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Long-term survival of encapsulated GDNF secreting cells implanted within the striatum of parkinsonized rats

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Abstract

Several findings suggest that glial cell line-derived neurotrophic factor (GDNF) may be a useful tool to treat parkinsonism by acting as a neuroprotective and neurotrophic factor for dopaminergic neurotransmission systems. In the present study, we implanted alginate-poly-L-lysine-alginate microcapsules containing immobilized Fischer rat 3T3 fibroblasts transfected to produce GDNF *in vitro* into the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats. Microencapsulated GDNF secreting cells were stable for at least 3 weeks *in vitro*. Intrastriatal implantation of microencapsulated GDNF secreting cells into 6-OHDA lesioned rats resulted in a decrease in apomorphine-induced rotations by 84%, 64%, 84%, 60% and 52% (2, 5, 8, 16 and 24 weeks, respectively) with respect to the value before implantation and with respect to the value obtained from the empty microcapsule implanted-group at each time point. Six months after transplantation, immunohistochemical detection of GDNF revealed strong immunoreactivity in the striatal tissue surrounding the microcapsules in the absence of tissue damage due to microcapsule implantation. No changes in the levels of dopamine and its metabolites or of tyrosine hydroxylase immunoreactivity were detected in the striatum. In summary, the implantation of microencapsulated GDNF secreting cells allows the delivery of this molecule into the rat striatum for at least 6 months and results in substantial behavioral improvement.

Keywords: Microencapsulation; 6-OHDA lesion; Immunohistochemistry; Rotational behavior; Glial cell line-derived neurotrophic factor (GDNF)

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by the loss of mesencephalic dopaminergic neurons in the substantia nigra pars compacta and the concomitant reduction of dopamine in the striatum (Smeyne, 2005). The cardinal symptoms of PD, which include bradykinesia, tremor, rigidity and postural imbalance, can be improved initially by treatment with levodopa (L-dopa), but its therapeutic effects decrease progressively and side effects, such as dyskinesia and "wearing-off" and "on-off" phenomena appear (Obeso et al., 2000). Moreover, L-dopa does not block the degenerative process affecting dopaminergic neurons. Indeed, no currently available therapy is able to impede or at least slow down the pathological process.

The administration of several neurotrophic factors has been intensively investigated in recent years as a possible therapeutic strategy for PD. In particular, one of these factors, GDNF has been shown to exert prominent actions on dopaminergic neurons, by enhancing their survival and stimulating the outgrowth of dopaminergic fibers (Tomac et al., 1995; Kirik et al., 2004). It has been also shown that GDNF improves 6-hydroxydopamine (6-OHDA) or 1-methyl-4-

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phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced behavioral deficits in rodents and monkeys (Hoffer et al., 1994; Tomac et al., 1995; Kirik et al., 2004) and can protect nigrostriatal dopaminergic neurons against the effect of 6-OHDA (Fox et al., 2001; Kordower et al., 2000; Grondin et al., 2003). Thus, when GDNF was intracerebroventricularly administered to PD patients, no improvement and several adverse effects were observed (Nutt et al., 2003); in contrast, when it was infused in the putamen of PD patients in a phase I clinical trial, significant efficacy and safety was observed (Gill et al., 2003; Slevin et al., 2006). However, a recent, randomized, controlled blinded clinical trial has failed to show any significant advantage of intraputamenal GDNF over placebo (Lang et al., 2006). These contradictory results have been the subject of recent controversies concerning the future of GDNF as a possible tool for PD treatment (Barker, 2006; Penn et al., 2006). One of the critical problems which has remained unresolved is the way to continuously and directly apply the factor to the dopaminergic pathway, since the effects of GDNF are transient, this neurotrophic factor does not cross the blood-brain barrier and the receptors GDNF interacts with are widely distributed in the brain (Kirik et al., 2004; Dass et al., 2006).

The transplantation of encapsulated cells, genetically modified to produce GDNF, may be a way to circumvent the problem of repeated or continuous administration. Cells are included in a matrix and then surrounded by a semipermeable polymeric membrane (Orive et al., 2003a) which permits the entry of nutrients required for cell viability and the exit of the therapeutic molecule, obtaining in this way a sustained release and delivery of GDNF. Moreover, the membrane isolates the enclosed cells from the host immune system, preventing the recognition of the immobilized cells as foreign (Orive et al., 2003b). Indeed, the use of these systems reduces or eliminates the need for chronic immunosuppression of the host. This encapsulated cell technique has already been tested in patients with PD (Mínguez-Castellanos and Escamilla-Sevilla, 2005; Linazasoro, 2003) and with other diseases of the nervous system, such as amyotrophic lateral sclerosis (Zurn et al., 2000) and Huntington's disease (Bloch et al., 2004).

Recently, we showed that GDNF secreting Fischer rat 3T3 fibroblasts maintain their viability and functionality *in vitro* after their immobilization in capsules elaborated with a different polymer (Ponce et al., 2005). Since alginate is the most widely used polymer for this purpose (Hortelano et al., 1996; Schwenter et al., 2003), we encapsulated cells in alginate poly-L-lysine-alginate (APA) microcapsules using an alginate which we had previously identified as being the most biocompatible (Ponce et al., 2006). Biocompatible microcapsules can thus be obtained by using this alginate under our conditions, leading to the production of microcapsules with a spherical shape and suitable size, these being major challenges for optimal immunocompatibility (Orive et al., 2006). In fact, a critical microcapsule diameter of around 400–450 μm is the preferred device geometry for cell immobilization from a biocompatible, functional and technological point of view (Orive et al., 2006).

In the present study, we aimed to investigate the ability of GDNF-secreting microencapsulated fibroblasts to deliver GDNF *in vitro* and *in vivo* over a long period of time. To this end, we determined (1) the viability and GDNF production of microencapsulated cells *in vitro* and (2) the behavioral, biochemical and immunohistochemical modifications in parkinsonized rats after microcapsule implantation.

2. Material and methods

The study was performed in male Sprague-Dawley rats weighing 150 g at the beginning of experiments. Rats were housed under standard laboratory conditions (22 °C, 12 h light-dark cycle, with *ad libitum* access to food and water).The treatment of the animals and their conditions was in accordance with the relevant Spanish legislation and the European Community Council Directive on "Protection of Animals used in Experimental and Other Scientific Purposes" of 24 November 1986 (86/609/EEC).

2.1. Materials

All reagents for culture were purchased from Gibco BRL (Invitrogen, Spain). The following reagents were purchased from the indicated suppliers: alginate (FMC Biopolymers, Drammen, Norway); poly-L-lysine hydrobromide, 6-hydroxydopamine, chloral hydrate, desipramine HCL, pargyline, ascorbic acid, apomorphine hydrochloride, D-amphetamine, dopamine, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 3,4-dihydroxyphenylalanine (Sigma, St. Louis, MO, USA); goat anti-GDNF antiserum (R&D systems, MN, USA); mouse anti-TH antibody (Diasorin, MN, USA); donkey anti-goat horseradish peroxidase labeled antiserum and rhodamine labeled anti-mouse antiserum (Jackson Immunoresearch, PA, USA); tyramide signal amplification kit (Perkin Elmer, Boston MA, USA); glycergel media (DakoCytomation, Glostrup, Denmark).

2.2. Microcapsule elaboration

Fischer rat 3T3 fibroblasts transfected to produce GDNF were kindly donated by the Laboratory of Molecular Neurobiology, Karolinska Institute, Stockholm (Sweden). Cells were maintained in culture at 37 °C in a humidified 5% CO_2/95% air atmosphere and passaged every 2–3 days (Arenas et al., 1995).

Empty alginate-poly-L-lysine-alginate (APA) microcapsules and microcapsules containing GDNF secreting 3T3 fibroblasts were elaborated using an electrostatic droplet generator following the procedure described by Lim and Sun with some modifications (Lim and Sum, 1980). Two different potentials, 400 and 800 kV, were used to obtain microcapsules of different diameters around 1.7 mm for compression resistance assays and around 400 μm for the rest of the studies.

For the elaboration of empty microcapsules, an alginate solution (1.5%) was extruded into 55 mM anhydrous calcium solution. The microbeads obtained were maintained in agitation for five minutes in the solution of calcium chloride in order to complete ionic gelation. Finally, the microbeads were suspended in 0.05% (w/v) poly-L-lysine for five min and afterwards they were again coated with another layer of 0.1%

(w/v) alginate for another five min. The capsules were washed once with culture medium afterwards. Subsequently, they were maintained under normal culture conditions. Microcapsules containing Fischer rat 3T3 fibroblasts, transfected to produce GDNF (4.5×10^6 cells per ml) were elaborated by suspending cells in an alginate solution (1.5% w/v). The cell-gel suspension was extruded into 55 mM anhydrous calcium solution and the procedure described above was followed.

2.3. Microcapsule characterization

A uniform shape and a smooth microcapsule membrane without irregularities are essential to ensure the viability of the microencapsulated cells and the biocompatibility of the immobilization device after implantation (De Vos et al., 1994). Therefore, a detailed morphological characterization was carried out before implantation using an inverted optical microscope (Nikon TMS) equipped with a camera (Sony CCD-Iris).

2.4. Stability of the microcapsules: compression resistance studies

The stability of the microcapsules was evaluated by incubating approximately 30 capsules in 10 ml of culture medium and maintaining them in the normal culture conditions described above for 24 h. On the day of elaboration of microcapsules and after 24 h of culture, the compression resistance of the microcapsules was determined as the main force (g) required to generate 75% compression of the sample using a Texture Analyzer (Model TA-T2i, Stable Microsystems, Surrey, UK). The force exerted by the probe on the beads was recorded as a function of the displacement leading to a force versus strain curve.

2.5. Determination of the viability of the enclosed cells and GDNF production

Microencapsulated fibroblasts were maintained in culture for 21 days in order to study their viability and GDNF secretion *in vitro*. Cell viability was determined by the tetrazolium assay (Uludag and Sefton, 1990). In summary, 25 μl of a 5 mg/ml solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in PBS was added to approximately 40 microcapsules placed in a 96 well cell culture cluster and incubated at 37 °C for 4 h. Afterwards, the MTT solution was removed by vacuum aspiration and 100 μl of dimethylsulphoxide was added. The purple solution was read 5 min later on a microplate reader (Titerteck Multiscan Lab Systems) at 560 nm test wavelength with reference at 690 nm.

GDNF production by the non-encapsulated cells and the microencapsulated cells was measured with the GDNF E_{max}® Immunoassay system (Promega, Madison, USA) (Ponce et al., 2005). It detects GDNF in the range of 16-1000 pg/ml. Briefly, approximately 100 microcapsules were put into a 24 well cell culture cluster with 500 μl of culture medium and maintained under normal culture conditions for 24 h. Afterwards, the media was collected and undiluted samples were assayed for GDNF content.

2.6. Parkinson's disease model

Rats received unilateral injections of 6-OHDA into the right medial forebrain bundle (MFB). They were anesthetized with chloral hydrate (400 mg/kg, i.p.) and treated 30 min prior to 6-OHDA injection with desipramine HCl (25 mg/kg, i.p.), a noradrenergic reuptake blocker, and pargyline (50 mg/kg, i.p.), an inhibitor of the monoamine-oxidase enzyme. They were then positioned in a Kopf stereotaxic instrument and a hole was drilled in the skull. Lesions were produced by microinjection of 6-OHDA (8 μg, 2 mg/ml, dissolved in milli Q water containing 1% ascorbic acid) into the right MFB with a 22-gauge Hamilton syringe, at a rate of 1 μl/min. The stereotaxic coordinates according to the Paxinos and Watson rat brain atlas were: anterior: −2.5; lateral: −1.8, and vertical: −8.6 from bregma and the tooth-bar set at 0 mm (Paxinos and Watson, 1996). The rats were maintained in cages with free access to food and water for 2 weeks before the first rotational test.

2.7. Behavioral effects of microcapsule implantation

Four weeks after the 6-OHDA lesion, animals were deeply anesthetized with choral hydrate (400 mg/kg, i.p.) and placed in a Kopf stereotaxic instrument for microcapsule implantation. Skull skin was cut along the midline and a burr hole was drilled. Microcapsules were implanted into the right striatum. The stereotaxic coordinates for the microcapsule graft were: +0.2 mm anterior to the bregma, 3.2 mm lateral to the sagittal suture, and 6.5 mm ventral to the surface of the brain with the tooth-bar set at 0.0 mm. Before implantation, the microcapsules were washed several times with phosphate buffer (PBS 0.01 M, pH 7.4). Around 30–40 microcapsules were grafted into each rat. One group received empty microcapsules (empty-treated group) ($n = 11$) and the other GDNF secreting microencapsuled cells (GDNF-treated group) ($n = 16$).

Determination of the success of nigrostriatal denervation was assessed with a rotational behavior test previously described (Bilbao et al., 2006; Kemmerer et al., 2003). Briefly, the total number of full turns (360° rotation) was counted using rotometer bowls after rats were injected with the dopamine agonist apomorphine hydrochloride (0.5 mg/kg s.c.) 2 weeks after the 6-OHDA lesions and with amphetamine (3 mg/kg i.p.) 3 weeks after the 6-OHDA lesions. Apomorphine-induced rotational behavior was examined again at 2, 4–5, 8, 16, and 24 weeks post implantation, while amphetamine-induced rotational behavior was examined again at 2–4, 7–12 and 18 weeks post implantation. We used different test periods of time for apomorphine and amphetamine in order to ensure thorough washing out of drug and therefore avoiding drug interaction.

2.8. Immunohistochemistry and neurochemistry

Some animals from each group were sacrificed 24 weeks after implantation for immunohistochemical examination. To this

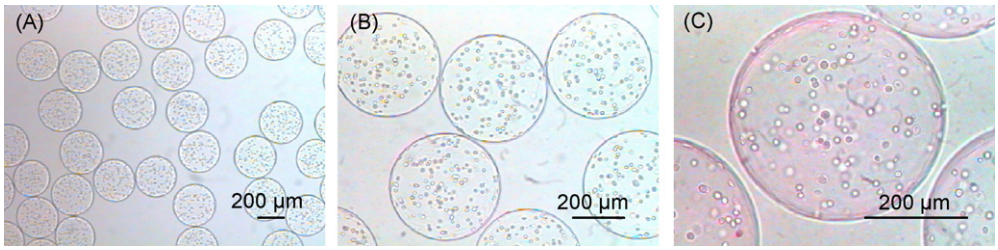

Fig. 1. Phase contrast images obtained by bright field microscopy, showing GDNF secreting Fischer 3T3 fibroblasts enclosed in alginate-poly-L-lysine-alginate microcapsules. (A) ×5 Magnification. (B) ×10 magnification. (C) ×20 magnification.

end, animals were anesthetized with chloral hydrate (400 mg/kg i.p.). The cardiovascular system was flushed transaortically with heparinized 0.9% NaCl solution, followed by fixation with 4% paraformaldehyde in phosphate buffer (PBS, 0.1 M, pH 7.4). Brains were extracted, postfixed by immersion in the same fixative for 2–3 h at 4 °C and cryoprotected by immersing the tissues in a 20% sucrose/PBS solution overnight. Then, tissues were frozen and kept at −80 °C. The brains were cut on a Microm cryostat to obtain 12 μm sections. These were mounted on gelatin-coated slides for tyrosine hydroxylase (TH) and GDNF immunohistochemistry. Adjacent sections were collected for Nissl staining to facilitate identification of the brain nuclei and to pinpoint the precise location of APA microcapsules containing GDNF secreting 3T3 fibroblasts. In addition, a counterstain with Hoechst 33258 (Sigma, B2883) at 1 μg/ml, 15 min was performed to detect the cell nucleus inside the microcapsules. Sections were washed by immersion in PBS (0.1 M, pH 7.4). Goat anti-GDNF antiserum was diluted (1:500) in PBS containing bovine serum albumin and milk powder. Mouse anti-TH antibody was diluted (1:1000) in PBS containing bovine serum albumin. Sections were incubated overnight in the presence of the primary antibodies at 4 °C. Tyramide signal amplification was used to amplify the signal of the GDNF antibody. Sections were washed for 15 min in TNT buffer (0.1 M Tris–HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.5). Thereafter, sections were blocked with TNB (TNT buffer, 0.5% blocking reagent) for 30 min and incubated for 30 min with donkey anti-goat horseradish peroxidase (HRP) labeled antibody at 37 °C. Finally, sections were washed in TNT for 20 min, and then incubated with tyramide fluorophore (fluorescein) (1:200) for 10 min. For the TH antibody, a donkey anti-mouse antibody labeled with rhodamine was used. The slides were mounted with Glycergel medium. Observation and photography of the slides were performed using a Zeiss Axioskop 2 plus microscope. The atlas of Paxinos and Watson (1996) was used to identify brain structures. Immunofluorescent intensity was measured using the NIH-Image analysis software (NIH, Bethesda, MA, U.S.A.).

The content of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), together with 3,4-dihydroxyphenylalanine (L-dopa) accumulation levels were determined in the rat striatum. Thirty minutes before sacrifice, animals received one injection of 3-hydroxybenzylhydrazine (100 mg/kg, i.p.), an inhibitor of the

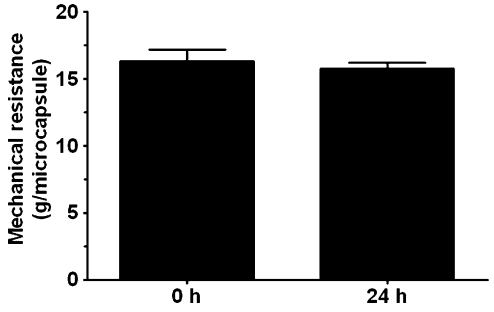

Fig. 2. Stability of alginate-poly-L-lysine microcapsules containing GDNF secreting Fischer 3T3 fibroblasts. Results are expressed as mean ± S.D.

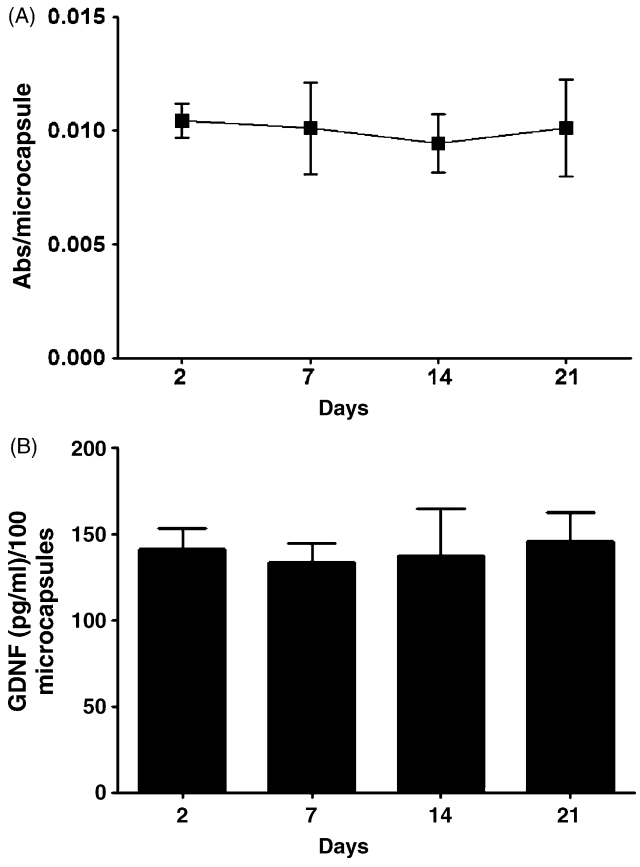

Fig. 3. (A) Viability of GDNF secreting Fischer 3T3 fibroblasts microencapsulated in alginate-poly-L-lysine-alginate for up to 21 days *in vitro*. (B) GDNF production by microencapsulated Fischer 3T3 fibroblasts transfected to produce this molecule. Results are expressed as mean ± S.D.

aromatic L-amino acid decarboxylase, to determine the accumulation of L-dopa as a measure of the *in vivo* activity of TH. The dissection of the striatum was performed on an ice-cooled plate; the tissue was homogenized in 0.1 M perchloric acid, centrifuged (30 min, 12,500 × *g*) and the supernatant was spin-filtered. Finally, one aliquot was assessed by high performance liquid chromatography with electrochemical detection (Waters Associates, USA) (Andia et al., 1994).

2.9. Statistical analysis

All values are expressed as mean ± S.E.M. with the exception of values of viability, GDNF production and stability, which are expressed as mean ± S.D. for 10 replicates. Comparison of viability, GDNF production and stability was carried out using the Student's *t*-test. Rotational data from unilaterally 6-OHDA lesioned rats were analyzed using a one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Comparisons between the groups were carried out using two-way ANOVA followed by the Bonferroni test. Comparison of behavior and weight values between two groups was made using the Student's *t*-test. Histological results were also analyzed using the Student's *t*-test. The accepted level of significance was $P < 0.05$.

3. Results and discussion

3.1. Characterization of microcapsules

GDNF secreting 3T3 fibroblasts were microencapsulated in APA, since alginate is the most widely used polymer for this purpose (Hortelano et al., 1996; Schwenter et al., 2003). It is necessary that the microcapsules have a good stability *in vitro* to improve and prolong the *in vivo* performance of the encapsulated cells. Thus, the cells need to adapt to the new microenvironment of the alginate matrix, being particularly important that microencapsulated cells are maintained in normal culture conditions for 24 h before their implantation (Shen et al., 1994). We observed that microcapsules presented a totally spherical and uniform shape with a visible and defined membrane formed by the interaction of alginate and poly-L-lysine (Fig. 1). All the capsules showed a similar diameter with a little dispersion (405.33 ± 9.90 μm). The mechanical resistance of the capsules was maintained after 24 h *in vitro* and was not altered by the culture conditions. In fact, their resistance to compression just after their elaboration was 16.33 ± 0.83 g/microcapsule and after 24 h was 15.79 ± 0.40 g/microcapsule (Fig. 2).

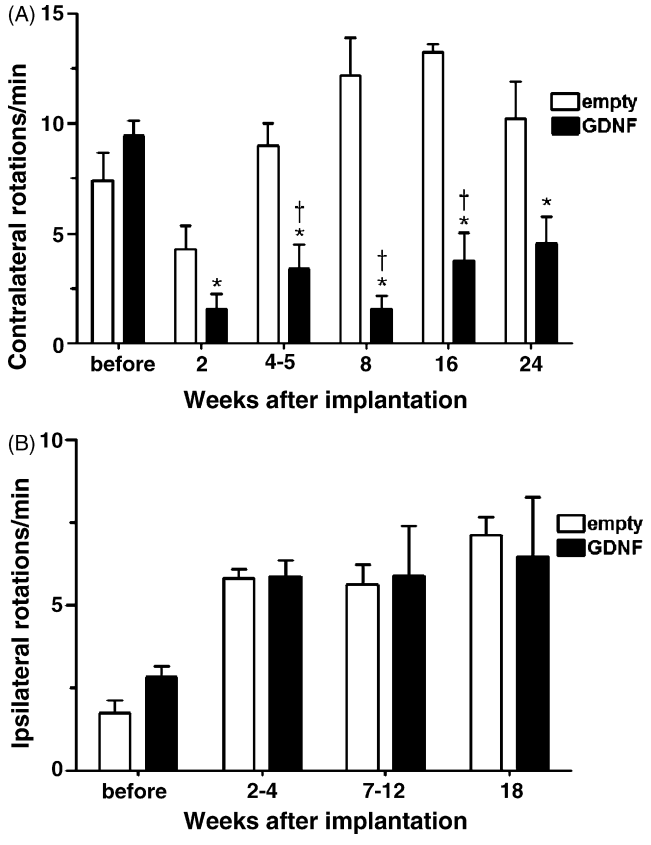

Fig. 4. Histograms illustrate the data obtained from the rotational behavior test of rats with unilateral 6-OHDA lesions after apomorphine (A) and amphetamine (B) administration before and after implantation. Each bar represents the mean ± S.E.M. (ANOVA *$P < 0.01$, when compared with the rotational behavior before implantation; two-way ANOVA †$P < 0.0001$, GDNF-treated group compared to control group).

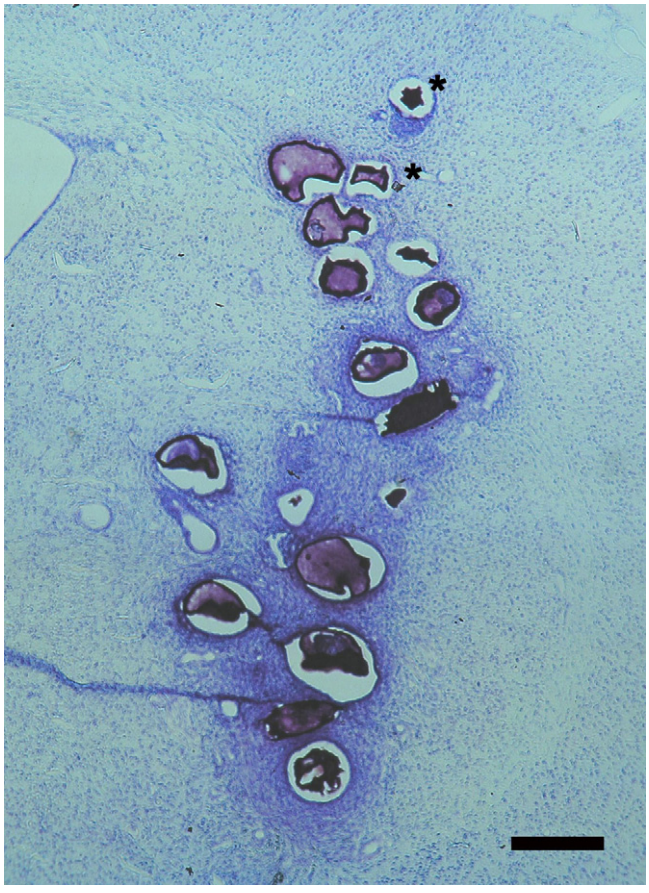

Fig. 5. Localization of capsules in a 12 μm slice with Nissl staining. The image shows the medial part of the caudate-putamen nucleus. Most of the capsules are located in the striatum. However, in a few animals, as in this representative case, some of the capsules were located close to the corpus callosum or even in the inner part of the cortex. Scale bar, 500 μm.

3.2. Cell microencapsulation: viability and GDNF production

Cell viability and GDNF secretion by the cells were analyzed before and after microencapsulation. Before microencapsulation, 1×10^6 cells released 20.67 ± 4.82 ng/ml of GDNF over 24 h. As shown in Fig. 3A and B, after microencapsulation, cells maintained a constant and stable metabolic activity and GDNF production (around 140 pg/ml of GDNF/100 microcapsules) for the 21 days of the study which indicates good adaptation of the cells to the new microcapsule environment. This is important since it has been reported that improvement in motor behavior is more gradual and maintained over time when GDNF administration is constant, while the administration of only one dose of drug provokes early but not long-lasting changes (Kirik et al., 2004).

3.3. Evaluation of in vivo effects of implanted microcapsules

In vivo functionality of microencapsulated GDNF secreting cells was assessed in 6-OHDA lesioned rats. Only animals that exhibited contralateral and ipsilateral rotation after injection of apomorphine (0.5 mg/kg, s.c.) or amphetamine (3 mg/kg i.p) two and 3 weeks post MFB lesion, respectively, were included in the study. No statistically significant differences in the number of rotations between the two experimental groups were observed before microcapsule implantation ($P > 0.05$, Student's t-test) (Fig. 4A and B). All rats survived to microcapsule implantation which was well tolerated and no significant differences were detected in the mean weight value between empty and GDNF-treated groups at 2 weeks (347 ± 9 g versus 344 ± 7 g), 5 weeks (365 ± 6 g versus 371 ± 7 g, $P > 0.05$) and 8 weeks (394 ± 8 g versus 414 ± 21 g, $P > 0.05$) after implantation.

At the end of the experiment, the localization of the microcapsules in the rat brain was confirmed by Nissl staining. Around 90% of implants had been placed in the medial part of the caudate-putamen nucleus. In some animals, a few microcapsules were detected either near the dorsal part of the striatum, close to the corpus callosum and cortex, or in the ventral part, close to the accumbens nucleus (Fig. 5). Microcapsules were surrounded by tissue without evidence of tissue damage, which indicates good acceptance of the microcapsules without immune responses.

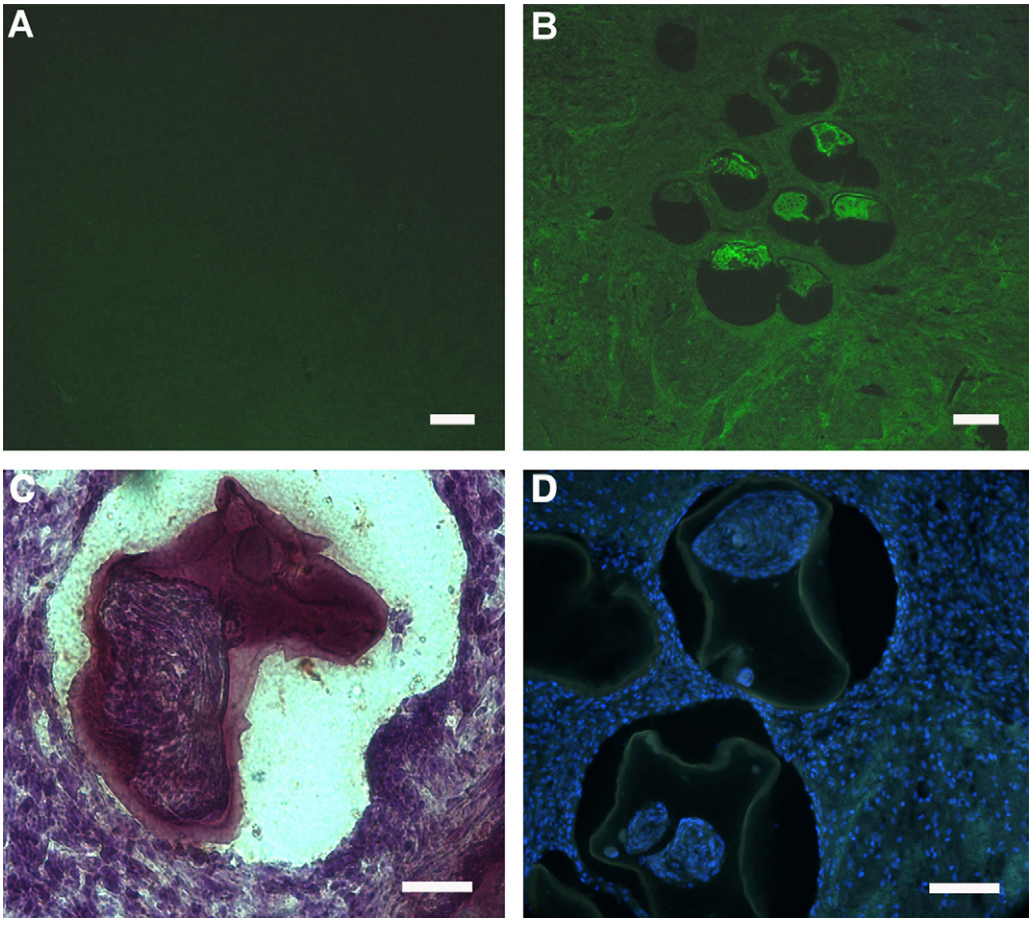

Fig. 6. GDNF immunoreactivity in the striatal nucleus on both sides of the same rat. (A) The image corresponds to the non-lesioned side, which does not show GDNF immunoreactivity. (B) GDNF secretion from encapsulated cells implanted in the lesioned side, showing GDNF immunoreactivity around the microcapsules. (C) Nissl staining showing fibroblasts surrounded by the microcapsule fragments (pink) and the round hole which was occupied. (D) Hoechst-Nissl counterstaining. Fibroblast cell nuclei can be observed in fluorescent blue color within the alginate cover of two microcapsules after 6 months implantation. No morphological signs of inflammation could be detected in the striatum, as can be observed in the images. Scale bars: (A and B) 300 μm; (C) 50 μm; (D) 100 μm.

The implantation of microcapsules containing GDNF secreting cells significantly reduced the number of rotations induced by apomorphine (2, 5, 8, 16 and 24 weeks after microcapsule implantation) in the GDNF-treated group when compared to the empty-treated group (two-way ANOVA, $F_{5,82} = 6.50$, $P < 0.0001$) (Fig. 4A). Thus, in the GDNF-treated group, the number of rotations per minute induced by apomorphine was reduced by 84%, 64%, 84%, 60% and 52%, respectively (ANOVA $F_{5,47} = 13.05$, $P < 0.01$), compared to the value before implantation (Fig. 4A). This effect was specifically due to GDNF delivery since no significant differences in the rotational behavior were found in the empty microcapsule implanted-group (ANOVA $F_{5,35} = 5.56$, $P > 0.05$, compared to the value before implantation) (Fig. 4A).

There were no differences in the number of rotations induced by amphetamine, comparing the initial values and those obtained 2–4, 12 and 18 weeks after implantation in the GDNF-treated group (ANOVA, $F_{3,46} = 1.977$, $P = 0.0713$). No differences were observed when the values obtained in the GDNF-treated group were compared with those obtained in the empty-treated group (two-way ANOVA, $F_{2,78} = 0.23$, $P = 0.8731$) (Fig. 4B).

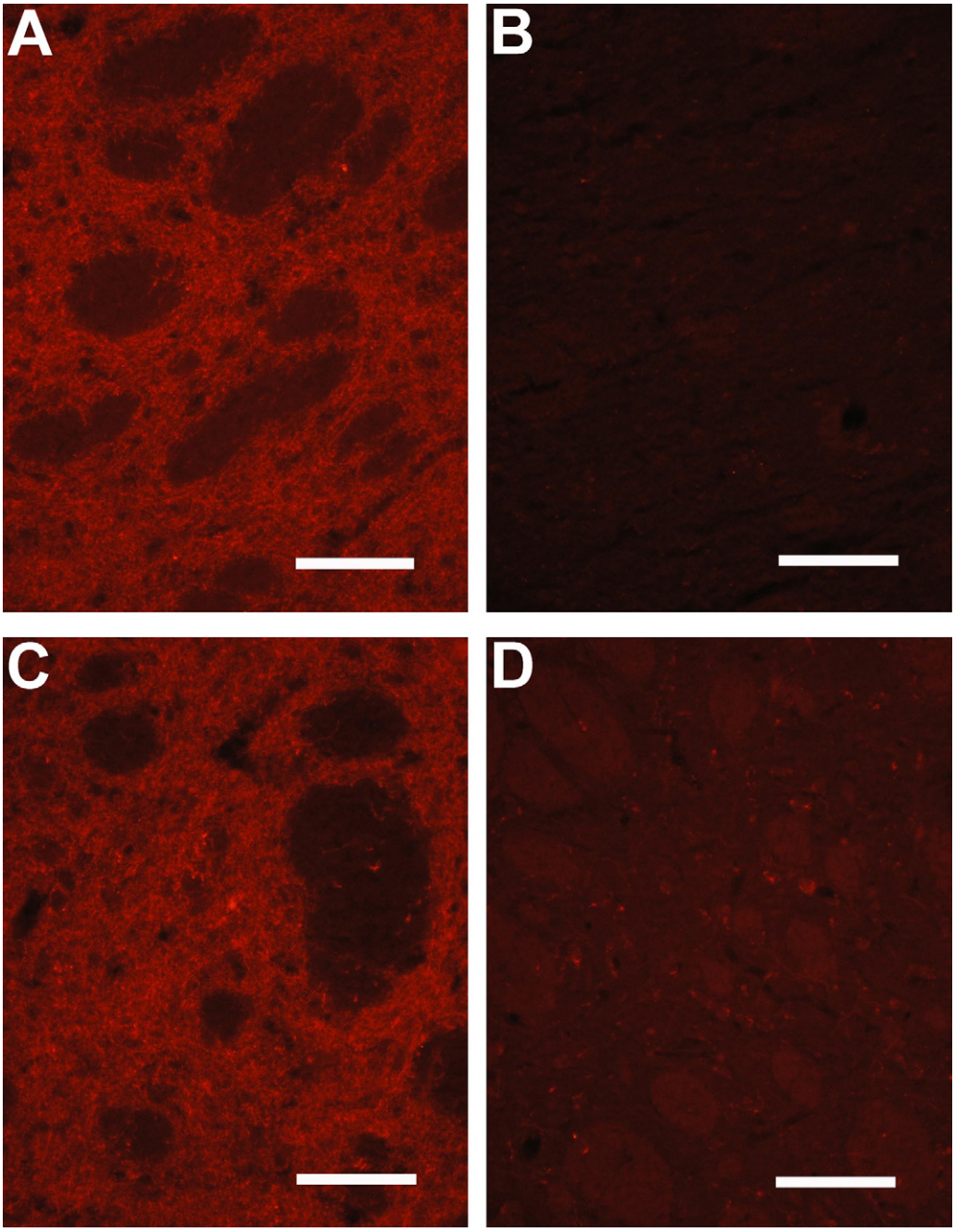

Fig. 7. Tyrosine hydroxylase immunoreactivity (TH-ir) in the striatal area of a control rat (A and B) and in a GDNF-treated rat (C and D). (A and C) non-lesioned side; (B and D) lesioned side. TH-ir was observed to decrease in the lesioned side when compared with the non-lesioned side for both groups of rats. Scale bar, 75 μm.

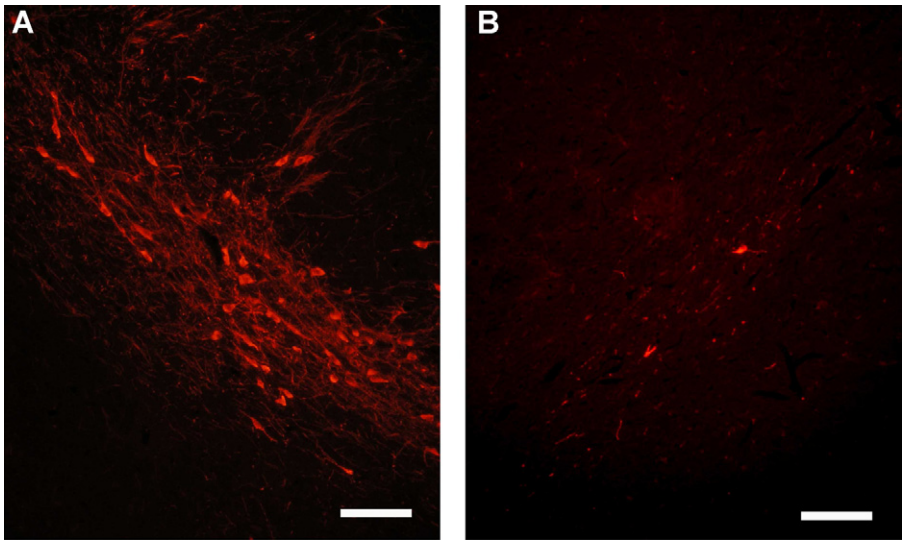

Fig. 8. Tyrosine hydroxylase immunoreactivity (TH-ir) in the substantia nigra of a representative rat. (A) Non-lesioned side. (B) Lesioned side. A decrease in the number of TH positive cells in the substantia nigra is observed in the lesioned side. The extent of this decrease was comparable for all the animals and treatments considered in the present study. Scale bar, 100 μm.

This improvement of behavioral abnormalities is compatible with a partial functional recovery of nigrostriatal dopaminergic neurotransmission. Rotational behavior with apomorphine requires a complete loss of dopaminergic neurons in order to occur; in contrast, only 50% neuronal loss is required for amphetamine effects to become apparent (Bilbao et al., 2006). Since no sprouting and neuronal recovery was observed (see below), these results are also compatible with a direct action of GDNF on striatal GDNF receptors as suggested by Yasuhara et al. (2005).

3.4. Immunohistochemical analysis

Immunohistochemical detection of GDNF showed intense GDNF immunoreactivity in the striatal tissue surrounding the capsules. GDNF was detected to diffuse a few hundred microns from the microcapsules and could still be detected 6 months following microcapsule implantation (Fig. 6A and B), indicating a continuous release of GDNF by the encapsulated cells into the striatum and that cells were viable and maintained their function inside the capsules during at least 6 months. Nissl staining revealed the continuing survival of the fibroblasts after the 6 months period as can be observed in Fig. 6C. Furthermore, counterstaining with bisbenzimide (Hoechst 33258) demonstrated that these fibroblasts were not apoptotic, since their nucleus showed a fluorescence intensity which was incompatible with chromatin condensation or nuclear fragmentation (Fig. 6D). No morphological evidence of inflammation was detected 6 months after implantation; in fact, the microcapsules were found to be completely surrounded by striatal cells (Fig. 6B, C and D).

TH immunoreactivity in the striatal area was used not only to evaluate the dopaminergic nigrostriatal pathway, but also as an indicator of possible sprouting of dopaminergic fibers and/or dendritic spines in the striatal area (Kirik et al., 2004). Changes in the optical density of TH immunoreactivity 24 weeks following implantation were similar in both the empty-treated group and the GDNF-treated group. Thus, TH immunoreactivity decreased in the striatal area of the lesioned side compared to the non-lesioned side. The decrease observed in the striata of the lesioned side of the GDNF-treated group was 25.17% ± 7.56 %, while that of the empty-treated group was 34.82% ± 10.42% (Fig. 7) (two-tailed, unpaired Student's t test, $P = 0.48$; $n = 4$). In addition, a progressive loss in the number of TH immunoreactive neurons was observed in the substantia nigra corresponding to the lesion side (TH immunoreactive neurons decreased in the

Table 1
Determination of 3,4-dihydroxyphenylalanine (L-DOPA) accumulation concentration and dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) content in the striatum of lesioned rats

Treatment	L-DOPA (ng/mg tissue)[a]		DOPAC (ng/mg tissue)		DA (ng/mg tissue)		HVA (ng/mg tissue)		n
	Right (lesion)	Left	Right (lesion)	Left	Right (lesion)	Left	Right (lesion)	Left	
Lesion and implantation of empty capsules	0.52 ± 0.48	2.34 ± 0.43	0.07 ± 0.07	0.55 ± 0.26	0.94 ± 0.11	16.08 ± 3.51	0.06 ± 0.10	0.71 ± 0.09	3
Lesion and implantation of GDNF capsules	0.10 ± 0.09	1.31 ± 0.33	0.04 ± 0.06	0.96 ± 0.66	0.57 ± 0.33	12.31 ± 3.67	0.01 ± 0.02	0.61 ± 0.21	5

Each value represents the mean ± S.D. in ng/mg wet tissue of n rats per group.
[a] L-DOPA accumulation was measured 30 min after administering NSD-1015 (100 mg/kg, i.p.).

GDNF-treated group by 78.04 ± 11.56% and in empty-treated group by 87.47 ± 4.44%) (Fig. 8).

3.5. Neurochemical analysis

No significant changes in the levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) or homovanillic acid (HVA) in the striatum of the empty-treated group and the GDNF-treated group were observed in the 4 weeks after implantation of microcapsules containing GDNF secreting cells (Table 1). Moreover, no significant difference was found in L-dopa accumulation in the GDNF-treated group versus the empty-treated group (Table 1). Results of previous studies are quite contradictory in this regard. Sprouting has been observed after the administration of higher quantities of GDNF before or in the days subsequent to the lesion (Kirik et al., 2004; Love et al., 2005) or after a partial DA degeneration (Jollivet et al., 2004), in which neuroprotective effects may occur. However, in the present study GDNF secreting cells were implanted after 2–3 weeks of complete nigrostriatal DA depletion; thus the present study is more concerned with neuroregenerative rather than neuroprotective effects of GDNF administration. In any event, additional research of a moderate model of parkinsonism will provide useful information in this regard.

4. Conclusion

Encapsulated and transplanted cells which were genetically modified to release GDNF into the striatum of parkinsonian rats were able to deliver continuous and reliable levels of GDNF over a long period of time (during at least 24 weeks), resulting in a recovery of rotational behavior and good biotolerance. These results will be useful when designing new systems which allow the continuous delivery for long periods of time of GDNF and other therapeutic molecules for the treatment of human neurodegenerative disorders. Further studies concerning the area and time of implantation and the degree of DA depletion are warranted.

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