Modification of the number and phenotype of striatal dopaminergic cells by carotid body graft

W. San Sebastián,1 J. Guillén,2 M. Manrique,1,3 S. Belzunegui,1 E. Ciordia,2 A. Izal-Azcárate,1 P. Garrido-Gil,1 M. Vázquez-Claverie1 and M. R. Luquin1,3

1Laboratory of Regenerative Therapy, 2Animal Services Unit, Center for Applied Medical Research (CIMA) and 3Department of Neurology and Neurosurgery, Clínica Universitaria de Navarra, University of Navarra, Avenida de Pío XII, 55 Pamplona Navarra, Spain

Correspondence to: M. Rosario Luquin, Laboratory of Regenerative Therapy, Center for Applied Medical Research (CIMA), University of Navarra, Avenida de Pío XII, 55, Pamplona, Navarra, Spain
E-mail: rluquin@unav.es

In non-human primates, striatal tyrosine hydroxylase-immunoreactive (TH-ir) cells are increased in number after dopamine depletion and in response to trophic factor delivery. As carotid body cells contain the dopaminotrophic glial cell line-derived neurotrophic factor (GDNF), we evaluated the number, morphology and neurochemistry of these TH-ir cells, in the anterior and posterior striatum of five monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which received a graft of carotid body cell aggregates (CBCA) (n = 3) or sham surgery (n = 2), and six MPTP-monkeys that were sacrificed 6 months and 3 years after the last MPTP dose [MPTP I (n = 3) and MPTP II (n = 3), respectively]. Three intact monkeys served as controls. A disability rating scale was used for the assessment of parkinsonism in all lesioned animals, both before and after surgery. For the neurochemical examination, tissue sections were double-labelled with antibodies to TH, dopamine transporter, dopa decarboxylase-67, vesicular monoamine transporter 2, glutamic acid decarboxylase-67, calbindin, parvalbumin, calretinin, neuronal nitric oxide synthase and GDNF. Only animals receiving CBCA graft showed a moderate but significant recovery of parkinsonism that persisted 12 months after the graft. The grafted striatum contained the greatest TH-ir cell density (120.4±6.3 cells/100 mm²), while the control striatum displayed the lowest (15.4±6.8 cells/100 mm²), and MPTP I, MPTP II and sham-operated monkeys showed a similar intermediate value (66.1±6.2, 58.3±17.2 and 57.7±7.0 cells/100 mm², respectively). In addition, in the post-commissural striatum, only CBCA graft induced a significant increase in the TH-ir cell density compared to control animals (47.9±15.9 and 79±3.2, respectively). Phenotypically, TH-ir cells were striatal dopaminergic interneurons. However, in the grafted animals, the phenotype was different from that in control, MPTP and sham-operated monkeys, with the appearance of TH/GDNF-ir cells and the emergence of two TH-ir subpopulations of different size as the two main differentiating features. Our data confirm and extend previous studies demonstrating that striatal CBCA grafts produce a long-lasting motor recovery of MPTP-monkeys along with an increase in the number and phenotype changes of the striatal TH-ir interneurons, probably by the action of the trophic factors contained in carotid body cells. The increased number of striatal TH-ir cells observed in the grafted striatum may contribute to the improvement of parkinsonism observed after the graft.

Keywords: striatum; Parkinson’s disease; dopaminergic cells; carotid body graft; Macaca fascicularis

Abbreviations: BDNF = brain derived neurotrophic factor; CaBP = calbindin-28kD; CB = carotid body; CBCA = carotid body cell aggregates; CR = calretinin; DA = dopamine; DAT = dopamine transporter; DDC = dopamine decarboxylase; GABA = gamma-aminobutyric acid; GAD67 = glutamic acid decarboxylase 67; GDNF = glial cell line-derived neurotrophic factor; IF = immunofluorescence; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGF = nerve growth factor; nNOS = neuronal nitric oxide synthase; 6-OHDA = 6-hydroxydopamine; PBS = phosphate buffer saline; PD = Parkinson’s disease; PV = parvalbumin; SVZ = subventricular zone; TH = tyrosine hydroxylase; TH-ir = tyrosine hydroxylase-immunoreactive; VMAT2 = vesicular monoamine transporter 2

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Introduction

The striatum is the major input nucleus of the basal ganglia and is primarily composed of GABAergic spiny projection neurons in both rodents (95%) and primates (77%) (Graveland and DiFiglia, 1985). The remaining striatal neurons are interneurons that have been classified into a diversity of morphological and neurochemical subtypes (Kawaguchi et al., 1995; Wu and Parent, 2000). Among this variety of interneurons, there is a small cell population which is immunoreactive for tyrosine hydroxylase (TH-ir). These cells were first described by Dubach et al. (1987) in the striatum of non-human primates, subsequently in rats (Tashiro et al., 1989a, b; Mura et al., 1995) and ultimately in the striatum of mice and humans (Cossette et al., 1999, 2003, 2005a; Prensa et al., 2000; Baker et al., 2003; Huot et al., 2007). These striatal dopaminergic (DAergic) cells are particularly abundant in the head of the caudate nucleus and pre-commissural putamen (Betarbet et al., 1997). Interestingly, the number of TH-ir cells is increased up to 3-fold in monkeys chronically exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Betarbet et al., 1997; Smith and Kieval, 2000; Palfi et al., 2004; Mazloom and Smith, 2006), in 6-hydroxydopamine (6-OHDA) rats (Tashiro et al., 1989b; Meredith et al., 1999; Lopez-Real et al., 2003; Jollivet et al., 2004) and in patients with Parkinson’s disease (PD) (Porritt et al., 2000; Huot et al., 2007). However, the significance of these striatal cells in the function of the basal ganglia in health and in PD still remains controversial.

There is a general agreement that these TH-ir cells are striatal interneurons. The vast majority of them are aspiny medium-sized bipolar cells and a small percentage (<1%) are spiny multipolar cells (Betarbet et al., 1997). Additionally, double immunofluorescence studies have revealed that TH-ir cells colocalize with glutamic acid decarboxylase (GAD), thus showing their striatal origin, and dopamine transporter (DAT), confirming their DAergic nature. However, some of the most common markers of the striatal neurons such as calcium-binding proteins or nitric oxide synthase are not expressed by these DAergic cells (Betarbet et al., 1997).

Trophic factors, such as nerve growth factor (NGF) or glial cell line-derived neurotrophic factor (GDNF), are proven to exert a strong influence on the survival and proliferation of the striatal TH-ir cells (Jollivet et al., 2004; Kishima et al., 2004). In fact, lentiviral delivery of GDNF into the striatum of MPTP-monkeys has been shown to elicit a further enhancement in the number of these cells with no significant changes in their neurochemical profile (Palfi et al., 2002).

Carotid body (CB) has been used as donor of DAergic cells in PD patients, since it is composed of glomus cells that contain and secrete dopamine and trophic factors such as GDNF (Nosrat et al., 1996). Previous studies from our own laboratory and from other groups have demonstrated that striatal grafts of carotid body cell aggregates (CBCA) elicit a marked motor improvement in animal models and PD patients (Espejo et al., 1998; Luquin et al., 1999; Hao et al., 2002; Toledo-Aral et al., 2002; Arjona et al., 2003; Shukla et al., 2004; Villadiego et al., 2005). We here investigate the long-term recovery of MPTP-monkeys grafted with CBCA as well as the number and phenotype of the striatal TH-ir cells contained in the grafted striatum and their possible implication in the alleviation of parkinsonism.

Materials and methods

Animals

Fourteen macaques (Macaca fascicularis) were included in the study. Eleven animals were rendered parkinsonian by systemic administration of MPTP (Sigma) until they developed stable and severe parkinsonism (one weekly injection of 0.1–0.6 mg/kg for a cumulative dose of 5–12 mg/kg). All MPTP-treated animals completed a period of lesion stabilization (3 months) to ensure that the motor deficit would not experience a spontaneous recovery (Rose et al., 1989). Three of them were subsequently treated with a striatal, unilateral autotransplant of CBCA (CYN-U1, CYN-U3 and CYN-U4, grafted group), and two other MPTP-monkeys underwent sham surgery (sham-operated group). One received unilateral sham injection and the other was bilaterally operated. The other six animals were also chronically exposed to MPTP but received neither graft nor sham surgery. These animals remained drug-free during the experiment until sacrifice 6 months (n = 3, MPTP I group) and 35 months (n = 3, MPTP II group) after the last MPTP injection. In the following sections (Results and Discussion), and whenever MPTP I and MPTP II groups are included in a unique group, they will be referred to as ‘MPTP-monkeys’. Similarly, as TH-ir cells of sham-operated group behave like those of MPTP-monkeys, they are also included in that group (morphological and neurochemical data). Animals were housed in an animal room under standard conditions of air exchange (161/min), humidity (50%), and light/night cycles (8 a.m. to 8 p.m.), and were fed fresh fruit and commercial pellets, with free access to water. Experimental protocols were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the care and use of animals for experimental procedures, and under guidance of the Ethics Committee for Animal Experimentation of the University of Navarra.

Surgery

Surgical resection of the CB and the procedure followed for performing the striatal grafts of CBCA have been described elsewhere (Luquin et al., 1999). Briefly, after the surgical removal of the CB, it was divided into several small pieces and the cell aggregates thus obtained were stereotaxically injected into two different sites of the post-commissural putamen (7–9 aggregates per injection site). We chose this area because this is the motor region of this nucleus and this is the striatal region in which grafts and GDNF infusion have been performed in PD patients (Freed et al., 2001; Olano et al., 2003; Patel et al., 2005; Slevin et al., 2006). Sham-operated animals underwent identical surgical procedure but the CB was not removed and they received two...
injections of tyrode solution (physiological medium where aggregates were suspended) instead of CBCA. Animals that received either unilateral autotransplants of CBCA or tyrode were sacrificed 12 months after surgery.

**Behavioural assessment**

Motor deficits induced by MPTP were assessed according to a non-human primate disability rating scale, which independently scores from 0 (normal) to 3 (maximum disability) parkinsonian features such as tremor (intensity and duration), balance, feeding and freezing; from 0 (normal) to 4 (maximum disability) bradykinesia and posture, and from 0 (normal) to 5 (maximum disability) the reduction in spontaneous activity, thus giving a total maximum score of 28 (Luquin et al., 1999).

**Preparation of tissues**

After deep sedation with a mixture of ketamine (10 mg/kg) and midazolam (1 mg/kg), animals were transperurally perfused with 0.01 M phosphate buffer saline (PBS) and 4% paraformaldehyde (PFA, Sigma) in 0.01 M PBS. Brains were immediately removed, blocked and postfixed overnight in 4% PFA. They were then cryoprotected in a 30% sucrose solution in 0.01 M PBS until processing. Forty micrometer coronal tissue sections were cut on a freezing microtome (Leica, Germany) and collected in 0.125 M PBS containing 2% dimethylsulphoxide (Sigma) and 20% glycerin (Panreac). They were stored at –20°C until ulterior analysis.

**Histology**

Free-floating immunocytochemistry and immunofluorescence techniques were used for histological analysis of the brain tissue sections.

**Immunocytochemistry**

Tissue sections containing either the anterior or posterior striatum were washed in bidistilled water and 0.01 M PBS to remove the cryoprotectant solution and incubated in PBS with 0.02% hydrogen peroxide (H2O2) for peroxidase inhibition. After that, they were incubated in 0.01 M citrate buffer pH 6.0 for 30 min at 80°C for antigen retrieval. Then, tissue sections were rinsed in 0.1 M PBS and incubated in 5% normal goat serum with 0.2% Triton X-100 (Sigma) for 30 min. They were incubated overnight in the same solution containing a primary antibody to TH (polyclonal antibody, Table 1), rinsed in 0.01 M PBS and incubated for 30 min in 0.01 M PBS containing the corresponding secondary antibody. Subsequently, they were incubated with the Vector avidin–biotin complex (1 : 200, Vectastain Elite ABC kit, Vector Laboratories), for 30 min. Staining for peroxidase was performed in buffer acetate–imidazole (0.125 M acetate and 0.01 M imidazole, pH 7.2) using 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma), 0.001% H2O2 (Merck) and 0.1% nickel ammonium sulphate (BDH, UK). Finally, the sections were rinsed in bidistilled water, mounted on gelatin-coated slides and coverslipped using DPX (BDH). The following day, sections were counterstained with Nissl.

**Immunofluorescence**

Double-label immunofluorescence (IF) was performed to detect neurochemical markers, other than TH, in the striatal TH-ir cells.

### Table I: Primary antibodies used for immunocytochemical and immunofluorescent techniques

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host species</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td>Mouse</td>
<td>Chemicon Intl</td>
<td>1 : 25000</td>
</tr>
<tr>
<td>TH</td>
<td>Rabbit</td>
<td>Chemicon Intl</td>
<td>1 : 6250 (IF)</td>
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<td>DAT</td>
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<td>Sigma</td>
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<td>Mouse</td>
<td>Sigma</td>
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</tr>
<tr>
<td>VMAT2</td>
<td>Rabbit</td>
<td>Chemicon Intl</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>GAD67</td>
<td>Mouse</td>
<td>Chemicon Intl</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>CaBP</td>
<td>Mouse</td>
<td>Swant</td>
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<tr>
<td>CR</td>
<td>Rabbit</td>
<td>Sigma</td>
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<tr>
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<td>Sigma</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>nNOS</td>
<td>Rabbit</td>
<td>Chemicon Intl</td>
<td>1 : 1000</td>
</tr>
<tr>
<td>GDNF</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1 : 500</td>
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</table>


The following antibodies against different markers of striatal and DAergic neurons were used to better characterize these cells: DAT, GAD67, calbindin-28kD (CaBP), calretinin (CR), parvalbumin (PV), neuronal nitric oxide synthase (nNOS), dopa decarboxylase (DDC) and vesicular monoamine transporter 2 (VMAT2) (Table 1). In addition, we used anti-GDNF antibodies, as this is the most dopaminotropic factor of those contained in CB cells. Coronal striatal tissue sections were rinsed and permeabilized following the protocol used for peroxidase immunocytochemistry (see earlier), except for the DAT, which is located in the cytoplasmatic membrane. They were then incubated in 0.06% potassium permanganate to reduce the autofluorescence of primate tissues or lipofuscin. After some rinses, sections were incubated overnight at 4°C in a solution containing the following mixtures of primary anti-TH antibodies with anti-DDC, anti-VMAT2, anti-DAT, anti-GAD67, anti-nNOS, anti-CaBP, anti-CR, anti-PV or anti-GDNF antibodies (see Table 1 for antibody details). After rinsing with 0.01 M PBS, sections were incubated for 2 h in 0.01 M PBS containing normal goat serum (1 : 20) and the corresponding combination of secondary antibodies coupled to fluorescent markers Cy3, Alexa Fluor 568 or Alexa Fluor 488. Finally, sections were counterstained with a nucleic acid stain (TO-PRO-3 iodide, Molecular Probes, Netherlands) and coverslipped with mounting medium (Immuno-mount, Thermo-Shandon). Doubly labelled cells were detected in confocal images obtained by using a laser scanning microscope 510, equipped with three lasers (LSM 510/Meta; Zeiss, Germany).

**Cell quantification and morphological features of striatal TH-ir cells**

TH-ir cell counting was performed in both right and left striatum (caudate and putamen) with bright-field microscopy and using a 20× objective lens (Olympus BX51, Denmark). For each animal, cell counts were performed on eight anatomically matched brain tissue sections taken at different levels of the striatum: four sections containing the pre-commissural striatum (AP: from
CBCA graft effect on striatal TH-ir cells

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Results
Long-term recovery of parkinsonism in CBCA-grafted animals

All MPTP-treated animals developed a stable bilateral parkinsonism characterized by loss of spontaneous activity, bradykinesia, impairment of balance, resting tremor and freezing. The severity of parkinsonism remained stable along the survival time in the MPTP I, MPTP II and sham-operated groups with disability scales of 19 ± 5, 12 ± 2 and 13 ± 1, respectively. In contrast, disability motor score of the CBCA-grafted animals significantly decreased 3 months after grafting and remained stable until sacrifice 12 months later (18 ± 1 and 11 ± 1, respectively; P ≤ 0.05), indicating that CBCA autograft elicits a moderate and sustained long-term motor recovery.

Quantitative analysis of the striatal TH-ir cells

We only quantified the TH-ir structures with a Nissl-labelled nucleus to ensure they were indeed cells. TH-ir cells were found within the striatum of control animals, MPTP I, MPTP II, sham-operated groups and parkinsonian monkeys treated with unilateral autotransplant of CBCA.

The greatest density of striatal TH-ir cells was detected in the grafted striatum, while the striatum of control animals displayed the lowest density of TH-ir cells (Fig. 1). As previously reported, in the pre-commissural striatum of MPTP-monkeys, the density of striatal TH-ir neurons was increased up to 3-fold with respect to controls (P ≤ 0.05). In addition, MPTP I and MPTP II groups showed similar cellular density indicating that the increased number of striatal TH-ir cells is not a transient effect induced by the nigrostriatal damage but is maintained over a period of time as long as 3 years. Thus, in the control monkeys, the average cell density in the anterior striatum was 15.4 ± 6.8 cells/100 mm² on processed tissue sections, while in the MPTP I and MPTP II groups the average density of TH-ir cells was increased to 66.1 ± 6.2 and 58.3 ± 17.2 cells/100 mm², respectively. The average cell density in sham-operated animals was very similar to that observed in MPTP-monkeys (sham-operated: 57.7 ± 7.0, MPTP I: 66.1 ± 6.2 and MPTP II: 58.3 ± 17.2 cells/100 mm²) and significantly increased when compared to control group (57.7 ± 7.0 and 15.4 ± 6.8 cells/100 mm², respectively; P ≤ 0.05). In monkeys treated with unilateral graft of CBCA, the grafted striatum exhibited a significantly greater density of TH-ir cells than the non-grafted, the control, MPTP I, MPTP II and sham-operated striata (120.4 ± 10.3, 96.9 ± 12.7, 15.4 ± 6.8, 66.1 ± 6.2, 58.3 ± 17.2 and 57.7 ± 7.0 cells/100 mm², respectively; P ≤ 0.05). Interestingly, the striatal TH-ir cell density was also significantly enhanced in the non-grafted striatum of the animals that received a unilateral graft of CBCA compared with the intact, the denervated (MPTP I and II groups) and the sham-operated striatum, (96.9 ± 12.7, 15.4 ± 6.8, 66.1 ± 6.2, 58.3 ± 17.2 and 57.7 ± 7.0 cells/100 mm², respectively; P ≤ 0.05). In other words, unilateral CBCA grafts resulted in a bilateral increased number of striatal TH-ir cells.

In the post-commissural striatum, all experimental groups displayed a larger number of striatal TH-ir cells than control group (control: 7.9 ± 3.2, MPTP I: 23.4 ± 9.3, MPTP II: 24.2 ± 5.9, sham-operated: 23.1 ± 6.7 non-grafted striatum: 24.3 ± 9.5 and grafted striatum: 47.9 ± 15.9 cells/100 mm²). However, only in the grafted striatum, this value reached statistical significance (P ≤ 0.05). In other words, only in the grafted striatum the TH-ir cell density was significantly enhanced both in the pre- and post-commissural striatal region indicating a direct and spread effect of CBCA graft on the number of TH-ir cells.

Statistical analysis

Score of the disability scale, perikaryal diameter, perikaryal area and cell density were calculated independently for each experimental group. Comparisons among groups were made using the analysis of variance (ANOVA) and subsequent TukeyB multiple comparison tests and Kruskal–Wallis non-parametric ANOVA followed by the Mann–Whitney U-test for unpaired data when corresponding. Those statistical analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD). Probability values (P) smaller than or equal to 0.05 were considered to indicate significant differences.

In this formula m3 and m4 are the skewness and the kurtosis of the distribution, respectively. Values >0.555 indicate bimodal or multimodal distributions.

\[
\text{b} = \frac{m_3^2 + 1}{m_4 + \left(\frac{(k-1)^2}{k-3m-2}\right)}
\]

where m3 and m4 are the skewness and the kurtosis, respectively.
Morphological features and regional distribution of striatal TH-ir cells

We subsequently studied the distribution and morphology (perikaryal diameter and area) of the striatal TH-ir cells in the anterior striatum, where they are particularly abundant, using bright-field microscope images. In all groups of animals, almost all the TH-ir cells (99%) were aspiny and bipolar neurons with a round or oval perikaryon (Fig. 2) and one or more deep invaginations in the nucleus membrane (Fig. 3A). As has already been described, these TH-ir neurons displayed a particular pattern of distribution, being especially abundant in the rostro-dorsal region of the caudate nucleus and putamen (Dubach et al., 1987; Betarbet et al., 1997). However, in MPTP-monkeys, sham-operated and grafted animals, TH-ir cells were also detected in more medial and posterior regions of the striatum, although they still maintained their typical distribution pattern.

Interestingly, the morphological features of the striatal TH-ir neurons were slightly different in all the experimental groups. In the control animals, the striatal TH-ir neurons exhibited a soma of 9.55 ± 1.75 μm in diameter and 49.62 ± 16.35 μm² in area. We found no statistical differences in the perikaryal diameter of TH-ir cells among the experimental groups, although this parameter was slightly increased in grafted animals (Table 2 and Fig. 4A). In contrast, perikaryal area of TH-ir cells was significantly larger in MPTP-monkeys and in the contralateral striatum of the grafted animals. However, the perikaryal area of the TH-ir cells in the grafted striatum showed a non-significant increase as compared to the rest of the groups, probably due to a larger dispersion of data (Table 2 and Fig. 4B).

To analyse the perikaryal area of the striatal TH-ir cells in the normal human striatum, Cossette et al. (2005a) used the bimodality coefficient and discovered that the prevalent group of TH-ir cells comprises two neuronal subtypes: a group of small-sized neurons and another group of larger cells. Hence, we used the same bimodality coefficient to examine the data distribution of perikaryal diameter and area of the TH-ir cells in the striatum of all experimental groups (Table 3). We found a unimodal distribution of the TH-ir cells in the control, denervated and non-grafted striatum while, in the grafted striatum, the data distribution of both perikaryal diameter and area of TH-ir cells showed two peaks (modes). One peak corresponded to the small-sized cells and the other to a small pool of larger TH-ir cells (Fig. 4). This finding would explain the fact that the perikaryal size increase of the TH-ir cells of the grafted striatum was not statistically significant. Although in grafted monkeys the TH-ir cell density significantly increased in both striata, the presence of this small group of larger cells only in the grafted side suggests that the CBCA graft exerts a specific effect on the morphology of striatal TH-ir cells.

Neurochemical characterization of striatal TH-ir cells

In an attempt to better characterize the striatal TH-ir cells, we performed double-IF methods to confirm whether they also expressed other DAergic markers (DAT, DDC, VMAT2), some common markers of striatal neurons (GAD67, CaBP, PV, CR, nNOS) and also GDNF, since this molecule is contained in the CB glomus cells (Nosrat et al., 1996; Villadiego et al., 2005) and has...
a proven strong effect on the proliferation and survival of DAergic cells (Lin et al., 1993; Airaksinen and Saarivaara, 2002; Krieglstein, 2004).

As degenerating swollen DAergic axons might be mistaken for bipolar cell bodies, we firstly verified that all the TH-ir structures had TO-PRO3-positive nuclei confirming that they were indeed cells (Fig. 3B–D) (Cossette et al., 2005b; Tande et al., 2006). In all groups of animals, the totality of the striatal TH-ir cells expressed DDC and almost 50% expressed VMAT2. In addition, the vast majority of the TH-ir cells of MPTP-monkeys and control animals also expressed DAT, indicating that they can synthesize, release and uptake dopamine (Weihe et al., 2006). However, in grafted monkeys, the proportion of cells colocalizing TH/DAT was mildly decreased, (Table 4 and Fig. 5). In all animals, virtually all the striatal TH-ir cells expressed GAD67 and were negative for CaBP. Furthermore, in all animals, a large proportion of TH-ir striatal cell bodies expressed CR and around one-fourth of them stained for PV. On the other hand, in the striatum of control animals, no TH-ir cell expressed nNOS, but a few TH/nNOS-ir cells were found in the striatum of MPTP-monkeys and both striata of grafted animals (Table 4 and Fig. 5). These findings demonstrate that striatal TH-ir cells display the phenotype of some striatal interneurons. Finally, we found few TH-ir cells also labelled with GDNF in both striata of the CBBC-grafted animals. However, none of the striatal TH-ir cells in control and MPTP-monkeys expressed this neurotrophic factor (Table 4), which suggests that GDNF might act on striatal dopaminergic cells of the grafted striatum and consequently modulate their phenotypic profile (Villadiego et al., 2005).
Discussion

The results here presented demonstrate the long-term motor recovery (12 months) induced by CBCA graft in chronic MPTP-monkeys, along with the presence of remarkable changes in the number, morphological and neurochemical characteristics of the intrinsic striatal TH-ir cells. More importantly, our findings indicate that all of these effects are specifically induced by the CBCA graft, probably by means of the trophic factors contained in the CB cells (Paciga and Nurse, 2001; Cao et al., 2003; DiGiulio et al., 2005; Villadiego et al., 2005; Wang and Bisgard, 2005), as an increased number of TH-ir cells has been reported in parkinsonian monkeys after lentiviral delivery of GDNF into the striatum (Palfi et al., 2002). Taken together, these data suggest that, among other mechanisms, the increase of striatal TH-ir cellular population might be

![Graphical representation summarizing the quantitative analyses of perikaryal diameter (A) and perikaryal area (B) of TH-ir cells in control, MPTP and grafted animals. The curves include values for all TH-ir neurons detected in the striatum of each animal. Note that in both diameter and area, we can observe a gap (around 15 μm and 95 μm², respectively) that separates the two modes detected by the bimodality coefficient. Arrows indicate distribution gap observed only in the grafted striatum.](image)

| Table 2 | Quantitative analysis of the perikaryal diameter (μm) and perikaryal area (μm²) of striatal TH-ir cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Control animals | MPTP animals    | Grafted animals (CYN) |
| U1              | U3              | U4              | Non-grafted side | Grafted side    | Non-grafted side | Grafted side    | Non-grafted side | Grafted side    |
| Diameter        | 9.55 ± 1.75     | 9.90 ± 1.48     | 10.14 ± 1.29     | 10.09 ± 1.80    | 9.99 ± 1.14     | 10.82 ± 3.16    | 10.40 ± 1.74    | 10.50 ± 2.76    |
| Area            | 49.62 ± 16.35   | 60.07 ± 16.20*  | 60.06 ± 13.12*   | 59.52 ± 22.03   | 62.32 ± 15.15*  | 61.75 ± 22.08   | 63.22 ± 16.86** | 58.67 ± 25.88   |

Note: Data are shown as mean ± SD. Asterisks indicate significant differences compared to control group, *P ≤ 0.05; **P ≤ 0.01.

| Table 3 | Bimodality coefficient (b) from the distribution of perikaryal diameter and area of striatal TH-ir cells in each experimental group |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Control animals | MPTP animals    | Grafted animals |
| Non-grafted side | Grafted side    | Non-grafted side | Grafted side    |
| Diameter        | 0.259           | 0.403           | 0.363           | 0.595           |
| Area            | 0.251           | 0.409           | 0.428           | 0.560           |

Note: In bold, b values above 0.555, indicating a bimodal distribution.

| Table 4 | Neurochemical characterization of striatal TH-ir cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Dopaminergic markers | DDC | VMAT2 | DAT | GDNF |
| Control animal   | ++++ | +++   | +++  | +    |
| MPTP animals     | +++  | +++   | +++  | +    |
| Grafted animals  | ++++ | ++++  | +++  | +    |

Striatal and interneuronal markers

<table>
<thead>
<tr>
<th>GAD67</th>
<th>CaBP</th>
<th>CR</th>
<th>PV</th>
<th>nNOS</th>
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involved in the improvement of parkinsonism observed in grafted animals.

**Morphological and neurochemical characteristics of the striatal TH-ir cells**

Although there are some differences, the phenotypical profile of TH-ir cells here described, in control and MPTP-monkeys, is consistent with previous reports. Differences in monkey strains, ages or experimental paradigms used (i.e. MPTP administration schedule or route) might account for the slight discrepancies in the neurochemical contents of the striatal TH-ir cells described in our animals as compared to other studies (Betarbet *et al.*, 1997; Kishima *et al.*, 2004). In agreement with previous reports (Betarbet *et al.*, 1997; Cossette *et al.*, 2005b), we found that virtually all the striatal TH-ir cells belonged to the type of small-sized aspiny neurons. Compared to control animals, both DAergic denervation and CB transplants elicited an enlargement in the perikaryal size of the TH-ir cells. Surprisingly, only in the grafted striatum we found two different somatic sizes of TH-ir cells, indicating that the CBCA graft induces not only an increase in the number, but also provokes some phenotypical

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Fig. 5 Confocal immunofluorescent images of striatal TH-ir cells showing that these cells also express other neurochemical markers. (A–C, in rows) Expression of dopaminergic markers in striatal TH-ir cells: (A) One TH-ir cell double-labelled for dopa decarboxylase (DDC) and (B) another cell stained for vesicular monoamine transporter 2 (VMAT2). In addition, their nuclei are stained in blue with TO-PRO3. (C) One TH-ir cell that also expresses dopamine transporter (DAT). Note the double TH/DAT labelling of both perikaryon and processes of the TH-ir cell. (D–G, in columns) TH-ir cells that display neurochemical markers for striatal interneurons: (D) one of the two DAergic neurons expressing glutamic acid decarboxylase-67 (GAD	extsubscript{67}) and TH-ir cells expressing (E) parvalbumin (PV), (F) calretinin (CR) and (G) neuronal nitric oxide synthase (nNOS). (H) A TH-ir striatal cell positive for glial cell line-derived neurotrophic factor (GDNF) in the CBCA-grafted striatum. Scale bars, 5 μm (A) and 10 μm (B–H).
changes in these cells. A distal effect of some trophic factors contained in the grafted CB cells (Paciga and Nurse, 2001; Cao et al., 2003; Di Giulio et al., 2005; Villadiego et al., 2005; Wang and Bisgard, 2005) could explain this observation, as a similar effect on striatal TH-ir cells has been described in previous studies with GDNF (Bjorklund et al., 2000; Kishima et al., 2004).

On the other hand, our results are in agreement with previous reports in primates demonstrating that no or <1% of striatal TH-ir cells express CaBP, a marker of striatal projection neurons (Betarbet et al., 1997; Cossette et al., 2005b; Tande et al., 2006). In addition, few of these cells also contained calcium-binding proteins (as CR and PV) and nNOS, suggesting that they belong to some other type of striatal interneurons (Kawaguchi et al., 1995; Cossette et al., 2005b). However, the larger proportion of TH/CR-ir cells with respect to other interneuronal markers is consistent with the fact that CR-ir cells are the most abundant striatal interneurons in primates (Wu and Parent, 2000). Consequently, the lack of expression of CaBP in the striatal TH-ir cells along with the small-sized soma, the aspiny dendrites and the striatal and interneuronal marker expression, strongly support the belief that these TH-ir cells are striatal interneurons.

One remarkable and important finding of this work is the demonstration that in all groups the striatal TH-ir cells are similar to classic DAergic neurons, as the majority of them contain the essential machinery for the DAergic neurotransmission, such as TH, DDC, VMAT2 and DAT. However, some of these TH-ir cells (around 50%) lack VMAT2, indicating that they might not be fully functional DAergic neurons but instead may secrete DA in a non-vesicular manner, as recently has been described to occur in the striatum of rhesus monkeys (Weihe et al., 2006). Similarly, and especially in the grafted striatum, some TH-ir cells do not express DAT. This membrane DA transporter plays a central role in determining the time that the released dopamine remains in the synaptic cleft (Horn, 1990). Hence, it is conceivable that the lack of DAT in these TH-ir cells will extend the lifetime of the extracellular DA in an attempt to compensate the striatal DA deficiency (Garris et al., 1994) thus further contributing to the recovery of DAergic deficit. Interestingly, the presence of DAT expression in the majority of TH-ir cells does not seem to confer on these cells greater vulnerability to degeneration. In fact, the majority of these striatal TH-ir cells express the membrane DAT, and they not only are not destroyed by the repeated administration of the toxin but their number is increased after MPTP exposure.

Finally, we found that some of the striatal TH-ir cells of the grafted striatum but none of the cells of other groups expressed GDNF. Although we cannot provide a direct evidence for the implication of GDNF or other trophic factors in the phenotypic change detected in the TH-ir cells of the grafted striatum, the large amount of GDNF contained in the CB cells (Nosrat et al., 1996) and the existence of doubly TH/GDNF-labelled cells only in the grafted animals support the idea that this trophic factor could have exerted a certain effect on this cell population.

Long-term motor recovery

We previously reported a significant motor recovery in MPTP-monkeys treated with unilateral striatal CBCA graft (Luquin et al., 1999). Here we not only confirm these previous results, but also demonstrate that this motor improvement is not a transient effect. In contrast, the motor benefit of the grafted animals was maintained over the 12 months after surgery. This alleviation of parkinsonism cannot be ascribed either to the spontaneous recovery that occurs after the initial doses of MPTP (Rose et al., 1989) or to a non-specific effect of the surgery, since all animals were allowed to recover from the last MPTP dose for at least 3 months and sham-operated animals showed no motor recovery during the follow-up.

One interesting finding of this work is the demonstration that the number of striatal TH-ir cells markedly increased after the CBCA graft and this increment may be involved in the motor recovery of the animals. As previously reported by others, we found that striatal TH-ir cells of the non-human primate increase especially in the anterior striatum, after DAergic denervation (Betarbet et al., 1997; Porritt et al., 2000). In our study the number of TH-ir cells in the anterior striatum was similar in both groups of MPTP-monkeys in spite of different survival times. This finding rules out the possibility that this increment is a transient effect induced by acute nigrostriatal denervation. Surprisingly, in the anterior striatum this increment was further enhanced in the grafted and non-grafted striatum of CBCA-grafted animals as compared to MPTP and sham-operated animals, indicating that unilateral CBCA graft is able to elicit a bilateral increase in the number of the striatal TH-ir cells by a possible underlying crossover mechanism (Bankiewicz et al., 1986). In fact, the observation that unilateral delivery of trophic factors, like GDNF and brain-derived neurotrophic factor (BDNF), is able to reach both brain hemispheres, probably by retrograde transport, supports this hypothesis (Palgi et al., 2002; Kishima et al., 2004; Gash et al., 2005). As CB contains different trophic factors (Paciga and Nurse, 2001; Cao et al., 2003; Di Giulio et al., 2005; Villadiego et al., 2005; Wang and Bisgard, 2005), the bilateral increase in the number of striatal TH-ir cells in our grafted animals might be explained by a similar mechanism. Nevertheless, on the basis of present results, we cannot establish that the enhanced number of striatal TH-ir cells is solely due to the effect of trophic factors like GDNF.

When analysing the number of TH-ir cells in the post-commissural striatum, we found that the number of these cells was only increased in the grafted striatum, indicating a wide and extended effect of the CBCA within the grafted striatum and also, the specificity of the graft in the
induction of such phenomenon. However, in the grafted striatum, the increment in the number of TH-ir cells was higher in the anterior striatum than in the post-commissural region. This was an unexpected finding as CBAC graft was deposited in the posterior putamen, and consequently the highest increment in the number of TH-ir cells would be anticipated to occur in this striatal region. However, and because under physiological conditions TH-ir cells are more abundant in the anterior striatum, it is possible that the number of these cells is more easily regulated (for instance, by trophic factors) in areas where these cells are ordinarily more numerous, thus explaining the more marked increment in the anterior striatum observed in our animals.

The role that these TH-ir cells play in the motor recovery of our grafted animals is uncertain. However, as the number of these TH-ir cells is not increased in the striatum of sham-operated animals, it can be assumed that they are directly involved in the long-lasting motor improvement of our animals. In fact, all of these cells exhibit the critical DAergic machinery required to synthesize and release dopamine and consequently, they might compensate the loss of dopamine and the motor deficits induced by the nigrostriatal lesion. It is possible that any manoeuvre able to further promote the increase of these TH-ir cells can elicit a greater improvement of parkinsonism and will become a therapeutic approach to treat PD patients.

### Origin of the striatal TH-ir cells

Previous observations demonstrated that the striatal TH-ir cells found in aged MPTP-monkeys are not new neurons but rather result from the phenotypic shift of pre-existing GABAergic striatal interneurons (Tande et al., 2006). However, the possibility that striatal TH-ir cells, or at least some of them, are newly generated neurons cannot be completely excluded, as recent studies have shown that 30% of DAergic neurons in the human striatum coinexpress β-tubulin class III, a marker of early committed neurons (Cossette et al., 2005b). Additionally, several reports have confirmed the neurogenic potential of the striatal parenchyma and also the migration of neural progenitor cells from the subventricular zone (SVZ) to the striatum (Reynolds and Weiss, 1992; Pencea et al., 2001; Bedard et al., 2002). As we did not perform studies of cell proliferation in our animals, we cannot establish whether the striatal TH-ir cells detected in the grafted striatum are the result of the phenotypic shift of pre-existing striatal GABAergic interneurons, the consequence of increased proliferation of SVZ progenitor cells that can originate new neurons or come from striatal quiescent stem cells.

In summary, the present study provides evidence that the CBCA autograft in parkinsonian monkeys induces specific and long-lasting cellular changes on striatal TH-ir cells that probably contribute to the sustained motor recovery found in our parkinsonian animals. Further studies are necessary to better understand the effect of the CB grafts on striatal DAergic cells and the intrinsic mechanisms involved in recovery from parkinsonism.

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