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Increased Survival of Dopaminergic Neurons in Striatal Grafts of Fetal Ventral Mesencephalic Cells Exposed to Neurotrophin-3 or Glial Cell Line-Derived Neurotrophic Factor

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The transplantation of fetal mesencephalic cell suspensions into the brain striatal system is an emerging treatment for Parkinson's disease. However, one objection to this procedure is the relatively poor survival of implanted cells. The ability of neurotrophic factors to regulate developmental neuron survival and differentiation suggests they could be used to enhance the success of cerebral grafts. We studied the effects of neurotrophin-3 (NT-3) or glial cell line-derived neurotrophic factor (GDNF) on the survival of dopaminergic neurons from rat fetal ventral mesencephalic cells (FMCs) implanted into the rat striatum. Two conditions were tested: (a) incubation of FMCs in media containing NT-3 and GDNF, prior to grafting, and (b) co-grafting of FMCs with cells engineered to overexpress high levels of NT-3 or GDNF. One week after grafting into the rat striatum, the survival of TH+ neurons was significantly increased by pretreatment of ventral mesencephalic cells with NT-3 or GDNF. Similarly, co-graft of ventral mesencephalic cells with NT-3- or GDNF-overexpressing cells, but not the mock-transfected control cell line, increased the survival of graft-derived dopaminergic neurons. Interestingly, we also found that co-grafting of GDNF-overexpressing cells was less effective than NT-3 at improving the survival of fetal dopaminergic neurons in the grafts, and that only GDNF induced intense TH immunostaining in fibers and nerve endings of the host tissue surrounding the implant. Thus, our results suggest that NT-3, by strongly enhancing survival, and GDNF, by promoting both survival and sprouting, may improve the efficiency of fetal transplants in the treatment of Parkinson's disease.

Key words: Fetal ventral mesencephalic cells; Transplant; Neurotrophin-3; GDNF; Dopamine; Tyrosine hydroxylase; Parkinson's disease

INTRODUCTION

Intrastriatal grafting of fetal mesencephalic cells (FMCs) containing dopaminergic neurons has been proposed as treatment to alleviate dopaminergic cell loss in Parkinson's disease (10,18,23). However, the low survival of the transplanted dopaminergic neurons (12) indicates that this procedure should be considered with caution. The identification of growth factors that improve the survival and activity of nigral dopaminergic neurons has suggested that these molecules may improve the survival of transplanted FMCs (22).

Here we focus on growth factors of two different families: the neurotrophins and the glial cell-line derived neurotrophic factor (GDNF) family. The neurotrophin family is formed by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), -4/5 (NT-4/5), and -6 (NT-6). They bind and activate two different types of receptor: a common p75 neurotrophin receptor, and a specific tyrosine kinase receptor: trkA for NGF, trkB for BDNF and NT-4/5, and trkC for NT-3 (28).

The GDNF family of neurotrophic factors is formed by GDNF (37), neurturin (NTN) (33), persephin (PSP) (40), and artemin (ART) (6). Ligands of this family bind to high-affinity glycosylphosphatidylynolsitol (GPI)-anchored receptors, collectively named GDNF family receptor α (GFRa), to form a complex that subsequently activates the receptor tyrosine kinase, c-ret (29,31,53, 54). GFRα1 is the high-affinity receptor that is preferen-

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tially bound by GDNF (31), GFRα2 by NTN (31), GFRα4 by PSP (16), and GFRα3 by ART (6).

A large number of growth factors stimulate the survival and differentiation of dopaminergic neurons in primary cultures. This includes members of the neurotrophin family, such as BDNF, NT-3, and NT-4 (26,27,35,58), and members of the GDNF family, such as GDNF (8,14,24,37), NTN (25), PSP (40), and ART (6). In vivo, endogenous dopaminergic neurons also respond to several of these factors, including the neurotrophins BDNF, NT-3, and NT-4 (2,20,35), and GDNF (7–9,11,13,45). In addition, fetal dopaminergic neurons grafted into the rat striatum respond to BDNF (44,59), NT-4 (21), and GDNF (3,19,43,46,48,50,51,56). Because of the peptidic nature of growth factors and the presence of extracellular peptidases and uptake systems, a steady production of neurotrophic factors is required. One procedure for the continuous administration of growth factors is to implant genetically modified stable cells that produce them. This strategy has shown that factors of the neurotrophin (4,35) and GDNF families (1,5) enhance the survival and differentiation of central catecholaminergic neurons in vivo.

Here we studied the effects of NT-3 and GDNF on fetal mesencephalic dopaminergic neurons grafted into the striatum. FMCs were either pretreated with NT-3 or GDNF prior to grafting or were co-grafted with stable cell lines overexpressing NT-3 or GDNF (1,5). Our results show that both GDNF and NT-3 treatments improved the viability of fetal dopaminergic neurons in the graft.

MATERIALS AND METHODS

Animals and 6-Hydroxydopamine Lesions

Male Sprague-Dawley rats, weighing about 250 g, were housed and treated according to the policy on the use of animals in neuroscience research published by the Society for Neuroscience. The experimental protocols were approved by a review committee of the University of Barcelona under supervision of the Government of Catalunya. Animals were anesthetized with ketamine hydrochloride (100 mg/kg, IP) + 5,6-dihydro-2-(2,6-xylidino)-4h-1,3-thiazine hydrochloride (Rompun®, Bayer) (2 mg/kg, IP), and placed in a stereotaxic instrument with the upper incisor bar set 3.3 mm under the interaural line. Animals were unilaterally injected with 6-hydroxydopamine (4 μg/μl in saline with 0.02% ascorbic acid) into the right medial forebrain bundle (A: −4.4, L: −1.3, H: −7.8, from bregma) (42), in a volume of 2 μl and a rate of 1 μl/min. Animals injected with an equal volume of saline + ascorbate were used as controls.

Preparation of Fetal Mesencephalic Cell (FMC) Suspensions

Cell suspensions were prepared from the ventral mesencephalon of 14-day-old embryos. The mesencephalic area was dissected out as described by Dunnett and Björklund (15), incubated at 37°C for 20 min in Dulbecco's modified essential medium (DMEM) supplemented with 2 mM glutamine, containing 0.05% DNase and 0.1% trypsin. The cell suspensions were gently disagregated, washed in DMEM, and sedimented by centrifugation. Cells were resuspended in 5 μl of DMEM per piece and embryo (17). The final concentration was adjusted to 100,000 cell/μl with DMEM. In some experiments, suspensions of FMC were preincubated for 3 h with NT-3 or GDNF (both at 50 ng/ml, from RBI, Natick, MA) or in medium without neurotrophins. To evaluate the viability of the cell suspensions, the percentage of surviving cells was determined by fluorescence microscopy with acridine/ethidium bromide (1:1), as described (15).

Transfected Fibroblasts

Rat 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) overexpressing NT-3 (4) or GDNF (5), and mock-transfected fibroblasts were used. Transfected 3T3 cells were grown in DMEM supplemented with 10% fetal calf serum, 1 mg/ml penicillin/streptomycin, 1 mg/ml glutamine, and 200 μg/ml G-418 at 37°C and 5% CO_2. Cells in active growth phase were washed and collected in serum-free medium at a concentration between 0.5 and 2.0×10^5 cells per microliter for grafting.

Grafting

Three weeks after the 6-OHDA lesion of the medial forebrain, animals were grafted with a suspension of fetal mesencephalic cells introduced into the right striatum (coordinates A: +0.7, L: −3.2; H: −5, from bregma) with a flow rate of 1 μl/min, according to one of the following procedures:

a. 3 μl of suspensions containing about 400,000 FMC was implanted after preincubation for 3 h at 37°C in a shaking bath with or without NT-3 or GDNF (50 ng/ml in both cases). Four animals were used in each group: FMC without neurotrophins, FMC + NT-3, and FMC + GDNF
b. 4 μl of a mixture containing FMC and 3T3 was implanted in the right striatum. Different proportions of FMC and 3T3 were assayed (1.5×10^5:2×10^5 and 3×10^5:1.5×10^5), in a final injected volume of 4 μl. Four animals were used in each group. Animals implanted only with FMC, mock-transfected 3T3, and FMC + mock-transfected 3T3 were used as controls.

Animals were processed in all cases for the study of dopaminergic cell survival, 7 days after grafting, in order to avoid excessive proliferation of the fibroblasts.

TH Immunocytochemistry

The animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer. The brains were rapidly removed and postfixed for 24 h, and selected coronal sections were embedded in paraffin. Sections (5 μm) through the substantia nigra or the striatum were processed for tyrosine hydroxylase immunocytochemistry and developed with the avidin-biotin-peroxidase method (Vectastain, ABC Kit from Vector). Briefly, tissue sections of rat brain were treated with 0.05% saponin, followed by methanol and hydrogen peroxide, and finally normal horse serum. The sections were incubated overnight at 4°C with an anti-TH monoclonal antibody (Sigma), at a dilution 1:200. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide.

Cell Counts and Statistics

TH-positive neurons were counted in the ipsilateral striatum in six sections per animal and in four animals per condition, at a ×250 magnification in a Zeiss-Jenalumar microscope. The total number of cells was calculated according to the volume of the graft: approx. 0.06 mm^3. For statistical evaluation, data were subjected to one-way analysis of variance (ANOVA) and DMS post hoc test.

RESULTS

All studies were carried out in 6-OHDA-lesioned animals. The extent of the lesion was assessed in each animal by TH immunostaining. All animals included in the study showed almost complete loss of TH+ cells in the ipsilateral substantia nigra.

Viability of FMC Suspensions Before Grafting

Because FMCs are known to survive poorly after preparation of cell suspensions, we first examined whether preincubation of FMCs with neurotrophic factors known to promote the survival of dopaminergic neurons could prevent the loss of TH+ cells. Preincubation of FMCs at a concentration of 100,000 cells/μl in DMEM at 37°C for 6 h resulted in a decrease in the cell viability from 85% to 60%. In contrast, preincubation of FMCs in the same medium with NT-3 or GDNF (at 50

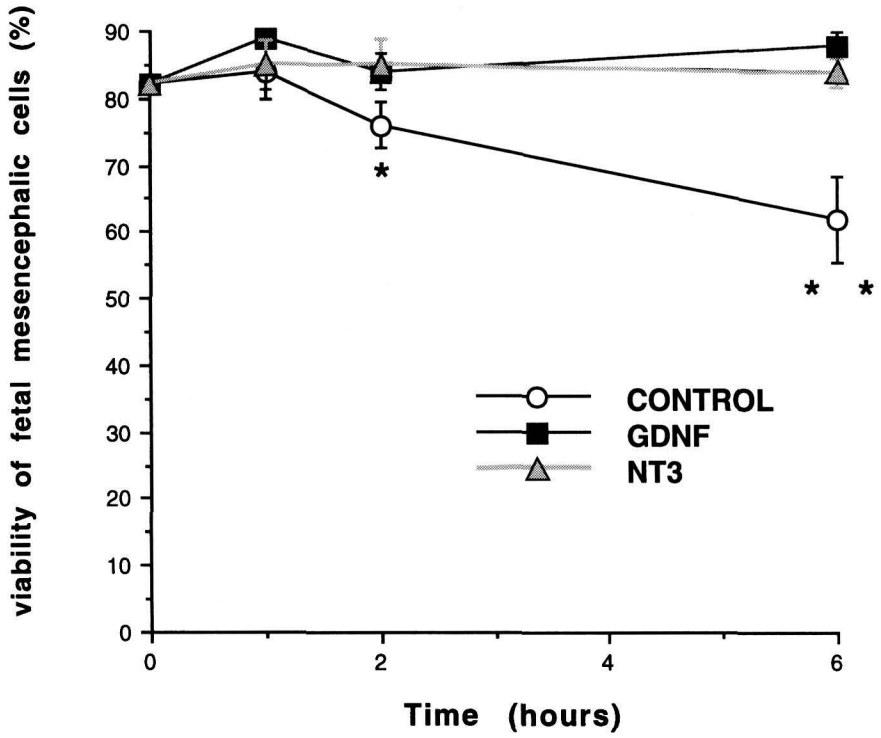

Figure 1. Effects of neurotrophin-3 (NT-3) and glial cell line-derived neurotrophic factor (GDNF) on the percentage of viable rat fetal mesencephalic cells in suspension, as assessed by acridine orange (see Materials and Methods). Each point is the mean ± SEM of three separate experiments. $*p < 0.05$, $**p < 0.01$ with respect to the corresponding control values.

ng/ml) for 6 h maintained the viability of mesencephalic cell suspensions at about 85% (Fig. 1).

Survival of Dopaminergic Cells After Preincubation With Neurotrophins and Grafting

Next, we tested whether preincubation of FMCs with NT-3 or GDNF enhanced the survival of TH+ cells in vivo, 1 week after intrastriatal grafting. Pretreatment with NT-3 or GDNF (at 50 ng/ml) increased the numbers of TH+ cells more than fourfold, compared to FMCs preincubated without the factor (Figs. 2 and 3), suggesting an early neuronal survival promoting effect of NT-3 and GDNF on TH+ cells grafted in the host striatum.

Survival of Dopaminergic Cells Co-Grafted With Transfected Fibroblasts

To examine whether constantly delivered NT-3 or GDNF in vivo was more efficient in preventing the loss of TH+ cells grafted in the host striatum, we performed co-grafts of FMCs and fibroblast cell lines known to express high levels of recombinant, biologically active NT-3 (4) and GDNF (5). One week after grafting a mixture of FMCs and transfected 3T3 fibroblasts (2:1), the survival of TH+ cells in the striatum was significantly increased by both GDNF- and NT-3-transfected fibroblasts, compared with mock-transfected fibroblasts or FMCs alone (Fig. 4). All the measures were performed 1 week after grafting, when only small tumors and focal damage were caused by the fibroblasts. Figure 5A shows the appearance of an implant of FMCs plus fibroblasts, 1 week after intrastriatal grafting. The survival of dopaminergic cells was much higher in co-grafts with NT-3-producing fibroblasts. This effect was also observed when different ratios of FMCs and 3T3 cells were co-grafted (Table 1). Grafting of a larger number of 3T3-NT-3 cells resulted in a slightly higher number of surviving TH+ cells compared to the total number of cells grafted (0.9% and 0.6% when 200,000 or 150,000 3T3-NT-3 cells were grafter, respectively). In GDNF-producing grafts, the number of TH+ cells was lower than in NT-3-producing grafts, but a marked increase in TH+ fibers was observed in the host striatal tissue surrounding the implant (Fig. 5C) at a distance of about 525 μm from the border of the graft.

DISCUSSION

Many studies have suggested that graft survival in a host brain depends, among other parameters, on the access of transplanted neurons to growth factors (34). Here we describe, by two different procedures, a significant improvement in TH+ FMC survival mediated by two neurotrophic factors—NT-3 and GDNF—at relatively short times after transplantation (1 week). This period

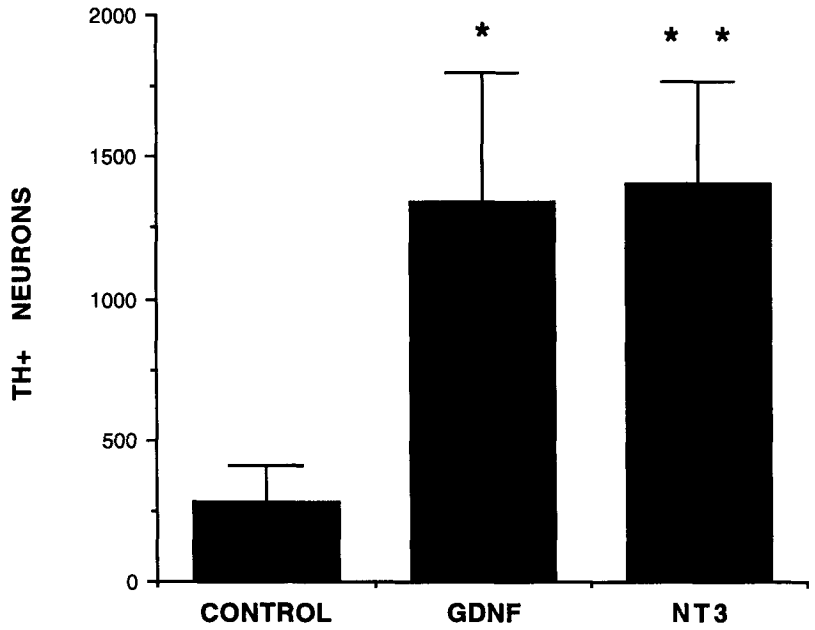

Figure 2. Preincubation of fetal mesencephalic cells for 3 h with NT-3 (50 ng/ml) or GDNF (50 ng/ml), but not vehicle (DMEM, control), increased the number of tyrosine hydroxylase-positive neurons 7 days after striatal grafting. The results are the mean ± SEM of four animals for each group. Significances were determined by ANOVA, $F(3, 12) = 4.08$, $p = 0.03$, and post hoc DMS test ($*p < 0.05$, $**p < 0.01$ compared with controls).

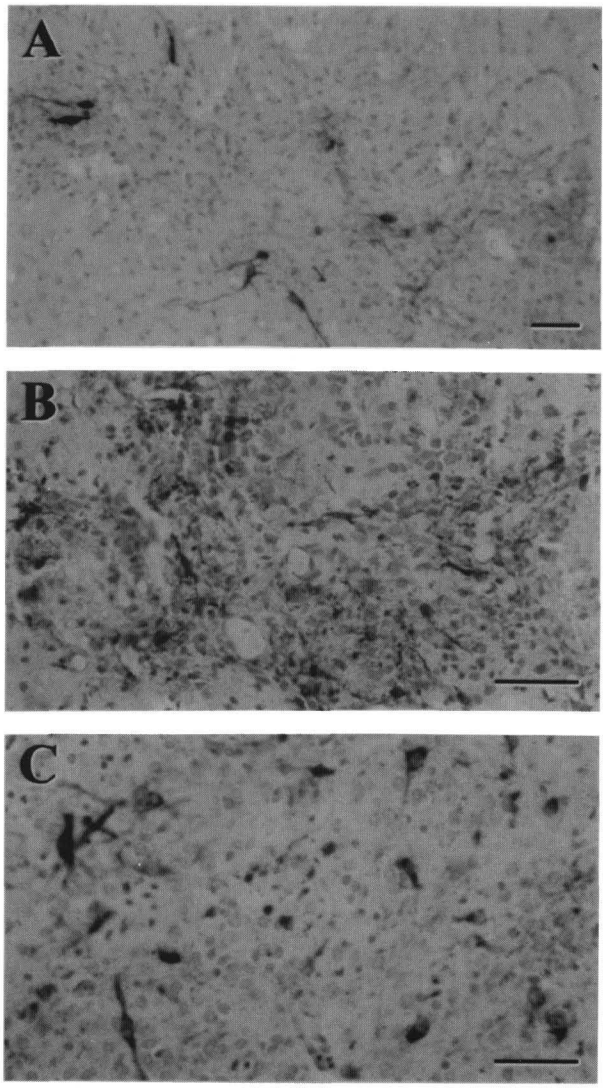

Figure 3. Microphotographs of tyrosine hydroxylase-positive neurons in striatal grafts of fetal mesencephalic cells, 7 days after grafting and preincubation with (A) DMEM without neurotrophic factors, (B) medium containing neurotrophin-3 (50 ng/ml), or (C) medium containing glial cell line-derived neurotrophic factor (50 ng/ml). Sections were counterstained with hematoxylin. Scale bar = 50 μm.

has been chosen to avoid an excessive proliferation of the transfected fibroblasts and compression of the FMC. Thus, our results suggest that cells other than fibroblasts might be better suited to implement for clinical use and to exploit the beneficial effect of GDNF and NT-3 on fetal cells after transplantation.

The expression of both NT-3 (38) and its receptor, trkC (30), is high during fetal development (between E14 and E16) but reduced in the adult brain. NT-3 is expressed in many brain areas, including the mesencephalon and striatum, during the perinatal period (32). NT-3 immunoreactivity has been detected both in glia and neuronal populations of developing substantia nigra (58), and it has been shown to increase the survival of ventral mesencephalic dopaminergic neurons in primary cultures (27). Our data also suggest that NT-3 is a survival factor for embryonic dopaminergic neurons in vivo, because preincubation of immature dopaminergic cells with NT-3, or direct supply of NT-3 to grafted FMCs in vivo, enhances the survival of TH+ cells in the host brain.

In addition, GDNF was initially characterized by its ability to promote the survival of mesencephalic dopaminergic neurons in vitro (37), and more recently, it was reported to reduce the apoptosis of dopaminergic neurons (14). Because GDNF is also expressed in the developing striatum and mesencephalon (49,52,54), and because its receptors, the proto-oncogene c-ret and the co-receptor GFRαl, are present in developing nigral neurons (54,55,57), it has been suggested that GDNF plays a trophic role in the developing nigrostriatal system. In the adult brain, GDNF mRNA is expressed at low levels both in the substantia nigra and in the striatum (5,47), suggesting a possible local and target-derived role of GDNF in the support of striatal and dopaminergic neurons. Interestingly, mechanical injury increases GDNF expression in the striatum (36), and administration of GDNF in vivo protects striatal neurons (41). Moreover, the degeneration of nigrostriatal dopaminergic neurons can also be prevented in vivo by direct injection of GDNF protein (20,45,52), adenovirus-mediated GDNF gene transfer (7,9,13,39), or cell-mediated delivery of GDNF (1,41).

Our results show that treatment of FMCs with NT-3 or GDNF prior to or during grafting enhanced the survival of dopaminergic neurons in the striatum. Indeed, the exposure of FMCs to trophic factors maintains in vitro their viability at least 6 h (Fig. 1), and the in vivo survival of the dopaminergic cells, 1 week after grafting into the brain, was fourfold higher than that of non-preincubated cells (Fig. 2). That indicates that prior treatment of a cell suspension at early stages with trophic factors is crucial for the late survival of these cells.

Although both neurotrophic factors were beneficial, surprisingly, continuous administration of NT-3 in vivo was more effective than GDNF in maintaining the survival of dopaminergic neurons after grafting. Many studies have examined the beneficial effects of pretreatment or treatment of FMCs with neurotrophic factors in vivo. Coadministration of GDNF and FMCs (3,19,50) or administration of GDNF in the vicinity of the intrastriatal (19,43,46,48) or nigral (51,56) FMC grafts has been shown to: (a) promote the survival and sprouting of TH+ neurons from FMCs grafted either in the stria-

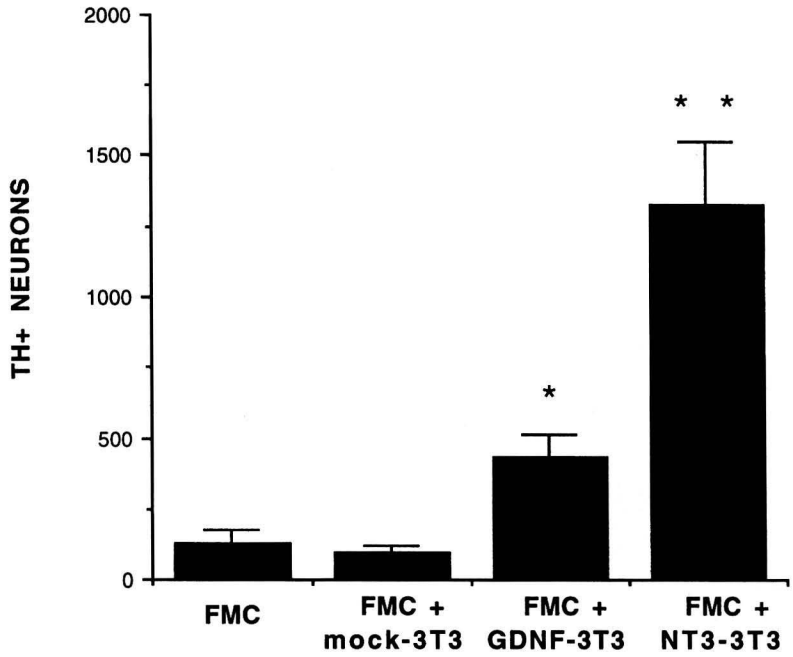

Figure 4. Co-grafting of 300,000 fetal mesencephatic cells (FMC) and 150,000 fibroblasts overexpressing neurotrophin-3 (NT3-3T3) or glial cell line-derived factor (GDNF-3T3), but not mock-transfected fibroblasts (mock-3T3), increased the number of surviving tyrosine hydroxylase-positive neurons in the rat striatum compared to the grafting of FMCs alone. The results are the mean ± SEM of four animals for each group. Significances were determined by ANOVA, $F(3, 12) = 6.3$, $p = 0.005$, and post hoc DMS test ($*p < 0.05$, $**p < 001$, compared with FMC values).

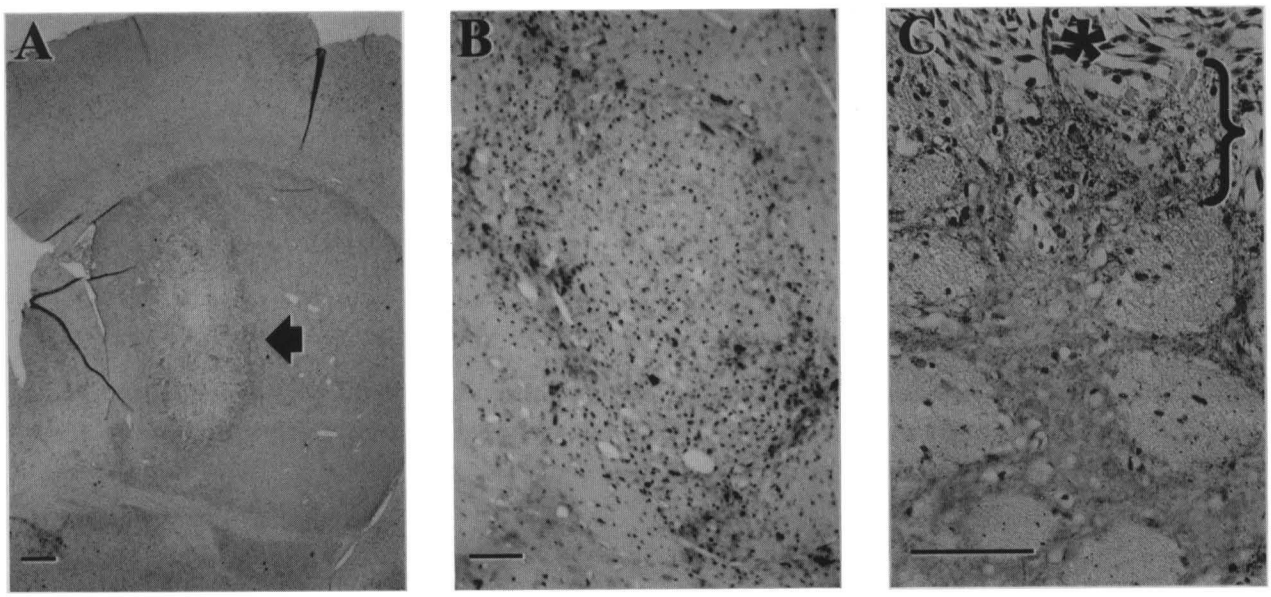

Figure 5. Distribution of TH immunostaining in striatal co-grafts of 300,000 fetal mesencephalic cells (FMCs) and (A) 150,000 mock-transfected fibroblasts, (B) NT-3-producing fibroblasts, or (C) GDNF-producing fibroblasts. Arrow in (A) indicates the position of the graft. Asterisks and arrowheads in (B) indicate the position of fibroblasts and high density of TH+ neurons, respectively. Note in (C) the increase in TH immunoreactive fibers (brackets) surrounding the graft of FMCs and GDNF-producing fibroblasts. Sections were taken 7 days after grafting and were counterstained with hematoxylin. Scale bars = 100 μm.

Table 1. Number of TH+ Neurons in the Rat Striatum After Co-Grafting of Different Amounts of Fetal Mescephalon Cells (FMC) and Neurotrophin-3 (NT-3) or Mock-Transfected-3T3 Cells per Graft

	FMC + 3T3/NT-3	FMC + 3T3/Mock
150,000 FMC + 200,000 3T3	1303 ± 233* (10.8%)	92 ± 30 (0.8%)
300,000 FMC + 150,000 3T3	1905 ± 387* (7.9%)	287 ± 92 (1.2%)

Percentages refer to surviving TH+ cells with respect to the dopaminergic cells grafted (8% of the total FMCs).
*$p < 0.01$ compared with mock-transfected 3T3 co-grafts; $n = 4$ for each group.

tum or in the substantia nigra, and (b) to improve motor asymmetry in 6-OHDA-lesioned animals. Although other neurotrophic factors, such as BDNF, improve behavioral performance, they have not shown increased survival or sprouting from TH+ FMCs (44,51,59). It has also been reported that NT-4, but not NT-3, induces sprouting and behavioral recovery from 6-OHDA lesions (21). However, there is a discrepancy concerning the effects of NT-3 as a survival factor for TH+ neurons derived from FMCs (21,27). While treatment of FMCs with NT-3 promotes the survival of TH+ cells in vitro (27) and in vivo (present results), intraparenchymal injections of NT-3 failed to enhance the survival of the TH+ cells in the grafts (21). Thus, our results suggest that the constant and local delivery of NT-3, newly made and delivered by the cell line, is more effective at maintaining TH+ FMCs.

Interestingly, and unlike the effects of NT-3, our results also show that GDNF is able to induce sprouting of TH+ fibers in the host striatal tissue surrounding the graft of FMC + GDNF-3T3. These findings are consistent with previous reports showing the induction of sprouting of TH+ FMCs by GDNF (3,19,43,45,48, 50,51,56), but not by NT-3 (21). Similar effects on dopaminergic neuron sprouting have been described for GDNF-expressing fibroblasts grafted either in the striatum or the substantia nigra (1,41). Moreover, because high doses of GDNF (or proximity to the GDNF graft) are required for the induction of sprouting of TH+ fibers (1,5), our results suggest that high levels of GDNF were available in the tissue, and that differences between GDNF and NT-3 reflect either differences in the responsiveness of TH+ cells at a certain development stage or diverse biological activities of these molecules. Both GDNF and NT-3 may act sequentially on developing dopaminergic neurons to sculpt their final mature phenotype. In that case, strategies including a sequential treatment of FMCs with neurotrophic factors or exposure of FMCs to a combination of factors, including GDNF, NT-3, and probably others like BDNF (2,35,44,59), either before and during the early phases of transplantation, may enhance the survival, integration, and performance of dopaminergic cells in the host brain. In that way, NT-3 and GDNF could thus be candidates for the transplantational treatment of Parkinson's disease.

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