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Research Report

Striatal carotid body graft promotes differentiation of neural progenitor cells into neurons in the olfactory bulb of adult hemiparkisonian rats

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ABSTRACT

Progenitor cells generated in the subventricular zone (SVZ) migrate toward the olfactory bulb (OB), where they differentiate into neurons. Growth factors have been shown to promote neurogenesis in the SVZ/OB-system while dopaminergic lesion exerts an opposite effect. As carotid body (CB) cells express growth factors here we study the impact of intrastriatal CB graft on migration and differentiation of neural progenitor cells in the hemiparkinsonian rat SVZ/OB-system. Bromodeoxyuridine (BrdU) was given to intact, 6-hydroxydopamine (6-OHDA)-lesioned and 6-OHDA-lesioned animals transplanted with vehicle or rat CB cells. The migration of progenitor cells was assessed by the quantification of BrdU-labeled cells in the SVZ/OB-system and the neuronal differentiation by the proportion of newborn neurons in the OB. The graft survival was confirmed by CB cell morphology and their tyrosine hydroxylase expression. Some of these CB cells were stained with BrdU, thus indicating their ability for self-renewal. Grafted glomus cells also expressed brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). The migration of neural progenitor cells was significantly decreased in 6-OHDA-lesioned respect to intact animals. We found a similar number of BrdU-labeled cells in sham-operated than in CB-grafted animals, suggesting that CB graft has no effect on progenitor cell migration. CB-grafted animals exhibited a significantly larger percentage of newborn cells (BrdU/Neuronal Nuclei-labeled cells) respect to 6-OHDA-lesioned and sham-operated animals. This study suggests that striatal CB graft might promote differentiation of SVZ progenitor cells into neurons, probably by the growth factors contained in CB cells.

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1. Introduction

It is widely accepted that the adult mammalian brain has the capacity to continuously generate new neurons in at least two specific areas: the subgranular zone (SGZ) of the hippocampus (Palmer et al., 1997; Gage et al., 1998; Kempermann and Gage, 2000) and the subventricular zone (SVZ) of the lateral ventricle (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Garcia-Verdugo et al., 1998). The newborn cells in the SVZ migrate along the rostral migratory stream (RMS) and reach the olfactory bulb (OB) where they mature into local interneurons (Lois and Alvarez-Buylla, 1993; Lois et al., 1996). Thus, SVZ/OB-system seems to constitute an interesting cell reservoir of neural progenitor cells in the adult brain that might be used for regenerative therapy in neurological disorders.

The mechanisms implicated in the proliferation of the neural progenitor cells in the SVZ/OB-system and their subsequent migration and differentiation into neurons remain unknown. Numerous studies have revealed that growth factors are involved in the generation and support of new neurons, indicating a substantial role of trophic factors in the control of neurogenesis. In fact, administration of brain derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF) into the lateral ventricle increases the number of newborn neurons in the rat OB whereas epidermal growth factor (EGF) reduces this process (Kuhn et al., 1997; Zigova et al., 1998). On the other hand, intraventricular administration of vascular endothelial growth factor (VEGF) also results in a larger number of new mature neurons in the rat OB (Schanzer et al., 2004).

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive degeneration of the dopaminergic neurons in the substantia nigra (SN). Direct dopaminergic projections from the SN to the SVZ cells have been recently demonstrated in non-human primates (Freundlieb et al., 2006), thus indicating that the proliferation of the neural progenitor cells in the SVZ is directly governed by dopaminergic fibers arising from the SN. In keeping with this, it is worth mentioning that decreased proliferation of adult progenitor cells, both in the SGZ and in the SVZ/OB-system, has been found in PD patients and experimental models of the disease (Baker et al., 2004; Hoglinger et al., 2004). Interestingly, this deficit is reversed by drugs acting at D3 dopamine receptor sites (Van Kampen and Eckman, 2006).

The carotid body (CB) is a neural crest-derived organ that has been used as a cell donor source to treat PD with promising

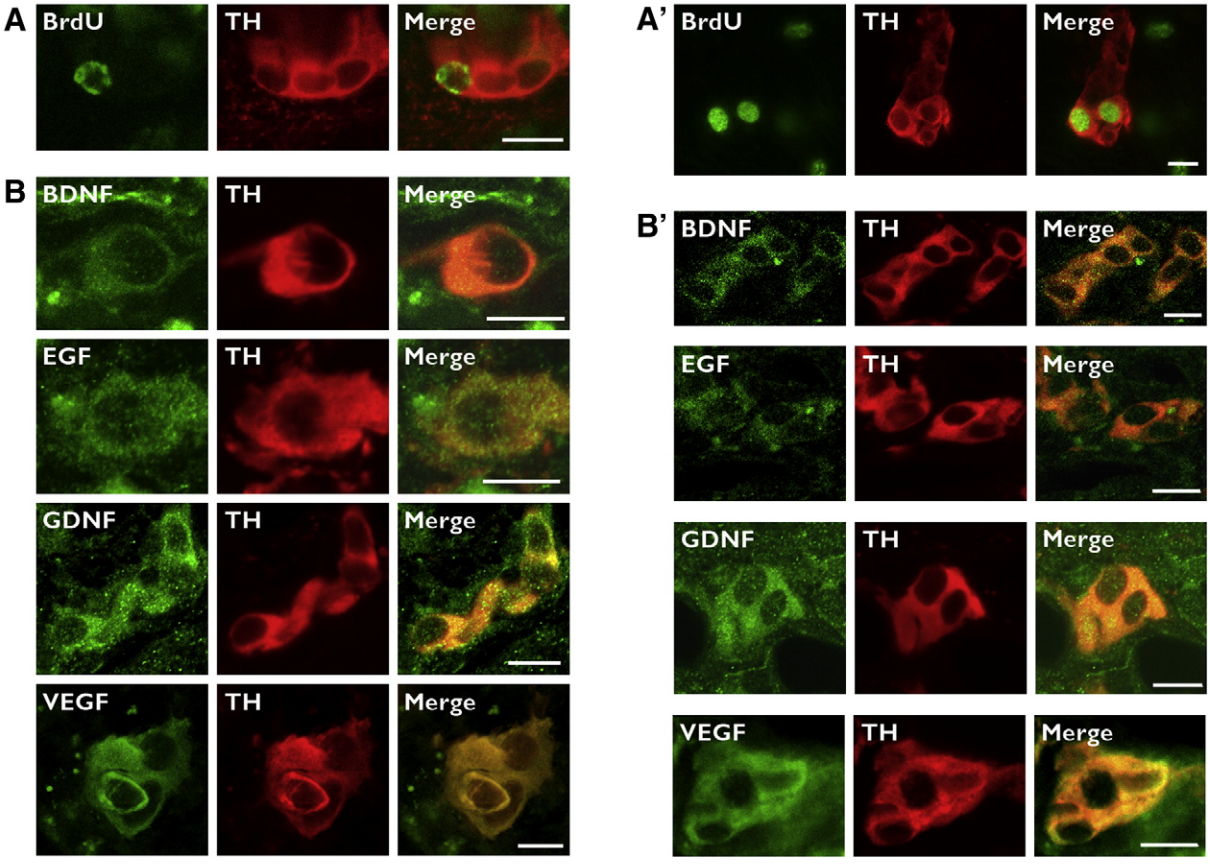

Fig. 1 – Double-immunofluorescence of grafted striatum of animals transplanted with carotid body (CB) cell aggregate and sacrificed 1 (A, B) and 2 (A', B') months after the graft. (A, A') Several CB-grafted cells were doubly labeled with TH (red) and 5-bromo-2′-deoxyuridine (BrdU) (green) in all of CB-grafted animals either with 1 (A) or 2 (A') months survival. (B, B') Long-term expression of several growth factors in grafted glomus cells. In animals sacrificed 1 (B) and 2 (B') months after the graft the glomus cells expressed derived neurotrophic factor (BDNF), epidermal growth factor (EGF), glial derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF). Scale bar, 10 μm.

results. Mechanisms involved in the motor recovery induced by CB graft seem to be due to trophic factors released by the grafted CB cells (Toledo-Aral et al., 2003; Villadiego et al., 2005). CB consists of two types of cells: type I (glomus cells) that express tyrosine hydroxylase (TH), adrenomedullin, BDNF and glial derived neurotrophic factor (GDNF) and type II (supporting cells) that mainly express glial fibrillary acidic protein (GFAP) (Chiocchio et al., 1966; Hertzberg et al., 1994; Kameda, 1996; Martinez et al., 2003; Wang and Bisgard, 2005). In addition, under hypoxic conditions the rat glomus cells are capable of expressing VEGF and both cellular types can also be renewed, indicating that CB cells have the ability to proliferate (Bee et al., 1986; Di Giulio et al., 2003). Although CB cells contain growth factors able to induce the proliferation, migration and differentiation of neural progenitor cells, the effects of striatal CB cells grafts on SVZ/OB-system neurogenesis have not been reported. Here we investigated the impact of striatal CB transplants on the migration and differentiation of neural progenitor cells in the SVZ/OB-system of hemiparkinsonian rats grafted with CB cells.

2. Results

2.1. *Survival and proliferation of grafted glomus cells*

To identify the CB graft and confirm whether grafted CB cells still expressed TH, we processed coronal tissue sections of the grafted striatum for TH immunofluorescence. In all processed striatal sections we found many glomus cells stained with TH within the graft showing the typical morphology of glomus cells that consisted of cell clusters with large round nucleus. Accordingly, we identified the survival of the CB aggregate in the striatum of all transplanted animals (5 and 9 weeks' survival) by the expression of TH and the characteristic morphology of the glomus cells. Our results demonstrate not only the survival of the glomus grafted cells but also the maintenance of their dopaminergic phenotype.

The CB cells can undergo mitosis in conditions of low oxygen concentrations (Bee et al., 1986; Pardal et al., 2007). As transplant might have created a hypoxic condition in the CB cells, we investigated whether grafted glomus cells experienced mitosis. Grafted striatal tissue sections processed for double immunofluorescence with TH and 5-bromo-2′-deoxyuridine (BrdU) displayed some TH immunoreactive cells with BrdU-labeled nucleus within the graft of all grafted animals (5 and 9 weeks' survival) (Fig. 1A, A', respectively), demonstrating that grafted glomus cells are capable of self-renewal.

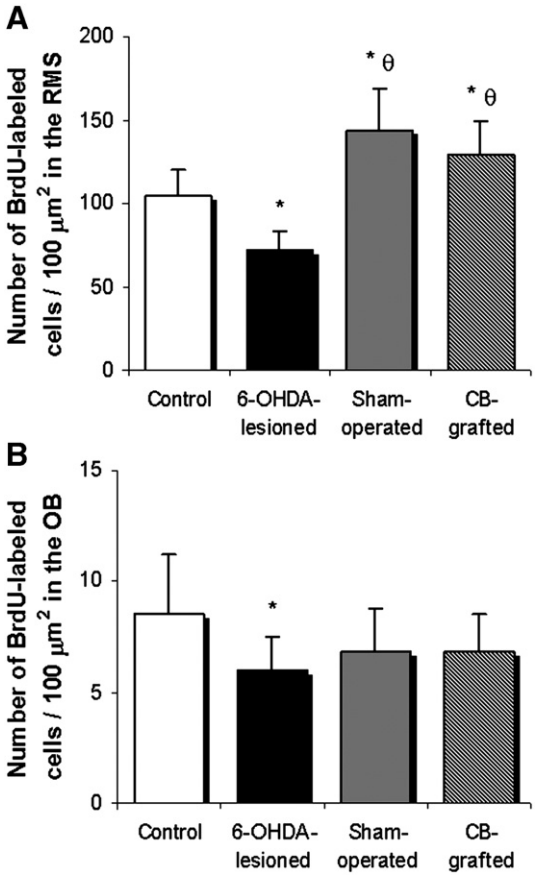

Fig. 2 – Quantification of 5-bromo-2′-deoxyuridine (BrdU) labeled cells in left rostral migratory stream (RMS) of animals sacrificed 1 month after grafting and in the left olfactory bulb (OB) of animals with 2 months' survival. (A) Dopaminergic lesion caused 30% decrease in the number of BrdU-labeled cells compared with control animals. The number of BrdU-labeled cells in the RMS of sham-operated was enhanced with respect to control and 6-OHDA-lesioned animals (37% and 94% respectively). In the carotid body (CB)-grafted animals this number also was increased with respect to control (24% and 76% respectively). (B) Only 6-OHDA-lesioned animals exhibited a significant decrease of 34% in the number of olfactory BrdU-labeled cells. No significant differences were observed in the sham-operated and CB-grafted animals compared with control or 6-OHDA-lesioned animals. $^{*}p<0.05$ versus control animals and $^{\theta}p<0.05$ versus 6-OHDA-lesioned animals.

2.2. *Grafted CB cells express a variety of growth factors*

One of the aims of this study was to investigate the ability of CB cell grafts to promote cell migration and neuronal differentiation in the SVZ/OB-system, since CB cells contain growth factors (Izal-Azcarate et al., 2008) with a proven neurogenic effect on the SVZ/OB-system (Kuhn et al., 1997; Zigova et al., 1998; Schanzer et al., 2004). For this reason, we first confirmed that grafted CB cells still retained the ability to express some of these growth factors. As trophic factors are expressed in glomus CB cells, striatal tissue sections that contained the graft were doubly immunostained with TH and the antibodies against the following growth factors: BDNF, EGF, GDNF and VEGF. Confocal imaging analysis demonstrated expression of BDNF, EGF and GDNF in grafted TH-labeled cells irrespective to the survival time (Fig. 1B, B'). Since hypoxia is the main stimulator of VEGF (Di Giulio et al., 2003), we also investigated whether the grafted CB cells expressed this growth factor. We found that dopaminergic cells displayed immunoreactivity to VEGF in animals sacrificed both 5 and 9 weeks after the transplant (Fig. 1B, B', respectively). These results indicate the long-term expression of several growth factors in the grafted

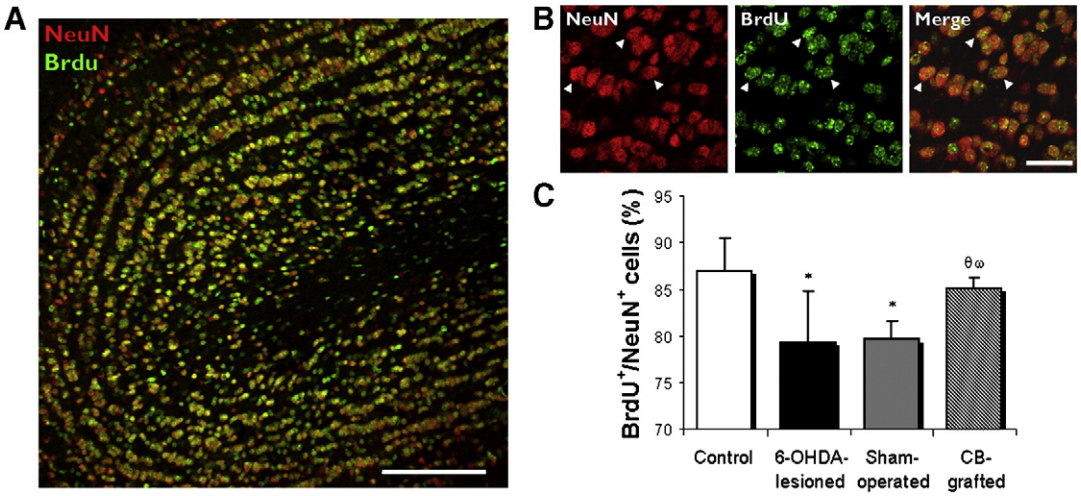

Fig. 3 – Illustration of the quantification of newborn neurons in the granular cell layer olfactory bulb (OB) of animals sacrificed 2 months' after the graft. (A) Low magnification confocal image demonstrating the presence of BrdU-labeled cells (green) co-expressing the neuronal nuclei (NeuN) marker (red). Scale bar, 40 μm. (B) High magnification confocal image of these newborn neurons. The arrowheads indicate double-labeled cells. Scale bar, 20 μm. (C) The proportion of doubly labeled cells (newborn neurons) was enhanced in animals transplanted with carotid body (CB) cell aggregate (CB-grafted) with respect to 6-OHDA-lesioned and sham-operated animals. No significant differences were observed in CB-grafted animals as compared to control animals. $^{*}p<0.05$ versus control animals, $^{\theta}p<0.05$ versus 6-OHDA-lesioned animals and $^{\omega}p<0.05$ versus sham-operated animals.

glomus cells with proven effect on the proliferation, migration and neuronal differentiation of the SVZ/OB-system.

2.3. *Intrastriatal CB graft does not increase cell migration in the SVZ/OB-system*

Although the majority of studies focusing on the proliferation of neural progenitor cells have preferentially studied the SVZ, we performed the quantification of BrdU-labeled cells in the RMS and in the OB since our particular interest was the migration of the SVZ progenitor cells toward the OB and their ulterior differentiation into mature neurons. For this reason, we analyzed the number of BrdU-labeled cells in the RMS of animals sacrificed 1 month after the initiation of BrdU administration and in the OB of animals with 2 months' survival. For the quantification of BrdU-labeled nuclei, we selected and quantified only those cells intensely stained as an indicator of mitotic activity.

In agreement with recent studies (Baker et al., 2004; Hoglinger et al., 2004), we found that nigrostriatal dopamine depletion elicited 30% reduction of BrdU-labeled cells in the RMS with respect to control animals. However, the number of BrdU-labeled cells in the RMS of sham-operated and CB-grafted animals was higher than in control (37% and 24%, respectively) and was further increased when comparing to 6-hydroxydopamine (6-OHDA)-lesioned animals (94% and 76%, respectively). There was no significant difference in the total number of BrdU-labeled cells in the RMS between sham-operated and CB-grafted animals (Fig. 2A). Quantification of BrdU-labeled cells in the OB was performed in the animals sacrificed 2 months after the initiation of BrdU administration. We found the number of BrdU-labeled cells significantly decreased (34%) in the OB of 6-OHDA-lesioned rats. The number of BrdU-labeled cells in the OB of sham-operated and CB-grafted animals was similar to that of 6-OHDA-lesioned group and smaller than in control animals although this difference was not statistically significant (Fig. 2B). As the number of BrdU-labeled cells in the RMS and OB was identical in sham-operated and CB-grafted animals, we conclude that intrastriatal CB graft does not influence progenitor cells migration in the SVZ/OB-system.

2.4. *Intrastriatal CB graft increases neural progenitor cells maturation into neurons in the GCL of the OB*

In order to determine the potential of intrastriatal CB graft to promote the neuronal differentiation in the OB, we analyzed the number of BrdU-labeled cells that co-localized with NeuN in the animals sacrificed 2 months after the initiation of BrdU administration. We used a confocal laser microscopy to determine the ratio of newborn cells that had differentiated into mature neurons. An example of BrdU-labeled cells co-expressing NeuN in the granule cell layer (GCL) of the OB is illustrated in Fig. 3A, B.

The control animals displayed 87±3% of cells doubly labeled with BrdU and NeuN in the GCL of the OB, while in the 6-OHDA-lesioned animals and in the sham-operated animals this proportion was significantly reduced. Interestingly, the CB-grafted animals showed a significant increase in the proportion of BrdU-labeled cells co-localizing with NeuN (85±1%) as compared to 6-OHDA-lesioned (79±5%) and sham-operated animals (80±2%) but the percentage of doubly BrdU/NeuN-labeled cells was similar to that of control animals, indicating the ability of CB graft to reverse the decreased neuronal differentiation of progenitor cells created by the nigrostriatal lesion (Fig. 3C).

3. Discussion

Our results are consistent with previous studies showing the long-term survival of CB grafts in the brain parenchyma (Espejo et al., 1998; Toledo-Aral et al., 2003; Villadiego et al., 2005). In fact, animals sacrificed 5 and 9 weeks after transplant exhibited TH-labeled cells within the graft, thus reflecting not only the survival of the transplanted CB cells but also their ability to maintain their dopaminergic phenotype. In addition, the organization in glomeruli characteristic of the native CB and the TH expression in their cells supported the survival of the graft.

One notable finding here reported is the demonstration of cell division in the grafted glomus cells and the expression of growth factors in these cells, up to 9 weeks after the graft. Several studies have described that hypoxia is a potent stimulator of the mitosis of CB cells (Bee et al., 1986; Wang and Bisgard, 2005). However, others claimed that no signs of mitosis of type I cells were detected in rats exposed to hypoxia for 1–3 weeks (Pequignot et al., 1984). Our results showing mitosis of glomus cells 9 weeks after graft demonstrate that these cells are able to proliferate in supposed hypoxic environment.

The expression of growth factors in the glomus cells of our CB-grafted animals might have contributed to the survival of the graft. The expression of GDNF in the grafted glomus cells here described is consistent with previous reports revealing the long-term expression of GDNF in the glomus cells after transplant (Villadiego et al., 2005). However, other growth factors expressed by the natural CB cells such as BDNF and EGF (Izal-Azcarate et al., 2008) were also expressed by the grafted CB cells indicating that these cells are able to maintain their phenotype even under non physiological conditions. In fact, BDNF and GDNF are neurotrophic factors involved in regulating survival and differentiation of many neurons during development (Birling and Price, 1995; Schwartz et al., 1997). Finally, VEGF has the ability to stimulate the proliferation of neural precursor cells *in vitro* and *in vivo* and its expression in the CB cells is promoted by hypoxia (Tipoe and Fung, 2003; Di Giulio et al., 2003). Our results demonstrating VEGF expression in the grafted glomus cells are consistent with the *in vivo* studies and point the existence of a certain hypoxic condition in the grafted CB cells. More importantly, our results further support the validity of these cells as source of several growth factors for the treatment of PD.

Taken together, our results suggest that the expression of growth factors by the glomus cells might contribute to the maintenance of the dopaminergic phenotype and survival of the grafted CB cells. In addition, the expression of these growth factors might also account for proliferation of type I cells within the graft, as reflected by doubly TH/BrdU-labeled cells in the grafted CB. In keeping with this, the presence of dopaminergic cells within the graft up to 1 year after transplant (Toledo-Aral et al., 2003) would also reflect the ability of glomus cells to proliferate, rather than the survival of the original grafted cells. However, we cannot ascertain whether these "new dopaminergic cells" are originated from the recently described stem cells contained in the graft (Pardal et al., 2007).

Although several authors have described the existence of intrinsic dopaminergic cells within the striatum of rats (Tashiro et al., 1989) and primates (Betarbet et al., 1997) is unlikely that the TH-labeled cells observed in the striatum of the grafted animals belong to this cell population. First, they appeared forming clusters of cells with the morphological characteristics of the glomus cells and secondly these striatal TH-labeled cells expressed the trophic factors that usually contain the natural CB cells but no by the striatal dopaminergic cells.

As we found several growth factors expressed in the CB cells, we investigated the ability of the graft to promote the migration and differentiation of progenitor cells in the SVZ/OB-system. In agreement with previous findings in PD patients and in animal models of PD (Baker et al., 2004; Hoglinger et al., 2004), we found 30% decreased cell proliferation in the SVZ/OB-system of rats with unilateral lesion of the nigrostriatal pathway. However, the number of BrdU-labeled cells in the RMS of 6-OHDA-lesioned rats similarly increased after either CB graft or vehicle injection (24% and 37%, respectively as compared to controls). The similar increase observed in these two groups of animals might be explained by the unspecific effect created both by the sham and the CB graft. It is worth mentioning that a similar effect has also been described in the SVZ/OB-system of rats after brain insults such as ischemia and chemical injury (Zhang et al., 2004; Gotts and Chesselet, 2005). A delayed effect of the CB graft on cell migration in the SVZ/OB-system can be ruled out as the number of BrdU-labeled cells found in the OB 2 months after the initiation of BrdU administration was similar in all groups of animals with 6-OHDA lesion irrespective to the treatment followed after the dopaminergic lesion. Consequently, our results indicate that the CB graft does not increase the migration of SVZ progenitor cells. We hypothesized that the contain by the grafted CB cells of dopamine and growth factors with opposite effects on the migration of newborn cells might explain the lack of effect on cellular migration.

For the study of neuronal differentiation of SVZ progenitor cells, we determined the proportion of BrdU/NeuN-labeled cells in the OB, and we found a greater percentage of BrdU/NeuN-labeled cells in the OB of CB-grafted animals than in 6-OHDA-lesioned and sham-operated animals, but similar to that of control animals. Taking into account that the number of BrdU-labeled cells in the OB was similar in all 6-OHDA-lesioned animals, the increased proportion of BrdU/NeuN doubly labeled cells observed in the CB-grafted animals clearly support a neurogenic role of the CB graft. One explanation for that can be found in the growth factors contained by the grafted CB cells which would promote neuronal differentiation in the OB acting as infusion pump of growth factors. However, we cannot completely ascertain that the enhanced differentiation here documented is due to growth factors contained in the CB cells since the detected expression of these substances by immunohistochemistry do not guaranty that they are released in adequate amounts to exert a biological effect. Nevertheless, the increased number of striatal TH-labeled cells reported in the striatum of parkinsonian monkeys grafted with CB cell aggregates supports the hypothesis that grafted CB cells are able to release some growth factors (San Sebastian et al., 2007), since a similar effect on the intrinsic dopaminergic cells has been

documented after striatal lentiviral delivery of GDNF (Palfi et al., 2002).

In summary, our results confirm and extend previous findings demonstrating the ability of grafted CB cells to survive and express several growth factors up to 9 weeks after graft. Although we performed no quantitative analysis, the long-term expression of different growth factors in the grafted glomus cells partially supports the functionality of these cells. Consequently, we suggest that the effect observed in our animals with respect to differentiation of neural progenitor cells in the SVZ/OB-system, could be ascribed to the growth factors contained in the grafted glomus cells. Since CB grafts could enhance the neuronal differentiation, it is possible that the transplant of CB cells might play an important role in the search for new strategies focusing on brain self-repair.

4. Experimental procedures

4.1. *Animals*

All experiments were performed on 9 week-aged male Sprague–Dawley rats ($n=48$, Harland, Barcelona, Spain) weighing approximately 270 g at the start of the experiment. Animals were housed at a regulated temperature (22±1 °C) in a 12-h light/dark cycle with access *ad libitum* to food and water. Experimental protocols were performed in accordance with European Communities Council Directive 86/609/EEC regarding the care and use of animals for experimental procedures and under the guidance of the Ethics Committee for Animal Experimentation of the University of Navarra.

4.2. *Surgical procedures and treatments*

Animals were anesthetized intraperitoneally (i.p.) with a mixture of ketamine (75 mg/kg; Merial) and xylazine (10 mg/kg; Bayer). They were subsequently placed in a stereotaxic frame (David Kopf Instruments, USA) and stereotactically injected into the left SN (coordinates from lambda: AP=+3.2 mm, ML=+2.1 mm and VD=−7.4 mm) with 5 μg of 6-OHDA dissolved in 3 μl of 0.9% sterile saline containing 0.2 mg/ml ascorbic acid. 6-OHDA was infused at a rate of 1 μl/min and the needle was left in place for 2 min after the injection to minimize the retrograde flow along the needle track.

Two weeks after the surgery, 6-OHDA-lesioned animals were assessed for rotational behavior in response to D-amphetamine sulfate (5 mg/kg i.p.; Sigma) administration. Rats were placed into automated rotometer bowls (LE 3806; Panlab-Letica, Spain) and left and right full-body turns were counted over 90 min after the injection by a computerized activity monitor system. Animals that exhibited ranges from 3 to 10 turns per minute were selected for the ulterior study. The parkinsonian rats were randomly allocated into these 3 groups. 6-OHDA-lesioned group consisted of rats treated with 6-OHDA injections into the SN that received no subsequent striatal injection ($n=12$). Transplanted animals comprised a group of 6-OHDA-lesioned rats treated with striatal grafts of CB cell aggregate (CB-grafted group; $n=12$) and another group of 6-OHDA-lesioned rats that were treated with an injection of vehicle into the striatum (sham-operated group; $n=12$).

The surgical procedure used for CB grafting has already been described elsewhere (Espejo et al., 1998). Briefly, after the surgical removal of the rat CB it was divided in four pieces and one of them suspended in 1 μl of Tyrode's solution and stererotaxically injected into the left dorsal striatum (coordinates from bregma: AP=+0.2 mm, ML=+2.6 mm and VD=−5.4 mm). Sham-operated animals were given 1 μl of Tyrode's solution into the same striatal coordinates.

All groups of rats received daily injections of 50 mg/kg (i.p.) of the thymidine analog BrdU (Sigma). Treatment was initiated 3 weeks after the dopaminergic lesion and was maintained for the following 24 days. To study the effect of the striatal CB graft on cell migration through the RMS one-half of the BrdU-treated animals of each group ($n=6$) was sacrificed 1 month following BrdU administration (5 weeks after graft). The rest of the animals ($n=6$ per group) was sacrificed 2 months (9 weeks after graft) after the initiation of BrdU administration and served for the study of the CB graft effect on neuronal differentiation of progenitor cells. A group of intact animals ($n=12$) was used as control and also treated with the same schedule of BrdU administration.

4.3. *Histological processing*

At the end of the experiment, animals were deeply anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde (PFA; Sigma) in phosphate buffer (PB), pH 7.4 at 4 °C. Brains were incubated overnight in 4% PFA and were transferred the next day to PB, pH 7.4. Coronal 40 μm sections were cut on a vibratome (VT1000 S; Leica, Germany), collected in PB and stored at 4 °C until they were processed for immunohistochemistry.

To identify the CB graft, striatal tissue sections were processed for immunofluorescence with TH and analyzed with a confocal microscope. Free-floating brain sections were incubated at 4 °C overnight with mouse anti-TH, 1:1000 (Chemicon International) in PB, 0.2% Triton X-100 (Sigma) and 5% normal sheep serum and the immunoreaction was visualized with the Cy 3-conjugated sheep anti-mouse, 1:500 (Sigma). Finally, samples were mounted on gelatin-coated slides and coverslipped with Immu-mount (Thermo-Shandon) mounting medium. We subsequently examined whether cell division occurred in the transplanted glomus cells. For double BrdU/TH immunofluorescence we followed the same process than that for double BrdU/Neuronal Nuclei (NeuN) immunofluorescence (see below). The primary antibody of TH was mouse anti-TH (1:1000, Chemicon International).

To detect growth factors in the grafted glomus cells, striatal tissue sections containing the graft were processed for double immunofluorescence. Free-floating brain sections were incubated at 4 °C overnight with the following primary antibodies in PB, 0.2% Triton X-100 and 5% normal serum: rabbit anti-BDNF, 1:500 (Abcam); rabbit anti-EGF, 1:500 (Abcam); rabbit anti-GDNF, 1:500 (Santa Cruz Biotechnology); mouse anti-TH, 1:1000 (Chemicon International); rabbit anti-TH 1:1000 (Chemicon International); and mouse anti-VEGF 1:500 (Santa Cruz). The immunoreaction was visualized with the following fluorescent secondary antibodies: Cy 3-conjugated sheep anti-mouse, 1:500 (Sigma); Alexa Fluor 488 or 568-conjugated goat anti-rabbit IgG, 1:500 (Molecular Probes); Alexa Fluor 488-

conjugated goat anti-rat IgG, 1:500 (Molecular Probes); and Alexa Fluor 488-conjugated goat anti-mouse IgG, 1:500 (Molecular Probes). Finally, samples were mounted on gelatin-coated slides and coverslipped with Immu-mount mounting medium.

For BrdU immunostaining, free-floating tissue sections containing the RMS and OB were pretreated with 1N HCl at 60 °C for 30 min. Samples were incubated at 4 °C overnight with rat anti-BrdU 1:1000 (Abcam) in 0.1 M PB, 0.2% Triton X-100 and 5% normal rabbit serum. Biotinylated rabbit anti-rat was applied for 30 min at 1:200 (Dako), followed by avidin–biotin complex (ABC) kit (Vectastain Laboratories) for 30 min at 1:100. Staining for peroxidase was performed using 0.05% 3-3′ diaminobenzidine (DAB; Sigma) and 0.001% hydrogen peroxide (H_2O_2). Sections were mounted on gelatin-coated slides and coverslipped with DPX (BDH, England) mounting medium. We further investigated whether BrdU-labeled cells also expressed the neuronal marker, NeuN. Free-floating OB sections were first blocked for 30 min in PBS, 0.2% Triton X-100 and 5% normal serum. Samples were incubated at 4 °C overnight with mouse anti-NeuN 1:500 (Chemicon International) in PBS, 0.2% Triton X-100 and 5% normal sheep serum, followed by fluorescent secondary antibody Cy 3-conjugated sheep anti-mouse (Sigma) at 1:500 for 2 h. Then, sections were fixed in 4% PFA for 10 min and BrdU staining as described above. Finally, samples were mounted on gelatin-coated slides and coverslipped with Immu-mount mounting medium.

Fluorescent signals were detected and processed using a confocal laser scanning microscopy equipped with three lasers (LSM510/Meta; Carl Zeiss, Germany).

4.4. *Quantification*

The number of BrdU-labeled cells was assessed by using a semiautomatic stereology system (C.A.S.T. Grid) and applying the optical fractionator (Gundersen et al., 1988). To quantify the number of BrdU-labeled cells in the left RMS and in the GCL of left OB, we used four tissue sections of each animal taken from one of 11 parallel series of 40 μm thick coronal sections. BrdU-labeled cells were blindly counted in predetermined areas of the RMS (from 4.2 to 2.8 mm anterior to bregma) and OB (from 6 to 7.4 anterior to bregma). The quantification was performed at 100× objective and BrdU-labeled cells were counted when the nuclei came into focus and did not touch the exclusion lines of the counting frame. Gundersen coefficients of error were generally less than 0.1.

In order to estimate the ratio of BrdU-labeled cells that mature into neurons in the left OB, we analyzed the cells doubly labeled with BrdU and NeuN. Three coronal sections, taken one of 11 parallel series, were examined blindly at 40× objective using a confocal laser microscopy. The ratio of doubly labeled cells was counted in three random fields per GCL in each of the three coronal sections (from 6.5 to 7.4 mm anterior to bregma).

4.5. *Statistical analysis*

Comparisons among groups were made using the analysis of variance (ANOVA) and subsequent TukeyB multiple comparisons test and Kruskal–Wallis non-parametric ANOVA followed by the two-tailed Mann–Whitney *U* test for unpaired data when corresponding. Those statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD). Differences were considered statistically significant at $p < 0.05$.

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