ \text{mm}^3$), the estimated number of grafted chromaffin cells was 3466 ± 213 cells per graft. Regarding the adrenal gland, the estimate of the volume of the adrenal medulla ($n = 5$) was $8.2 \pm 1.2\ \text{mm}^3$ (mean $\pm$ SEM; mean CE = 0.18), and the estimate of the number of adrenal chromaffin cells, as measured by the physical disector method, was $219,806 \pm 21,847$ cells (mean CE = 0.14). Because the mean volume of the pieces used for transplantation was $0.74 \pm 0.2\ \text{mm}^3$, the estimated number of grafted adrenal chromaffin cells was $19,836 \pm 1963$ cells per graft.

Neurotransmitter phenotypic expression in chromaffin cells of pieces of paraganglia and adrenal medulla used for grafting was studied by staining for immunofluorescence to TH, the dopamine synthesizing enzyme, and DBH, the noradrenaline synthesizing enzyme. As revealed by confocal fluorescent microscopy of sections taken from the paraganglia used for transplantation, the Zuckerkandl's organ contained rounded or polymorphic TH+/DBH+ chromaffin cells (20–30 μm in diameter) and mesenchyma (Fig. 1), and the adrenal medulla contained many rounded TH+/DBH+ chromaffin cells (15–25 μm in diameter) (Fig. 1d).

Figure 1. Morphological features of the Zuckerlandl's organ (*a–c*) and adrenal medulla (*d*). *a*, Coronal section of the Zuckerlandl's organ used for transplantation, without enzymatic treatment, after double labeling for TH (red) and DBH (green). Confocal fluorescent micrograph shows dispersed cell groups and cell nests (*Cn*) of noradrenergic chromaffin cells (TH+/DBH+; yellow-green) surrounded by mesenchyma (*Ms*). *b*, Higher magnification of a cell nest labeled for DBH showing many DBH+ rounded chromaffin cells of 20–30 μm in diameter (green). *c*, Higher magnification of chromaffin cells with green and yellow patches in the cytoplasm indicative of the presence of DBH and TH-DBH, respectively, and hence norepinephrine. *d*, Piece of an adrenal medulla used for transplantation, without enzymatic treatment, after double labeling for TH and DBH immunofluorescence. Confocal fluorescent micrograph shows many rounded chromaffin cells (15–25 μm in diameter) with green and yellow patches in the cytoplasm, indicative of the presence of DBH and TH-DBH. Scale bars: *a*, 200 μm ; *b*, *d*, 100 μm ; *c*, 50 μm .

In the Zuckerlandl's organ, TH+/DBH+ chromaffin cells were found to be associated in cell clusters and bigger “cell nests” (Fig. 1*a*, yellow-green color), in accordance with other authors (Testut and Latarjet, 1978; Bohn et al., 1982), and dopaminergic or TH+/DBH– cells were not observed (absence of red fluorescent cells). Fluorescent microscopy hence confirmed that the Zuckerlandl's organ belongs to the type of extra-adrenal chromaffin paraganglia with mesenchyma (Testut and Latarjet, 1978), and that its chromaffin cells are not dopaminergic, but noradrenergic and/or adrenergic.

Progressive amelioration of parkinsonian deficits after grafts of the extra-adrenal Zuckerlandl's organ, and transient recovery after adrenal cell transplants

One month after grafting, drug-induced turning was reduced by 36% (Zuckerlandl group, nonsignificant) and 65% (adrenal group, $p < 0.05$), and the right forepaw use ratio was not significantly different from controls in both grafted groups. Sensorimotor deficits were significantly ameliorated in both groups (whisker test, 77%, Zuckerlandl; 72.2% adrenal; odor test, 60%, Zuckerlandl; 75%, adrenal) versus sham-grafted parkinsonian rats ($p < 0.01$). However, although behavioral recovery was transient in rats grafted with adrenal cells, functional recovery was sustained and gradually enhanced over time in the Zuckerlandl group, as observed in Figure 2. Thus, the adrenal group showed a significant recovery in motor and sensorimotor deficits up to the second month after grafting, but thereafter the salutary effects wore off, the rats' behavior being similar to that found in sham-grafted parkinsonian rats that did not otherwise show any significant amelioration. Regarding the Zuckerlandl group, 5 months after transplantation, drug-induced turning was significantly ameliorated by 80% ($p < 0.01$), right forepaw use ratio was 42% ($t = 14.2$; $p < 0.01$ vs sham-grafted and adrenal groups without significant differences with control nonparkinsonian rats), and sensorimotor orientation fully recovered (whisker, 91.2%; odor, 94% vs sham-grafted rats, without significant differences with controls). Noteworthy, the degree of functional recovery depended on the intensity of the parkinsonian syndrome, as measured through the number of drug-induced turnings. Thus, motor asymmetry was completely abolished in 5 of 12 rats, those with a number of rotations $< 650/\text{hr}$ after nigra lesion.

Striatal reinnervation and long survival of grafted extra-adrenal cells of the Zuckerlandl's organ

At 5 months after grafting, different degrees of TH-positive reinnervation of the grafted striatum were observed in rats with Zuckerlandl's organ grafts (Fig. 3*a,b*). Thus, stereological methods revealed that the mean percentage of volume of reinnervation was 68.3% ($26 \pm 0.3 \text{ mm}^3$ of reinnervated area of a mean dorsal striatal volume of $38.1 \pm 0.4 \text{ mm}^3$; mean CE = 0.12). Densitometry measurements also showed a significant increase in striatal TH innervation in the grafted striatum, although to a lesser degree, from $12.8 \pm 1\%$ of normal in denervated sham-grafted striata (Fig. 3*e*) to $49.9 \pm 12\%$ of normal in grafted animals, as shown in Table 1. Grafts were stained with TH and DBH antisera or thionin, being located in the dorsal striatum presenting a fusiform or oval morphology (Fig. 3). Immunohistochemistry staining revealed the presence of TH+ and DBH+ chromaffin cells inside grafts, many of them with long neuritic processes emerging from the soma (Fig. 3*c*). Confocal fluorescent microscopy also showed the presence of DBH+ cells and neuritic processes running out the graft into the surrounding striatum (Fig. 3*d*). No grafted cells were observed out of the grafting site. The mean number of chromaffin cells inside the grafts was 461 ± 56 cells at this stage, and considering that grafted paraganglion pieces contained 3466 ± 213 chromaffin cells, this indicates that $\sim 13.3\%$ of chromaffin cells survived the grafting trauma. The number of surviving transplanted cells in five grafted rats as measured through stereology is shown in Table 1. In contrast, at 5 months after grafting, brain sections with adrenal grafts stained for immunohistochemistry to TH revealed the presence of grafts with a necrotic appearance without surviving TH+ chromaffin cells, and lack of TH+ positivity of the host striatum, except for the presence of a TH+ halo in the immediate neighborhood of the graft and, in some cases, a small TH+ striatal area at the border of the left ventricle, as shown in Figure 3*f*.

Grafted chromaffin cells of the Zuckerlandl's organ express and release GDNF and TGF- β_1

To detect if grafted extra-adrenal chromaffin cells expressed neurotrophic factors, striatal sections containing grafts ($n = 3$) were stained for immunoreactivity to the neurotrophic factors

Figure 2. Time course of drug-induced turning, right forepaw use (cylinder test), and sensorimotor orientation (whisker-touch and odor tests), after grafts of the Zuckerkandl's organ and adrenal tissue. Groups: ●, control (nonparkinsonian rats); ■, 6-OHDA-induced lesion and Zuckerkandl's organ graft (Zuckerkandl group); ▲, 6-OHDA-induced lesion and sham graft (sham-grafted group); and ▼, 6-OHDA-induced lesion and adrenal transplant (adrenal group). Two-way ANOVA for repeated measures indicated significant group (3, 41 df) and interaction effects (15, 210 df) for drug-induced turning (group, $F = 12.4$; interaction, $F = 7.3$; $p < 0.001$), whisker-touch latency (group, $F = 133.2$; interaction, $F = 36.7$; $p < 0.001$), and odor latency (group, $F = 80.8$; interaction, $F = 26.6$; $p < 0.001$). Maximum latency in sensorimotor tests, 25 sec. Mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ versus sham-grafted rats (Newman-Keuls test, or Student's t test in the cylinder test). *pre*, Before lesion; *les*, 2 weeks after lesion; *1m*, *2m*, *3m*, *5m*, 1, 2, 3 and 5 months after extra-adrenal, adrenal, or sham grafting, respectively; *ctrl*, control group; *adrl*, adrenal group; *Zuck*, Zuckerkandl group; *sham*, sham-grafted group. Grafts were implanted 2 weeks after nigra lesion (arrow). The cylinder test corresponds to values 5 months after grafting.

GDNF, TGF- β_1 , and aFGF. Grafted paraganglionic cells presenting GDNF and TGF- β_1 positivity, but not aFGF, were observed as shown in Figure 4. This indicates that extra-adrenal chromaffin cells expressed the neurotrophic factors GDNF and TGF- β_1 after grafting *in vivo*. The presence of these neurotrophic factors in host striatal tissue ($n = 4$) was then discerned by means of ELISA. Detectable levels of GDNF and TGF- β_1 proteins were measured in the left striatum of grafted parkinsonian rats, as shown in Table 2. GDNF and TGF- β_1 protein levels were threefold and fivefold higher, respectively, in the striatum of grafted rats in comparison with that of sham-grafted rats ($U = 0$; $p < 0.05$). These findings indicate that extra-adrenal chromaffin cells chronically released these neurotrophic factors into the host striatum after grafting.

Dopamine neurotransmission is improved in denervated striatum after grafts of the Zuckerkandl's organ

To establish whether functional recovery in rats was linked to enhanced levels of intrastriatal dopamine, postmortem contents of this amine and their metabolites DOPAC and HVA

Figure 3. Morphological features of 5-month-old transplants of the Zuckerkandl's organ (*a–d*), as well as striatal appearance 5 months after sham-grafting (*e*) or adrenal cell transplants (*f*). *a*, *b*, Coronal sections of the striatum grafted with extra-adrenal cells of the Zuckerkandl's organ where a TH-positive transplant (arrow) and a broad TH+ reinnervated area in the host left striatum (brown) can be clearly observed. *c*, Higher magnification of the transplant labeled for DBH where DBH+ chromaffin cells (arrows) with long neuritic processes (arrowheads) are observed. *d*, Confocal fluorescent micrographs of the graft revealing the presence of DBH+ cells (green, arrows) and many neuritic processes running out of the graft. *e*, Coronal section of a sham-grafted striatum showing the nearly absent TH positivity in the left denervated striatum. *f*, Coronal section through the brain striatum with an adrenal medullary transplant, where a necrotic graft can be observed (arrow), as well as lack of striatal TH positivity except for a halo of TH positivity surrounding the graft, and a small TH+ area at the border of the left ventricle. *g*, Graft. Scale bars: *a*, *b*, *e*, *f*, 1 mm; *d*, 100 μ m; *c*, 50 μ m.

were measured by HPLC in left striata of four rats per group (Table 3). Denervated striata of sham-grafted and adrenal groups showed very low levels of DA (–82, –75.6%, respectively, vs normal striata) and strongly enhanced levels of DOPAC and HVA, DA metabolites (sham = DOPAC, +361.9%; HVA, +318.3%; adrenal = DOPAC, +332.4%; HVA, +330.3%). Besides, DA “turnover” was strongly enhanced (sham, +2,541%; adrenal, +1,712%). In this context, it is supposed that the increases in DOPAC and HVA levels and DA “turnover” reflect enhanced dopamine release and metabolism in the surviving DA neurons (Robinson and Wishaw, 1988; Sarre et al., 1994; Wachtel and Abercrombie, 1994). After extra-adrenal grafting, the striatal content of dopamine was significantly enhanced (+140.7%), and the levels of DOPAC and HVA were reduced (DOPAC, –40.2%; HVA, –47.5%) versus sham-grafted denervated striata. The DA

Table 1. Densitometry measurement for TH+ signal in the left striatum of sham-grafted animals and rats with extra-adrenal cell grafts, along with the estimate of the number of surviving extra-adrenal cells at 5 months after grafting

	Individual values					Mean $\pm$ SEM
Sham graft ($n = 5$)						
TH+ density	13.7	11.2	9.7	14.4	15.2	12.8 $\pm$ 1
Extra-adrenal cell graft ($n = 5$)						
TH+ density	27.3	66.7	89.1	34.1	32.3	49.9 $\pm$ 12
Surviving grafted cells	381	489	655	328	452	461 $\pm$ 56

The sections taken from five sham-grafted brains used for TH immunohistochemistry and five brains with extra-adrenal cell grafts of the Zuckerkandl's organ (used for Nissl staining and TH/DBH immunohistochemistry and immunofluorescence) were analyzed. Densitometry values are expressed as a percentage of the intact contralateral striatum in every rat. The estimate of the number of surviving chromaffin cells was calculated by using estereological methods.

“turnover” was also reduced (-75%), indicating an improvement of DA neurotransmission and metabolism in grafted striata. In comparison with dopaminergic levels within non-denervated normal striata of control rats, the Zuckerkandl's organ grafts induced DA levels to be increased by 43% of normal at 5 months after grafting (a quite similar percentage value to that obtained for TH+ density in grafted striata).

DISCUSSION

Long-term functional recovery after extra-adrenal cell grafts of the Zuckerkandl's organ versus transient recovery after adrenal cell grafts

Remarkably, cell aggregates grafts of the extra-adrenal Zuckerkandl's organ induced a progressive and sustained functional recovery in parkinsonian rats. These beneficial effects were related to the survival of 13.3% of grafted extra-adrenal chromaffin cells, a finding otherwise quite similar to the survival rate of grafted mesencephalic dopamine neurons (3–20%; Brundin et al., 2000). This indicates that those grafted chromaffin cells that survived the grafting trauma then survived for a long period, making them suitable for transplantation. Although the survival rate can be considered as low, representing a potential limitation for the therapeutic value of these grafts, it is always possible either to increase the amount of implanted tissue or, as revealed by studies with other grafting cells such as neural ones, to treat the grafting tissue before transplantation with antioxidants such as lazaroids or neurotrophic factors to increase the survival rate of grafted cells (Brundin et al., 2000). These approaches could be applied to Zuckerkandl's organ grafts, a fact that requires further investigation in animal Parkinson's models. However, of note is that the long-surviving grafted cells induced an important functional recovery, together with a significant enhancement of TH+ density in the host striatum (indicative of dopaminergic reinnervation), and a reliable increase in the intrastriatal dopaminergic

content, as measured through HPLC. This latter fact surely led to functional improvements of drug-induced turning, forepaw use, and sensorimotor orientation in parkinsonian rats, which are dependent on the recovery of the dopaminergic tone of dorsal striatum (Björklund et al., 1980; Brundin et al., 1987). An improvement of dopamine metabolism was also revealed by HPLC through the reduction of dopamine “turnover,” which was found to be highly enhanced in denervated striata, surely as a reactive response of surviving nigra neurons (Robinson and Whishaw, 1988; Sarre et al., 1994; Wachtel and Abercrombie, 1994). The increase of striatal dopaminergic content after grafting cannot be explained by dopamine cell release, because grafted cells were not dopaminergics (TH+/DBH– cells), and it is known that only minute amounts of dopamine can be released from TH+/DBH+ adrenal and extra-adrenal chromaffin cells, as reported by other authors (Lyon et al., 1987; Missale et al., 1988; Pupilli et al., 1994). The findings indicate that the improved dopaminergic tone after grafting can be better accounted for by striatal regeneration because of sprouting of spared dopaminergic fibers, as indicated by the broad TH+ area and the enhanced TH+ density in the grafted striata. This is consistent with the time course of functional recovery, which developed progressively over the 5 months after the lesion, as seen in motor and sensorimotor tests. A possible participation on sprouting of the neuritic processes arising from grafted extra-adrenal cells cannot be discarded, although its number was limited and cannot account for all the gain of diffuse (and distant to the graft) TH staining in the striatum.

In contrast, adrenal medullary transplants only induced a transiently behavioral recovery, and the histological study revealed that, at 5 months after grafting, grafts presented a degenerate appearance, and there were no surviving grafted cells. This finding is in accordance with previous studies demonstrating that adrenal chromaffin cells present a poor survival in the brain and induce a transient amelioration in parkinsonian rats (Yurek and

Figure 4. Immunohistochemistry for GDNF and TGF- β_1 in transplants of the Zuckerkandl's organ at 5 months after grafting, showing the presence of GDNF-positive (*a*) and TGF- β_1 -positive chromaffin cells (*b*). *g*, Graft. Scale bars, 100 μ m.

Table 2. GDNF and TGF- β_1 protein levels (nanograms per milligram of tissue) measured by ELISA in punches from left striatum in controls, sham-grafted animals, and rats with grafts of adrenal cells or extra-adrenal cells of the Zuckerkandl's organ (at 5 months after grafting)

Group	GDNF	TGF- β_1
Control ($n = 4$)	0.03 $\pm$ 0.02	0.04 $\pm$ 0.01
Sham-grafted ($n = 4$)	0.06 $\pm$ 0.02	0.05 $\pm$ 0.02
Adrenal ($n = 4$)	0.04 $\pm$ 0.02	0.06 $\pm$ 0.02
Zuckerkandl ($n = 4$)	0.19 $\pm$ 0.08*	0.24 $\pm$ 0.09*

Mean $\pm$ SEM. Kruskal–Wallis indicated significant group effects (GDNF, $H = 9.9$; TGF- β_1 , $H = 9.4$; $p < 0.01$).

* $p < 0.05$ versus the other three groups (Mann–Whitney U test).

Table 3. Levels of dopamine, DOPAC, and HVA (micrograms per gram wet weight tissue) and DOPAC/DA ratio in punches from left striatum in controls, sham-grafted animals, and rats with grafts of adrenal cells or extra-adrenal cells of the Zuckerkandl's organ (at 5 months after grafting)

Group	DA	DOPAC	DOPAC/DA	HVA
Control ($n = 4$)	6.02 ± 0.5	1.05 ± 0.1	0.17 ± 0.01	1.42 ± 0.1
Sham-grafted ($n = 4$)	$1.08 \pm 0.2^{***}$	$4.85 \pm 0.5^{***}$	$4.49 \pm 0.7^{***}$	$5.94 \pm 0.6^{***}$
Adrenal ($n = 4$)	$1.47 \pm 0.2^{***}$	$4.54 \pm 0.3^{***}$	$3.08 \pm 0.6^{***}$	$6.11 \pm 0.7^{***}$
Zuckerkandl ($n = 4$)	$2.6 \pm 0.3^{***}$	$2.91 \pm 0.3^{***}$	$1.12 \pm 0.1^{***}$	$3.12 \pm 0.4^{***}$

Mean $\pm$ SEM. Kruskal–Wallis test indicated significant group effects (DA and DOPAC, $H = 9.8$; ratio, $H = 10.1$; HVA, $H = 9.9$; $p < 0.01$).

* $p < 0.05$ versus sham-grafted and adrenal groups; ** $p < 0.05$; *** $p < 0.01$ versus control group (Mann–Whitney U test).

Sladek, 1990; Bresjenac et al., 1997). Furthermore, the dopaminergic content of grafted striata remained very low, and there were no signs of striatal reinnervation, except for a halo of TH+ fibers immediately around the graft. This perigraft sprouting has already been observed after adrenal medullary grafts implanted in the striatum (Bohn et al., 1987; Bankiewicz et al., 1994; Bresjenac et al., 1997), and it has been demonstrated to be attributable to infiltration of microglia and macrophages that exert a limited trophic action that persists for many months after injury or grafting (Batchelor et al., 1999). In this context, in dead parkinsonian patients with 3- to 40-month-old adrenal medullary grafts, autopsy revealed that there was often a perigraft dopaminergic sprouting response without surviving adrenal cells inside the graft (Hirsch et al., 1990; Kordower et al., 1991). The data of the present study hence indicate that transient recovery after adrenal transplants would be accounted for by the beneficial action of adrenal chromaffin cells before cell death and graft degeneration.

Grafted extra-adrenal cells of the Zuckerkandl's organ express and release GDNF and TGF- β_1 , and induce striatal regeneration

It is well known that intrinsic dopaminergic reinnervation of the denervated striatum is a constant feature after grafting chromaffin cells (Freed et al., 1981; Bohn et al., 1987; Espejo et al., 1998; Dunnett and Björklund, 1999; Luquin et al., 1999). The broad TH+ reinnervation measured in striata with extra-adrenal chromaffin cells suggests that grafted cells exerted a trophic action on the denervated striatum leading to sprouting of host dopaminergic fibers. In this context, the mechanisms underlying the trophic action of chromaffin cells of the adrenal lineage is poorly understood, but it has been recently shown that adrenal medullary cells contain and release trophic factors such as GDNF and TGF- β_1 , both *in vitro* and *in vivo* (Unsicker and Krieglstein, 1995, 1996; O'Connor, 1999; Combs et al., 2000). GDNF is an extraordinarily potent neurotrophic factor (active at picomolar concentrations) which, when delivered by injection or via transplanted cells or viruses, protects dopaminergic neurons in animal models of Parkinson's disease (Lin et al., 1993; Beck et al., 1995; Tomac et al., 1995; Tseng et al., 1997; Gash et al., 1998; Hagg, 1998; Krieglstein et al., 1998; Rosenblad et al., 1999; Kirik et al., 2000; Aebischer and Ridet, 2001). TGF- β_1 is also known to protect dopaminergic neurons when delivered *in vitro* (Unsicker et al., 1996) and, interestingly, TGF- β_1 is an important cofactor that potentiates the neurotrophic actions of GDNF *in vitro* and *in vivo* (Krieglstein et al., 1998; Schober et al., 1999). The immunohistochemical data of the present study revealed that grafted extra-adrenal chromaffin cells also expressed GDNF and TGF- β_1 , and significant levels of these neurotrophic factors were detected in the striatal tissue as measured by ELISA, strongly suggesting that extra-adrenal chromaffin cells chronically released them after

grafting *in vivo*. Hence, the well known neurorestorative action of GDNF on dopaminergic neurons, together with a TGF- β_1 -induced potentiation of GDNF activity, could account for the striatal reinnervation of the host tissue. Clearly, the main advantage of grafts of the Zuckerkandl's organ appears to be the long survival of extra-adrenal chromaffin cells (in contrast to adrenal chromaffin cells) that allow them to exert a chronic trophic action based on the delivery of GDNF and TGF- β_1 , and likely other neuroprotective agents such as neuropeptides and cytokines that are known to be released by chromaffin cells as well (Unsicker, 1993). These findings strongly enhance the potential therapeutic value of the grafts of the Zuckerkandl's organ. Another advantage of grafts of the Zuckerkandl's organ is that there are two paraganglia in humans, hence the surgical resection of one organ would not have significant side effects.

In conclusion, our study has documented that intrabrain grafts of the Zuckerkandl's organ, an extra-adrenal paraganglion without dopaminergic cells, induced progressive and sustained improvement of functional deficits in parkinsonian rats. In contrast, grafts of adrenal chromaffin cells only induced a transient recovery. The beneficial effects of grafts of the Zuckerkandl's organ were related to long survival of grafted cells, striatal reinnervation, enhancement of dopamine levels in the host striatum, and the cell delivery into the host striatum of glial cell line-derived neurotrophic factor and transforming growth factor- β_1 . These neurotrophic factors have neurorestorative properties on dopaminergic neurons, which could account for the tissue regeneration. Because more available and less controversial alternative sources for antiparkinsonian therapy need to be developed (Jennings, 2000), this work should stimulate research on the clinical applicability of transplants of the Zuckerkandl's organ in Parkinson's disease.

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