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ARTICLE

# Neurotensin Polyplex as an Efficient Carrier for Delivering the Human GDNF Gene into Nigral Dopamine Neurons of Hemiparkinsonian Rats

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**Recently we showed that the neurotensin polyplex is a nanoparticle carrier system that targets reporter genes in nigral dopamine neurons *in vivo*. Herein, we report its first practical application in experimental parkinsonism, which consisted of transfecting dopamine neurons with the gene coding for human glial cell line-derived neurotrophic factor (hGDNF). Hemiparkinsonism was induced in rats by a single dose of 6-hydroxydopamine (30 μg) into the ventrolateral part of the striatum. We showed that transfection of the hGDNF gene into the substantia nigra of rats 1 week after the neurotoxin injection produced biochemical, anatomical, and functional recovery from hemiparkinsonism. RT-PCR analysis showed mRNA expression of exogenous hGDNF in the transfected substantia nigra. Western blot analysis verified transgene expression by recognizing the flag epitope added at the C-terminus of the hGDNF polypeptide, which was found mainly in dopamine neurons by double immunofluorescence techniques. These data indicate that the neurotensin polyplex holds great promise for the neuroprotective therapy of Parkinson disease.**

**Key Words: gene delivery, gene therapy, nanoparticles, neuroprotection, neuroprotective therapy, nonviral vector, parkinson disease, receptor-mediated endocytosis**

## INTRODUCTION

Gene therapy protocols for Parkinson disease have paid little attention to the nonviral systems that depend on receptor-mediated endocytosis for gene transfer because their efficiency had not been improved to provide sustained gene expression *in vivo* [1,2]. Neurotensin polyplex is the first such nonviral system for gene delivery that has proved able to transfect dopamine neurons of the substantia nigra *in vivo* [3–5]. Neurotensin polyplex consists of biodegradable nanoparticles resulting from the electrostatic binding of the conjugate neurotensin–poly-L-lysine to a plasmid DNA (pDNA) [5,6]. The conjugate of neurotensin with poly-L-lysine is the carrier that uses the endocytosis of neurotensin with its high-affinity receptor (NTS1) to shuttle the transgene into dopamine neurons [3–5,7]. Without compromising its specificity, the coupling of the hemagglutinin-HA2 fusogenic peptide (FP) and the Vp1 SV40 karyophilic peptide (KP) improves the transfection efficiency of neurotensin polyplex *in vivo* [4,5]. Moreover, the tissue-specific promoter for dopamine neurons, hDAT [8], prolongs reporter-gene expression for more than 3 months and additionally provides another point of selectivity to the neurotensin polyplex [5].

The glial cell line-derived neurotrophic factor (GDNF) is a survival factor for midbrain dopamine neurons and



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a strong candidate for the treatment of Parkinson disease [9–11]. Chronic infusion of GDNF into the putamen of patients with Parkinson disease has been effective in increasing dopamine storage and reducing signs of motor impairment [12–14]. To reduce risks caused by chronic disruption of the blood–brain barrier, gene therapy strategies are under development in an attempt to provide sustained GDNF protein levels with a single gene transfer. Transduction of the GDNF gene by viral vectors into cells of the substantia nigra or the striatum to protect tyrosine hydroxylase-immunoreactive (TH-IR) cells has been extensively studied in animal models of Parkinson disease [15–19]. To date they are the preferred vehicles for gene transfer because of their higher efficiency of gene transfer *in vivo* [20,21]. With the incorporation of FP and KP, neurotensin polyplex significantly improved its transfection efficiency *in vivo* while preserving its biodegradability and low immunogenicity, the basic features of polymer-based nonviral vectors [6,22]. The combination of the potent effect of GDNF and the capability of the neurotensin polyplex for gene delivery into dopamine neurons *in vivo* is likely to retain the advantages of each approach and result in a more effective treatment for reducing dopamine neurodegeneration. Thus, we propose that the neurotensin polyplex-mediated transfection of the human (h)GDNF gene into surviving dopamine neurons of hemiparkinsonian rats will reduce their motor deficits. To test this hypothesis, we injected a single dose of neurotensin polyplex containing the plasmid pEF-Bos-hGDNF into the substantia nigra of hemiparkinsonian rats 1 week after a 6-OHDA injection into the ipsilateral striatum. We compared the effects of the transfection with pEF-Bos-hGDNF with two control groups of rats: a group

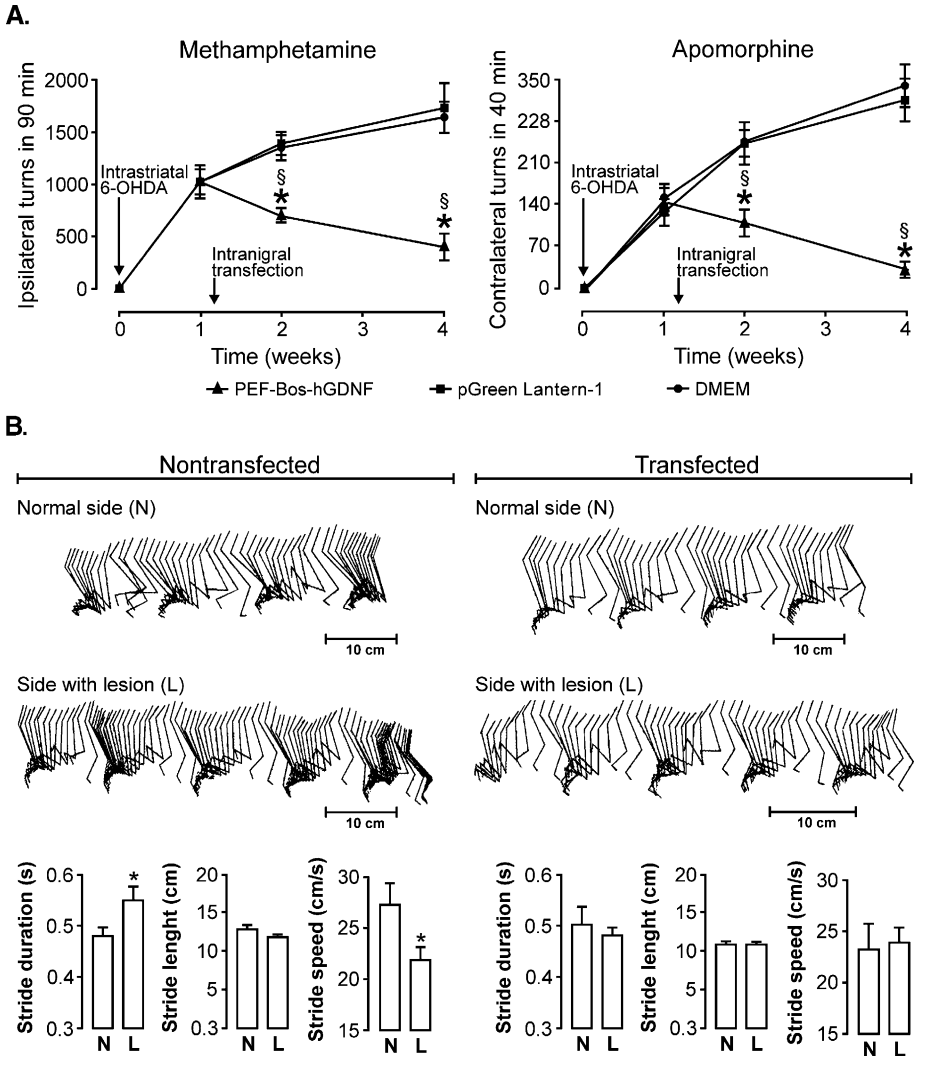


**FIG. 1.** Transfection of pEF-Bos-hGDNF reduces the motor impairment of hemiparkinsonian rats. (A) The reduction in methamphetamine-and apomorphine-induced turning behavior in transfected animals. §Significantly different from turns shown by the same group before transfection; *significantly different from control groups ($n$ = 20 in each experimental condition). (B) The analysis of the unrestrained gait of nontransfected ($n$ = 3) and transfected ($n$ = 3) hemiparkinsonian rats made at the end of the study. The line drawings show the spatiotemporal sequence of movements of both hind limbs in a series of four and five strides (see supplementary video for a representative motion analysis of the gait). The graphs show the mean ± SEM values corresponding to three parameters of at least four hind-limb strides per animal and experimental condition. *Significantly different from the hind limb without lesion. $P < 0.05$, repeated-measures two-way ANOVA and Bonferroni posttest.

microinjected with the plasmid solvent and another group transfected with pGreen Lantern-1 coding for the green fluorescent protein (GFP). To validate the hGDNF transgene expression and to discriminate it from the endogenous GDNF gene product, we added the flag sequence coding for an artificial octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) to the C-terminus of the hGDNF.

## RESULTS

### Reversal of Motor Impairment of Hemiparkinsonian Rats with the Intranigral Transfection of pEF-Bos-hGDNF

Hemiparkinsonian rats exhibiting equivalent values of turning behavior (1000 ± 200 ipsilateral turns/90 min induced by methamphetamine and 150 ± 50 contralateral turns/40 min induced by apomorphine) received different treatments into the substantia nigra 1 week after ipsilateral–striatal 6-OHDA injection. Rats treated with DMEM alone showed a rising ipsilateral and contralateral rotational behavior over time. Transfection of pGreen Lantern-1 had an effect similar to that of DMEM on rotational behavior. A significant reduction in both methamphetamine-and apomorphine-induced turning behavior occurred with transfection of either hGDNF or hGDNF-flag cDNA (Fig. 1A). Compared with the turning behavior of control hemiparkinsonian rats, the reduction in methamphetamine-induced turning behavior was 41 ± 4% (2 week) and 73 ± 7% (4 weeks) after transfection, whereas the reduction was 49 ± 5 and 90 ± 2% for apomorphine-induced turning behavior at the same two time points (Fig. 1A).

The kinematic analysis of unrestrained gait at the end of the study (4 weeks after causing the lesion or 3 weeks after transfection) also showed reversal of motor impairment of hemiparkinsonian rats with the transfection of the plasmid pEF-Bos-hGDNF (Fig. 1B). Compared with the contralateral side control, the hind limb of the side with a lesion of untreated hemiparkinsonian rats showed a significant reduction in stride speed (~26%; two-way ANOVA, $P < 0.001$; $n = 3$; Fig. 1B) that decreased the velocity of the unrestrained gaits (see supplementary video for a representative motion analysis of the gait). The gait slowness was not associated with changes in stride length but with a noticeable decrease in stride duration (Fig. 1B). In contrast, the pEF-Bos-hGDNF-transfected hemiparkinsonian animals showed no significant differences in stride duration, length, and speed, comparing both hind limbs, that

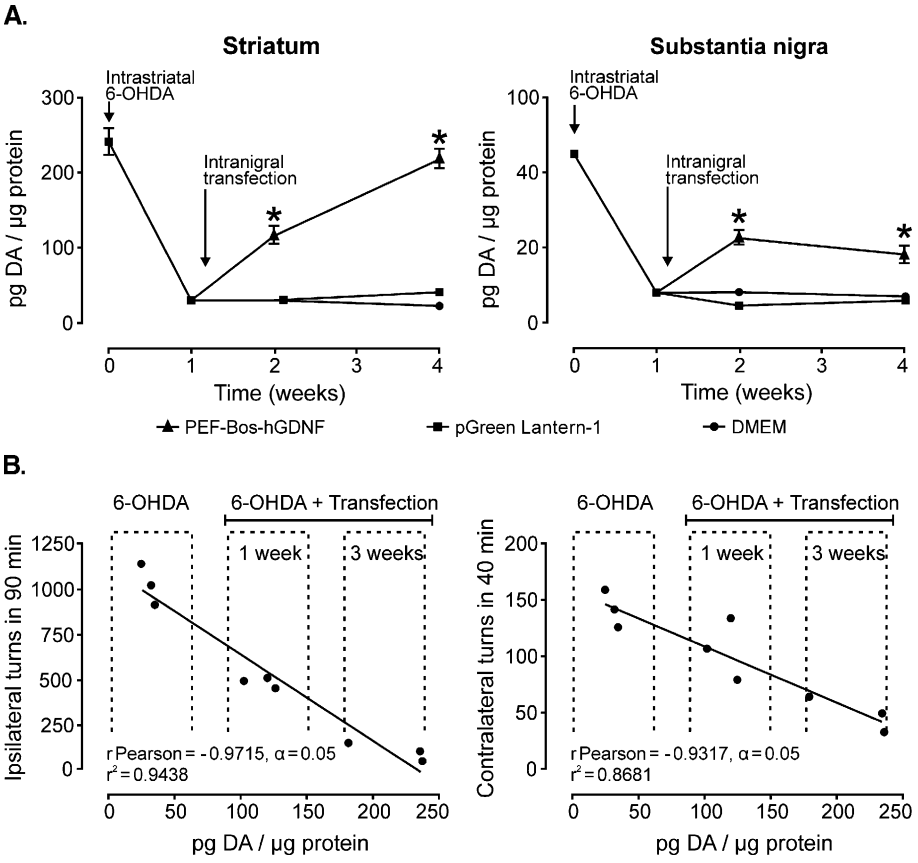


**FIG. 2.** Dopamine content recovery and its correlation with the hemiparkinsonism remission following intranigral transfection of pEF-Bos-hGDNF. (A) The partial recovery of dopamine content. Each value represents the mean ± SEM of independent measurements in three rats. (B) Correlation analysis between the recovery of dopamine levels in the striatum and the reduction of drug-induced turning behavior in hemiparkinsonian rats with pEF-Bos-hGDNF transfection. Values from nine rats were included in the correlation analysis; three animals were control rats with lesions caused by 6-OHDA injection, three animals were pEF-Bos-hGDNF-transfected rats evaluated 1 week after transfection, and three animals were pEF-Bos-hGDNF-transfected rats evaluated 3 weeks after transfection. *Significantly different from DMEM and pGreen Lantern-1-injected control groups; $P < 0.05$, two-way ANOVA and Bonferroni posttest.

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with a lesion and the control, during the unrestrained gait (Fig. 1B).

### Recovery of Dopamine Content in Hemiparkinsonian Rats Following Intranigral Transfection of pEF-Bos-hGDNF

Striatal injection of 6-OHDA reduced by 87% the dopamine levels in the striatum and substantia nigra and they remained as low as 30 ± 2.9 pg/μg (striatum) and 8.0 ± 0.5 pg/μg (substantia nigra) at the end of the study (Fig. 2A). Neurotoxin-induced reduction of dopamine levels was not altered by transfection with pGreen Lantern-1 or DMEM injection (Fig. 2A). We found a significant rise in dopamine content both in the striatum and in the substantia nigra in hemiparkinsonian rats transfected with pEF-Bos-hGDNF. We observed differential recovery at the end of the study; dopamine levels recovered by 90 ± 8% in the striatum but only by 50 ± 4%

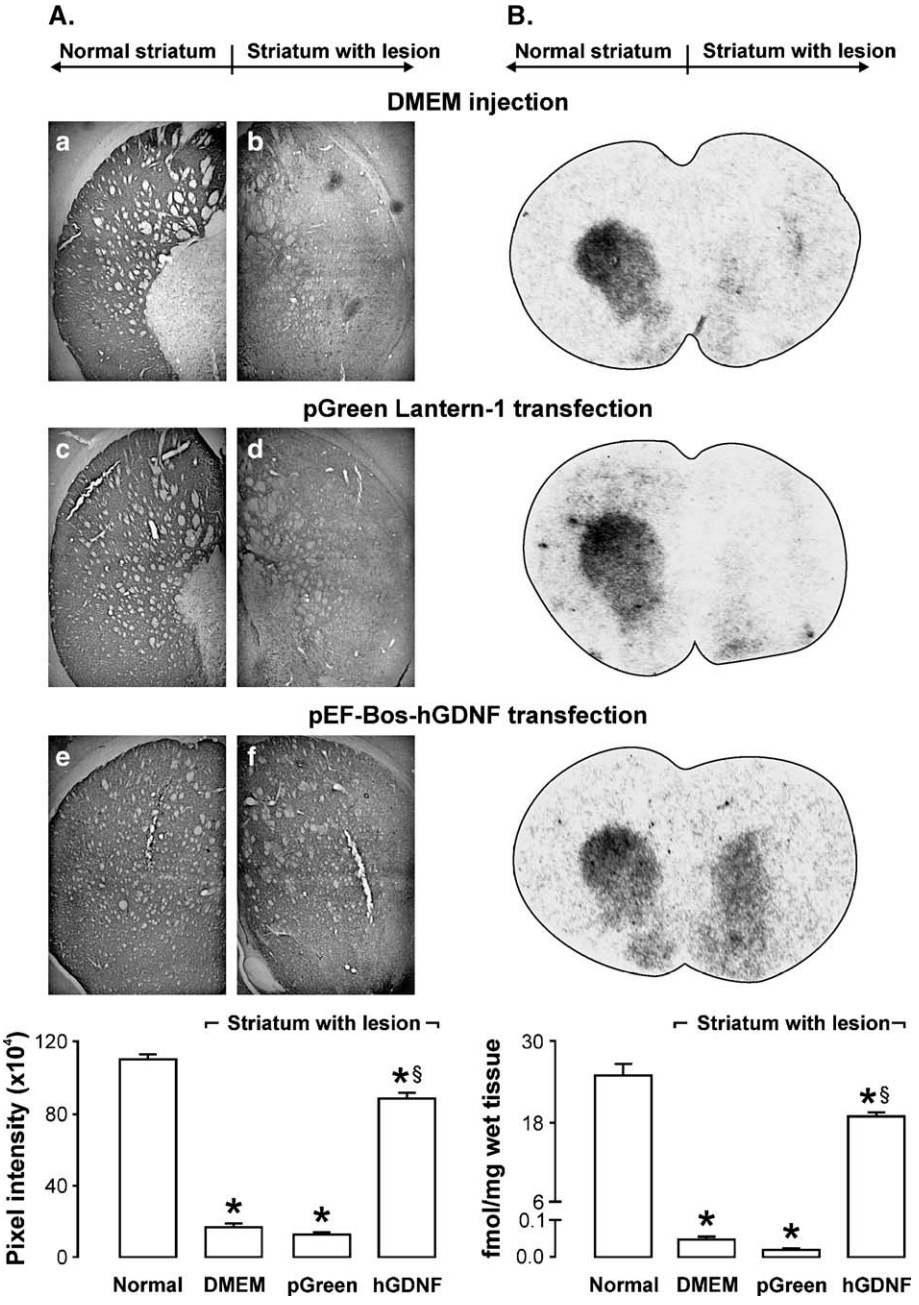


**FIG. 3.** Partial recovery of dopamine innervation of the striatum in hemiparkinsonian rats at 3 weeks after intranigral transfection of pEF-Bos-hGDNF. (A) A representative micrograph of TH-immunostained striatal slices. The paired photographs, a and b, c and d, and e and f, correspond to the same striatal level. Original magnification 3.15×. The graph shows the pixel intensity of the digital images of TH immunostaining in one of every six collected serial slices (35 μm) of the whole striatum ($n$ = 3 rats in each group). (B) A single analysis of the autoradiography of dopamine transporter density in the striatum of hemiparkinsonian rats. The graph shows the means ± SEM of binding density of the whole striatum.

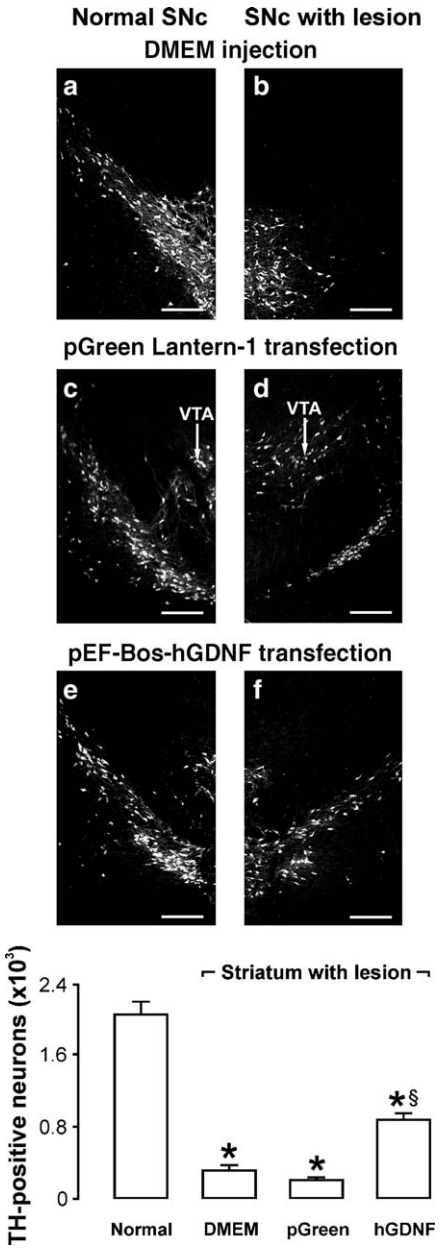


**FIG. 4.** Partial rescue of TH-immunoreactive neurons of the substantia nigra with pEF-Bos-hGDNF 3 weeks after transfection. The paired photographs, a and b, c and d, and e and f, correspond to the same nigral level. Calibration bars, 100 μm. The graph shows the count of TH-IR neurons on one of every six collected serial slides (35-μm) of the whole substantia nigra ($n$ = 3 rats in each group). SNc, substantia nigra pars compacta; VTA, ventral tegmental area.

in the substantia nigra (Fig. 2A). A high degree of correlation between rising dopamine levels in the striatum and decreased methamphetamine-($r^2$ = 0.9438) or apomorphine-induced ($r^2$ = 0.8681) turning behavior could be established in hemiparkinsonian rats transfected with pEF-Bos-hGDNF (Fig. 2B).

### Recovery of Dopamine Innervation to the Striatum in Hemiparkinsonian Rats Following Intranigral Transfection of pEF-Bos-hGDNF

We assayed the density of TH immunoreactivity and autoradiographic levels of dopamine-transporter (DAT) binding sites in striatal slices from hemiparkinsonian rats 3 weeks after transfection. As expected, 6-OHDA injection reduced both TH immunoreactivity (Fig. 3A) and DAT levels in the ipsilateral striatum (Fig. 3B). Neither DMEM injection nor pGreen Lantern-1 transfection was able to modify the reduction in TH immunoreactivity (83 to 87%) and DAT levels (99%) in the striatum with lesions. By contrast, the decrease in TH immunoreactivity (Fig. 3A) and DAT levels (Fig. 3B) was 18 and 19%, respectively, in the lesioned striatum of animals with nigral transfection of pEF-Bos-hGDNF.

### Effect of Nigral pEF-Bos-hGDNF Transfection on the loss of TH-Immunoreactive Neurons in the Substantia Nigra

We measured a significant loss (76%) of TH-IR neurons of the substantia nigra 4 weeks after 6-OHDA injection into the ipsilateral striatum (Fig. 4). Neither DMEM injection

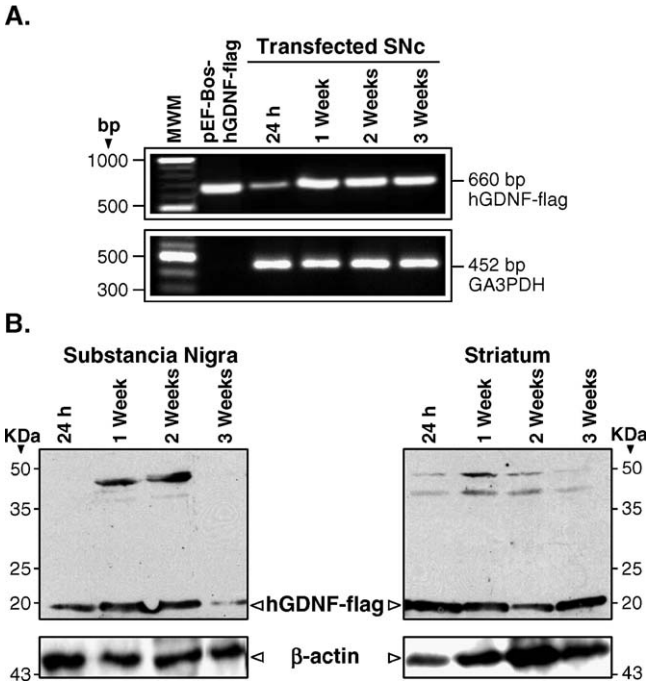


**FIG. 5.** Time course of hGDNF-flag gene expression in the substantia nigra of hemiparkinsonian rats following intranigral transfection of pEF-Bos-hGDNF-flag. (A) A representative photograph of RT-PCR products showing hGDNF-flag mRNA expression. (B) hGDNF-flag protein expression. Representative immunoblots from substantia nigra or striatum homogenates of the transfected side show immunopositivity to antibodies against the flag epitope and β-actin. Eight rats ($n$ = 2 at each time point) were used for RT-PCR and Western blot analysis.

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nor pGreen Lantern-1 transfection was able to reduce the 6-OHDA-induced neuronal loss, which was 83% (DMEM) and 89% (pGreen Lantern-1). However, the loss of TH-IR neurons was 57% in the substantia nigra transfected with pEF-Bos-hGDNF, which was significantly different (one-way ANOVA, $P < 0.001$) from DMEM and pGreen Lantern-1 injections (Fig. 4).

### Transgene Expression Following Intranigral Transfection of pEF-Bos-hGDNF-flag in Hemiparkinsonian Rats

We assessed expression of the hGDNF-flag by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot in eight hemiparkinsonian rats ($n = 2$ in each experimental group) that showed reversal of motor impairment with the pEF-Bos-hGDNF-flag transfection. The expression of the hGDNF-flag was not detected in the nontransfected substantia nigra of hemiparkinsonian rats by either RT-PCR or Western blot analysis (data not shown). RT-PCR showed the hGDNF-flag mRNA expression in tissue from the substantia nigra as early as 24 h after transfection (Fig. 5A), which remained stable until the end of the study (3 weeks after transfection). Western blot assays (Fig. 5B) verified that the hGDNF-flag protein was expressed in the transfected substantia nigra of all the animals ($n = 2$ at each time point) studied. They also showed the presence of the hGDNF-flag protein in tissue from the ipsilateral striatum, thus suggesting its axonal transport from the transfected nigral neurons (Fig. 5B).

Double-immunofluorescence analysis showed a clear colocalization of flag-and TH-immunostaining in cells of the substantia nigra transfected with the hGDNF-flag, thus suggesting that dopamine neurons are the main type of cell expressing the transgene (Fig. 6).

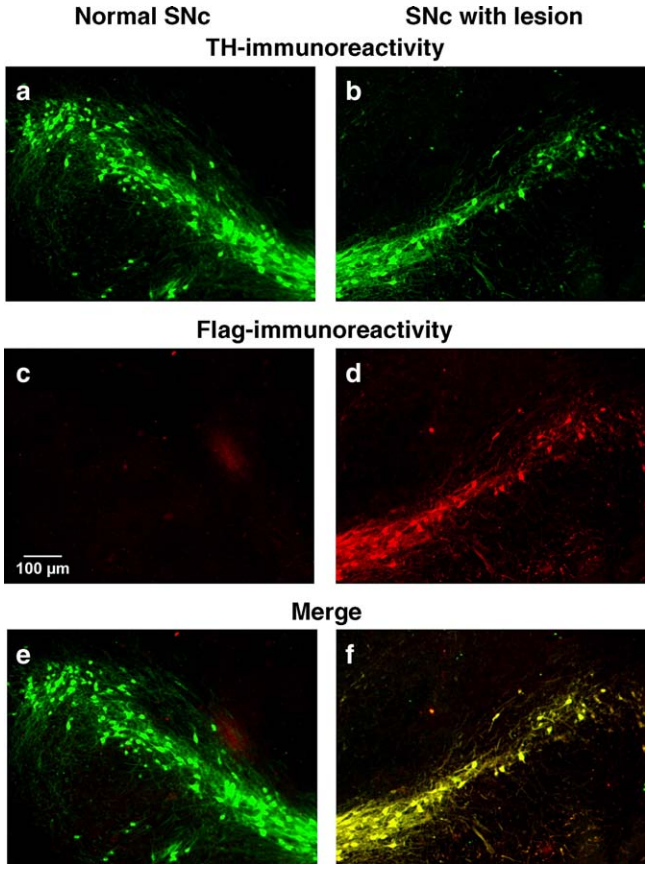


**FIG. 6.** GDNF-flag expression in dopamine neurons of the substantia nigra of hemiparkinsonian rats at 3 weeks following intranigral transfection of pEF-Bos-hGDNF-flag. The paired photographs, a and b, c and d, and e and f, correspond to the same nigral level. The calibration bar in c is valid for all the images. The micrographs are representative of three independent experiments.

## DISCUSSION

This study shows for the first time the ability of the neurotensin polyplex to deliver a therapeutic gene into nigral dopamine neurons in a rat model of Parkinson disease. The evidence of hGDNF expression in the transfected substantia nigra is provided by findings with RT-PCR, Western blot, and double immunolabeling techniques. The general agreement among results obtained with these three independent techniques strongly indicates the effectiveness of this transfection strategy, even in dopamine neurons under the influence of 6-OHDA-induced oxidative stress. The human EF-1α promoter might cause the relatively long-term expression of hGDNF-flag. Accordingly, hDAT promoter provides much longer transgene expression [5] than viral promoters do [3] when using the neurotensin polyplex for gene delivery. Because the endogenous hEF-1α and hDAT promoters are normally active in dopamine neurons, their replicas in pDNAs might evade the methylation process that inactivates viral promoters [23,24] longer. The presence of hGDNF protein was shown in the substantia nigra and in the striatum. As expected, the 20-kDa protein corresponds to the molecular mass of GDNF when its glycosylation [9] and the contribution of the molecular mass of the flag (1013 kDa) are taken into account. The other bands detected between 37 and 50 kDa are likely to correspond to unprocessed forms [25] or protein interactions that are observed even under denaturing conditions [25–28]. In agreement with a previous report [11], the presence of hGDNF in the striatum and susbtantia nigra suggests that the neurotrophic effect is exerted simultaneously on the terminals and soma of the remaining dopamine neurons.

Transgene expression occurred with a concomitant reversal of motor impairment only in rats transfected with the pEF-Bos-hGDNF or pEF-Bos-hGDNF-flag plasmids. The motor impairment was closely related to the vast denervation to the striatum and the reduction of TH-

immunoreactive neurons in the substantia nigra. In this study, the drug-induced turning behavior appeared when the striatal dopamine depletion was 87 ± 1%. This result agrees with the idea that behavioral signs appear when the striatal dopamine depletion approaches 90% [29–33]. The injection of a higher dose of 6-OHDA (30 μg) than usually used in previous work (<20 μg) into the ventrolateral part of the striatum, an area innervated exclusively by the substantia nigra [29,34], might explain the progressive dopamine neurodegeneration in control hemiparkinsonian rats. In contrast, remission of hemiparkinsonism signs occurred after the transfection of pEF-Bos-hGDNF or pEF-Bos-hGDNF-flag. The increased dopamine content in the striatum can account for the reduction of methamphetamine-and apomorphine-induced turning behavior, as shown by the correlation analysis between these variables.

The neurotensin polyplex, self-assembled at an optimum molar ratio between its components, is safe because it neither affects cell viability *in vitro* [5] nor produces signs of hemiparkinsonism when injected into rats without lesions (data not shown). Despite the biodegradability of neurotensin [35] and poly-L-lysine [36], cytotoxicity has been shown *in vitro* although at molar ratios greater than the optimum [5]. Poly-L-lysine of low and high molecular mass, both in free and in pDNA-complexed forms, is also known to induce apoptosis in cultured cells [37]. Finally, control of transgene expression is essential to avoid deleterious effects caused by the excessive expression of GDNF [38–40]. In summary, the neurotensin polyplex will be only a powerful and useful tool for the screening of therapeutic genes in animal models of Parkinson disease until it proves to be safe for clinical use.

## Materials and Methods

***Synthesis of the neurotensin carrier and polyplexes.*** The detailed procedure of neurotensin-carrier synthesis and of neurotensin polyplex formation at an optimum molar ratio and its biophysical properties are reported in Ref. [5]. Briefly, neurotensin (Sigma, St. Louis, MO, USA) and FP (GLFEAIAEFIEGGWEGLIEGCAKKK; purity >90%; SynPep, Dublin, CA, USA) were cross-linked with poly-L-lysine (48 kDa mean molecular mass) using LC-SPDP as the cross-linker [41]. Suitable gel-filtration chromatography was used to purify the SPDP derivatives and the neurotensin–SPDP–(FP-SPDP)–poly-L-lysine conjugate, the neurotensin carrier. This conjugate was concentrated to 1 ml, further dialyzed against phosphate-buffered saline solution, pH 7.4 (PBS), and sterilized by filtration. The neurotensin polyplexes were made by electrostatically binding the mutant Vp1 SV40 KP (MAPTKRKGSCPGAAPNKPK; 90% purity; Synpep Corp.) to pDNA [4]. Retention and retardation microassays [4,5] were used to determine the optimum molar ratios of polyplex components and were 30 nM pDNA:20 μM KP:200 nM neurotensin carrier for pGreen Lantern-1 and 30 nM pDNA:20 μM KP:300 nM neurotensin carrier for pEF-Bos-hGDNF and pEF-Bos-hGDNF-flag.

***Plasmids.*** Plasmid pEF-Bos-hGDNF (5.9 kb) coding for human GDNF under the control of human EF-1α promoter was obtained by cloning the human GDNF cDNA (636 bp) in the sense orientation into the *Xba*I–*Xba*I sites of the mammalian expression vector pEF-Bos [42,43].

Plasmid pEF-Bos-hGDNF-flag (5.9 kb) is a derivative of pEF-Bos-hGDNF in which the *Bst*XI fragment carrying the hGDNF cDNA was exchanged by a *Bst*XI fragment of hGDNF with a flag epitope added in frame at the 3′ end by PCR. The primers forward, 5′-CCAGTGTGATGGATGAAGTTATGGGATGTCGTGGC-3′, and reverse, 5′-CCAGTGTGCTGGCTACTTGTCATCGTCGTCCTTGTAGTCGATACATCCACACCTTTTAGCGGA-3′, were used for amplification with pEF-Bos-hGDNF as a template. Full hGDNF sequence and correct flag addition were confirmed by automatic sequencing (BigDye v3.1.; Applied Biosystems, Foster City, CA, USA).

Plasmid pGreen Lantern-1 (5.031 kb) coding for GFP under the control of CMV promoter was obtained from a commercial source (Gibco BRL, Grand Island, NY, USA).

***Stereotaxic procedures for injections of 6-OHDA and of NT polyplex.*** Experiments were done on adult male Wistar rats bred in our facilities. All procedures were in accordance with the Mexican current legislation, NOM-062-ZOO-1999 (SAGARPA), based on the *Guide for the Care and Use of Laboratory Animals*, NRC. The CINVESTAV Institutional Animal Care and Use Committee approved our animal use procedures (Protocol 0109-02). All efforts were made to minimize animal suffering. Anesthetized rats (220 ± 10 g) were subjected to the stereotaxic procedures as described previously [3–5]. Three microliters of 6-OHDA solution (10 μg free base/μl PBS with 0.02% ascorbic acid) was injected into the striatum at the coordinates AP, −0.3 mm from bregma; ML, +4 mm; DV, −5.5 mm from dura mater [44]. Different neurotensin polyplexes were injected into the ipsilateral substantia nigra at the coordinates AP, −4.6 mm from bregma; ML, +1.5 mm, DV −6.8 mm from dura mater. After injection of 1 μl of 50 mM kelatorphan [3], 1 μl of neurotensin polyplex solution was injected at a flow rate of 0.1 μl/min. The total amount of pDNA injected was 118 ng for pEF-Bos-hGDNF, 118 ng for pEF-Bos-hGDNF-flag, and 100 ng for pGreen Lantern-1.

***Behavioral testing.*** Ipsilateral or contralateral turning behaviors were induced consecutively (1 day after another) by methamphetamine (8 mg/kg body wt, total dose, ip) or by *R*-(−)apomorphine sulfate (0.5 mg/kg, total dose, ip). Drug-induced turning behavior was recorded at 1-min intervals over 90 min (methamphetamine) or 40 min (apomorphine) as described previously [45].

The unrestrained gait of rats was recorded with two video cameras placed on either side of a transparent acrylic passageway. Waist (iliac bone), hip (ischiac bone), knee, ankle, and the first phalanx joint of both the right and the left hind limbs were marked with indelible ink points on shaved skin. The geometrical coordinates of articulations were measured frame by frame from recorded videos to obtain the spatiotemporal sequence of movements of both hind limbs during several strides of normal gait. The duration, length, and speed of at least four strides were determined.

***Dopamine content and dopamine transporter autoradiography.*** Dopamine content was determined by reverse-phase HPLC and electrochemical detection in supernatants from homogenates of the striatum or substantia nigra, as described elsewhere [46].

Dopamine transporter binding was assayed using $^{3}$H-WIN-35428 (84 Ci/mmol; New England Nuclear, Boston, MA, USA) as a specific ligand and GBR-12909 dihydrochloride (Sigma) as a binding blocker [47]. After the binding assay, 16-μm striatal slices (plates 10–12 of the atlas [44]) were apposed to $^{3}$H-Hyperfilms, alongside microscale calibrated $^{3}$H standards (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 8 weeks. The films from autoradiography assays were analyzed using a computerized image-analysis system (MCID System; Imaging Research, Inc., St-Catherines, ON, Canada).

***Immunohistochemistry and immunofluorescence.*** Striatal sections were obtained and processed using immunohistochemistry and nigral sections with indirect immunofluorescence techniques as reported previously [3–5]. Immunohistochemistry was done using a mouse monoclonal anti-tyrosine hydroxylase TH2 antibody (1:6000; Sigma) and a biotinylated anti-mouse horse IgG (H+L) (1:100; Vector Laboratories, Burlingame, CA, USA). The immunohistochemical staining was developed using the

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avidin–biotin–peroxidase complex (1:10; ABC Kit; Vector Laboratories) and DAB (Sigma). Immunohistochemical labeling was observed with a Leica DMIRE2 microscope and images were digitized with a Leica DC300F camera (Nussloch, Germany). Quantification of intensity of the immunohistochemistry image was estimated by the pixel intensity in the striatum area with the GNU Image Manipulation Program (GIMP 1.2; http://www.gimp.org; Free Software Foundation, Inc., Boston, MA, USA). The indirect immunofluorescence was done with a goat polyclonal anti-tyrosine hydroxylase (C-20) antibody (1:60; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody was a fluorescein (FITC)-conjugated donkey anti-goat IgG (H+L) (1:100; Jackson Immunoresearch, Palo Alto, CA, USA). Colocalization of the hGDNF-flag expression in dopamine neurons was shown by double indirect immunofluorescence against TH as described above and the flag. The primary antibody was a mouse monoclonal antibody against Flag M2 (1:100; Sigma). The secondary antibody was a rhodamine (Rho)-conjugated rabbit anti-mouse IgG $F(ab')_2$ (1:100; Pierce Chemical Co., Rockford, IL, USA). The fluorescence was detected at excitation–emission wavelengths of 488 and 522 nm (green for FITC) and 568 and 585 nm (red for Rho). One of six nigral slides (an average of 10 sections per rat) was used for the counting of TH-IR cells. Digital images of the substantia nigra compacta, projected on a screen monitor, were divided in 16 quadrants by using the GNU Image Manipulation Program, and the TH-IR cells were counted manually. Negative controls were brain sections processed under similar conditions in the absence of the primary antibody.

***Analysis by RT-PCR and Western blot of hGDNF-flag expression.*** RT-PCR was performed as described elsewhere [48]. To amplify the full-length (660 bp) hGDNF-flag, the forward primer, 5′-TATGAAGTTATGGGATGTCGTGGCT-3′, and the flag reverse primer, 5′-CACTTGTCATCGTCGTCCTTGTAGTC-3′, were used with an annealing temperature ($T_a$) of 60°C. To amplify 452 bp of GA3PDH, the forward primer was 5′-ACCACAGTCCATGCCATCAC-3′ and the reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′, with a $T_a$ of 55°C. PCR products were analyzed on 2% agarose gel electrophoresis and their ethidium bromide-stained bands were digitized with a Kodak DC290 camera.

Western blots were done as described previously [49] in homogenates from striatal and nigral tissues. Proteins (50 μg) were subjected to electrophoresis in 12% SDS–PAGE gels and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) to be immunolabeled with a mouse monoclonal antibody to Flag M2 (1:100; Sigma) and then with a peroxidase-labeled goat anti-mouse IgG (H+L; 1:6000; Zymed, San Francisco, CA, USA). To normalize the total amount of protein per lane, membranes were stripped and incubated with a monoclonal mouse antibody against β-actin (1:200 dilution) [50] and the peroxide-labeled goat anti-mouse IgG (1:6000).

***Statistical analysis.*** All values are expressed as the mean ± SEM. The repeated-measures two-way ANOVA was used to analyze the difference of the turning and the unrestrained gait values. Comparison of dopamine content values was analyzed with the two-way ANOVA. Differences of TH-immunostaining densitometry and neuron counting were analyzed with a one-way ANOVA. The difference between pairs of group means was shown by the Bonferroni posttest. The correlation analysis between dopamine levels and drug-induced turning behavior was done by using Graph Pad Software (San Diego, CA, USA). In all analyses, the null hypothesis was rejected at the 0.05 level.

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2006.09.001.

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