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# Dissociation between short-term increased graft survival and long-term functional improvements in Parkinsonian rats overexpressing glial cell line-derived neurotrophic factor

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## Abstract

The present study was designed to analyse whether continuous overexpression of glial cell line-derived neurotrophic factor (GDNF) in the striatum by a recombinant lentiviral vector can provide improved cell survival and additional long-term functional benefits after transplantation of fetal ventral mesencephalic cells in Parkinsonian rats. A four-site intrastriatal 6-hydroxydopamine lesion resulted in an 80–90% depletion of nigral dopamine cells and striatal fiber innervation, leading to stable motor impairments. Histological analysis performed at 4 weeks after grafting into the GDNF-overexpressing striatum revealed a twofold increase in the number of surviving tyrosine hydroxylase (TH)-positive cells, as compared with grafts placed in control (green fluorescent protein-overexpressing) animals. However, in animals that were allowed to survive for 6 months, the numbers of surviving TH-positive cells in the grafts were equal in both groups, suggesting that the cells initially protected at 4 weeks failed to survive despite the continued presence of GDNF. Although cell survival was similar in both grafted groups, the TH-positive fiber innervation density was lower in the GDNF-treated grafted animals (30% of normal) compared with animals with control grafts (55% of normal). The vesicular monoamine transporter-2-positive fiber density in the striatum, by contrast, was equal in both groups, suggesting that long-term GDNF overexpression induced a selective down-regulation of TH in the grafted dopamine neurons. Behavioral analysis in the long-term grafted animals showed that the control grafted animals improved their performance in spontaneous motor behaviors to approximately 50% of normal, whereas the GDNF treatment did not provide any additional recovery.

## Introduction

Behavioral recovery after intrastriatal transplantation of fetal ventral mesencephalic (VM) cells in the rat Parkinson model has so far been most pronounced when the grafted cells are spread evenly throughout the target structure (Winkler *et al.*, 2000). We have previously shown that the extent of denervation in the host striatum critically influences the survival, fiber outgrowth and functional efficacy of VM grafts (Winkler *et al.*, 1999; Kirik *et al.*, 2001). Thus, in animals with mild to intermediate dopamine (DA)-denervating lesions, graft-induced fiber outgrowth is less pronounced as compared with animals with more severe or complete lesions. Overall, there seems to be a maximal tyrosine hydroxylase (TH)-positive fiber innervation provided by the graft, with a ceiling at about 50–60% of normal innervation density, and accordingly motor performance of grafted animals in tests of forelimb use (such as the stepping test) levels off at approximately 50% of normal (Kirik *et al.*, 2001). It is possible that the ability of the denervated striatum to promote further fiber outgrowth from the grafts may be lost at about this level due to lack of long-lasting trophic support.

Glial cell line-derived neurotrophic factor (GDNF) was initially discovered as a growth- and survival-promoting factor for developing DA neurons *in vitro* (Lin *et al.*, 1993) and has since then been shown to support the survival and fiber outgrowth of fetal DA neurons after transplantation *in oculo* (Strömberg *et al.*, 1993; Johansson *et al.*,

1995) or into the 6-hydroxydopamine (6-OHDA)-lesioned rat striatum. The latter has been shown in studies where GDNF was added either to the cell suspensions of embryonic VM tissue (Apostolides *et al.*, 1998; Mehta *et al.*, 1998; Sullivan *et al.*, 1998) or delivered in the vicinity of intrastriatal VM grafts by (i) intermittent injections or continuous infusion of recombinant GDNF protein (Rosenblad *et al.*, 1996; Sinclair *et al.*, 1996; Yurek, 1998) or (ii) cotransplantation with GDNF-expressing cell lines (Sautter *et al.*, 1998; Wilby *et al.*, 1999; Ostenfeld *et al.*, 2002). In these studies, the effects of transient GDNF treatment of transplanted DA neurons were evaluated at 4–8 weeks after transplantation.

Although the initial improved survival of DA cells by GDNF is well documented, it remains unclear whether the rescued cells could contribute to a long-term restoration of motor function, beyond what can be achieved with grafts in the absence of trophic support. For long-term delivery of GDNF, two different strategies are being pursued: (i) transplantation of cells genetically modified *in vitro* to express GDNF (*ex vivo* gene therapy) and (ii) direct delivery of the GDNF gene using recombinant viral vectors (*in vivo* gene therapy). The latter approach, in particular, has the advantage that it can provide a stable GDNF expression over many months and at tissue levels that efficiently protect nigral DA neurons in both rodent and primate models of Parkinson's disease (Déglon *et al.*, 2000; Kirik *et al.*, 2000a; Kordower *et al.*, 2000; Georgievska *et al.*, 2002a,b). In the present study, we have used the recombinant lentiviral (rLV) vector system for GDNF gene delivery to the striatum, in order to test whether continuous long-term GDNF support could increase the maximal

innervation density and thereby also the functional benefits from intrastriatal VM grafts.

## Materials and methods

### Subjects

A total of 85 adult female Sprague-Dawley rats (B & K Universal, Stockholm, Sweden) were used in this study. The animals were housed under a 12 h/12 h light/dark cycle with *ad libitum* access to food and water, except during the performance of the staircase test when the food consumption was limited to 10–12 g/rat/day. The housing of the animals and all surgeries were performed according to the rules set by the Ethical Committee for use of Laboratory Animals at Lund University.

### Experimental design

A total of 70 animals were used to study the long-term effects of continuous GDNF overexpression on the survival, fiber outgrowth and function of VM grafts (Fig. 1). The animals received intrastriatal 6-OHDA lesions ($n = 61$) or remained intact ($n = 9$) throughout the experiment (normal controls). At 8 weeks after the 6-OHDA lesion, 54 animals were divided into two groups and received injections of rLV-GDNF ($n = 29$) or rLV-GFP ($n = 25$) into the striatum, while the remaining lesioned animals ($n = 7$) were anaesthetised and perfused in order to evaluate the extent of the DA denervation. At 15 weeks after the rLV injections, 18 animals received VM transplants into the striatum (GDNF-trpl, $n = 9$; GFP-trpl, $n = 9$) while 15 were sham operated (GDNF-sham, $n = 8$; GFP-sham, $n = 7$). In addition, the remaining animals ($n = 21$) were divided into two subgroups to determine the expression levels of GDNF at the time of grafting (i.e. at 15 weeks after vector injection; rLV-GDNF, $n = 6$; rLV-GFP, $n = 4$) and at the end of the experiment (i.e. 9 months post-transduction; rLV-GDNF, $n = 6$; rLV-GFP, $n = 5$). The grafted or sham-operated animals were tested on a battery of drug-induced and spontaneous motor behaviors, as indicated by the time-points given in Fig. 1 and were anaesthetised and perfused for histology at 6 months after transplantation.

A separate experiment was designed to study the short-term effects of continuous GDNF overexpression on the survival of and outgrowth from VM grafts. For this purpose, 15 lesioned animals received injections of rLV-GDNF ($n = 8$) or rLV-GFP ($n = 7$) into the striatum, followed by transplantation of VM cells 2 weeks later. These animals were anaesthetised and perfused for histology at 4 weeks after the transplantation.

### Surgical procedures

All surgical procedures were performed under Hypnorm (0.14 mg/kg; Apoteksbolaget, Sweden) and Dormicum (3.4 mg/kg; Apoteksbolaget) anesthesia using a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and a Hamilton syringe fitted with a glass capillary (outer diameter 60–80 μm). The anterio-posterior (AP) and medio-lateral (ML) coordinates were calculated from bregma, according to the atlas of Paxinos & Watson (1998), and the dorso-ventral (DV) coordinate was calculated from the dural surface. The tooth bar was kept at 0.0 for all surgical procedures.

### 6-Hydroxydopamine lesions

The animals received injections of 6-OHDA (3.5 μg/μL free-base 6-OHDA dissolved in 0.2 mg/mL ascorbic acid-supplemented saline) at four sites into the right striatum, according to Kirik *et al.* (1998). A volume of 2 μL per site (7 μg 6-OHDA in total) was injected at a rate of 1 μL/min into each of the following coordinates: (i) AP +1.3, ML −2.6, DV −5.0; (ii) AP +0.4, ML −3.2, DV −5.0; (iii) AP −0.4, ML −4.2, DV −5.0 and (iv) AP −1.3, ML −4.5, DV −5.0. The needle was left in place for an additional 3 min before withdrawal.

### Lentiviral vector production and surgery

The rLV vectors encoding for the GFP or GDNF genes were generated as previously described (Georgievska *et al.*, 2002a). Briefly, the transfer constructs pHR.CMV.GFP.W and pHR.CMV.GDNF.W and the helper plasmids pCMVΔR8.91 and pMD.G were cotransfected into 293T cells and virions released into the media were collected at 48 and 72 h after transfection. The viral suspensions were concentrated by two rounds of ultracentrifugation at 116 000 *g* for 1.5 h/round and the final virus pellet was dissolved in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% glutamine. The number of transducing units (TU) for the final rLV vector stocks was determined as previously described (Georgievska *et al.*, 2002a). The titres of the
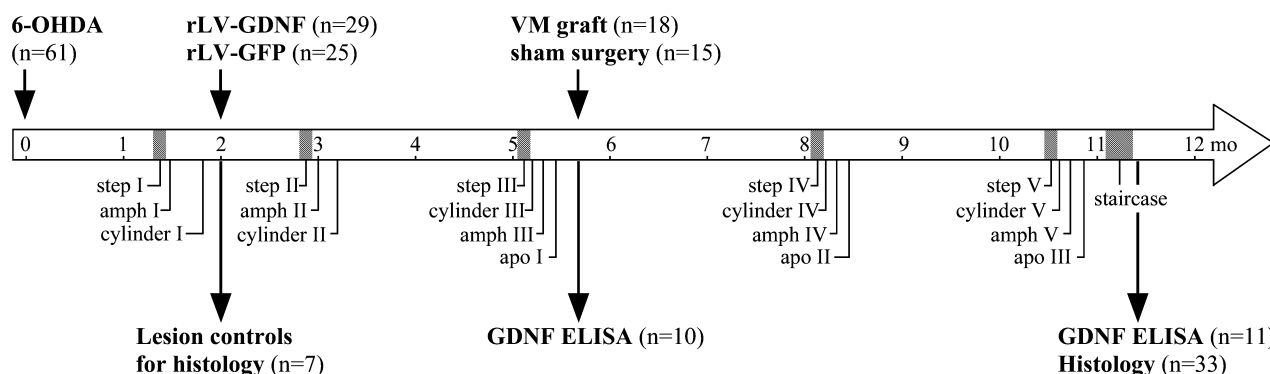


FIG. 1. Time-course of surgery and testing. Behavioral assessments were performed in five different sessions: at 6 weeks after the 6-hydroxydopamine (6-OHDA) lesion and just before injection of recombinant lentiviral (rLV) vectors (session I), at 1 and 3 months after rLV injection (sessions II and III) and at 3 and 6 months after transplantation (sessions IV and V). During sessions III–V a group of intact animals (normal controls, $n = 9$) were tested in parallel to the lesioned animals. At 8 weeks after the 6-OHDA lesion, seven animals were perfused (lesion controls) for histological evaluation of the extent of dopamine denervation. Subgroups of lesioned animals that had received injection of rLV-glial cell line-derived neurotrophic factor (GDNF) or rLV-GFP were killed at 15 weeks and 9 months after vector injection in order to determine the GDNF tissue levels by ELISA. The remaining animals were perfused at 6 months after transplantation (i.e. at 9 months after the rLV injection and at 11 months after the 6-OHDA lesion) for histological evaluation. VM, ventral mesencephalic.

rLV-GFP vector batches were estimated to $1.0 \times 10^8$ and $6.8 \times 10^8$ TU/mL and the rLV-GDNF vector batches were $1.0 \times 10^8$ and $3.2 \times 10^8$ TU/mL. The rLV-GDNF batches used in the two different experiments were first tested *in vivo* to ensure a similar level of GDNF production.

Before injection into the striatum the rLV vector stocks were diluted fivefold in saline. Approximately $6 \times 10^4$–$2 \times 10^5$ TU of rLV-GDNF and $6 \times 10^4$–$4 \times 10^5$ TU of rLV-GFP were injected in total into the right striatum at three tracts (1 μL/site) with two 0.5-μL deposits at each of the following sites: (i) AP +1.4, ML −2.6, DV −4.5, 3.5; (ii) AP +0.4, ML −3.2, DV −4.5, 3.5 and (iii) AP −0.8, ML −4.4, DV −4.5, 3.5. The viral vector was injected at 1 μL/min and the needle was left in place for an additional 3 min before withdrawal.

## Transplantation surgery

The VM cell suspensions were prepared according to Nikkhah *et al.* (1994). VM tissue from embryonic day 14 rat embryos was dissected and incubated in 0.1% trypsin, 0.05% DNase in Dulbecco's modified Eagle's medium at 37 °C for 30 min and mechanically dissociated into a single cell suspension. The suspension was then concentrated by centrifugation and resuspended to about 100 000 cells/μL with a viability of 98–99% at the beginning of the grafting session.

A total number of about 450 000 cells were distributed over five injection sites into the right striatum. Three 0.3-μL deposits were placed at each of the following coordinates: (i) AP +1.1, ML −3.4, DV −5.0, −4.3, −3.5; (ii) AP +0.6, ML −2.5, DV −5.0, −4.3, −3.3; (iii) AP +0.3, ML −3.8, DV −5.0, −4.3, −3.3; (iv) AP −0.5, ML −4.2, DV −5.0, −4.3, −3.3 and (v) AP −1.5, ML −4.5, DV −5.0, −4.3, −3.5. The viability of the cells in the remaining suspension after completion of the transplantation surgery was 95–98%.

## Behavioral analyses

### Drug-induced rotations

Rotational behavior was assessed in automated rotometer bowls after injection of D-amphetamine sulfate (2.5 mg/kg; Apoteksbolaget) or apomorphine-HCl (0.25 mg/kg; Sigma-RBI, St Louis, MO, USA) by monitoring the total number of full-body turns over 90 and 40 min, respectively. The rotational values are expressed as net 360° turns/min, with rotations towards the lesioned side (ipsilateral) assigned a positive value.

### Cylinder test

Forelimb use was assessed using the cylinder test (Schallert *et al.*, 1997) as described by Kirik *et al.* (2000b). Briefly, forelimb use was analysed by videotaping the animal in a transparent glass cylinder, where it was allowed to move freely and explore the environment. Mirrors were placed behind the cylinder at an angle in order to allow forelimb placements when the animal was turned away from the camera. An observer blinded to the identity of the animals scored the number of forelimb touches with the cylinder wall and a total number of 20 touches were recorded for each animal. The values are expressed as the percentage of left touches of total number of contacts.

### Forelimb akinesia test (stepping test)

Forelimb side stepping was analysed using the stepping test (Olsson *et al.*, 1995) as described by Kirik *et al.* (1998). Briefly, the animal was held by the experimenter in order to fix the hindlimbs with one hand and the forelimb not to be tested with the other hand, thus allowing the animal to support its weight on the free forepaw. By slowly moving the animal sideways along a table surface (90 cm in 4– 5 s) stepping movements in the forehand direction were initiated in order for the animal to adjust the balance. The test was repeated twice for each forelimb over four consecutive days and the average of the last three days was calculated.

### Paw reaching (staircase test)

A modified version of the staircase test described by Montoya *et al.* (1991) was used. Briefly, the animals were food deprived for 2 days before starting the test. They were then placed individually in Plexiglas test boxes for 15 min and allowed to retrieve pellets that were placed on each side of the staircase (10 pellets in each of four different levels, 40 pellets in total). The test was performed on nine consecutive days and the number of pellets taken and missed was counted on each side after each test session. The difference between the two constituted the number of successful retrievals for each animal.

## Glial cell line-derived neurotrophic factor ELISA

The GDNF tissue levels were determined at 15 weeks (rLV-GDNF, $n = 6$; rLV-GFP, $n = 4$) and 9 months (rLV-GDNF, $n = 6$; rLV-GFP, $n = 5$) after the rLV injections. The animals were deeply anesthetized with sodium pentobarbital (Apoteksbolaget) and decapitated. The brains were rapidly removed and placed into a brain slicer and two coronal sections covering the head and tail of the striatal region and one section covering the midbrain were obtained. The striatum was dissected dorsal to the anterior commissure and freed from the septum and overlying cortex, while a tissue punch centred on the substantia nigra (SN) was taken from the midbrain section. The striatal and nigral tissues were later sonicated in a homogenization buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1.7 μg/mL phenyl-methylsulfonyl fluoride, 1.0 μg/mL leupeptin, 10 μg/mL aprotinin and 1.0 μg/mL pepstatin A) at a tissue concentration of 30 mg/mL and centrifuged at 20 000 *g* for 10 min at 4 °C. The GDNF tissue levels were determined on homogenates by ELISA according to the supplier's recommendations (G3240; Promega, Madison, WI, USA).

## Histological analysis

The animals were deeply anesthetized with sodium pentobarbital (Apoteksbolaget) and transcardially perfused with 50 mL of isotonic sodium chloride, followed by 250 mL ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and post-fixed for an additional 2 h in the same fixative and transferred into 25% sucrose for cryoprotection. The brains were cut on a freezing slide-microtome into 40-μm-thick sections and divided into eight series at the level of the striatum and five series at the level of the midbrain. Immunohistochemistry was performed using specific anti-bodies for GDNF (goat IgG, 1 : 2000; R & D Systems, Minneapolis, MN, USA), GFP (chicken IgG, 1 : 5000; Chemicon, Temecula, CA, USA), TH (mouse IgG, 1 : 4000; Chemicon) and VMAT-2 (vesicular monoamine transporter; rabbit IgG, 1 : 2000; Chemicon). The sections were first quenched for 10 min in a potassium–phosphate buffer containing 3% $H_2O_2$ and 10% methanol and pre-incubated for 1 h in 5% serum, 0.25% triton in potassium–phosphate buffer (blocking buffer) before incubation with the primary antibody overnight at room temperature (22 °C). This was followed by a 1-h incubation with a biotinylated secondary antibody, diluted 1 : 200 in 0.25% triton in potassium–phosphate buffer (horse anti-goat BA9500,

rabbit anti-chicken BA9010, horse anti-mouse BA2001 and goat anti-rabbit BA1000, respectively; Vector Laboratories, Burlingame, CA, USA). The reaction was visualized using avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories) and the chromogen 3′,3′-diaminobenzidine. The tissue sections were mounted on chrom-alum-coated glass slides and later dehydrated in ascending alcohol solutions, cleared in xylene and coverslipped with Depex mounting medium.

## Microscopic analyses

### Stereological estimation of cell numbers

Estimation of the total numbers of TH- and VMAT-2-immunoreactive cell bodies in the intrastriatal grafts and SN pars compacta was performed using the optical fractionator principle (West, 1999). All striatal sections containing TH- or VMAT-2-positive cells in one of eight series and all sections covering the SN pars compacta in one of five series were used for stereological cell counting. The borders of the SN pars compacta were defined as previously described (Kirik *et al.*, 1998). Briefly, the medial border of the SN was defined as a vertical line passing through the medial tip of the cerebral peduncle and by the medial terminal nucleus of the accessory nucleus of the optic tract (thereby excluding the TH-positive cells in the ventral tegmental area); the ventral border was defined dorsally to the cerebral peduncle (including the TH-positive cells in the pars reticulata) and the lateral border included the pars compacta and pars lateralis. The sampling was performed with a $100 \times$ oil objective using the CAST system (Version 2.0; Olympus, Denmark A/S, Albertslund, Denmark), which places the first counting frame randomly over the counting area and systematically moves through all fields using an X–Y step length defined by the experimenter.

### Striatal fiber density measurements

Images of striatal sections were captured by a digital camera (ProgRes C14; Jenoptik, Germany) from an illumination table and the optical density of TH-positive and VMAT-2-positive fibers in the striatal images was determined using the ImageJ program (a free software developed by NIH, rsb.info.nih.gov/ij/). For each animal the optical density was measured at seven rostrocaudal levels, covering the entire head and tail of the striatum, corresponding to: (i) AP +1.60; (ii) AP +1.00; (iii) AP +0.20; (iv) AP −0.30; (v) AP −0.90; (vi) AP −1.40 and (vii) AP −2.10) relative to bregma according to the atlas of Paxinos & Watson (1998). The measurements were corrected for non-specific background by subtracting the optical density for corpus callosum (as measured in each section) and the values are presented as a percentage of the control side value (intact striatum).

### Statistical analysis

Differences between groups were assessed using unpaired *t*-test or ANOVA where appropriate. Post-hoc analysis was performed using the Student Newman-Keuls test. Significance was accepted at the 95% confidence level.
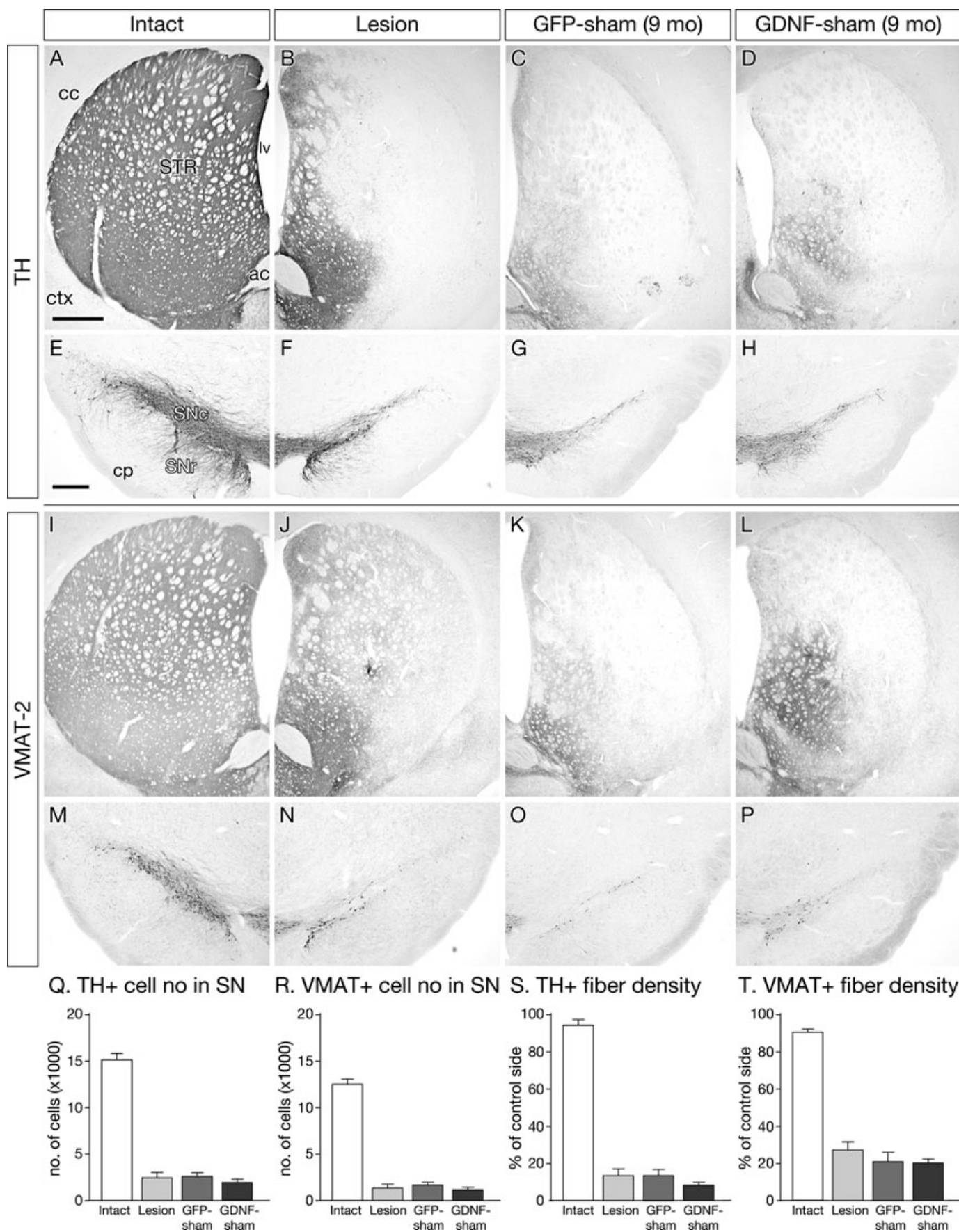
## Results

### Continuous glial cell line-derived neurotrophic factor over-expression in chronically 6-hydroxydopamine-lesioned rats

In the first experiment, our aim was to investigate the long-term behavioral effects of VM grafts placed in the host striatum over-expressing GDNF. However, as the four-site intrastriatal 6-OHDA lesion paradigm employed in this experiment leaves a small fraction of the nigrostriatal DA projection system intact, there is a possibility that GDNF overexpression *per se* might lead to a boosting of function or regeneration from spared nigral DA neurons, thereby confounding the assessment of the graft-induced functional recovery. Therefore, in order to assess the contribution of GDNF, we included two levels of controls. First, at 8 weeks after the lesion surgery and upon completion of the baseline behavioral tests (test session I in Fig. 1), 33 lesioned and behaviorally characterized animals were divided into two balanced groups (based on their scores in the stepping test) to receive injections of rLV-GDNF ($n = 17$) or rLV-GFP ($n = 16$) vectors. These animals were followed over 15 weeks and their motor performance at 1 month (test session II in Fig. 1) and 3 months (test session III in Fig. 1) was compared with the baseline testing scores before vector injection. These test sessions included the amphetamine rotation test (Fig. 3A) and forelimb use in the stepping (Fig. 3B) and cylinder (Fig. 3C) tests. In none of these tests did the GDNF-overexpressing animals (GDNF-sham) differ from the GFP-sham group suggesting that, in the chronically lesioned animals, different from the acute lesions, the surviving nigral DA neurons are not able to respond to GDNF and provide an improvement in motor function.

The second level of control included an additional group of lesioned animals that were killed at the time when the other animals received viral vector injections (see Fig. 1). Histological analyses of the short-term lesion group and the vector-injected groups at the end of the experiment (i.e. after 9 months of transgene expression) helped us to compare the extent of nigrostriatal degeneration. Inspection of TH-immunostained sections from the lesion control animals (Fig. 2B and F) and the GFP-sham group (Fig. 2C and G) indicated that the depletion of nigrostriatal DA neurons after the intrastriatal 6-OHDA lesion was stable, at least between 2 and 11 months post-lesion. Furthermore, the extent of the nigrostriatal lesion was similar in animals overexpressing GDNF in the striatum (Fig. 2D and H). The total number of TH-positive cells in the intact SN (Fig. 2E) was estimated at $15\,133 \pm 738$, while the nigral TH-positive cell numbers in the lesion controls (Fig. 2F), GFP-sham (Fig. 2G) and GDNF-sham (Fig. 2H) treated groups were estimated at $2453 \pm 571$, $2597 \pm 328$ and $1841 \pm 334$ cells, respectively (Fig. 2Q).

In addition to TH, we quantified the number of nigral cells expressing VMAT-2, a second protein expressed in DA neurons and found on the membranes of specialized intracellular vesicles sequestering the newly synthesized DA. We found previously that,

FIG. 2. Evaluation of the extent of dopamine (DA) denervation after the intrastriatal 6-hydroxydopamine (6-OHDA) lesion. Photomicrographs of sections immunostained for tyrosine hydroxylase (TH) (A–H) or VMAT-2 (I–P) illustrating the significant loss of nigral cells and striatal fiber innervation in animals killed at 8 weeks after the 6-OHDA lesion (B, F, J and N) compared with the intact (contralateral) side (A, E, I and M). Subsequent injection of recombinant lentiviral (rLV)-GFP (C, G, K and O) or rLV-glial cell line-derived neurotrophic factor (GDNF) (D, H, L and P) into the striatum did not affect the extent of nigrostriatal lesion at 9 months after the vector injection. Quantification of the number of TH-positive (Q) and VMAT-2-positive (R) cells in the SN showed an 80–90% loss of nigral DA neurons in all lesioned groups (lesion only, GFP-sham and GDNF-sham) and similar reductions (80–90%) were observed in densitometry measurements of the striatal TH-positive (S) and VMAT-2-positive (T) fiber innervation. Scale bar in A represents 1 mm in A–D and I–L and scale bar in E represents 0.4 mm in E–H and M–P. cc, corpus callosum; cp, cerebral peduncle; ctx, cortex; ac, anterior commissure; lv, lateral ventricle; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STR; striatum.

Figure with columns labeled Intact, Lesion, GFP-sham (9 mo), GDNF-sham (9 mo); rows labeled TH (panels A–H) and VMAT-2 (panels I–P). Bottom row of bar graphs: Q. TH+ cell no in SN; R. VMAT+ cell no in SN; S. TH+ fiber density; T. VMAT+ fiber density.

although TH may be down-regulated in DA neurons that are continuously exposed to GDNF, VMAT-2 levels do not change and could thus be used as an independent marker to visualize and quantify the DA neurons and their fibers (Georgievska *et al.*, 2002a). The numbers of nigral VMAT-2-positive cells were $12\,523 \pm 623$ in the intact SN (Fig. 2M) and $1335 \pm 355$, $1683 \pm 388$ and $1130 \pm 201$ in the lesion control (Fig. 2N), GFP-sham (Fig. 2O) and GDNF-sham (Fig. 2P) groups, respectively (Fig. 2R), similar to the TH-positive cell counts (see above). Overall, there was an 80–90% depletion of nigral DA neurons (as detected with both TH and VMAT-2 antibodies) in all lesioned groups, including the GDNF-overexpressing animals (GDNF-sham), suggesting that, in the chronically 6-OHDA-lesioned rats, GDNF does not provide protection of cells or recruitment of new cells to express phenotypic markers in the SN.

We also determined whether sprouting of terminal fibers occurred at the striatal level by measuring the total fiber density (expressed as percentage of control side) using TH (Fig. 2A–D) and VMAT-2 (Fig. 2I–L) as markers. The striatal TH- and VMAT-2-positive fiber innervation was reduced by 80–90% in all groups and there was no difference between the GDNF-sham and GFP-sham groups on either measure (Fig. 2S and T).

The striatal tissue levels of GDNF in animals injected with rLV-GDNF in the striatum were determined to be $1.61 \pm 0.36$ ng/mg tissue at 15 weeks after transduction and $1.92 \pm 0.92$ ng/mg tissue at the end of the experiment (i.e. at 9 months post-transduction). These values were not significantly different from each other ($P > 0.05$), suggesting that GDNF production from the transduced cells persisted at a stable level throughout the experiment. The GDNF levels detected in the contralateral non-transduced striatum in rLV-GDNF-injected animals were not different from the endogenous levels detected in rLV-GFP-injected control animals (0.01–0.03 ng/mg tissue). Furthermore, the presence and distribution of GDNF protein in the entire striatum was confirmed using immunohistochemistry in all GDNF-treated animals, both sham and transplanted groups (data not shown).

### Functional impact of fetal dopamine neuron grafts placed in a glial cell line-derived neurotrophic factor-overexpressing host striatum

The observation that motor impairments remained stable for 15 weeks under continuous GDNF production allowed us to design the study to assess the functional impact of VM grafts placed in the GDNF-overexpressing host striatum. Therefore, the animals in the GDNF-sham and GFP-sham groups were divided into two subgroups to receive either sham surgery or transplantation of fetal VM cells (GDNF-trpl and GFP-trpl). The animals were then followed for an additional 6-month period, during which further assessments of motor behavior were conducted at 3 and 6 months post-transplantation (test sessions IV and V in Fig. 1).

Before transplantation, all 6-OHDA-lesioned animals showed clear impairments in the various motor tests, including amphetamine rotation, stepping test and cylinder test (Fig. 3A–C), as well as in the apomorphine rotation test and the skilled paw use in the staircase test (data not shown). In the amphetamine-induced rotation test, all lesioned animals turned ipsilateral to the lesion side before grafting, while the normal controls showed no side bias despite induction of motor activity (Fig. 3A). At 3 and 6 months post-transplantation, the turning behavior was overcompensated, i.e. reversed to the contralateral side in the grafted animals, irrespective of vector treatment (−2.6 to −7.5 contralateral turns/min).

In the stepping test (Fig. 3B), the GFP-trpl animals improved significantly over time from $1.2 \pm 0.8$ steps before grafting to $5.1 \pm 1.5$ steps at 3 months and $6.1 \pm 1.2$ steps at 6 months post-transplantation [ANOVA effect of time; $F(2,16) = 11.4$; $P < 0.001$], corresponding to $\approx 50\%$ of normal performance (11–12 steps). This effect was significantly different from the GFP-sham group at 6 months after transplantation [ANOVA effect of group; $F(4,37) = 33.4$; $P < 0.0001$]. The GDNF-trpl group, however, only
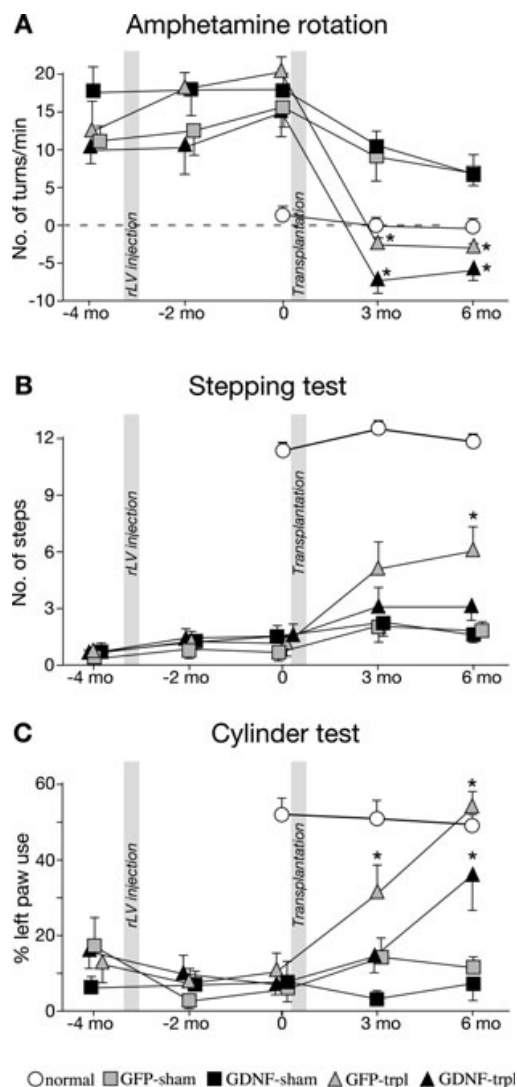
FIG. 3. Drug-induced and spontaneous motor behavior. Behavioral assessment of the animals was performed at 6 weeks after the 6-hydroxydopamine lesion (−4 months in graphs), at 1 and 3 months after recombinant lentiviral (rLV) injection (−2 months and 0 in graphs) and at 3 and 6 months after transplantation (as indicated in graphs) using amphetamine-induced rotation (A), stepping test (B) and cylinder test (C). Injection of rLV-glial cell line-derived neurotrophic factor (GDNF) into the striatum did not improve the lesion-induced behavioral impairments in any of the tests compared with the rLV-GFP-injected control animals. In the amphetamine rotation, the grafted animals [GFP-transplant (trpl) and GDNF-trpl] reversed the turning to the contralateral direction (overcompensation) and were significantly different from their sham-operated control groups (GFP-sham and GDNF-sham). In the stepping test, the GFP-trpl group improved significantly at 6 months after transplantation, compared with the GFP-sham group, while no significant difference was observed between the GDNF groups. In the cylinder test, the GFP-trpl group showed a significant increase in percentage left paw use at 3 months and improved further at 6 months after transplantation, while the GDNF-trpl group showed significant improvement at 6 months only. *Significantly different from sham-operated control group, $P < 0.05$.

improved from $1.6 \pm 0.5$ steps before grafting to $3.1 \pm 0.7$ steps at 3 months and $3.2 \pm 1.0$ steps at 6 months ($\approx$25% of normal). This improvement did not reach significance [ANOVA effect of time; $F_{(2,16)} = 3.4$; $P = 0.058$] nor did it differ from the GDNF-sham group.

In the cylinder test (Fig. 3C), both grafted groups showed significant improvements over time, whereas the GFP and GDNF sham-operated control groups remained impaired [ANOVA effect of time; $F_{(2,12)} = 0.9$; $P = 0.418$ and $F_{(2,14)} = 0.7$; $P = 0.519$, respectively,]. In the GFP-trpl group, the percentage of left touches increased from $10.9 \pm 4.9\%$ before grafting to $31.4 \pm 7.9\%$ at 3 months and $53.9 \pm 4.1\%$ at 6 months post-transplantation [ANOVA effect of time; $F_{(2,16)} = 15.9$; $P < 0.001$], representing a full recovery in this group of animals. In contrast, in the GDNF-trpl group, graft-induced recovery in the cylinder test was delayed (not significant at 3 months) and only partially recovered at 6 months after transplantation [$7.2 \pm 2.5\%$ left touches before grafting to $35.9 \pm 9.7\%$ left touches at 6 months post-transplantation; ANOVA effect of time; $F_{(2,16)} = 5.1$; $P < 0.05$].

## Impact of glial cell line-derived neurotrophic factor overexpression on grafted fetal dopamine neurons

The survival and growth of the grafted DA neurons was analysed at 6 months after transplantation using immunohistochemistry for TH and VMAT-2. The total number of surviving TH-positive cells in the control grafts was $5586 \pm 911$ (Fig. 4A), indicating a good overall graft survival (about 12.4% survival rate based on the calculation that approximately 10% of the initially grafted cells were TH positive). In the GDNF-trpl group, the number of surviving TH-positive cells was $4742 \pm 717$ and did not differ from the control grafted group (unpaired $t$-test; $t = -0.7$, $DF = 16$, $P = 0.48$) (Fig. 4A). To rule out the possibility that the GDNF treatment induced a down-regulation of TH expression in the transplanted DA neurons at the cell body level, the number of VMAT-2-positive cells in the grafts was also quantified and found to be $4444 \pm 1116$ in the GDNF-trpl group (Fig. 4B), similar to the TH-positive cell counts (see above). In the GFP-trpl group, the VMAT-2-positive cell count was $6643 \pm 1172$ and did not differ significantly from the GDNF-trpl group (unpaired $t$-test; $t = -1.4$, $DF = 16$, $P = 0.19$).

Determination of the density of TH-positive fiber innervation in the grafted striatum (Fig. 4C) showed that the GFP-trpl control group had an increased TH-positive fiber density in the striatum ($54.4 \pm 8.1\%$ of control side). This was well above the residual fiber innervation in the lesioned non-grafted animals ($13.6 \pm 3.3\%$ of control side), suggesting that the surviving TH-positive cells in the grafts contributed significantly to the innervation of the host striatum. In the GDNF-trpl group, by contrast, the density of TH-positive fibers appeared significantly lower ($31.7 \pm 4.4\%$ of control side; unpaired $t$-test; $t = -2.4$, $DF = 16$, $P < 0.05$) compared with the GFP-trpl control group. However, the VMAT-2-positive fiber density was similar in both groups (64–69% of control side; unpaired $t$-test; $t = 0.5$, $DF = 16$, $P = 0.60$) (Fig. 4D), suggesting that continuous GDNF overexpression over 6 months induced a down-regulation of the TH protein in the graft-derived fibers.

Two different possibilities may account for the lack of improved DA neuron survival in grafts placed into the GDNF-overexpressing striatum at 6 months. (i) GDNF expressed at this dose (1.5–2.0 ng/mg tissue) and through this mode of delivery (through release from striatal cells) was unable to protect DA neurons after grafting. (ii) Although the DA neurons were initially protected, they failed to
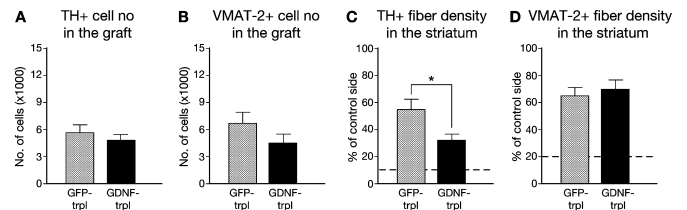


FIG. 4. Analysis of dopamine neuron survival and striatal fiber innervation at 6 months after transplantation. Quantification of tyrosine hydroxylase (TH)-positive (A) and VMAT-2-positive (B) cells in the grafts showed that there was no significant difference in cell survival between the two treatment groups. Densitometry measurements of the TH-positive fiber innervation (C) revealed a significant reduction in the glial cell line-derived neurotrophic factor (GDNF)-transplant (trpl) group, whereas the VMAT-2-positive fiber innervation density (D) was equal between the two groups. The dashed line in C and D indicates the average striatal fiber density in the non-transplanted control groups (GFP-sham and GDNF-sham), presented in Fig. 2. *Significantly different from the GFP-trpl group, $P < 0.05$.

survive long term despite the continued presence of GDNF. In order to address this issue, a second experiment was designed where 15 chronically lesioned rats received injections of either rLV-GDNF ($n = 8$) or rLV-GFP ($n = 7$) into the striatum. Two weeks after vector injection, all animals received grafts of dissociated fetal VM tissue and were allowed to survive for an additional 4 weeks before perfusion and subsequent histological analysis.

Inspection of the TH-immunostained sections indicated that the graft size in GDNF-overexpressing animals was larger, as compared with the control grafts, containing densely packed TH-positive cells and fibers within the grafts (compare Fig. 5A and B). Quantitative analysis of the total number of TH-positive cells in the 4-week-old grafts showed that the number of surviving TH-positive cells in the control (rLV-GFP) group was $5453 \pm 197$, a similar survival rate (12.1%) to that observed in the long-term experiment. In the GDNF-overexpressing animals, the number of TH-positive cells in the grafts was twofold higher ($10\,300 \pm 408$) (unpaired $t$-test; $t = 11.2$, $DF = 13$, $P < 0.0001$) (Fig. 5C), demonstrating that GDNF initially increased the survival of grafted DA neurons as seen at 4 weeks after transplantation; however, these cells failed to survive at 6 months. These results were confirmed by quantification of VMAT-2-expressing cells in the grafts (Fig. 5E and F). Significantly more VMAT-2-positive cells were counted in the GDNF-overexpressing animals ($10\,546 \pm 712$), as compared with the control grafted animals ($7754 \pm 646$ cells) (unpaired $t$-test; $t = 2.9$, $DF = 13$, $P < 0.05$) (Fig. 5G).

In contrast to the long-term experiment, the TH-positive fiber outgrowth from the grafts at 4 weeks appeared to be the same in the GFP- and GDNF-treated animals ($45.7 \pm 3.5$ and $40.5 \pm 5.5\%$ of control side, respectively; unpaired $t$-test; $t = -0.8$, $DF = 13$, $P = 0.43$) (Fig. 5D). In addition, the VMAT-2-positive fiber density was similar between the two groups ($81.3 \pm 3.1$ and $80.1 \pm 2.5\%$ of control side, respectively; unpaired $t$-test; $t = -0.8$, $DF = 13$, $P = 0.43$) (Fig. 5H). These data indicate that exposure to GDNF for the first 4 weeks increased the DA cell survival (as assessed by TH and VMAT-2 immunostaining) and did not seem to affect the TH protein expression in the surviving DA neurons.

## Discussion

The effects of continuous GDNF overexpression on grafted DA neurons were studied using an rLV vector to deliver the GDNF gene
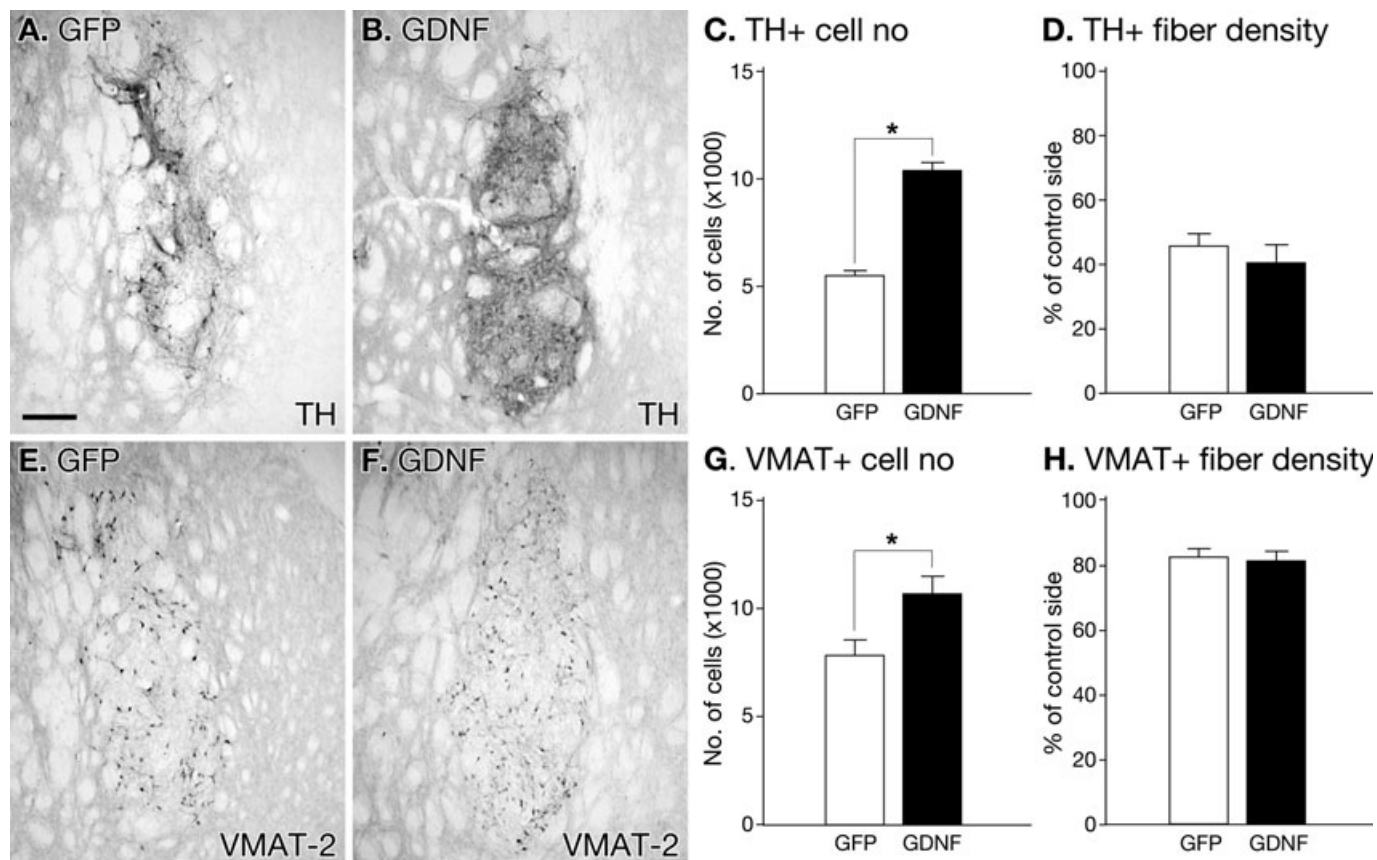
FIG. 5. Analysis of dopamine neuron survival and striatal fiber innervation at 4 weeks after transplantation. Immunohistochemistry for tyrosine hydroxylase (TH) (A and B) revealed an increased graft size in the glial cell line-derived neurotrophic factor (GDNF)-treated animals (B) compared with the control (GFP) grafts (A). Quantification of the number of TH-positive cells in the grafts (C) showed a twofold increase in the number of surviving cells in the GDNF group, whereas the TH-positive striatal fiber density was similar in both groups (D). Immunohistochemistry for VMAT-2 (E and F) showed a significant increase in the number of VMAT-2-positive cells in the GDNF-treated grafts (G) and the VMAT-2-positive fiber density in the striatum was not different between the groups (H). Scale bar in A represents 0.2 mm in A, B, E and F. *Significantly different from the GFP group, $P < 0.05$.

into the chronically DA-denervated striatum. The results show that GDNF was able to support the survival of transplanted DA neurons at 4 weeks post-grafting but that rescued cells did not survive at 6 months, despite the continued presence of GDNF around the VM grafts. In addition, the transplant-induced functional recovery in the GDNF-treated grafted animals was delayed in the cylinder test and completely blocked in the stepping test as compared with grafted control animals. This was accompanied by a GDNF-induced down-regulation of the TH enzyme in the grafted DA neurons at 6 months post-grafting.

### Short-term increased survival of grafted dopamine neurons by glial cell line-derived neurotrophic factor

At 4 weeks after transplantation we observed a twofold increase in the number of surviving TH-positive cells in the GDNF-treated animals. Similar results have been obtained in studies where recombinant GDNF protein was either added to the cell suspension medium before grafting (Apostolides *et al.*, 1998; Mehta *et al.*, 1998; Sullivan *et al.*, 1998) or delivered near the VM grafts by repeated intrastriatal injections (Rosenblad *et al.* 1996; Sinclair *et al.*, 1996) or continuous infusions (Yurek, 1998). In these studies, GDNF was delivered at high doses (0.5–5 µg) over 2–3 weeks, resulting in an overall two- to 10-fold increase in the survival of TH-positive cells in the VM grafts at

4–8 weeks after transplantation. However, the overall graft survival (irrespective of treatment) is quite low in some of these studies (Sinclair *et al.*, 1996; Apostolides *et al.*, 1998; Mehta *et al.*, 1998; Yurek, 1998) and they have been limited to fairly short (4–8 weeks) survival times.

Similar effects on DA cell survival have been observed after cotransplantation of VM grafts with different cells genetically modified *in vitro* to express GDNF (Sautter *et al.*, 1998; Wilby *et al.*, 1999; Ostenfeld *et al.*, 2002). However, the GDNF expression from the *ex vivo* transduced cells is down-regulated by 8 weeks after transplantation into the striatum (Ostenfeld *et al.*, 2002), suggesting that these grafts only provide a transient GDNF support. In contrast, the rLV vector used in this study was able to express GDNF in the denervated striatum at high and stable levels (1.5–1.9 ng/mg tissue) over at least 9 months. Interestingly, at 6 months after grafting, the number of TH-positive cells in the GDNF-treated grafts was equal to the control grafts, suggesting that the initial protection of the grafted DA neurons was no longer present at this time-point, despite the continued GDNF support.

### Long-term effects of glial cell line-derived neurotrophic factor on graft-derived dopaminergic fibers

We have previously observed that long-term continuous GDNF overexpression can induce a selective down-regulation of TH in both

6-OHDA-lesioned and intact adult animals (Georgievska *et al.*, 2002a, 2004; Rosenblad *et al.*, 2003). In the intact nigrostriatal DA system, continuous GDNF overexpression (about 2 ng/mg tissue) induced a 60–70% reduction in the striatal TH protein levels (as determined by western blot) without affecting the DA tissue levels in the striatum (Georgievska *et al.*, 2004). By contrast, the expression of VMAT-2, a protein expressed on synaptic vesicles in dendritic and axonal processes of midbrain DA neurons (Nirenberg *et al.*, 1996, 1997), did not appear to be regulated by GDNF (Georgievska *et al.*, 2002a, 2004; Rosenblad *et al.*, 2003) and was therefore used in this study as a second (independent) marker for the grafted DA neurons. At 6 months post-grafting, the striatal TH-positive fiber density was significantly reduced in the GDNF-treated animals compared with the grafted control animals (31.7 vs. 54.4% of normal). This was not due to a reduction in the actual fiber outgrowth from the grafts, as the VMAT-2-positive fiber innervation remained equal in both groups (64–69% of normal). This indicates that continuous expression of GDNF induces a selective down-regulation of TH in the grafted DA fibers, which was observed at 6 months but not at 4 weeks after transplantation. This would be in line with our previous observations in intact rats, where down-regulation of TH is time dependent and only occurs after 6 weeks of continuous rLV-mediated GDNF overexpression (Georgievska *et al.*, 2004), which would explain why this effect was not observed at 4 weeks.

### Functional impact of glial cell line-derived neurotrophic factor-treated ventral mesencephalic grafts

We used the partial 6-OHDA lesion model to assess the functional effects of the intrastriatal VM grafts exposed to GDNF over 6 months. This lesion model resulted in an overall 80–90% reduction in nigral DA cell numbers and striatal fiber innervation, accompanied by severe impairments in both drug-induced and spontaneous motor behaviors, that remained stable over 11 months. Vector-mediated delivery of GDNF into the striatum at 2 months after the 6-OHDA lesion did not improve motor function in these animals. At this time-point, the 6-OHDA-induced cell death in the SN was already complete and expression of GDNF over the next 9 months did not provide any protection and/or regeneration of the nigral DA cell bodies and nor did it promote sprouting of remaining striatal DA terminals. In contrast, in the acute 6-OHDA lesion, rLV-mediated overexpression of GDNF at similar tissue levels ($\approx$1–2 ng/mg tissue) efficiently protects the nigrostriatal DA system against the lesion (Georgievska *et al.*, 2002a,b). This suggests that, in the chronically lesioned animals, the few surviving nigral DA neurons are not able to respond to GDNF, which is different from when GDNF is delivered acutely after the lesion. This is in accordance with previous studies, where delayed GDNF protein infusion into the striatum provides limited protection of the injured nigral DA neurons (Kirik *et al.*, 2001).

We have previously reported that the magnitude of transplant-induced functional recovery in the partial 6-OHDA lesion model is highly dependent on the axonal outgrowth from the grafted DA neurons (Kirik *et al.*, 2001). Transplantation of the same number of fetal VM cells (450 000) into the lesioned striatum resulted in an average TH-positive innervation density of about 50% of normal at 7 months post-grafting and the graft-induced recovery in motor function reached an average of 50–60% of normal performance in the stepping test. In the present study, we used both drug-induced and spontaneous motor tests (mainly the stepping and cylinder tests) to assess the functional efficacy of intrastriatal VM grafts exposed to GDNF over a 6-month period. The grafted control animals showed the expected improvement in the stepping test (50% of normal), matched by a similar level of striatal TH-positive fiber innervation (54% of normal). By contrast, animals receiving grafts into the GDNF-overexpressing striatum failed to show any significant improvement in the stepping test. In the cylinder test, transplant-induced recovery was significantly delayed in the grafted GDNF-treated animals and only reached $\approx$70% of normal, whereas the grafted control animals recovered completely in this test. In addition, complete recovery was observed in the amphetamine-induced rotation in both grafted groups. This is consistent with the fact that only 100–200 surviving DA neurons are sufficient to reverse the turning bias (Brundin *et al.*, 1985). Therefore, correction of amphetamine rotation is a sensitive indicator of graft survival which, however, provides limited information about the size or function of the graft.

Although the TH-positive fiber density appeared to be significantly reduced in the GDNF-treated animals (31 vs. 54%) at 6 months after transplantation, the actual graft-derived fiber innervation was equal between the groups (as determined by VMAT-2 immunostaining). Based on this observation, we would have expected the transplant-induced recovery in the GDNF-treated animals to reach at least the same level as in the grafted control animals, rather than being reduced or completely blocked. The reason for this discrepancy is not clear. It is possible that the GDNF-induced down-regulation of TH in the graft-derived fibers compromises the production and/or release of DA into the denervated striatum. This does not seem to be the case in intact animals, where striatal DA tissue levels remain normal despite extensive GDNF-induced reductions in TH protein levels in the striatum (Georgievska *et al.*, 2004). However, we cannot rule out that immature DA neurons respond differently to continuous GDNF stimulation or that they are unable to compensate for the down-regulation of TH. Another possibility is that the constant presence of high levels of GDNF in the denervated striatum impairs the establishment of proper synaptic connections between the graft-derived DA fibers and the surrounding host tissue, thus compromising the function of the VM grafts. In the developing brain, GDNF does not appear to be required for the nigrostriatal DA fibers to reach the striatum (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996) but is rather involved in regulating the DAergic innervation pattern post-natally (López-Martín *et al.*, 1999). During the first post-natal week, GDNF expression in the striatum is patchy and coincides strongly with the presence of TH-immunoreactive fibers. This suggests that GDNF has not only trophic but also tropic actions on developing DA neurons. Accordingly, the expression of GDNF in the striatum is highest during the first post-natal week, when the DAergic fiber network is established, and subsequently decreases to low levels in the adult striatum (Strömberg *et al.*, 1993; Choi-Lundberg & Bohn, 1995). This would suggest, at least during development, that the initial presence of GDNF in the striatum is involved in guiding fiber outgrowth into the striatal target neurons, whereas a natural down-regulation of GDNF is needed for the establishment of mature and functional synaptic contacts. If this model is correct, it would mean that any attempt to boost growth and function of intrastriatal VM grafts using GDNF should use transient, rather than continuous, delivery of the trophic factor.

## Abbreviations

AP, anterio-posterior; DA, dopamine; DV, dorso-ventral; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; ML, medio-lateral; 6-OHDA, 6-hydroxydopamine; rLV, recombinant lentiviral; SN, substantia nigra; TH, tyrosine hydroxylase; trpl, transplant; TU, transducing unit; VM, ventral mesencephalic; VMAT, vesicular monoamine transporter.

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