

**LONG-TERM NEUROPROTECTION AND NEURORESTORATION BY
GLIAL CELL-DERIVED NEUROTROPHIC FACTOR MICROSPHERES FOR
THE TREATMENT OF PARKINSON'S DISEASE**

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ABSTRACT

Background: Glial cell-derived neurotrophic factor (GDNF) is a survival factor for dopaminergic neurons and a promising candidate for the treatment of Parkinson's disease (PD). However, the delivery issue of the protein to the brain still remains unsolved.

Purpose: The aim of the study was to investigate the effect of long-term delivery of encapsulated GDNF within microspheres.

Methods: A single dose of microspheres containing 2.5 µg of GDNF was implanted intrastrially in animals 2 weeks after a 6-hydroxydopamine lesion.

Results: The amphetamine test showed a complete behavioral recovery after 16 weeks of treatment which was maintained until the end of the study (week 30). This effect was accompanied by an increase in dopaminergic striatal terminals and neuroprotection of dopaminergic neurons.

Conclusions: The main achievement was the long-term neurorestoration in parkinsonian animals induced by encapsulated GDNF, suggesting that microspheres may be considered as a means to deliver GDNF for PD treatment.

KEYWORDS

Parkinson's disease

GDNF

Microspheres

Neurodegeneration

6-OHDA rat

Tyrosine hydroxylase.

ABBREVIATIONS

GDNF: glial cell-derived neurotrophic factor

PD: Parkinson's disease

SNpc: substantia nigra pars compacta

VTA: ventral tegmental area

PLGA: poly-lactic-co-glycolic acid

TH: tyrosine hydroxylase

pTH: phosphorylated tyrosine hydroxylase

6-OHDA: 6-hydroxydopamine

BDA: biotinylated dextran amine

INTRODUCTION

Glial cell derived neurotrophic factor (GDNF) was initially described as a potent neurotrophic factor for dopaminergic neurons, regarded as a promising therapeutic agent for PD¹. Several animal studies and a few clinical trials have set the requirements for proper GDNF treatment²⁻⁴. Intracerebroventricular injection of GDNF in PD patients caused severe side effects, without improving parkinsonian symptoms^{5, 6}, probably because GDNF did not reach the target tissues⁷. For these reasons, GDNF should be delivered locally to the caudate-putamen in humans or striatum in rodents⁸. The results from two independent open-label trials showed that the sustained delivery of GDNF within the putamen using a mechanical pump reduced the motor symptoms and achieved a partial restoration of the nigrostriatal pathway^{9, 10}. These promising results were in contrast to the findings in the first double-blind placebo-controlled study in which GDNF infusion, at a lower dose and using a wider catheter, failed to improve motor symptoms in patients¹¹. The lack of efficacy in this trial, the appearance of antibodies against GDNF and cerebellar lesions in monkeys receiving very high doses of GDNF, resulted in the withdrawal of GDNF from all clinical tests. These studies concluded that a new delivery system should be developed before considering GDNF infusion in human patients. This has prompted researchers to seek alternative methods for long-term protein delivery.

In the present study we propose the use of biodegradable and biocompatible non-toxic poly-lactic-co-glycolic acid (PLGA) microspheres for sustained GDNF delivery into the brain. The goal of this work was to assess whether a single low dose of microencapsulated GDNF would be sufficient to maintain long-term beneficial neurorestorative and/or neuroprotective effect in the partial 6-hydroxydopamine (6-OHDA) rat model of PD. We provide evidence that encapsulated GDNF may be

delivered to the striatum, inducing a long-lasting recovery of lesion-induced motor impairment. Restoration of functional behavior is accompanied by striatal dopaminergic fiber sprouting, neuroprotection of dopaminergic cells at the SNpc level and an increase in tyrosine hydroxylase phosphorylation.

MATERIAL AND METHODS

Microsphere preparation.

Rat recombinant glycosylated GDNF was expressed and purified as previously described¹². GDNF-loaded microspheres were prepared using the solvent extraction/evaporation method utilizing TROMS technology¹³. Fluorescent microspheres were prepared similarly but encapsulating rhodamine B isothiocyanate (0.1 mg) in the inner aqueous phase instead of GDNF.

Animal procedures.

Animals. Female Sprague-Dawley rats of 220-240 g were obtained from Harlan (Barcelona, Spain). Animals were kept in standard animal facilities with free access to food and water, in a temperature and humidity-controlled room with 12 h on–off light cycle. Procedures involving animals were in accordance with the guidelines established by the normative of the European Council (86/609/EEC).

Experimental parkinsonism. Animals were anesthetized via an intraperitoneal injection of a 1:1 mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). Rats received two 6-OHDA (Sigma) injections, each one of 10 µg in 5 µL of saline containing 0.1% ascorbic acid, into the striatum at the following coordinates relative to bregma¹⁴: AP: +0.5 mm, ML: 2.5 mm DV: 5 mm and AP: -0.5 mm, ML: 4.2 mm, DV: 5 mm.

Microsphere implantation. Stereotaxic surgery was performed 15 days after the 6-OHDA lesion using the same coordinates. Each rat received a dose of 2.5 µg of GDNF in two implantations, each one comprising 1.5 mg of microspheres (1.25 µg of GDNF).

Amphetamine test. The rotational behavior of the animals induced after an intraperitoneal injection of 5 mg/kg D-amphetamine (Sigma) was assessed on a computerized rotometer (Panlab).

Histological techniques

Animals were perfused transcardially with a fixing solution containing 4% paraformaldehyde in 0.125 M phosphate buffer, pH 7.4. Coronal sections (40 μ m thick) were obtained. Coronal sections (40mm thick) were use for immunohistochemistry. Histological techniques and optic density measurements were performed as described previously¹⁵.

Statistical analysis

Those data following a normal distribution were analyzed using independent Student's t test and non-parametric data using a Wilcoxon test for independent samples. A *p* value < 0.05 was considered significant.

RESULTS

Assessment of biodegradability of PLGA microspheres in vivo

We first evaluated whether GDNF-microspheres were present 28 weeks after their implantation. Microspheres loaded with rhodamine were prepared and injected within the rat striatal parenchyma. Animals were sacrificed at different times and rhodamine-microspheres were visualized by confocal microscopy (Fig. 1A). Between the first and second week there was a large decrease in the microsphere injection size that continued to decrease, at a lower rate, over time. Importantly, a significant amount of microspheres were still detectable at week 28th post-injection.

Microencapsulated GDNF reverses altered motor behavior in a PD animal model

To evaluate the long-term effect of encapsulated GDNF the partial 6-OHDA animal model was used. The extent of the lesion was assessed 13 days after the toxic administration by the amphetamine-induced rotational behavior test. Two days later, animals received an injection of either empty or GDNF-loaded microspheres. Striatal implantation of GDNF-microspheres significantly reduced amphetamine-induced ipsilateral rotational behavior (Fig. 1B) after 8 weeks of treatment. Furthermore, the imbalance in rotational behavior completely disappeared at week 16 and the recovery was maintained up to week 30.

Effect of GDNF-microsphere treatment on TH-positive cells

The effect of GDNF-microspheres at the striatal level was evaluated by TH immunohistochemistry. Intrastratial injections of GDNF-microspheres induced profuse TH-immunoreactive (TH-ir) fiber sprouting surrounding the injection areas (Fig. 1C). In contrast, only the injection tract was visible around the injection sites of empty microspheres. Aberrant sprouting in the globus pallidus was not observed¹⁶. Densitometric analysis at three different striatal rostrocaudal levels (Fig. 1D) showed a

22% increase in the area of TH immunoreactivity at the most caudal striatal levels. Our results suggest that these fibers are likely to arise from sprouting by the remaining terminals located within the ventral striatum and the nucleus accumbens.

TH-ir neurons were counted in three sections throughout the central portion of the SNpc, at the level of the medial terminal nucleus of the accessory optic tract (Fig. 2A). A statistically significant 1.7-fold increase in the number of TH-ir cells was observed within the SNpc of animals treated with GDNF-microspheres (Fig. 2B). The degree of TH activity within these cells was studied by double immunofluorescence to detect the form of the enzyme TH phosphorylated at ser31^{17, 18} (Fig. 2C). All cells detected were double labeled and fluorescence signal corresponding to pTH with respect to TH-positive cells was quantified. A significant increase in this ratio at the SNpc level was found in GDNF-treated animals (Fig. 2D). This was not observed at the VTA level (Fig. 2E). These results suggest that the improvement in behavior induced by GDNF-microspheres could be due to both a neuroprotective effect on dopaminergic cells and an increase in TH activity.

DISCUSSION

The results of the present study demonstrate that PLGA microspheres are an efficient vehicle for sustained delivery of neurotrophic factors to the brain. Biodegradable and biocompatible PLGA microspheres offer an interesting alternative and have already been used in clinical practice for the treatment of human gliomas^{19, 20}. Microspheres release drugs locally for an extended period of time, have a low risk of side effects and protect the encapsulated protein from degradation by tissue proteases.

Several studies to determine neuroprotection have been performed by administering GDNF prior to performing the 6-OHDA lesion^{16, 21-24}. While these studies are interesting for testing the efficacy of GDNF, the administration of the trophic factor after a lesion would have greater clinical relevance. For this reason, GDNF-microspheres were implanted intrastrially 14 days after the 6-OHDA lesion. At 28 weeks post-implantation, microspheres were still present and the GDNF beneficial effect was preserved. GDNF was clearly detected by immunohistochemistry 5 weeks after microparticles implantation¹⁵. In the present study, the immunohistochemistry procedure failed to detect the presence of GDNF, probably because the remaining protein was under the detection threshold of the technique. It may be assumed that very low GDNF doses are enough to maintain the beneficial effect as previous studies have demonstrated that protein withdrawal in PD patients²⁵ or GDNF viral expression switch off, reversed GDNF effect²⁶. The distribution of the trophic factor is another important issue. In this study, two intrastriatal microparticle injections were sufficient to promote a stable functional recovery of the 6-OHDA animal model. The number of microparticle injections may be increased for non-human primates or human brains with a larger putaminal volume.

An additional issue with GDNF-based therapy is the production of neutralizing antibodies against GDNF¹¹. An immune reaction against the *E. coli* unglycosylated recombinant protein used in the clinical trial could be responsible for the production of autoantibodies²⁷. To overcome this potential problem, glycosylated recombinant rat GDNF was used in our study¹² and the absence of anti-GDNF antibodies was confirmed¹⁵.

The low dose required in the present approach to achieve a functional effect was also remarkable. A total amount of 2.5 µg of GDNF was sufficient for the long-term effect of GDNF treatment. Considering that the human putamen volume is 171 times larger than the rat striatum^{28 29}, an estimation of the dose necessary for patients for a 28 week treatment would be 427 µg.

The main achievement of this study was the stable long-term functional recovery in parkinsonian animals induced by GDNF treatment initiated after the 6-OHDA lesion. The results of this work indicate that microspheres should be considered as a method to deliver GDNF directly to the striatum for the treatment of PD.

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LEGENDS

Figure 1: Neurorestorative effect of GDNF-microspheres. (A) Degradation of rhodamine microspheres implanted intrastrially. The same formulation of microspheres used for GDNF treatment was prepared and loaded with rhodamine. Rhodamine-microspheres were injected intrastrially and the extent of the deposit was followed at different time intervals. Microspheres are still present at week 28. Scale bar: 100 μm . (B) Amphetamine-induced rotation was performed 13 days after the 6-OHDA lesion. All animals that fulfilled the inclusion criteria (>9 full body turns per minute) received the injection of either empty or GDNF-loaded microspheres 48 h later. At 10 weeks post-6-OHDA and 8 weeks after beginning the GDNF treatment, animals receiving GDNF-microspheres showed a significant improvement in their rotational behavior when compared to animals that received empty microspheres. The rotational response was completely reversed at week 16 and the recovery maintained until the end of the study (week 30). Control animals treated with empty microspheres (open circles), $n = 6$; animals treated with GDNF microspheres (filled circles), $n = 8$. $*p < 0.05$. (C) TH immunohistochemistry was performed in animals sacrificed 30 weeks after 6-OHDA lesion. Images at the striatal level of the two injection sites show an increase in TH-positive fiber density surrounding the GDNF-microspheres deposits that is not detectable around the empty-microspheres deposits in lesioned animals. Scale bar: 1 mm. (D) Quantification of the TH-positive fiber density at three different rostrocaudal levels. $*p < 0.05$.

Figure 2: GDNF effect in the substantia nigra *pars compacta*. (A) Immunohistochemistry for TH revealed an increase in the number of surviving cells in the SNpc of GDNF-treated compared to empty-microspheres-treated animals 30 weeks

after the 6-OHDA lesion. (B) Quantification of TH-positive neurons from the SNpc showed a statistically significant increase in the number of cells present in animals treated with GDNF microspheres compared to animals that received empty microspheres. Scale bar: 1 mm. Wilcoxon test $*p<0.05$. (C) Double immunohistochemistry for TH and pTH showed that all cells were double labeled and presented an increase in pTH level. Quantification of fluorescence intensity of pTH with respect to the total amount of TH at the level of SN (D) and VTA (E) showed a significant increase of pTH in TH-ir neurons from the SNpc of GDNF-treated animals compared to the contralateral side. Scale bar: 20 μm . t-Student $*p<0.05$.